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Bringing a Preclinical Candidate to the Clinic

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Early Drug Development

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Volume 1

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Volume 2

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Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at <http://dnb.d-nb.de>.

Print ISBN: 978-3-527-34149-8
ePDF ISBN: 978-3-527-80176-3
ePub ISBN: 978-3-527-80177-0
Mobi ISBN: 978-3-527-80178-7
oBook ISBN: 978-3-527-80175-6

Cover Design Schulz Grafik-Design, Fußgönheim, Germany
Typesetting SPi Global Private Limited, Chennai, India
Printing and Binding

Printed on acid-free paper

Salir all'Infinito

A Michele, per Anna

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Preface

Modern research in drug discovery and development (DDD) resulted in enormous progress in understanding disease-underlying mechanisms on a molecular level via systems biology strategies and in developing advanced methodological tools [1]. Regrettably, however, this progress did not translate into higher rates of successful approvals for new chemical entities (NCEs). Only one out of 5000–10 000 NCEs is approved, and only one out of nine compounds in clinical development reaches the market [1].

To counteract this unsatisfactory situation and to reduce the number of late-stage failures of clinical candidates, current pharma research dedicates an increased attention to a particular step in the DDD path: the early or preclinical drug development step [1–3] that comprises all activities aimed at bringing optimized lead structures to first-in-human trials considering pharmacological and toxicological characterization as well as GLP and GMP activities according to regulatory guidelines. The goal is to optimally filter out "detrimental" compounds at a very early state of the process and thereby to increase success rates.

In the introduction of this book, Fabrizio Giordanetto gives an overview of the general early drug development (early DD) workflow. In four follow-up sections, the sequential steps of early DD are described in detail, focusing on the availability of the drug substance according to GMP guidelines and the solid phase characterization, the availability of the drug product after preformulation work, the prediction of PK/PD, and the *in silico*, *in vitro*, and *in vivo* prediction of drug safety. All sections include several case studies to further exemplify the respective early DD steps under consideration. Finally, strategic aspects of patenting are addressed.

Drug substance: Drug substance is defined as an active ingredient intended to furnish pharmacologic activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease or to affect the structure or any function of the body; it does not include intermediates used in the synthesis of such an ingredient. Chapters in this section particularly concern process chemistry including route-finding and up-scaling environmental aspects such as green chemistry and costs of goods.

Drug product: The drug product is defined as the finished dosage form, often comprising the drug substance formulated with inactive ingredients optimized for the intended application route with a suitable ADME profile. Drug formulation and their delivery into the human body represent a central

part of the DDD process. Formulation aspects may impact lead design as well as nonclinical and clinical evaluations.

Formulating drug substances into drug products serves to optimize stability and absorption for oral products and solubility for systemically administered drugs. Numerous DDD programs search for new ways of formulating marketed drugs and drugs under development in order to improve their pharmacokinetic (PK) profiles, thereby enhancing their safety and/or efficacy characteristics or improving the dose regimen.

Evaluating/predicting PK/PD characteristics: Determining the PK/PD properties of drug candidates is another main part in the DDD workflow. Previously, these characteristics were the major cause of attrition during DDD or of marketed drug withdrawals. Nowadays, novel technologies help to eliminate NCEs with poor solubility or poor drug-like properties at early discovery steps.

Critical in project planning is to decide which studies should be done early and which later. There is an obvious need for an early *in vitro* assessment of metabolic stability and CYP450-mediated enzyme inhibition in human preparations, as well as some information on oral bioavailability in laboratory animals. As most drugs undergo at least some biotransformation, a decision is needed regarding how much metabolism work should be conducted at this state to overcome metabolic deficits.

Preclinical drug safety: Provided an NCE is selected for further development, its profile of acute and chronic toxicity is evaluated *in vitro* and *in vivo*. Prominent aims are to identify organs targeted by the NCE and to test for teratogenic and carcinogenic effects. Preclinical safety is evaluated according to *good laboratory practices*. Safety evaluations are among the first development studies applied to an NCE and continue during clinical development. Preclinical *in vivo* studies last from a few weeks to months, depending on the planned duration of use in humans. Such studies are performed in a rodent and nonrodent species, choice of which is based on the closest resemblance to humans.

Toxicogenomics is viewed as an alternative to animal toxicology testing. These cell-based assays might exhibit sincere advantages concerning speed of testing and reduced use of whole animals. It is unclear, however, whether such *in vitro* models might replace animal testing. Perhaps toxicogenomics may best be used during early screening as a predictive toxicology tool to eliminate NCEs in the discovery phase. Currently, it seems that the classical testing of a rodent and a nonrodent species for toxicity properties will remain the gold standard for at least the near future. Topics, briefly ascribed above, are in detail discussed in this section.

The series editors are grateful to Fabrizio Giordanetto for organizing this volume and to work with such excellent authors. Last, but not the least, we thank Frank Weinreich and Waltraud Wüst from Wiley-VCH for their valuable contributions to this project and to the entire book series.

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October 2017

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A Personal Foreword

As a medicinal chemist, I always transition to early clinical development with great anticipation and excitement. The leap from the adventurous challenge of defining a lead series and establishing efficacy proof of concept to the exacting process of enabling the selection of a compound for human clinical studies is an exhilarating one, as virtually all project paradigms change. Experimental screening, compound synthesis, data analysis, decision making, human interactions, financial consequences, and strategic commitments all move suddenly to a new level. And no matter how much I try to pace, prepare, and plan for it, the moment it happens has always a project-specific, surprising element that I cherish enormously.

Successfully mastering early clinical development requires a conspicuous amount of tenacity, pragmatism, knowledge, experience, and intuition. It demands a growing number of different business functions, experts, and personalities to be perfectly aligned while constructively challenging and complementing each other. Being part of such a diverse yet united team, working together under ever-increasing stringencies and demands, and progressively approaching the interim goal of first-in-man testing are simply the ultimate treats for a drug discovery scientist.

It can nevertheless be challenging to navigate this space effectively, to resolve complex scientific intricacies in the tight planning and scheduling regimes of early drug development, all against mounting competition. This prospect can be particularly daunting for researchers facing early drug development for the first time, especially given the paucity of bibliographic material on the subject, the anecdotal nature of the information being shared, and its limited applicability outside the context of a specific project it was developed for.

This book attempts to provide a relevant and much needed early drug development resource to drug discovery scientists. It dissects key contributing disciplines and points out their relationships and dependencies. It draws heavily on real-life project case studies to emphasize potential early drug development strategies, their requirements, risks, advantages, and limitations. Importantly, each chapter is structured against the notion of a project target product profile, an essential scientific planning and decision-making tool with implications and impact reaching far beyond the realm of early drug development. A project-specific, fit-for-purpose target product profile exerts technical, execution, and strategic

clarity across boundaries and experience levels, and I trust readers will appreciate the value of such a compass throughout the book.

Repeated exposure of young drug hunters to the complexity and allure of the early clinical development environment and associated way of thinking are crucial to their professional and personal development. I am certain that the collective knowledge contributed by seasoned industrial and academic drug hunters to this book will provide them with a helpful early drug development stepping stone.

Personally, I hope this book will inspire young scientists to step outside the comfort of their own discipline, to proactively build bridges to ancillary functions and to maintain a passionate, meticulous, and truth-seeking outlook as prerequisites to their early drug development successes.

November 2017
New York

Fabrizio Giordanetto

1

Early Drug Development: Progressing a Candidate Compound to the Clinics

Introductory Remarks

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Drug discovery and development is a fascinating, challenging, and multidisciplinary process where ideas for therapeutic intervention are devised, evaluated, and translated into medicines that will ultimately benefit society as a whole. As the name implies, it consists of mainly two elements: an initial discovery phase, followed by a development phase. These two phases differ significantly from each other with respect to scope, challenges, and approaches. As an example, while discovery experiments are typically executed in a laboratory setting using isolated and approximate systems (e.g. recombinant protein, cells, animals), development experiments consist of clinical trials in hospitals with human subjects and their full pathophysiological complexity. Differences notwithstanding, discovery and development must be integrated into a coherent whole for the process to be successful. Accordingly, much thought has been devoted to ensure scientific, logistical, and organizational aspects of such integration are taken into consideration and optimized [1–4].

Thankfully, the early view (and practice) of a discovery unit tasked with the delivery of a compound, typically termed a "preclinical candidate," which is then "thrown over the fence" to the development organization responsible for its clinical progression as a candidate drug, is a memory from a (not so) distant past. Alignment of research objectives and outcomes relevant to the discovery phase with clinical imperatives relevant to the development phase and commercial viability is not always straightforward, especially in new sectors of the pharmaceutical research environment where innovative therapeutic hypotheses are speculative and not clinically validated. Nevertheless, such an alignment is absolutely required for success, and a joint understanding and ownership of the practical implications of such alignment needs to be fostered within the project teams and their organizations.

Conceptual tools to support the initial definition of discovery and development alignment at a project level, and the strengthening of this alignment as the drug hunting program evolves, have been developed and provide a useful framework [5, 6]. Unsurprisingly, early drug development is where this alignment between discovery and clinical requirements is crystallized, normally by the selection of

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

one or more compounds that fulfill a predefined profile, that will be progressed to clinical studies.

The definition of this so-called target product profile (TPP [7]) affects all research activities during lead optimization, including focused compound design in order to reach the set TPP standards, and planning of a screening cascade in order to maximize the number of testing cycles on key TPP parameters. Some salient TPP properties such as toxicological risks, predicted human dosing, and pharmaceutical properties can only be effectively, and practically, assessed for the first time in a project timeline during early drug development. TPP definition and compliance have therefore far-reaching effects across the drug discovery–drug development value chain: they dictate which compounds are made in the first place, which compounds will be selected for clinical development, and ultimately which compounds will be successful at the end of the development cycle.

This book is structured around the TPP to highlight its importance as an early drug development compass. Here, we set the compound(s) of interest – one of which is destined to become the new drug substance – front and center because the experimental quantities relevant to the TPP, regardless of testing paradigms and screening technologies used, are all properties inherent to the compound itself and are set when the compound is first designed. By taking this approach, we hope to stimulate readers along three main axes: (i) achieving a clear line of sight between preclinical measures and the desired clinical outcomes; (ii) the variability, uncertainty, and realm of applicability of the data generated and the methods used; and (iii) the integration of diverse data and disciplines. These three elements are constantly pondered and discussed by early drug development scientists as part of the TPP definition and fulfillment process. They provide an evidence-based approach to defining and refining the TPP and to selecting the best possible compounds to meet the TPP requirements.

The parameters comprising a TPP are more important than the specific target values of any particular TPP parameter. To highlight this concept, an example TPP is shown in Table 1.1. TPPs are, by definition, project and time specific, and they should be viewed as living documents. Project teams should strive to define the TPP as early as possible, with the attitude to refine the TPP as more data are generated, typically when pharmacological efficacy measures or early toxicity signals are established, or in response to external stimuli such as results from competitors or clinical validation studies, to name but a few examples. Similarly, even within the same overall project, the TPP for a backup compound will very likely be different from the one used for the clinical front-runner; additional insights, knowledge, and differentiation properties gleaned during lead optimization, early drug development, and clinical development will be incorporated into the revised TPP.

When considering the importance of the TPP to early drug development, it is striking that all of its parameters are, at best, surrogates of clinical readouts, each characterized by its own uncertainty and variability based on the underlying data and methods used. Although major advances have been made in predicting human pharmacokinetics from animal data, there is still ample room for surprises in Phase I pharmacokinetic studies due to the intrinsic

Table 1.1 Target product profile (TPP) example as an essential early drug development tool.

	Description	Target value	Comparator/ standard of care	Planned studies	FDA's TPP template section [8]
Project goal	A statement that the drug is indicated for the treatment, prevention, relief, or diagnosis of a particular indication of a recognized disease or condition and their associated manifestations or symptoms alone or in conjunction with a primary mode of therapy				Indication and usage
Drug substance	Physicochemical properties (e.g. crystallinity, thermal property, hygroscopicity)				
	Synthetic and purification risks				
	Estimated cost of goods				
Drug product	Route of administration				Dosage and administration
	Recommended usual dose (maximum absorbable dose)				
	Dose range shown to be safe and effective				
	Dosage intervals or titration schedule				
	Usual duration of treatment course when treatment is not chronic				
	Dosage form				Dosage forms and strengths
	Dosage strength				
	Special handling and storage conditions, chemical stability of formulation				How supplied/ storage and handling
Pharmacokinetics and pharmacodynamics	*Mechanism of action*: Summarize established mechanisms of action in humans at various levels (e.g. receptor membrane, tissue, organ, whole body)				Clinical Pharmacology
	Pharmacokinetics: Describe clinically significant pharmacokinetics of a drug or active metabolites (i.e. pertinent absorption, distribution, metabolism, and excretion parameters). Document their compatibility with intended magnitude and duration of effect (e.g. include results of pharmacokinetic studies that establish the absence of an effect, including pertinent human studies and *in vitro* data)				

(Continued)

Table 1.1 (Continued)

	Description	Target value	Comparator/ standard of care	Planned studies	FDA's TPP template section [8]
	Pharmacodynamics: Include a description of any biochemical or physiologic or pharmacologic effects of the drug or active metabolites related to the drug's clinical effect or those related to adverse effects or toxicity. Include data on exposure–response relationship and time course of pharmacodynamics response				
Toxicology	Results of long-term carcinogenicity studies – species identified				Nonclinical toxicology
	Mutagenesis results				
	Reproduction study results				
	Include a description of clinically significant adverse reactions and potential safety hazards and limitations of use because of safety considerations, as reasonable evidence of these issues is established or suspected as from, e.g. safety pharmacology and GLP toxicological studies				Warnings and precautions
	Describe overall adverse reaction profile of the drug based on available safety database (e.g. safety pharmacology, GLP toxicology studies). List adverse reactions that occur with the drug and with drugs in the same pharmacologically active and chemically related class, if applicable. Within a listing, adverse reactions should be categorized by body system, severity of the reaction, or in order of decreasing frequency, or by a combination of these, as appropriate. Within a category, adverse reactions should be listed in decreasing order of frequency				Adverse reactions
	Describe clinically significant interactions, either observed or predicted (i.e. other prescription drugs or over-the-counter drugs, class of drugs, or foods such as grapefruit juice or dietary supplements); practical advice on how to prevent drug-drug interactions (description of results from studies conducted or observations from the integrated safety summary); drug-laboratory test interactions (known interference of drug with lab test outcome)				Drug interactions
Intellectual property	Patent status and plans				
	Freedom to operate analysis outcome				

variability of human absorption, metabolic, and excretion properties, especially with compounds characterized by low-to-moderate bioavailability [9]. When it comes to predicting pharmacological efficacy and toxicity, the current dismal clinical attrition statistics and the corresponding breakdown as to the primary reason for failure are sobering reminders of to what little extent we can predict clinical results [10], although having human-validated biomarkers and genetics evidence for a given target can help to mitigate these risks [11, 12]. Furthermore, the various TPP parameters cannot be dealt with in isolation but are intimately connected. Integration of TPP parameters so as to provide clinically useful estimates such as starting dose, dose frequency, and therapeutic windows adds an additional layer of complexity and uncertainty during early drug development. Given these premises, early drug development is where the multidisciplinary nature of drug discovery and development makes the biggest impact. Successful integration of scientific data from disciplines such as medicinal chemistry, process chemistry, pharmacology, toxicology, and pharmaceutics requires discipline experts to work seamlessly as a team, fluent in each other's vocabulary, able and willing to challenge and support each other. Their ability to proactively anticipate and address TPP-related issues, to master the interdependencies between TPP parameters, and to distill diverse inputs into actionable plans and schedules is as important to success as the quality of the scientific data generated and the validity of the therapeutic hypotheses being tested.

Part I presents practical considerations related to preparing sufficient quantities of selected compounds to enable their evaluation against the TPP. Chapters 1–3 introduce critical strategic, financial, planning, and organizational aspects of scale-up and production of sufficient drug substance so as to allow the TPP-based selection process and initial clinical development activities. Chapter 4 discusses how integration of novel chemistry methods and technologies can reduce the timelines associated with drug substance delivery, afford higher structural complexity to satisfy the constant drive for drug substance differentiation, and minimize the environmental impacts of manufacturing processes. The last two chapters describe real-life case studies of enabling chemical synthesis for early drug development purposes, with a view to manufacturing, that neatly integrate the various elements previously discussed.

Although most TPP-relevant properties of a drug substance are inherent to its chemical structure, some compound properties can nonetheless be significantly optimized or mitigated when the drug substance is engineered into a given drug product. Part II details the preparation, assessment, and selection of drug products that fulfill TPP and developability criteria. Solubility and permeability – two essential parameters of the drug substance – are categorized according to the Biopharmaceutics Classification System (BCS) framework [13]. Both parameters carry significant implications for a compound's exposure in efficacy and toxicology studies and key early drug development activities; engineering of the drug substance into a drug product involves a wide variety of techniques, most aimed at tailoring these two essential parameters. Three chapters present how the experimental characterization of solid-state properties, the selection of (co)crystal and salt forms, and traditional formulation methods enable the practical development of a wide array of drug products. The benefits of physical state manipulations such

as particle size and nanodispersions are also discussed. Examples from late lead optimization and early drug development projects are presented to showcase the flexibility provided by *ad hoc* drug substance investigation activities.

Part III introduces pharmacokinetics (PK) and pharmacodynamics (PD) as dual cornerstones of early drug development. Rather than devoting two independent chapters to each, a single chapter sets forth vital guidelines for their integration into an overarching PK/PD framework. These guidelines include not only essential scientific PK/PD principles and strategies but also the holistic mind-set and cross-disciplinary practice required for their effective implementation. A specific chapter has been dedicated to prediction of human PK/PD relationships, with an eye toward satisfying TPP and clinical parameters; particular importance is given to the applicability, uncertainty, and translational risk elements associated with the approach taken and the available data. Several case studies further anchor the usefulness of the PK/PD paradigm and expose some practical implications in PK/PD study design, compound selection and synthesis, TPP definition, and reference compound benchmarking.

Toxicology, a crucial aspect tackled during early drug development, is described in Part IV. Strategies and methods consistent with current rational and efficient industrial standards are discussed first as a key part of the project TPP. In keeping with the previous PK/PD section, a quantitative and integrated approach to assess toxicological risk throughout early drug development is presented. Advantages and limitations of the various methods are discussed, especially from a translatability and risk management point of view. Safety pharmacology activities are addressed as complementary and dependent upon efficacy-based studies so as to allow the derivation of safety margins via toxicokinetic–toxicodynamic (TK/TD) approaches. Available computational approaches to predict toxicological outcomes are surveyed and described based on their applicability domain and predictive power. Given the difficulty in precisely predicting toxicological endpoints, several real-world project examples in risk assessment and mitigation are presented to highlight the diversity of the chosen approaches.

Part V completes the TPP-centered motif of this book by describing intellectual property (IP) matters and requirements. After a review of patent law relevant to early drug development, a number of patent protection strategies are discussed in terms of their impact and implications for adequately safeguarding a specific invention. Two additional perspectives, in line with recent changes in the drug discovery and development environment, are then presented. The first details IP challenges and opportunities associated with the development of generic drugs and the attendant consequences for companies developing first-in-class or best-in-class products. Here, an elaboration on generic companies' drivers and IP approaches is offered to support innovators in evaluation of their own IP strategy. The second describes special considerations that need to be assessed when developing drugs – as is increasingly commonplace – as part of a collaborative venture, which brings additional IP complexity and consequences for ownership and IP rights.

Another important aspect to be considered during an early drug development program is the regulatory environment in which the project operates. While

a detailed discussion of regulatory agencies and associated practices is beyond the scope of this book, each section and chapter describes, whenever possible, the fundamental regulatory principles that need to be considered as part of the process. This is of particular relevance during toxicology-based assessments, as the safety risk each new drug product will impose upon the patient is an area of intense regulatory scrutiny. Accordingly, the chapters in Part IV list relevant International Congress on Harmonization (ICH) guidelines, with direct links to the original sources to support the reader in addressing these regulatory elements. Here, special emphasis has been placed on framing a regulatory discussion rather than providing a checklist of data to be generated. Each development program will have to develop a fit-for-purpose data package (as opposed to a standardized one) for discussion, negotiation, and agreement with the regulatory agencies. Early discussions with regulatory agencies are of the utmost importance, as they provide mutual buy-in into acceptable and not acceptable risks, help the agencies to familiarize themselves with novel scientific and therapeutic approaches, and help the project team to focus its resources and efforts on the most critical (from a regulatory viewpoint) issues.

Integration and alignment of the many disciplines and activities presented in this book is a prerequisite to successful early drug development. Each project is challenged with defining and achieving competitive requirements for progression to clinical studies while factoring in associated data variability, risks, and uncertainties. Accordingly, early drug development scientists need to devise the best possible set of studies that are feasible and relevant with respect to risk reduction and decision making. A common understanding of the advantages and limitations specific to a proposed early drug development plan allows its effective execution and builds in the necessary flexibility to respond and adapt to the data generated. Against a backdrop of mounting clinical attrition, unmet medical need, and patient safety concerns, early drug development is the most critical gate to success.

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Part I

Drug Substance

2

Early Phase API Process Development Overview

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2.1 Introduction

Process Development of active pharmaceutical ingredients (APIs) continues to evolve to meet the changing business and regulatory environment. Large pharmaceutical companies (Pharma) have been under pressure to reduce cost in research and development, driving new paradigms in process development in order to maintain project support with fewer resources [1, 2]. This is especially relevant to early development, wherein risk of attrition is higher, and funding is lower compared to late development. The increased use of Contract Research Organizations (CROs) and Contract Manufacturing Organizations (CMOs) from emerging markets, along with internal contractors, has also contributed to create a more complex integration of external and internal development capabilities. In addition, the pharmaceutical companies themselves have become more complex organizations, often with specialized capabilities and technologies that offer advantages to speed and efficiency, quality of process understanding and control, and reduction in overall long-term costs. While Pharma continues to adapt to new resource constraints and operational models, they must also respond to changes in the Chemistry, Manufacturing, and Control (CMC) expectations made through multiple regulatory bodies around the world.[1]

In this chapter, an overview of early API process development will be provided with an attempt to cover a range of current paradigms and constructs. The primary drivers and constraints in early process development will be discussed, followed by a general discussion of the transition from discovery to development. A fully functionalized organization construct will be provided, followed by a section on equipment that is indicative of an API Process Development Organization. As one reads through the chapter, it should be kept in mind that all

1 Examples include the relatively recent International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use guidance for elemental impurities (ICH Q3D), and the guidance on genotoxic impurities (ICH M7(R1)), which were finalized in December, 2014 and May, 2017, respectively. For a complete list of ICH guidelines, see: http://www.ich.org/products/guidelines.html (accessed 13 September 2017).

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

Process Development Organizations are different, and the exact composition and interactions thereof will vary.

2.2 API Process Development Overview

2.2.1 Early Process Development

For the purposes of this book, early process development will be considered the time frame starting from the planning for the delivery of bulk API to support Good Laboratory Practice (GLP) toxicology studies (often referred to as Regulatory Toxicology, or Reg–Tox studies) to the delivery of bulk API supporting Phase I clinical trials (Figure 2.1). The focus of early process development is enabling the first process to synthesize API on a scale that meets GLP toxicology and First-in-Human (FIH) supply needs and associated quality standards. Typically, given the likely multikilogram scale to enable Reg–Tox and FIH studies processes transition from lab glassware to Kilogram Laboratory (Kilo Lab) equipment, and processes that have been used for lab scale synthesis may no longer be feasible on larger scale.

Following FIH, process development continues to be applied toward the resupply of API supporting clinical and development activities and ultimately transitioning to late process development, where there is sufficient clinical data to initiate commercial manufacturing process development and ensure readiness to supply a drug immediately upon regulatory approval. The goals and timelines for these activities are different than early development and are outside the scope of this book.

2.2.2 Early Development Drivers and Constraints

Quality is paramount in the delivery of API throughout all stages of development, as the goal is to enable clinical studies wherein the safety of the patient is not

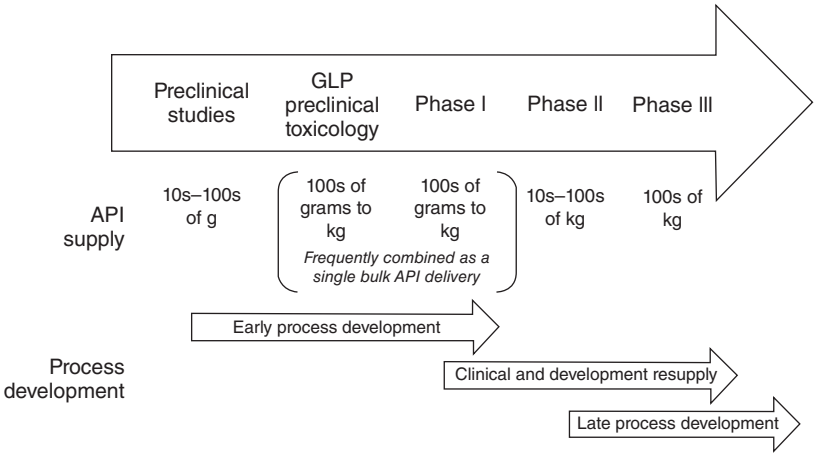

Figure 2.1 Typical API supply quantities and Process Development stages for a standard drug filing pathway.

compromised by the quality of the API. The API used in these studies must be of high purity and produced from a process wherein quality implications have been considered (e.g. control of process impurities and those present in the starting materials). Consequently, developing the scalable purification strategies in the first API batch to meet the required high purity standards makes up a large percentage of early development activities. Chromatography is a common strategy of purification in discovery. Some process development organizations will choose to chromatograph late stage intermediates, or even the final API, to meet those standards. However, this can introduce a purification control strategy that can be expensive, rate-limiting, and difficult to match in profile when another purification strategy, such as crystallization, is ultimately developed.

The second most important driver of early drug development is speed. Speed is one of the constant forces that affect all project decisions. There are several reasons for the need to be fast and nimble at this stage. The pharmaceutical industry is a competitive one, where the norm is that many companies are working on the same or similar therapeutic targets. Given the increased value of a product that is first to market, efficient, and rapid execution is essential to staying competitive [3, 4]. This phase of development is also marked by a high degree of momentum and excitement about the project. The early clinical studies offer an opportunity to significantly advance the understanding of the candidates (e.g. selectivity, pharmacokinetics, and pharmacodynamics) and hence improve the probability of success (despite all the effort and years of research that have already been spent, the probability of any candidate nominated for clinical trials successfully becoming an approved drug is less than 10%) [5]. In order to speed drug development and maximize the efficiency of resources, the goal is to move through this learning cycle as rapidly as possible while minimizing the investment in an individual candidate. It is also at this phase of the project wherein API supply is usually rate-limiting toward advancing the candidate to market. The Phase I clinical trials cannot begin until GLP toxicology studies are complete, and, in turn, these cannot start until sufficient API is available to supply those studies.[2]

Cost is an important driver and constraint in early development. The priority is maximizing project execution through balancing resources deployed to projects (people and equipment) and the actual synthetic cost per kilogram of an individual API (expense budget). Consequently, early process development teams are relatively small compared with late development teams and may consist of only 1–2 process chemists with minimal support from the other lines within the API development organization. As the transition occurs from the discovery team, the process development team is working to understand the synthetic routes used in earlier studies. The technology used by the discovery team was developed for different purposes, with an emphasis on making many compounds at small scale, so it is not likely to be the most efficient synthetic route to synthesize the single candidate that moves forward into development. The decision to maintain the

2 In general, the API quantities for the GLP toxicity studies represent the largest demand for API and greatly exceed the amounts needed for the actual FIH studies. This is a natural outcome of the goal of GLP toxicity, to determine the highest NOAEL (no-observed-adverse-effect-level) dose. Exceptions include studies where open-label extensions of phase I studies are planned (e.g. oncology FIP studies) and when extensive drug product development is planned.

current route versus rerouting will be based on a number of factors, such as risks associated with scaling the current technology, efficiency gains associated with alternate routes (e.g. significant decrease in number of steps will help mitigate the time penalty associated with developing those steps), and scale of operations needed to maintain the supply of API for project progression. This decision will impact both the time and cost to advance the candidate.

2.3 The Transition from Discovery to Development

Where discovery ends and process development begins will depend upon the business strategy coupled to the organizational structure.[3] Process development could be said to start when changes to a synthetic process are made specifically to enable a larger-scale preparation of bulk API. This often occurs within the discovery organization to support pre-GLP toxicology studies requiring hundreds of grams of API. Generally speaking, however, process development represents the handoff of supply responsibilities from a group focused on discovery and identification of potential development candidates to another group that has as its mission to develop processes that provide bulk supplies of API intended for studies that will be included in regulatory submissions. This is also the point where the analytical scrutiny of the process and product needs to be significantly escalated to ensure appropriate decisions on impurities are made during the development phase. Considering a typical development timeline (Figure 2.2), most large pharmaceutical companies have used the supplies supporting the first GLP toxicology studies as the transition point between discovery and process development. This was a logical transition point because it represented a significant step change in quantities of API prepared, typically from hundreds of grams supporting pre-GLP studies to kilograms supporting GLP toxicology studies. In addition, the impurity profile of the lot of API-supporting GLP toxicology studies must be representative of the API that will be used in the FIH studies to ensure patient safety. To minimize risk of unqualified impurities and potential exposure differences arising from new forms of the API, the same lot of API is often used to supply both GLP toxicology and FIH studies. Logistically this also makes sense since the quantities needed for GLP toxicology far exceed the quantities needed for Phase I studies in most cases. Under these circumstances, the lot of API must be made under current Good Manufacturing Practices (cGMP), which require another level of process control to ensure that patient safety is not impacted by the process to prepare the API. The substantial infrastructure and training required to support cGMP synthesis is not found in discovery groups, nor would it make sense for it to.

Some organizations have chosen to externalize all API supply up through clinical Phase II, when proof of concept (POC) is demonstrated, or a similar milestone

3 Pharmaceutical organizations sometimes incorrectly use the terms "research" to mean the group supporting discovery and identification of molecular targets to advance through clinical trials. This is a misleading term as research occurs in both the discovery phase and development phase, as evidenced by the large number of primary literature publications and new technology arising from development groups.

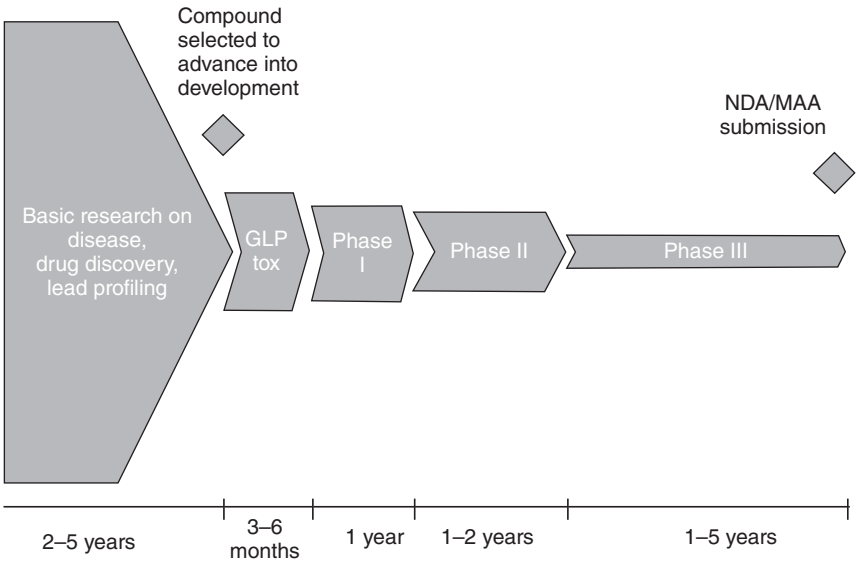

Figure 2.2 Typical drug development timeline.

has been achieved that increases the confidence that the asset will receive market approval [6]. Process development still occurs through the CMO network but is typically short term and focused on near-term deliveries, and as long as API quality specifications are met, reduction in cost becomes the primary driver for process changes.

Other organizations have chosen to move development further into the discovery space. This can be a minimalistic approach, wherein process development chemists act as consultants to discovery chemists. Alternatively, teams consisting of both discovery chemists and the Process Development Organization are responsible for ensuring a smooth supply chain through FIH and Phase II.

At Pfizer, the process development[4] and discovery[5] groups create a structured Synthesis Management Team (SMT) for each research project team[6] that operates as a multidisciplinary team with members from both organizations. At the kickoff of these teams, there are typically multiple series in play for a specific molecular target. This team is responsible for developing a rapidly scalable process to supply API supporting preclinical safety evaluation and initiation of FIH clinical studies, and providing speed to establishment of the Proof of Mechanism for the target. To achieve those goals, the SMT works together to define units of work within each line that will enable a rapid scale-up of API once a lead is identified. This could include wholesale rerouting of the synthetic route, but usually involves targeted changes to address identified scale-up risks, such as high-energy reagents, technology screening to improve catalytic processes, or

4 Chemical Research and Development (CRD).
5 Worldwide Medicinal Chemistry Synthesis.
6 The discovery team responsible for delivering one or more lead candidates for a specific molecular target.

continuous processes when there is a clear benefit to the early delivery of API [7]. The advantage of this approach is improvements in early process design that result in a timeline reduction to FIH. The disadvantage of this approach is a larger investment of resources at a stage where there is a high attrition rate.

2.4 Process Development Organizational Construct

2.4.1 Core Functions

A fully functional process development organization consists of many components, including both personnel with varied skill sets, and equipment that enables both the development and execution of processes on scale (Figure 2.3).

In early development, the goal of developing a practical synthesis of bulk API means that organic synthetic chemists make up the core of early API process development projects. These chemists are typically referred to as process chemists as it both defines their purpose, and identifies these chemists as a subset of chemists that have specialized skills sets, knowledge, and experience [8].

Both discovery and process chemists must have a thorough and contemporary knowledge of synthetic methods. However, whereas the discovery chemist goal is to prepare small quantities of numerous diverse molecules for testing, often by any methodology necessary to achieve this, the process chemist is focused on synthetic methods that can translate readily to scale. The latter requires an understanding of scale robustness, market availability and cost of substrates and reagents, safety implications, and the potential impact on API quality. While it

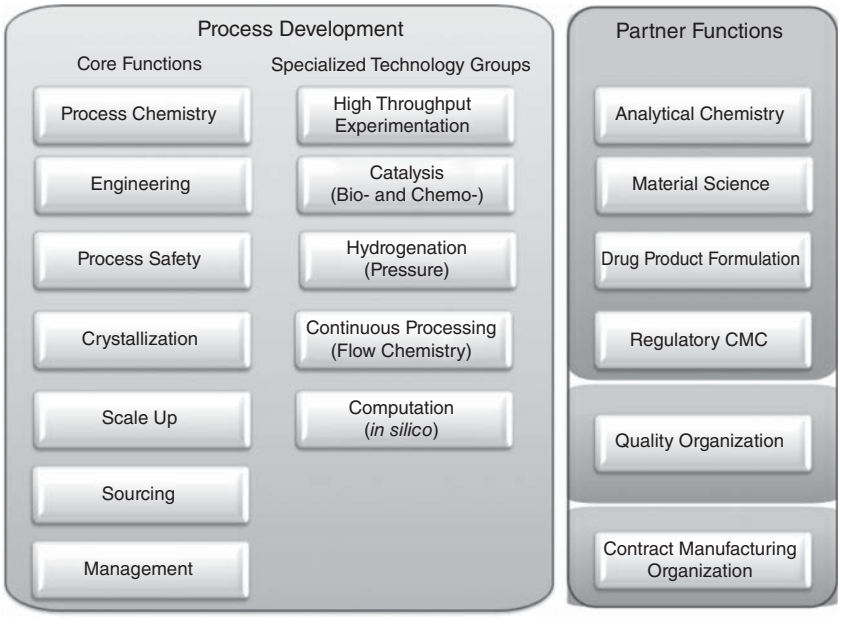

Figure 2.3 Process Development Organization and partner functions.

is not always possible, some common transformations in discovery would be replaced with methods that offer better scalability or quality advantages. For example, the palladium-catalyzed Suzuki–Miyaura cross-coupling reaction is a staple in discovery chemistry because it is a great transformation for building libraries due to its high success rates across a broad range of substrates and use of readily accessible, stable boronic acids that are generally easy to work with. However, it is less attractive for a scale-up process compared to an iron-catalyzed Kumada reaction that provides the equivalent overall transformation. Palladium itself is an impurity that must be reduced to parts per million to ensure safety to the patient. In addition, the starting boronic acid or derivative thereof typically requires at least one additional synthetic step to prepare, either internally or at a vendor, and is often a potential genotoxic impurity [9, 10]. If the boronic acid is an actual or potential genotoxic impurity (Class 2 or 3 impurity as defined by the International Council for Harmonization for Pharmaceuticals for Human Use (ICH) M7(R1) guideline [11]), it may require the development of a custom analytical method and reduction in content to the threshold of toxicological concern (TTC), typically measured in parts per million in the API.[7] By comparison, a similarly performing iron-catalyzed Kumada reaction, using a Grignard reagent prepared *in situ* from the same boronic acid precursor, would be a better option as both the metal and reagent are nontoxic, and the process results in at least one step reduction in the synthesis to the API.[8]

The process chemist in early development needs to have sufficient scale-up knowledge and experience to make key, strategic decisions regarding where, and how much, to invest in route development. It is very unlikely that the synthesis process provided by discovery is ready for preparing the first kilogram quantities of API, and some enabling will be needed. However, timeline and resource constraints in early development will not be sufficient to develop the idealized commercial route. Thus, the process chemist will make strategic decisions about what should change versus what remains the same. To do this, the process chemist must be able to assess a route and identify challenges to scale-up, often before having any experience with the chemistry other than the information provided by Discovery. In the extreme case, a completely new route is required. However, new route development almost invariably adds additional resources and time investments, and the drivers for a wholesale change need to be compelling. More typically, there are targeted adjustments made to the route, such as the substitution of reagents or reaction conditions, reordering the synthetic sequence of steps, and the development of new routes to key intermediates. In addition, the reactions are optimized, and scalable post-reaction processing with intermediate isolation points is developed to improve efficiency and establish impurity control.

Early process development chemists not only need to be adept at identifying and gauging risk but also must be more comfortable accepting risk as part of the

7 Actual TTC depends upon dose and duration of study or treatment.

8 Palladium-catalyzed reactions are still the most prevalent in both discovery and process chemistry due to the broad applicability of the established technology compared to the less-developed cross-coupling of alternatives such as iron- or nickel-catalyzed options. However, internally we have observed that the non-precious metal-catalyzed transformations can outperform palladium for some transformations.

process. There is comparatively little time to define and ready a process for the first kilogram-scale delivery supporting GLP toxicology studies and FIH supplies, and some risks cannot be obviated while maintaining a reasonable timeline on a limited resource budget.[9] While accepting of some risk, the process chemist has to be skilled in identifying risk, gauging probability of an unexpected event occurring against the potential impact of that event, incorporating de-risking strategies, and understanding the potential solutions available prior to beginning the campaign. Even with careful planning for potential deviations, unexpected events can still occur, and the early process development chemist must be flexible and nimble to ensure that the delivery of bulk API is completed successfully. For example, one very common risk in early development is the presence of either new impurities, or higher levels of impurities previously observed in lab development runs, arising from the sourced starting materials or upon scale-up of the downstream process. Unlike late phase process development, wherein both the impurities in starting materials and reaction parameter space are thoroughly correlated to impurity profiles, the first kilogram deliveries may be based upon point correlations derived from a small number of experiments using a single lot of starting material. The bulk of sourced custom raw materials for the first kilogram delivery can contain new and/or unexpected levels of known impurities, as the vendors who are making the intermediates are in a similar predicament of having little process experience prior to scale-up and are likely to have scaled up the process to a custom raw material for the first time. Since the raw materials typically arrive just prior to scheduled scale-up, the chemist must rapidly decide if the material needs to be further purified using procedures that are developed in real time or if the impurities can be rejected downstream. Both approaches invoke designing key experiments that can be rapidly executed to define the path forward, as the process is typically on a tight schedule in the scale-up facilities, and anything that adds additional time to execution can not only impact the final delivery date for that program but also impact the scheduling and timing for other programs scheduled to run in the same scale-up facility.[10] The early development chemist is fully aware of these all too common scenarios and typically incorporates impurity purging crystallization points as part of the scale-up de-risking strategy.

Engineers are an important resource in early Process Development Organizations. The transition from laboratory glassware to larger-scale manufacturing equipment brings significant changes to heat and mass transfer effects that can lead to very different reaction times and purity profiles. The engineering skill set is targeted to understanding the impacts of these changes on the process at hand

9 Once a target is identified for Reg–Tox, API supply becomes rate limiting. Thus, finalizing a route to initiate the purchase of raw materials (advanced intermediates) is of utmost urgency and drives timelines to reaching clinical studies.

10 Chromatography, which is powerful purification approach used extensively in discovery, is possible, but is not a preferred method for several reasons including the time it takes to develop and complete the process on scale, the lack of capabilities on scale, the high cost to run externally, and perhaps most significantly, chromatography may provide a purity profile that will be hard to replicate with alternative purification methodology in subsequent scale up campaigns.

and developing scale-up solutions. However, timelines for early development projects often do not allow for an extensive engineering analysis. Therefore, many of the simple engineering principles will need to be imbedded into the process chemistry group as part of their training. Examples of common scale-up issues that can be readily identified by the chemist are: gross mixing sensitivity for fast reactions (identify with high and low level agitation experiments), solids suspension for heterogeneous reactions (identify with barely suspended agitation experiment), heat transfer issues with highly exothermic reactions (identify with experiment run at a temperature 5–10 °C higher than target), poor filtration behavior (measure approximate *k*-values for filtration of isolated intermediates, and APIs). If these simple experiments reveal potential issues, it is important to bring in the engineering skill set to better define the edge of failure and potential scale-up solutions. Given the short development timelines, it will be important for the engineer to adopt a fit-for-purpose approach that brings the risk of scaling up a process to an acceptable level as rapidly as possible. The appropriate use of *in silico* process modeling tools to drive an efficient experimental program can also significantly accelerate the development of the needed process understanding [12–14].

An area of primary importance when contemplating scaling up any chemistry is the safety to those conducting the chemistry as well as to the surrounding labs and communities. Thus, a Process Safety function is a core function within early Process Development Organizations. Careful consideration must be given as to how the potential hazards vary at each scale of process chemistry (i.e. laboratory, kilo lab, pilot plant, and commercial manufacturing). On a laboratory scale, the focus is the reagent hazards and compatibilities, generation of a balanced equation to assess products and byproducts, and assessment of any specific high-energy functional groups. Differential scanning calorimetry (DSC) is a simple test that provides significant information about the innate safety. As scale increases, addition safety information, e.g. thermal screening unit (TSU) and reaction calorimetry testing, will be gathered to understand the potential for exotherm, runaway reaction, and off-gassing. As required, additional tests can be conducted in specialized process safety laboratories to ensure the safety of a process and trigger redesign where necessary.

Crystallization is one of the most important purification techniques in API process development. In addition to purification of intermediates, designing a crystallization process to consistently produce API of the targeted form and appropriate quality requires an understanding of crystallization principles and applications thereof. Most large pharmaceutical Process Development Organizations will have a Crystallization Group with expertise in the fundamental principles and applications of crystallization. The Crystallization Group will have specialized equipment to support solubility and particle size measurements, microscopes to characterize crystal habit, and various tools to understand the kinetics of crystallization and definition of metastable zones. However, the Crystallization Group is usually focused on the crystallization of API in late phase projects, and it is the process chemist who develops crystallization processes for intermediates and API in early development, with the Crystallization Group providing guidance and experimental support on an as-needed basis. Thus, the

process chemist is also expected to develop a level of expertise in crystallization as one of their core competencies.

Supplies of API designated for nonclinical studies, including GLP toxicology studies, do not need to be prepared using procedures and equipment that conforms to strict cGMP guidance. Small quantities, in the 100s of grams, can be prepared using laboratory equipment, which could include 10–20 l jacketed reactors located in walk-in hoods within a standard laboratory environment. Safely preparing kilogram quantities to support GLP toxicology studies typically requires larger equipment located in facilities specifically designed for this purpose. Clinical studies supplies must be prepared using cGMP. Thus, most companies engaged in the internal preparation of API supplies have dedicated scale-up facilities staffed with cGMP-trained personnel. In some organizations, the bulk API is prepared externally through CMOs, who provide the infrastructure and trained staff to support non-GMP and cGMP large-scale manufacturing.

An external sourcing function serves to establish a third-party network of suppliers to prepare the bulk quantities of chemicals needed to prepare the API. Outsourced chemicals can be broken down into three main categories: commodity, custom intermediates, and API. Commodity chemicals are chemicals that are offered in supplier catalogs. The synthetic routes to commodity chemicals are often unknown, as the supplier may choose to retain this information as a trade secret in order to be competitive in the market. The lack of this information can become problematic if new impurities are introduced late in the development process due to unexpected changes in the synthetic process occurring at the same, or a new, different vendor supplying the material. Custom synthesis chemicals are not available in catalogs and are intermediates in route to the API. These custom intermediates can be as little as one synthetic step from raw materials (commodity chemicals) or can require a complex multistep synthesis to prepare. The custom intermediates are often the cGMP starting materials for early development products and therefore do not fall under the cGMP guidance (i.e. pre-GMP), which facilitates speed and lowers development and production costs [15]. However, the quality of these intermediates must still be maintained such that it will not negatively impact the quality of the final API. Since these are non-catalog items, and typically structures that are specific to an individual API, the Process Development Organization or the CMO must develop a synthesis technology package that can be used to prepare them in bulk quantities. This can add significant time to the delivery of the first bulk batch of API. In addition, it takes time to purchase and receive the commodity raw materials used in the preparation of custom intermediates. Sourcing the API itself is another category of outsourced bulk chemical. As well as the synthesis of custom intermediates prepared from commodity chemicals, this would include the cGMP synthetic steps that convert those intermediates to the final API. The cGMP steps require more infrastructure, resources, and training. Consequently, these steps are more expensive to run externally.

In the current worldwide ecosystem, in which low cost vendors in Asia play an important role, sourcing of advanced pre-GMP intermediates or cGMP API is much more common as compared 10–20 years ago. The sourcing function is

critical to ensure the right third-party CMOs are employed to deliver the amount of intermediate or API needed, on time, and with agreed upon quality. In the early phases of development, technology packages provided to prospective vendors are often sparse on details about the process, and some organizations may rely on the vendors to propose and develop processes to intermediates. Consequently, there is an inherent risk that the material will arrive late, in insufficient quantities, and/or not meet target specifications. The organization has to respond to resolve the issues and keep the program on plan to the best of their ability.

Along with the scientific and scale-up staff, all organizations will have a management component to allocate resources, support development of the staff, manage budgets, and direct the strategy toward the implementation of changes, leading to continuous improvement and alignment with the larger company strategy.

2.4.2 Specialized Technology Groups

Specialized technology groups are becoming increasingly common in large pharmaceutical Process Development Organizations and represent some of the most innovative changes occurring in the business. The investment in capital and staff to construct these groups can yield large returns in all phases of development because they have the knowledge and infrastructure to rapidly identify and develop powerful near-term enabling and long-term commercial processes.

One of the most impactful technologies that have enabled rapid identification of reaction processes has been the relatively recent use of automated (or semiautomated), high-throughput screening (HTS), or in a broader sense, high-throughput experimentation (HTE). Whereas once considered a technology applied to compound screening in discovery, modern applications of HTE platforms can screen hundreds of reagents and conditions in a single run on very small scale (e.g. as little as 1–2 mg/experiment for some applications). If designed well, HTE platforms will outperform traditional manual screening by at least 1–2 orders of magnitude. The small quantities of substrate required to produce large decision-making data sets render these platforms particularly valuable in early phase development, where substrate and time are most limited. However, to operate these platforms effectively requires skill in the design of the workflow such that it is efficient and uses a protocol that gives consistently high quality results that translate to successful, scalable processes. The personnel who excel in these groups tend to be those who are adept at working with automation, applying a range of potentially complex software and software interfaces, developing and interpreting statistically designed experiments, translating complex chemistry to very small scale and vice versa, and maintaining an attention to quality and detail to validate protocols and detect potential deviations while retaining a focus on the primary goal – the rapid development of a scalable process.

Catalytic reactions are highly valued in both early and late development routes. Many organizations are finding that catalytic reactions are best developed in a group that has the infrastructure to rapidly screen broad libraries of catalysts and

can develop a deep level of understanding and experience in the development of scalable catalytic processes, the learnings of which pay dividends in future projects. These can include hydrogenation and reactions using gases under pressure in general, biocatalysis, organocatalysis, and organometallic catalysis. Catalysis expertise is either developed internally and/or hired in. The catalysis experts are often colocated or embedded within the HTS/HTE group due to the synergistic nature of the two disciplines. HTS is a logical first step in identifying principle components of a catalytic reaction (e.g. metal, ligand, and solvent), which is followed by experiments designed at acquiring more detailed understanding of the reaction. These can be performed as targeted experiments (e.g. kinetic modeling or identification of key intermediates in a catalytic cycle) or with a screening platform approach (e.g. catalyst loading studies and Design of Experiments (DOE)).

Reactions conducted with gas phase reagents under pressure require specialized equipment and training to ensure safe execution. These transformations are most commonly hydrogenations using hydrogen gas and metal catalysts but can include hydroformylations, carbonylations, and high temperature reactions with other volatile small molecules such as ammonia or acetylene [16]. Most Process Development Organizations have dedicated facilities and trained staff specifically to address safety concerns and to build a level of expertise that can be parlayed into projects in the future.

Biocatalysis has become a mature field in many ways. The range of transformations and scope of substrates is ever increasing, especially with the ability to improve substrate scope and enzyme performance through genetic engineering. The processes are both safe and inexpensive, so much that for some transformations, they have essentially replaced established chemocatalysis as the preferred mode for scale-up [17, 18]. Consequently, many Process Development Organizations have invested in building biocatalysis groups with experts in this field. In early development, biocatalysis has a more limited range of applications, as some enzymes are not available in bulk on short notice, and genetic engineering is not feasible within an early development time frame. Consequently, the focus in early development is on using well-established, commercially available enzyme technologies that can be rapidly scaled, such as the lipase, ketoreductase, and transaminase classes of enzymes.

Flow chemistry, or in a broader sense, continuous processing, is becoming increasingly more common in API Process Development.[11] The application of continuous processing in pharmaceutical companies is still highly variable, from no applications to companies that have fully committed to continuous processing for commercial processes. In the early development space, the value of flow chemistry can be enabling chemistry that could not otherwise be scaled, especially when that chemistry is the key step for a process with greatly reduced steps compared with the next reasonable batch alternative. In addition, the application of flow may obviate the need to add resources and time developing an alternative route. Another advantage of flow processes in early development is to de-risk

11 Flow chemistry typically refers to the reaction process only, while continuous processing is a more accurate term to reflect all applications of continuous processes, including both the reaction as well as the post-processing unit operations.

scale-up. When a batch process is run on scale, one usually commits the entire batch to a process, and if a deviation occurs, the entire batch is impacted. In early development, a single batch can represent the entirety of the API delivery. In a flow process, it is easier to implement In-Process Control (IPC) tests and Process Analytical Technology (PAT) that can identify an issue as it develops and allows either the operation to be discontinued or a small subdivision of the stream that was impacted to be redirected from the bulk, thereby minimizing the impact. Consequently, some API Process Development Organizations have hired or internally developed personnel with flow chemistry expertise, along with the equipment to support development and execution.[12] A similar trend is occurring in the third-party CMO network, wherein these organizations are building facilities to support both pre-GMP and cGMP continuous processing capabilities [19, 20]. There are still challenges applying flow processes in early development. Developing flow processes usually requires more experimentation and materials compared with a batch process. Correspondingly, CMOs often charge additional costs to develop flow processes, and sufficient quantities of material to support flow may not be available in early development.

Having tools that can quickly provide synthetic route proposals and the ability to predict which routes are likely to be successful can bring a lot of value in early development, where time and resources, including availability of key intermediates, are too limited to formulate and experimentally test all proposals. Toward these goals, computational chemistry and retrosynthesis software are having an increasing impact on process development [21]. When several synthetic routes are under consideration, computational tools can be used to help predict the likelihood of success through calculations of transition states, HOMO and LUMO orbital energies and coefficients, molecular conformations, pK_a, heterolytic and homolytic bond strength, and other computational approaches. In addition, there are retrosynthesis tools that use large databases of primary literature to generate synthetic routes [22, 23]. The challenge with the retrosynthesis tools can be identifying what is truly useful from the large quantities of output the tools generate. All of the *in silico* approaches have varying degrees of accuracy and precision, which must be considered when interpreting the output. While computational tools are becoming increasingly easier to use, an organization typically needs access to computational expertise to use them effectively, either from within or through external liaisons.

2.4.3 Partner Functions

Analytical chemistry is a close partner to process development, and some organizations include this function within the process development construct. High quality analytical methods for evaluating intermediates and products from rapidly evolving synthetic routes are essential to success, given that impurities

12 Most chemical engineers are trained in the fundamentals of continuous processing as part of a standard university curriculum due to the common application of these processes in other industries. In contrast, flow chemistry is not part of the standard chemist curriculum, and most chemists are taught to think in terms of batch chemistry. That has been changing, but is still far from the standard.

in API must be controlled to very low levels, typically well below 1%, and in the parts per million concentration for some metals (e.g. transition metals such as Pd) and genotoxic impurities (e.g. genotoxic arylboronic acids). Additionally, the rapid identification of side products and impurities can inform the chemist as to what conditions will reduce their formation. Analytical methods are often used to understand impurity purge potential of intermediate crystallizations that can influence the route strategy to ensure the most effective crystallizations are incorporated, and the synthetic route places them at the optimal point in the synthesis. Analytical chemists also provide the expertise to execute and interpret specialized PAT, such as *in situ* infrared (IR) and Raman spectroscopy, as well as Flow NMR. Analytical chemistry is often called upon to develop customized methods for particularly difficult analytes, such as volatile compounds that do not have a UV chromophore.

The identification of the first crystalline form can occur prior to or following the transition from discovery to process chemistry. In either case, a material sciences partner will screen and characterize early development candidate for polymorphs, solvates, and cocrystals in an early effort to identify the most stable, pharmaceutically acceptable form. In some organizations, the form screening and characterization occurs within the Crystallization Group described previously.

A separate function within the organization supports drug product formulation development. In early development, the relationship is predominantly based upon API supply needs for the clinical study, where some programs may need additional bulk API to support advanced formulations, such as topical formulations and accelerated programs filing NDA submission based upon Phase II clinical data. Particle size distribution can be an important contributor to drug product performance, and the API team works with the drug product team to define the most appropriate particle size distribution for a given API.

Another important partner interface is with the regulatory group, who coordinates and provides guidance in the preparation of the CMC section of regulatory filings, including investigational new drug (IND) files, investigational medicinal product dossiers (IMPDs), and query responses.

The quality group is a critical partner in the manufacture of API intended for clinical use. The quality unit is an independent organization that works with the manufacturing group to establish a quality system and ensures that manufacturing is conducted in accordance with the quality system. In the pharmaceutical industry, the quality system refers to a set of procedures, training, and facility requirements that "assures that the desired product quality is routinely met, suitable process performance is achieved, the set of controls are appropriate, improvement opportunities are identified and evaluated, and the body of knowledge is continually expanded" [24]. The quality unit is also responsible for release of API intended for clinical studies.

The third-party CMO network could be considered as a "partner function," as they are often integrated into the early development strategy. On one end of the spectrum, small companies and some larger pharmaceutical companies outsource all early development of API. More commonly, a CMO will be used to add flexible capacity to the organization, primarily focusing on activities that are considered of lower risk to the program, such as the preparation of custom

intermediates to support lab development, the synthesis of metabolites and reference standards, and primarily, the preparation of bulk custom intermediates used in the preparation of API for the GLP toxicology and FIH studies. Managing these relationships successfully requires significant effort on the part of the organization, not just to establish the business contracts through the sourcing group but also to help enable the successful delivery of bulk. Successful partnerships tend to include regular dialogue between the internal process chemists and those of the CMO to help guide decisions and troubleshoot issues as they arise. It can be tricky to find the balance wherein the internal resources do not become overburdened supporting a contractual delivery responsibility of the CMO.

2.5 Process Development Equipment

2.5.1 Lab Equipment

The goal for the process chemist is to develop technology that will work in the real world of a manufacturing environment as rapidly and efficiently as possible. In recent years, there has been a significant evolution of the process chemistry laboratory to help maximize the information that is derived from each experiment [25]. While the traditional tools of round-bottom flasks and rotary evaporators (rotovaps) still have some utility for early screening type experimentation, these have largely been replaced for more detailed enabling experimentation with automated, jacketed reactors that offer significantly improved temperature control and overhead stirring to better mimic mixing and thermal profiles at scale (Figure 2.4). The new reactors offer the benefit of being able to add reagents under precisely controlled rates, and the reaction progress can be monitored in real time with PAT for both chemistry (Raman and IR spectroscopy) and particle information (focused beam reflectance measurement (FBRM)). The parameters important to successful scaling of the technology to the manufacturing area can be isolated and studied, leading to improved confidence at scale.

Much of the early development work is centered on determining the feasibility and efficiency of various bond connections. In order to make the assessments, it is imperative to have the appropriate analytical tools readily available that enable rapid structure and purity determination. For the day-to-day use of laboratory tools such as nuclear magnetic resonance (NMR) spectroscopy and ultra-high performance liquid chromatography coupled with mass spectroscopy detection (UPLC/MS), rapid turnaround is critical, so they need to be close to (if not in) the laboratory where the process development is occurring. UPLC provides the ability to resolve and quantify low levels of impurities to enable the design of appropriate control strategies in the delivery of high quality API for clinical studies. The mass spectroscopic detection aids in identification of impurities that have been formed, which is important information to intelligently develop the synthetic process. NMR provides an analytical technique that is orthogonal to the UPLC and also helps with structure determination.

One of the differences between process chemistry and medicinal chemistry is the emphasis process chemistry places on developing a process that has higher

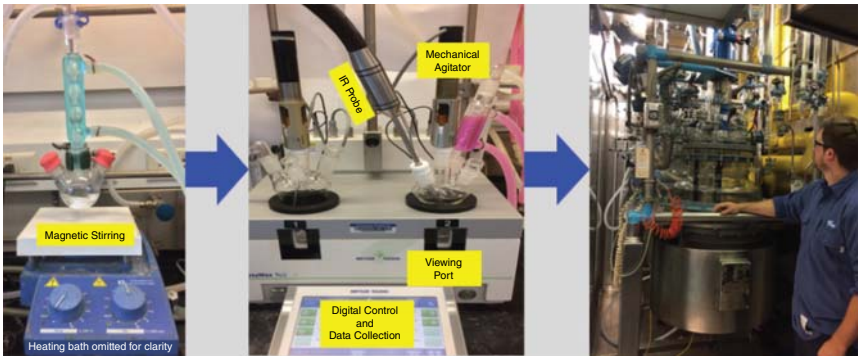

Figure 2.4 Evolution of equipment for process chemistry.

space–time throughput due to the impact on vessel size requirements and number of batches required to deliver bulk API. Toward this, preparative chromatographic isolations are usually replaced with crystallizations as the primary means of purification. Crystalline intermediates usually function as key gates in the control strategy for manufacture of a quality API. The API itself is most often delivered as a single crystalline form of defined physical attributes. To ensure the robustness of these crystalline intermediates and API, it is important the process chemist has access to analytical tools that characterize the solid form, such as powder X-ray diffraction (PXRD) and polarizing microscope, as well as tools for developing the crystallization process such as FBRM. This equipment is often located and managed in the Crystallization Group within the Process Development Organization.

2.5.2 Scale-up Equipment in the Laboratory

During the early development stage, there are many activities that will require API (e.g. early *in vivo* toxicology studies, formulation development, and stability assessment). The requested amounts of API can vary from a few grams for standards or further biological characterization of the compound to hundreds of grams to enable *in vivo* toxicology studies. The amount of API will also be highly dependent on the potency of the compound. Therefore, the process chemist needs access to a range of equipment sizes to deliver the requested quantities of API. Ideally, whatever the scale of manufacturing needed, the work would be performed in a stirred vessel reactor with appropriate instrumentation to allow appropriate control over the processing conditions and collection of data to maximize learnings from the larger-scale runs.

Another consideration for the equipment and facilities is the appropriate control of exposure of the active compounds being synthesized to the scientists. Often the potency of the compounds can be quite high, and airborne concentrations will have to be controlled to below $1\,\mu g\,m^{-3}$. This will require special containment equipment beyond the normal laboratory fume hood [26].

2.5.3 cGMP Manufacturing Equipment

Within the United States the cGMP requirements for the manufacture of drugs are enshrined within 21 Code of Federal Regulations Part 211. The cGMP regulations contain minimum requirements for facilities, methods, and controls used in the manufacture of drugs to ensure the products are safe for human use. The International Conference on Harmonization (ICH) is a collaboration of regulatory authorities from around the world and the pharmaceutical industry who discuss technical and scientific aspects of drug registration with the goal of achieving greater worldwide harmonization aimed at ensuring safe, effective medicines are developed in an efficient manner. ICH has developed guidance on the requirements for the cGMP manufacture of API in ICH Q7 (see Footnote 7). The guidance covers all phases of development and commercialization. The section that specifically covers clinical API is Section 19. The guidance acknowledges that the level of knowledge of the manufacturing process for API is growing during the clinical trial periods, and all information expected for commercial products will not be available during the early phases. "The controls used in the manufacture of APIs for use in clinical trials should be consistent with the stage of development of the drug product incorporating the API." The FDA has also issued a "how-to" guide on complying with the ICH Guidance [27].

The amounts of API that will be needed to support early clinical studies vary significantly depending on many factors such as therapeutic area and doses to be studied in the clinic and toxicology studies. This means there needs to be a broad array of cGMP compatible manufacturing equipment available (from large laboratory-scale through to pilot plant facilities). The commonly used equipment for scale-up include fixed vessel and variable temperature batch reactors that can be used from many operations including reactions, extractions, crystallizations, and distillations. Filters and dryers (or combined filter dryers) are needed for crystallization processes. Large-scale preparative chromatography equipment can provide a powerful purification approach in early development, wherein time and material constraints may not allow the development of more efficient alternatives. Chromatography can also provide a rapid, scalable solution to unexpected deviations in purity that may come from sourced raw materials and custom intermediates or arise from unanticipated scale-up effects. As the program advances, however, chromatography usually becomes throughput limiting and very expensive to scale, driving development of more efficient alternatives such as crystallization.

Large-scale continuous processing equipment can also be useful in early development, as it can often access conditions not available in batch reactors, as well as de-risk scale-up as described previously. There are many types and configurations of continuous processing equipment, the most common in early development include plug flow reactors (PFRs) and continuous stirred tank reactors (CSTRs).

The manufacture of early development clinical API plays an important role in the continued learnings about the synthetic process since it is the first opportunity to observe the process at scale. To help ensure that learning is efficient, the manufacturing area should have access to many of the same analytical tools that

were used to develop the process in the laboratory. Given the short timelines and limited experience with the process, these tools can provide invaluable insight into processes that have not scaled up as planned.

2.6 Summary

Early development is a very dynamic time in drug development, and API supplies supporting regulatory toxicology studies and the first clinical trials in human subjects are on the critical path for most programs. Accessing speed to development of processes that deliver high quality API with limited resources is a key challenge for the early Process Development Organization. The discovery process development interface can be an opportunity to accelerate through collaborative development and information sharing. A fully loaded Process Development Organization, including core functions and specialized technology groups, along with partner functions, provides a highly trained, multiskilled staff and technology infrastructure to deliver a large portfolio of high quality APIs as quickly as feasible in an overall resource efficient manner.

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3

The Discovery/Development Transition

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3.1 Introduction

This chapter will focus on the conviction that minimizing the time to the first dose in man is an important competitive advantage during drug development. The time from the first synthesis (FS) of a compound in discovery to the first dose in man (*FIM*) can be minimized during the discovery/development transition. The strategies that one company (Bristol–Myers Squibb, BMS) used to effect this time in the mid-1990s, as well as the rationale for doing so, will be detailed. Their application today in BMS, small biotech, and contract manufacturing and research organizations (CMOs, CROs) suggests that they are still relevant 20 years later and will likely remain relevant for the foreseeable future.

3.2 Discovery-to-development Transition Before 1980

In order to understand the present and predict the future, it is often useful to consider the past. One of the authors joined the pharmaceutical industry in 1969, fresh from a postdoctoral appointment. The Organic Chemistry Department of the Squibb Institute for Medical Research followed two approaches to drug discovery that were reflected in the organization of the department: a medicinal chemistry section and a natural products section.

In the medicinal chemistry section, chemists made heterocyclic compounds as potential animal health, CNS, and anti-inflammatory and antihypertensive agents. Though a few programs were based on some naturally occurring molecule, most were engaged in me-too analogs (e.g. β-blockers, NSAIDS). In more than a few instances, the driving force for compound choice was the kind of chemistry that the chemist was most familiar and comfortable with. A group leader that I met in an unnamed company during an employment interview highlighted this. He confided that he was most interested in the cyclopropylcarbinyl/cyclobutyl/homoallyl cation system; if I joined his team, I would be expected to make an exploration of that system a central feature of the compounds to

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

be made. Though hard to believe by today's standards and practice, he never explicitly mentioned the biologic activity I was expected to pursue!

The Natural Products section was directly involved in modifying structures based on naturally occurring molecules: steroids as topical anti-inflammatory agents, β-lactams as antibacterial agents, and peptides as cardiovascular agents. Though I joined the cardiovascular group of the medicinal chemistry section, my first project involved making compounds related to the protoveratrine alkaloids as antihypertensive agents [1].

As different as these two approaches were, they shared a common approach to biological testing. An *in vivo* screen for the activity is featured prominently in the initial testing paradigm. Potential antibiotics were tested in mouse models of infection, potential antihypertensive agents in the spontaneously hypertensive rat (SHR) model, potential anti-inflammatory agents in a carrageenan edema mouse model, and potential topical steroids in the vasoconstrictor model on the backs of human female subjects [2]. Additionally, the very first test performed at Squibb on all synthetic compounds in the medicinal chemistry section was the so-called *rat Q and Q* model. This usually involved oral dosing of the compound to a rat followed by close **Q**uantitative and **Q**ualitative observation. A simple readout for a potential analgesic/anti-inflammatory agent was the length of time the rat stayed on a hot plate.

Whatever the test and scoring system, the activity in these models meant that the compound was active *in vivo* and, most often, by the oral route. Having this knowledge early in the evaluation of compounds provided significant impetus to move the compounds into development very quickly. After all, the models used allowed direct comparison to the standard agents approved for that indication by the route intended for commercialization.

Detailed knowledge of the absorption, distribution, metabolism, and excretion (ADME) properties was the remit of the development program as the compounds were already demonstrated to be active in *vivo* and, very often, as active or more active than the comparator. At this time, pharmaceutical properties were not a major concern. Most compounds were of relatively low molecular weight (<500 Da), had at least one basic amine moiety, and were invariably evaluated as water-soluble salts.

As an example of this paradigm, consider the nadolol development program. This had its origin in a prediction made by Dr Frank Weisenborn (Director of Organic Chemistry Department) that a 1,4-dihydroaromatic moiety in a molecule would have similar biologic activity to the molecule containing its aromatic counterpart [3]. This prediction had already led to the synthesis and rapid development of epicillin and cephradine as the 1,4-dihydroaromatic analogs of ampicillin and cephalexin, respectively. Though 5,8-dihydropropranolol was inferior to its aromatic parent, hydroxylating the double bond induced interesting properties. The *cis*-dihydroxy compound was shown to be a potent β-blocker *in vitro* and was also potent in the SHR model after oral dosing. Based on these findings, the compound was advanced into development as a mixture of four diastereomers and eventually marketed as Corgard® (Figure 3.1).

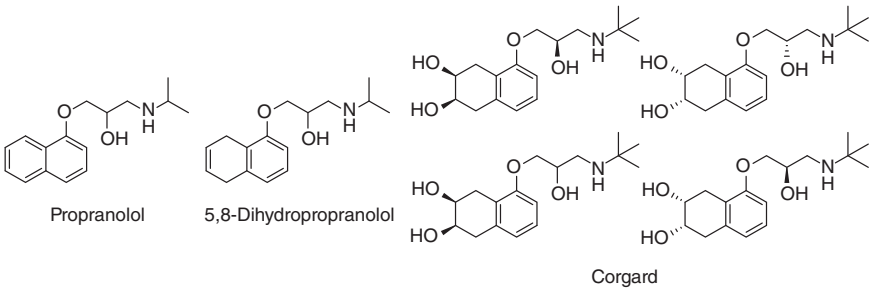

Figure 3.1 Chemical structures of propranolol, 5,8-dihydropropranolol, and Corgard.

3.2.1 Discovery/Development Handover

The handover from discovery to development was always made as a formal event. With little more than the structure–activity relationships (SARs) based on several *in vitro* and *in vivo* models of disease, compounds were handed off to the development function. An apt description of this practice was that the development candidate appeared as if it were "thrown over the wall." From 1970 to the early 1980s, there was a little change in this paradigm. In fact, Squibb built a new set of offices and laboratories in 1972–1973; the discovery functions moved while the development functions stayed put. Discovery and development were viewed as separate endeavors. Discovery was primarily focused on the SAR of disease biology; it was left to development to fill in the details of ADME, pharmaceutical, and toxicological properties.

3.3 Discovery-to-development Transition in the 1980s

There were few exceptions to the paradigm described above at Squibb; one of these was the second-generation angiotensin converting enzyme (ACE) inhibitor program that transitioned in the early 1980s. Molecules that contained a phosphinic acid zinc-binding moiety were shown to be very potent *in vitro* and *in vivo* after intravenous (IV) administration. However, the parent diacids were not sufficiently orally active. A number of prodrug approaches were investigated; the most useful involved making acyloxymethyl esters of the phosphinic acid moiety. The unsubstituted esters were not hydrolyzed enzymatically fast enough, and the disubstituted esters were too unstable to simple acid hydrolysis in the stomach. The monoalkyl esters were shown to have the best properties, and the final choice was made with the aid of data from an inverted rat intestine model [4]. Fortunately, one of our pharmaceutical staff members was familiar with this technique from his graduate school training and was able to contribute key data that led to the choice of the α-isopropylpropanoyloxymethyl ester of the phosphinic acid. This ester proved stable enough in the stomach to be absorbed and labile enough to the esterases of the intestinal mucosa to be hydrolyzed to the active phosphinic acid.

We had previously found it impossible to choose between highly active (*in vitro* against ACE) and poorly orally absorbed analogs using blood pressure lowering in whole animals.

The 1980s proved to be a remarkable time in the pharmaceutical industry. A billion dollar annual sales for a single compound became a reality. R&D investment increased dramatically, and the size and capability of discovery and development research groups grew as well. Though R&D is a universally well-accepted and understood term, it does not serve well at the interface of discovery and development. Indeed, as we shall see, discovery groups need to keep development imperatives in mind when selecting drug candidates, and development groups are involved in much research when pursuing them.

3.4 Discovery-to-development Transition in the 1990s

By the early 1990s, several large mergers had already taken place: Glaxo Wellcome and BMS. The discovery and development portfolios of these combined companies were large and expensive to pursue. Additionally, the number of new targets available began to increase dramatically. For the first time, bringing a novel therapeutic modality into development had the prospect of actually lengthening development times, especially clinical ones. Many of the new targets lacked clinical validation or even appropriate animal models with which to judge their *in vivo* activity. In some cases, closely related targets forced chemists and biologists to probe SAR differences across tens or even scores of assays to sort *good* activity from potential toxicity from one or more closely related enzymes. Eventually, even the promise of huge rewards (e.g. blockbuster drugs that had annual sales in the multibillion dollar range) was not enough to continue a "business as usual" approach to R&D.

Faced with this reality, many companies developed and publically stated ambitious goals for development time reduction and portfolio productivity. In 1994, Hoechst stated that it would "…reduce time-to-market by 30–40%." Glaxo aimed at a development time of "…5–7 years against 9–12 years taken now…" and Novo Nordisk's 1995 goal was to "…halve its development time." In 1996, Glaxo Wellcome aimed to produce "...three significant new medicines per annum by the year 2000" and Hoechst Marion Roussel's goal was "...two major new NCE's per year…" Two years later, these companies had undergone further consolidation. New goals were set as six NCEs per year by Glaxo SmithKline and four NCEs per year by Aventis.

With outputs like these, the size of the required R&D portfolios would be quite large, since the portfolio success rates of compounds entering phase I were then and are now in the range of 10% at best [5]. Reduction of portfolio size can be achieved in two main ways: by increasing the overall success rate and by decreasing the overall development time. The rest of this chapter's discussion will focus on the late discovery and early development period, recognizing that there are potentially equal or larger rewards available from optimizing late development and regulatory programs.

3.4.1 Development Time

A Tufts benchmarking study[1] (Table 3.1) at this time reported the times in months from the first pharmacological testing to the first study in humans. Note that the average of the 1980s and 1990s was 32.5 months, 10 months slower than the 1960–1970 average. As mentioned above, this was probably due to several factors including novel targets and less validated therapeutic modalities, increased regulatory scrutiny, and more complex molecular entities entering development.

A contemporaneous A.D. Little benchmarking study (see also footnote 1) of 96 projects reported similar conclusions for the time from the first discovery synthesis (*FS*) to the first dose in humans: 31 months for the decade of the 1990s.

Development time reduction can be placed in an appropriate perspective by the following look at US market exclusivity in novel therapeutic classes (Table 3.2). Squibb's captopril, the first marketed ACE inhibitor for hypertension and congestive heart failure, enjoyed a 5.8-year period of marketing exclusivity. In stark contrast, by the end of the 1990s, the first neuraminidase inhibitor for influenza virus infections had only several months of exclusivity. Since both compounds were available for the next US influenza season, the period of exclusivity was essentially nonexistent.

Table 3.1 Time to the first dose in humans.

Decade	Time (months)
1960s	18
1970s	27
1980s	34
1990s	31

Table 3.2 Exclusivity times.

Time span	Initial US entry	Second US entry	Exclusivity time
1976–1981	Cimetidine	Ranitidine	5.8 years
1981–1986	Captopril	Enalapril	4.7 years
1987–1990	Lovastatin	Pravastatin	2.8 years
1990	Celecoxib	Vioxx	0.5 years
1999	Relenza	Tamiflu	2 months

1 The authors regret that the original references to the A. D. Little and KMR benchmarking studies described in this chapter cannot be located after almost 30 years. The data in Ref. [5] above and that found in the 1998 KMR Discovery Benchmark Presentation (Chicago, August 4, 1998) leave little doubt that the situation described in the body of this chapter has not changed much. As one example, the 1998 KMR presentation gave the range of times in preclinical development for 12 companies as 8.0 months to 29.8 months!

Considering the difference between the fastest and slowest projects from the A. D. Little study (>2 years), it is apparent that there is a significant competitive advantage to be gained by minimizing early development time.

3.4.2 The BMS IND Initiative

BMS was no exception to the need of increasing productivity in the 1990s. Several years after the 1989 merger, a corporate-wide productivity initiative was started with ambitious goals to be set in development, manufacturing, and marketing. The senior R&D management responded by chartering two related projects: the *PLP-to-IND* initiative and *IND-to-NDA* initiative. At that time at BMS, a preclinical lead profile (PLP) document was prepared by Discovery and presented to the senior management. The approval of this document marked the formal start of development. Interestingly (and fortunately), the senior management did not provide an explicit set of goals for these initiatives; their charter was to examine the current state of affairs and to improve it.

One of us (CMC) volunteered to lead the PLP-to-IND initiative. Responsible for development chemistry at the time, my colleagues and I had long believed that an earlier compound supply would be an important component of a successful early development program. This belief was bolstered by the A.D. Little benchmarking study mentioned above that showed the following reasons for project *delay* (Table 3.3; see also footnote 1).

We began with a retrospective look at projects done at BMS. One initial finding was that the start of development did not occur at the same time (*in a compound's life*) from company to company. Each company had its own requirements for the discovery/development transition, and each had a more or less formal pathway for approval. This made it very difficult to compare the available benchmarking data. However, unlike the start date of development, which might vary considerably from company to company, the date of *FS* of a given compound was unambiguous.

Though the date of IND filing is definite and easily knowable, it is not as important as the date of first dosing to a human subject/patient (the first-in-man or *FIM* date). *FIM* dosing in the United States can occur 30 days after filing an IND; however, a company could very well not be prepared for *FIM* dosing for several weeks or months after the 30-day minimum. For that reason, we decided to widen the initial charge to the team: from the initial *PLP-to-IND* period, we choose to look at the date of *FS* to the date of *FIM* dosing (*FS* to *FIM*). As we have seen above,

Table 3.3 Early development delay factors.

Delay factor	% of total delays (96 projects)
Portfolio prioritization	23
Compound supply	22
Protocol preparation	12

Table 3.4 *FS*-to-*FIM* times.

Project	*FS* to *FIM* time (months)
Captopril	12.4
Aztreonam	10.2
Fosinopril	15.2
Nefazodone	8.3
Gadoteridol	17.8

this decision allowed us to find appropriate benchmarking data; it also mirrored a key finding and eventual recommendation of the team.

We first looked at the recent projects at Bristol–Myers and Squibb that were deliberately *accelerated*. Table 3.4 contains the data for five projects that had an average *FS*-to-*FIM* period of 12.8 months. Interestingly, of these projects, captopril had the simplest initial synthetic sequence (four steps from commercial materials) that supported the *FIM* dosing, and aztreonam and fosinopril had the most complex (15 steps and 13 steps, respectively) from commercial materials.

This was compared with the most recent benchmarking data from A.D. Little and KMR available in the mid-1995 (see footnote 1). Unfortunately, this date was not part of the benchmarking metrics gathered by KMR at the time. A related parameter was nevertheless collected: the date of first pharmacological testing. At Squibb and at BMS, the first pharmacological testing occurred only days after the *FS*. For the times we were considering, we decided to equate the date of entry into a company compound registration system with the date of first pharmacological testing and the date of *FS*.

Comparing these five projects with an *FS*-to-*FIM* average time of 12.66 months against the average A.D. Little and KMR data (*FS*-to-*FIM* times of 31 months) revealed that they were much faster. Our analysis then focused on the reasons why these projects had such short *FS*-to-*FIM* times. We found that there were four characteristics of these *accelerated* projects:

1) Development activities began well before the formal discovery-to-development transition.
2) Issues were addressed as soon as they were recognized.
3) Resources were immediately made available to prevent these issues from becoming obstacles.
4) Teamwork and enthusiasm around these projects abounded.

The team eventually made more than 100 detailed recommendations that generally fell into three significant categories: parallel activities, integration, and optimization. The following paragraphs will illustrate examples from each category.

3.4.2.1 Parallel Activities

Of the three major reasons for project delay cited above, the one most likely to be shortened by parallel activities is compound supply. Discovery chemists use syntheses that are designed to be expedient. The most important data to be obtained

is the relationship between compound structure and biological activity. Synthetic schemes are often designed to allow many analogs to be made from an advanced intermediate; sometimes these analogs are made and purified robotically. These protocols for synthesis and chromatographic purification, though well suited to the needs of discovery, are not often well suited to prepare much larger amounts needed for *FIM*-enabling studies.

Recognizing this, the team recommended that process research should become common during late discovery programs (a strategy we termed *prospective process research*). At a certain point in a discovery program (usually during the lead development or optimization phase), development chemists evaluated the route(s) being used in a given discovery program and, when necessary, improved these to the point of being able to support rapid preparation of supplies for the rate-limiting (to IND and *FIM*) good laboratory practice (GLP) toxicology studies. As an example at BMS, in the late 1990s, it became our stretch goal to have toxicology supplies ready at the time of compound entry into development. In order to do this, we stayed in close contact with discovery colleagues and began to evaluate routes in the lab no later than 6 months before the projected compound choice date.

One of our projects had a very long *FS*-to-*FIM* time: well over two years. This was due to the chemists supplying a salt that was not suitable for the intended route of administration. From this and related findings, the team recommended that a *f*inalize *o*ur *r*aw *m*aterial (FORM) team should be constituted usually during the lead optimization phase of the discovery program. The FORM team's remit was to follow the progress of the SAR, with the goal of assuring that the salt and/or polymorph chosen for development was suitable for discovery, development, and marketing purposes. A member of the pharmaceutical function usually led these teams.

3.4.2.2 Integration

A second team was also constituted when a late discovery program was in the compound optimization phase: a DCT or *d*evelopment *c*oordination *t*eam. The core members of this team were from the CMC disciplines supplemented by ADME, drug safety evaluation (DSE), and regulatory and clinical supply team members. The object of this team was to coordinate all activities that would lead to the shortest *FS*-to-*FIM* time. This team was usually led by a scientist from the CMC discipline that had the most significant challenges with that particular lead series during the final lead optimization. We eventually provided formal training to our staff that aspired to be DCT leaders. In some cases these leaders were eventually chosen as project team leaders based on previous exceptional performance leading their DCT(s).

Development Coordination Team Charter and Mission The DCT is a multidisciplinary team of frontline scientists,[2] unencumbered by territorialism and

2 DCT charter, mission statement, and training agenda are courtesy of Dr. Pushpa D. Singh who, together with her colleagues in the BMS Pharmaceutical Development Strategic Operations, developed these materials.

bureaucracy, who embrace new technologies and processes with the aim of proactively managing the development timeline in accordance with established plans while optimizing the use of development resources. The DCT is responsible for the planning, coordination, and communication of activities among development functional areas that support the approved strategy and timelines for individual projects, especially as they relate to CMC. The initial development plan is the starting point for DCT activity. The team will develop and communicate integrated plans to *flesh out* the approved development scenario and monitor and report progress, especially when modification of project milestones and/or timelines is necessary. It will decide among alternative strategies and tactics with the dual goal of minimizing timelines across the development portfolio and optimizing the use of resources within the CMC, DSE, and MAP functional areas. The team will assure that subsequent functional area plans are integrated and fully support the development strategy.

A two-day agenda for DCT leader training (circa 2000) is included below to indicate the potential scope and remit for similar groups' initiatives.

Day 1

1) Development team business process
2) Matrixed teams
3) First-in-human (FIH) approaches
4) Genotoxic impurities
5) Radiosynthesis and ADME supplies
6) Case study sessions

Day 2

1) Technology transfer process
2) Team leadership
3) Effective meetings
4) Development team simulation
5) Development team challenges roundtable

3.4.2.3 Optimization

BMS, like many pharmaceutical companies formed by mergers, had multiple departments in all functional areas, some that were separated by state and national boundaries. Our DSE function had four sites that routinely performed IND-enabling GLP studies. When we looked at the metrics for the time to produce the safety package from the availability of the drug substance batch, we noticed that one site was several weeks slower than the others. This was traced back to their practice of not ordering animals for the GLP toxicology studies until the drug substance batch was on site. The other three departments would closely coordinate the availability of the animals for dosing with the *projected* availability of the API. This added an element of risk that we judged was acceptable in order to reduce by two weeks the *FS*-to-*FIM* timeline.

3.4.2.4 Teamwork

The two new teams described above that grew out of this initiative were critical to its success. These teams quickly established themselves as integral parts of development governance at BMS. When the initiative formulated its recommendations, it also predicted the effect on three project attributes (quality, resources, and time). We projected that there would be an increase in project quality with no increase in resources. Importantly, we projected a five-month decrease in *FS*-to-*FIM* times. Within the first year, we were astounded to learn how much we had underestimated the decrease in project times.

Before the initiative at BMS, discovery and development were viewed as largely independent activities. The discovery function was responsible for bringing a PLP candidate forward, and the development function was responsible for bringing it to *FIM* dosing. On average, a discovery program led to a formal PLP approval 15 months after the *FS*, and an IND was filed 20 months later, totaling an average of 35 months.

After the initiative, the *FS*-to-*FIM* time was viewed as a company responsibility. Within the first year, these were drastically reduced. The major reason for this was the people who were involved: the same staff that made up the initiative teams became the first DCT and FORM team leaders. While the initiative was still underway, they began to implement the recommendations in programs and projects in which they worked. *FS*-to-*FIM* times less than two years became common; our best project was delivered in 367 days, one day longer than the leap year 1996.

3.4.2.5 Enthusiasm

This *category* was not one of those that had multiple recommendations for each functional area in development. Rather it was an outgrowth of the initiative work itself. Staff was eager to apply what they had recommended; they approached new projects with renewed enthusiasm. Communication and camaraderie between discovery and development, and between chemists and analysts and pharmacists, improved dramatically. Together, these fostered a genuine sense of ownership and enthusiasm for projects. All teams celebrated their success with inscribed paperweights, pens, and other small but significant mementos on display in many labs and home offices to this day.

This was not surprising to the authors. Early in the monobactam project that led to Azactam [3] (circa 1980), a business development colleague had hundreds of white buttons made with a red heart that simply stated, "I love monobactams." Seeing these buttons throughout the company provided significant encouragement to the team involved when tackling the scientific challenges and delivery goals.

3.5 Present Practice at BMS

Over the last decade, building on the foundations set forth by the PLP-to-IND initiative previously described, pharmaceutical development at BMS has sought to maintain an aggressive approach to enable the earliest possible initiation

of IND toxicology and phase 1 studies. Challenges to consistently achieving speed-to-patient for preclinical and phase 1 assets include increasingly stringent regulatory requirements, for example, in the area of genotoxic impurity control (discussed in Section 3.5 of the book), and the increasing molecular complexity and structural diversity of new drug development candidates transitioning from the discovery organization to development.

In the face of these challenges, the achievement of the earliest possible initiation of clinical programs has been made possible through careful attention to key work processes. In the chemical development area, these include placing a premium on developing and maintaining a department of top talent geared toward rapidly identifying first principles-based solutions to complex chemistry problems, including a very rapid identification and development of new and innovative synthetic approaches to early drug candidates. Concurrently, pharmaceutical development began to increase external collaborations and formed partnerships with a variety of high quality organizations.

It is noteworthy that most development candidates do not require a totally revamped synthesis at the IND stage, and the majority of APIs required for IND-enabling studies are prepared using at least the overall theme of the discovery synthesis. However, employing a very lengthy discovery synthesis, or one requiring extraordinary development efforts at the outset, is often not a sound approach from a portfolio management perspective since the associated resource needs can be injurious to the progression of other assets entering or in development. This decision, i.e. to employ the discovery route or invest in new synthesis technology, is the first strategic decision made on a new project and is key to achieving the goal of rapid phase 1 starts (at the end of this section is a short narrative that serves to illustrate our approach to a project that did require an early change in synthesis). Typically, this decision is taken 6–9 months ahead of the anticipated transition to development, with the exact timing usually dependent on an assessment of emerging biology, pharmacokinetics (PK), and toxicology data as well as the priority for the program under study. These variables, which essentially represent the likelihood of transition of an asset to development, intersect with the anticipated difficulty in synthesizing and formulating the target API and the associated staff and financial investment. The decision to initiate prospective chemical development is taken after consideration of all of these factors.

We began to employ a standard 2–3 kg *API output goal* for all IND toxicology API campaigns, which was also sufficient to provide material for early formulation development. While the actual toxicology material requirements often turn out to be lower, this standard approach allows for the initiation of early prospective development and scale-up without waiting for formal material requests from the drug safety organization and also serves as a buffer to the yield fluctuations often encountered in early stage chemical development work. In line with this, we also embraced the concept of at-risk commitment of resources to multiple drug candidates within the same programs, accepting that generally only one compound would transition to the development organization. To ensure the earliest possible initiation of phase 1 studies, we also began to initiate, at risk, a follow-up API campaign for the delivery of phase 1, clinical-grade API with a delivery goal

of three to six months subsequent to the delivery of the IND toxicology API supplies, with the exact timing determined by the clinical development plan.

Integration of talent with the latest technologies is also crucial, and BMS Pharmaceutical Development has continued to increase investments in high-throughput screening capabilities (e.g. chemical reaction screening and crystallization). In addition, targeted formulation platforms to fund early toxicology and clinical formulations have received increased emphasis and are important components to the rapid lift-off of development programs.

3.5.1 The Role of Chemical Complexity

The concepts and implications of chemical complexity are important to understand in the sense that there is often a direct correlation between complexity and the difficulty in synthesizing and developing a drug candidate. Understanding complexity trends should also help to predict future project staffing requirements and recruiting needs.

Although several complexity models have been developed over the years in different laboratories, when tested against the BMS portfolio, they fell short of adequately articulating and predicting observed complexity phenomena with respect to the practical impact related to chemical development (for a review on the subject the reader is referred to Ref. [6] and references therein). As a result, several years ago, the BMS Chemical Development organization undertook to develop a new quantitative model to probe the complexity of drug candidates transitioning from the BMS Discovery organization. This effort resulted in a new model that mathematically predicts chemical complexity based on the sum of both inherent molecular (i.e. structural) complexity and extrinsic complexity, i.e. the difficulty in synthesizing a compound based on the available chemistry technology at a given point in time [6]. In other words, the model views complexity as a time-dependent, dynamic phenomenon rather than as a static concept. When applied to the BMS portfolio, the model indicates a clear trend toward increasing complexity of drug candidates entering development. To this point, one of the key roles of a process chemistry group is to address extrinsic complexity and reduce the overall complexity of a given portfolio asset during the development process.

Perhaps an obvious corollary to the trend of increasing API complexity is the underlying reality that the *intermediates* leading to a given API will also likely follow the same general trend toward increasing complexity (the obvious exception is the *perfect convergent synthesis*, wherein very simple building blocks are rapidly assembled into a more complex entity). This concept takes on greater importance in today's world of ever-increasing reliance on external API supply chains and places a premium on establishing close partnership relationships with high quality, reliable CMOs.

This complexity model has been used to articulate the challenges faced by the chemical development organization as well as to predict department staffing needs. Of course, the reasons for this observed increase in complexity are open to discussion. In the opinion of one of the authors, the key factors include more sophisticated biology knowledge and testing paradigms, resulting in significantly

expanded (diversity and complexity) arrays of ever more challenging molecular targets. Furthermore, today's chemists have access to an expanded chemistry toolbox, fueled to a great extent by the ongoing revolution in catalysis chemistry. At the same time, the advancement of formulation science and the associated increase in the utilization of spray-dried dispersion technologies have enabled the advancement of new, highly insoluble molecular entities, which at one time, would have been considered undevelopable.

3.5.2 An Example of Early Prospective Chemical Development

An informative recent example of prospective chemical development at BMS is the JAK2 inhibitor, BMS-911543 (Figure 3.2) [7].

In mid-2009, it became clear that, pending successful completion of some early toxicology and animal efficacy studies, the BMS Discovery organization was targeting transition of this asset to development by early 2010. Accordingly, prospective chemical development activities were initiated. The synthesis used by the medicinal chemistry team consists of 19 linear steps starting from 2,6-dichloropyridine. While suitable for the identification of a drug candidate, development of a synthesis of this length and inefficiency along rapid timelines would have been a severe resource drain resulting in a negative impact on the progression of other assets in our portfolio [8].

Accordingly, we decided to staff a small team of chemists to quickly propose and evaluate alternative routes. If these efforts didn't rapidly achieve fruition, we would have been forced to reconsider the discovery approach. Two new routes were proposed; both emanated from a common, commercially available starting material (as opposed to the dichloropyridine precursor used in the discovery synthesis). While the initial route scouting work was underway, we ordered, at risk, sufficient material to enable the scale-up of the early steps.

Fortunately, within the space of several months, a proof of concept was achieved for both new approaches, each with the clear potential to significantly shorten the original synthesis and deliver IND toxicology supplies by early 2010. The route selected for additional short-term development (Figure 3.3) had the advantage of brevity, better prospects for longer-term development, and fewer inherent process safety issues [8, 9].

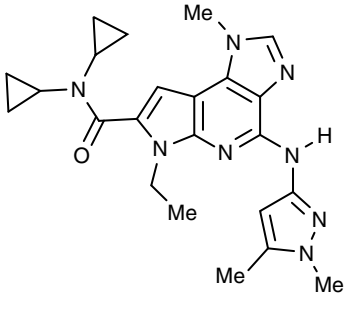

Figure 3.2 Chemical structure of BMS-911543. (*Source*: See also Ref. [7].)

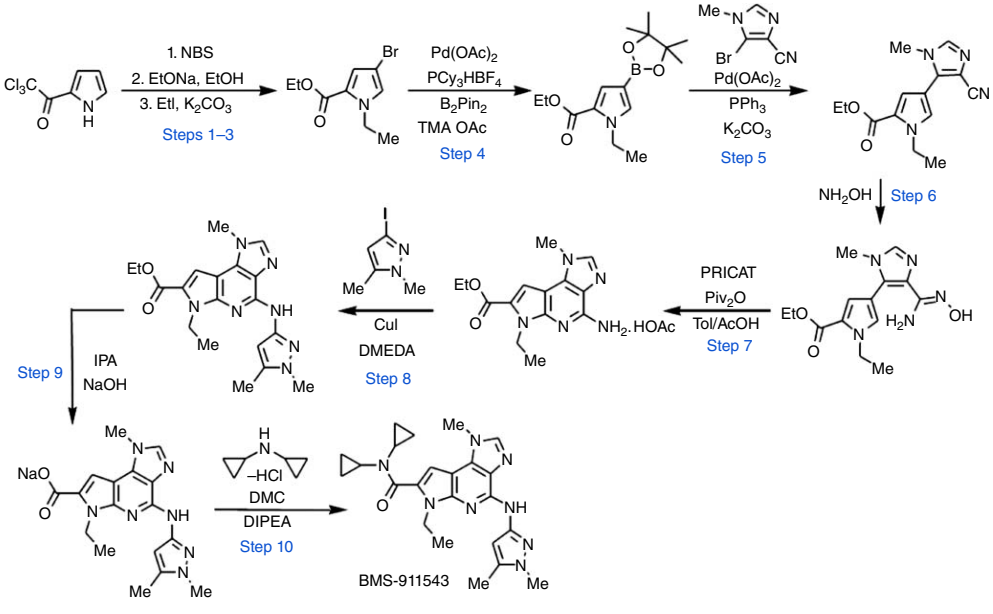

Figure 3.3 Development route devised for BMS-911543. (*Source*: See Ref. [8].)

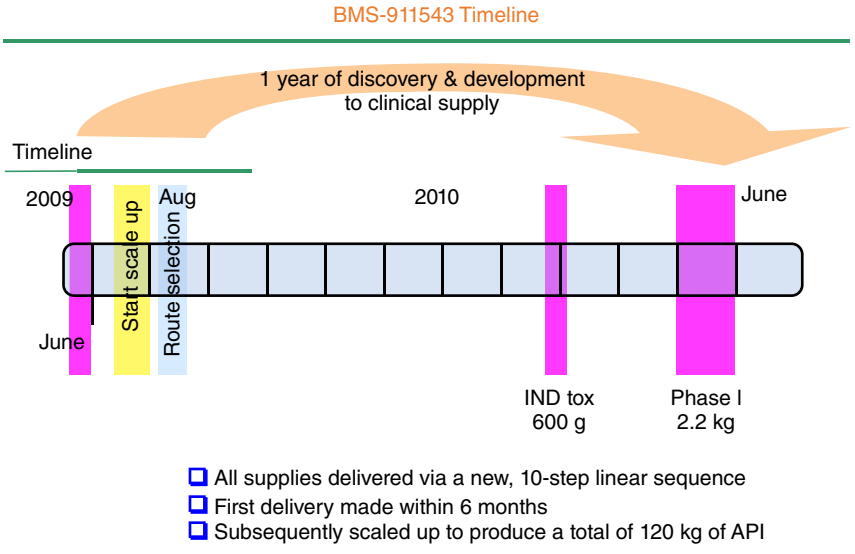

Figure 3.4 Process development timeline for BMS-911543.

Overall, IND-enabling supplies were generated in ~7 months and synchronous with the transition of BMS-911543 to development. This resulted from rapid identification and development of a totally new, 10-step synthesis eventually proceeding in 11% overall yield. New or improved chemistry was developed, which impacted literally every transformation in the sequence and significantly accelerated material delivery. The delivery of IND-enabling API was followed shortly by the delivery of phase 1 clinical supplies. All of these activities spanned just one year from inception to the delivery of phase 1 API supplies, as displayed in Figure 3.4.

It is important to re-emphasize that these results were made possible only through the full engagement of talented individuals who embrace first principle approaches to development, modern technology (i.e. catalyst and chemical reaction screening), and ongoing partnerships with high quality external collaborators. This type of integration is crucial in today's business environment for both rapid synthesis invention and all aspects of development, as discussed below.

3.6 Application in Small Biotechnology Companies Today

One of the fundamental changes in the industry has been the growth of the small biotechnology sector. Many of these companies are virtual, either completely or in many of the disciplines needed for full discovery and development programs. The corresponding change in the CRO space has allowed any and all of the missing disciplines to be provided *virtually*. The experience with several of these biotech companies has shown that the concepts of parallel activities and prospective process research can be applied even in this new *virtual* world.

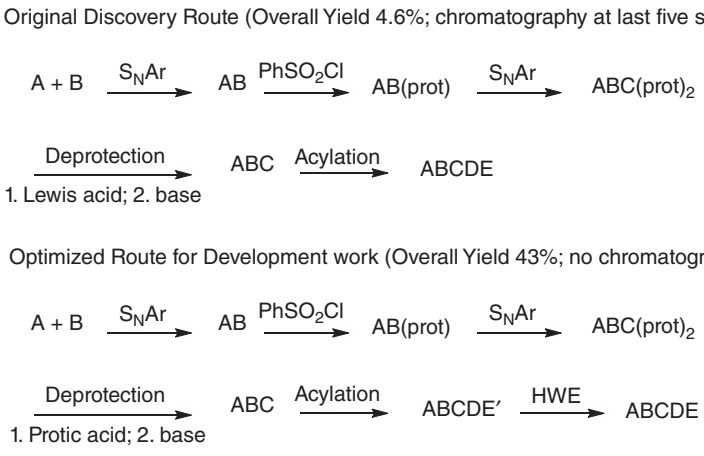

Figure 3.5 Schematic representation of the early discovery route and the optimized sequence used in development.

As an example, one of the authors began working with a small biotech in the context of a late discovery program that was optimizing molecules of structure ABCDE. Each of the A–E moieties had two or more examples that were of significant interest. The typical yield for a final molecule assembled at a CRO was much less than 10%. The analysis of the synthetic methodology underlying this program suggested the following prospective work:

1) Prepare kg quantities of the two C fragments at one current CRO.
2) Prepare the most likely AB fragment at the same CRO.
3) Divert some FTEs from a second CRO to look at two problematic areas of the methodology:
   a) Access to one of the three B fragments.
   b) Replace the ABC + D + E approach that proceeded in low yield.

The decision was made to use the existing preparation of the two C moieties since, though lengthy and inefficient, they were certainly practical and capable of producing the several kilogram of each moiety that were required. All of this work was completed during the final lead optimization phase and led to the much improved sequence illustrated in Figure 3.5.

The completion during the final stages of lead optimization that enabled GLP toxicology to start soon after with the final compound was selected. In parallel, a work that identified a crystalline final form of the API was also completed. Currently, a CRO has begun to prepare the necessary cGMP material for the initial clinical study while a second CRO is developing, in parallel, a suitable crystallization protocol for the API.

3.7 Application in CROs

A number of CROs emphasize capabilities that can be used to move client's compounds quickly and efficiently during an early development program. Several

examples are briefly discussed below to highlight the importance of CROs in today's pharmaceutical research and development ecosystem.

Patheon (www.patheon.com) is a contract development and manufacturing organization with operations in four continents**.** Its RES COM Unit located in Regensburg, Germany, was formerly part of BMS. Its laboratory and pilot plant staff were able to scale up chemistry very quickly to support initial toxicology and clinical studies. When the Regensburg site was sold by BMS, the group became part of DSM and has recently become part of Patheon. It has reportedly retained the ability to scale up chemistry in a timely and efficient manner. As discussed above, a rapid scale-up to production of the material needed to initiate the GLP toxicology program is a key component of shortening *FS*-to-*FIM* timelines.

Catalent, Inc. (Catalent Pharma Solutions, www.catalent.com) is broadcasting a webinar with the title "Expedite Your Early Drug Development with Unique Formulation Techniques."

Avista Pharma (Avista Pharma Solutions, http://www.avistapharma.com/), a CRO formed in 2015 by a venture capital firm, has the capabilities to support drug discovery and all of the CMC drug development activities necessary to file an IND.

3.7.1 Colocation of CMC Activities

At BMS, we were fortunate to have two sites with the capability to make drug candidate compounds, perform the GLP toxicology studies, do formulation development, and make cGMP clinical supplies. Even if the compound had to be transferred between two BMS sites, the ability to do that in a time-efficient manner was well worked out. The absence of *idle* time spent by the development candidate in delivery vehicles or custom offices is one more component of short *FS*-to-*FIM* timelines. Recently, Dalton Pharma Services (www.dalton.com) communicated by e-mail the following: "We accelerate your drug development programs by integrating formulation, process optimization and scale-up and API and sterile or solid dose manufacturing at a single location."

Translational Pharmaceutics® is a platform developed by Quotient Clinical (www.quotientclinical.com) that is based on the colocation of drug product formulation development and analytical facilities, cGMP manufacturing suites, and a clinical unit [10]. This configuration allows the manufacture of GMP clinical trial materials within days of dosing, providing sufficient time to analyze and release drug product. One major CMC advantage of this model is that shelf life requirements to support the *FIM* study are significantly reduced, and as a result the timelines to generate the regulatory submission data package are accelerated.

In an example recently described (K. Crowley of Quotient Clinical, personal communication), a lipid-based solution administered in a capsule was selected for the clinical study. A regulatory data package including short-term (21-day) stability data, bracketing a 5- to 200-mg unit dose range, was generated. Translational Pharmaceutics® allowed all drug products to be manufactured in real time, immediately prior to dosing. The platform was supported by an adaptive clinical protocol, enabling the determination of dose and number of cohorts in real time based on emerging data. This example shows how real-time manufacture of drug

product creates within-trial flexibility of dose strength, alleviating the need to premanufacture multiple dose strengths of drug product prior to the FIH study.

These approaches allow a small, virtual biotechnology company to choose one or more of the strategies and tactics that enabled BMS to drastically reduce its *FS*-to-*FIM* times without the multibillion dollar investment that created the facilities and capabilities that BMS (and other large pharmaceutical companies) generated. A biotechnology company should include the ability to expedite early drug development in the evaluation matrix of the CROs that they choose. Here, the strategic importance of finding the best CRO fit for a given program cannot be overemphasized.

3.8 Conclusions

The key concepts described above, developed in the context of a Big Pharma setting in the mid-1990s, are relevant today for biotech, small, medium, and large pharma as well as CROs and CMOs. Parallel activities, integration, and optimization of activities are applicable to innovator companies, whether big or small, as discussed above. The consolidation of the pharmaceutical industry has been followed by a similar consolidation of the CROs and CMOs that support them. We are convinced that adopting the strategies discussed above will provide as much benefit today as it has done for BMS during the past 20 years.

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4

Active Pharmaceutical Ingredient Cost of Goods: Discovery to Early Development

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4.1 Introduction

As a prospective drug candidate is advanced from the stage of discovery lead optimization through preclinical development and into clinical development, there are many short-term cost of goods (CoG) factors that should be taken into consideration. Short-term CoGs in this sense are focused on what it costs to produce a batch of the desired target molecule. CoGs include raw materials (RMs), unit operations, and production costs. In a general sense, there is a premium placed on speed and cycle time in the early stages of R&D. However, CoGs can rapidly have a major impact on preclinical and clinical development programs. The CoG factor is of paramount importance in the final stages of development as a commercial process is being defined. For the purpose of this chapter, CoG may refer to costs of:

a) *Active Pharmaceutical Ingredient Drug Substance (API DS) Campaign*. This is either a total campaign cost or cost per kg of API DS (or key intermediate) and is useful to drive improvements between campaigns and to plan key preclinical and clinical milestone strategies.
b) *API DS Produced to Support a Key Milestone*. This is a collective cost across either single or multiple campaigns. This will often be based on a route that is neither fully optimized nor the final manufacturing route. This COGs valuation provides program planning and historic cost information.
c) *API DS in the Manufacturing Process*. This is either the actual or projected cost per kg of API DS (or key intermediate). It is useful in helping define the manufacturing route and may be used in route selection, net present value forecasting, vendor selection, and various cost calculations.

This chapter focuses on small molecule new chemical entities (NCEs). Many principles from small molecule NCEs will apply to other modalities, such as linkers and conjugation to monoclonal antibodies. However, generics, biocatalysts [1], biosimilars, vaccines, and monoclonal antibodies [2] are out of scope.

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

4.2 Stages of Research

The attrition rate of small molecule NCEs is generally higher in early upstream development as compared with later downstream development [3]. Thus, CoG considerations may play a secondary role compared with speed in early preclinical or clinical development. With that being said, judicious financial stewardship is a core value that should be inculcated throughout all aspects of research. This chapter discusses direct and indirect considerations for CoGs as molecules are advanced from discovery research through preclinical development and into clinical development.

In general, the synthetic routes used to produce API in each stage of biopharmaceutical research have a distinct purpose (Figure 4.1) [4]. The amount of API needed to support these activities can range from milligrams to metric tons. Through this process and by necessity, the synthetic route needs to be made progressively more efficient, which translates to a decrease in CoGs.

A key consideration, as molecules move into development, is determining what piece of preclinical or clinical data can be considered as a GO/NO GO decision node for the prospective drug candidate [5]. This knowledge, as determined in a cross-functional scientific sense, serves as an anchor point for the active pharmaceutical ingredient supply chain. Two immediate questions are:

1) How much API drug substance is needed to support the program to arrive at each GO/NO GO point [quantity]?
2) What is the knowledge base around the existing synthetic route [route information]?

These two questions feed directly into the next rung of attributes for consideration, which are depicted in Figure 4.2. This framework provides a systematic series of discussion points that should be considered when planning financial aspects of advancing molecules into clinical development. Each of these will be

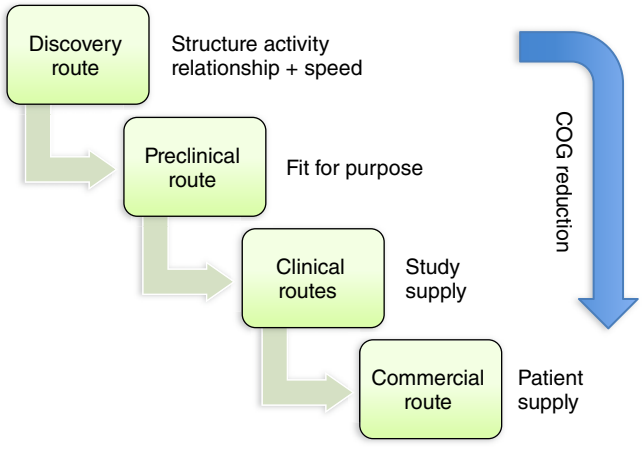

Figure 4.1 Synthetic route focus by stage.

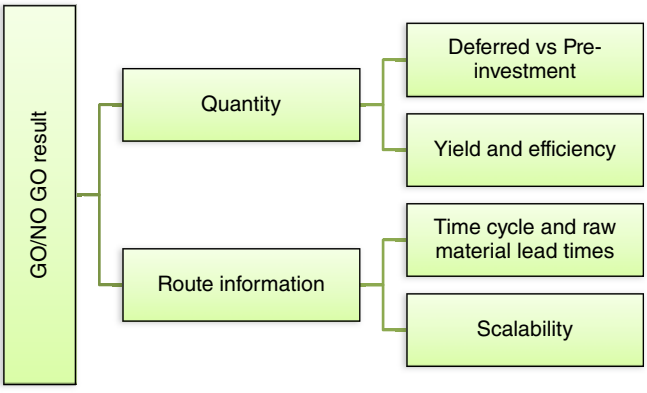

Figure 4.2 API drug substance cost of good attributes.

addressed in this chapter. These points are generally independent of whether internal or external personnel, capabilities, and capacities are used in the API production.

4.3 Synthetic Route Translatability and Scalability: Strategy

There is obviously a direct correlation between API production cost and the quantity of API needed to support a GO/NO GO decision (Figure 4.2). The program and project development strategy is of critical importance in determining the amount of API to be produced in any given delivery. Committing to a synthetic campaign in order to produce only the amount of material needed to obtain data necessary for the GO/NO GO decision can be viewed as a resource-sparing investment strategy. The typical concern with solely producing the minimal amount of API needed for any given GO/NO GO milestone arises if the molecule meets the preclinical or clinical GO criteria, and it is then desired to advance the molecule to the next stage of development. In this case, a program delay will occur and another production campaign will be required. The flip side of this argument is that an increased amount of money and effort can be spent in preparing a surplus of API to have on hand in case the molecule meets the GO criteria. Alternatively, a second API campaign may be initiated prior to the decision point. This may be the result of encouraging preclinical- or clinical-related data readouts or may be simply a strategic decision to pre-invest in the project or program. Regardless, this hybrid approach can balance the risk posture between CoG expense and API resupply availability. Another option is that an excess of an intermediate is synthesized and only a portion of this is carried on to API. The remaining intermediate is staged for a potential resupply campaign or to prepare a similar compound. These two last scenarios provide options for the program to advance at an intermediate rate. As a result, if a

NO GO decision for the project is reached, then the CoG expense will only be fractionally realized.

The second CoG attribute related to API quantity is the yield and efficiency in which the target molecule is manufactured (Figure 4.2). In the simplest terms, the less productive the chemical route, the higher the CoG. A common strategic question is whether to commit the existing synthetic route to a scale-up campaign without first investing in improvements or modifications. Doing so may provide a cost-effective strategy, but this needs to be balanced by the risk of any of the synthetic steps or chemical operations not being reproducible and of having a minimal understanding of environmental, health, and safety (EHS) factors. In general the goal is to produce the NCE or API with a minimum of 98% purity, and the amounts produced will increase as the project moves into commercialization. As a result, even in these fit-for-purpose (FFP) campaigns, there may be some requisite R&D work required to better understand the process safety aspects of the route prior to or during the API campaign. If R&D work is conducted outside the actual API campaign window, this investment will not be captured in the CoG calculation. Another important aspect of planning campaign costs is assumptions around the overall yield. Consideration should be given to whether improvements are likely to be made during the campaign or whether to assume the overall campaign yield will be reflective of the existing yield. This can greatly influence the CoG of API produced per campaign or for a given milestone. Care must be given in not being overzealous in yield planning, and a risk assessment should be made regarding the likelihood of underproducing the requisite amount of API DS in any given campaign.

The third component is understanding the relationship between the existing campaign time cycles, RM availability, and other route cost drivers relative to the projected campaign values. Some materials may be readily available on small scale but will be prohibitively expensive or be in limited supply as development continues. Coupled to this is the overall time cycle of the campaign. Manpower resources are also a significant driver to the API production cost. Decreasing the number of steps and operations required will, in general, positively affect the CoG. An example of the inverse situation arising happens when any of the replacement operations or chemical reagents possess significant cost drivers.

The fourth consideration is how much development work is needed to support the safe and reliable production of the required amount of material (Figure 4.2). In a tangential sense, the question of whether pre-investing in API R&D to advance the compound past the desired GO/NO GO decision point is also a factor that may influence the CoG. There may be some R&D or other associated costs that are closely bundled with campaign CoGs. Some associated costs may include EHS testing and/or monitoring, unique capital assets, and waste management costs. Some of these R&D costs may be tied intimately to campaign costs and, as such, may be included in the CoG calculation. Depending on the criticality, required route improvements may not be triggered until a campaign commitment is reached. Such improvements may include critical unit operation optimization, solvent replacement, reaction concentration, reaction work-up modification, crystallization optimization, and running through processes (telescoping) in which crude intermediates are carried directly into the subsequent step.

4.4 Raw Material Considerations

Other key cost factors in advancing compounds from discovery to the clinical supply chain are the reagents and starting materials that are chosen. There are a few ways that this may affect the CoG. First, the starting material may be readily available to support synthetic efforts in discovery research but may be of limited supply when advancing the asset through campaigns to support preclinical and clinical API supplies. In cases in which this occurs and the chemists are unable to procure the required reagent in either a timely or cost-effective manner, then additional work must be done, which leads to an increase in the CoG for that particular campaign. The starting material may need to be synthesized, which will add production cost, or a modified route must rapidly be discovered, which will add R&D cost.

Catalysts can often be significant cost drivers to a campaign. In transitioning a molecule from discovery and into clinical development, it is prudent to rapidly see if the catalyst loading can be decreased. A few simple experiments in this vein can result in large CoG dividends. Another point of fact is that the best catalyst may not always be the best choice from a CoG and timely campaign synthesis perspective. For example, if catalyst A gives a 100% yield and 100% selectivity, it will rapidly become the reagent of choice in a medicinal chemistry program. This will ultimately be prescribed in the experimental procedures passed into early development. As the amounts of catalyst A needed in preclinical and clinical development campaigns increase, it may become prohibitively expensive and require a long lead time to source.

It is often prudent for the development chemists to know the details of other catalyst options. If in this example catalyst A provides the desired product quantitatively and with absolute selectivity but is not available on scale, and it is known that catalyst B provides the desired product in a 97% yield and 97% selectivity but is readily available or inexpensive, then this may actually qualify as the catalyst of choice. Some additional R&D may be needed to ensure that the performance of the product is adequate in downstream processing, so that any unwanted impurities can be readily removed and the product purity upgraded. If unwanted impurities are readily rejected without a concomitant yield loss of the target molecule, consideration may be given to accepting an even slightly lower yield, if the catalyst is extremely inexpensive (e.g. Catalyst C).

4.5 Continual Assessment of Alternative Routes and Technologies, Including Preparative Chromatography

Throughout the development of an NCE or API, process chemists and engineers critique how compounds are prepared. Often process chemists immediately reorder the sequence of steps in the route used by drug discovery to make a lead compound. These changes may be carried out for reasons of safety, to incorporate more convergent and practical steps and to position steps generating

troublesome impurities further away from the final step, or for other reasons. Later in development, process chemists and engineers also consider alternative technologies for practical scale-up; for instance, membranes may be employed to remove troublesome residual solvents. Another example would be substituting continuous flow operations for batch operations, with the primary drivers being safety, quality, throughput, and economics [6]. Purification by simulated moving bed (SMB) chromatography, also called multi-column continuous chromatography or MCC chromatography [7], is another technology, which may be selected when crystallization cannot be used or is not practical (*vide infra*). Routes are changed and new technologies are introduced to make operations more cost-effective and reduce the CoG.

Further discussion on SMB is pertinent because it can be used as a practical alternative to both classical resolution of enantiomers and preparative chromatography. Classical resolution involves adding a chiral salt-forming compound to a mixture of a desired compound and its enantiomer and crystallizing and isolating a salt of the desired compound. If the desired enantiomer, without the salt-forming material, is recovered in about 45 % yield wt/wt, additional attempts to recover additional product from the mother liquors may be abandoned.[1] SMB can afford a cost-effective alternative to a classical resolution. As with any chromatography, purification is simplest if only one impurity is to be separated from the desired product; hence separation of enantiomers by chromatography over a chiral stationary phase is a logical approach, provided that all other impurities are essentially nonexistent. SMB is economical and practical for manufacturing APIs [7–11]. Purification by SMB has been part of manufacturing operations for intermediates for several APIs, including escitalopram [12] and sertraline [13]. In each of the latter two cases, SMB was used to resolve a key intermediate early in each route. Under large-scale continuous operations, more than 99% of the solvent used for separations has been recovered and reused, thus essentially removing the cost of solvent from CoG calculations [9]. The cost of a chiral stationary phase can be amortized over years, provided that the quality of the input streams can be controlled and the chiral stationary phase can be reused. The cost of resolving enantiomers by SMB, a relatively simple purification if essentially only one impurity is present, has been as low as $100–200/kg of purified product [10].

SMB can also be used in lieu of preparative column chromatography. Purification by preparative column chromatography is labor intensive and can require large volumes of solvents, thus raising the CoG. Preparative HPLC columns are available [14, 15], and details for developing practical separations on large scale have been discussed [16]. Preparative chromatography can be economically useful, especially when the productivity of the separations is at least 1 kkd (kilograms isolated per kg of stationary phase per day) [17]. Some other alternatives for purification include high-speed countercurrent chromatography to purify oily lipids, although preparative HPLC may be preferred for final purifications [18].

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1 Weight % yields, wt/wt, are ratios of the product relative to the initial weight. In the case of a resolution, a 45% yield wt/wt indicates that 90% of the desired enantiomer was recovered.

Solid phase extraction (SPE) has been shown to be effective in biochemistry lab courses [19], and guides are available for SPE [20], so purification by adsorbents may prove practical on scale. Supercritical fluid chromatography (SFC) can provide rapid separation of impurities, especially undesired enantiomers [21]. Here, the mobile phase has low viscosity, and hence smaller particles with correspondingly higher surface area and improved resolution are practical for use. However, SFC is not usually employed on large scale because of the cost and inherent risk of running large operations under 100–250 bar of pressure [10]. SMB is another option that has been used to purify complex molecules, including artemisinin [22] and monoclonal antibodies [23]. SMB has also been coupled with continuous enzymatic processes [24].

Predicting the cost of a chromatographic purification on scale is difficult. A cost of $1000/kg of intermediate that is purified by SMB may be a reasonable estimate in the beginning of developing a CoG estimate; this amount is five times greater than the stated cost of a chromatographic resolution by SMB in routine manufacturing [10]. If a contract development and manufacturing organization (CDMO, sometimes called a contract research organization/contract manufacturing organization (CRO/CMO)) were to examine the chromatographic separation of the target molecules, a more realistic value could be assigned. It may be necessary to consider SMB purifications at several points in a route in order to estimate at which step the optimal chromatographic separation could be obtained. The earlier the separation of enantiomers is performed in the route, the more cost-effective is the process [25]. Furthermore, racemization of the undesired enantiomer will significantly decrease the CoG. If the cost of specialized equipment is considered, the CoG will drop as more material is made using that equipment.

4.6 Initial CoG Projections

Regardless of whether it is for internal considerations or a key factor in making an outsourcing decision, CoG projections are commonly done for each campaign of an intermediate or API (Figure 4.3). A large component of the overall cost driver

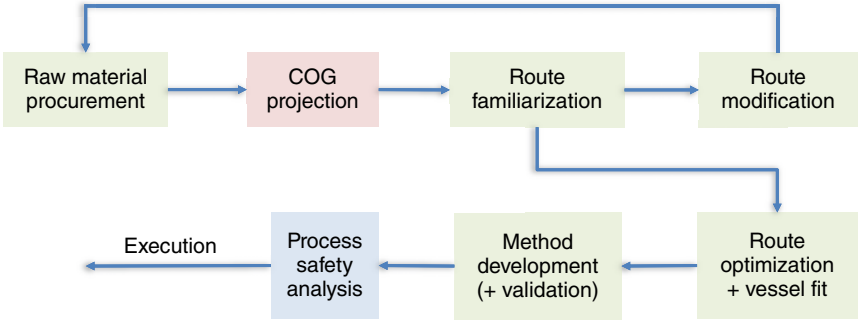


Figure 4.3 Cost of goods: iterative projections.

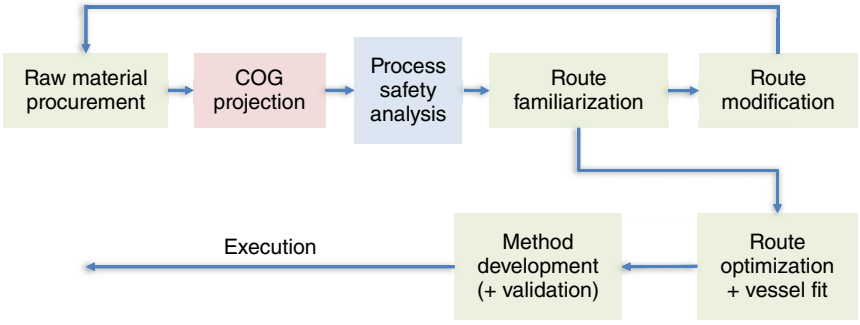


Figure 4.4 Cost of goods: iterative projections – front-loading process safety.

is typically the RM, which can be identified immediately. The RM cost analysis will allow for a first draft CoG projection based on the current experimental procedure. Of course this is simply a paper exercise at this point. Enhanced CoG projections can subsequently be made after brief familiarization with the chemistry. Depending on the state of the chemistry or projected scalability of the chemical processes, the general sequence of key activities may change. For example, some process safety considerations may need to be front-loaded in the evaluation process (Figure 4.4). These CoG analyses need not be done in a formal sense and can simply be understood from the purchase order cost if the API or intermediate is being outsourced.

4.7 CoG Versus Campaign Time Cycle

As previously mentioned, the focus in discovery research is primarily on diversity and speed. A route that is efficient to meet discovery chemistry objectives may rapidly become inefficient and a new or improved route will be needed for either preclinical or clinical support.

Figure 4.5 depicts four CoG/time cycle quadrants. Obviously, it is desirable to have a route that is reflective of quadrant C through all phases of preclinical and clinical development. This would translate to an inexpensive and on demand R&D API supply chain. Realistically, however, many small molecule NCE projects fall short of C in the early phases of development and are often defined by the less desirable quadrants A, B, or D. These three scenarios present more challenging decision-making processes in regard to the commitment of resources to the project.

The most common type of project in category A revolves around having a fairly straightforward synthetic route but extremely expensive RMs. The negative driver is generally the nonavailability of requisite amounts of RMs or reagents. This can be driven by the absolute lack of the reagent in sufficient quantities. Likewise, affordable production of the reagent on scale may exist but may be limited or unavailable within the constraints of the projected API campaign timelines. This may also be driven by shipping restrictions for certain reagents

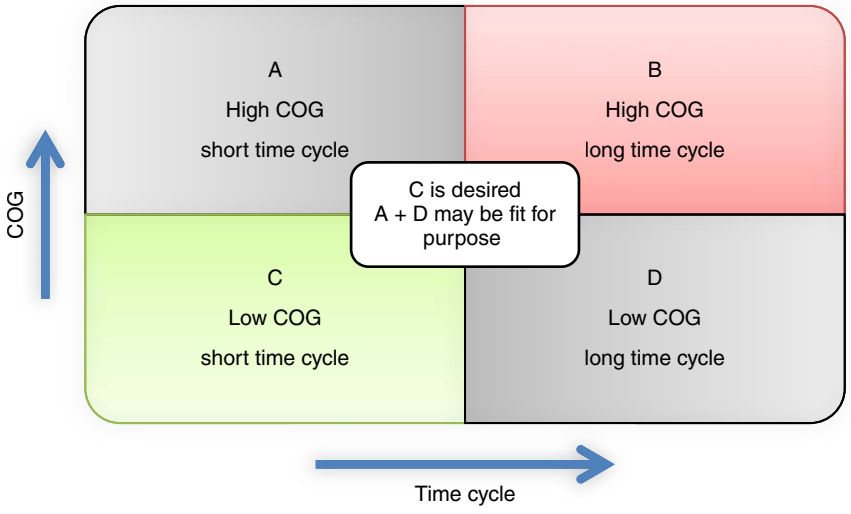


Figure 4.5 CoG versus time cycle.

within defined geographies. There may also be company restrictions on using certain reagents within defined facilities. So while the operational cost may be low, the time cycle from triggering an API campaign to having API DS in hand is long. As this may be prohibitive in some cases, this may actually become an undesired category B program. R&D investment would be required to discover a way to shorten the long time cycle that arises from a long synthetic route, lengthy operational time cycles, or lengthy reagent lead times.

4.8 Synthetic Route Translatability and Scalability: Tactics

A given API delivery may have a low CoG that will not necessarily translate to a favorable CoG for commercial supply. Situations that can give rise to this are:

a) An intermediate available as surplus from a prior campaign – of the same or related API.
b) An intermediate available by degradation or chemical conversion of an available late stage intermediate or API.
c) An intermediate available from an ongoing campaign from a related but different target molecule.

If the target molecule can utilize an intermediate that has been prepared for a project in which that intermediate is no longer needed, then the short-term CoG may be dramatically reduced. This intermediate may become available when a surplus was made or when less than the expected amount of the intermediate was consumed in another project. Causes for these include better than expected

performance in the original synthesis of the intermediate, a project delay or termination, or identification of an alternative route that obviated the need for that intermediate. Using common intermediates from structurally similar molecules can also reduce a CoG if a single quantity of this intermediate can be purchased instead of several smaller quantities. The timing of the purchase must be harmonized with the timing of the campaigns under consideration. Another possibility is that a related molecule becomes available, which could readily be converted to the desired intermediate; for instance, an ester could be converted to the corresponding amide. Of course these opportunities may not be available for long-term production and CoG considerations.

At each stage of scale-up, there should be an understanding of whether any reagents, chemical transformations, unit operations, or intermediates would pose an EHS concern. If so, these may affect the campaign CoG as additional R&D may be needed to understand and execute within safe operating ranges. However, once established and assuming this element of the synthetic route is maintained in subsequent campaigns, the cost is not necessarily translated to downstream campaigns, as the R&D required to develop this understanding is adsorbed in the campaign in which it was done. Exceptions occur when it becomes necessary to have unique, lengthy operational or capital equipment costs in order to progress the chemistry on scale. Waste disposal costs are usually not a considerable cost factor in preclinical and early clinical development, but can rapidly become a cost driver in later stage development programs.

In order to deliver API in the most efficient fashion, each step of the campaign would be run in a single batch. Not only is this the most cost-effective way of delivering API, but also it is the most expeditious. Running multiple batches, with all other parameters being equal, will increase the CoG for the campaign. There will be exceptions to this, especially when transferring the chemical process to a new facility. For example, it indeed may be faster to do two batches in a laboratory fume hood than one batch in a kilo lab. Likewise, it may also be faster to do two kilo lab batches than one batch in a pilot plant facility. In addition, there may be reasons to do multiple batch processing for some chemical transformations in preclinical and early clinical development. There are three major reasons for multiple batch processing. First, multiple batch processing increases data and knowledge around a particular step or unit operation. Second, this may be desired when there is a general lack of understanding about the process or there is a lack of reproducibility in any of the critical to quality parameters of a synthetic step. The final reason for multiple batch processing is to employ a conservative supply chain risk posture. The thought process behind this is that if something happens during operations that negatively affect the outcome of the batch, then the entire stockpile of starting material, reagents, and/or substrate would not be affected. For all of these scenarios, the previously mentioned facility differential does not typically apply. As a result, the decision to conduct multiple batch processing will generally increase the CoG and extend the campaign timeline.

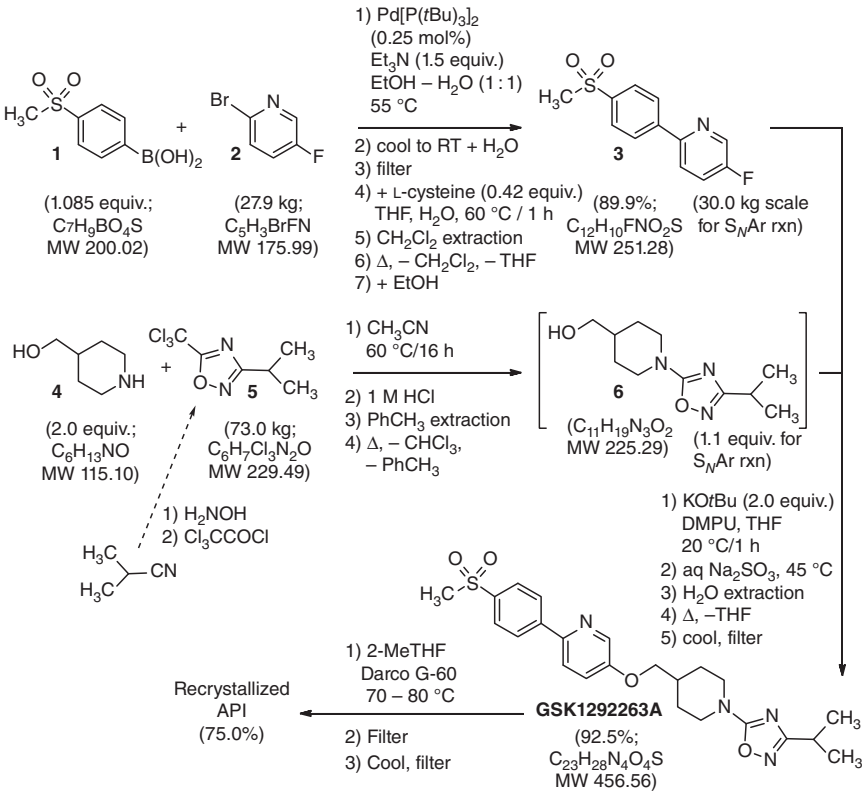
4.9 Preparing a CoG Estimate

Initial CoG estimates often will not consider work-up and isolation of intermediates and the API, even though these aspects are critical to maintaining high quality of the product and high productivity of operations. Although process chemists usually anticipate that simple and rugged work-ups can be developed, the physical state of an intermediate or product can pose difficulties and prompt development of an alternative route.

Process chemists in the pharmaceutical industry prefer isolating solids over oils and liquids. Distillation of liquids can be time consuming, and many intermediates and APIs decompose at high temperatures [26]. Crystalline solids can be recrystallized to upgrade the quality of intermediates and the API, although obtaining high recovery of compounds that melt below 50 °C may prove difficult. Compounds that are oils at room temperature may be challenging to purify. In these cases, SMB may offer a valid alternative, as previously discussed.

Many variables can be employed in a CoG spreadsheet, resulting in CoG estimates that can be pictured as multidimensional response surfaces. The impact of changing variables can be judged by changing the values of parameters in the spreadsheet.

Because a CoG estimate depends on the values of many variables, clearly defining both variables and any assumptions is critical for deriving a meaningful estimate. Some obvious variables are the yields of individual reactions, the cost of starting materials, and the number of equivalents of reagents that are charged in each reaction. One may consider the cost of any key component that has been sourced in bulk and the estimated cost of RMs required in bulk as a fraction of catalog cost. Any purification such as preparative chromatography may be factored into the CoG, as discussed above. The labor rate and the cost of labor can be factored into a CoG: if a company has sufficient unused capacity the process owners may disregard the cost of labor, but a CDMO will include the cost of labor into a CoG, since occupied equipment prevents them from running other processes that could generate revenue. Economy of scale can be seen in most scale-up operations, as bigger batches can raise the process productivity ($kg\,h^{-1}$), and hence the contribution of labor to the CoG is proportionally less. Often the production of an API will entail several batches being conducted for each intermediate; such campaigning may be required to utilize the equipment that is available. Campaigns also spread out the risk of missing the production goals if a batch fails, with the trade-off that economy of scale has a smaller impact. Ultimately the cost of inorganic reagents and solvents can be included and whether these solvents can be recovered and recycled. Another layer of parameters can include the costs of quality control (QC) analyses, the cost of waste disposal, and any extra material prepared as a contingency in case of unforeseen difficulties in meeting the desired quantity or quality of materials. If the target molecule is being made by a CDMO, it is beneficial to define the terms of purchasing potential overage amounts prior to campaign initiation.



Scheme 4.1 Preparation of GSK1292263A.

Following is a hypothetical CoG estimate prepared for the three-step sequence and recrystallization of GSK1292263A, an NCE [27], as shown in Scheme 4.1. In this CoG estimate oxadiazole **5** is assumed to be purchased, as it can be prepared in only two steps [28] and could be outsourced. Intermediate **6** is telescoped into the next step. No chromatography was employed. The times for operations, necessary for calculating the labor costs, were estimated from the experimental data provided.[2]

A spreadsheet program is a helpful means to create a CoG estimate, with pages linked together for ease of calculations, and to separate areas of thought. The following analyses use four pages, with the first detailing the variables and showing the calculated CoG. Ideally all parameters to be changed iteratively during a CoG estimate can be changed on this one page, and key values required on the second and third pages are provided by links to this first page. The second page calculates the cost of RMs and chromatography, which are considered in this analysis

2 In the experimental details provided in Ref. [27], 16 hours of reacting was required to make compound **6**, and one hour of reaction at 20 °C was required to generate GSK1292263A. The Suzuki coupling to make compound **3** was estimated to occur over 5 hours. Times for cooling suspensions were included when specified. Other processing times, e.g., charging, heating, cooling, extractions, and concentrating off solvents, were estimated for those scales.

as elements that are not changed upon scale-up. A third page, linked to the first, shows the labor costs for a given scenario. The final page is optional and tabulates the results of changing variables, clearly demonstrating that changes in a CoG are nonlinear. These pages follow.

Table 4.1 details the values that are used and assumed in the CoG estimate. It shows hypothetical ranges, and points out where values must be entered. It also lists for clarity any assumptions that are employed, which might cause certain parameters to otherwise be overlooked.

The entries in Table 4.2 ignore the cost of solvents and reagents used for work-ups, which may be appropriate early in the development of a compound. Solvent costs in bulk quantities could be estimated from the price of MeOH and multipliers for various solvents [25]. Table 4.2 clearly shows that boronic acid **1** and bromopyridine **2** are major contributors to the CoG. Competitive bidding could source less expensive supplies of these intermediates [29].

One significant item in Table 4.2 is the low contribution to the overall cost of the RMs from an expensive component, $Pd[P(t\mathrm{Bu})_3]_2$. The authors noted that this catalyst was substituted for $Pd[PPh_3]_4$, which was prepared before use. By employing such a low charge on a molar basis, the overall contribution of this reagent would be very small; however, it would be necessary to control the quality of the input streams so that catalyst poisoning would not require additional charges of this catalyst for reaction completion.

In order to calculate the labor costs (Table 4.3), the processing times for the four steps shown in Scheme 4.1 were estimated from the experimental details provided by the GSK authors. To extrapolate to the times needed for operations on scales different from those for the scales described, a scale-up factor was used. In general a 10-fold scale-up of batch operations requires a doubling of processing time. Although this rule of thumb was calculated based on the ability of heat to be transferred from reactors through external jackets, as needed for batch operations, it generally holds for scale-up of batch operations [30].[3] To calculate a factor for the times for batch operations in the spreadsheet, the scale-up (wt/wt) was raised to the 0.3 power; the value of 10 raised to the 0.3 power is 1.995. This calculation does not apply for continuous operations.

In Table 4.4 the CoGs for four scenarios at three different outputs are shown. Greater economy on scale is evident for larger batches. It can also be seen that both improving the yield of the final step (recrystallization) and decreasing the cost of starting materials can have big impacts on the yield (Scenario 4). This would be considered an early CoG estimate, as it does not include costs for solvents, reagents to work up reactions, preparing any extra material for contingencies, or surcharges for QC and waste disposal.

3 Calculations are based on a model of a multi-purpose batch reactor as a sphere, and the ratios of volume to surface area for spheres. The volume of a sphere increases as a function of the radius cubed while the surface area increases proportionally to the radius squared; hence more time is needed to transfer heat to or from an external fluid as the volume of a reactor increases. Aside from the modelling, on scale more time is needed for routine operations such as charging reaction components and the separation of phases during extractive workups.

Table 4.1 CoG calculation, with variables.

	A	B	C	D	E	F
1		Range	Selected	Cost		
2	Amount of GSK1292263A to prepare/batch		200		kg/batch	(Enter value in C2)
3	Bulk cost of raw materials as percentage of lowest catalog cost	2.5–25%	10%			(Enter value in C3)
4	Price of oxadiazole intermediate **5**, per kg	$200–500	$400			(Enter value in C4)
5	Cost of raw materials/kg of purified product			$5651	/kg purified product	(From Table 4.2)
6	Cost of raw materials/batch			$1 130 223	/Batch	
7	Labor rate	$200–750/h	$250		/h	(Enter value in C7)
8	Hours of labor required (from "labor costs" sheet)		144		h	
9	Cost of labor			$35 982	/Batch	
10	Total cost to prepare target amount (w/o chromatography)			$1 166 205	/Batch	
11	Cost of GSK1292263A w/o chromatography			$5831	/kg	
12	Total number of chromatographic purifications		0		/Route	
13	Cost of each chromatographic purification per kg of purified intermediate or API	$200–5000/kg	$1000		/kg	(Enter value in C13)
14	Total cost of chromatographic purifications			$0	/Batch of GSK1292263A	
15	Cost of GSK1292263A including chromatography			$5831	/kg	
16	Factor for QC surcharge	0–30%	0%			(Enter value in C16)
17	QC cost, additional			$0	/kg	
18	Contingencies added	0–25%	0%	$0		(Enter value in C18)
19	Waste disposal surcharge	10% of raw materials, or up to $500/drum	0%	$0		
20	Subtotal for QC costs, contingencies, waste disposal			$0	/kg	
21	Cost of GSK1292263A including chromatography, QC, contingencies, and waste disposal			$5831	/kg	
22	Total weight of intermediates to prepare			485	kg	
23	*Assumptions and variables*					
24	For every 10-fold scale-up, processing times increase by 2 (mathematically, the wt/wt scale-up factor raised to the 0.3 power)					
25	Assume that all of one intermediate is converted into the next intermediate in one step. Campaigns reduce risk but increase labor costs					
26	Economy of scale: larger batches reduce CoG through proportionally smaller labor costs					
27	Economy of scale may not apply if extensive chromatography is needed					
28	Cost of solvents and water for reactions and work-up included: yes/no		No			
29	Cost of inexpensive reagents for work-up included: yes/no		No			

Table 4.2 Costs of raw materials and chromatographies.

Costs of raw materials and chromatography required to generate 1 kg of API											
Product	**Reaction yield**	**Starting (raw) material**	**MW of raw**	**Catalog cost**	**Density ($kg\,l^{-1}$)**	**Estimated bulk cost**	**Raws: eq. per reaction**	**Raws: mol**	**Amount required**	**Cost of RM or chromatography**	**Contribution to total raw cost**
Aryl fluoride (Step 1)	89.9%	Boronic acid	200.02	$274.50/5 g		$5490/kg	1.09	3.18	0.64 kg	$3487	61.7%
		Bromopyridine	175.99	$101.00/5 g		$2020/kg	1.00	2.93	0.52 kg	$1040	18.4%
		$Pd[P(t\,Bu)_3]_2$	511.06	$264.00/1 g		$26 400/kg	0.0025	0.007	0.004 kg	$99	1.7%
		Et_3N	101.19	$441.50/18 L	0.726	$3/kg	1.50	4.39			
		EtOH									
		L-Cysteine	121.16	$187.50/500 g		$38/kg	0.42	1.23	0.15 kg		
		THF									
		CH_2Cl_2									
		EtOH									
Primary alcohol (Step 2)	100.0%	4-Piperidinemethanol	115.10	$285.00/25 g		$1140/kg	2.00	5.79	0.67 kg	$759	13.4%
		Trichloro oxadiazole	229.49			$400/kg	1.00	2.89	0.66 kg	$266	4.7%
		CH_3CN									
		1 M HCl									
		$PhCH_3$									
Preparative chromatography to purify intermediate/product in step above: yes = 1, no = 0						0			0.00 kg	$0	0.0%
GSK1292263A (Crude) (Step 3)	92.5%	Aryl fluoride	251.28				1.00	2.63	0.66 kg		
		Primary alcohol	225.29				1.10	2.89	0.65 kg		
		KO*t*Bu	112.21	$82.60/500 g		$ 17/kg	2.00	5.26	0.59 kg		
		DMPU									
		THF									
		Na_2SO_3									
Preparative chromatography to purify intermediate/product in step above: yes = 1, no = 0						0			0.00 kg	$0	0.0%
GSK1292263A (Recrystallized) (Step 4)	90.0%	GSK1292263A	456.56				1.00	2.43	1.11 kg		
		2-Me-THF									
		Darco G-60									
GSK1292263A required (mol)			456.6					2.19	1.00 kg		
	74.8%	Overall yield (longest linear sequence)									
	Total weight of intermediate to prepare 1 kg of GSK1292263A								2.42 kg		
	Total cost of raw materials to prepare 1 kg of GSK1292263A									$5651	100.0%
	Number of chromatographic purifications/1 kg batch of GSK1292263A					0					
	Cost of chromatography to prepare 1 kg of GSK1292263A									$0	0.0%
	Total cost of raw materials + preparative chromatography to prepare 1 kg of GSK1292263A									$5651	

Table 4.3 Labor costs.

Amount of API to be prepared		200 kg (from CoG and variables sheet)					
Product	Operations on scale	Cited scale, starting material (kg)	Approx. time for all operations on cited scale (h)	kg of starting material, kg, to prepare X kg of API	wt/wt scale-up factor, times	Multiplier for scale-up of operation times (factor raised to 0.3 power)	Approx. time for all operations all operations for X kg API (h)
Aryl fluoride (Step 1)	16 h reaction, extract, heat, and cool; concentrate; telescope into next step	73	30	103.0	1.4	1.11	33
Primary alcohol (Step 2)	5 h Suzuki reaction, filter off product, treat w/cysteine to remove Pd, phase splits, concentrate, isolate	27.9	36	132.8	4.8	1.60	57
GSK1292263A (Crude) (Step 3)	1.5 h reaction, quench, phase splits, concentrate, isolate	30	19	132.2	4.4	1.56	30
GSK1292263A (Recrystallized) (Step 4)	Hot polish filtration, concentrate, seed, cool, isolate	30	12.9	222.2	7.4	1.82	24
	Total time for operations						144

Table 4.4 CoGs for various scenarios.

	Scenario 1	Scenario 2	Scenario 3	Scenario 4
Raws as % catalog costs (%)	10	10	5	5
Yield of step 1 (%)	90	90	90	90
Yield of step 2 (%)	93	93	93	93
Yield of step 3 (%)	100	100	100	100
Yield of step 4 (%)	75	90	75	90
Number of chromatographies	0	0	0	0
CoG/kg, 50 kg API batch including labor ($)	7283	6126	4052	3433
CoG/kg, 100 kg API batch including labor ($)	7090	5943	3859	3251
CoG/kg, 200 kg API batch including labor ($)	6971	5831	3740	3138

4.10 Ancillary Expenses

Some additional cost factors of CoG estimates may come into play, both as one-time charges and ongoing charges, as detailed in the following:

4.10.1 Analytical Considerations

Robust in-process controls (IPCs) and analytical methods are usually not available early in development, although they can be essential to reliable and reproducible processes. How data are expressed is one example. For instance, data may be shown as area % (area under the curve (AUC); GCAP may be used for gas chromatography area % and LCAP may be used for HPLC area %) or as weight % (GCWP/LCWP for gas chromatography weight %/HPLC weight %). When GCAP/LCAP are not established, some efforts may rely on liquid chromatography–mass spectrometry (LCMS) or even thin-layer chromatography (TLC). More expense will accrue if data need to be expressed as GCWP/LCWP. Developing robust IPCs and analytical methods can increase the initial CoGs and may be deferred until later in development, or the costs of method development and validation may not be included in a CoG.

4.10.2 Polymorph Screening and Salt Screening

Polymorphs may occur unexpectedly for intermediates and APIs, and the physical state of compounds can adversely affect processing. Early screening is best to preclude surprises. Polymorph screening, usually only for the API, may not be included in a CoG and is usually not conducted early in process development. Notable exceptions may occur for high priority projects or programs: NCEs in which downstream API phase changes can cause significant delays or challenges (e.g. inhalation programs), projects in which early and broad intellectual property protection is critically important, or cases in which bioavailability is critically important.

Salt screening of API follows a similar theme to that of polymorph screening, and both activities are described in details in Chapter 11. In fact, some level of salt screening, if inherent pK_a(s) and pK_b(s) are suitable, may occur in the discovery space. Identifying an appropriate phase to advance from discovery consists of deciding on the polymorph, salt form, and hydrate/solvate. These attributes should support the early toxicology work of maximizing exposure and the early clinical work of possessing appropriate stability. A sample of the desired phase of API typically undergoes some level of stability and accelerated stability studies early in preclinical or clinical development. The costs associated with this work are often excluded from CoG calculations.

4.10.3 cGMP Surcharges

Some CDMOs routinely conduct all operations under cGMP, while other CDMOs may add surcharges for conducting operations under cGMP conditions. Adding surcharges for cGMP operations will increase the CoG, and the impact of such surcharges should be considered during contract negotiations. cGMP processing is required for all steps within the cGMP pocket of the synthetic process, which is defined as all processing downstream of the intermediate identified as the regulatory starting material (RSM). The NCE's sponsor chooses and declares an RSM. Paying for work to be done under cGMP conditions for intermediates before the RSM is superfluous and can result in an inflated CoG for a given delivery. Being aggressive and pushing the RSM designation close to the API can minimize apparent cGMP processing requirements but may also have belated and significant consequences. As regulatory bodies typically do not comment on the decision on an RSM until later in development, an RSM may be challenged by a regulatory agency after batches of an API intended for human dosage were prepared. In this case, if only the steps downstream of the originally defined RSM were done under cGMP conditions, then the program may have used non-cGMP API for cGMP clinical studies. One way to balance these risks is to ensure that one or two steps prior to the declared RSM are run under cGMP conditions. In any case, the cost of cGMP work is a definite factor that must be considered in any CoG calculation.

4.10.4 Critique of the Abilities of Process Groups and Drug Discovery Groups to Advance Development of APIs

Figure 4.6 illustrates some additional considerations contributing to CoGs. These will become more heavily weighted toward the right as an NCE advances from discovery to clinical development. The exact importance of any one of these factors for a given delivery may vary by project, delivery purpose, and financial resources. The diamond-shaped indicator between the medicinal chemistry scale-up end of the spectrum and the process chemistry end of the spectrum in Figure 4.6 shows some arbitrary and relative importance of these attributes for a generalized project. These factors are not meant to be a comprehensive listing of all possible factors and are agnostic of whether the API or intermediate is being produced internally or via a CDMO. API is typically on the critical path

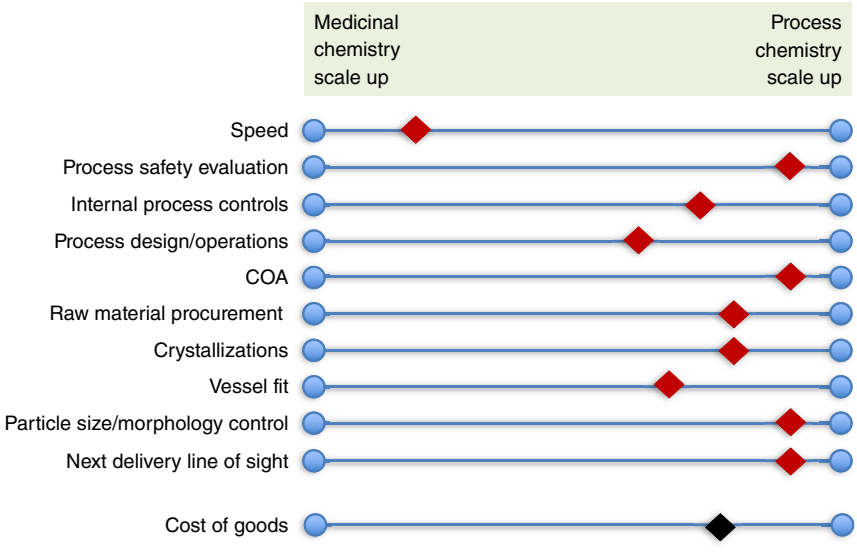


Figure 4.6 Additional considerations contributing to CoGs.

of early discovery and early clinical development support. As such, the speed in which API of acceptable quantity and quality can be made is a constant driving force. Some medicinal chemistry scale-up groups may have limited resources to flag, evaluate, and address safety concerns for a synthetic step, process, or unit operation. This attribute may be a driver to rapidly transition from a medicinal chemistry scale-up group to a process chemistry scale-up group. IPCs are also more robustly defined in a process chemistry scale-up group, as are the certificates of analysis (COA) for the final intermediate or API.

In general, process chemistry support groups will have a much more detailed and quantitative understanding and analysis of the deliverable than will a medicinal chemistry support group. Process groups are better situated to do process and operations design work. Likewise, the procurement teams in place to support process chemistry groups are generally more advanced and focused on scalable, reliable, and inexpensive sources than those used for medicinal chemistry. Process groups are more likely to develop processes that crystallize intermediates and API, which is of paramount importance in any chemical process. This provides reproducibility with regard to the physicochemical attributes and also is an important operation by which to reject unwanted impurities and concomitantly upgrade the purity of the isolated compound. Chromatographic purifications used in medicinal chemistry support are often replaced by telescoping processes in development or by creating a crystallization protocol.

As chemistry advances, consideration should be given to using the correctly sized vessels. For example, if a maximum batch volume would require use of a 100 L reactor, consideration should be given to using a process group that can accommodate this versus a medicinal chemistry scale-up group that may be limited to smaller reactor sizes, which would ultimately lead to multiple batch processing. In a similar fashion, prior to a campaign, it is sometimes useful to

understand how vessels are being utilized. For example, if vessels are going to be used and the maximum volume in each step is well below the maximum volume of the vessel, thought should be given as to why the scale-up group is using larger vessels than are necessary. On the flip side of this point, if the larger vessels are available or necessary, then additional API or intermediate can be prepared by maximizing the reaction volumes in the vessels. This can be done cost-effectively if the production costs greatly exceed the RM costs. This is not a prudent consideration if the RM costs are significant cost drivers relative to the production costs. The maximum process volume/starting material ratio (V_{max}) may also be added to a CoG spreadsheet to help guide scale-up.

As discussed earlier in this chapter, physicochemical attributes of APIs are important criteria to understand and deliver upon scale-up. This may also include particle size control or reduction or even delivering amorphous API, as discussed in Chapters 11 and 13, respectively. Many medicinal chemistry scale-up groups are not situated to deliver against these objectives, and if these attributes are important, use of a process chemistry scale-up group would be appropriate. Finally, a vast amount of institutional knowledge is garnered during any delivery. This knowledge, when generated by process chemists, can readily be utilized in further design, development, and optimization of a chemical process. This first-hand knowledge and hands-on experience are beneficial from a line of sight perspective into subsequent deliveries.

To conclude, as an NCE progresses into development, development activities should be performed by development chemists. The amount of work on any one of these attributes may vary from delivery and project relative to another attribute. Each of these components will impact the CoG for any given API or intermediate campaign. For efficient development of an NCE it is essential to utilize an appropriate process chemistry team, internal or external to the company advancing the NCE, to support scale-up campaigns.

4.11 Long-Term Considerations

The technology needed for a key operation may prompt the development of alternative routes. For instance, organizations without large Hastelloy reactors in-house may not develop projects involving batch operations at −70 °C. Similarly, organizations without reactors for high-pressure hydrogenations on scale may develop alternative steps.

Many factors are important in selecting a CDMO and hence can affect the CoG. Organizations that have the capabilities for process research, process development, and manufacture should be prepared for a more facile technology transfer with CDMOs. If a CDMO is not well versed in the technology needed, then additional research and costs may accrue. In writing a contract with a CDMO, it is important to describe the quantity of material needed, the timelines for the product, and the desired quality of the product, e.g. LCAP or LCWP. The timing of production batches can be critical, and some flexibility by the CDMO is often needed since research rarely proceeds as expected. Attorneys may be involved with the wording of contracts involving large sums of money.

Some "soft" considerations in selecting a CDMO can dramatically influence the success of a project and the CoG. Approaching a CDMO as a partner rather than another pair of hands can lead to more inputs that aid process development and manufacturing. To this end, meeting the personnel of the CDMO face to face can be very helpful.

When manufacturing an API, having reproducible operations is crucial. Reproducible operations should lead to control of the API and hence ensure the safety of the drug product. Reproducible operations will also ensure routine productivity on scale. Applying a numerical value to the reproducibility of processes is difficult early in the development of an NCE, although chemists and engineers developing the processes may understand the troublesome points of the operations. During routine manufacturing operations, reproducibility can be judged by the portion of batches that are out of specification and by the cycle times of batches. A process that has wider proven acceptable ranges (PARs) but lower productivity may be preferred if it is more reproducible.

An illustration of this is outlined in the following examples. If one step of a process is run on laboratory scale five times prior to scale-up in a kilogram lab and the yields (either isolated or assay) are 51%, 53%, 50%, 51%, and 53%, then the reaction is "in control." although the yield is poor. A heightened confidence level is attained when planning to scale up reactions that are in control, as the risk that the yield (save for operational error) will deviate significantly from the laboratory runs is lessened relative to a reaction that is not in control. An example of this may be a reaction that provides laboratory scale yields of 51%, 53%, 50%, 51%, and 83%. In this case, one of the reactions provided a yield that was significantly higher than the others. In the absence of an understanding of why one run provided a significantly higher yield of product, another run may produce a yield lower than 50%. Planning for the first case is relatively straightforward; however, dealing with an out-of-control process can result in higher CoGs by primarily two mechanisms. First, additional time and resources may be dedicated to developing a process that is "in control," which will correspondingly add to the CoGs for a given campaign. The payoff, however, may be huge. In the example above, it is usually a matter of time and rigorous process research and mechanistic understanding to ultimately attain a reproducible yield of 83% or greater. Second, it may be prudent to assume that less than a 50% yield will be obtained upon scale-up of the "out-of-control" process. In this case, the risk of missing the target deliverable quantity is mitigated, but an increased RM and supporting reagent burden is incurred, which likewise feeds into a higher campaign CoG.

4.12 Summary

CoG estimates are an important tool of developing APIs, thus serving a crucial and strategic need during early drug development. Many CoG aspects must be considered, such as route selection, operations, and emerging technologies [31]. In preparing a CoG estimate, it is essential to clarify the variables and assumptions. Throughout multiple stages of development, CoG estimates can

be prepared to guide the development of cost-effective processes and be used as a tool when evaluating different routes or processes to prepare an intermediate or API.

Acknowledgments

N. G. A. thanks Olivier Dapremont for discussions on SMB. N. G. A. is grateful for tours of SMB facilities at Lundbeck in Denmark (2001) and at AFC in California (2016). The authors also thank R. Scott Hoerrner for review of this manuscript.

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5

New Technologies in Process Development

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5.1 Introduction

The pharmaceutical industry is undergoing rapid changes in the way in which it seeks to bring new medicines to market. There are considerable increasing cost pressures arising from the need to treat more people for less cost. This is being addressed in a multitude of different ways: for example, by seeking to develop new molecules that are better defined as quality candidates, with less chance of costly failures, as the candidate is progressed, and by establishing faster ways to develop new drugs, particularly with respect to clinical trials using smaller numbers of subjects with higher quality data and, of course, cheaper ways to actually synthesize the new drugs. For these reasons, the hunt for cheaper, more sustainable synthetic approaches is becoming an increasingly important factor in process development.

It is becoming increasingly difficult to find transformational new approaches to the synthesis of small molecule active pharmaceutical ingredients (APIs) using the same organic chemistry approaches that have been used over the last century. Instead newer technologies are being considered and developed into processes. This chapter will focus on synthetic biocatalysis, catalysis, and continuous chemistry as these are the three technologies that are rapidly gaining traction within the industry and are transforming process chemistry during early clinical development and beyond.

Fermentation to give natural products has long been used to access cheap products. However, it is limited to give materials that are final products from natural biochemical pathways or close analogs. Metabolic engineering can be used to knock out a gene responsible for an unnecessary part of the pathway. The resultant product of the truncated pathway is still obtained by following the biochemical pathway to this certain point where the machinery for the next step is no longer present. This allows the buildup, and isolation, of this "pathway" material.

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

A more versatile approach is biocatalysis. Changes in the way in which molecular biology is conducted, alongside an ever-increasing number of available enzymes and associated functional knowledge of these enzymes, are driving a considerable resurgence of biocatalysis as a synthetic tool. An industry has grown out of the increasing knowledge of genes and proteins (enzymes) whereby the gene that can produce the desired protein can be ordered online at a rapidly decreasing cost. The gene is usually ordered in a vector, often a plasmid, which can be routinely transformed into a production organism for expression and subsequent isolation. This process is now so simple that most enzymes can be rapidly obtained in sufficient quantity for development purposes.

There has also been a fundamental shift in the way in which enzymes are being deployed as biocatalysts. Originally, one would "fit the process to the enzyme" by altering the process to enable a limited number of nonideal enzymes to enact the desired chemical transformation. This concept is rapidly being overtaken by the new paradigm of "fitting the enzyme to the process," as the speed and efficiency of evolving a poorly active/stable/stereo- or enantioselective enzyme into one with the desired properties becomes faster and more efficient through the industrialization (automation) of the enzyme evolution process.

Synthetic biology has vast potential for the future of effective syntheses of small molecules but is currently being used in an opportunistic sense. We cannot yet create pathways to all the desired small molecules we might wish to obtain as effectively (predictably) as we can use a mature science like organic chemistry. However, the tools being developed for this burgeoning new applied science are progressing rapidly, and this is highly likely to be seen as a favored approach in the future.

The use of a biocatalyst (either as a free or immobilized enzyme) in conventional glassware (either a flask in a laboratory or a reactor in a pilot plant) is becoming commonplace. Although taking advantage of the enzymes intrinsically high level of control in a variety of different reactions can now be readily achieved, it does still suffer from many of the issues associated with batch processes: (i) mixing starting materials with reactants and catalysts/biocatalysts mixed with solvent, (ii) stirring to allow a chemical transformation to occur, and (iii) isolating the intermediate/product from the other components of the reaction via an often laborious work-up. One new technology that addresses these issues is flow or continuous chemistry.

Continuous chemistry, like biocatalysis, has been successfully used by a variety of chemical industries for decades, but its widespread use within the pharmaceutical industry has been slow to progress. It has very significant advantages in that intermediates are not isolated and the inventory of reactive materials undergoing a chemical transformation is restricted and can therefore be handled with much greater control. This can take the form of performing reactions that chemists would not routinely conduct in a batch sense due to the serious safety risks associated, e.g. high temperature chemistry on scale, effectively adding new reaction types to the chemists' repertoire. The high level of control achieved with continuous chemistry is apparent once steady state has been achieved, where it leads to a consistently high degree of quality control (e.g. impurity profile).

Chemical catalysis is perhaps the technology most chemists are comfortable using. It is increasingly seen as a way to achieve transformational changes to chemical routes. There are a huge variety of catalysts that the chemist can employ, and there are many established processes that already use catalysis. The increasing access to novel ligands to impart new reactivities to metal-centered catalysts, preceded by considerable academic interest, has really led to a marked increase in the use of catalysis for a great variety of chemistry, especially for hydrogenations and coupling processes (e.g. Suzuki reactions).

These "new" technologies are all being progressed today with an increasing emphasis on automation. The introduction of reliable, easy to operate automated systems is adding a new dimension to the way many scientists conduct their work. Design of Experiments and Quality by Design are now common phrases to development chemists. The need to create clear operating ranges for chemistry conducted at manufacturing sites is reflected in the changing expectations from a regulatory perspective. The need to be able to define clear operating ranges for all parts of a chemical process such that any small changes are understood in terms of the change in yield and especially quality is essential. Knowing these ranges and being able to justify them in regulatory filings is a prerequisite for launching new drugs to the market. Statistical design of experiments to get the maximum amount of data from the minimum number of experiments, seeking to understand which factors influence the quality of intermediates and ultimately the API, is widely used within the pharma industry. The ongoing paradigm shift is to ensure that the highest quality data is achieved in this process by using automated equipment that introduces less variance into the experiments than when conducted by hand. Automation has long been a principle of many full manufacturing processes, but this principle is now being driven backward into the development process and is used widely for screening, routine analysis, and optimization of reaction conditions.

Sustainable manufacturing is no longer seen as a nice-to-have. Developing processes that use "green chemistry," where the principles laid out by Anastas and Warner are routinely considered and incorporated into new routes for API synthesis [1], is nowadays an expectation, not an exception. The new technologies (synthetic biochemistry, catalysis, and flow) are all exemplars of green technologies. Biocatalysis is frequently conducted in water at temperatures that are close to ambient (safe, energy efficient, and minimizes organic waste). Catalysis performs chemistry with increased selectivities and thus higher yields often with catalysts that can be recycled and metals that can be recovered. Continuous chemistry allows chemistry that could be hazardous to perform in a batch process to be performed safely. It also reduces the amount of waste by introducing a high level of control of impurities for each chemical reaction such that isolation of intermediates can be avoided.

Perhaps the most significant aspect of all these technologies, from a sustainable perspective, is that all of them open up new chemical opportunities that would not be readily available using "conventional" chemistry. All of them can lead to entirely new routes that are shorter, safer, and more mass efficient. They can be truly transformational to a drug discovery program transitioning to clinical development.

5.2 Synthetic Biochemistry

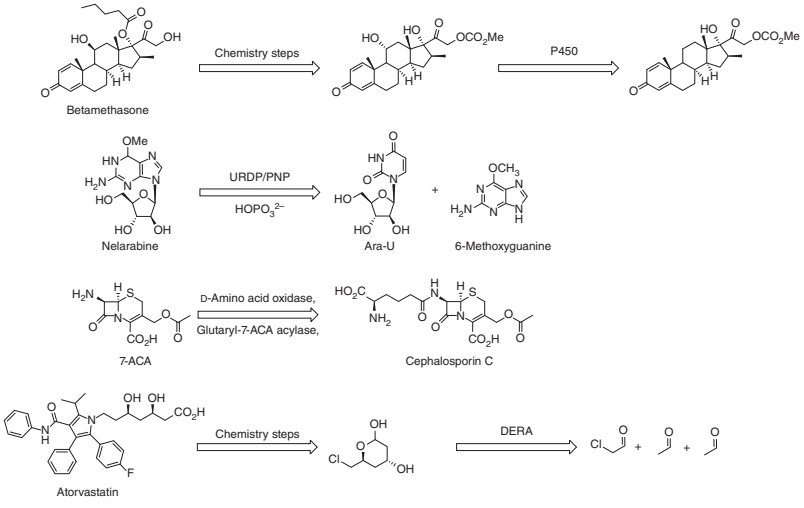
Synthetic biochemistry is an umbrella term used (within GSK, GlaxoSmithKline) to encompass the use of biocatalysis to perform chemical synthesis. This can range from the use of single, partially purified, free, or immobilized enzymes in single synthetic steps (single-step biotransformations) to the production of natural products or their analogs from cheap raw materials (fermentation). This section will focus only on single and multistep biotransformations, using biocatalysts in the form of whole cells, partially purified enzymes, or immobilized enzymes, as these processes can be readily added to the chemist's armory, given a little training, and can often be employed in standard apparatus.

Due to the high selectivity, the relatively benign nonhazardous reaction conditions, and the availability of biocatalysts from renewable feedstocks, biocatalysis has been viewed as an attractive technology for decades. In fact, within the pharmaceutical industry biocatalysis has already delivered huge successes toward the production of a range of major drug classes and has been extensively reviewed elsewhere [2–4]. For example, the microbial hydroxylation of steroids through whole cell biocatalysis, which has enabled the efficient production of precursors of steroidal antiasthma drugs, the production of semisynthetic beta-lactam antibiotics using immobilized enzyme technology, the access to antiviral nucleoside analogs through dual enzyme cascades, and the use of enzyme-catalyzed domino reactions for the production of statins represent just a few high profile cases (Scheme 5.1). All of these products would have required substantially lengthier approaches using traditional synthetic chemistry that could have provided a significant barrier to their commercial viability due to cost, quality, or security of supply.

Fermentation technology is well established as an efficient approach to access highly complex scaffolds. In the fine chemical industry, 35% of chiral building blocks were reported to be produced using generic biocatalytic technologies in 2007 [5], and many industrial processes have been developed to meet pharma needs for chiral building blocks [6]. In contrast, biocatalysis has not yet been broadly adopted by either medicinal or process chemists within pharma itself, despite the above successes, for a variety of reasons such as:

- A restricted number of biocatalysts available and narrow substrate scope.
- Long process development times.
- Typically, high costs and poor reproducibility between batches of many commercial biocatalysts, particularly for use in early phase kilo scale syntheses.
- The requirement for specialized skills.
- The high cost of biocatalyst improvement that is typically required to meet production needs.

This situation is now rapidly changing and offers new opportunities to the pharmaceutical industry. Here we will discuss the current state of the art in established and emerging biocatalysis capability, attempt to address the perceived issues highlighted above, and outline some of the limitations and future needs.



Scheme 5.1 Retrosynthetic steps involving some well-known industrial biocatalytic steps. Enzyme acronyms: P450, cytochrome P450 monooxygenase; URDP, uridine phosphorylase; PNP, purine nucleoside phosphorylase; DERA, 2-deoxyribose-5-phosphate aldolase.

5.2.1 Current State Biocatalysis

Figure 5.1 shows a breakdown of the 2013 product portfolios of three major pharmaceutical companies by reaction type.[1] These data are broadly in agreement with data compiled in 2005 by Carey et al., where notably, 74% of the chiral centers were bought in [7]. Schneider et al. arrived at a similar conclusion by the utilization of data mining techniques to survey the reactions and products published in granted US patents and patent applications between 1976 and 2015 [8], thereby further validating these "snapshot in time" analyses.

In past decades, in the absence of dedicated micro- and molecular biological support, a chemist might only have realistically expected to identify suitable biocatalysts toward a narrow range of reaction types – notably, ester/amide/carbamate protection or deprotection reactions – based on the screening of a limited number of commercial hydrolase enzyme kits available at the time. These would typically have been employed for kinetic resolutions or desymmetrizations and then only as a method of last resort where traditional approaches had been exhausted. Compared with a chemical reagent that might be expected to perform a target reaction on >95% of occasions, these hydrolase enzyme kits were far from adequate, hence their poor adoption. Worse still, as many of these commercial enzymes are produced for larger markets (e.g. detergents), a biocatalyst that is available from one supplier during one campaign might no longer have been available, or the specification might have changed by the next, leaving the project team with the task of developing a new process or substantially altering their current one. Alternatively, the enzyme hit might have been of mammalian origin that adds an additional level of complexity or, more likely, would prevent its use due to the need to navigate complex transmissible spongiform encephalopathy (TSE) risks [9].

Many of the other major reaction types, such as N-, O-, or C-alkylation, C–C bond formation, halogenation, and various oxidation and reduction reactions, were known to exist in biochemical pathways or had been demonstrated in whole cell biotransformations using microbial cultures. Most chemists, while designing a synthetic route, will have at some time identified such a biotransformation from the literature only to find that the desired enzyme is not available or have ordered small quantities of an enzyme with a similar name from a catalog company only to find that it does not give the desired reaction, suffers from other process related limitations, or is unavailable on scale.

The emergence of high-throughput genome sequencing enabled by an exponential increase in computing power, bioinformatics, cheap gene synthesis, improved high-throughput screening technology, improved molecular biology, and enzyme engineering techniques and the emergence of metagenomics to access DNA from the vast array of uncultivable microorganisms have all been fundamental in transforming this situation. In fact, it is now possible to rapidly mine a huge number of enzyme and genome sequences online, thus avoiding

1 The work leading to these results received funding from the Innovative Medicines Initiative Joint Undertaking project CHEM21 under grant agreement n°115360, resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' in kind contribution.

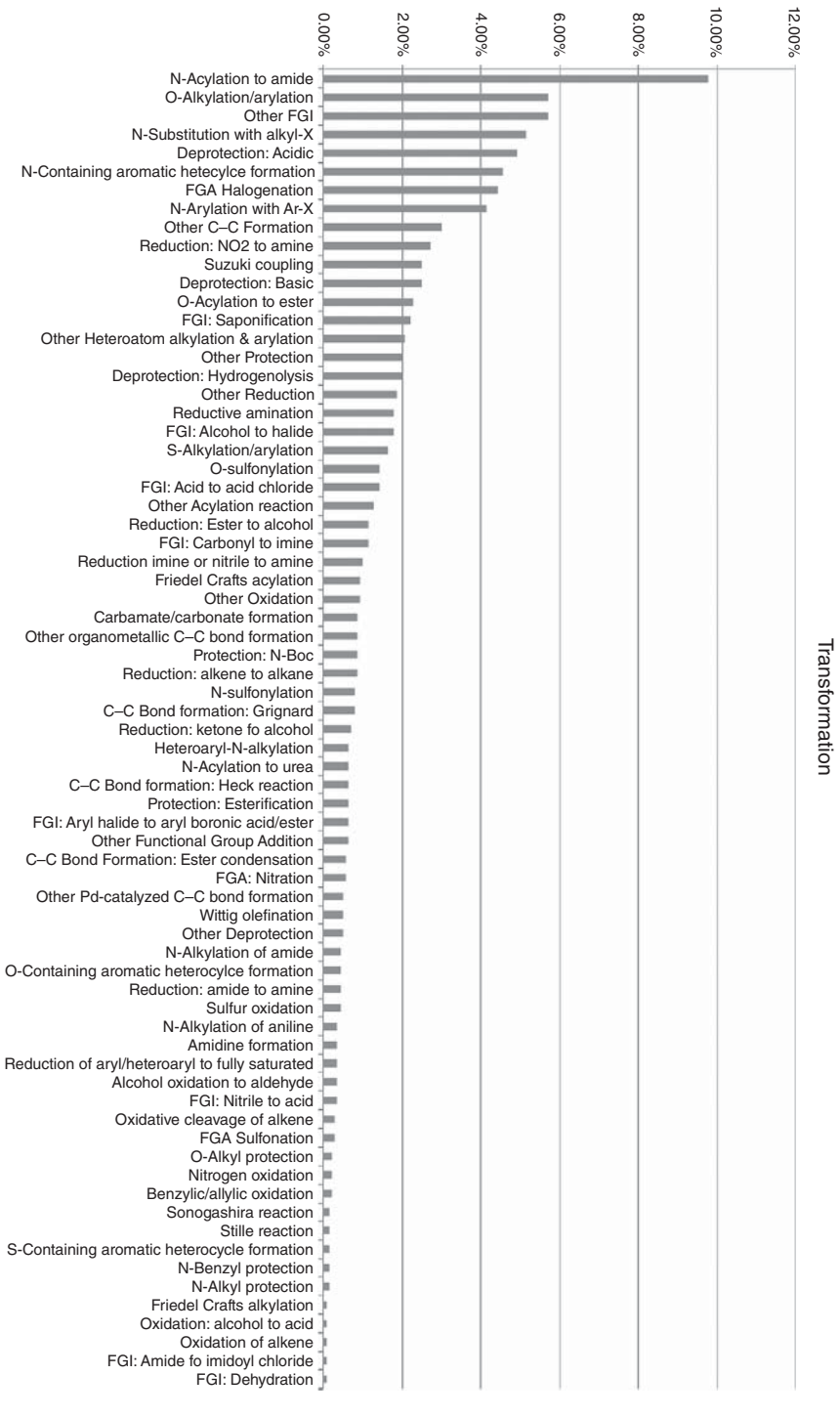
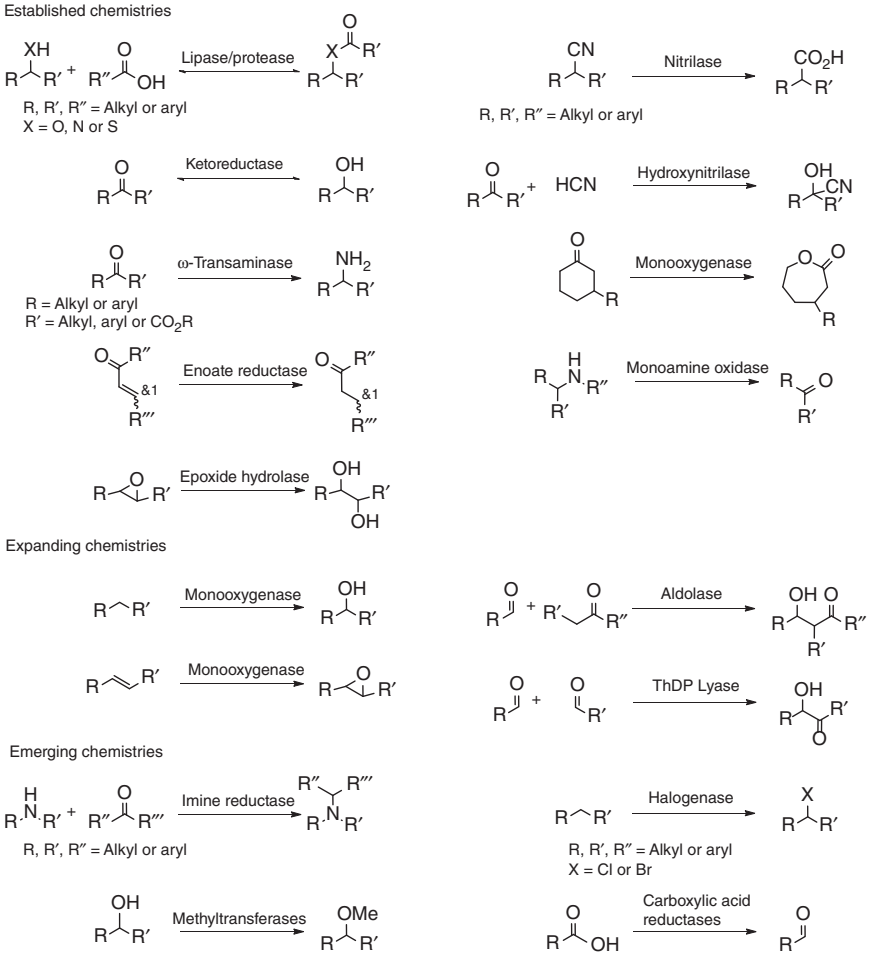


Figure 5.1 Graph prepared from the CHEM21 EFPIA members portfolio review of synthetic routes to prepare active pharmaceutical ingredients (see footnote 1).

the need to source the original microorganisms, order them online in suitable vectors, and transform and express them within a well-characterized host a couple of weeks later after they have been delivered [10]. The explosion in the number of enzymes that can be quickly obtained has led to the creation of enzyme panels covering huge substrate space, containing highly selective wild-type and/or variant enzymes that are often enantiocomplementary, thus addressing past wishes for "enantiomeric" enzymes [11]. Furthermore, some enzymes of mammalian origin are now also available in recombinant form, avoiding TSE risks by expression of the synthetic gene sequence in a microbial host [12]. This increase in accessibility has led to a significant rise in the range of enzyme classes under investigation and an expansion in the number of academic and industrial groups performing the research [13].

Numerous commercial suppliers are now available that produce kits or panels of the most established classes of enzyme. These have become the "workhorses"



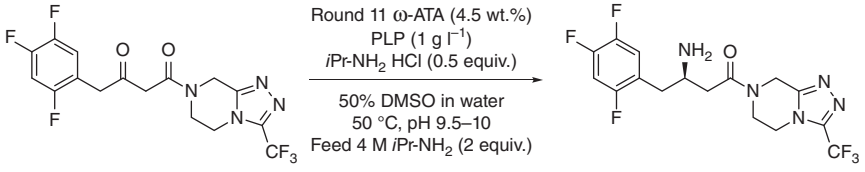
Scheme 5.2 Status of various biotransformations (not exhaustive).

of biocatalysis and have all been extensively reviewed elsewhere (Scheme 5.2) [14]. A list of suppliers has recently been compiled by Rozzell [15]. With these enzyme suppliers in hand, a significant number of major reaction classes, some of which have been highlighted by the American Chemical Society Green Chemistry Institute Pharmaceutical Roundtable (ACS GCIPR) as in need of more sustainable solutions, can now be targeted using biocatalysis [16]. Furthermore, the researcher can be relatively confident in finding an enzyme that will provide the desired activity and selectivity toward a given substrate. Single sourcing risks may be avoided either by identifying suitable hits from different suppliers, by buying the gene for in-house enzyme production or production using a third party, or by setting up an agreement with the supplier should they discontinue production themselves.

Many of these suppliers will provide either sets or panels of the appropriate enzymes, depending on the screening capabilities of the customer, or even provide screening services and are capable of supplying any hit enzyme on a multi-kilo scale. Substantial effort is ongoing to hone these enzyme panels by fingerprinting them toward diverse substrate sets in order to weed out enzymes with degenerate selectivity and cover gaps.

Most chemists have the view that enzymes can only be used in dilute aqueous environments and are unstable to high temperature. However, this is not always the case, and many wild-type enzymes have been used in neat organic solvent. Furthermore, enzymes from thermophilic microorganisms have proven to be a good source of high temperature and solvent-tolerant enzymes [17, 18]. In fact, the current record for high temperature biocatalysis is held by the protein bovine serum albumin (BSA) that is capable of hydrolyzing *p*-nitrophenyl palmitate at temperatures as high as 160 °C [19]. Therefore, enzyme kits and panels, even those containing wild-type (or natural) enzymes, often provide hits that can be used as a starting point for the preparation of gram or even kilo quantities of product, even if not ideal for use in a production process that can require extreme conditions for an enzyme to operate under to meet the necessary criteria [20]. To meet production needs, now well-established directed evolution and/or rational enzyme engineering approaches [21–23] can be used to modify the enzyme to fit a desire. This was elegantly demonstrated through a Codexis/Merck collaborative effort that resulted in the development of a highly engineered ω-transaminase (ω-ATA), through 11 rounds of directed evolution, to perform the final asymmetric chemistry stage in the production of sitagliptin (Scheme 5.3) [24]. Notably in this case, no enzyme initially gave activity toward the desired substrate and so had to be evolved through a process of substrate walking, starting from a model substrate, ultimately to afford a highly active and enantioselective enzyme capable of withstanding highly denaturing organic solvent/aqueous reaction conditions. Eleven rounds of directed evolution might take up to 9 months to complete, but suitably improved variants can often be identified in just a couple of weeks by performing fewer rounds and targeting particular residues within the protein to mutate.

Availability of such a broad range of enzymes on scale allows the chemist to use these materials in reactions in a similar fashion to any chemocatalyst, with many similar issues encountered during process development en route



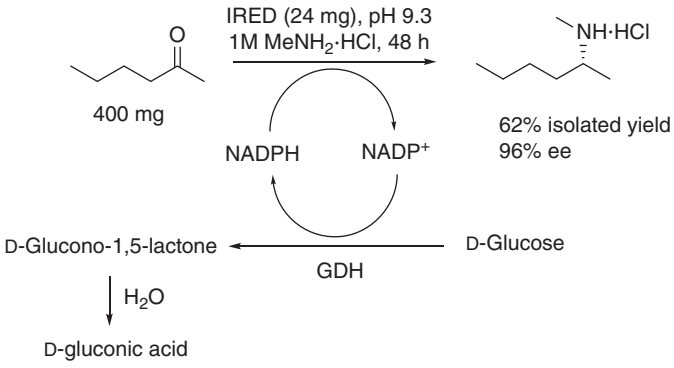
Scheme 5.3 Transaminase-catalyzed preparation of sitagliptin.

to the identification of a robust process that maximizes product yield, quality, and productivity while minimizing cost. Product isolation can frequently be a challenge due to the tendency of many enzymes to form an emulsion during extraction. Given their allergenic nature, it is also important to develop suitable analytical methods to ensure that the product is not contaminated with protein, particularly if the biotransformation is the final chemistry stage in the synthesis of an API [9]. Emulsion formation can often be minimized or avoided by enzyme precipitation (with a water miscible organic solvent or pH change) and filtration, followed by removal of the solvent by distillation, and subsequent extraction of the product from the remaining aqueous portion. Alternatively, more efficient approaches that avoid the need to remove precipitated enzyme through filtration or centrifugation are also being developed such as the use of catastrophic phase inversion [25]. Enzyme immobilization has frequently been used to solve product recovery issues, minimize protein contamination, and reduce costs where enzyme recovery and recycle is a key cost factor, at least for enzymes that do not require cofactors [26–28]. Practical implementation of immobilized cofactor-dependent enzymes is far less developed (although examples using ω-transaminases [29] and ketoreductases (KREDs) [30] are now being realized) and examples using oxygen-dependent enzymes even less so. However, neither of these approaches is ideal, and as directed evolution and rational enzyme engineering strategies mature, it is becoming increasingly realistic to consider the production of highly active enzyme variants that are present in such low quantities within the reaction mixture that they no longer impede product extraction. For example, while engineering a highly enantioselective and robust KRED variant for the production of a Montelukast intermediate [31], Ulijn et al. only selected variants between rounds that gave improved separation properties in addition to the main target properties.

5.2.2 New Single-step Biotransformations

Whereas the repertoire of enzyme types available and approaches to modify them to suit production needs are rapidly progressing, there are still significant gaps in the transformation types that are currently available within the biocatalysis toolbox. Efforts to fill these gaps are progressing at varying rates depending on the enzyme class in question. Some particularly exciting recent developments are described below.

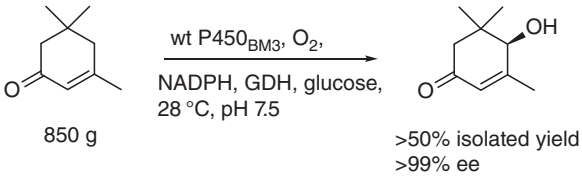
Secondary and tertiary amines are present in a significant proportion of drugs and are typically accessed using traditional N-alkylation/arylation or reductive amination procedures, each of which frequently suffers from selectivity issues



Scheme 5.4 Preparative scale IRED-catalyzed reductive amination of hexan-2-one.

or the use of hazardous reagents. Following initial work by Mitsukura et al. who identified enantiocomplementary imine reductase (IRED) activity toward the cyclic imine 2-methyl-1-pyrroline in *Streptomyces* species, a broad range of NADPH-dependent (*R*)- and (*S*)-selective cyclic IREDs that demonstrate activity toward both imine and imminium reduction have since been identified from databases, and this rapidly expanding field has recently been reviewed by Grogan and Turner [32]. Particularly exciting is that some of these IREDs display reductive aminase activity. In a recent example, Wetzl et al. screened a set of 28 IREDs for reductive amination activity, diastereoselectivity, and enantioselectivity toward a small set of cyclic and acyclic ketone and primary amine substrates [33]. All expected products were afforded to some extent by at least one enzyme, with variable yield and selectivity. On a preparative scale, in the presence of a 24 mg loading of crude IRED from *Streptomyces tsukubaensis*, a 100 mM solution of hexan-2-one (400 mg) in a 1 M aqueous solution of methylamine (5 equiv.) at pH 9.3 containing a glucose dehydrogenase (GDH) cofactor recycling system and catalytic NADPH gave a moderate yield of (*R*)-amine product in 96% ee (Scheme 5.4). The treatment of (*R*)-3-methyl-cyclohexanone under similar conditions gave a 50% yield of the *syn*-product in 94% de.

Oxidation does not feature highly in the pharma reaction repertoire shown in Figure 5.1 because, until recently, there have been few efficient and sustainable reagents available. As a result, synthetic routes that avoid such transformations have typically been sought. Progress in the development of chemocatalytic oxidation methods is discussed later in this chapter, and a general review on progress in the development of biocatalytic oxidation methods has been published elsewhere [34]. Of the biocatalytic approaches available, the cytochrome P450 (CYP) hydroxylation of nonactivated C–H bonds has inspired the most interest, but its high potential has yet to be fully realized due to numerous issues such as low activity, stability, uncoupling, and complex processing issues due to the requirement for oxygen and an electron source [35]. However, the application of directed evolution techniques over the last few years have led to rapid advances in this field, which hold significant promise for the future, as recently reviewed by Roiban and Reetz [36].



Scheme 5.5 *In vitro* biohydroxylation of α-isophorone using wt-$P450_{BM3}$.

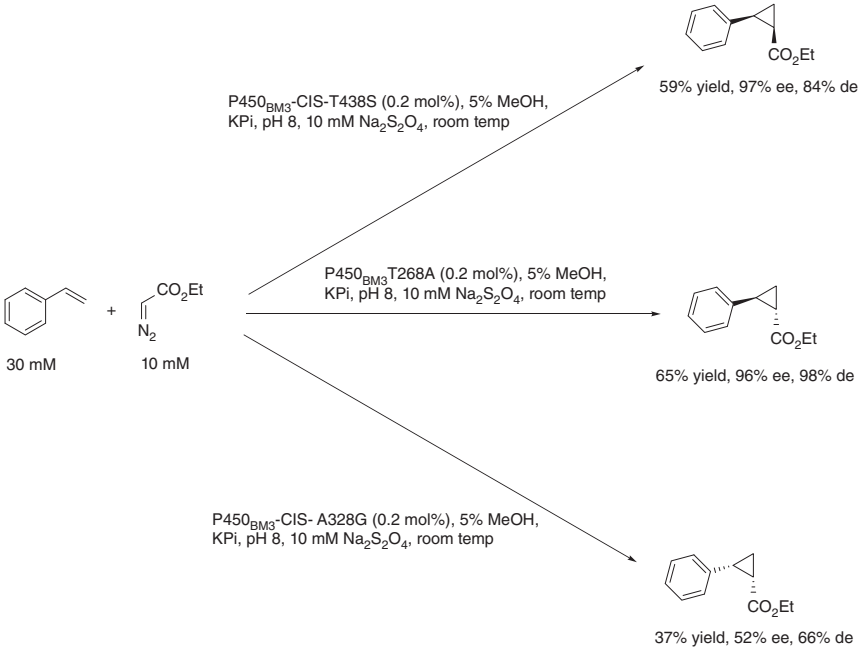
Using wild-type P450 from *Bacillus megaterium* ($P450_{BM3}$), Kaluzna et al. of DSM recently reported the first successful large-scale demonstration of an *in vitro* P450-catalyzed biohydroxylation [37]. Following optimization, a 10 g l^{-1} solution of the lipophilic substrate α-isophorone was transformed to (*R*)-4-hydroxyisophorone on a 100 l scale over a 10 h period using a freeze/thawed whole cell preparation containing co-expressed $P450_{BM3}$ and GDH (in order to minimize mass transfer limitations and maximize coupling efficiency, enzyme stability, and cofactor recycling efficiency) in the presence of excess glucose and exogenous NADP under pH-controlled conditions (Scheme 5.5). This reproducibly led to >80% conversions with 94% regioselectivity, affording the desired product in >50% isolated yields and >99% ee following work-up. The key to success was the use of a pure oxygen feed that avoided foaming and enzyme denaturation as seen with an air feed and also increased oxygen transfer by fourfold. The use of pure oxygen can be extremely hazardous and required careful monitoring of the oxygen content in the reaction mixture and headspace. The oxygen feed was set to ensure that levels did not exceed 5% in the reactor and a nitrogen sweep maintained the headspace oxygen level below 20%.

Liese and coworkers published a very similar approach using α-ionone as substrate, a crude cell-free extract of a $P450_{BM3}$ triple mutant, and GDH cofactor recycling system [38]. The use of surfactant and low cosolvent volumes aided low substrate solubility, leading to a positive impact on reaction rate, as did the use of pure oxygen supplied using a gassed stirred tank reactor. The main limitation to the use of this technology was found to be stability of the P450, with total turnover numbers typically in the range of 5000 irrespective of the conditions used, which restricts its use to high value processes.

Following the discovery of *Agrocybe aegerita* (*Aae*UPO) and demonstration of its synthetic utility by Ullrich et al. [39], unspecific peroxygenases (UPOs) are attracting much attention as potential alternatives to P450s due to their ability to catalyze the same reaction classes but without gas transfer limitations (they use hydrogen peroxide as oxidant), without the need for cofactor and with activity and stability toward solvents including neat organic solvent. Such properties could make them an ideal easy to use tool, particularly for the functionalization of lead compounds by the medicinal chemist. UPOs have recently been reviewed by Bormann et al. [40], and interestingly, the well-known chloroperoxidase from *Caldariomyces fumago* (*Cfu*CPO), which displays excellent enantioselectivities but poor activity, falls into this enzyme class. Whereas *Aae*UPO is significantly more active and stable than *Cfu*CPO, difficulties in heterologous expression of the former is hampering its wider evaluation. Recent success in evolving a

peroxygenase variant that confers soluble expression of active and stable enzyme from *Saccharomyces cerevisiae* could aid this goal [41].

It has long been known that many enzymes are promiscuous, catalyzing additional reaction types to those of their biological function. P450s are one such example, catalyzing hydroxylation, epoxidation, and dealkylation reactions, to name but a few. Inspired by chemistry, Arnold and coworkers were interested to know whether P450s could also catalyze aqueous phase cyclopropanation reactions that are isoelectronic with epoxidation reactions, but not seen in nature due to the absence of the necessary carbene [42]. Using the metal-catalyzed conversion of styrene and ethyl diazoacetate to ethyl-2-phenylcyclopropane-1-carboxylate as a model cyclopropanation reaction, most heme-containing enzymes tested gave similar low conversions and selectivities to those previously seen with the chemical catalyst, hemin, whereas wt-$P450_{BM3}$ gave a lower conversion, but a shifted diastereoselectivity along with some enantioselectivity, albeit low. Testing a range of 92 $P450_{BM3}$ variants that were already available within the group, hits were identified that could almost exclusively produce either *cis*- or *trans*-diastereomer of the (2*S*)-enantiomer in moderate yield and with total turnover numbers of >200 within a 2 h reaction period (Scheme 5.6). Concentrating on the more difficult to produce *cis*-products, further active-site engineering of $P450_{BM3}$ led to a variant $P450_{BM3}$T268A with inverted stereoselection. Importantly, it was found that oxygen is not required for the reaction to progress and, in fact, that it inhibits it, necessitating the need to perform reactions under an



Scheme 5.6 Cyclopropanation reactions catalyzed by $P450_{BM3}$ lysates.

inert atmosphere. A chemical reductant was also necessary to reduce spent catalyst back to the catalytically competent Fe(II) species. Testing a small range of substrates, both electron withdrawing and accepting *para*-substituted and branched styrenes were tolerated. The group has recently reviewed this work and other P450-catalyzed carbene reactions, including S–H, N–H, and nitrene insertions and aziridinations that they have since identified [43].

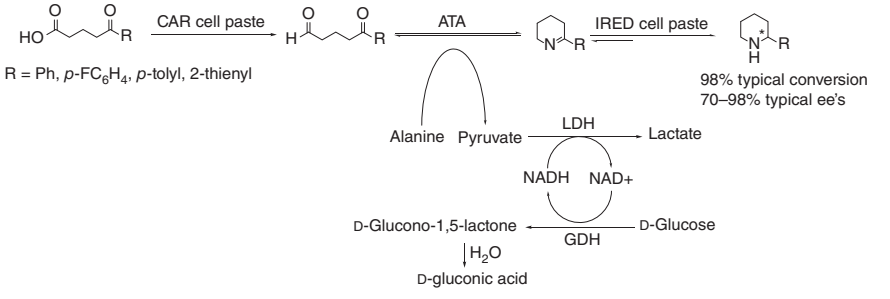
With these established and emerging biotransformations in hand, the theoretical percentage of pharma reactions that are accessible has grown from about 25% a decade ago to around 40% today and is likely to achieve about 65% in the future (GSK, unpublished data). The number of opportunities that are practically achievable is also expected to grow as kit and panel coverage improves, although the value in pursuing each of these opportunities will of course require pragmatic analysis on an individual basis against other available technologies.

5.2.3 Cascade Biotransformations

Single-step biotransformations using isolated enzymes can provide significant advantages over chemical methods as discussed above. However, whole cell processes allow the use of a broader range of enzymes that are less amenable to isolation or require use of the cellular machinery to supply substrates or cofactors that are, respectively, too expensive to produce by other means or cannot be efficiently recycled *in vitro*. However, such processes usually need to be run on a far higher volume than their chemical counterparts (100 vols rather than 10 vols), require the use of specialist fermentation kit and specialist skills, and require complex downstream processing as previously mentioned. All of these points can make such processes prohibitively expensive to introduce, particularly into pharmaceutical or fine chemical settings where a multipurpose kit is generally preferred, except in cases where this approach offers particularly high added value over the alternatives. Further harnessing the natural selectivity of enzymes by the catalysis of sequential reactions using more than one enzyme in tandem (now most commonly termed as an enzyme cascade) [44] offers to increase the value ratio of product to substrate achieved within a single chemistry stage, therefore offsetting some of these limitations in the same manner as a fermentation. By doing so, this approach also offers the opportunity to substantially reduce labor and overhead costs that can contribute as much as 50% total cost of goods for a pharmaceutical product by reducing the number of isolation stages and reducing solvent consumption for which there is typically a greater demand during reaction work-up and reactor cleaning than during the reaction itself. A cascade approach can also allow the control of unfavorable equilibria, facilitate cofactor regeneration by using a more efficient sequential rather than tradition approach, and enable the application of routes that require unstable or toxic intermediates through transient *in situ* generation. This rapidly expanding field has been the topic of several recent reviews that cover a plethora of different cascade approaches from the reconstitution of artificial pathways or truncated pathways in heterologous hosts to the development of artificial pathways both *in vivo* and *in vitro* using free and immobilized enzymes or even artificial enzymes [45–47]. Given the breadth of

this rapidly expanding field, only a couple of artificial pathways are shown below that have been recently developed to overcome well-known chemistry issues.

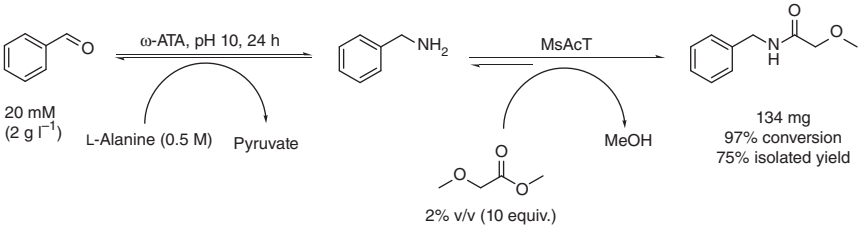
Aldehydes can be unstable, toxic, and challenging to produce chemically, often requiring the use of hazardous reagents. Carboxylic acid reductases (CARs), which have recently been reviewed [48, 49], offer an attractive alternative but need to be activated using a phosphopantetheinyl transferase (PPTase) enzyme, which in turn requires ATP that is expensive and not readily recycled *in vitro*. As a consequence, carboxylic acid reduction would need to be performed *in vivo* on scale, but the resultant aldehyde products, which are highly reactive and toxic to the host cells, are typically produced in low conversions/product concentrations due to either further reduction by endogenous enzymes or death of the host cells. An efficient *in situ* product recovery (ISPR) method or further conversion to a more manageable high value product by use of an enzyme cascade is therefore required to overcome these limitations. To this effect, a number of cascade approaches have recently sought to incorporate CARs. France et al. used a retrosynthetic approach that led to the design and development of highly efficient CAR/ATA/IRED cascades toward the synthesis of a range of mono- and bifuntionalized pyrrolidines and piperidines [50]. This approach is particularly attractive as it harnesses many of the key benefits of biocatalysis such as chemoselectivity, enantioselectivity, and diastereoselectivity in a single pot. Focusing on the simplest case, either enantiomer of a range of 2-aryl-substituted piperidines could be accessed in excellent conversion and enantioselectivity using 75 mg ml^{-1} of wet cells containing CAR from *Mycobacterium marinum* (mCAR) activated with PPTase from *Bacillus subtilis* to afford an intermediate ketoaldehyde from a 5 mM solution of ketoacid in pH 7 buffer (Scheme 5.7). This could then be converted *in situ* with high chemoselectivity to an achiral aminoketone using an isolated transaminase in the presence of 250 mM of *rac*-alanine as amine donor. The resultant cyclic imine was finally converted to the desired (*S*)- or (*R*)-piperidine enantiomer with high enantioselectivity using 50 mg ml^{-1} wet cells containing either the (*R*)-selective IRED from *Streptomyces* sp. GF3587 or the (*S*)-selective IRED from *Streptomyces* sp. GF3546, respectively. The formation of disubstituted piperidines was more complex due to balance between substrate and enzyme control of diastereoselectivity in the IRED reduction step.



Scheme 5.7 CAR/ATA/IRED cascade for the preparation of chiral 2-aryl piperidines.

The key to successful implementation of CARs in these cascades, other than the need to co-express PPtase, was the incorporation of a GDH/lactate dehydrogenase system to ensure that the highly chemoselective transamination from the L-alanine amine donor to aldehyde acceptor was strongly driven toward amine product formation. In the absence of this system, significant irreversible over-reduction of the aldehyde occurred due to endogenous enzymes present in the host. Finally, the group went on to demonstrate these cascades on a preparative scale, affording >50 mg quantities of product in high yield.

Amidation is the most prominent transformation performed in the pharmaceutical industry, often for the coupling together of individual fragments, and is typically performed using hazardous reagents or bulky coupling agents. Non-ribosomal methods of enzymatic amide synthesis from esters and acids have recently been reviewed by Goswami and Van Lanen and could offer a more efficient method of amide synthesis, compatible with other enzymes for use in cascades [51]. Enzymatic ester to amide synthesis is attractive because many chemical methods often require an additional ester hydrolysis step prior to coupling. Such transformations are typically performed by lipases or proteases in neat organic solvent, but it can be challenging to develop a process due to the need to minimize water in order to reduce the high level of unwanted enzyme-catalyzed hydrolysis while at the same time trying to allow for sufficient residual water content to retain amidation activity. Alternatively, there are hydrolase enzymes capable of transacylation of more than just amino acid substrates in aqueous solution, such as the acyltransferase from *Mycobacterium smegmatis* (*Ms*AcT) [52], penicillin G acylases [53], and CapW from the capuramycin biosynthetic pathway [54]. Berglund and coworkers have recently employed *Ms*AcT, a member of the relatively recently discovered multifunctional GDSL esterase/lipase family [55], in combination with an ω-ATA to convert aldehydes to amides using an *in vitro* concurrent cascade performed in aqueous solution. The optimum pH for the amidation step was found to be 11 and so an ω-ATA from *Silicibacter pomeroyi* was selected for use in the cascade due to its high pH optimum (pH 9.5). Under optimized conditions, using a 20 mM concentration of benzaldehyde, 2% v/v methylmethoxy acetate (10 equiv.), and a 0.5 M concentration of L-alanine in 0.4 M CHES buffer, pH 10, the desired amide was produced in 97% conversion over a period of 12 h using a twofold excess of ω-ATA to *Ms*AcT in terms of units activity to maximize the synthesis to hydrolysis ratio (Scheme 5.8). In the absence of *Ms*AcT, amine was only



Scheme 5.8 ω-Transaminase/acyltransferase cascade for the conversion of aldehydes to amides.

produced in 40% conversion, demonstrating that the acyltransferase is driving the equilibrium. Presumably the excess of L-alanine is required to enhance the transamination reaction rate, assuming that the pyridoxal-5′-phosphate (PLP) cofactor content had also been optimized.

Linear aliphatic aldehydes also produced amide, but in lower yield. Methyl acetate was the only other acyl donor tested, giving high rates of reaction, but slightly lower maximum conversions than with methyl methoxyacetate. Although the substrate scope is limited, this could provide an excellent opportunity for enzyme engineering.

For *in vivo* cascades, due to the potential for ester hydrolysis, acid to amide transformations using ATP-dependent amide ligases might ultimately offer a more versatile approach to amide formation [44].

5.2.4 The Future of Synthetic Biochemistry

In 2012 a group of 15 companies from the pharmaceutical, fine chemical, and specialist biocatalysis sectors reported prioritized lists of issues that would enhance the adoption of biocatalysis and synthetic biology in industry were they to be progressed [13].

More high quality transaminases, high performance monooxygenases, and "high quality" hydrolases to improve the substrate scope of existing hydrolysis/acylation panels all featured as strong requirements within the established biotransformation category.

IREDs and C–C/C–N bond forming activities (e.g. for Knoevenagel, Suzuki, Heck, Strecker, Mannich reactions) all featured as biotransformations with little or no precedent that would be of high value to industry were they to be developed. Amide- and nitrile-reducing activities also featured highly. Significant progress has clearly been made toward the development of IREDs as discussed above, and some interesting C–C bond forming methodologies such as asymmetric Stetter reactions catalyzed by thiamine diphosphate-dependent lyases [56] and asymmetric Michael-type reactions of nitroolefins and acetaldehyde catalyzed by 4-oxalocrotonate tautomerase (4-OT) have also been developed [57]. However, on the whole, many of these desired transformations within this category remain elusive to biocatalysis. One interesting approach to bridge this gap, while retaining compatibility with natural enzymes for future cascade development, might be to develop artificial enzymes as recently reviewed by Pordea and Ward [58] and Heinisch and Ward [59]. Ward and coworkers have recently reported the first demonstration of *in vivo* directed evolution, assembly, and activity of an unnatural metalloenzyme catalyst for olefin metathesis [60]. Directed evolution of protein ligands could provide a powerful and rapid alternative to traditional chemical approaches. In fact, the best metathesis catalysts developed in this work competed favorably against commercially available benchmark olefin metathesis catalysts when compared *in vitro*.

Because of increasing successes in expanding the biocatalyst toolbox, new cascade development is also expanding rapidly as are chemoenzymatic cascades (GSK, unpublished data) and the application of biocatalysis in flow [61]. However, this is highlighting other limitations that will require future solutions

such as the development of standard and reliable expression systems, hosts, methods to analyze pathways, machine learning, and downstream processing technologies to fully harness these advances, all of which are enjoying increased focus through the expanding field of synthetic biology. As these approaches aim to generate synthetic routes within cells, there is clearly a need for more rational approaches to the retrosynthetic design of pathways that might be best achieved through the teaching of biocatalysis and biosynthesis alongside traditional chemistry methods within university chemistry degree courses, as recently proposed by Turner and O'Reilly [62].

5.3 Chemical Catalysis

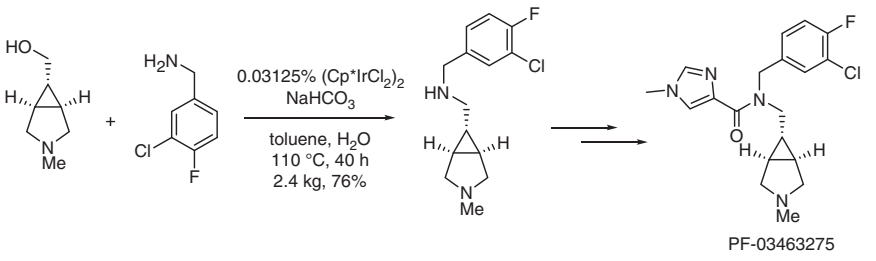
Catalysis is one of the 12 principles of green chemistry [1, 63] due to the potential for a catalyst to decrease the energy barrier for a reaction to occur, leading to faster reactions with increased efficiency and reducing the energy requirement. Use of catalysis in synthesis can give increased selectivity, improving the yield and reducing waste, as well as allowing access to single enantiomer products and avoiding the need for resolution. It may also enable new reactions and therefore open up the potential for decreased step count, again leading to improvements in cost and process mass intensity. It is these features that make it a technology of increasing importance in the pharmaceutical industry [64].

The types of reactions most commonly used in the pharmaceutical industry are shown in Figure 5.1. Within this classification, several reaction types are frequently performed catalytically. Heteroatom arylation is frequently carried out by copper- or palladium-mediated cross-coupling [65, 66], with recent developments allowing the use of aryl chlorides for copper coupling, making this method appear more attractive [67]. Heteroatom alkylation can in certain cases be performed by hydrogen borrowing [68], as in the Pfizer example below (Scheme 5.9) [69]. An alternative to this is to carry out reductive amination by condensation with an aldehyde and hydrogenation of the resulting imine over palladium or platinum. Both of these methods have the advantage of avoiding the need for genotoxic alkylating agents requiring tight control of residual reagent [70].

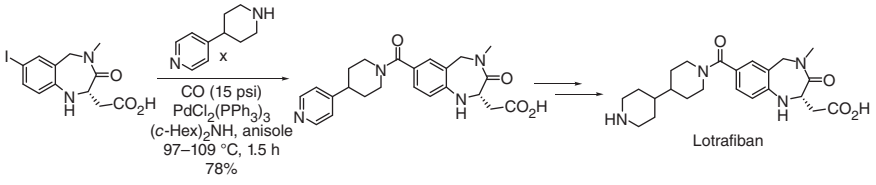
There are a number of catalytic acylation methods that have been reviewed extensively [71]. An alternative approach to access the same products is carbonylative coupling with palladium catalysis to transform an aryl halide to an amide or ester as in the synthesis of lotrafiban (Scheme 5.10) [72].

Within the C–C bond formation class (Scheme 5.11), a large proportion is made up of palladium-catalyzed cross-couplings. The Suzuki reaction is the most commonly used of these couplings, mainly due to the stability and ease of access to the boronic acid component, and numerous reports exist where the boronic ester or acid is formed and telescoped into the Suzuki stage. These types of coupling have been extensively reviewed [73, 74].

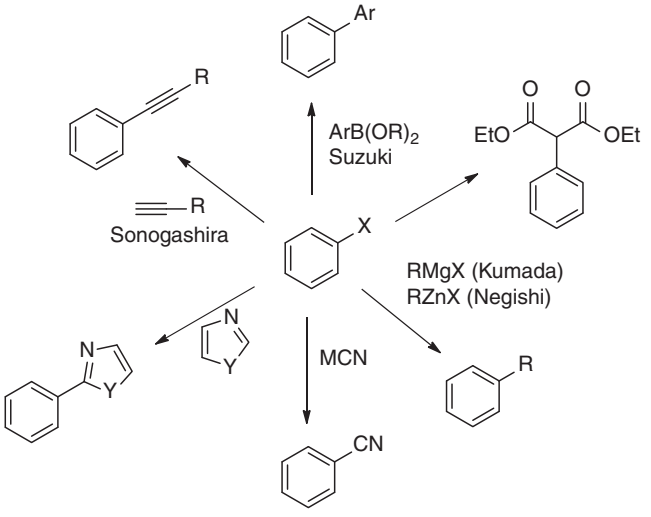
A number of these cross-couplings (Suzuki, Kumada, Negishi, etc.) still require preparation of a functionalized intermediate, often through cross-coupling with a halide. Examples are now coming through process development of direct C–C



Scheme 5.9 Example of hydrogen borrowing used in pharmaceutical synthesis.



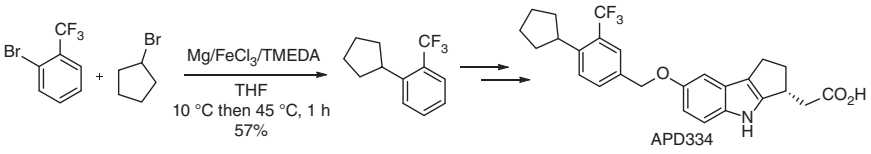
Scheme 5.10 Amide formation by carbonylation in synthesis of lotrafiban.



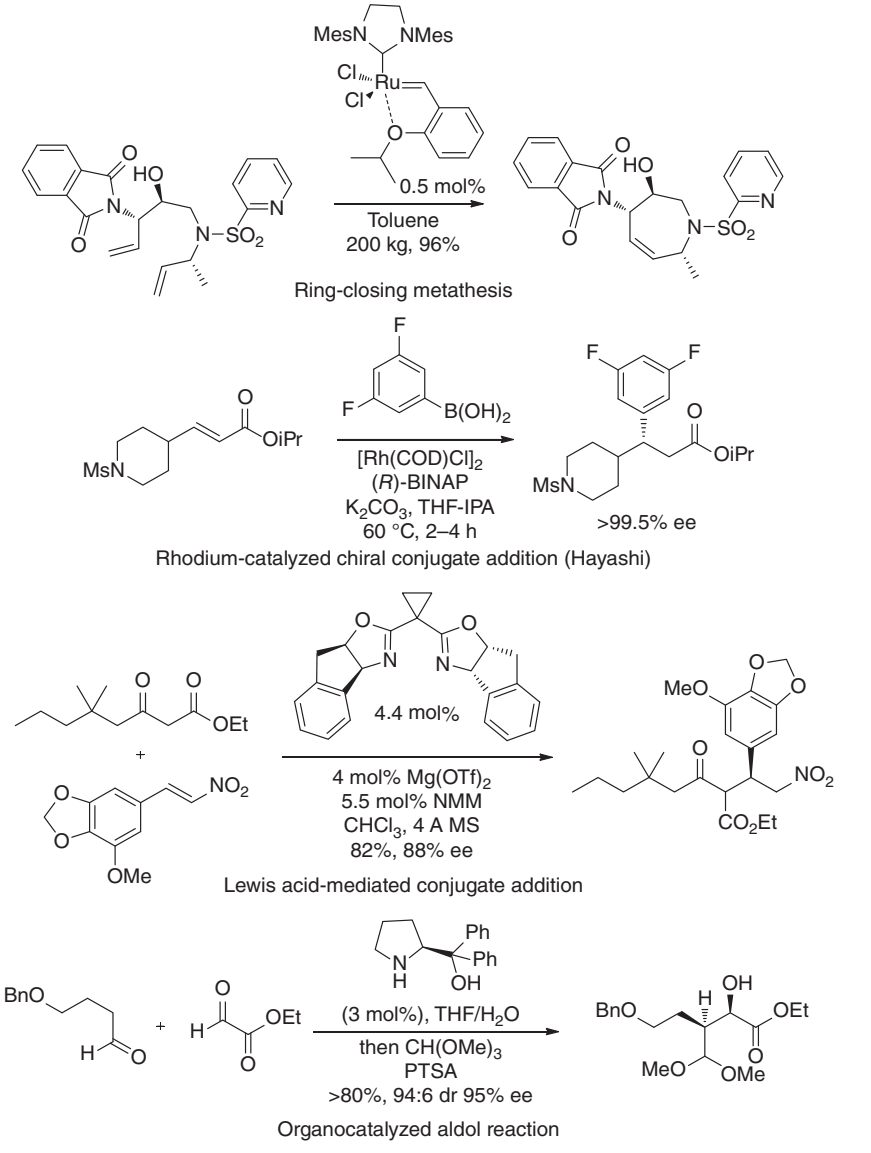
Scheme 5.11 Typical classes of transition-metal-catalyzed C–C bond formation.

coupling through C–H functionalization [75, 76]. Although Suzuki couplings are typically performed best on sp^2–sp^2 couplings, sp^2–sp^3 coupling is also possible [77, 78]. For these sp^2–sp^3 cases, alternative couplings may be better, and in fact palladium may not necessarily be the best metal; iron-catalyzed cross-couplings, for example, are useful for cross-coupling of Grignard reagents [79, 80], and examples have now been reported of this being run on development scale, including *in situ* formation of the Grignard reagent (Scheme 5.12) [81].

Further examples of catalyzed C–C bond formations include metathesis [82], enantioselective Hayashi [83] or Lewis acid-mediated [84] conjugate additions or



Scheme 5.12 Example of direct reductive coupling of an aryl with alkyl bromide.



Scheme 5.13 Further examples of catalytic C–C bond formation applied to pharmaceutical synthesis.

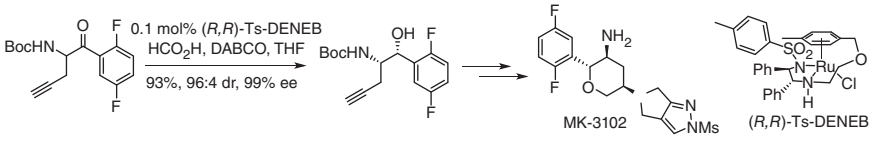
organocatalyzed reactions [85], and chiral phase-transfer-catalyzed alkylations (Scheme 5.13) [86].

In terms of deprotection, 20% of this reaction class from the assessment of Carey et al.[1] was made up of debenzylations, frequently carried out by hydrogenolysis over palladium on carbon support. The reduction class also comprises many reactions possible by hydrogenation over transition metal catalysts, including reductions of nitro groups, imines and nitriles, alkenes/alkynes, and saturation of aromatic heterocycles [87]. Some of these reactions may be done asymmetrically using homogeneous catalysis to set a stereocenter in a molecule [88–91], such as the example below from Merck of a ruthenium-catalyzed asymmetric hydrogenation with dynamic kinetic resolution (Scheme 5.14) [92].

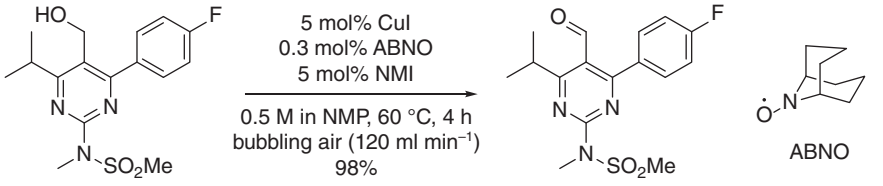
It is also starting to be possible to reduce amides and esters by hydrogenation that would be advantageous over traditional hydride-based reagents due to the production of less waste, although this tends to require high pressures [93, 94].

The oxidation class encompasses a number of reactions that may be carried out catalytically, including alcohol oxidations to aldehydes, ketones or carboxylic acids, dihydroxylations, epoxidations, and oxidation of heteroatoms. Large-scale oxidations employed in the pharmaceutical industry have previously been reviewed [95], with phase-transfer-catalyzed bleach oxidations of alcohols being among the most reported catalytic oxidation [96]. A notable development has been the application of aerobic oxidation, particularly by the Stahl group employing catalytic TEMPO or ABNO with a copper catalyst for oxidation of alcohols, reported in both batch and flow modes of operation [97, 98]. This oxidation method has been demonstrated for oxidation of a precursor to rosuvastatin on a 10 mmol scale in NMP at 60 °C (Scheme 5.15).

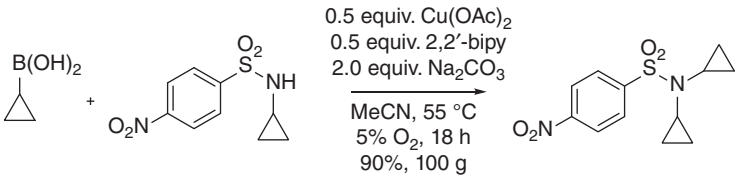
Although this reaction involved heating an organic solvent in the presence of air in a batch vessel, the authors performed the experiment 25 °C below the flash point of NMP under 1 atm of air as a basis of safety, noting that any significant scale-up would require a full safety assessment. Alternative measures could include sweeping the headspace of the reaction vessel with an inert gas to prevent reaching the limiting oxygen concentration for the solvent, leading



Scheme 5.14 Application of asymmetric hydrogenation with DKR in synthesis of MK-3102.



Scheme 5.15 Application of catalytic oxidation to a Rosuvastatin precursor.



Scheme 5.16 Application of Chan–Lam coupling on 100 g scale.

to flammability [99], or running the chemistry in a flow reactor, either with no headspace or under sufficient pressure to limit the solvent vapor concentration.

Another oxidative procedure that has received attention is the Chan–Lam coupling [100, 101], an alternative copper-catalyzed oxidative carbon–heteroatom bond formation between an N–H or O–H source and a boronic acid. The reaction requires the presence of oxygen, complicating the scale-up; however, a recent report from Mudryk, Zheng, and coworkers at Bristol–Myers Squibb describes scaling up this reaction on 100 g scale in 5% oxygen in nitrogen following appropriate safety studies (Scheme 5.16) [102].

5.3.1 Considerations for Application on Process Scale

The amount of effort put in to implement catalysis in a route will depend on the phase of the project and also the amount of time available to incorporate any change. For discovery, the aim is to discover a clinical candidate as rapidly as possible, so any synthetic plan must contain reactions with a good chance of success. Discovery routes will also often have the potential for variation at a number of points in the molecule as they are designed for discovering the best molecule [103, 104]. Once that candidate has been selected, focus can transition to the best way to synthesize it. For the early phase of development, the key thing is to be able to operate safely and have a route in place that will deliver sufficient material that meets specification. This may be the original discovery route or may involve some modifications. As the molecule progresses through the development phases, a significant effort is committed to ensuring the best manufacturing route, which is typically in place prior to Phase III. This will evaluate many different potential disconnections to give the most efficient way of constructing the molecule. To be able to fully evaluate disconnections, particularly early on when minimal material may be available, it is important to be able to explore reaction conditions in the most resource-efficient way possible. A number of methods exist for doing this, such as making use of statistical design [105] and high-throughput experimentation [106], including, as recently described by scientists at Merck, through the use of nanoscale screening methodology [107]. Application of cutting-edge catalysis is complicated by the fact that most drug molecules are functionalized, with structural features such as heteroatoms, heterocycles, carboxylic acids, and so forth, so methodology used must tolerate these functionalities. It is not uncommon for the substrate scope in literature reports of novel chemistry to be limited and for this type of functional groups to be absent from the examples discussed. This leads to reduced uptake due to lack of confidence in success or outright failure when the transformation is attempted.

Although there is a challenge in that it is difficult to have anything other than excellent results accepted by the highest impact factor journals, it is actually the information about which reactions do not work that informs on reactions that are still a challenge and truly differentiates methods and catalysts for a given transformation. It would of course be inappropriate to suggest that all newly developed methodology should be applicable to the fine chemical and pharmaceutical industries; however, novel methodology that works on this type of compound is likely to be taken up if it demonstrates a significant benefit. A number of screening methods have been reported for understanding how likely it is that reactions would tolerate the presence of certain functional groups [108–110], and this, in combination with the high-throughput experimentation described above, can allow rapid evaluation of conditions even on novel chemistry to understand whether it is applicable to the molecule under consideration.

Once the route is in place, a large body of work is undertaken to ensure the best process, i.e. the best combinations of reagents and the optimal set points for reaction parameters to ensure the necessary purity. This is a complex multidisciplinary undertaking between chemistry, engineering, analysis, particle sciences, process safety, physical properties, materials sourcing, and other groups representing pilot plant and manufacturing functions. Scale-up comes with additional considerations related to how chemistry would be run in a manufacturing environment: everything from the types of vessel (traditionally batch vessels of fixed size for a given module) to how the reagents would be charged. Any catalyst would have to be stable to weighing and charging, although it is possible to set up in specific ways when required. Order of addition of reagents, heating ramp rates, and so forth are also very important, since heat-ups will take significantly longer on larger scale that can lead to side reactions happening before or competing with the desired transformation. In the case of catalytic reactions, effort will be dedicated to understanding formation of the active catalyst and potential modes of catalyst deactivation [111] to try and ensure the catalyst keeps working as desired in the reaction. A number of key factors for consideration for a good process are described by the SELECT criteria [112], and these and other factors have been discussed by Dach and coworkers from Boehringer Ingelheim [20]. The SELECT criteria are as follows:

Safety. Any safety issues must be manageable, including toxicity, flammability, reactive intermediates, and so on. This is particularly noticeable when carrying out work with gases under pressure; oxygen/air when used in a system requires use of flow chemistry or if using air, must at the very least be below the solvent flashpoint. This is critical for safe scale-up of oxidative processes and indicates why uptake of this technology is slow. Hydrogen and carbon monoxide also require extremely careful handling and sophisticated detection systems. This is on top of the usual process safety considerations necessary for running any process at scale. Significant precautions also need to be taken when charging or filtering flammable catalysts out of systems and also when handling toxic catalysts.

Environmental. This refers to meeting current and anticipated future environmental regulations, including avoiding materials of concern that will soon be

banned from use (such as dichloromethane), as well as minimizing waste and the energy required to carry out a process. Waste needs to be transported and treated, so efforts are made to reuse or recycle, such as refining waste containing platinum group metals to get the value back or recycling solvent used in a process.

Legal. Freedom to operate means IP considerations, especially for patented ligands and technology, must be understood and taken into account at an early stage. Technology requiring a royalty payment is much less likely to be taken up than one with a simple licensing model, for example.

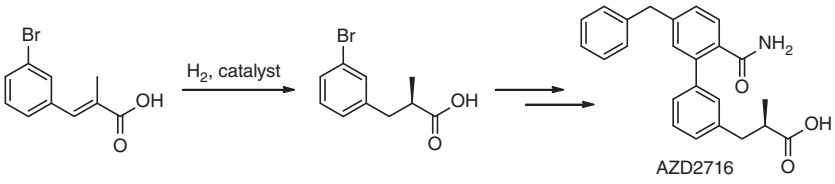
Economy. The route should have the minimum number of steps and meet the long-term cost target.

Control. All steps should be reproducible and tolerant of variation within defined limits of process parameters. Pharmaceutical production is highly regulated, with binding legal controls over the quality of the material generated, and is one of the most heavily regulated activities of all business. "Quality by Design" ensures quality based on thorough understanding and control of the production process, including in-depth study of process boundaries and mechanism and an understanding of the origin and control of impurities. As a very general rule, any impurity (including regio- and stereoisomers, intermediates, byproducts, residual ligands, reactants, reagents, etc.) must be <0.15% w/w of the API, unless they have a structural alert for genotoxicity, such as alkylating agents. These are much more tightly controlled down to ppm levels. A number of metals also fall into this category, particularly those used for catalysis: the platinum group (Pd, Pt, Ir, Rh, Ru) are typically limited to 10 ppm, nickel to 20 ppm, and copper to 300 pm [113]. Given that 10 ppm is 0.00001% w/w, this is clearly a very tight target to meet and can complicate synthetic sequences if the metal-catalyzed step is late on. A lot of work can go into making sure that this specification is met. Ligands also count as impurities so must also be removed; this is a further reason to seek the minimum possible catalyst and ligand loading to facilitate meeting specification.

Throughput. All processes should have high throughput and be high yielding. This includes time scale of manufacture and availability of starting materials: long dilute routes with slow reactions are disfavored, as are those starting with rare complex input materials.

5.3.2 Examples of Recent Catalysis Developments Applied in an Industrial Setting

Scientists at AstraZeneca recently reported the use of an enantioselective alkenoic acid hydrogenation in the synthesis of AZD2716 (Scheme 5.17) [114]. Several hundred grams were required for toxicological study with limited time for optimization, so the majority of the original discovery route was used. However, this route employed resolution of a racemic mixture, resulting in more than 50% loss of yield, so significant effort was put into developing an asymmetric hydrogenation route. Although such reductions of alkenoic acids are well precedented, with broad substrate scope, literature review revealed α-methyl cinnamates



Scheme 5.17 Use of asymmetric hydrogenation in synthesis of AZD2716.

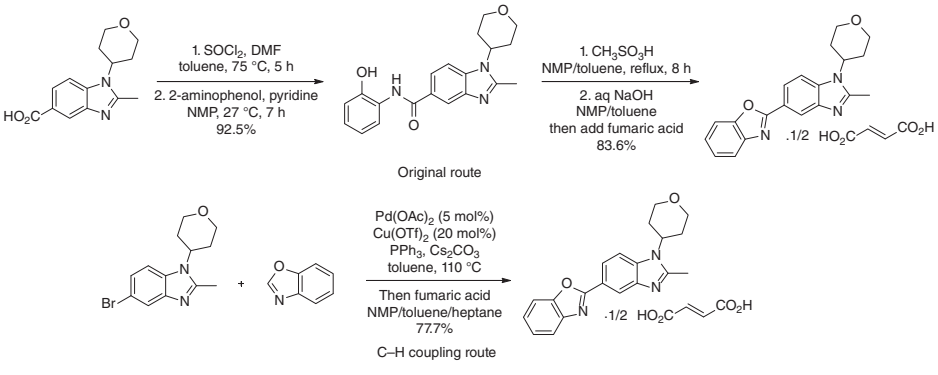
to be the exception, with only a few examples reported that utilized non-commercially available ligands. Therefore, high-throughput experimentation was used to screen a wide range of chiral ligands with both rhodium and ruthenium precursors, identifying the Josiphos ligand (*R*)-1-[(*S*)-2-(di-*tert*-butylphosphino)ferrocene-1-yl]ethylbis(2-methylphenyl)phosphine with a rhodium precursor as being competent to furnish the desired compound in 75% yield and 90% ee. This could be upgraded to 97% ee by crystallization of the (*R*)-1-phenylethylamine salt and was improved to >98% ee in the final API sufficient for that time. This outcome would not have been achievable within the timelines without the use of high-throughput experimentation.

A significant development recently has been in the use of direct C–H functionalization. This is starting to be reported in pharmaceutical synthesis, allowing shorter syntheses by obviating the need for pre-functionalization. Three examples published since 2015 are discussed below. Kuroda et al. at Sumitomo Dainippon Pharma Company recently described the use of a Pd–Cu-cocatalyzed C–H/C–Br coupling in their synthesis of a PDE4 inhibitor (Scheme 5.18) [115]. Application of this disconnection not only shortened the synthesis but also avoided use of the mutagenic 2-aminophenol in the original route. It should be noted that while the original conditions of $Pd(OAc)_2$, PPh_3, and Cs_2CO_3 reported by Miura and coworkers [116] afforded the desired product in 73% HPLC yield, addition of copper salts led to an improved HPLC yield of 87%.

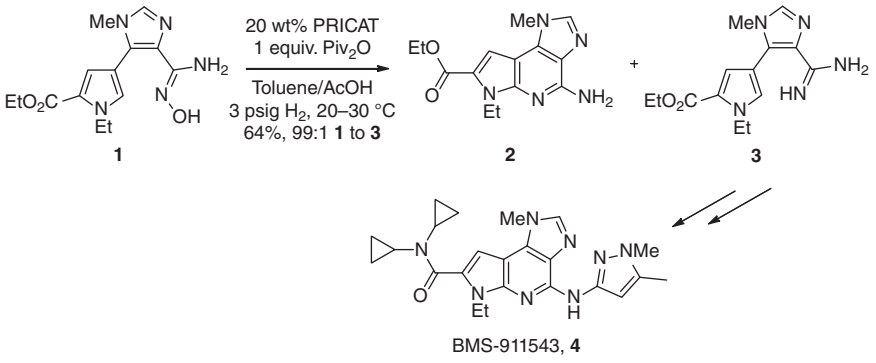
The second example comes from Eastgate and coworkers at Bristol–Myers Squibb and new route work carried out toward synthesis of the JAK2 inhibitor BMS-911543 **4** (Scheme 5.19) [117]. This involved developing the key electrocyclization reaction from **1** to **2** to effect the desired intramolecular C–H functionalization. Originally the plan had been to perform the cyclization from **3**; however, this proved unsuccessful so the researchers proceeded to consider a radical approach, activating the hydroxylamine and screening a range of mediators.

From initial screening work, Raney nickel was the only successful mediator, and it was quickly determined that the presence of hydrogen was crucial along with the O-acylation of the amidine **1** to avoid side reactions including cleavage to inactive **3**. Screening of nickel precursors revealed PRICAT (nickel oxide supported on silica) to be particularly effective, with significant parameter screening, resulting in the conditions shown in Scheme 5.19. Another interesting point to note from this work is the study of the synthesis of intermediate **1** (Scheme 5.20).

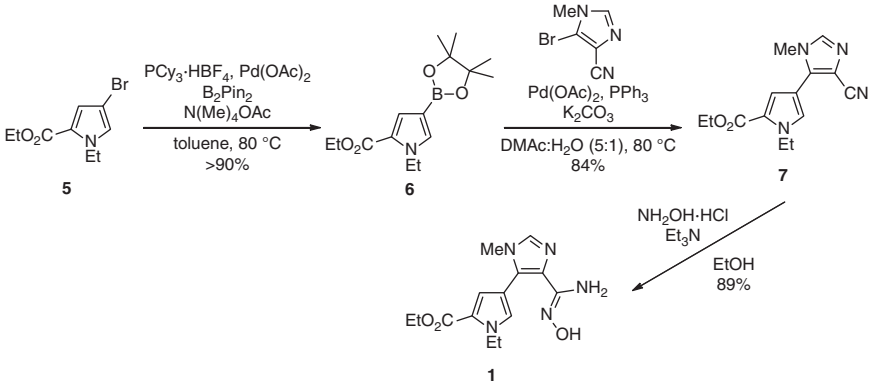
This required access to **6** through Miyaura borylation followed by Suzuki coupling. While initial screening indicated that $KOAc/Pd(OAc)_2/PCy_3$ in toluene



Scheme 5.18 Original and improved routes to PDE4 inhibitor.



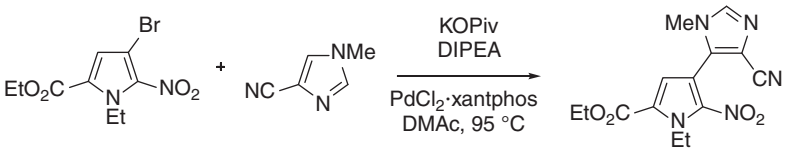
Scheme 5.19 Intramolecular C–H functionalization in synthesis of BMS-911543.



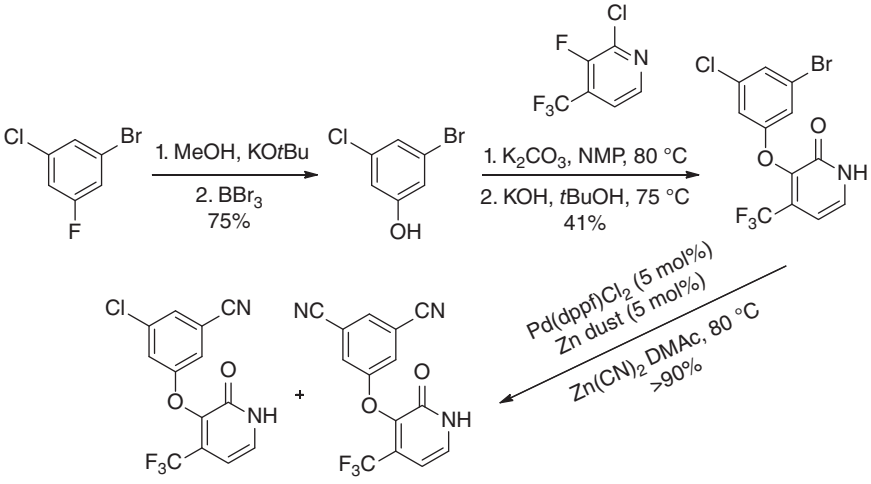
Scheme 5.20 Synthesis of intermediate **1**.

could give a high yield and ~99 : 1 selectivity for borylation over debromination, this proved problematic on scale-up and gave up to 50% debromination on a gram scale. A thorough study of formation of the active catalyst indicated that the debromination was due to formation of unligated palladium during catalyst activation and that two reduction pathways to access reduced palladium(0) catalytic species were in operation [118]. The nature of the catalyst complex was affected by the order of reagent addition, and this had a significant impact on the reaction outcome. This discussion is relevant as it highlights the importance of mechanistic understanding in scale-up of catalytic reactions to give a robust reaction.

The same group has reported synthesis of a very similar molecule through direct C–H coupling (Figure 5.21) [119]. The authors note that ligand screening revealed the bidentate ligand Xantphos to be optimal for this coupling, commenting that this was surprising for this transformation as a bidentate ligand would not be expected to leave enough vacant coordination sites on palladium for the Fagnou concerted metalation–deprotonation mechanism. Following detailed studies with Blackmond and coworkers at Scripps, it was determined that mono-oxidation of the ligand was crucial for forming the active catalyst, giving a hemilabile bidentate ligand for palladium (Scheme 5.21).



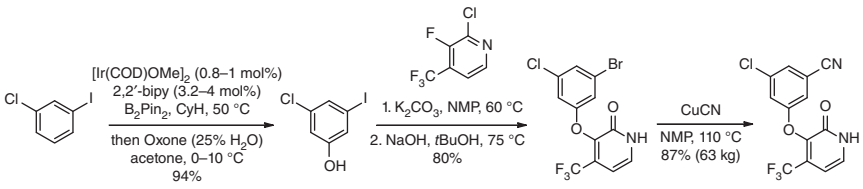
Scheme 5.21 Direct C–H activation using Xantphos as ligand.



Scheme 5.22 Synthesis of a nitrile intermediate in the synthesis of Doravirine.

A further example of C–H functionalization in synthesis comes from Campeau et al. at Merck, in the synthesis of Doravirine [120]. The original synthesis is shown in Scheme 5.22.

The original route was dependent on the availability of substituted 1,3,5-benzene starting materials. A double-cyanated impurity was observed in the cyanation reaction that was not purged later on in the synthesis, leading to a specification for this species of <1%. Conditions were identified through screening to give less than 1% of the dinitrile impurity on small scale in a glove box, but on transferring these conditions to the laboratory outside of the glove box, >1% of dinitrile by HPLC was observed. This led to consideration of an alternative cyanation substrate, namely, the iodide that would allow milder cyanation conditions. A test reaction using commercially available 3-chloro-5-iodo-phenol confirmed that copper cyanide gave complete conversion to the desired mononitrile with no observation of the double-cyanated impurity. The copper-mediated reaction was not effective on the corresponding bromide, requiring forcing conditions, leading to formation of a number of impurities including dinitrile. This is of note as an example where an iodide was the preferred precursor, as opposed to the typical case where cheaper bromides or chlorides are used where possible; ultimately, the best precursor is one that allows formation of the product in the desired quality. As the desired phenol was not available on large scale, a scalable synthesis was developed using iridium-catalyzed C–H



Scheme 5.23 New conditions for synthesis of a nitrile intermediate in the synthesis of Doravirine.

borylation followed by oxidation, demonstrating applicability of this chemistry on 85 kg scale (Scheme 5.23).

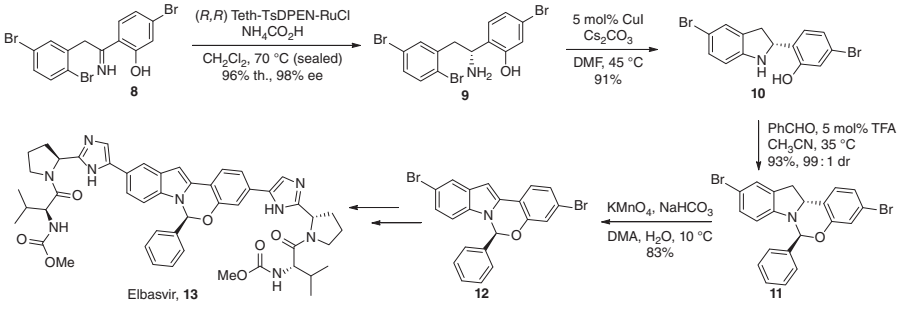
The final example of new applications of catalysis to be discussed here is a further example from Merck (Scheme 5.24) [121]. Synthesis of HCV NS5a antagonist Elbasvir proceeded through asymmetric transfer hydrogenation of imine **8**, followed by copper-catalyzed intramolecular C–N coupling to form indoline **10**, then dynamic diastereoselective condensation of **10** with benzaldehyde to afford aminal **11** in high diastereoselectivity through reactive crystallization. This was then treated with potassium permanganate to give the indole in >99% ee. Numerous other sets of conditions, including catalytic manganese, were investigated but led to significant racemization.

This oxidation process gave MnO_2 as by-product, complicating the isolation and leading to a significant amount of metal waste with a consequent negative impact on sustainability. As a result, an evaluation of alternative methods employing visible light catalysis was carried out in collaboration with the Knowles group at Princeton [122]. High-throughput screening identified Ir photocatalyst $[Ir(dF\text{-}CF_3\text{-}ppy)_2(dtbpy)](PF_6)$ in conjunction with *tert*-butyl peracetate as a competent system for this oxidation, without erosion of enantioselectivity. Scale-up experiments were performed in a simple flow reactor to increase the solution surface area, which would increase the light flux and therefore the reaction rate. These experiments revealed *tert*-butyl perbenzoate to be more effective than the peracetate that required a longer residence time for completion, leading to a decrease in enantioselectivity. This process on a lab scale produced 100 g in 5 h in 85% isolated yield and in 99.8% ee (Scheme 5.25).

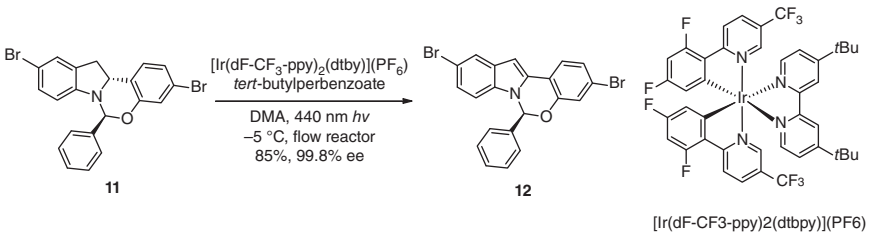
This report gives significant confidence in the potential for future application of this type of catalysis on a process scale.

5.3.3 The Future of Chemical Catalysis

Much has been reported on the trend for more polar, three-dimensional molecules to reduce attrition in the drug development process [123, 124]. This will require methods to access these compounds, particularly in the presence of a significant number of heteroatoms, and where a stereogenic center is present, any methods must allow for selective access to a single enantiomer. Use of high-throughput experimentation and statistical design techniques will allow evaluation of a large number of reaction components to reach the optimal conditions for a given transformation and enable application of even unprecedented transformations on pharmaceutical molecules of interest. In the near term,



Scheme 5.24 Synthesis of Elbasvir.



Scheme 5.25 Photocatalytic indoline dehydrogenation to access intermediate **12**.

newer reactions that are not precedented on complex, later stage compounds in the synthesis may still be applicable early on in the sequence to access starting materials; as more information is obtained on the scope of these transformations, through fragment-based screening, the use of informer libraries, or reports describing scope and limitations in more detail, it may be possible to apply these techniques later in the synthetic sequence. Numerous methods exist for sp^2–sp^2 cross-coupling, but sp^2–sp^3 couplings can be complicated by β-hydride elimination. This is where methods such as that reported in Scheme 5.12 are important, since this seems to be less prevalent with non-platinum group metals, and not requiring preformation of the organometallic can lead to a reduction in step count. Utilization of non-precious metals is also increasing in asymmetric hydrogenation reactions, where they are able to function with single-point binding [125–127]. They are also able to do some interesting chemistry with redox-active ligands [128–130], and it is for these reasons that non-precious metal chemistry will become more prevalent, not simply due to metal cost. It is noteworthy that the ligand has generally a more significant cost than the metal, unless that ligand is triphenylphosphine or a simple amino acid. Other chemistry to access these types of systems may come from use of photocatalysis. Here, numerous methods have recently been reported [131, 132], and it will be interesting to see how many of these are employed on complex molecules and on scale in the coming years. Although direct C–H coupling and hydrogen borrowing are starting to show some applicability, these are so far still limited, but it is expected that they will become more widespread in the future as catalytic systems improve, particularly those that are less sensitive to air. Oxidation chemistry is also likely to be incorporated into synthetic routes more often following the studies by Stahl and coworkers to demonstrate copper-catalyzed alcohol oxidation safely on scale [94, 95, 99]. Collaborations between industrial and academic groups interested in working on industrially relevant chemistry will be able to accelerate the development of new reactions, which can then be tested using the screening methods described and their evolution [133].

5.4 Continuous Chemistry

Continuous processing is a widely used and long-established technology for manufacture in the bulk chemical and petrochemical industries as well as many other manufacturing industries. However, in its application to the

pharmaceutical industry, it is not yet widely used and still considered to be a new technology. Albeit one that has seen a considerable surge in interest from pharmaceutical companies and academic groups over the last decade, the benefits that continuous processing can bring to the pharmaceutical industry are realized, and the technology and knowledge to apply it grow.

The application of continuous processing to process development can largely be broken down into two broad categories: single-stage and multistage continuous processes. Single-stage continuous processes are often used for technical reasons, allowing the scale-up of chemistry that may not be viable in batch, facilitating either the rapid scale-up of a route for early supplies, or allowing the use of chemistry that would not typically be considered appropriate for batch manufacture, enabling more efficient routes of manufacture. Multistage continuous processes are processes where several stages of chemistry are coupled together, sometimes with work-up operations between steps and can offer many advantages from a manufacturing perspective that can include, but are not limited to, reduced foot print of plant, reduced capital expenditure for new plant, reduced cycle time, reduced energy requirements and labor costs, and consistent quality of output. The subsequent sections will further discuss some of the key benefits of both single and multistage continuous processing, illustrating this with selected examples from the literature.

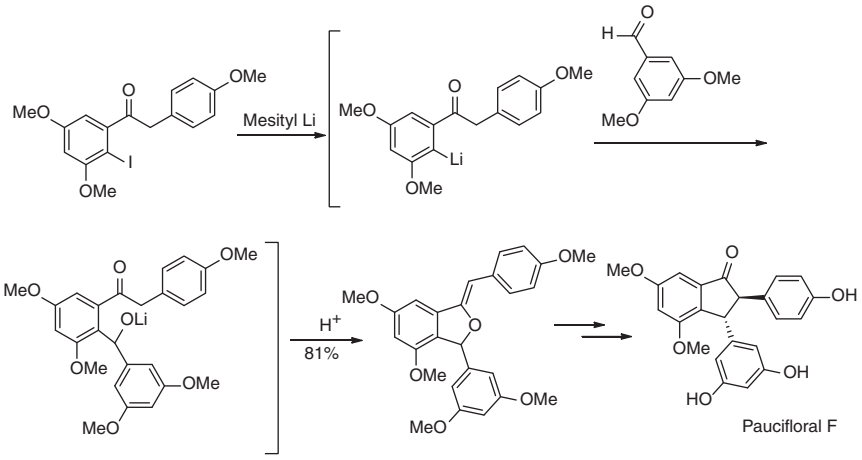
5.4.1 Single-stage Continuous Processing

As a result of the increased vessel size and subsequent reduced surface area to volume ratio and increased mixing times, scaling up processes in batch reactors invariably results in increased addition time for starting materials and reagents; increased time to heat up, cool down, or control exo/endotherms; and changes in reaction profile for mixing sensitive reactions. At best this will lead to longer cycle times but can also result in reduced quality or yield should a change in these parameters play a critical role in the success of the reaction. Similarly, as the size of a reaction vessel increases, so do the associated hazards due to the larger inventory of materials present.

Other more specialist areas of chemistry, such as photochemistry and electrochemistry, can be difficult to incorporate into the general purpose batch vessels available in most manufacturing facilities and can also suffer from the undesirable batch scale-up effects previously discussed. The following section will describe in more detail where continuous processing has been used to overcome some of these issues.

5.4.2 Fast Reactions with Unstable Intermediates

The ability to rapidly form and quench unstable and reactive intermediates in flow can allow reactions to be carried out that would fail when transferred into larger batch vessels as addition and mixing times increase and heat transfer capacity is reduced. Flow can even enable reactions to be carried out that would be impossible to perform in batch on any scale due to the instability of the intermediates involved. Work published by the group of Yoshida perfectly demonstrates this



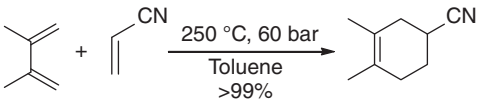
Scheme 5.26 Microreactor generation of an unstable aryl lithium species.

concept where [134], in one example shown in Scheme 5.26, it was demonstrated that the aryl iodide could undergo metal halogen exchange with mesityl lithium to produce a highly unstable aryl lithium intermediate, in the presence of a ketone. If this intermediate were quenched very rapidly (within 0.003 s) with the aldehyde, then the desired product could be produced in 81% isolated yield. Holding the corresponding aryl lithium species for longer than this resulted in increasing amounts of decomposition products, such as dimer compounds. The formation and subsequent reaction of such an unstable intermediate could only be successfully achieved using flow chemistry, and this instance required a microreactor, specifically designed to achieve the rapid mixing and residence time required for the successful execution of the chemistry.

5.4.3 High Temperature and Pressure

The smaller size of flow reactors over batch reactors means that it can be simpler and safer to perform reactions under high temperatures and pressures, and the improved heat transfer results in much faster heating and cooling of the reaction mixture. One area where this can be of benefit is when reactions developed in a microwave under elevated temperatures and pressures need to be scaled up beyond what is practical in research scale microwave equipment. As the majority of homogenous reactions carried out with microwave heating can be reproduced using thermal heating at the same temperatures and pressures, flow is an ideal technology to scale up this chemistry. An example of this comes from the group of Kappe [135], where they demonstrated that the Diels–Alder cyclization of acrylonitrile and 2,3-dimethylbutadiene in flow at 250 °C under 60 bar pressure closely mimicked the results obtained in a batch microwave under similar conditions (Scheme 5.27).

In some cases, where volatile reagents or starting materials are present, the ability to carry out reactions under pressure in the absence of headspace can result in improved yields and/or selectivity over batch reactors where the concentration of



Scheme 5.27 High temperature Diels–Alder reaction in flow.

the volatile component can be reduced in the reaction mixture due to losses to the headspace. This concept was illustrated in the kinetic and scale-up investigations of epoxide aminolysis in microreactors by Jamison and Jensen [136].

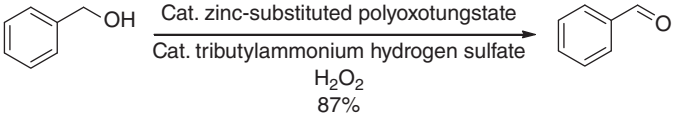
5.4.4 Mixing of Biphasic Reactions

The rate of biphasic reactions can be impacted by the mass transfer of reagents across the two phases that can lead to difficulties in scaling up some biphasic reactions. The well-characterized and controlled mixing in flow reactors can allow mass transfer to be controlled and reproduced successfully, and in some cases the intense mixing can remove the mass transfer limitation altogether. Work published by Jensen on the biphasic oxidation of alcohols to aldehydes using a zinc-substituted polyoxotungstate catalyst, a phase-transfer catalyst and hydrogen peroxide showed that the mass transfer limitation of this system could be removed by running in an appropriate flow reactor, resulting in a significant increase in productivity compared with the batch alternative (Scheme 5.28) [137].

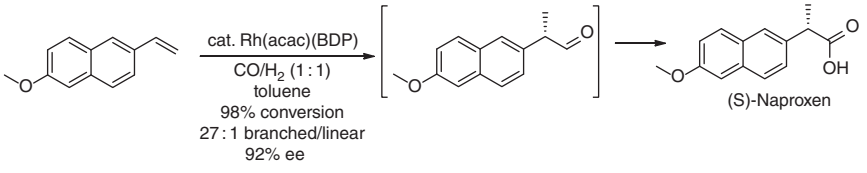
As with liquid–liquid biphasic reactions, gas–liquid biphasic reactions can also suffer from mass transfer limitations and thus benefit from being performed in a flow system. For example, the selectivity of a hydroformylation reaction described by Eli Lilly can be affected by the mass transport of the carbon monoxide from the gas into the liquid phase (Scheme 5.29). Using a carefully designed flow reactor to ensure appropriate mass transfer, they were able to successfully scale-up the hydroformylation of a substituted styrene to provide an efficient synthesis of (*S*)-naproxen [138].

5.4.5 Safety

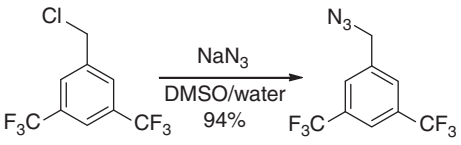
The smaller scale and greater containment of continuous reactors can often enable reactions to be carried out that are deemed too hazardous for batch



Scheme 5.28 Application of flow to a biphasic alcohol oxidation reaction.



Scheme 5.29 Hydroformylation of a (*S*)-naproxen intermediate.



Scheme 5.30 Use of sodium azide in flow.

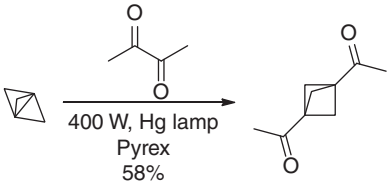
scale-up. It should be noted however that unsafe reactions performed in a flow reactor can still be extremely hazardous, so should not be run unless a thorough process safety evaluation and appropriate controls have been put in place to ensure safe operation. An example of this is given in Scheme 5.30 [139], where process development scientists carried out the displacement of a benzyl chloride with sodium azide using a tubular flow reactor. The key advantage of the tubular flow reactor over a batch reactor is that there is no headspace, so potential condensation of the extremely hazardous hydrazoic acid in the headspace is avoided. This, in addition to the smaller reactor volume, enabled safer operation of this step due to the lower inventory of the hazardous reagents present.

5.4.6 Photochemistry

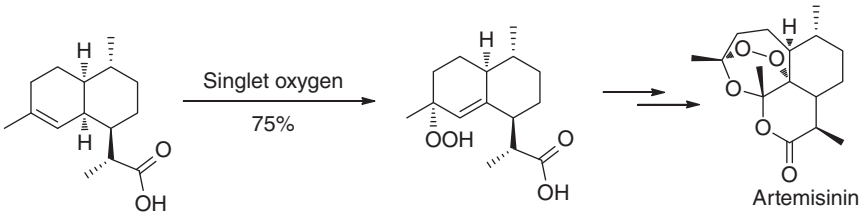
Photochemistry relies on the ability to transmit light into the reaction mixture. In batch, as the surface-to-volume ratio decreases with scale, this can become more difficult, potentially resulting in longer reaction times and side reactions. In addition, the smaller inventory of flow reactors offers a safety advantage when using flammable solvents with lamps that can generate a significant heat output or when hazardous compounds are being irradiated. This was demonstrated for the photochemical opening of propellane to produce the synthetically valuable diketone (Scheme 5.31) [140]. This was operated in flow due to safety concerns of handling large quantities of this energetic compound in a batch photochemical reactor.

Seeberger and coworkers have also demonstrated the continuous photochemical generation of singlet oxygen and subsequent ene reaction en route to the synthesis of the antimalarial drug artemisinin (Scheme 5.32) [141].

A rapidly growing area of interest is the use of visible light photocatalysis. Here visible light is used to excite a catalyst to a higher energy state, allowing it to take part in subsequent reduction and oxidation reactions. For many of these reactions, the rate of reaction is limited by the light flux of the reactor, so the greater surface-to-volume ratio of flow reactors is a key to allow these reactions to be scaled up successfully. This is exemplified by the photocatalytic



Scheme 5.31 Photochemical ring opening of propellane.



Scheme 5.32 Generation and reaction of singlet oxygen in a continuous photoreactor in order to prepare an artemisinin intermediate.

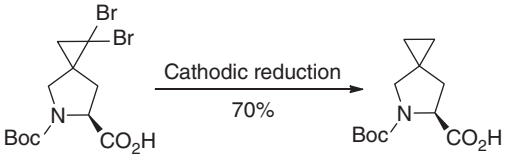
indoline dehydrogenation used in the synthesis of Elbasvir as discussed above (Scheme 5.25). The photo redox approach to the oxidation coupled with scaling up in flow allowed the key intermediate in the synthesis of Elbasvir to be produced while maintaining the stereochemical integrity of the hemiaminal and avoiding the use of undesirable oxidants such as $KMnO_4$ [122].

5.4.7 Electrochemistry

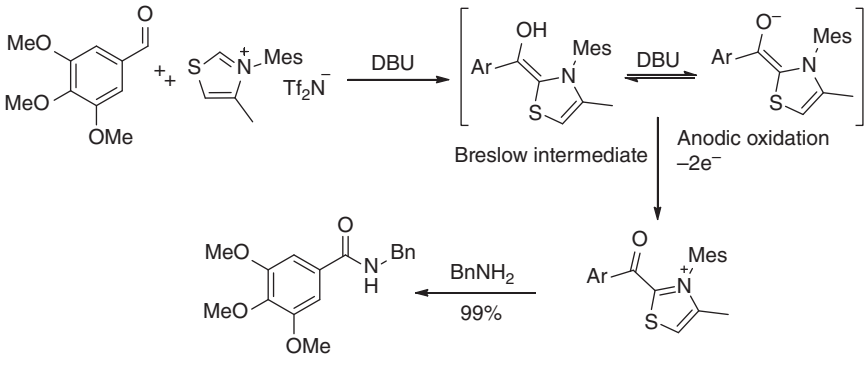
With the increasing availability of electrochemical flow cells that facilitate the development and scale-up of electrochemical processes, more research groups are turning to electrochemistry as an efficient way to carry out redox reactions, avoiding the use of traditional stoichiometric oxidizing agents.

Waldvogel, in collaboration with Novartis, has developed the electrochemical reduction of a geminal dibromocyclopropane (Scheme 5.33) [142]. Non-electrochemical methods of dehalogenation, such as Birch reduction, or palladium/hydrogen systems resulted in ring opening of the sensitive cyclopropane group, but the electrochemical reduction method allowed the bromines to be reduced out of the molecule at the leaded bronze cathode while leaving the cyclopropane intact. The process was initially developed using a batch electrochemical cell, but the batch system used was only appropriate for a few liters of reaction mixture, so to scale beyond this, the authors turned to a split electrochemical flow cell that offered improved electrode area to volume ratio's, improved temperature control, and continuous operation mode, allowing a greater volume of material to be processed.

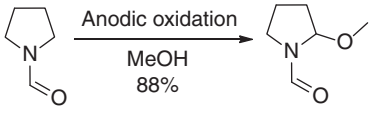
In addition to reactions at the cathode, reductions can also be carried out at the anode of an electrochemical cell. Brown reports several examples of anodic oxidations using a whole electrochemical flow cell, such as the anodic oxidation of the Breslow intermediate, allowing the synthesis of amides from aldehydes as



Scheme 5.33 Debromination of a geminal dibromocyclopropane using a split electrochemical flow cell.



Scheme 5.34 Conversion of aldehydes to amides by anodic oxidation.



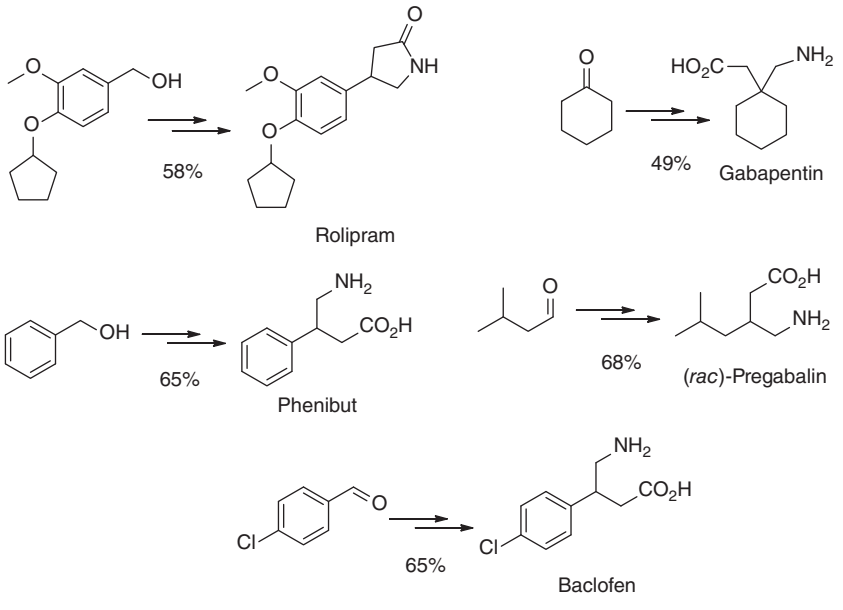
Scheme 5.35 Methoxylation of *N*-pyrrolidine using a microelectrolysis flow cell.

detailed in Scheme 5.34 [143] and the methoxylation of *N*-formylpyrrolidine as shown in Scheme 5.35 [144].

5.4.8 Multistage Continuous Processing

Coupling several stages of chemistry together utilizing continuous reaction, work-up, and isolation operations can allow a much greater level of process intensification and process robustness compared with traditional batch manufacture. Benefits can include smaller equipment footprint, leading to reduced building costs, capital costs, and energy consumption, as well as access to chemical transformations not possible in batch (as discussed in the earlier single-stage section). In addition, greater levels of automation coupled with continuous monitoring of system performance and the potential to use real-time online analytical techniques can lead to greater process robustness. This later example has lead regulatory agencies, such as the FDA through the Pharmaceutical Quality for the twenty-first century Initiative (https://www.fda.gov/downloads/drugs/developmentapprovalprocess/manufacturing/questionsandanswersoncurrentgoodmanufacturingpracticescgmpfordrugs/ucm176374.pdf), to encourage pharmaceutical manufacturing companies to modernize how pharmaceuticals are produced, including switching from batch to continuous manufacture to ensure a more secure and consistent supply of medicines. As a result of this drive, examples of multistep processing from both academic and industrial groups are rapidly increasing.

Examples include Seeberger's chemical assembly system where five synthesis modules (oxidation, olefination, Michael addition, hydrogenation, and hydrolysis) could be configured in the appropriate order with the appropriate



Scheme 5.36 Access to five different APIs through alternative configuration of a chemical assembly system.

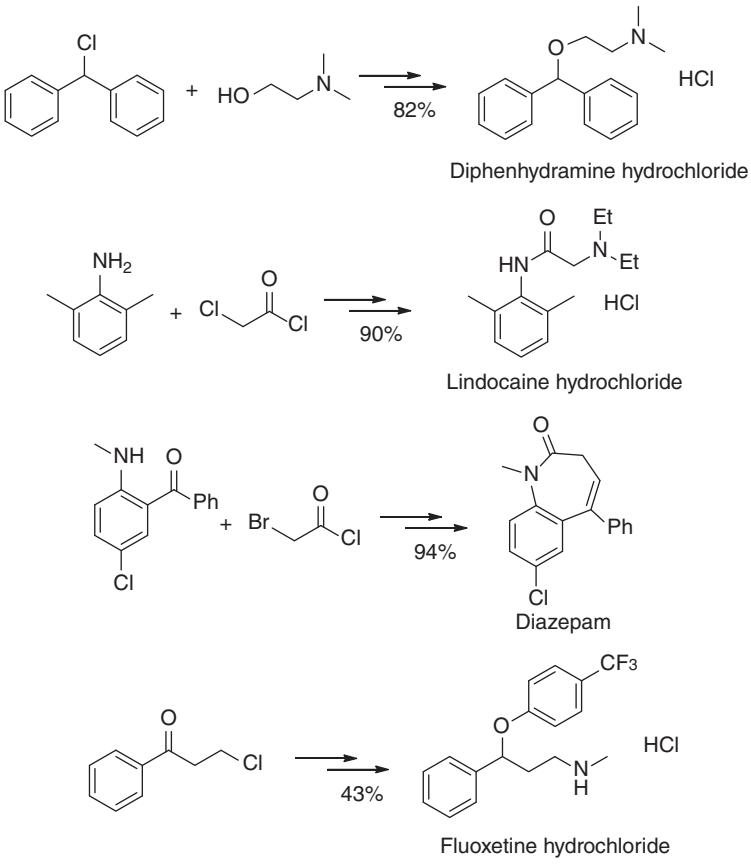
starting materials and reagents to synthesize five different APIs: Rolipram, Lyrica, Phenibut, Baclofen, and Gabapentin (Scheme 5.36) [145].

Jensen has also demonstrated this concept [146]. Using reconfigurable modules the continuous manufacture of four API's, diphenhydramine hydrochloride, lidocaine hydrochloride, diazepam, and fluoxetine hydrochloride, was demonstrated (Scheme 5.37), and the solids dissolved within the same system to prepare liquid oral and topical dosage forms.

MIT, in collaboration with Novartis, has demonstrated the continuous manufacture of Aliskiren hemifumarate (Scheme 5.38) [147]. This process integrates three steps of chemical synthesis that utilizes both continuous reactive, work-up, and isolation operations to produce API, which then fed into the continuous formulation stage. Here the API is blended with excipient and tablets produced through a hot melt extrusion process. The overall cycle time from starting material to tablets was 48 h, significantly lower than the cycle time for the corresponding batch process that is in excess of 300 h and utilizes a significantly larger footprint.

5.4.9 The Future of Continuous Chemistry

Despite the many advantages that continuous processing can offer to the pharmaceutical industry, its current utilization among many pharmaceutical companies and CMOs remains low compared with batch processing. However, as skills, experience, and knowledge in developing continuous processes increase and equipment across all scales becomes available, then the barrier to developing

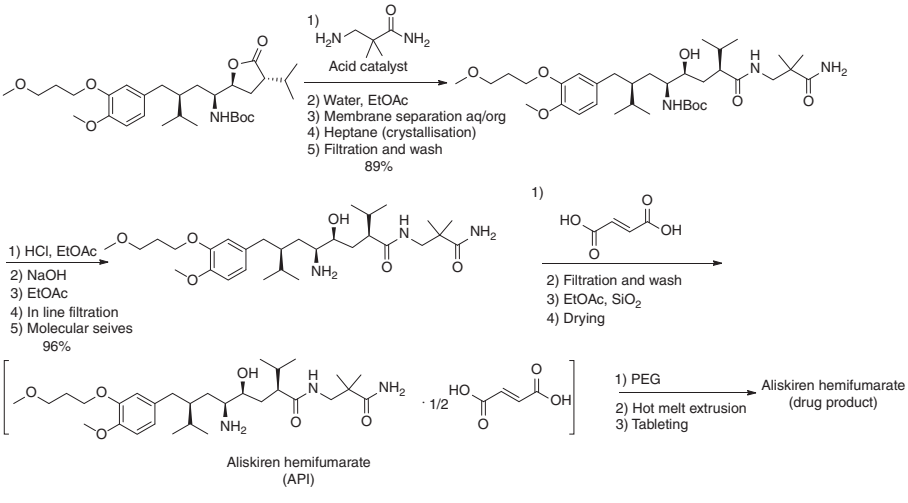


Scheme 5.37 Access to four different APIs through alternative configuration of a chemical assembly system.

and industrializing a continuous process over a batch process will be lowered. This along with developments in technology to allow a greater variety of reactions to be run continuously, together with simple and robust work-up operations, alongside developments in automation, and online analytics will expand the range of molecules that can be synthesized using this technology. This will allow the benefits of continuous processing to be fully realized, bringing the pharmaceutical industry in line with the majority of the other manufacturing industries where continuous manufacturing is considered the default mode of production.

5.5 Conclusion

Synthetic biochemistry provides access to a range of highly controlled chemistry methodologies including remote hydroxylations with P450s, halogenations, or amine formation from secondary alcohols with very high enantioselectivities. These new methods, along with the many biocatalytic possibilities arising from



Scheme 5.38 Continuous manufacture of Aliskiren hemifumarate.

expansion of new enzyme classes for biocatalysis, e.g. EREDs and IREDs, provide access to new routes that would be difficult and possibly commercially nonviable if conventional multistep organic methodologies were to be employed.

Chemical catalysis, like synthetic biochemistry, provides access to tools that can be transformational to the way in which small molecules are synthesized. Ring closing metathesis (RCM) can provide access to medium-size ring systems in a high yielding and predictable fashion; coupling processes such as Heck or Suzuki reactions have been realized at all scales from early phase studies up to and including full manufacturing processes and have been demonstrated to be highly effective for the syntheses of multiple commercial entities. Hydrogen borrowing is becoming more widely used as it continues to be developed as a synthetic tool and already shows significant advantage in that it can remove the risk involved in the use of N-alkylations with respect to potential genotoxic alkyl halides.

Continuous chemistry can be used for chemistry where batch reactors simply are not viable, unstable intermediates are used, or large inventories of hazardous materials at high temperature and pressure present an unacceptable high safety risk. It is increasingly seen as a methodology that allows greater quality control and a more consistent impurity profile of products. The technology itself is highly amenable to photo- and electrochemical transformations, presenting new opportunities for these underutilized methodologies.

To maintain a competitive manufacturing edge within the pharma industry, the introduction of new ways of conducting small molecule synthesis is a requirement. The new technologies discussed in this chapter are becoming progressively more embedded into the strategic workflows of pharma companies as they seek to improve the way new small molecule APIs are synthesized. They consistently provide safer, more sustainable methods with improved synthetic routes over those used initially for discovery routes, and this, in turn, is the driver for their continued and increasing implementation within the pharmaceutical industry.

Acknowledgments

We would like to thank Dr Gheorghe-Doru Roiban and Dr Glynn D. Williams for their help in proofreading this chapter.

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6

Vortioxetine and Early Drug Development Considerations at the Interface of R&D

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6.1 Introduction

Vortioxetine (**1**, Lu AA21004, 1-[2-(2,4-dimethylphenylsulfanyl)phenyl]piperazine, Figure 6.1) is a multimodal serotonergic antidepressant that was approved for the treatment of major depressive disorder (MDD) by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in 2013, and it was launched in the United States in January 2014. Vortioxetine was in 2016 registered in more than 60 countries around the world and is sold under the trade names Brintellix® or Trintellix®. Vortioxetine is an inhibitor of serotonin (5-HT) reuptake at the serotonin transporter (SERT) and is also an agonist at 5-HT_{1A} receptors, a partial agonist at 5-HT_{1B} receptors, and an antagonist at 5-HT_3, 5-HT_{1D}, and 5-HT_7 receptors [1, 2]. Vortioxetine is the only compound on the market with this combination of pharmacodynamic activities.

Vortioxetine was discovered by researchers at H. Lundbeck A/S in Denmark, and it was developed in collaboration with Takeda. The research program was started in January 2001, and the objective was to discover a rapid-acting antidepressant, although this was not the focus of the clinical program. However, a number of observations and a continued preclinical and clinical research effort throughout the development program led to the notion that vortioxetine, in addition to treating mood symptoms, had the potential to treat cognitive dysfunction in MDD [3]. To date regulatory agencies in >50 jurisdictions, including Europe, have included data in the vortioxetine label to reflect the effect on the aspects of cognitive dysfunction in MDD.

The first human dose of vortioxetine was given in 2003, only two years after the initiation of the project. It was possible to support the project with sufficient amount of high quality active pharmaceutical ingredients (APIs) early on because of a very close collaboration between chemists in medicinal chemistry

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

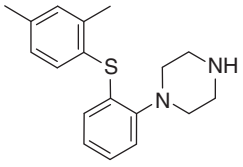

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Figure 6.1 Structure of vortioxetine (**1**, Lu AA21004, Brintellix® or Trintellix®).

and process chemistry, enabling a smooth transition from the research to the development organization. Here we summarize the different synthetic routes to vortioxetine and its isotopes, its salts as well as the synthesis of putative metabolites, and their importance for metabolite identification. Another objective is to highlight the importance of working closely together across organizational borders to secure the right competencies and capacities to move a drug candidate into clinical studies.

6.2 Synthesis of Vortioxetine

The vortioxetine structural class was prepared in several ways during the research stage of the project wherein numerous analogs were synthesized and studied before the compound itself was nominated for early development. The methodology development continued in the medicinal chemistry team during the backup discovery program. The synthesis strategies are illustrated in Figure 6.2.

- *Iron-mediated Route.* Access to the dichlorobenzene iron **2** adduct was key to the iron-mediated synthetic route in which one of the chlorines was displaced by polymer-supported piperazine **3** before the remaining chlorine in **4** was reacted with thiophenol **5** to afford the polymer-bound, protected final compound **6** in two sequential iron-mediated reactions. Decomplexation of the iron and cleavage from the solid support concluded the synthesis. This route was superb for expedient structure–activity relationship (SAR) studies based on *in vitro* assays. However, at that point in time it could not be scaled to more than milligram quantities of material, which was insufficient for *in vivo* studies.
- *Mustard Route.* Since the iron-mediated synthetic route did not allow the synthesis of analogs in which the central benzene ring was nonsymmetrically substituted, we decided to develop a new route to address this issue. This work culminated in the synthesis of single compounds and focused libraries according to a three- or five-chemical step protocol based on nucleophilic aromatic substitution reaction between thiophenol **5** and 2-fluoro-nitrobenzene **7** to afford thioether **8**, reduction to the corresponding aniline **9**, and cyclization of the piperazine ring using mustard reagent **10** in a single step or via a cyclic imide **12** that required additional reduction and deprotection steps. These routes were useful to prepare gram quantities of single test compounds and for parallel chemistry applications, respectively. But they were deemed suboptimal for broader SAR explorations.
- *Palladium-mediated Route.* The use of two subsequent palladium-catalyzed formations of aromatic carbon–heteroatom bonds formed the basis of the third route employed in medicinal chemistry research. This chemistry traced the target molecules back to mono-protected piperazine **14**, an *ortho*-dihalogenated benzene **16**, and a thiophenol **5**. This modular strategy was flexible. Importantly, and unlike the iron-assisted original synthesis, it

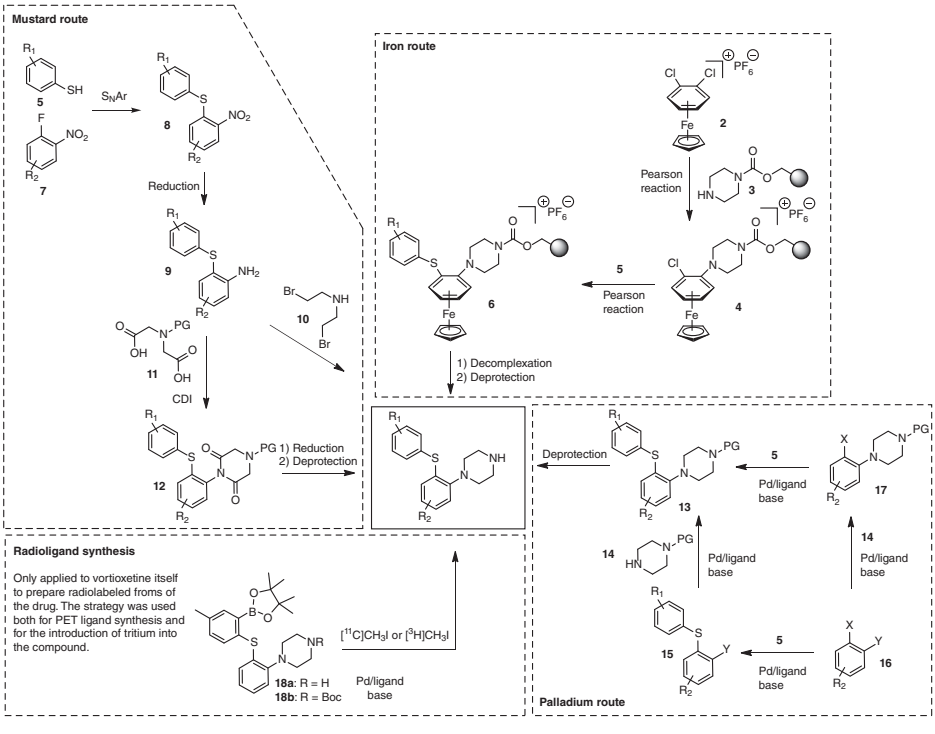


Figure 6.2 The three main synthesis strategies employed in the medicinal chemistry program leading to vortioxetine.

allowed for the synthesis of analogs with nonsymmetrically substituted central rings. This approach was useful both for parallel chemistry applications and for single-compound synthesis. It was readily scalable, and despite being developed after the nomination of vortioxetine for early development, the process chemistry team studied and optimized this route in parallel with the mustard method.

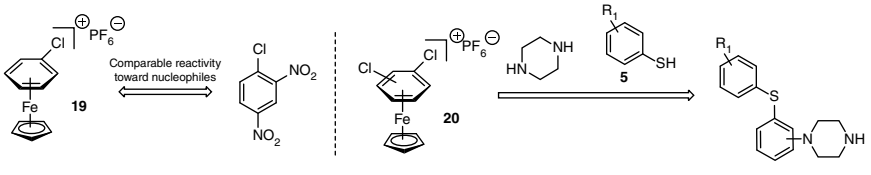
- *Radioligand Synthesis*. A Suzuki–Miyaura cross-coupling strategy was successfully employed to prepare vortioxetine both as a PET ligand and as a tritium-labeled material. The reaction of **18** with either form of radioactive methyl iodide in the presence of a suitable palladium catalyst afforded the desired compounds.

6.2.1 Iron-mediated Synthetic Route

The vortioxetine class originated from an effort at H. Lundbeck A/S to target monoaminergic receptors and transporters as an overall strategy to identify novel antidepressants.

The need for a rapid and general method to support a broad SAR exploration prompted the development of a route to this class of compounds that would enable parallel synthesis of exploratory combinatorial libraries. Toward this end, iron-activated nucleophilic aromatic substitution chemistry (for a review on the subject, see Ref. [4]) appeared to be a promising strategy for the synthesis of these compounds [5]. Given a previous observation that the reactivity of the chlorine in complex **19** is comparable with that of 1-chloro-2,4-dinitrobenzene (for a detailed discussion on this topic, see Ref. [6]), it was envisioned that the three possible intermediates **20** (one of which is **2**) would participate in two sequential nucleophilic displacement reactions to afford the final compounds as outlined schematically in Scheme 6.1. This strategy was inspired by the reported procedures for the corresponding *meta* [7] and *para* [8] dichlorobenzene complexes and their reactivity toward nucleophiles.

There were two main concerns about this chemistry: (i) the photodecomplexation of the iron from the final product was known from in-house experimentation to be a tedious and, at times, messy transformation and (ii) the use of two different nucleophiles was perceived as a major issue given that, in the majority of syntheses reported in the literature, the synthesis of unsymmetrically disubstituted benzenes in this manner could not be achieved with satisfactory yields using standard solution-phase chemistry [9]. The solution to these problems was found by using an immobilized variant of the first nucleophile that



Scheme 6.1 Foundation of the iron-mediated synthetic route.

would separate the second reactive chlorine from the immobilized nucleophile. Firstly, this avoided the formation of the undesired symmetrical double substitution product, and, secondly, it allowed the photo-decomplexation step to be followed by a simple filtration to remove the organometallic by-product.

The strategy outlined in Figure 6.2 and exemplified in Scheme 6.2 involved immobilization of the piperazine nucleophile to a Merrifield resin via a carbamate linker. The two aromatic nucleophilic substitution reactions were conducted in DMF, THF, or mixtures thereof at 60–70 °C. Photo-decomplexation took place in the presence of 1,10-phenanthroline under UV irradiation in a ligand–ligand exchange transformation. Due to the formation of the intensely red-colored phenanthroline-iron complex **21**, the progress of the reaction could be monitored visually. Subsequent cleavage and simultaneous piperazine deprotection was accomplished by treatment with trifluoroacetic acid in dichloromethane. The prerequisite iron-1,2-dichlorobenzene complex **2** was prepared as previously described [10].

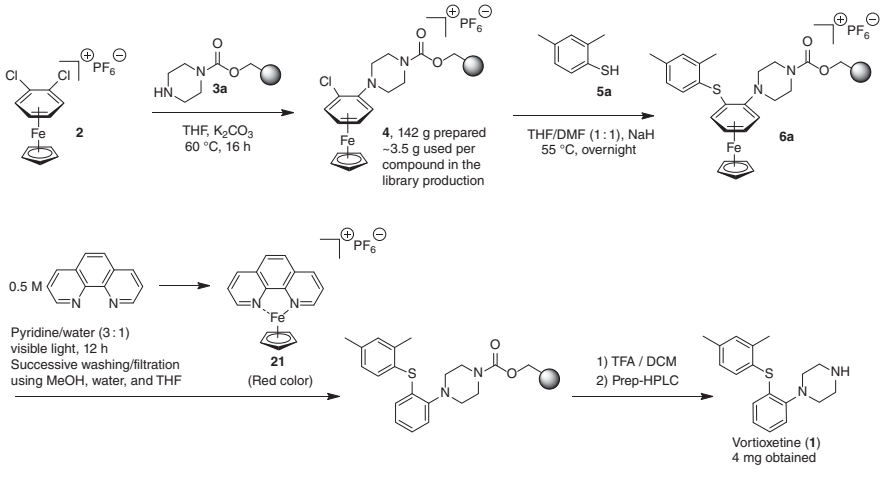
This chemistry was broadly applicable and allowed for the synthesis of approximately 2000 compounds using a range of sulfur, oxygen, nitrogen, phosphorus, and selenium nucleophiles. Some of these results have been published using a different protective group strategy [11]. Full experimental details on the work related to the vortioxetine project were previously disclosed in a patent [10].

As shown for vortioxetine in Scheme 6.2, the key immobilized reagent **4** was produced in a large amount to enable subsequent combinatorial chemistry campaigns. Upon successful optimization, this chemistry was executed toward the synthesis of many compounds and was generally effective in preparing milligram quantities of the test compounds.

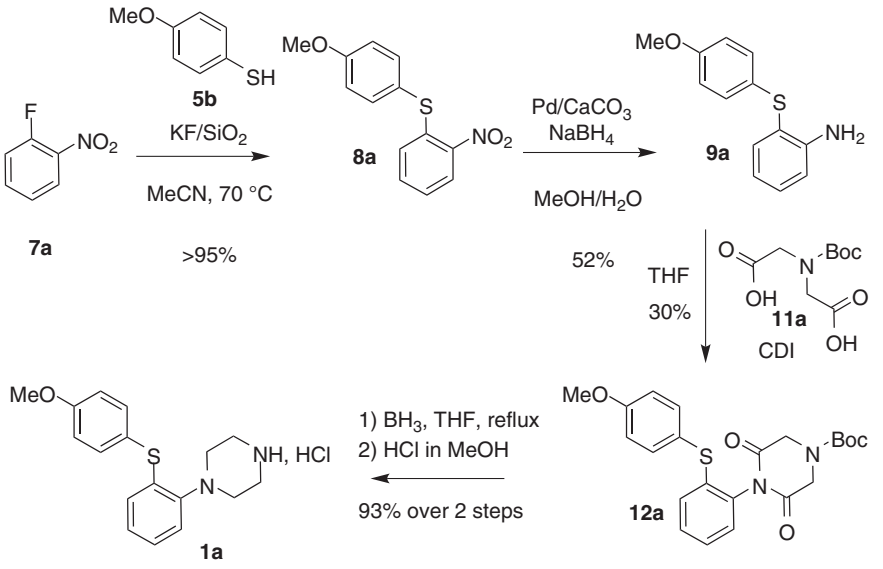
In general the iron-mediated synthetic approach efficiently addressed the needs of the early hit-to-lead campaign and played a critical role in the project in that vortioxetine itself was first prepared using this chemistry. However, the example of vortioxetine in Scheme 6.2 serves to illustrate the major problem associated with this synthesis strategy: the low amount of the test compound that was obtained. This was clearly insufficient to support the *in vivo* pharmacology studies. The other key limitation of this strategy was the inability to prepare analogs wherein the central benzene ring was substituted. However, later work by the process chemistry team did show that the iron-mediated chemistry was indeed scalable, so it would have been possible to prepare larger amounts of the anticipated mixtures and separate them during work-up. Nevertheless, the synthesis of such analogs was better addressed using either the mustard route or palladium-mediated synthesis.

6.2.2 Mustard Route

The need for parallel synthesis of larger quantities of material to support more elaborate pharmacological profiling and in particular *in vivo* studies prompted the development of a new synthesis approach. Initially, a small (12-member) focused library was prepared using parallel chemistry. This work was done by optimizing each step along the way as discussed in the following. As an example, the synthesis of compound **1a** is shown in Scheme 6.3.



Scheme 6.2 Preparation of vortioxetine using the iron-mediated synthetic route on solid support.

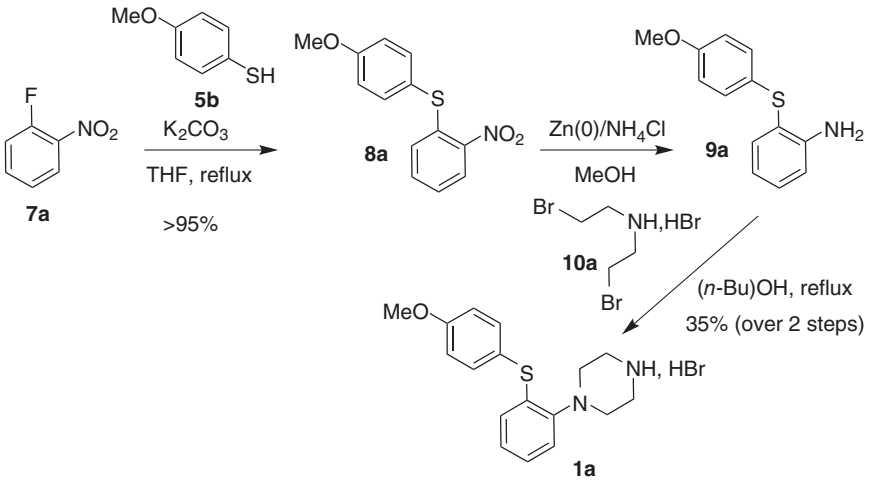


Scheme 6.3 Synthesis of vortioxetine analog **1a** using parallel chemistry.

The first step proceeded in nearly quantitative yield, and the use of silica-supported potassium fluoride reduced the purification to a simple filtration. Optimization of the remaining reaction conditions in this first library was conducted as the chemistry was performed. Specifically, the reduction of the nitro group in **8a** to the corresponding aniline **9a** and the cyclization step leading to **12a** were challenging steps. In the former case, a number of classic conditions including iron dust in acetic acid and tin metal in concentrated hydrochloric acid were evaluated, but both were found to be producing complex mixtures. The use of a catalytic amount of $Pd/CaCO_3$ and sodium borohydride as the terminal reductant proved more useful. The reduction did not always proceed to completion, and two rounds were, for example, performed *en route* to the final compounds. Furthermore, the cyclization step often failed to proceed to completion, and the reaction typically stopped at the amide intermediate instead of progressing to the imide **12**.

Compound **1a** was also synthesized using the chemistry summarized in Scheme 6.4. As in the original focused library, the synthesis started with the nucleophilic aromatic substitution reaction between 4-methoxybenzenethiol **5b** and 2-fluoro-nitrobenzene **7a**, this time with potassium carbonate as the base and THF as the solvent, to provide the product **8a** in near-quantitative yield. The subsequent reduction of the nitro group was achieved with zinc metal and ammonium chloride in methanol. The crude aniline **9a** was carried on in the next step where it was subjected to mustard reagent **10a** to afford the final compound **1a** as the hydrobromide salt. The reported yield of 35% is for the two last steps, including the precipitation of the final compound during work-up. No attempts were made to isolate more material from the filtrate.

The synthesis strategy using the mustard analog was based on classic chemistry and worked very well both for focused library production and for



Scheme 6.4 Improved synthesis of vortioxetine analog **1a** using the mustard route.

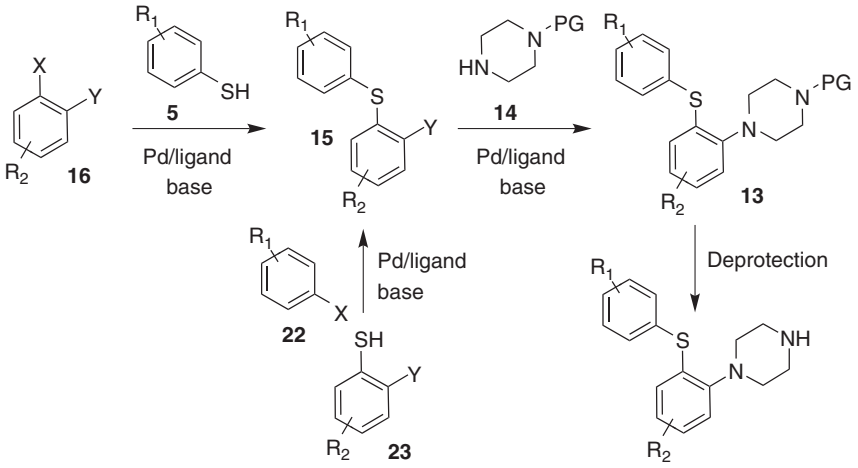
single-compound synthesis. As such it satisfied the need for a more scalable alternative to the iron-mediated synthetic route.

6.2.3 Palladium-mediated Route

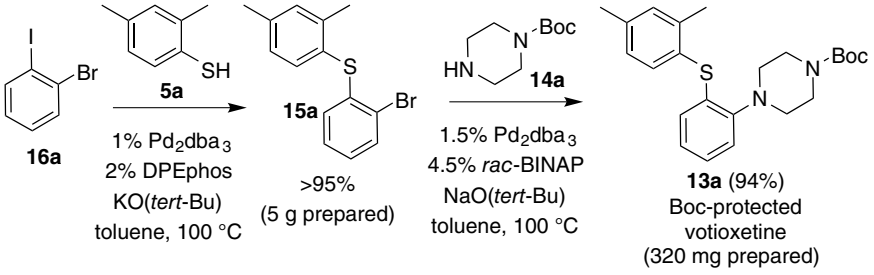
Supporting *in vivo* pharmacology via the iron-mediated chemistry was impractical, and the mustard route was hampered by other issues such as difficulties in reducing the nitro group or low yields when forming the piperazine ring. Therefore, it was decided to develop a synthesis that would both be scalable and allow access to target compounds with a more complex substitution pattern on the central benzene ring. To this end the formation of aromatic carbon–heteroatom bonds via palladium-catalyzed coupling of thiols or amines to aryl halides had been reported in the literature by the research groups of Hartwig and Louie [12] and Buchwald and coworkers [13]. As outlined in Scheme 6.5, the basic idea was to prepare the thioether linkage in **15** from either "end" (i.e. from **5** + **16** or from **22** + **23**) and to introduce piperazine **14** on the central benzene ring leading to **13** via a subsequent aryl amination reaction.

At the start of the experimental work on this strategy, we had an extensive experience with the synthesis of brominated diaryl sulfides **15** (Y = Br) according to the protocol reported by Schopfer and Schlapbach [14]; these aryl bromides had also been used as substrates in bromine–lithium exchange chemistry *en route* to tetrahydropyridine and piperidine variants of the vortioxetine structural class of antidepressants (see, for example, Refs [15, 16]). Based on this approach, **15a** was prepared from **5a** and **16a** and subsequently found to react in near-quantitative yield with Boc-protected piperazine (**14a**) in the presence of a catalytic amount of Pd_2dba_3 ligated by racemic BINAP under the general conditions reported by Wolfe and Buchwald (Scheme 6.6) [17].

The result in Scheme 6.6 prompted the process chemistry team to focus on this new route despite the fact that they had already prepared more than 1 kg



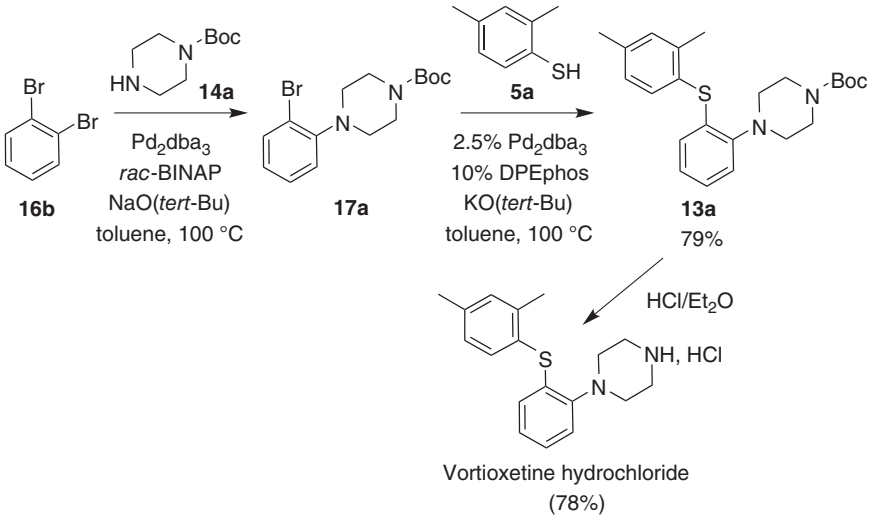
Scheme 6.5 Initial version of the palladium-mediated synthetic route.



Scheme 6.6 First synthesis of Boc-protected vortioxetine via the palladium-mediated synthetic route.

of API using an optimized version of the mustard route. The results of these efforts are discussed in detail later in this chapter. However, there was a close and continuous interaction between the two chemistry teams at this point in the project, and about a month after the synthesis in Scheme 6.6, the process chemistry group had prepared almost 100 g of API using this approach. Today, this project stands out as the model example at Lundbeck for how the two chemistry teams from research and development should interact in order to deliver API in a timely and efficient manner, serving the need for API in a project moving toward first-in-man studies.

Having established that both the aromatic C–N and C–S bonds could be formed via palladium catalysis, the team next focused on an extension of the palladium-mediated synthetic route by forming the phenyl piperazine portion of the target molecules prior to the formation of the diaryl thioether linkage and to expand the SAR from the resulting synthesis intermediate **17a** (Scheme 6.7). These building blocks allowed expansion of the SAR around vortioxetine by enabling synthesis of hitherto unavailable target compounds with substituted central benzene rings. In most cases no efforts were made to maximize the



Scheme 6.7 Synthesis of vortioxetine via the "reversed" palladium-mediated synthetic route.

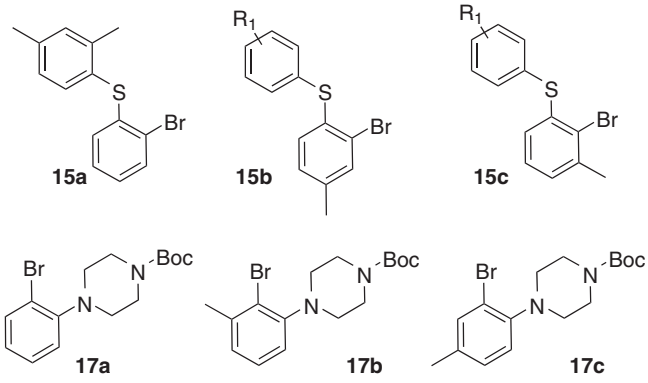


Figure 6.3 Key building blocks for the palladium-mediated synthetic strategy.

isolated yields as the focus was on SAR expansion. The medicinal chemistry applications of the palladium-mediated synthetic route have been published in detail previously [1].

The synthesis of the key building blocks like **15a–c** (Figure 6.3) was readily performed on multigram scale. In what was in part an extension of this work, we further developed conditions for the monoamination of symmetrical dibromobenzenes and the chemoselective monoamination of nonsymmetrical dibromobenzenes to provide access to intermediates like **17a–c** on multigram scale [18]. The chemistry exemplified in Schemes 6.6 and 6.7 provided access to test compounds from small scale, focused libraries to larger-scale single compounds to support a more extensive profiling by the pharmacologists. The chemistry was readily performed without the use of glove box or Schlenk techniques. All reagents were weighed out in air, and reactions were performed in nondistilled toluene.

As vortioxetine was progressed through preclinical and early clinical studies, the process chemistry and medicinal chemistry teams continued to collaborate on the API process development and production. The initial efforts focused on the mustard route to support the safety and toxicological studies required to go into clinical phase 1 studies. One of the primary concerns was the fact that the mustard reagent **10** and related compounds such as its *N*-benzyl derivatives are lachrymators and require precautions to be handled safely. It was also an issue that the reagent of choice, **10a** itself, was prepared from the corresponding diol by treatment with concentrated hydrobromic acid, and this process gave variable amounts of morpholine hydrobromide as a by-product.

The process team found that it was possible to prepare both aromatic carbon–heteroatom bonds using a single catalyst in a one-flask transformation. Consequently, the crude API was contaminated with palladium impurities, and there were also concerns about the inorganic products sodium iodide and sodium bromide due to the heavy halogens. Both of these challenges were solved with an optimized purification protocol. The *iso*-propyl alcohol solvate of the hydrobromide salt of vortioxetine was of particular importance in this context due to the improved purification characteristics of this compound. This gave a fast useful production method yielding vortioxetine in 84% yield in just one step. By use of this solvate purification process, it was possible to reduce or remove the problematic impurities as well as palladium residues.

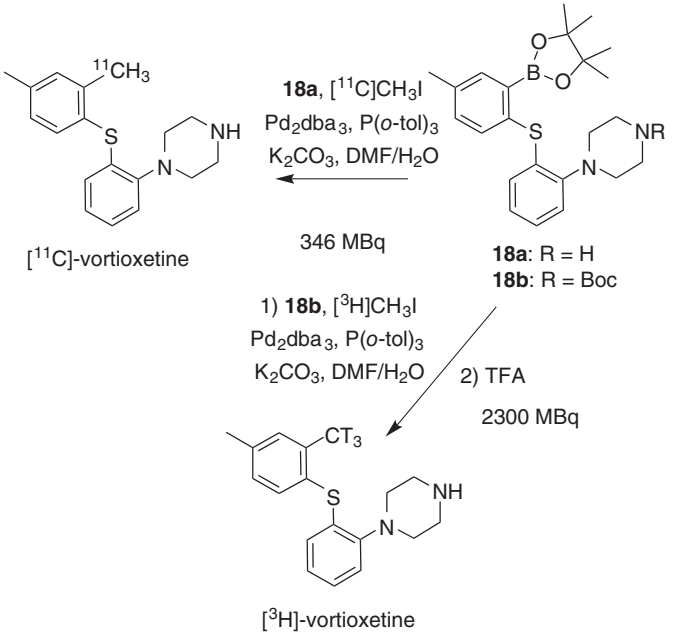
6.2.4 Radioligand Synthesis

Vortioxetine has been labeled with carbon-11 and evaluated as a positron emission tomography (PET) ligand [19]. The radioligand was prepared using a last step Suzuki–Miyaura cross-coupling using unprotected boronate ester **18a** as the substrate in the reaction with [^{11}C]methyl iodide (Scheme 6.8). Using very similar conditions it was possible to extend this chemistry to the preparation of tritiated vortioxetine. This radioligand has been applied for probing the binding site of vortioxetine at SERT [20].

6.3 Metabolites of Vortioxetine

The major metabolic pathways for vortioxetine are presented in Figure 6.4. Vortioxetine is extensively metabolized by N-oxidation of the secondary amine moiety, oxidation of the core benzene ring para to the piperazine ring, oxidation of the *para*-methyl group on the distal benzene ring, and subsequent glucuronic acid conjugation (Figure 6.4). To a lesser extent, oxidation at the sulfur atom is observed [21].

A total of seven metabolites were found in humans, all of which were also identified in the animal species studied. The carboxylic acid derivative Lu AA34443 (**24**), resulting from oxidation of the *para*-methyl group, was the major metabolite in all tested species, while no modifications of the *ortho*-methyl group were observed [21].



Scheme 6.8 Synthesis of ^{11}C- and ^{3}H/tritium (T)-labeled vortioxetine.

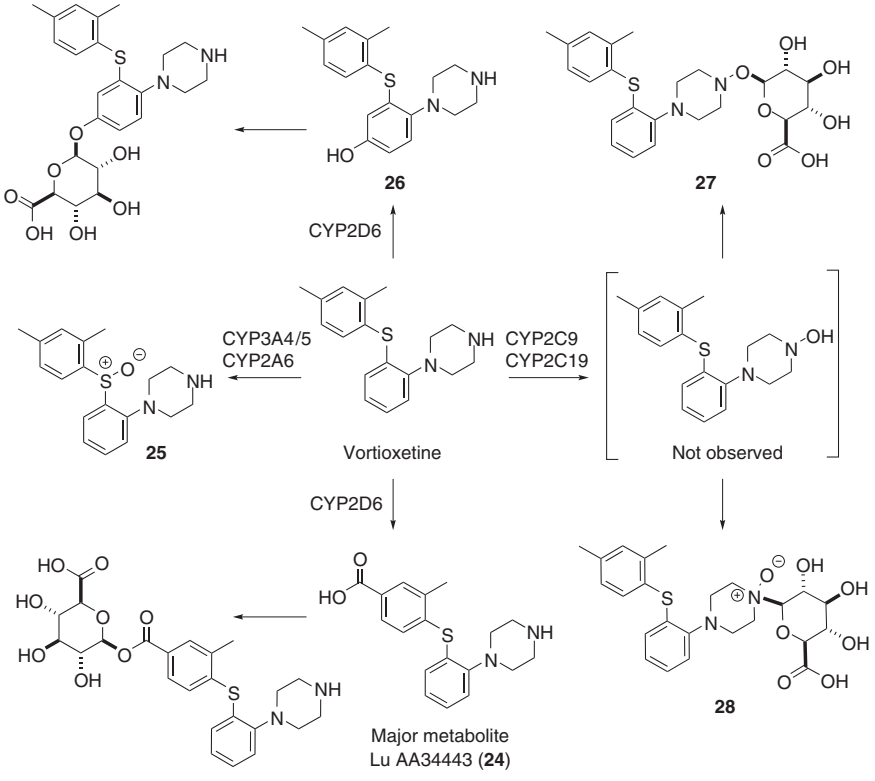
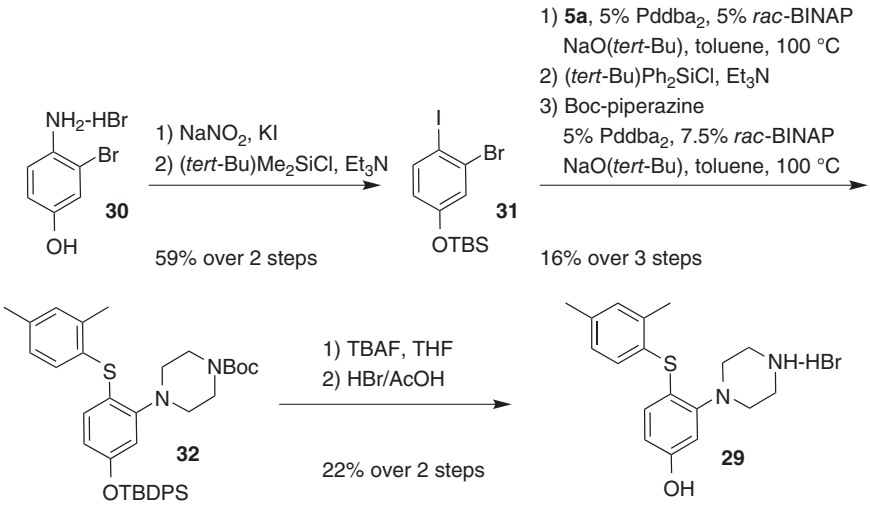


Figure 6.4 Overview of metabolic pathways for vortioxetine.

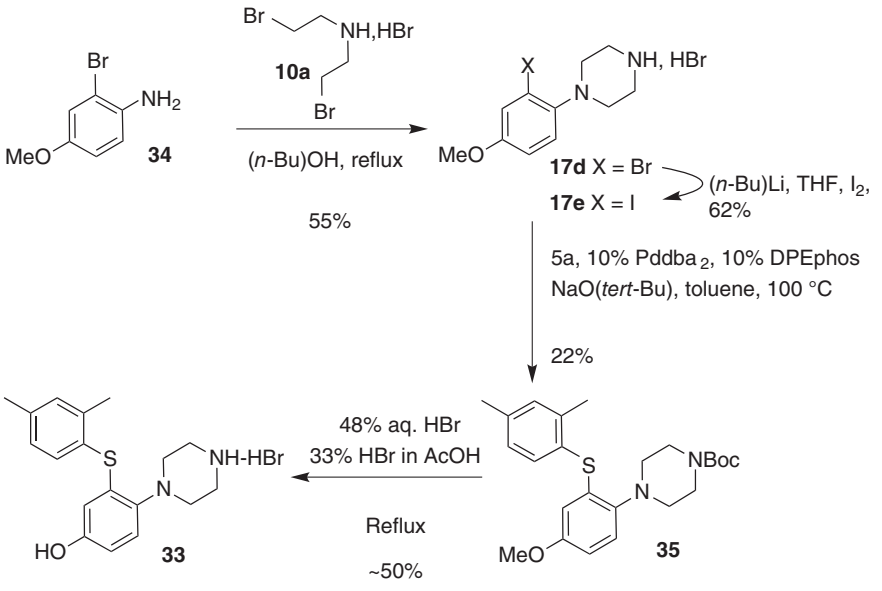


Scheme 6.9 Synthesis of the proposed mono-hydroxylated vortioxetine metabolite **29**.

Access to authentic samples of putative metabolites of drug candidates is critical to the development of a novel drug. Metabolites can be difficult to prepare, and the *a priori* knowledge about their chemical structure is often limited to generic structural information, such as "a mono-hydroxylation of the left-hand part of the molecule." Thus, chemists typically prepare several potential metabolites in order to confirm the identity of the ones observed experimentally. In the vortioxetine case, access to sufficient material from rat urine guided the synthesis of one of the main metabolites. The structure **29** was initially assigned to the metabolite based on nuclear magnetic resonance (NMR) data (Scheme 6.9).

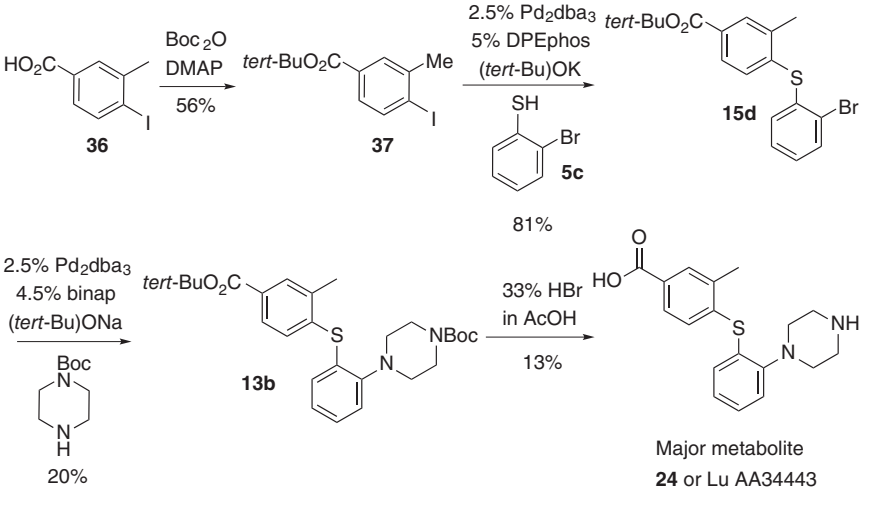
The strategy applied in the medicinal chemistry team was to prepare **29** as quickly as possible and in a regioisomeric manner. Therefore, it was decided to rely on an easily cleaved phenol protective group and to use the established palladium-mediated chemistry route. Diazotization of aniline **30**, itself readily prepared by regioselective lithiation [22], afforded iodo-bromo anisole **31**. Chemoselective thioarylation proceeded well, but the dimethyl-*tert*-butyl silyl protective group was lost and subsequently replaced with a more stable diphenyl-*tert*-butyl silyl group before aryl amination afforded **32** in low yield over the three steps. Deprotection over two steps afforded **29** that precipitated in low yield during work-up. Disappointingly, NMR studies showed that **29** was *not* the isolated metabolite. Therefore, we focused on the synthesis of the regioisomer **33** whose synthesis is summarized in Scheme 6.10.

The synthesis of the regioisomeric putative metabolite **33** started from the aniline **34**. Reaction with mustard reagent **10a** gave the piperazine **17d**. Unlike other aryl bromides in this project, this material did not react with thiophenol **5a** in the presence of one of our routinely applied palladium catalyst systems. Therefore, a slight detour via the more reactive iodide **17e** was necessary. This afforded the coupled product **35** in low but sufficient yield. Global deprotection concluded the synthesis of **33**, which was indeed the desired metabolite.

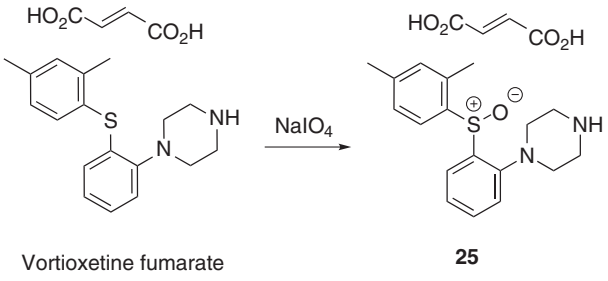


Scheme 6.10 Synthesis of the actual mono-hydroxylated vortioxetine metabolite **33**.

All metabolites (Figure 6.4), except the glucuronides of **24** and **26**, were synthesized. Metabolite **24** or Lu AA34443 was prepared in four steps from carboxylic acid **36** (Scheme 6.11). The carboxylic acid was protected as the *tert*-butyl ester, using Boc anhydride as the *tert*-butanol source and DMAP as the catalyst. The corresponding *tert*-butyl ester **37** was coupled with *ortho*-bromothiophenol **5c** to the diphenyl sulfide **15d** using Pd_2dba_3 and DPEphos. Subsequently, a palladium-catalyzed C–N coupling was implemented to afford the Boc-protected



Scheme 6.11 Synthesis of major vortioxetine metabolite **24** or Lu AA34443.



Vortioxetine fumarate **25**

Scheme 6.12 Synthesis of vortioxetine metabolite **25**.

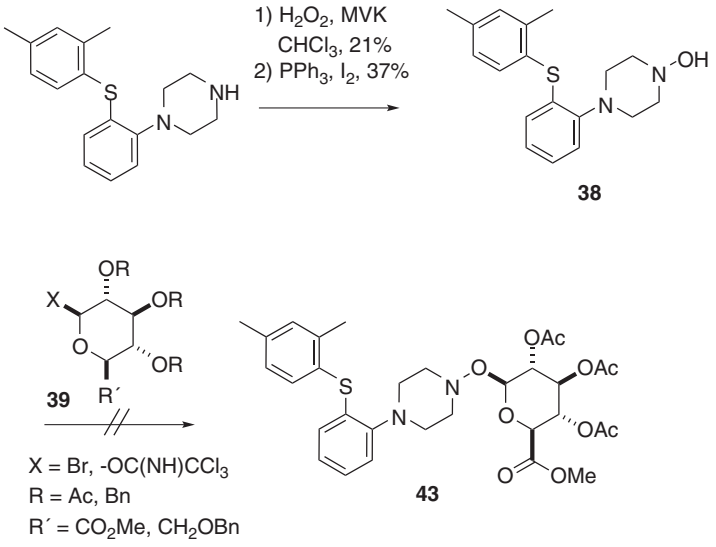
piperazine **13b**, which was Boc-deprotected using hydrobromic acid in acetic acid to afford the desired metabolite **24** or Lu AA34443.

The synthesis of the sulfoxide metabolite **25** was achieved by exposing the fumarate salt of vortioxetine to sodium perchlorate in a water/methanol mixture affording the free base of **25** in 94% yield (Scheme 6.12). Precipitation using fumaric acid afforded **25** as a 1.5 : 1 salt in a modest 21% yield.

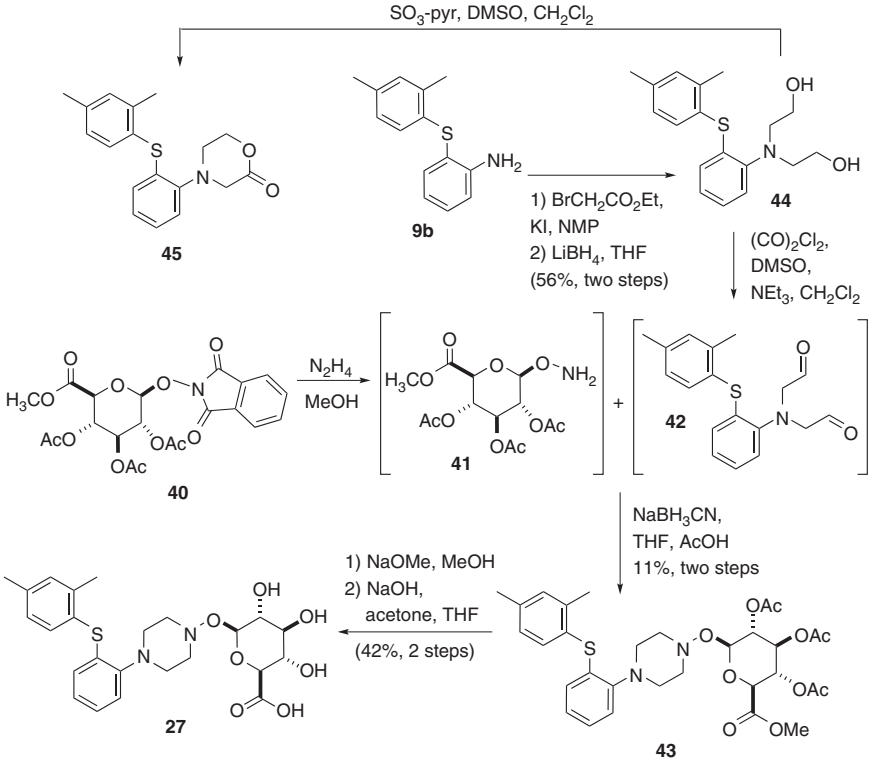
Oxidation of a secondary amine, if present, to form the corresponding hydroxylamine intermediate is seldom observed *in vivo* for piperazines or other nitrogen-containing heterocycles. An explanation for this may be found in reports that this intermediate is rather unstable [23–26] although observations of stable heterocyclic hydroxylamines have occurred [27–30]. Consequently, there is only a limited number of examples of glucuronic acid conjugation of heterocyclic hydroxylamines in the literature [31–33], and the structures of these metabolites have largely been deduced via mass spectrometry and ^{1}H NMR analysis.

Humans dosed orally with vortioxetine afforded two less common compounds verified to be hydroxylamine glucuronide **27** and *N*-oxide *N*-glucuronide **28**. These two metabolites represented significant challenges with respect to structure elucidation and identification. Extensive efforts went into the structure identification of these two metabolites. Organic synthesis of *N*-oxide *N*-glucuronide **28** was unsuccessful, but *in vitro* biosynthesis using human liver microsomes and subsequent semipreparative HPLC–MS resulted in the production of approximately 10 mg of this metabolite. Initial efforts to synthesize the hydroxylamine glucuronide **27** via a coupling strategy between a hydroxylamine **38**, itself prepared from vortioxetine by N and S oxidation with hydrogen peroxide followed by chemoselective reduction of the sulfoxide with triphenylphosphine and iodine, and the electrophilic glucuronic acid derivatives **39** were met with disappointment (Scheme 6.13). After extensive experimentation, metabolite **27** was unambiguously prepared using a novel approach for the synthetic preparation of the compound (Scheme 6.14), and various NMR experiments were performed for structural identification purposes.

The synthetic strategy to **27** was to incorporate the N–O-hydroxylamine moiety as early as possible from starting materials known in the literature. Thus, phthalimide **40** was chosen as the starting point [34]. The phthalimide hydroxylamine-protecting group was removed via brief exposure to hydrazine



Scheme 6.13 Failed attempt to synthesize hydroxylamine glucuronide **27**, via direct coupling of hydroxylamine **38**.



Scheme 6.14 Synthesis of **27** via a double reductive amination strategy.

in methanol. An observation was that liberated hydroxylamine was inherently unstable under the reaction conditions. The free hydroxylamine **41** was subjected to a double reductive amination where coupling with dialdehyde **42** using sodium cyanoborohydride afforded **43**. An interesting observation was observed *en route* to **42**, when oxidation of diol **44** (prepared from **9b**) was performed using Parikh–Doering conditions (i.e. SO_3·Pyr, DMSO, DIPEA) [35]. Under these conditions, lactone **45** instead of the desired dialdehyde **42** was afforded. Several conditions were screened in order to oxidize diol **44** to the corresponding dialdehyde **42**. This problem was solved using a Swern oxidation [36]. One could possibly envision epimerization at the anomeric position under the slightly acidic conditions during the reductive amination, but due to anchimeric assistance from the neighboring acetyl moiety, the β-anomer is favored. Coupling product **43** was then deprotected using a two-step protocol to afford the N–O-glucuronide **27**. The synthesis unambiguously allowed structural assignment of the structure of metabolite **27**, as well as indirect identification of metabolite **28**.

6.4 Conclusion

Vortioxetine is a multimodal serotonergic antidepressant that was discovered by scientists at H. Lundbeck A/S in Denmark. Initially, an iron-mediated synthetic route was developed, and approximately 2000 compounds were synthesized in milligram scale using this route. In the vortioxetine drug project, alternative synthetic routes were necessary in order to scale up the synthesis of key compounds and to allow for more variation in the substituent pattern of target compounds, especially in the central benzene ring. Therefore, two new routes (i.e. mustard route and palladium-mediated route) were developed. For vortioxetine itself, API supply was never an issue because medicinal chemists and process chemists worked together very early on to develop and scale up the chemistry in an interactive manner. Toward this end, process chemists finally optimized all three synthetic strategies to a level where they were capable of delivering the API, based on the project's demands. Similarly, putative metabolites and metabolites were synthesized early on mainly by the medicinal chemists and later on and in the more challenging cases by process chemists. The case highlights the importance of working closely together across organizational borders to secure availability of the right competencies and capacity to progress a drug candidate from research to clinical studies.

Abbreviations

5-HT	serotonin
AcOH	acetic acid
ADME	absorption, distribution, metabolism, and excretion
API	active pharmaceutical ingredient
rac-BINAP	(±)-2,2′-bis(diphenylphosphino)-1,1′-binaphthalene

Boc	*tert*-butyl-oxy-carbonyl
dba	dibenzylideneacetone
DCM	dichloromethane
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DPEphos	bis[(2-diphenylphosphino)phenyl] ether
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
HPLC	high performance liquid chromatography
MDD	major depressive disorder
MS	mass spectrometry
NMP	*N*-methylpyrrolidine
NMR	nuclear magnetic resonance
PET	positron emission tomography
Pyr	pyridine
SAR	structure–activity relationship
SERT	serotonin transporter
TFA	trifluoroacetic acid
THF	tetrahydrofuran
UV	ultraviolet

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7

Development of a Practical Synthesis of 4′-Azido-2′β-Methyl-2′-Desoxycytosine and Its Prodrugs as HCV Chemotherapeutic Agents

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7.1 Introduction

The introduction of the azide functionality into nucleoside scaffolds has led to important contributions in the area of HIV [1, 2] and HCV [3–5] therapy. We were asked to support a medicinal chemistry program aimed at developing a novel class of anti-HCV agents represented by 4′-azido-2′-deoxy-2′-C-methyl-cytidine MV064274 and its diester prodrugs, e.g. MV075379 (Figure 7.1) [6].

Structurally, our synthetic targets bear much similarity to natural nucleosides, as their mode of action relies on their ability to fit in the binding site of enzymes that recognize RNA and DNA building blocks. With respect to uridine (**1**), a reasonable starting material, our targets showcase several modifications: first, at the C-2′ carbon center, the α-hydroxyl function in uridine has been replaced by a β-methyl group; second, at C-4′ an azide has been introduced for a hydrogen atom with retention of configuration. Both these modifications are synthetically daunting and result in complex linear strategies to reach our targets. The third modification, i.e. amination of the uracil moiety to the corresponding cytosine base, is synthetically straightforward.

The medicinal chemistry route (Schemes 7.1 and 7.2) had been scaled with great difficulty to provide a few hundred grams of material for *in vitro* and preliminary animal studies (M. Nilsson, private communication). The next step in development was the delivery of a 5 kg GMP batch for GLP toxicology and Phase I clinical studies. The first part of the synthesis (Scheme 7.1) addresses the introduction of the methyl group at C-2′ to prepare (2′*R*)-2′-deoxy-2′-*C*-methyl uridine (**10**). This single synthetic manipulation is achieved in nine chemical steps, including nine isolations and several chromatographic purifications, and proceeds in an overall yield of 3.4%.

The second part of the synthesis of MV075379 is composed of six chemical steps, with six chromatographic purifications, and delivers the target in 11.5% yield as an amorphous free base. The overall yield of our target prodrug over 15 steps is 0.39%. In some cases, medicinal chemistry syntheses can be used to

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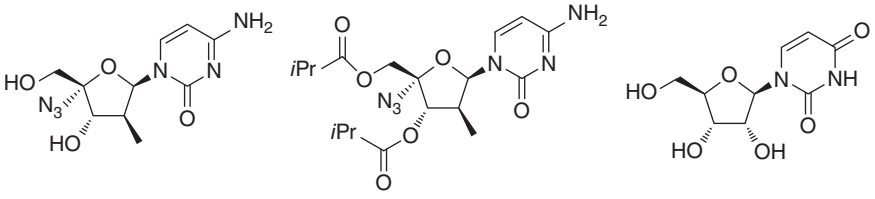


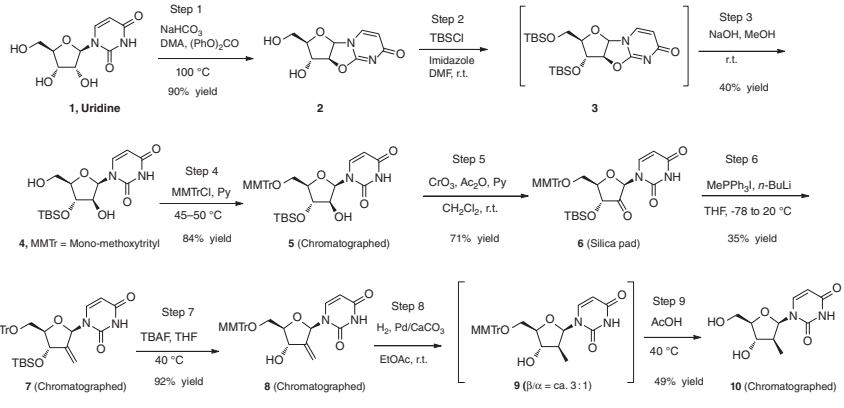
Figure 7.1 Targets of synthetic interest and a reasonable precursor (**1**).

deliver initial GMP materials, while process chemists endeavor to develop a more practical synthesis, if the program is sufficiently funded.

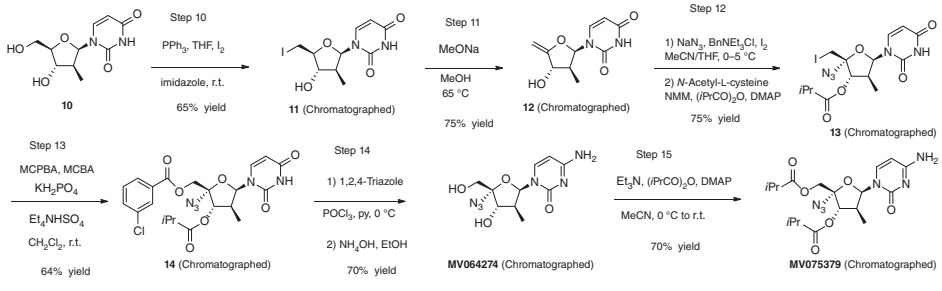
Clearly, this strategy was not directly applicable to the MV075379 case. It is relatively straightforward to assess that the existing synthesis cannot possibly deliver multikilo amounts of the new drugs in an acceptable time frame, using reasonable human and financial resources. In fact, when allowing about 50% safety margin in case of lost batches, one would need 1.7 tons of uridine to prepare 5 kg of our target. Assuming that three separate batches will be run, the first few steps will have to be run in production equipment. Production equipment is hardly available for preparing Phase I batches to which kilo lab or pilot facilities are dedicated. Assuming one week of work per step and two weeks for each chromatography, a team of 15–20 chemists may expect to deliver the target in 79 weeks. Clearly, the synthesis as such is not worth executing, and it needs a lot of improvement. The rest of this chapter details how those improvements were implemented.

There are several strategic considerations that militate against using a *quick and dirty* approach to prepare the first GMP batch. During chemical development, it is crucial to arrive as soon as possible (if possible with the first GMP batch) at an *enabling route*, i.e. a synthesis that could deliver the batches for the entire clinical programs without delays. If needed, a separate team can proceed to design a cost-effective commercial process, but the timing of this depends on funding and perhaps on the achievement of some clinical milestone. It is therefore worth spending sufficient time to develop an enabling route not only to deliver the batch in reasonable time but also to ensure that the next batches can be reproducibly made by a practical, scalable approach. There are risks associated with changing the synthesis during development: any major changes could generate new process impurities, which may take unusually long times to be addressed and removed. Developing an enabling route before delivering the first GMP batch minimizes the risks associated with too many process changes between Phases I and III.

Often, the enabling route is just a modification of the medicinal chemistry route, optimized for yields, and safety of operations and purification schemes. In this case the team evaluated the medicinal chemistry synthesis and identified its weaknesses. Given the time and cost involved in chromatographic purifications, a development group usually sets as immediate goal the elimination of all chromatographies. These can be replaced by crystallizations or precipitations, operations that are more easily reproducible and less time consuming. In addition, a strategy employed in early development is to limit the number of isolations. Isolations are costly in terms of plant occupancy and solvent usage. A well-developed,



Scheme 7.1 Original synthesis of (2′*R*)-2′-deoxy-2′-*C*-methyl uridine **10**.



Scheme 7.2 Original synthesis of MV075379.

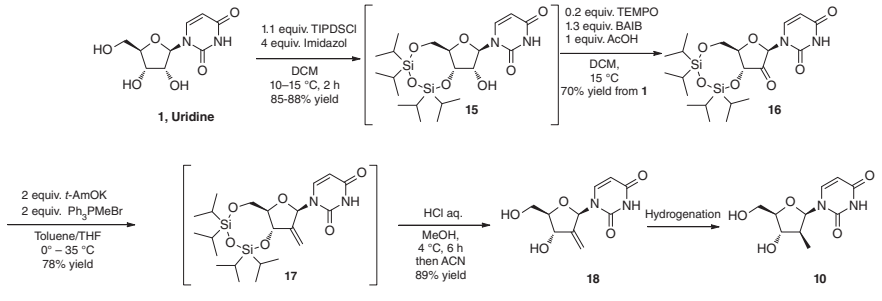
robust 15-step process should not contain more than 6–8 isolations. Often, a clean reaction can be *telescoped*, in a suitable solvent, into the next chemical step, running only an in-process control (IPC), to make sure the quality and the yield of the intermediate in solution is as expected.

Our initial analysis focused on the actual chemistry involved in Schemes 7.1 and 7.2. Steps 1–4 are used just to achieve differential protection of the hydroxyl groups of uridine at C-3′ and C-5′, leaving C-2′ free for the oxidation reaction. Such orthogonal protection is not needed because the bis-ester derivative was selected as final active pharmaceutical ingredient (API), and the goal became to just protect the two hydroxyl groups simultaneously in one chemical step. Step 5 involves oxidation of intermediate **5** with chromium trioxide, followed by Wittig reaction and a poorly diastereoselective hydrogenation. It was considered that this synthetic strategy could be kept if the yields could be improved and the very toxic chromium-based oxidant could be replaced by a milder, less toxic agent. This would require a better understanding of the low-yielding Wittig reaction and a careful optimization of the hydrogenation step. Given that a stereochemical bias already exists (circa 3 : 1) in favor of hydrogen attack from the α face, a homogeneous hydrogenation catalyst with suitable bulky ligands could afford selectively the β-methyl analog **10**, thereby removing the need for a chromatographic separation of the two epimers. In summary, the plan was to produce intermediate **10** in complete diastereoselectivity in five chemical steps instead of nine.

The synthetic pathway from **10** to the API is less subject to streamlining. The dehydration to **12** is straightforward, and iodoazidation is effective at introducing the azide function with complete regio- and stereoselectivity. Clearly, enough safety data must be obtained before considering the scale-up of an azidation step. The completeness of the face selectivity remained of course to be established, and synthesis of the 4′-*epi*-isomer was required. The replacement of the 5′-iodo group with a carboxylate anion requires, apparently, an oxidative activation due to the quasi-neopentyl character of the iodide, hence its resistance to S_N2 displacement. Undaunted by the early experience of our medicinal chemistry colleagues, we decided to try to optimize a nonoxidative substitution. Indeed, *m*-CPBA represents a handling hazard, especially in a plant setting, and its use is best avoided. Also quite wasteful are the hydrolytic steps for both esters of **14**, groups that have to be introduced again later to obtain the API. In conclusion, we supposed that the process could be shortened by 4–6 steps, and our additional goal was to improve the yield at least by a factor of 10. Finally, a thorough crystallization screen on the API and its salts would be carried out in an attempt to optimize the solid form for further development [7].

7.2 New Synthesis of (2′*R*)-2′-deoxy-2′-*C*-methyl uridine (10)

The preparation of compound **10** was already described in the literature [8] in five steps starting from cheap and naturally available natural uridine (Scheme 7.3) [9–15]. This approach allows selective protection of the 3′–5′ hydroxyl groups,



Scheme 7.3 Scalable synthesis of compound **10**.

leaving the desired 2′ OH function available to manipulations, such as oxidation, homologation, and hydrogenation. However, the key hydrogenation step of the *exo*-methylene group displays modest face selectivity in the presence of palladium catalysts (e.g. $\beta/\alpha = 3:1$ with Pd on $CaCO_3$) [8].

The initial protection step to yield **15** was reported in pyridine as solvent [16]. Avoiding the use of toxic pyridine was necessary. Here, the use of imidazole in DCM allowed the smooth formation of the 3′,5′-protected intermediate. It was found that 4 equiv. of imidazole are necessary to drive the reaction to full conversion (**1** < 0.3%). The use of 1.1 equiv. of pure TIPDSCl leads to complete conversion. Nevertheless, the slight excess of silylated reagent triggers the formation of a number of impurities (Figure 7.2).

Reduction of the stoichiometry to either 0.98 equiv. of pure TIPDSCl or further dilution of the reaction mixture using 1.1 equiv. of the reagent limits the formation of these impurities: the unreactive starting uridine, if present, can be easily purged by an aqueous wash at the same time as imidazole. Water in the reaction mixture was identified as a parameter leading to the formation of the monoprotected impurity (**imp. 2**, Figure 7.2). Due to this observation, azeotropic distillation of the starting reaction mixture containing **1** imidazole in DCM was implemented (KF < 0.05 w/w%). A closer monitoring of the reaction showed full conversion in 1 h at 10–15 °C. Extending the reaction time beyond 5 h was deleterious for the purity and further impurities (**imp. 4-a** and **4-b**, Figure 7.2) formed, lowering the purity to 70% after 50 h. In total, eight impurities were identified, totaling 12–17 area % (Figure 7.2).

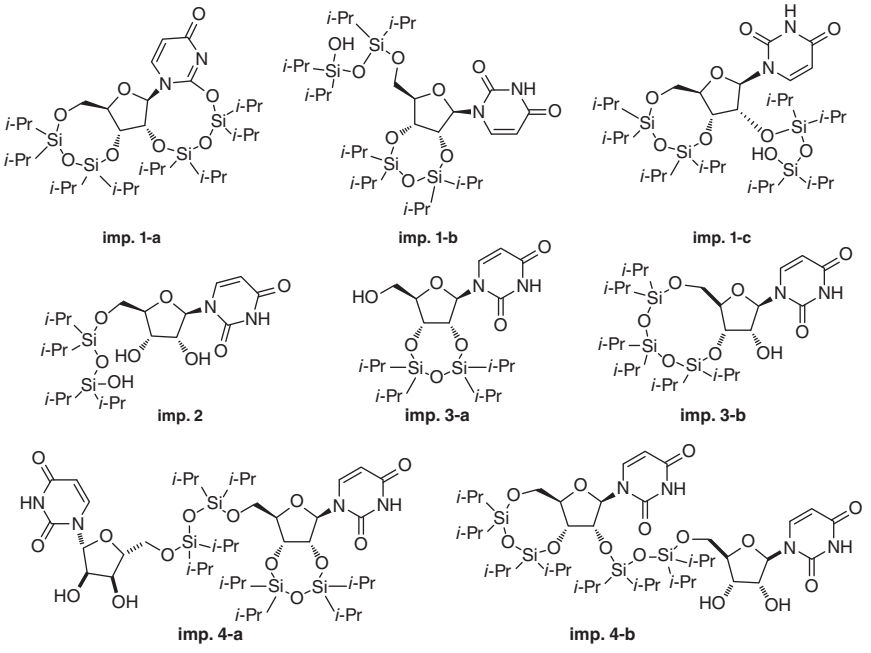


Figure 7.2 Proposed structures of impurities in uridine protection.

Under these conditions, the assay yield was consistently 85–88% up to 10 kg scale, with 87–88 area % purity. Because compound **15** was amorphous, thereby preventing ready isolation by crystallization, we processed the crude product further and found that all process impurities are conveniently removed in the oxidation step.

This oxidation step has been described in the literature using CrO_3-based reagents [17], characterized by low yields and toxic or the Dess–Martin reagent [18], which is hazardous to scale up. We have developed a TEMPO/bis(acetoxy)iodobenzene (BAIB) oxidation to solve the problems associated with the above reagents. The DCM solution obtained in Step 1 is treated directly with 1.3 equiv. of BAIB and 20 mol% TEMPO at 15 °C [19]. The reaction produces crystalline **16** in about 70% yield (over two steps), after aqueous work-up and solvent switch to heptane, from which the product crystallizes in >99.8% purity, leaving behind all impurities and the by-product of the oxidation, iodobenzene.

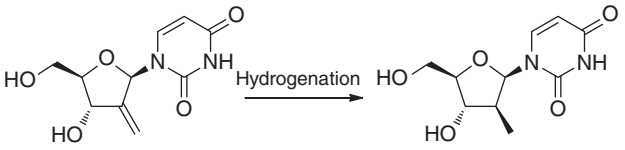
The main problem in this reaction was the lack of reproducible reaction times. Reactions could be complete in 8 h, but sometimes took 1–2 days. Increasing the temperature to 25–30 °C caused the loss of catalyst. Reaction monitoring showed an initiation phase during which <1% reaction takes place, and this had variable duration (all reagents are in solution when using DCM). Addition of 1 equiv. of acetic acid reduced this initiation phase by catalyzing the disproportionation of TEMPO to the activated oxoammonium intermediate [20–23], which $PhI(OAc)_2$ is unable to produce.

After water and aqueous sodium thiosulfate washes and then partial solvent removal, the desired ketone was crystallized by antisolvent addition (*n*-heptane) in 91% yield and with a purity of 99.2%. A normal-phase analytical method (silica gel, methanol/heptane) was developed because the ketone tends to form a stable *gem*-diol under reverse-phase LC conditions, leading to poor peak shape. Despite this tendency, the ketone **16** could be isolated as a crystalline product as water was azeotropically removed during solvent distillation.

The known Wittig reaction conditions using the dimsyl or tBuOK as base [17, 24] were made more practical by using potassium *t*-amylate (a 25w/w% solution in toluene) as base. The [2 + 2]-cycloaddition step occurs rapidly at room temperature to afford a stable oxaphosphetane that slowly extrudes TPPO at 30–35 °C for about 5 h [25]. Removal of TPPO entailed a solvent switch to heptane, from which the bulk of TPPO crystallizes after seeding, and filtration of the supernatant through a silica gel pad in ethyl acetate/heptane. The residual level of TPPO was systematically below 10 mol%. This step was scaled up to 89 kg, producing 78% solution yield of **17** with a purity of 95–98%.

The subsequent deprotection was successfully achieved by treatment in 2 M HCl aq. in methanol at 40 °C for 6 h. The traditional fluoride reagents were excluded because of the impossibility of extracting the diol derivative into organic solvents and the sensitivity of organometallic catalysts to fluoride (next steps). After complete deprotection and careful solvent switch to acetonitrile at 10–15 °C, the diol **18** was isolated by crystallization in 84–87% yield, purging residual TPPO and the chloride salts as well.[1] A final reslurry in MTBE was

1 TPPO and chloride ions were powerful inhibitors of the next step, the homogeneous hydrogenation.

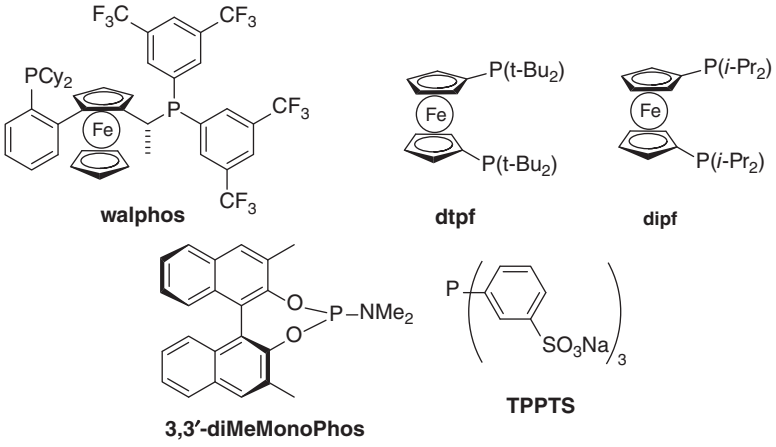


18 **10**

Initial conditions:
$[Rh(nbd)_2]BF_4$, Walphos 1:1, S/C 800/1, 40 bar, 40 °C, de 98.1%

Modified conditions:
$[Rh(nbd)_2]BF_4$, dtpf 1:1, S/C 800/1, 20 bar, 50 °C, de >98%

Alternative conditions:
$[Rh(nbn)_2]BF_4$, **3**,3′-diMeMonoPhos, TPPTS 1:2:1, S/C 1000/1, 25 bar, 70 °C, de 94%



Scheme 7.4 Diastereoselective reduction of compound **18**.

needed to remove traces of silanol, also identified as a catalyst inhibitor of the hydrogenation. This step was performed on 50 kg without any deviation from the laboratory scale, and **18** was obtained with a purity of 99.6%.

The cornerstone of our strategy to access diol **10** efficiently was based on a face-selective hydrogenation; unfortunately, no diastereoselective methods had been reported at that time.[2] A limited number of metal catalysts based on Rh(I), Ru(II), and Ir(I)) were screened in connection with 43 phosphorus ligands, both chiral and achiral, in different alcoholic solvents. The S/C ratio was set at 25, temperature at 40 °C, and the hydrogen pressure at 40 bar. Excellent activities were observed for several Rh(I) and Ru(II) salts, whereas Ir(I) was completely ineffective [13]. After optimization, we selected the chiral Walphos ligand/$[Rh(nbd)_2]BF_4$ (1 : 1 ratio) catalyst system with an S/C ratio of 800 in degassed MeOH at 40 bar and 40 °C for over 12–16 h. The latter was applied for the first campaign on 7 kg scale (Scheme 7.4).

With the increasing demand for the API, the hydrogenation became the bottleneck of the process: the large-scale availability of high-pressure reactors (40–50

2 In preliminary tests, hydrogenation of **8** was sluggish and poorly diastereoselective.

bar) was problematic, and the ligand cost was a major problem. Lowering the pressure to 20 bar impacts negatively the output of the hydrogenation, especially diastereoselectivity (from 94% to 87% de) even if the catalyst loading was increased (S/C from 800 : 1 to 200–100 : 1). A second phase of screening was therefore initiated, focusing first on the less *performant* ligands (such as dipf, 91% de) from the initial screening and other inexpensive nonchiral ligands.

To our delight, di-*t*-butylphosphinoferrocene (dtpf) in combination with $[Rh(nbd)_2]BF_4$ shows equivalent selectivity as the Walphos catalytic system and even superior performance at 20 bar and 50 °C with S/C 800 : 1 (Scheme 7.4) [26].[3]

In parallel to this screening reinvestigation, the phosphoramidite ligands were considered because of the lower price compared to chiral ferrocene ligands (especially Walphos). Selectivity of 94% de was reached by treatment with $[Rh(nbd)_2]BF_4$/3-3′-diMeMomophos/TPPTS (1 : 2 : 1) in THF : MeOH at 25 bar and 70 °C with S/C of 1000/1 (Scheme 7.4) [27]. The higher temperature required to reach a complete conversion led to the formation of uracil in 5% yield due to thermal decomposition.

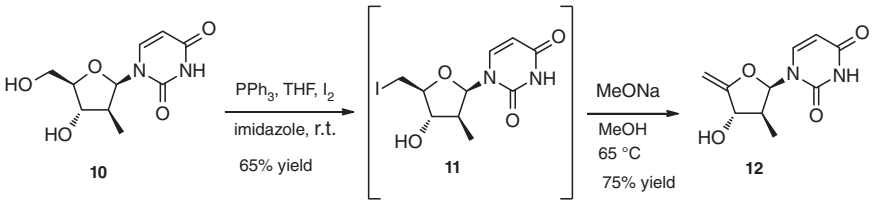
7.3 Dehydration and Iodoazidation Steps

The downstream steps of dehydration to **12** are straightforward and fairly efficient (Scheme 7.5).

Although in terms of atom economy, the iodination step is less attractive for large-scale purposes (waste generation), and the 5′-selectivity is excellent. Initially, intermediate **11** was isolated and purified, but a first round of optimization resulted in telescoping **11** into the next step. To intermediate **10** in THF, 1.2 equiv. PPh_3 and 1.2 equiv. imidazole were added, and then at 0–5 °C, iodine (1.1 equiv.) was dosed to the reaction mixture. Too large excess of iodine reduces the yield because a side reaction occurs, releasing uracil. Upon complete conversion (17 h at 18 °C), work-up consists in adding initially toluene followed by aqueous washes. Nevertheless some development was necessary to allow a scale-up of this chemistry at 40 kg scale and remove TPPO effectively. Eventually the work-up consisted in adding toluene and brine solution to the reaction mixture to avoid emulsion formation and twice back extraction of the aqueous layer with toluene/THF (1 : 1). The combined organic layers were concentrated by distillation under reduced pressure, carefully controlling the temperature below 45 °C. From the resulting mixture (containing 9–10% THF), the TPPO slowly crystallized and could be easily removed by filtration. The resulting solution, containing between 14% and 24% of **11**, was used as such in the next step. As the distillation process in the plant can be time consuming, confirming the stability of **11** under our distillation conditions was imperative.

A solvent switch was needed (THF to methanol) in the elimination step, which proceeded smoothly using an excess of NaOMe (4.1 equiv.) at 35 °C; the intermediate **12** was first isolated as a Na salt, followed by acidification and isolation of

3 By switching ligand, a fivefold cost reduction was achieved.



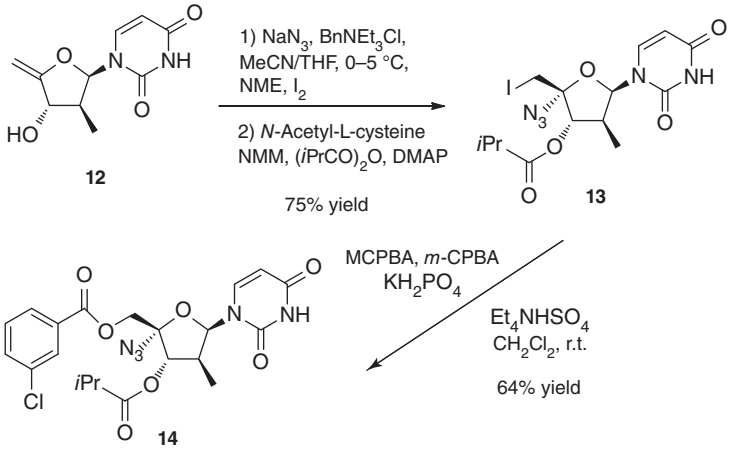
Scheme 7.5 5′-Dehydration of compound **10**.

the corresponding free from.[4] Following further development, the reaction mixture was quenched with acetic acid till pH 7–8, and the solvent was distilled off leading to the crystallization of the desired product. Compound **12** is prone to degradation (nucleoside C1′—N bond cleavage) and showed sublimation behavior, and for this reason the solvent distillation conditions should be controlled carefully (<40 °C, high vacuum).

In order to improve the quality of **12**, a recrystallization from methanol/water (1/1) was developed. The two-step process had overall yield of 88% (with an improvement of the diastereomeric ratio at 2′-position from 93.5 : 6.5 to 98.5 : 1.5). This implies small amounts of C-2′ epimer from hydrogenation could be tolerated.

7.4 Functionalization at C-4′

The following iodoazidation step is effective at introducing the azide function with high regio- and stereoselectivity (Scheme 7.6). This step is followed by oxidative substitution of the iodine atom by MCBA.



Scheme 7.6 Azidation/oxygenation sequence.

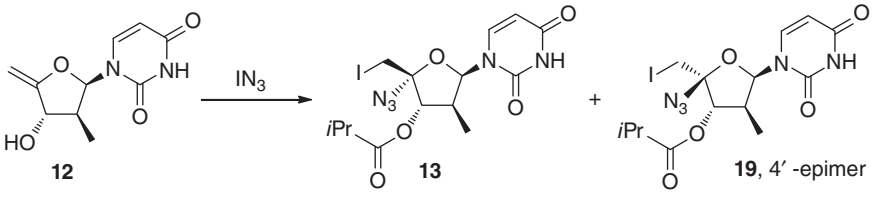
4 After solvent switch to 2-MeTHF and extra addition of NaOH till pH 12–13, intermediate **14** was soluble as sodium salt in water. Residual TPPO and major organic impurities were extracted in the organic layer. After acidification with HCl till pH 3–4, compound **14** was crystallized from 2-MeTHF/MeOH.

As the chemistry was well described in literature [28], limited development work was necessary; all efforts were focused on safety and robustness. According to the literature, in a separate vessel a soluble azide reagent (benzyltriethylammonium azide) is prepared by adding sodium azide and benzyltriethylammonium chloride to acetonitrile at room temperature. This suspension is slowly dosed to **12** in acetonitrile at 0 °C. Following complete addition, a small portion of *N*-methylmorpholine (NMM) is added. Then a solution of iodine in THF is slowly dosed to the reaction mixture, controlling the moderately exothermic reaction. Upon complete conversion of **12**, more NMM and DMAP are added followed by dosing of isobutyric anhydride. When the conversion reaches 99%, quenching with aqueous bisulfite solutions is carried out at 0 °C. The reaction mixture is slowly transferred to EtOAc and allowed to warm up to 25 °C for the extraction. The organic layers are carefully washed with water to ensure complete removal of the free azide. The organic layer is sequentially washed by aqueous solution of citric acid, sodium bicarbonate, and finally brine. Following distillation and crystallization from 2-propanol, intermediate **13** is isolated and dried.

The safety aspect of this operation must be addressed (*vide infra*). The reaction was studied in an RC-1 calorimeter: heat generation during addition of iodine/THF solution was largely dosing controlled; the accumulation potential was 10% and the reaction had a Q_{max} of 6 W kg^{-1}. The dosing of isobutyric anhydride showed higher accumulation potential (30%) and had Q_{max} of 18 W kg^{-1}. Samples for differential scanning calorimetry (DSC) measurements were taken at set points during process execution, but did not reveal any secondary decomposition. Overall, the iodoazidation–acylation sequence was found to be safe for use at larger scale in a semibatch mode. An interesting aspect in iodoazidation chemistry is the stereoselectivity (Scheme 7.7).

Initially, iodoazidation was believed to be completely stereoselective, but during the development work, an isomer was found to be present. Isolation led to its identification as the 4′-epimer (**19**) contained in crude **13** in amounts varying between 4% and 6%. In order to increase stereochemical purity, we used a water/methanol reslurry or recrystallization, and we were able to remove the C-4′ epimer **19** completely [26].

Iodoazidation reaction is key to the overall reaction sequence for this API. One of the key safety-related questions was whether IN_3 would accumulate *in situ* during the iodoazidation step, which would potentially be a major safety concern. IN_3 is a highly explosive compound when dry [29]. The chemistry of IN_3 is described by Hassner: [30, 31] electrophilic additions to alkenes are known to form products like **13**. IN_3 detection using *FTIR (ReactIR zirconium probe* – νIN_3 is 2040–2055 cm^{-1}) was explored and evaluated for *in situ* monitoring of this



Scheme 7.7 Focus on the iodoazidation step.

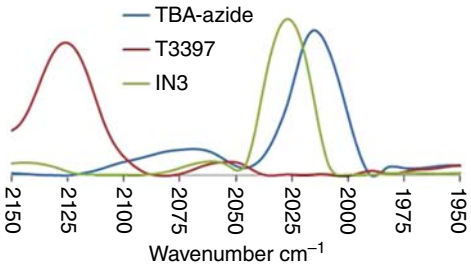


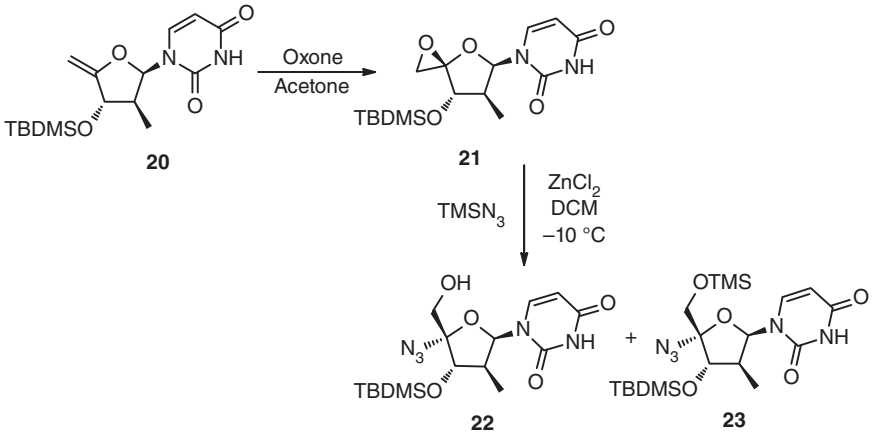
Figure 7.3 Reference spectra in neat THF.

compound. Small-scale preliminary experiments in a THF/MeCN matrix (50/50) at room temperature led to the observation that IN_3 absorbs at 2030–2035 cm^{-1}, clearly distinguishable from **12** and TBA azide (Figure 7.3).

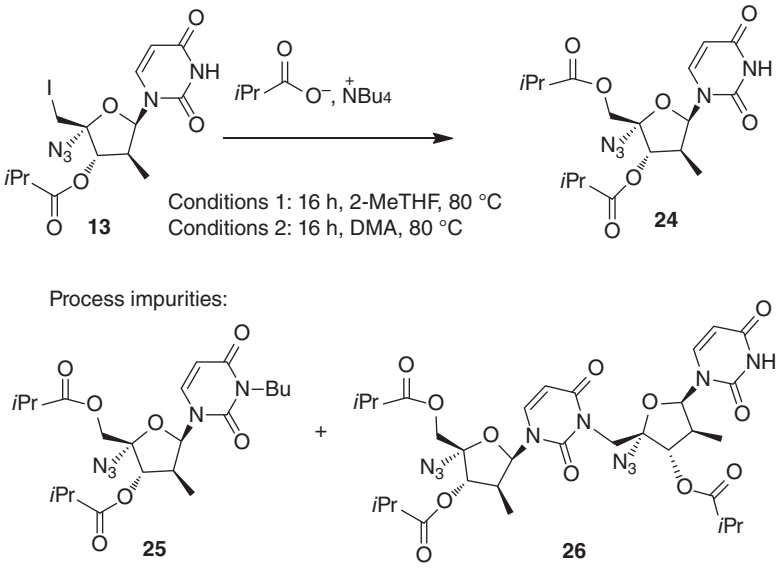
The IN_3 species was easily formed by reacting iodine monochloride with TBA azide, whereas the use of iodine under the same conditions was ineffective. Quenching of these reaction mixtures with **12** resulted in the formation of **13**. An *in situ* monitoring experiment under the actual reaction conditions detected hardly any IN_3, showing that the rate-determining step in this sequence is the formation of IN_3 and not its consumption**.** This alleviated the concern of IN_3 accumulation during further scale-up.

The second step of the sequence consists of converting **13** into **14**, using an excess of *m*-chlorobenzoic acid and *m*-CPBA in the presence of a phase-transfer catalyst (1 equiv. tetrabutylammonium sulfate, TBAS) in dichloromethane. This step suffered from robustness issues. We encountered major yields fluctuations (50–75%) and mismatch of laboratory versus plant results during scale-up. In attempt to improve the current route and to reduce cost of goods, development of alternative chemistry was initiated [26]. The first approach consisted in the formation of a spiroepoxide compound **21** followed by a Lewis acid-catalyzed ring opening with TMS azide to yield **22** and **23** (Scheme 7.8).

This chemistry worked well, achieving up to 70% overall yield over two steps, but the safety concerns in this route were unacceptable for further scale-up.



Scheme 7.8 Alternative azidation sequence via epoxide opening.



Scheme 7.9 Alternative synthesis of compound **24**.

Process safety evaluation showed that the reaction products were shock sensitive, and compound decomposition would result in gas evolution exceeding safe plant operation limits. The danger with TMS azide, which is rather stable by itself, is its sensitivity to hydrolysis (water or protic solvents) that results in hydrazoic acid (HN_3) formation. This should of course be avoided in all circumstances as HN_3 is a highly explosive and toxic gas. Only specialists having appropriate capabilities (e.g. bunkers) and monitoring devices (HN_3 detection) in place should be handling this reagent on scale. The use of Lewis acids such as $ZnCl_2$ could potentially lead to the formation of $Zn(N_3)_2$, a compound endowed with explosive and detonating properties.

Due to these considerations, the chemistry in Scheme 7.8 was abandoned, and we focused on a more robust and convenient conversion of **13** to **14**. In spite of the frequent use of *m*-CPBA for large-scale activities in pharmaceutical production [32], safety concerns demanded our attention for late development as batch size increases. The large excess of *m*-CPBA (3.9 equiv.) used in the oxidation step, its storage [33], and the intrinsic instability in DCM at high concentration [34] motivated our interest in exploring the direct substitution of the iodide [35].

A direct substitution was briefly described in analogous substrates in the presence of 15-crown-5 ether [5]. This led us to explore lipophilic ammonium isobutyrate salts and found that tetrabutylammonium isobutyrate displayed excellent reactivity (Scheme 7.9).

The best yield (95%) was obtained in DMA. Nevertheless, more practical conditions in the presence of 2-MeTHF were developed in a lower 79% yield because of the easier work-up and suppression of the formation of the two impurities **25** and **26**. These conditions were applied successfully on a 10 kg scale and were considered as long-term solution.

7.5 Synthesis of the API

Saponification of **14** was nonselective for the *meta*-chlorobenzoate when sodium hydroxide in ethanol was used (Scheme 7.10), and it was decided to carry out complete saponification. The sodium salt of the product was directly isolated by an antisolvent addition (MTBE) in 73–85% yield. The last three steps were telescoped to the API. The sodium salt was suspended in 2-MeTHF and acidified with 1 equiv. of isobutyric acid to circumvent acylation of the uracil moiety. Because of the low solubility of the diol derivative **26**, this reaction is extremely diluted (35 V). Finally the bis-isobutyrate is formed in almost quantitative yield by addition of the anhydride in the presence of Et_3N and catalytic 4-DMAP. The volume was reduced to 20 V, azeotroping the water out of the system. The resulting solution was treated with $POCl_3$, a large excess of 1,2,4-triazole (10 equiv.) and Et_3N (10 equiv.). After aqueous work-up to remove the excess of reagent, a simple treatment with 20 equiv. of aqueous ammonia allows the formation of the desired API in 91–93% yield and 90–92% purity. The overall yield for this telescoped sequence was about 70% on scale.

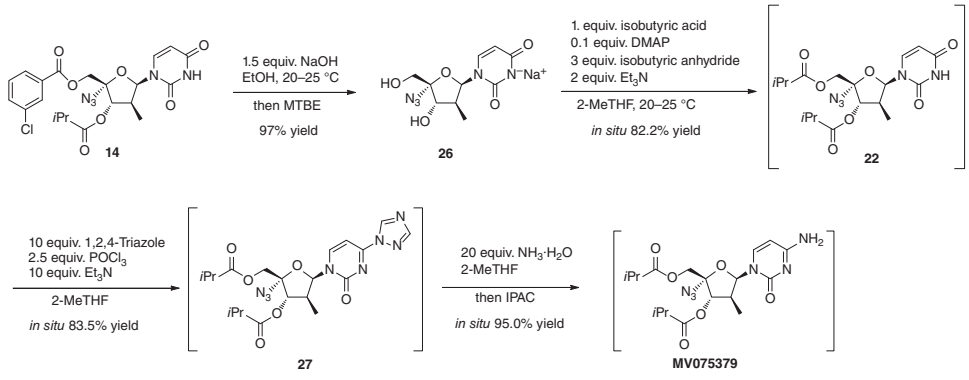
7.6 Solid Form Selection

It is quite important to understand the solid form characteristics of an API, even at the earliest stages of development because the selection of a particular solid form will affect bioavailability, at least for oral formulations, which was our foreseen administration route in the present case. A broad polymorph screen is necessary as early as possible, in addition to a salt screen if the molecule in question lends itself to salt formation (see Chapters 9 and 11 for a detailed discussion). The main parameter to be optimized and made robust is bioavailability, and in this sense particle engineering is of paramount importance [36], as detailed in Chapter 13. Other parameters that are just as important are processability and stability, in addition to safety and environmental factors. These considerations apply even to early development APIs, because changes in the final form during development will require lengthy bridging studies and will delay clinical development.

The free base of the API proved to be stable under ICH conditions [37] but amorphous or weakly crystalline. This was assessed via a broad screen encompassing a number of solvent systems. Such automated screens are standard in the industry.[5] Given the low potency of the API, it was assumed that a multigram oral tablet would have to be developed, and therefore high oral bioavailability was essential. Amorphous solids are usually much more bioavailable than crystalline ones [38], and therefore the development of the free base of the API was an attractive possibility.

In order to broaden our choice, crystalline salts were prepared. A salt screen was developed using a panel of biologically acceptable acids [39]. The amino

5 The Avantium Crystal 16™ system was employed.



Scheme 7.10 Overview of the end game.

Table 7.1 Selected properties of different API solid forms.

Form	Water solubility (mg ml^{-1})	Dissolution rate in 0.01 M HCl[a)]	Hygroscopicity	Polymorphs identified	Stability	Processability (filtration)
Amorphous free base	0.83	0.118	Low	0	Good	Very poor
Hydrochloride	6.84	0.270	Low	5	Good	Very poor
Tosylate	1.59	0.078	Very low	3	Good	Poor
Sulfate	0.63	<0.05	Moderate	1	Good	Very poor
Mesylate	8.21	2.038	Low	5	Good	Poor

a) In mg min^{-1}, from a 5 mg pellet in 20 ml 0.01 M HCl at 37 °C.

group of the API has a pK_a of 3.9, and therefore we were limited to strong acids. The screen produced several hits, and highly crystalline hydrochloride, sulfate, tosylate, and mesylate salts were found. Water solubilities as well as intrinsic dissolution rates in water and simulated gastric fluids were measured. As usual, thermal behavior was determined by DSC and thermal gravimetric analysis (TGA); crystallinity was probed by XRPD techniques and hygroscopicity by dynamic vapor sorption (DVS). In addition, a polymorph screen was run on all forms using a standard battery of solvents in order to determine how complex the phase diagram of each salt form would be. The number of polymorphs identified includes some solvates. Some properties of the salts are summarized in Table 7.1 (S. Stokbroekx, internal communication).

Although mesylate salts are usually not preferred because of their reaction with alcohols to form genotoxic impurities [40], we thoroughly evaluated the mesylate *in vivo* due to its high solubility, fast dissolution rate, and good stability. The hydrochloride was the alternative choice: although highly water soluble and stable, it crystallized in long needles, resulting in a bulky precipitate that could not be easily filtered. Extensive particle engineering, using Ostwald ripening techniques [41], did not result in a major improvement. Due to analogous problems encountered with the mesylate, eventually the choice fell on the amorphous free base, which could be isolated by spray-drying techniques [42]. The free base was endowed with good oral bioavailability, sufficient stability, and acceptable dissolution rate characteristics. Spray-drying is a core technology at Janssen and was selected due to the low *in vivo* potency of the API, which translated into multigram doses. In these cases amorphous APIs are almost always preferable to crystalline substances.

7.7 Process Safety

The presence of the azide functionality of the main core of the molecule implies specific attention for production, storage, and handling. Normally storage licenses need to be obtained, and compliance with specific regulations for

storage of highly energetic materials is required, especially due to the potential formation of HN_3. Therefore specific monitoring devices (HN_3 detection) and appropriate toxic and hazardous waste stream handling operations must be put in place.

As primary investigations to assess intrinsic stability of the API and its precursors, reaction calorimetry, thermal stability, and shock sensitivity for intermediates **13**, **14**, **API**, and crude **API** were studied prior to scale-up. All compounds were negative to the Lütolf test [43, 44] underlining the stability to shock. In spite of the expected intrinsic instability, no low decomposition temperatures were measured. Compound **13** showed strong exotherm upon decomposition (625 J g^{-1}) from 182 to 195 °C. Similar measurements were obtained for the API free base and mesylate salt, with decomposition above 160 °C and exotherm with 1090 and 635 J g^{-1}, respectively. Compound **14** showed the lowest stability, with two exotherms at 120 and 180 °C (765 J g^{-1}). Because DSC showed stability up to 100 °C, the compound was dried at 40 °C. The maximum process temperature was set at T_j 80 °C for the solvent switch.

7.8 Impurity Strategy

Our strategy includes evaluation of organic impurities at two different levels: normal process impurities and genotoxic impurities (GIs). Among the process impurities, the corresponding hydrolysis products, signaling transformation of the prodrug into the drug, could be tolerated at levels up to 0.5%, especially because they are formed slowly under the most severe ICH stability conditions.

All other impurities observed in the drug substance above the ICH Q3A reporting thresholds were dealt with as per guidelines [35]. For early development purposes, we used the following criteria:

IMP < 0.1%: no identification required.
0.1 < IMP < 0.3%: identification required.
IMP > 0.3%: identification and toxic qualification/risk assessment.

In early development, knowledge of impurity generation and purging is slowly built up, resulting in impurity management reports that are later updated as the process is locked. Our strong focus is always the potential impact on API quality. The analytical methods are not yet validated at this point: for API release the methods are qualified, and the intermediate methods are fit for phase.

During the present stage of early development, several large batches were produced, one of the amorphous free base and three batches of mesylate.

These batches formed the basis for our initial impurity assessment. The main impurities detected at levels >0.1% in some API batches are shown in Figure 7.4. Impurities **MV064274**, **27**, and **28** are related to the API through prodrug hydrolysis and activation. They are formed *in vivo* and found at fairly high levels during ICH stability studies. Their acceptance criteria were set at 0.5%, which can be defended with the argument of their formation *in vivo*.

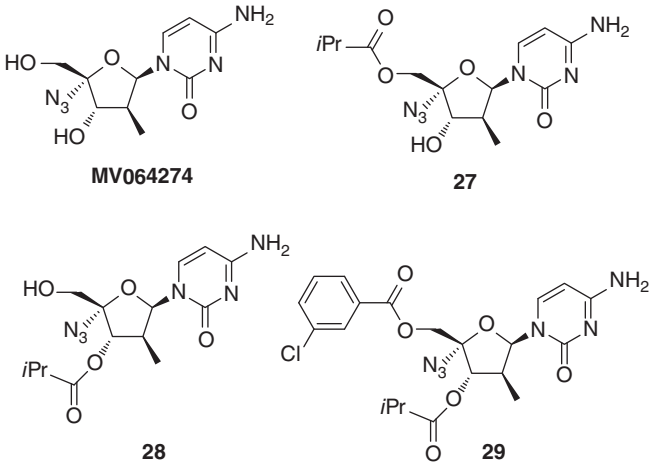


Figure 7.4 Main API process impurities.

Impurity **29** arose from intermediate **14**: evidently, the *m*-chlorobenzoyl moiety in intermediate **14** was not hydrolyzed completely and survived the amination intact without being effectively purged in the downstream purification steps. In later batches this impurity was reduced to levels <0.1% by close monitoring of hydrolysis step of **14** to **15**.

An important facet of chiral drug development is the discussion of potential stereoisomeric impurities; in this case, the following approach was taken, which is in line with a fit-for-phase approach in early development:

- 1′-Epimer and 3′-epimer: these stereocenters are derived from uridine and are not expected to be affected by the process.
- 2′-Epimer: as discussed in the paragraph on hydrogenation step, 2′-epimer is formed to a small extent during hydrogenation. It was carried up to the iodide formation and elimination stage. Here, it was demonstrated that its levels are <0.1%, and therefore there is no need to track this epimer all the way to the API. If the process is later changed, the purging of this epimer has to be reassessed.
- 4′-Epimer: as discussed in the paragraph on iodoazidation, 4–5% of this impurity is present. A monitoring and control strategy for future campaigns was put in place, and we demonstrated that by reslurrying the crude iodoazidation product in water/methanol, the impurity was purged to levels <0.1%. Given that epimerization at C-4′ during the next few steps is not possible, we do assume the corresponding impurity at the API level is not present.

In addition to controlling process impurities, a concern was the presence of free azide in the API (as result of degradation). The azide content was monitored on both API and drug product and was found to be below 3 ppm by ion chromatography.

Following ICH guidelines for impurity management, a genotoxic impurity assessment was made. All actual and potential process impurities were subjected

to an *in silico* analysis (DEREK) followed by a risk assessment. The API itself was found positive in DEREK and Ames testing due to the azide moiety in the structure. Following full evaluation (genotoxic analysis/mouse micronucleus test), the API was found to be not genotoxic. Further evaluation showed that our GMP starting material **14**, all subsequent intermediates, and actual impurities also gave a positive DEREK result due to the azide function in the molecule and therefore are related to the API (the same alert). Given the close structural similarity between API and potential impurities, it was deemed not necessary to conduct Ames tests on all these impurities. Another type of process impurity is isopropyl mesylate, a potential alkylating agent produced in the final salt formation as a result of a possible reaction between the solvent (*i*PrOH) and methanesulfonic acid. This compound was monitored closely and found to be below 1 ppm in all batches produced. After the free base was selected, this test became unnecessary.

7.9 Conclusion

In this chapter the focus has been on the development of a bulk-enabling route to 4′-azido-2′β-methyl-2′-desoxycytosine and its prodrugs. This reflects a precise philosophy in early development, which is based on controlling costs while preventing delays in the clinical program. The route described in this chapter was deemed safe and scalable to provide the bulk needed to support the entire clinical development without any major process changes that may not only improve the synthesis but also cause potential problems due to varying impurity profiles. It is acknowledged that the process is fairly effective and chromatography free, but it is not the best possible approach to the intended target. A major effort to develop a truly practical commercial process is usually deployed at clinical proof of concept, e.g. in Phase IIb. The selection of the solid form is, however, more critical even at the early stages of development, because changes in the final form during clinical development will affect formulation and bioavailability, therefore requiring time-consuming bridging studies. We have described the selection of an amorphous form for an API in order to maximize bioavailability. Although this is a rather unusual choice, it is considered that it is a fully justifiable selection in view of the particularly challenging situation. In conjunction with spray-drying, one of Janssen′s core technologies, formulation of amorphous APIs is a practical platform for drug development and commercialization.

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Part II

Drug Product

8

Solubility, Permeability, and Their Interplay

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8.1 Introduction

Nowadays, the identification of drug candidates that have low aqueous solubility is more likely attributable to the modern drug discovery tools, e.g. combinatorial chemistry, *in silico* modeling, and high-throughput *in vitro* experimental methods. Dissolution of the drug in the gastrointestinal tract (GIT) is an essential step toward good absorption and permeability, and therefore drug candidates that have low aqueous solubility may have insufficient absorption and bioavailability. A considerable effort has been put into developing new oral formulations for overcoming this obstacle and achieving adequate absorption.

Numerous physicochemical factors (e.g. the extent of ionization, solubility, lipophilicity, diffusion coefficient, and stability), physiological parameters (e.g. gastrointestinal (GI) pH, gastric emptying, GI motility, permeability mechanisms, small intestinal transit time), and factors related to the drug formulation make the drug absorption process a complex one. Some physiological/physicochemical/formulation examples include route of administration, gastric pH, GI content, blood flow through the absorption site, total surface area available for absorption, contact time in the absorption surface, lipid/water solubility and partitioning, molecular size, particle size, physical forms, chemical nature, and others [1–6]. In-depth understanding of the fundamental processes affecting drug absorption is necessary in order to successfully tackle different obstacles and improve the overall drug product performance. In the following, a brief introduction into the oral drug absorption process is presented.

Diffusion represents dissemination of particles (also atoms or molecules) through random motion, from regions with a higher concentration to regions of lower concentration. The idea of diffusion is bound to a mass transfer driven by a concentration gradient; nevertheless diffusion can also happen when there is no concentration gradient. A simple description of diffusion is given by Fick's law: the molar flux due to diffusion is proportional to the concentration gradient. Fick's first law describes passive diffusion (flux) of molecules down a concentration gradient; it can be applied to diffusion across the membrane of

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

the GI wall by the following equation:

$$J_W = P_W \times C_W = \frac{dM}{dt} \times \frac{1}{A} \tag{8.1}$$

where J_W is the flux across the GI wall (mass/area/time), P_W is the effective permeability of the intestinal membrane, C_W is the drug concentration at the GI membrane, M is the amount of drug in the body at a given time (t), dM/dt is a rate of diffusion, and A is the membrane surface area. This equation, and its analysis developed by Amidon et al. [7–9], pinpoints the permeability of the drug through the GI membrane, the solubility/dissolution of the drug in the GI milieu, and the drug dose as the fundamental factors that dictate mass transport through a membrane, i.e. absorption. This seminal analysis, that is, one of the most important predictive tools produced to assist drug development throughout the past two decades, is named the biopharmaceutics classification system (BCS) [10]. Since its introduction in 1995, BCS has become a pivotal regulatory tool worldwide by offering a new pattern in bioequivalence. It uses the solubility/dissolution of the drug dose in the aqueous GI milieu and the permeability of the drug through the GI membrane, to understand and predict the factors limiting oral drug absorption in a given case [11–13]. According to the BCS, all drug substances are classified into four categories based on their solubility and permeability characteristics (Figure 8.1):

- *BCS Class I*: high-solubility, high-permeability drugs. Class I drugs are very well absorbed. A drug substance is reported to be highly soluble when the highest dose strength of a single unit dose can be dissolved in 250 ml of water over the relevant GI pH range from 1 to 6.8. An immediate release (IR) product of this class is expected to achieve >90% intestinal absorption if at least 85% of the drug is dissolved within 30 min in the physiological pH range. Therefore, a waiver is approved for bioavailability/bioequivalence (BA/BE) studies of

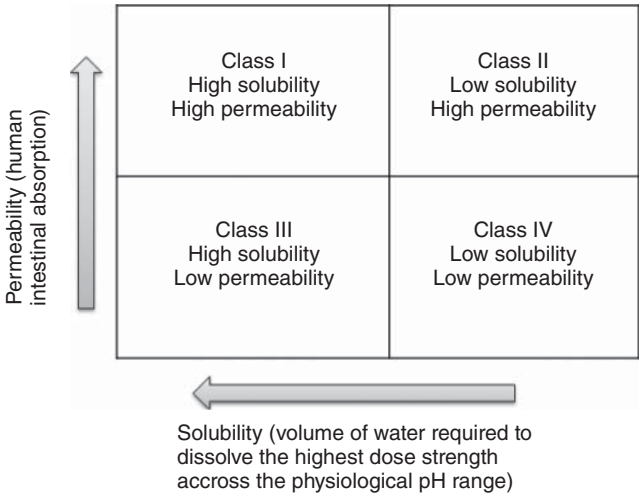

Figure 8.1 The biopharmaceutics classification system as was developed. (*Source*: Amidon et al. 1995 [10]. Reproduced with permission of Springer.)

BCS Class I IR drug products (with the exception of narrow therapeutic index drugs). Some examples of drugs belonging to this category include metoprolol, propranolol, buspirone, lidocaine, and minocycline.

- *BCS Class II*: low-solubility, high-permeability drugs. A drug substance is considered to have low solubility when the highest dose strength of a single unit dose cannot be dissolved in 250 ml of aqueous media over the relevant GI pH range from 1 to 6.8. Hence, the absorption of BCS Class II drug products may be restricted by the dissolution/solubility rate. BCS Class II drug examples include danazol, nifedipine, ketoprofen, ketoconazole, naproxen, carbamazepine, amiodarone, atorvastatin, glipizide, and itraconazole.
- *BCS Class III*: high-solubility, low-permeability drugs. The intestinal absorption of drugs from this class is expected to be restricted by the permeability rate, whereas the solubility will not be a limiting factor. Therefore, it has been recommended that as long as the drug formulation does not contain permeability-changing agents [14], a waiver for BA/BE studies for these drugs should be considered. In this case, the fact that the intestinal permeability is the rate-controlling step for oral drug absorption implies that the absorption kinetics of BCS Class III drugs from the GIT is controlled by the physicochemical and biochemical characteristics of the drug substance *per se* rather than formulation factors. Examples of BCS Class III drugs include atenolol, cimetidine, ranitidine, amoxicillin, and erythromycin.
- *BCS Class IV*: low-solubility, low-permeability drugs. The drugs from this class are expected to have poor oral bioavailability and a tendency to show very large inter- and intrasubject variability. Thus, unless the dose is very low, these drugs are generally poor oral drug candidates. Typical BCS Class IV examples include hydrochlorothiazide and furosemide.

According to the BCS, absorption can be characterized by three key dimensionless parameters: absorption number (A_n), dissolution number (D_n), and dose number (D_0) [12, 15]. These dimensionless numbers include physicochemical and physiological factors and characterize the most important view of GI drug absorption.

The absorption number (A_n) is the ratio between the effective permeability (P_{eff}) and the intestinal radius (R) multiplied by residence time (t_{res}), which can also be calculated as the ratio of residence time and absorptive time (t_{abs}):

$$A_n = \frac{P_{eff}}{R} \times t_{res} = \frac{t_{res}}{t_{abs}} \tag{8.2}$$

The dissolution number (D_n) is equal to the diffusivity (D) times the equilibrium solubility (C_S) divided by the initial particle radius (r_0), which is the ratio of residence time (t_{res}) and the dissolution time (t_{Diss}):

$$D_n = \frac{D \times C_s}{r_0} = \frac{4\pi {r_0}^2}{\left(\frac{4}{3}\right)\pi r_0^3 \rho} \times t_{res} = \frac{3t_{res}DC_s}{\rho {r_0}^2} = \frac{t_{res}}{t_{Diss}} \tag{8.3}$$

where ρ is the density.

According to the FDA guidance, an IR solid oral dosage form is considered rapidly dissolved if 85% or more of the labeled amount of the drug substance

dissolves within 30 min, using United States Pharmacopeia (USP) Apparatus I at 100 rpm (or USP Apparatus II at 50 rpm or at 75 rpm when correctly justified) in a volume of 500 ml or less in each of the following media: (i) 0.1 N HCl or simulated gastric fluid USP without enzymes, (ii) a pH 4.5 buffer, and (iii) a pH 6.8 buffer or simulated intestinal fluid USP without enzymes. An IR drug product is considered very rapidly dissolving if 85% or more of the labeled amount of the drug dissolves within 15 min with the abovementioned conditions [16].

The dose number (D_0) is equal to the dose (M_0) divided by the volume of water taken with the dose (V_0; 250 ml) and the drugs' equilibrium solubility (C_S):

$$D_0 = \frac{M_0/V_0}{C_s} \tag{8.4}$$

An extensive research in humans at Uppsala University and at the University of Michigan discovered the very good correlation between the drugs' fraction of dose absorbed and its effective permeability across the intestinal membrane (Figure 8.2) [17]. When the human P_{eff} of a drug is less than $\sim 2 \times 10^{-4}$ cm s^{-1} (the human permeability value of the commonly used low-/high-permeability class boundary standard metoprolol), the absorption is not expected to be complete. Full drug absorption will be probable for substances whose P_{eff} surpasses this value.

Extensive applicability including regulatory applications of the BCS scheme has been the focus of widespread research and discussion in recent years, as well as an effort to draw a BCS classification of many drug products [18–28]. BCS is one of the most important tools created to predict product performance and assist drug development. It has been accepted by the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), the World Health Organization (WHO), and other regulatory authorities worldwide for setting BA/BE standards for generic drug products approval. This chapter provides an overview

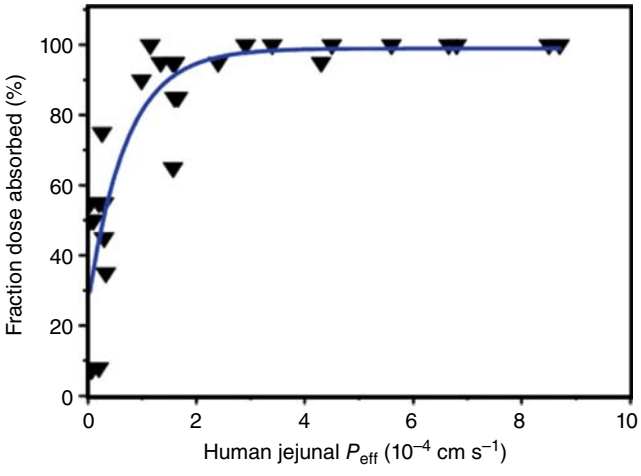

Figure 8.2 The correlation between human fraction of dose absorbed (F_{abs}) and the effective permeability (P_{eff}) across the human jejunal membrane. (*Source*: Lennernas 2007 [17]. Reproduced with permission of Taylor & Francis.)

of the key parameters that influence absorption: the solubility and permeability of the drug substances, as well as the solubility–permeability (S–P) interplay when using solubility-enabling drug formulations.

8.2 Solubility

Solubility is the maximal amount of substance that can be dissolved in a defined volume of solvent. In quantitative terms solubility is defined as the concentration of solute in a saturated solution at a certain temperature, and the solution is considered saturated when the solute is in equilibrium with the solid phase (solute). Solubility is one of the most important factors that influence absorption and consequent drug systemic circulation levels. The BCS solubility class of a drug is determined by dissolving the highest strength of a single unit dose of the drug in 250 ml of buffer adjusted between pH 1.2 and 6.8. The volume estimation of 250 ml results from typical bioequivalence study protocols that recommend administration of a drug product to fasting human volunteers with eight ounces (240 ml) glass of water, considering a 10 ml gastric resting volume. A drug substance is considered highly soluble when the dose/solubility volume of solution is less than or equal to 250 ml, that is, a dose number (D_0) ≤ 1. Poor water solubility is one of the main challenges in today's drug research and development, because the drug has to be dissolved at the site(s) of absorption in order to be absorbed. In different pharmacopoeias, a descriptive term is used to indicate solubility range category (Table 8.1). One problem with this traditional approach is that it ignores the dose, and so a potent compound may be categorized as *practically insoluble* according to its physicochemical characteristics; however attributable to its very low dose, it may still be completely dissolved in the available volume of liquid. On the contrary, by using the dose number (D_0) for solubility classification, the BCS takes into consideration the required dose and avoids such a mismatch between the theoretical definition and the actual behavior.

8.2.1 Solubility and Dissolution Rate

Dissolution refers to the process by which a solid phase (tablet/powder) goes into a solution phase such as water. In essence when a drug dissolves, solid particles

Table 8.1 Traditional definitions for estimating drug solubility.

Descriptive term	Parts of solvent for one part of solute
Very soluble	Less than 1 part solvent needed to dissolve 1 part solute
Freely soluble	From 1 to 10 parts solvent needed to dissolve 1 part solute
Soluble	From 10 to 30 parts solvent needed to dissolve 1 part solute
Sparingly soluble	From 30 to 100 parts solvent needed to dissolve 1 part solute
Slightly soluble	From 100 to 1000 parts solvent needed to dissolve 1 part solute
Very slightly soluble	From 1000 to 10 000 parts solvent needed to dissolve 1 part solute
Practically insoluble	More than 10 000 parts solvent needed to dissolve 1 part solute

separate and mix molecule by molecule with the liquid and appear to become a part of the liquid. Drug dissolution occurs when a tablet is introduced into a solution and is usually accompanied by disintegration and deaggregation of the solid matrix, followed by drug diffusion from the remaining small particles. Poor solubility and slow dissolution rate of drugs in the aqueous GI fluids often cause insufficient absorption and bioavailability, especially in oral delivery of poor water-soluble compounds [29]. The Nernst–Brunner and Levich variations of the Noyes–Whitney model show the factors that control the dissolution [30–32]:

$$\frac{dX_d}{dt} = \frac{A \times D}{h}\left(C_s - \frac{X_d}{V}\right) \tag{8.5}$$

where A is the surface area of the solid drug, D is the drugs' diffusion coefficient, h is the effective diffusion boundary layer thickness adjacent to the dissolving surface, C_s is the saturation solubility of the drug, V is the volume of water available, and X_D is the amount of the dissolved drug.

As the particles become smaller, dissolution rate (dX_d/dt) becomes faster, since surface area (A) increases with particle size reductions. Changing the pH of the solvent can have an impact on saturation solubility (C_S) of ionizable (both basic and acidic) drugs, which can lead to an increase or a decrease in the dissolution rate. It can also be seen that the rate of dissolution is largely affected by the physicochemical characteristics of the drug and by various physiological aspects of the GI.

In the case of BCS Class II or Class IV substances, the intestinal absorption can be considered dissolution or solubility limited. Class II substances are low-solubility, high-permeability compounds and as such are defined by a high absorption number (A_n) and a dose number (D_0) higher than one; when the dissolution rate of such compound is low, the dissolution number (D_n) is lower than one, whereas A_n and D_0 are high. On the other hand, if both A_n and D_n are low, the drug can be assigned to Class IV of BCS.

When the intestinal absorption is dissolution or solubility limited, the concentration of the drug in the GI milieu will be regulated by the relevant limiting factor, as can be learned for the classical case of digoxin and griseofulvin, which describes the effect of dissolution number and dose number on the fraction of dose absorbed (F_{abs}) for drugs with high permeability [10]. The solubility value of digoxin and griseofulvin is very similar ($\sim$20 mg ml^{-1}); however they have a very different dose (0.5 mg for digoxin and 500 mg for griseofulvin). Therefore, digoxin has a low dose number of 0.08, whereas griseofulvin has a high dose number of 133. As a result, more than 33 l of water is necessary to dissolve one dose of griseofulvin. The case of griseofulvin displays an equilibrium problem – there is simply not enough GI fluid to dissolve such a dose [33–35]. Therefore griseofulvin shows a high dose number and a low dissolution number. The fraction of the dose absorbed and bioavailability could be increased by lowering the administered dose, taking more liquid with the dose or by drug solubility enhancement [36–40]. The dose of a drug cannot be changed, due to pharmacokinetic and pharmacodynamic factors; the volume of water taken with a formulation is restricted by stomach anatomy and physiological capability; and lastly, solubility improvement via a suitable formulation is the only solution that could contribute to the

reduction of D_n and to increase the oral absorption of griseofulvin [41–44]. On the other hand, in the case of digoxin, the problem is of kinetic nature – the dose can be completely dissolved, as can be seen from the low D_0. However, attributable to, e.g. inadequate particle size, the dissolution of the drug may be very slow (D_n<1) and limit the dissolved amount of drug in the GI milieu and the overall absorption. For digoxin, it was calculated that particle diameter bigger than 10 μm will result in a dissolution rate limited absorption [45]. For this reason, a complete intestinal absorption could be possible if the particle size is decreased; indeed, micronized digoxin powder provides an adequate dissolution rate, and the intestinal residence time becomes sufficient for complete absorption. On the contrary, griseofulvin absorption is solubility limited, and improving its dissolution number cannot significantly increase its fraction of dose absorbed, so micronization is not likely to significantly increase the absorption of griseofulvin, not without reduction of dose number. Therefore a solubility-enabling formulation that allows sufficient solubilization in the gastrointestinal milieu will be needed [46].

8.2.2 Log *P*

One of the major factors dictating drugs' aqueous solubility is the ability of the drug to create hydrogen bonds with water molecules [47]. High water solubility is advantageous for dissolution in aqueous media, but at the same time such compounds often show low permeability as a result of their high polarity and hydrophilicity. The partitioning coefficient (Log *P*) is a measure of differential solubility of a drug compound in a lipophilic (*n*-octanol) and hydrophilic solvent (water). Logarithm of the two allows us to rank drugs in terms of hydrophilicity or hydrophobicity [48–51]. In addition to the *n*-octanol/water partitioning method, lipophilicity can also be described by the dynamic energy properties of the compound [52].

8.2.3 pH

The ability of a drug to partition from lipid to aqueous surroundings is often a function of solvent pH due to its effects on drug ionization. Basically, ionized drugs have a tendency to demonstrate better aqueous solubility than the unionized equivalent. Therefore, the rate of ionizable solute dissolution in aqueous media may be significantly affected by pH variation of the solvent. The Henderson–Hasselbalch equation is used to describe the effect of pH on drug ionization [53]:

$$\text{Weak acid}: \%\ \text{unionized} = 100/(1 + \text{antilog}\,(\text{pH} - \text{p}K_a)).$$

$$\text{Weak base}: \%\ \text{un-ionized} = 100/(1 + \text{antilog}\,(\text{p}K_a - \text{pH})).$$

Weakly basic drug compounds are likely to have a slower dissolution rate at pH higher than its dissociation constant, where more drug molecules are in their unionized form. On the other hand, weakly acidic drug compounds will show faster dissolution rate at pH higher than their acid dissociation constant (pK_a),

where more drug molecules are in their ionized form. Physiological values of gastric pH are 1.4–2.1, but they can be largely affected by food intake and can vary from 1 to 8 [54–56].

The pH values in the small intestine are higher than in the stomach, and the presence of food does not affect them as much. Small intestinal pH demonstrates a rising gradient from the proximal (duodenum) to the distal (ileum) segments. Intestinal pH values may range from 4 to 8 [57–59]. The raise in gastric pH after food intake increases the portion of alkaline drug that remains in unionized form and reduces the drugs' dissolution rate. For instance, sedimentation of weak bases like indinavir (with pK_a values of 3.7 and 5.9) will be expected if gastric pH is raised during a meal, resulting in a significant reduction of AUC and C_{max} values compared with a fasted human subject [60]. On the other hand, food can raise the dissolution rate of a weakly acidic drug, such as ibuprofen, by increasing the ionized fraction of the dose [61].

8.2.4 Bile Salts

Bile salts are amphipathic steroidal biological surfactants, derivatives of cholesterol [62–68], which are produced in the liver and stored in the gall bladder [69, 70]. The dipped side of the bile acid steroid skeleton is hydrophilic due to the presence of hydroxyl groups; however its convex side is hydrophobic due to the angular methyl groups. This particular structure differentiates them from traditional surfactants, which are usually resided from polar head and long nonpolar chain [71]. Their wetting effect [72–74] and micellization may considerably affect the solubility and dissolution of low-solubility drugs, playing a significant role in intestinal absorption of drugs. Beyond their critical micelle concentration (CMC), they aggregate and create micelles [75]; by forming submicron mixed micelles, bile salts increase the solubility of lipophilic drugs, making hydrophobic molecules more solubilized and more likely to reach the membrane of the enterocyte. For example, it was shown that the solubility of the lipophilic drug rifaximin increases, when the concentration of bile salts is increased with enhancing concentrations of bile acids [76], therefore increasing its antimicrobial effect.

8.2.5 The Particle Size

Particle size of the compound is an important physical parameter that may affect the dissolution rate. Smaller particle size results in a larger surface area available for dissolution, which, according to the Noyes–Whitney model, leads to a faster dissolution rate [77, 78]. The density of particles can also have an impact on the dissolution rate, since the density will change the *in vivo* particle dispersion and better dispersion will cause enhanced dissolution [79, 80]. The effect of particle size seems to be food dependent; it has been reported that under fasting conditions, smaller particle size greatly affects the dissolution rate and oral drug absorption of a poorly soluble antiretroviral agent, with no parallel effect in the postprandial (fed) state [81]. Since 1980s, nanonization (reduction in particle size to the nanoscale) has attracted considerable attention, particularly for increasing bioavailability of lipophilic drugs [30, 82–87]. The fact that the particle size is in

correlation with solubility, dissolution, and bioavailability was confirmed in many studies [88–90].

To improve the intestinal absorption of drugs, a modern biopharmaceutical approach uses amorphous solid dispersions (ASDs), which may achieve and maintain supersaturation for an appropriate period of time. Crystal formation in poorly soluble drugs can be inhibited by using different types of polymers. They maintain the supersaturated state and help avoid precipitation [91–96]. It has been shown that polymers have the ability to regulate particle size [97], enabling redissolution and decreased particle size of precipitated active pharmaceutical ingredients, which leads to better intestinal absorption and improved bioavailability [98–100].

For drug powders, particle size is not as important as the surface area that is available to the dissolution fluid. This is particularly significant when highly hydrophobic drugs have poor wetting properties in a dissolution medium and when a manufacturing process alters the particle size and hence the dissolution rate [101, 102].

For a detailed discussion on particle size reduction strategies and their impact on absorption, the reader is referred to Chapter 13.

8.2.6 Volume of Fluids

Fluid volume in the GIT is determined by the volume of water coadministered with the drug, secretions into the GI lumen, and fluid flux throughout the gut wall. Studying the rate and level of *in vitro* drug dissolution in physiologically applicable dissolution fluids may be used for better understanding and predicting drug absorption. It was shown that small gastric fluid volumes may decrease the dissolution of nifedipine and reduce its absorption in humans [103]. Advanced computational analysis using GastroPlus showed that the volume of intestinal fluid has an abundant effect on the prediction of the mean blood concentration profiles of poorly water-soluble compounds [104]. Mudie et al. have quantified the total volume and water distribution in the stomach and small intestine and revealed the existence of discontinuous fluid pockets in the small intestinal tract [105]. This study suggested that the highest percentage of liquid is present in the distal regions of the small intestine (distal duodenum, proximal, and distal ileum), and thus, in cases where the duodenum and proximal jejunum are the predominant site(s) of absorption, the drug needs to be dissolved before it reaches the small intestine.

8.3 Permeability

The dual function of the intestinal membrane includes allowing the permeation and absorption of essential nutrients while functioning as a barrier and preventing the entrance of toxins and pathogens to the body and potential cause of harm [106]. The permeability is associated with the level of intestinal absorption and with the rate of mass transfer through the GI membrane [107]. It is inversely

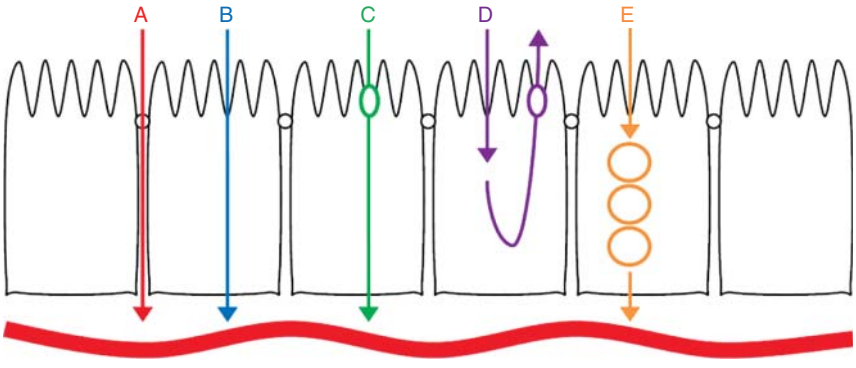

Figure 8.3 The different mechanisms for intestinal permeability. A, paracellular diffusion over tight junctions; B, transcellular simple passive diffusion; C, carrier-mediated transcellular transport; D, carrier-mediated efflux transport; and E, transcellular vesicular transport.

related to the resistance of transport across membrane or tissues: the higher the permeability, the lower the resistance to movement across the membrane. Parameters that affect the permeability include membrane structure, physicochemical properties of the drug (e.g. lipophilicity, molecular weight, hydrogen bonding, polar/nonpolar surface area, etc.), drug–solvent interactions, and others [108].

The lipid bilayer of the enterocyte brush border creates the epithelial barrier. This structure has significant affinity to lipophilic compounds. On the other hand, it is a strong barrier to hydrophilic molecules. The dual role of enterocytes is a reflection of absorptive surface for lipophilic molecules on one hand and a surface with transporters for hydrophilic molecules on the other. The membrane lipid bilayer consists of cholesterol and phospholipids; it offers stability to the membrane and governs its permeability characteristics. Drugs can pass through a biologic membrane via simple passive diffusion, facilitated passive diffusion, or active transport, as presented in Figure 8.3.

8.3.1 Passive Diffusion

Passive diffusion is the way by which molecules move down the concentration gradient without the use of energy. In order to efficiently pass the lipid bilayer of the cell membrane, the molecules have to be small and nonpolar. Passive diffusion can be completed with two pathways: transcellular and paracellular. The junctions between epithelial cells present a potential way for paracellular transport of molecules, which is not regulated by transporters or channels in the brush border membrane. A variety of factors (e.g. fasting or fed state, neuronal signals, mediators of inflammation, products derived from mast cells, etc.) may affect the epithelial tight junctions, resulting in their opening or closing [109–114]. Paracellular diffusion is more constrained in the colon than in the upper segment of the GIT, owing to the lower permeability of the tight junctions in this region. This pathway is charge and size dependent; the tight junctions are negatively charged, and therefore positively charged compounds penetrate this way more easily than do the negatively charged ones [115–117]. Tight junctions are also permeable to

non-charged compounds like water, serum albumin, or dextrans with a molecular size up to 0.43–0.45 nm in radius [118, 119].

On the other hand, various drug molecules enter biological membranes via passive transcellular transport. This transport is defined as a concentration gradient governed by mass transport of a permeant that passes through both the apical membrane and basolateral membrane. Flux is a parameter used for measuring the mass transport of drug molecules in a solution or movement of molecules through a barrier. The flux of a drug is the mass of drug molecules transferring across a given area during a given time:

$$J = \frac{M}{A \times t} \tag{8.6}$$

where the flux (J) is related to the area available for transport of molecules (A) and the mass of the molecules (M), at a given time (t).

Passive transport of drug molecules in solution or movement of molecules through barriers can be triggered by diffusion. Diffusion is the arbitrary thermal drive of molecules in a solution; it may only lead to a net movement of molecules down the concentration gradient. According to the Stokes–Einstein equation, the diffusion coefficient (D) is equal to the ideal gas constant (R) multiplied by the absolute temperature (T), divided by solution viscosity (η), the radius of spherical particle (r_A), and Avogadro's number (N_A):

$$D = \frac{R \times T}{6\pi\eta N_A r_A} \tag{8.7}$$

An increase in the solute molecular size and/or an increase in solution viscosity will lead to the decrease of diffusion coefficient.

The relationship between diffusion coefficient and flux can be written as Fick's first law:

$$J = -D \times \frac{dC}{dx} \tag{8.8}$$

where J is the flux, D is the diffusion coefficient, and dC/dx is the concentration gradient. The negative sign shows that flux is positive when molecular transport follows the concentration gradient.

Side-by-side diffusion chamber models can be used for transport studies of drug compounds. For carrying out these experiments, a donor section with a known starting concentration of the studied compound is needed, a barrier separating the donor and acceptor sections with a defined area and thickness, and a known volume of solution in both chambers. When the flux through the wall transfers a minor amount of the compound from the donor section (i.e. sink conditions), the concentration gradient through the wall is constant, and the flux will also be constant. In this case, the flux occurs as a function of the gradient. For these circumstances a simple form of Fick's law will be proposed to connect flux and concentration gradient. In this version of Fick's law, the flux (J) will be described as the difference between donor (C_D) and acceptor concentrations (C_A) multiplied by the permeability coefficient (P):

$$J = P(C_D - C_A) \tag{8.9}$$

Many years ago, Meyer and Overton suggested a simple law to predict membrane permeability [120, 121], which accounts only for passive diffusion as a way of molecular transport across lipid bilayer. Although this concept has been introduced 110 years ago, the number of molecules shown to disobey this law is very limited, proving that the following equation is more robust than previously assumed. In universal solutes and hydrocarbon environments, it shows a strong correlation of permeability upon the partition coefficients. According to Meyer and Overton, the permeability (P) is equal to the diffusion coefficient (D) multiplied by octanol/water partition coefficient (K) and divided by membrane thickness (h):

$$P = D \times \frac{K}{h} \tag{8.10}$$

8.3.2 Unstirred Water Layer

The idea of the unstirred water layer (UWL) was initially established by Noyes and Whitney [122]. UWL is a layer of water, mucus, and glycocalyx adjacent to the absorptive enterocyte membrane (Figure 8.4). It becomes more stirred as it moves from the intestinal wall to the lumen, and it can be considered as an additional barrier in both the donor and the receiver chambers.

The thickness of the human jejunal UWL was reported to be ~500 μm. Due to its hydrophilic nature and thickness, the UWL may become a significant barrier to the absorption of highly lipophilic compounds. In addition, the UWL greatly reduces the effective surface area compared to the underlying brush border membrane (1 : 500, respectively), presenting another mechanism by which the UWL may function as a barrier to drug absorption.

8.3.3 Membrane Transporters

Lipophilic drugs can pass through the intestinal membrane lipid bilayer by simple passive diffusion; however hydrophilic drugs require the presence of specialized transporters, which will mediate their cellular uptake.

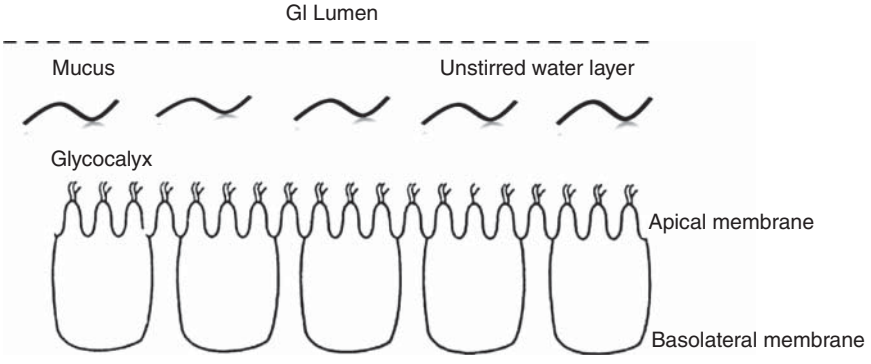

Figure 8.4 Illustration of the unstirred water layer (UWL) adjacent to the intestinal membrane.

Facilitated diffusion is a way of molecular transport that is enabled through membrane transport channels or by carriers, and this transport is not energy but concentration gradient driven. Glycoprotein channels are specific to a certain molecule or type of molecules. For example, GLUT4 is a glucose transporter found in muscles and adipose tissue. Insulin recruits transporters from intracellular stores, increasing their cell surface expression and consequent glucose uptake [123]. Carriers are a group of various fundamental membrane proteins that bind a certain substrate and undergo a transformation to disengage the substrate on the other side of the barrier.

Active transport is energy driven and transporters move substances against an electrochemical gradient. When the transport is tied to electrochemical energy derived from ion gradients, it is called a secondary active transport [124].

The two major families of membrane transporters (the ATP-binding cassette (ABC – efflux transporters) and the solute carrier (SLC – generally influx transporters)) include more than 400 kinds of transporters. ABC transporters are mainly active and need ATP hydrolysis to transfer substrates through the membrane, while SLC transporters include ion-coupled transporters, exchangers, or passive transporters [124–129]. A great variety of substances move across the cell membrane by ABC and SLC transporters, and the permeability of relevant drug compounds, their distribution, pharmacological effect, and toxicity may be affected by these transporters [130].

8.3.4 P-Glycoprotein (P-gp)

P-glycoprotein (P-gp) is one of the most significant and broadly studied ABC ATP-dependent transmembrane transporters, which acts as an exporter of xenobiotics and toxins out of the cells [131–135]. It was first defined as an agent, which facilitated the emergence of multidrug resistance (MDR) against anticancer drugs in tumor cells. As protector against toxins, P-gp exhibits its function in normal tissues like intestines, hepatocytes, renal tubules, placenta, capillary endothelial cells of brain, and peripheral blood cells. P-gp has the potential to impact the uptake of many drugs such as statins, anticancer drugs, and antibiotics and as a result may play an important role in their absorption and disposition. Examples of P-gp substrates include digoxin, loperamide, quinidine, vinblastine, talinolol, etoposide, and others [136, 137]. In the GIT, P-gp is highly expressed in the luminal membrane of enterocytes. Some evidence shows that P-gp expression gradually increases going from the proximal to the distal regions of the human small intestine [138–140]. This phenomenon was also shown in rats and mice [141–144].

The inhibition of efflux pump is primarily carried out in exchange for improving the transfer of drugs. Drugs that are known to inhibit P-gp include verapamil, quinidine, cyclosporin A, tamoxifen, laniquidar, and reserpine. P-gp inhibition can be performed by several mechanisms: (i) by blocking substrate binding competitively/noncompetitively, (ii) by interfering with ATP hydrolysis, and (iii) by changing the integrity of membrane phospholipids [145–148]. Blocking the P-gp-mediated inhibition of cellular uptake of drugs can enhance drug bioavailability and improve drug effect, e.g. in anticancer therapy. Some of the widely

used pharmaceutical excipients such as cosolvents (PEG 400) and surfactants (Tween 80, Pluronic 44, Pluronic 68, Cremophor EL) have been reported to have inhibitory effect on P-gp in addition to their ability to increase drug solubility [149]. These nonionic surfactants increase the intestinal absorption of several drugs, which are exported by a P-gp-mediated efflux mechanism in the intestine [150–154].

8.3.5 MRP2

ABCC2 gene-encoded efflux transporter is both a multidrug resistance-associated protein 2 (MRP2) transporter and a multidrug resistance protein. MRP2 is found on the apical membrane of the bile canaliculus in hepatocytes, enterocytes, renal proximal tubular cells, and placenta, where it facilitates the biliary elimination of substances. This transporter plays a role in detoxification by transferring various compounds, particularly conjugates of hydrophobic substrates with glutathione, sulfate, and glucuronate [155–157]. Moreover, MRP2 is able to convey uncharged substrates together with glutathione, and therefore it may also modify the pharmacokinetics of a wide range of drugs [158].

In some cases ABC transporters (both P-gp and MRP2) have an influence on drug disposition. It was shown that azithromycin is a substrate for P-gp and MRP2 and that its biliary and intestinal elimination is facilitated by these two main transporters [159]. The influence of the ketolide antibiotic telithromycin on the biliary elimination of doxorubicin, a substrate of P-glycoprotein and MRP2, was studied by Yamaguchi et al. [160]. It was revealed that telithromycin essentially reduced (80%) the biliary clearance of doxorubicin. Additionally, hepatobiliary elimination research exposed that cyclosporine almost completely inhibits the biliary excretion of telithromycin, suggesting that telithromycin is a substrate of P-gp and MRP2. It was reported that both P-gp and MRP2 have an effect on colchicine efflux [143], and together they reduce the intestinal absorption of this drug throughout the entire rat small intestine.

8.3.6 PEPT1

Peptide transporter 1 (PEPT1) represents an oligopeptide exchanger, placed in the intestinal brush border membrane, which offers a key tool for absorption of dipeptides, tripeptides, and peptidomimetic compounds in the human intestine [161–163]. The expression of this transporter rises from the duodenum to the ileum and depends on nutritional status (it increases in the fed state). Natural peptides, hormones, and drug substances can affect the distribution of PEPT1 in intestine [164]. In different medical conditions like ulcerative colitis or Crohn's disease, the presence of PEPT1 in the colon was found to be increased [165]. In order to exploit PEPT1 for optimization of drug delivery, more information about substrate-binding domain is required [166]. PEPT1 has two sites for phosphorylation by protein kinase C, whose activation has been reported to inhibit peptide transport in Caco-2 cells [167]. The enhancement of cAMP levels can also negatively affect the activity of PEPT1, probably due to cAMP-based stimulation of protein kinase C [168].

PEPT1 is a leading pathway for oral absorption of peptidomimetic pharmacological agents such as β-lactam antibiotics, antivirals, angiotensin-converting enzyme inhibitors, hypotensive agents, and others [169]. In order to improve oral delivery, attempts can be made to discover drugs that are PEPT1 substrates.

8.3.7 OATP

The organic anion transporter peptide (OATP) family includes influx transporters that appear in several tissues, including luminal cell membrane of small intestine, basolateral membrane of hepatocytes, kidney, blood–brain barrier, and placenta. They may significantly affect the pharmacokinetics of a wide range of drugs. For instance, OATP transporters were reported to have some effect on the absorption of fexofenadine, montelukast, talinolol, celiprolol, pravastatin, pitavastatin, rosuvastatin, levofloxacin, methotrexate, and saquinavir [170]. It was shown that the rat intestinal permeability of pravastatin remarkably decreases when coadministered with the effective OATP inhibitor naringin [171]. OATP also controls the intestinal permeability of atazanavir and therefore may cause the reduction of its oral bioavailability [172]. Constituents of grapefruit juice were described as OATP transporter inhibitors and hence may decrease the plasma levels of OATP substrates [173]. The blood concentration of fexofenadine [174], montelukast [175], and aliskiren [176] was reported to be affected by grapefruit juices due to inhibition of OATP2B2.

8.4 The Solubility–Permeability Interplay

As described above, solubility and permeability are the two critical factors that influence oral drug absorption. Separately, these two parameters have been extensively studied, but the interplay between them has been overlooked for a long time. The S–P interplay has significant applicability and influence on oral biopharmaceutics, by answering the question: what happens to the drug permeability when we increase the drug solubility via solubility-enabling formulation?

As denoted above, novel drug discovery methods lead to the presence of many drug candidates with low aqueous solubility [177–179]. This fact extremely complicates the development of these candidates into orally administered drugs, since dissolution of the drug in the aqueous GI milieu is almost always a precondition for permeation and absorption. In order to enhance the solubility of lipophilic drugs, different formulation techniques are employed, e.g. the use of cyclodextrins, surfactants, hydrotrope, cosolvents, ASDs, and others. These formulations surely enable significant increase in the drugs' apparent solubility; nevertheless their success to augment oral drug bioavailability is unpredictable, and reports of increased, unchanged, or even decreased absorption can be found in the literature. As noted above, the permeability is dependent on the diffusion coefficient, the membrane/aqueous partition coefficient, and the membrane thickness ($P = K \cdot D/h$). This definition of permeability describes how deep the drug will penetrate into the intestinal wall in a time unit. The presence of membrane/aqueous coefficient in this equation suggests a close relationship between solubility and

permeability, because this coefficient is directly linked to the solubility of the drug. The S–P interplay depends on the solubility-enabling formulation: different formulations may exhibit very different S–P interplay.

Cyclodextrin-based formulations may be tricky when relying only on solubility enhancement: while hosted inside the hydrophobic cavity of the cyclodextrin, the apparent solubility of the drug certainly increases; however these drug molecules are not available for permeation through the GI membrane. As a consequence, increased solubility but decreased permeability can be expected, attributable to reduction of the free drug fraction that is available for membrane penetration [180–182]. This type of S–P interplay has the nature of a tradeoff, and it is essential to hit the optimal S–P balance when developing such formulation, rather than relying solely on the solubility enhancement [183–185].

The chemical structure of surfactants – lipophilic tail and hydrophilic head – such as physiological bile salts or synthetic derivatives is the main reason for their ability to increase apparent drug solubility: they form micelles and host the lipophilic drug molecules inside their relatively hydrophobic core. Even though surfactants can significantly improve, the apparent solubility of lipophilic drugs, while inside the micelles the drugs' free fraction, decreases, resulting in permeability decrease, similarly to the case of cyclodextrins. On the other hand, surfactants have been known to disrupt the integrity of cell membrane, to intensify paracellular transport, and thus to enhance the intestinal permeability of the drugs with low permeability and high solubility [186–188]. Amidon et al. showed that surfactant levels higher than CMC reduce the permeability of the lipophilic drug progesterone [189]. According to the study of natural surfactants in humans by Hens et al., the solubility increase of fenofibrate via bile acids was accompanied by reduced plasma drug levels [190]. It was revealed that the low blood concentrations of fenofibrate can be explained by reduced permeability of the drug in the fed state due to micelle creation, therefore decreasing the fenofibrate free fraction that is available for permeation through the membrane. Again, an S–P tradeoff was evident, highlighting the complexity of using this formulation approach.

Solubilization by cosolvents is not associated with the creation of complexes; cosolvents increase the water solubility of lipophilic drugs by reducing the ability of water to squeeze them out via interfering with self-association of water molecules [191]. Hence, unlike the cases of cyclodextrins and surfactants, no decrease in the free fraction of the drug is obtained with this formulation approach. Surprisingly, when solubility enhancement is isolated from the free fraction considerations, the decrease in intestinal permeability was still observed. The effect of the commonly used cosolvent PEG-400 on carbamazepine intestinal permeability was tested, and decreased permeability of the drug with increasing cosolvent levels (and increase drug solubility) was found [192–194]. This shows that there is a direct relationship between solubility and permeability. The presence of the membrane/aqueous partition coefficient (K) in the permeability mathematical description ($P = K \cdot D/h$) is responsible for the S–P tradeoff, regardless of free fraction considerations. It can be concluded that with the formulation approaches described thus far, solubility and permeability need to be studied together in order to avoid wrong assumptions.

In recent years, ASD technologies became widespread in drug delivery studies. In contrast to the previously mentioned solubilization techniques, ASDs enhance the apparent solubility of lipophilic drugs by enabling them to reach and preserve an unstable level of supersaturation. While studying the ASDs of various lipophilic drugs, it has been shown that there is no permeability reduction associated with the solubility enhancement, and the intestinal permeability stayed constant during supersaturation [195–197]. ASDs increase the apparent solubility via supersaturation without simultaneous permeability loss, hence overcoming the S–P tradeoff and exhibiting a different and advantageous S–P interplay in which the permeability remains constant as the apparent solubility increases (Figure 8.5).

The lack of S–P tradeoff when using ASD could be explained in the following manner: the membrane/aqueous partition coefficient is governed by the equilibrium aqueous solubility of the drug, and while formulations like cyclodextrins, surfactants, and cosolvents affect the equilibrium solubility, ASD causes a time-dependent *kinetic* enhancement of the apparent solubility, with no effect on the equilibrium solubility. As a result, the membrane/aqueous partition coefficient remains unchanged during supersaturation, as well as the permeability. In contrast, solubility-enabling methods that impact the equilibrium solubility lead to reduced membrane/aqueous partition coefficient, thus resulting in the undesired S–P tradeoff.

As mentioned above, efflux transporters like P-gp may influence the intestinal permeability of drugs. We have recently revealed that significant supersaturation via ASD formulation can saturate P-gp mediated efflux transport in the GIT, resulting in an advantageous simultaneous increase of both the apparent solubility and permeability [194]. This case introduced a novel trend of S–P interplay: while a detrimental ↑S–↓P was shown for cyclodextrins, surfactant hydrotropy, and cosolvency, the favorable ↑S–↔P trend was evident for the use of ASD, and optimal ↑S–↑P was revealed for ASD formulations of low-solubility P-gp substrate compounds. Overall, it is clear that the S–P interplay cannot be ignored,

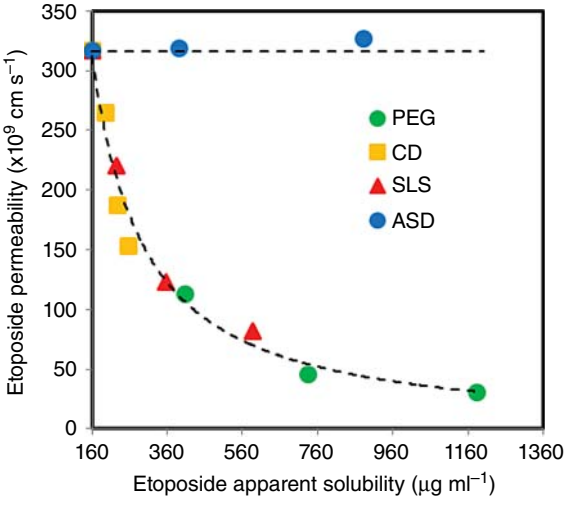

Figure 8.5 Etoposide's theoretical (dashed line) and experimental (markers) permeability as a function of the solubility enhancement afforded by the formulation. (*Source*: Beig et al. 2015 [195]. Reproduced with permission of Elsevier.)

and awareness of the influence of the solubility-enabling formulation on both the solubility and the permeability is prudent, especially during early clinical development when formulation decisions that will impact all other development activities are made.

8.5 Summary

This chapter provides an overview of the solubility, the permeability, the factors that influence both of them, and lastly their relationship and its importance for successful development of formulations for lipophilic drugs. The overview presented in this chapter highlights that the absorption of drugs, their effective delivery, and consequently their pharmacological effect is largely influenced by the solubility and the permeability. Moreover, it is essential to consider their interplay and to strike the optimal S–P balance in order to increase the overall drug absorption and bioavailability.

List of Abbreviations

GI	gastrointestinal
IR	immediate release
BA/BE	bioavailability/bioequivalence
BE	bioequivalence
P-gp	P-glycoprotein
MRP2	multidrug resistance-associated protein 2
PEPT1	peptide transporter 1
OATP	organic anion transporter peptide
S–P	solubility–permeability
ASD	amorphous solid dispersion

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9

Solid-State Properties

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9.1 Introduction

Solid-state properties affect the development of *every* drug product. Solid dosage forms such as tablets and capsules are the most popular drug products on the market [1]. Other dosage forms such as solutions, suspensions, creams, gels, and aerosols are (i) reconstituted or dissolved from solid dosage forms, (ii) formulated from drug substances or excipients that are stored as solids, or, lastly, but certainly not least, (iii) affected by the solubility and stability of certain components that are closely related to physical forms [2]. Therefore, solid-state properties have a profound impact on the development of a drug candidate at all stages.

The basic of solid-state properties is the physical form at the molecular level. The different conformation and spatial arrangement of molecules determine not only its physical form but also the energy state. The free energy of the solids further affects its solubility and stability; two properties that are vital to the development of drug products. The solubility of a drug substance relates directly to its bioavailability. For a compound whose bioavailability is limited by its poor solubility, pharmaceutical scientists proactively search for high-energy alternative physical forms – such as amorphous, metastable polymorphs, salts, or cocrystals – to boost the solubility and achieve the required bioavailability [3]. On the other hand, unexpected physical form change in the drug products may result in significant reduction of solubility and bioavailability, leading to clinical failures and product recalls [4]. A comprehensive physical form screening and selection of the most stable polymorphic form are always recommended for a drug candidate to minimize the potential of a late stage form change.

Stability of the drug substance is another key concern during the development of drug products. Degradation of a drug substance will not only diminish its biological activity but also generate impurities with potentially increased toxicity. Generally speaking, high-energy forms are not only physically metastable to the low-energy, thermodynamically stable forms but may be chemically more reactive and less stable. For instance, the photodegradation rate is more rapid for the metastable polymorph of the diuretic furosemide [5] or for the metastable glass

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

of the dye Disperse Orange 37 [6]. Therefore, it is a requirement that the physical form of a drug substance is chemically stable during the shelf life; while any form change that may reduce the chemical stability should be strictly avoided during manufacturing and storage.

In addition to solubility and stability, variations in the crystal structure may also affect the mechanical properties of the material, such as compressibility, elasticity, hardness, and flowability. Well-known examples in the inorganic realm include the hardness difference between graphite and diamond – two allotropes of carbon, or between glass and quartz-amorphous and crystalline silicon dioxide. For drug substances, changes in the physical forms may affect the easiness of tableting and bring challenges to the formulation development. For example, the compressibility of anhydrous theophylline can be modified through the formation of a monohydrate [7] or a methyl gallate cocrystal [8]. In both cases, differences in hydrogen bonding and molecular packing in the crystal lattice affect their mechanical properties. Furthermore, the performance of excipients are also affected by their physical forms. It has been reported that the hydration of lubricant magnesium stearate will improve its lubricity [9], while different crystalline phases of lactose will affect its aerosolization performance [10]. Therefore, careful selection of excipients and formulation processes is necessary for drug product development.

Besides the physical forms, other solid-state properties that exist at the particulate level may have a profound effect on pharmaceutical development. These properties include the size and shape of the particles, and the surface characteristics. These properties may alter the solubility, chemical stability, and mechanical properties of both the drug substances and the excipients. In this chapter, the basic concepts of physical forms related to early drug development including solid form screening and the form selection process will be covered. The impact of physical properties, namely, crystal habit and particle size, will also be discussed.

9.2 Amorphous and Crystalline States: Basic Concepts

Pharmaceutical solids can exist in multiple forms. A crystalline solid contains an ordered arrangement of atoms, ions or molecules, while an amorphous solid does not contain such long-range order. Liquid crystals, containing partial order between that of crystalline and amorphous forms, are less common [11]. A crystalline form may contain a single entity (the active pharmaceutical ingredient (API) molecule) or multiple entities, including the API and other adducts. Crystals containing only the API molecule are usually called anhydrate or ansolvate. The additional adduct to the API in the crystal could be a solvent, a neutral guest, or a counter-ion and the crystal form would be defined as a solvate, a cocrystal, or a salt, respectively. Hydrate is a special case of solvate in which the solvent molecule is water. Note that more than one adduct can accompany the API molecule in the crystal lattice; the crystal can exist in the forms of a salt solvate, a cocrystal salt, or even a cocrystal salt solvate [12].

The ability of organic molecule to exist in multiple crystalline states is known as polymorphism. In the broadest sense, polymorphism has been described

to encompass both amorphous and crystalline solids including solvates and hydrates [13, 14]. A more narrow definition is also commonly used, in which polymorphism refers specifically to the different crystalline structures of the same chemical composition [15]. In the latter definition, anhydrate/ansolvate, solvate, or salt/cocrystal may exhibit polymorphism. However, a solvate is not a polymorph of an anhydrate, as they have different chemical compositions, while an amorphous form is not a polymorph neither as it is not crystalline. We will adopt the latter definition in the remainder of this chapter.

9.2.1 Crystalline States: Polymorphs, Hydrates, Solvates, Salts, and Cocrystals

Polymorphism is a common phenomenon among pharmaceutical solids. To estimate the prevalence of polymorphism among organic molecules, two different approaches are commonly used by researchers. The first method is to search the data in the Cambridge Structural Database (CSD) [16]. Although a large data set could be surveyed by this method, it often underestimates the actual percentage of polymorphs since (i) its dependence on crystallographic data to judge polymorphism in which structural information for different solid forms may not be readily available; and (ii) the potential lack of extensive polymorph screening on each system. Nevertheless, a 2015 study showed that 36% of drug-like (as defined by Lipinski "rule of 5" criteria [17]) anhydrates exhibit polymorphism ($n = 4471$), comparable with 37% of all anhydrates regardless of drug-like properties ($n = 5941$) [18]. Another method is to survey internal data sets of polymorph screening results conducted by the pharmaceutical industry. While each system in the data set was more extensively screened and studied, much fewer cases can be presented for such a survey. Here, a Solid-State Chemical Information (SSCI) [19] survey of 245 compounds revealed that 50% exhibited crystal polymorphism [20], while solid form statistics from 229 solid form screens conducted at Roche and Eli Lilly revealed that the minimum polymorphism occurrence in neat (anhydrous) forms is at least 50% [18]. Given that the anhydrate is the most common solid form of an API, comprehensive form screening is necessary for the successful development and is recommended by the Food and Drug Administration (FDA) for new drug application (NDA) filling.

Hydrates are also common among pharmaceutical solids. According to a 1999 survey, approximately one-third of organic compounds in the European Pharmacopoeia can form hydrates ($n = 808$) [21]. Hydrate is a viable form of API for development given the non-toxic nature of water, although its physical stability under ambient condition is a concern. On the contrary, if an anhydrate is chosen for further development, its stability in aqueous or humid condition must be understood due to the ubiquitousness of environmental water. The physical form screening of anhydrate often includes an extensive search for hydrates. On the other hand, solvates other than hydrates are usually avoided as the final form of API owing to toxicity concerns and the limits on residual solvents set by the International Conference on Harmonization (ICH) [22]. However, solvates are sometimes exploited as an intermediate leading up to the synthesis of

the final API. Solvates may offer superior ability in rejecting chemical impurities than anhydrates [23–25], and sometimes a solvate is the only crystalline form available for the final API or a chemical intermediate. Furthermore, there are several examples where the final API form was derived via desolvation of a solvate. In such cases, the stability and the desolvation kinetics of solvates must be extensively studied and completely understood.

The formation of salts or cocrystals offers tremendous opportunities to expand the number of solid forms available for pharmaceuticals with a view to improve their physicochemical properties [26, 27]. Therefore, salt or cocrystal screening is often conducted when there is no available crystalline forms of the API or when the lead solid form does not possess satisfactory properties for further development. Salts and cocrystals are extensively covered in Chapter 10.

In addition to the API, many excipients also exhibit polymorphism that may affect the performance of drug products. For example, different crystalline forms of lactose and magnesium stearate have an impact on their performance as lubricant and inhalation drug carrier, respectively [9, 10]. Excipients and their residual water may also change the physical stability of API, forming hydrates or salts during formulation process. An excipient compatibility assessment is therefore recommended in order to exclude problematic excipients from future formulation development.

9.2.2 Polymorph Screening and the Solid Form Selection Process

Polymorph screening refers to a set of experiments to explore the phase realm and to identify possible solid forms of a chemical entity. Polymorph screening is usually followed by solid-state characterization and a selection process to identify which physical forms are considered developable. Therefore, it is critical to identify and understand the target solid-state properties as the focus and requirement varies with the development stage and the dosage form. Knowledge of the targeted properties will guide the form selection and the screen design.

A typical solid-state development process involved the following steps:

1) Establish the goal of form selection.
2) Characterization of the starting material.
3) Design and conduct a solid form (crystal, polymorph, salt, or cocrystal) screen.
4) Scale-up and characterize relevant solid forms.
5) Select a physical form for further development.
6) Develop a robust and scalable process to deliver the desired form and establish analytical methods for quality control.
7) Formulation of the drug substance (details are covered in Chapter 12).

The importance of each step is related to the specific drug substance and the stage of development while the order and necessity of each step is subject to change.

9.2.2.1 Goal of Form Selection

The requirement on solid forms is often dictated by the target formulation or delivery vehicle and may vary with the development stage. In the early discovery stage, after biological targets for a therapeutic area are identified

and validated, a series of candidate compounds are tested on those targets for their affinity, activity, and selectivity. High-throughput screening is commonly employed in this stage due to the large number of candidates and the repeating nature of testing. At this stage, simple vehicles like DMSO solutions are usually employed for compound dispensing, with little attention paid to the solid-state properties of candidates. Promising candidates are moved to the next stage, lead identification and optimization, for further evaluation. The purpose of this stage is to select and prepare one compound, with up to 1–2 backup compounds, for preclinical development. Therefore, more *in vitro* assays are conducted, focusing not only on activity but also on toxicity and physical properties for a full evaluation and comparison of all the leads. Solubility and permeability are evaluated for the first time, and solid-state properties start to play a prominent role. If no crystalline forms have been identified, a solid form screen is initiated at this time. This screen is sometimes conducted by synthetic chemists, material scientists or preformulation scientists. At the end of lead optimization stage, a formulation adequate for toxicological studies (tox-formulation) is designed based on the solubility and projected dose. The tox-formulation could be an aqueous-based solution or suspension. If a crystalline phase with acceptable solubility is available, the design of tox-formulation is simple. Otherwise, an amorphous material could be used to enable the delivery of maximum dose. The selected phase, crystalline or amorphous, is often described as a "fit-for-purpose" form or "safety assessment" form for the tox-formulation.

A single lead compound is then moved from discovery to the preclinical development stage. In this stage, data is collected for the purpose of an investigational new drug (IND) application in the United States (US) or a clinical trial application (CTA) filing in the European Union (EU), and the fate of the lead is much governed by its safety or toxicity evaluated in the animal models. Dose-limiting toxicity studies need to be carried on at least two animal species, one rodent and one non-rodent. At the same time, the first batch of compound prepared according to good manufacturing practice (GMP) is synthesized for the development of Phase I formulation. The target solid form needs to be established prior to the first GMP delivery. For this purpose, a manual polymorph screening is initiated. Stability assessments are initiated on the lead form (Phase I form or first-in-human form) or other relevant forms to evaluate both chemical and physical stability under stress conditions. In the clinical development stage, formulation development is focused on the final market formulation, which is established prior to Phase II development. To avoid any last-minute physical form changes, a comprehensive screen is conducted to ensure a robust, developable, and commercial form is chosen for development.

For the development of specialized formulation, the timeline of commercial form selection may be accelerated. This includes respiratory and parenteral formulations. As the requirements on the solid-state properties and the delivery vehicle itself are more specific, the commercial form needs to be selected much sooner. For parenteral formulation, the selected form should be soluble and stable enough to provide the required exposure. For inhaled formulation, the selected form needs to be physically stable enough under milling to provide the desired particle size (often less than 5 μm) otherwise a bottom-up process will need to be developed to deliver the target form with the desired particle size range.

9.2.2.2 Characterization of the Starting Material

Form and Thermal Properties If a crystalline form is not available with the starting material, a crystallization screen is initiated with the intent to identify a developable crystalline form. Starting materials that are amorphous or gel are often less chemically stable and tend to be more hygroscopic. If possible, glass transition temperature of amorphous solid should be determined to evaluate its crystallization tendency [28]. Amorphous materials with lower glass transition temperature are generally prone to crystallize. For crystalline starting materials, the most important thermal properties are melting point and enthalpy. Special attention should be paid to any thermal events observed during thermal analysis: a desolvation, a solid-solid transition, or a crystallization event may indicate a form change; while a decomposition event may alert the risk and indicate the upper boundary of the temperature range for physical form screening.

Aqueous and Organic Solubility Solubility in biorelevant media is one of the most important physical properties relevant to bioavailability and form selection. The choice of solid form could be critical for the development of poorly soluble compounds, and hence a high-energy form (metastable polymorph, salt, cocrystal, or amorphous solid dispersion (ASD)) could be desired. Solubility in organic solvents is also important for designing a crystallization process to deliver the desired solid form. If enough material is available, high throughput solubility screen can be exploited to generate a vast amount of solubility in a short period of time. Otherwise, visual solubility is commonly performed to obtain solubility estimates in relevant solvent systems.

Hygroscopicity Given the ubiquitous nature of water in the environment [21], it is essential to evaluate the physical stability of the starting material under different humidity conditions. This can be achieved by performing a moisture sorption analysis whereby the material is exposed to different humidity levels. If a form change is observed during the course of the study, the new form should be further characterized to understand its stability region. Knowledge of the hygroscopicity data is helpful in the design of a quality drug product since temperature and humidity are important factors during manufacturing, packaging and storage.

Chemical Stability and Chemical Purity The purity of starting material could affect the crystallization and polymorph screening outcomes, as certain impurities may promote or inhibit the crystallization of a specific polymorphic form [29]. In addition, solid-state characterization techniques are sensitive to crystalline impurities. Therefore, misleading results could be generated; while new forms could be missed if an impure material was used as starting material. The chemical purity of the material is often related to the synthetic process, and as the chemistry route develops and improves, the purity profile will also evolve. As a result, a more stable polymorphic form may appear due to improvements

in the quality of the API [30, 31]. It is critical that chemically pure API is used as the input to the solid form screen, and eliminates the potential of impurities inhibiting the formation of the more stable polymorph.

9.2.2.3 Polymorph Screening Methods

The intent of polymorph screening is to generate many different crystalline forms and evaluate relevant physical forms. Pertinent solid forms that emerged from polymorph screening can be scaled up and characterized. Therefore, a variety of crystallization methods are employed for polymorph screening. If a solvent is used in the physical form screen, it should not be limited to ICH class III solvents. In fact, exposure of the starting material to a diverse set of conditions will maximize the chance of discovering novel solid forms [32, 33].

A list of commonly used screening methods is summarized in Figure 9.1. Generally speaking, experiments can be divided into two categories: solvent-mediated experiments and solid-state experiments. Certain solid-state experiments are partially covered during the initial characterization of the starting material. For example, cooling from melt can be conducted during thermal analysis [34], while exposure to humidity is assessed during the dynamic vapor sorption experiment [35]. Any new forms emerged from initial characterizations should be treated as important relevant forms and reserved for further characterization.

Solvent-mediated experiments including crystallization from solution or slurry, are commonly used for all kinds of form screening. The selection of solvents is crucial for success as it may alter the crystallization outcome. Through dissolution, solvent molecules not only provided a media for the solid molecules to rearrange but could also interact with the solid molecules to form

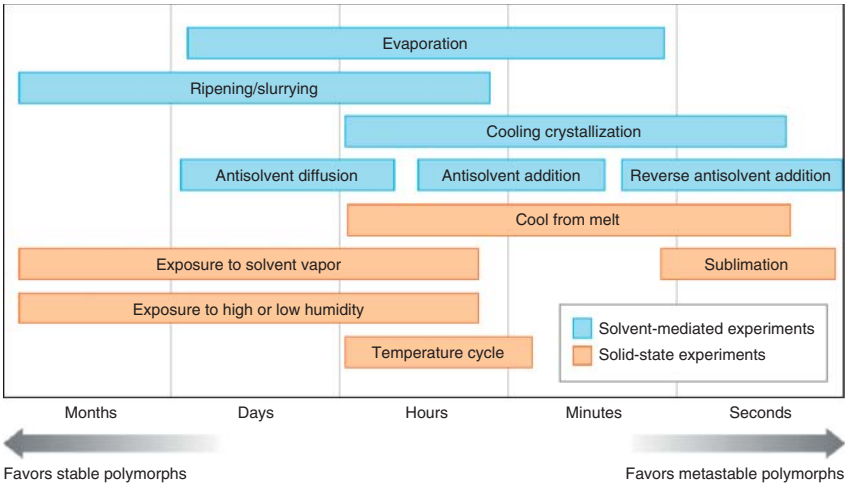

Figure 9.1 Timescales of different crystallization methods used to screen for different physical forms. *Source*: Anderton (2004) [59]. Reproduced with permission of Russell Publishing Ltd.

an intermediate complex or even a solvate. Solvent interaction may alter the conformation and hydrogen bonding of the solid molecule, thereby promoting or inhibiting the nucleation and growth of certain forms. It is therefore suggested that a variety of solvents with distinct structure and properties should be considered. Grouping of solvents based on their properties (e.g. hydrogen-bonding property, polarity, dielectric constant, etc.) is available and should be referred for experimental design [36]. On the other hand, crystallization or phase transition could also be hindered by low solubility, which may lead to miss solid forms in the experimental time frame [37]. It is also important to use aqueous-organic solvent mixtures to increase the possibility of finding hydrates than using neat water, especially when the aqueous solubility of the compound is limited [32]. Therefore, knowledge of solubility of the starting material in various solvents will help the solvent selection process. Solvents with poor solubility can be used as anti-solvent. Process relevant solvents for chemical synthesis and impurity rejection should also be included in the solvent list.

Among the screening solvents, special attention needs to be paid to the non-solvating solvents, those that will not form a solvate with the starting material. If a phase transition is observed in non-solvating solvents, the emerging form is thermodynamically more stable. Therefore, non-solvating solvents can be valuable as a media for slurry bridging to evaluate the relative stability of polymorphs. It should be emphasized that non-solvating solvents are sometimes misidentified. Solvates may be hidden in the initial screen due to their transient nature and the rapid desolvation upon isolation. To capture a transient solvate, the solids should be characterized as damp cakes with the mother liquor present.

If multiple forms are available as the starting material, the selection of starting form could have an impact on the screening. Generally speaking, it is better to start with amorphous than its crystalline counterpart as the pathway to metastable forms may be impeded by the memory of the initial crystalline form. Heteronuclei could be added during the screening to promote nucleation of new forms: isostructural solvates of different solvents may induce the crystallization of each other, while crystals of a structural-similar compound could be used to promote the crystallization of a hard-to-crystallize one. Polymer heteronucleation has been applied to multiple systems to discovery new solid forms or control the crystallization of metastable ones.

Temperature is another factor that needs to be considered during a screen design. The relative stability between solid forms could be temperature related, such as between enantiotropic polymorphs or between solvate and anhydrate. If possible, screening should cover a wide temperature range, at least to bracket process temperatures including crystallization, milling, and drying steps.

Many of the methods depicted in Figure 9.1 can also be performed in a high-throughput fashion using an automated platforms [38]. Starting materials could be dispensed as solids or solutions to a multivial plate, while addition of screening solvents or antisolvents, temperature cycling, solvent evaporation, slurry filtration, and residual solids collection and analysis could all be conducted on the same apparatus. With this tool, large sets of screening experiments could be conducted in a short period with less amount of starting material giving

more chances to discover new solid forms. New forms of ritonavir [39] and sertraline hydrochloride [40] have been identified through high-throughput screening.

Beyond experimental approaches, recent advances on crystal structure prediction have made possible the virtual polymorph screening of organic compounds. Predicted structures are usually grouped according to crystal systems and ranked by lattice energy. If a known polymorph is successfully predicted, its ranking in the list may provide assurance and guidance on the necessity for additional screening experiments. Computational simulation may also guide the solvent selection for nucleation of specific polymorphic form [41].

9.2.2.4 Assessing the Relative Stability of Multiple Physical Forms

If multiple forms were found during screening, the stability relationship of relevant physical forms should be evaluated. Relevant forms may include anhydrate with its polymorphs, hydrates, and solvates in the relevant process solvents. Slurry bridging is the most common experiment to investigate the relative stability. In a typical experiment, two or more forms are suspended in solvents that are presaturated with the compound. Alternatively, excess amount of solids could be added into the solvents to form a suspension of the relevant physical forms. The suspension is then agitated at a set temperature, while the unstable, more soluble form(s) will eventually dissolve and convert to the stable, less soluble form. Characterization of the residual solids will then reveal the stable polymorphic form.

Relative stability of anhydrate polymorphs should be carried in multiple non-solvating solvents and cover the process temperature range. The stability relationship between polymorphs should only be affected by temperature (assuming constant pressure) irrespective of the solvent system. If inverted slurry outcomes are encountered at two set of temperatures, the polymorphs are enantiotropically related with a transition temperature between these two temperatures. Further experiments could be carried to bracket the transition temperature range.

The stability of solvates/hydrates and anhydrate is affected by not only the temperature but also the solvent/water activity. Therefore, a set of binary solvents with different solvent activities are employed to evaluate the stability relationship of the anhydrate and solvate/hydrate. Similar to the transition temperature, the critical solvent activity refers to the transition point between these two physical forms. Slurry conditions should bracket the critical solvent activity at all relevant temperatures. Generation of a phase diagram consisting of solvent activity and temperature can guide the design of a crystallization process to deliver the target solid form.

Knowledge of the transition temperature and critical solvent activity are important thermodynamic data critical for process development. Therefore, the data should be confirmed by orthogonal methods. For slurry bridging, it's suggested that multiple solvents or solvent mixtures should be adopted. Orthogonal methods including solubility measurement (van't Hoff plot) [25], thermal analysis [42, 43], and dynamic vapor sorption have also been employed.

9.2.2.5 Form Selection Process

In the pursuit of a robust drug product, it is critical to select a physical form that has optimal properties for development. Given that solubility, dissolution and bioavailability are closely related to the physical form of the drug substance, it is important that the solid form is physically stable during the shelf life of the drug product to ensure that any variability in the bioperformance is not attributed to the crystalline form. Therefore, a general strategy is to select the most thermodynamically stable, lowest-energy polymorph to minimize the risk of any physical instability [1]. The thermodynamic stability is dependent on the environment, especially if the environment contains certain molecules that may co-crystallize with the drug substances to form a new physical form. These molecules can include the water, counter-ions, or cocrystal formers from formulation excipients. Although it could be easier to avoid any potential salt or cocrystal formers in the excipients, it is harder to exclude water from the formulation process or storage condition. For a drug substance that can potentially form hydrates, it is vital to understand the stability relationship and the long-term stability among the physical forms [44].

When solubility becomes a concern, the form selected for further development may not be the most thermodynamically stable one. Although there are strategies taking advantage of a low solubility form for developing modified release formulation [45], the concern is usually about insufficient exposure due to poor aqueous solubility. Therefore, a high-energy form such as amorphous (which will be discussed in the next section), salts or cocrystals (which will be covered in Chapter 10), and metastable polymorphs may be selected [3]. Since the requirement on physical stability remains the same, a detailed understanding of numerous factors including temperature, process-induced stresses, humidity, and processing variables impacting the physical stability of metastable form is required. Therefore, it is often more challenging to develop a metastable form.

Chemical stability is another decisive criteria in the solid form selection process. This is especially important when considering a metastable form as it may be more chemically reactive and less stable. Mechanical properties and other solid-state properties may also play a role in the form selection; however these factors are usually less vital than solubility and stability, as in most cases it could be overcome through careful selection of excipients and formulation processes [1].

9.2.3 Amorphous Solid Dispersions

The increasing number of Biopharmaceutics Classification System (BCS) class II and IV new molecular entities (NMEs) – pharmaceutical compounds that are limited by poor solubility and dissolution rates – demands the use of enabling formulations to meet the requirement of exposure high enough to support *in vivo* studies [46–49]. General formulation approaches to deliver molecules in the preclinical setting include nanoparticles, solubilizers, pH adjustment, cyclodextrin complexes, amorphous solid dispersions (ASDs), and others based

on the physicochemical properties of the API. ASDs have received a great deal of attention in the early preclinical development for its unique advantages. These formulations can offer faster dissolution rates and increased exposure in animal and human testing while deliver more conventional and robust solid oral dosage forms that are suitable for both small- and large-scale manufacture.

The term amorphous solid dispersions has been defined as a dispersion of an amorphous API stabilized by a carrier (polymer) in the solid state prepared by solvent, melting, or solvent–melting methods with improved physical and chemical stability [50]. The approach for preparing ASDs include solvent methods such as spin casting, electrospinning, lyophilization and spray drying, and thermal methods such as hot melt extrusion (HME). With the advancement of these technologies, the drug loading may vary with 30–40% API without any evidence of phase separation after the initial preparation of the drug product intermediate. A number of drug products containing amorphous solid dispersion have entered the market in recent years. Most of the products have been developed to overcome solubility limitations of the crystalline form of the drug substance (Table 9.1) [51, 52].

9.2.3.1 Spray Drying

One of the most common process to fabricate amorphous dispersion is spray drying. In the spray drying process, the API and formulation excipients (polymer, surfactant) are dissolved in a common solvent, and the resulting solution is pumped into a spray nozzle and atomized into a drying chamber. Hot drying gas (typically 60–100 °C) is introduced to the chamber and rapidly evaporates the solvent from the feed solution, ultimately reducing droplets to spray dried dispersion particles with insufficient time for phase separation or crystallization. In practice, spray drying method is readily scalable from milligrams to metric tons,

Table 9.1 Overview of marketed drug products using amorphous solid dispersions [51, 52].

Product	Drug	Carrier	Manufacturer	Dosage form
Certican	Everolimus	HPMC	Novartis	Tablet
Cesamet	Nabilone	PVP	Valeant	Tablet
Gris-PEG	Griseofulvin	PEG6000	Pedinol	Tablet
Intelence	Etravirine	HPMC	Tibotec	Tablet
Isoptin SR-E	Verapamil	HPC/HPMC	Abbott	Tablet
Kaletra	Lopinavir, ritonavir	PVPVA	Abbott	Tablet
Nivadil	Nivadipine	HPMC	Fujisawa	Tablet
Prograf	Tacrolimus	HPMC	Fujisawa	Capsule
Rezulin	Troglitazone	PVP	Sankyo	Tablet
Sporanox	Itraconazole	HPMC	Janssen	Capsule

enabling production of early development quantities through late stage manufacturing and commercialization. Spray drying is particularly beneficial for compounds with poor thermal stability compared with thermal methods due to the extremely fast solvent evaporation time (in seconds). The other advantages of spray drying include the ability to incorporate excipient into the process and the opportunity to adjust particle size and bulk powder properties by altering process parameters.

9.2.3.2 Hot Melt Extrusion

Hot melt extrusion (HME) is another widely used method to generate ASDs. It involves heat and pressure to melt the mixture of API and polymer, and the melt is forced through an orifice in a continuous manner. In the production of pharmaceutical formulations, a twin screw extruder is used to mix the drug substance and an appropriate polymeric excipient into a melt, which is extruded through a dye. Upon cooling, the amorphous and glassy extrudate is either shaped by calendaring or pelletized and milled to a desired particle size. The final milled extrudate is then typically blended with additional excipients and incorporated into traditional tablet or capsule dosage forms. Due to its continuous manner and the ease with scaling the process, HME is particularly attractive for large-scale manufacturing. In contrast to spray drying, HME does not require the use of organic solvents; however, it does have limitations as it may not be applicable for thermally sensitive materials, or high melting point drugs.

9.2.3.3 Solid Dispersion Workflow

The typical development process for amorphous solid dispersions in the preclinical development stage can be divided into three steps [53]. The first step is the early stage screening with the aim to identify the drug–polymer combination to reach the best dissolution profile. A large number of samples at mg-scale can be quickly prepared in parallel by solvent casting, which allows enough material for basic solid-state characterization and *in vitro* dissolution test. In the second step, 2–3 lead polymers are selected based on *in vitro* performance and the potential lead ASDs are then produced on a larger scale (e.g. mini spray dryer) for further evaluation, including chemical and physical stability studies and *in vitro* release characterization. In the third step, more in-depth investigations are conducted covering detailed *in vitro* performance assessments, drug loading optimizations, additional chemical and physical stability, and refinement of the ASD preparation. Finally, large-scale manufacture of ASD material is done to supply animal and clinical studies.

9.2.3.4 Dissolution and Stability Issue

Although amorphous solid dispersion has been applied in pharmaceutical development and manufacturing for years, the dissolution mechanisms of ASDs have not been studied and understood thoroughly. It has been reported that they can form supersaturated solutions upon dosing and possibly sustain the duration of supersaturation for several hours, thereby overcoming absorption limitations due to low equilibrium solubility. Based on the chemical nature of the components

and the drug loading (carrier to drug ratio), the drug release from amorphous solid dispersions can either be polymer controlled or drug controlled. During the dissolution of ASDs, the drug can remain dissolved or in an amorphous state for minutes or hours and then recrystallize either in the solid state or from a supersaturated state in solution, generating small particles at nano- or microscale suspended in the dissolution medium. The role of the polymer carrier to inhibit drug crystallization in this process is critical in maintaining the desired dissolution performance.

In contrast with other enabling formulation approaches, amorphous solid dispersions present a greater level of complexity and require careful evaluation of chemical and physical stability to ensure that they possess sufficient handling and storage characteristics for use in the desired study. Concerns surrounding physical stability (e.g. phase separation or crystallization of the API) is one of the primary reason why solid dispersion has not been widely adopted.

Numerous factors contribute to the success of solid dispersions including API characteristics, formulation parameters, manufacturing process parameters, and selection of excipients. The link between these factors and physical instability is still not completely understood. To address this question, deeper understanding of the physical chemistry of amorphous materials would be necessary. Although the mechanism for API–polymer miscibility and phase separation is not fully understood, there are practical rules of thumb to stabilize the amorphous solids. For example, lower storage temperature is recommended, usually 50 °C below the glass transition temperature (T_g) for extended shelf life. Moisture prevention and inclusion of an anti-plasticizer to increase T_g are also beneficial.

9.3 Physical Properties of Drug Substance

9.3.1 Particle Habit

The external or outer appearance of a particle is generally known as habit, morphology, or shape. It is an important solid-state characteristic that can influence drug product formulation, manufacturing, dissolution, and bioperformance. According to the United States Pharmacopeia (USP) and the National Formulary definition, as depicted in Figure 9.2, the particle shape can be categorized as:

- Acicular – Needlelike particles of similar width and thickness.
- Columnar – Long, thin particle with a width and thickness that are greater than those of acicular particles.
- Flake – Thin, flat particle of similar length and width.
- Plate – Flat particles of similar length and width; thickness greater than flakes.
- Lath – Thin and bladelike particles.
- Equant – Particles of similar length, thickness, and width.

The habit is highly dependent on the relative growth rate of individual crystallographic faces of a crystal with fast-growing faces having little or minimal

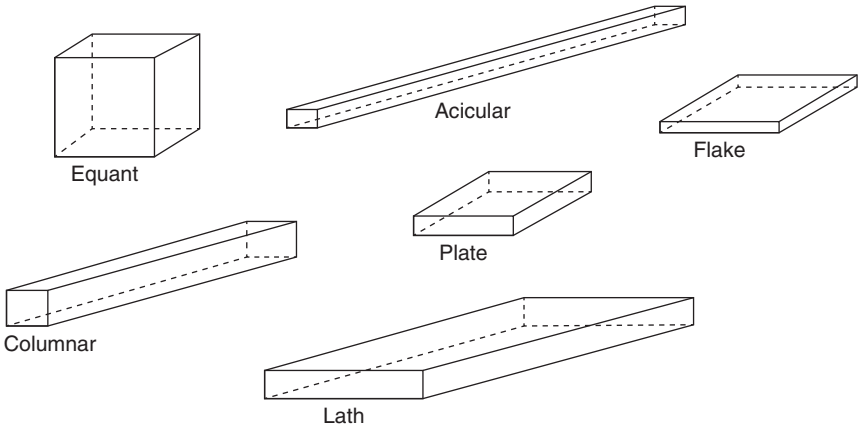

Figure 9.2 Particle shape descriptors. (*Source*: Adapted from National Formulary 2002 [54].)

effect on the overall growth morphology while slow-growing faces become more prominent. Generally, different crystal faces possess different surface chemistry (or functional groups), surface anisotropy, and surface energy. For similar crystal shapes of the same compound, it is possible to exhibit dissimilar solid-state behaviors. Jain et al. [55] examined the performance of two plate-shaped habits of aspirin of the same polymorphic form, form 1. The two morphologies only differ in their predominant facets: (100) and (001). The (100) crystal faces tend to be more hydrophilic as a result of the exposed polar carbonyl groups, whereas the (001) facets are composed of nonpolar aryl and methyl groups that render the surface more hydrophobic and a reduced potential for hydrogen-bonding interactions. Based on the different surface anisotropy, habits with the (100) plane as the dominant facet have higher degradation rates and hygroscopic behaviors. Due to the higher moisture uptake as a result of the exposed hydrophilic or polar surface, the (100) dominant plate-shaped particles degraded more readily than the hydrophobic (001) plates, likely a consequence of hydrolysis of the ester group. The surface anisotropy of aspirin crystals was also confirmed by Heng et al. [56] as wettability differences between the crystallographic facets were reported with the (100) facet ascribed to being less hydrophobic than the (001) facet due to the presence of polar carbonyl surface functions. Differences in the anisotropic surface chemistry of similar particle habits can have a profound effect on the performance of the material.

Many factors can influence the shape of the crystals. These include the choice of solvent for solution crystallization, the degree of supersaturation, and the presence of additives or impurities in the crystallizing solution. For instance, terephthalic acid crystallized as monoclinic needles from low supersaturations, while at high supersaturation rates, the growth is stunted giving rise to boulder-like particles. The most common approach to modify the particle habit is through changing the solvent system. Favorable or strong interactions between the solute and the solvent can lead to preferential adsorption of the solvent molecules onto specific crystallographic facets, which in turn inhibits their growth rate or can

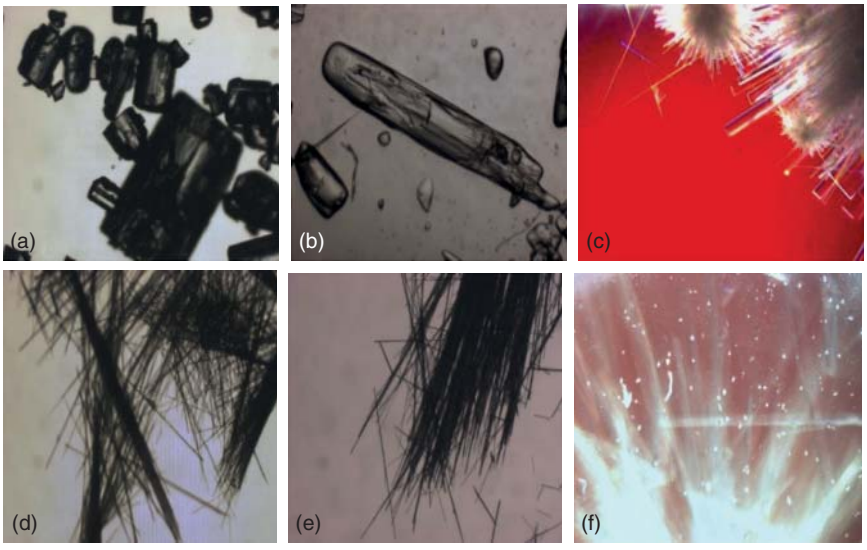

Figure 9.3 Microscopic images of (a) metformin HCl (as is); metformin HCl crystallized from (b) water, (c) formamide, (d) ethanol, (e) methanol, and (f) *n*-propanol. (*Source*: Benmessaoud et al. 2016 [58]. Reproduced with permission of Elsevier.)

lead to a reduced interfacial tension, which results in a transition from a smooth to rough interface and a simultaneous faster surface growth [57]. This is evident in the case of the antihyperglycemic drug, metformin HCl, where the crystal habit can vary based on the crystallization solvent (Figure 9.3) [58].

Chemical impurities or reaction by-products are another variable that can affect the crystal morphology. It is common to encounter changes to the particle habit as the chemistry route evolves due to the appearance and/or disappearance of impurities. In most cases, the habit-modifying abilities of the impurities are dependent on the levels or concentrations of the by-products in the crystallization medium. Careful control and design of the crystallization process can ensure the impurities are not generated at levels for the by-products to be effective habit modifiers. This was demonstrated with the control of the particle morphology and size of an oral cephalosporin antibiotic, cefmatilen hydrochloride hydrate, by having a robust crystallization process that afforded low levels of the habit-modifying impurity [60]. Intentional addition of excipients or additives into the crystallizing solution is a common strategy employed for altering the crystal shape. The additive should be pharmaceutically acceptable or generally recognized as safe (GRAS). Mirza et al. [61] demonstrated that the excipient, hydroxypropyl methylcellulose (HPMC), is an effective habit modifier for the macrolide antibiotic, erythromycin A dihydrate. With increasing HPMC concentrations, the particle habit evolved from irregular shape to platelike. Moreover, improved compaction and tableting performance were reported with the modified habit.

A common perception is that the external appearance of most APIs from solution crystallization is acicular or needle-like particles [62–64]. It is natural to

expect this given that molecular organic crystals tend to compose of anisotropic network of intermolecular interactions. A recent study by Hancock and coworkers at Pfizer revealed that the *typical* particle morphology of APIs are predominately equants with a median aspect ratio of between 0.6 and 0.8, and not necessarily needle-like [65]. Using a dynamic imaging analyzer, the Pfizer team gathered quantitative particle shape information of over a thousand API powder samples over the last decade. It was also revealed that the APIs have low surface roughness. The vast quantitative particle shape analysis is the first of its kind and provides a starting point to understand the typical particle shape characteristics of APIs.

The bulk behavior of powders such as flowability, cohesivity, and compressibility is influenced by the size and shape of the particles. Fu et al. [66] evaluated the powder flow characteristics of three different lots of lactose, two of which differ in size but possess similar habits, and the third lot has similar size but different particle habit. Both the size and shape of the lactose particles have a significant effect on the flow properties. One of the simplest measurements to assess powder flow is through determination of the Carr index or Hausner ratio, which are reliant on the tapped and bulk density. In 1970s, Riley and Mann first demonstrated the influence of particle shape on bulk density and on angles of repose [67]. As the shape of the particle moved from spherical to nonspherical, the Hausner ratio increased, which is indicative of flow characteristics becoming poorer. From a solid dosage form manufacturing standpoint, powder properties such as flow behavior and cohesion are critical attributes as the movement of bulk powder is necessary and occurs during blending, granulation, and tableting. Poor powder properties can ultimately lead to inefficient and costly processing.

An approach to overcome poor flow is through the formation of equant particles such as spherical crystals or agglomerates. Jitkar et al. [68] recently improved the flow behavior and compression of a nonsteroidal anti-inflammatory drug, etodolac, via generation of spherical agglomerates with a combination of polymers. Etodolac by itself crystallized as plates that lead to poor flow and have shown capping tendency (i.e. when the top (or cap) of the tablet fractures or splits from the body of the tablet). In contrast, the spherical agglomerates with the polymers exhibit plastic tendencies with improved hardness, which in turn overcome the capping issues. Approaches such as spherical crystallization or agglomeration are excellent morphological crystal engineering techniques to improve the micromeritic properties (e.g. compactability, flowability, and packability) of crystalline drugs. This has recently become a widely investigated subject especially since it can be carried out in a continuous manner [69, 70].

With tablet dosage forms, habit modification can be a lever to pull in terms of enhancing the tableting behavior and performance of crystalline solids and overcoming tablet sticking issues. In the former, this has been demonstrated for ibuprofen [71] acetaminophen [71], and erythromycin A dihydrate [72] by simply altering the particle morphology. In the latter, tablet picking and sticking can pose significant challenges especially when the material attached to the punch face or to the tablet press punch, respectively. Recently, Waknis and coworkers examined the sticking behavior of two distinct crystal morphology of mefenamic

acid by atomic force microscopy [73]. It was revealed that needle-shaped particles have higher sticking tendency to metal surfaces than platelike crystal irrespective of the particle size, and the higher sticking propensity is associated with greater surface exposure of the polar functional groups.

The crystal shape can also impact other type of dosage forms. For instance, in parenteral suspensions different particle habits can yield dissimilar suspension stability and syringeability [74]. Tiwary and Panpalia showed that the physical stability of trimethoprim suspension in terms of redispersability and sedimentation volume varies with crystal habit [75]. Anisometric crystals exhibited the best physical stability. In inhalation dosage form, needlelike particles with high aspect ratios are preferred as they have been demonstrated to have greater selectivity of airway deposition (compared with spherical particles) and provide better delivery performance [76]. Another important factor to consider, especially for dry powder inhalation (DPI), is the cohesion–adhesion behavior of the API and carrier excipient. In a way, this characteristic can vary with crystal habit and the presence of certain crystallographic facets, which is consistent with the nature of molecular crystals.

From a drug substance manufacturing perspective, the particle habit can also influence downstream operations such as filtration, milling, drying, storage, and handling. For instance, Chikhalia et al. [77] revealed that milling-induced disorder is more prone to occur in platelike crystal of β-succinic acid than particles that are needle shaped. Beck et al. [78] examined the influence of particle shape and size on pressure filtration on L-glutamic acid and an aromatic amine. It was observed that spherulites in general have higher cake resistance than needles and polyhedra in these two cases. Additionally, different crystal facets not only differ in surface energy but also generally possess different surface chemistry, which in turn can lead to dissimilar chemical stability and moisture sorption behavior such as the case of form 1 of aspirin [55]. Lastly, particle breakage can occur during drying especially when an agitated filter dryer is employed. This is potentially important as particle size and shape can vary during the drying process as a result of the crystal morphology having different fracturability. For instance, needlelike crystals are potentially more susceptible to fracture more readily than equant particles.

9.3.2 Particle Size

Particle size and size distribution are critical factors that must be considered during the development of oral solid dosage forms. They can knowingly impact characteristics of the drug product such as content uniformity and dissolution rate and also affect downstream processing especially as it relates to powder cohesion, flowability, and sticking. It is widely accepted that fine powder, with higher surface area to mass ratios, tend to stick, whereas larger particles flow more easily. The general rule is that particles larger than 250 μm are usually free flowing and particles below 100 μm become cohesive. As the particle size decrease further, below 10 μm, the powders are very cohesive and resistant to flow as the interparticulate cohesive forces arising mainly from van der Waals attraction become more dominant and the gravitational forces are less influential [79]. A common

tactic to improve flow is to add larger particles such as flow-aid additives to a fine powder.

Controlling the particle size of a material is crucial when considering that for oral administration of poorly soluble drugs, the size is directly linked to the dissolution rate and solubility. According to the Noyes–Whitney equation, the rate of dissolution of a solute is described by

$$\frac{dm}{dt} = \left(\frac{D}{H}\right) S(C_s - C)$$

where dm/dt is the dissolution rate of the solute, m corresponds to the mass of the dissolved material, t is the time, D is the diffusion coefficient, H is the thickness of the concentration gradient, S is the surface area of the particle, C_s is the equilibrium solubility, and C is the concentration of the solute in solution. The rate of dissolution is proportional to the specific surface area of the particle, which ultimately is directly related to the particle size. A reduction in the particle size will result in an increase in the surface area, thereby increasing the dissolution rate.

Additionally, the equilibrium solubility of a particular physical form increases with diminishing particle size. The effect of particle size on the thermodynamic equilibrium solubility is best described by the Gibbs–Thomson or Ostwald–Freundlich relationship. It is only when the particle size is in the submicron range where the effect of particle size on the solubility can be realized as a consequence of the increase in the surface area to volume ratio. Ultimately, this affects the absorption and bioavailability. This is demonstrated in the case of the water-insoluble drug, danzol, where bioavailability can be improved by reducing the danzol particle size to less than 200 nm [80]. The formation of nanocrystals and nanosuspensions is a common solubilization strategy for enhancing the oral bioavailability of poorly water-soluble compounds from a physical modification standpoint.

Another important aspect of particle size control relates to the content uniformity in the solid dosage form. Tablets and capsules are generally manufactured in an environment where the drug substance and excipients will be blended, granulated, and tableted (or filled for capsule dosage forms). Depending on the powder flow, cohesivity and sticking properties of the drug substance arising from differences in the size and shape of the particles, there is a potential for segregation within the powder blend that will likely impact the tablet compression and encapsulation processes. Ultimately, this can give rise to non-uniform distribution of particles in the dosage form or inconsistent content uniformity. With low dose compounds or high potent drug substance, this can be a problem. Through careful selection of the particle size distribution, content uniformity can be achieved to yield an acceptable drug product.

There are many theoretical approaches that predict content uniformity from the particle size distribution data. One of the early work involved determining the content uniformity by assuming a Poisson distribution for the particle size distribution for particles in the tablet [81]. Later, Yalkowsky and Bolton developed an analytical expression for the content uniformity involving the mean particle size

and relative standard deviation [82]. More recently, Rohrs et al. [83] modified the Yalkowsky–Bolton model and provided a reliable method based on mean particle size, size distribution width, and target dose to conform content uniformity criteria of the USP. Generally, smaller particles affect content uniformity to a much a lesser extent than larger ones. A small particle size and narrow size distribution tend to favor better content uniformity.

Particle size can also impact tableting properties such as tablet structure and tablet strength. Sun and Grant showed that for L-lysine monohydrochloride, powder with smaller particle size resulted in tablets with greater tensile strength [84]. Here, different size fractions obtained from sieving were compacted. Crystals with larger particle size packed more efficient and yielded lower porosities at low compaction pressures; however, differences in porosity between small and large particles diminished with increasing pressure. Two decades earlier, Mckenna and McCafferty examined the effect of particle size on tablet tensile strength for spray dried lactose and showed that stronger compacts occurred with smaller particle size [85].

In drug substance manufacturing, the particle size is typically controlled by crystallization or milling, as described in Chapter 11. The former involves formation of solids from solution via crystallization or precipitation. Particle size control through crystallization has been extensively discussed by Beckmann [86] and Braatz et al. [87]. The latter is a top-down approach where particle size is reduced via dry milling or wet milling. Wet milling with high shear mixers typically generates particles in the 20–50 μm range. For smaller particles, dry milling would be needed. The two most common dry milling technologies are pin or jet milling. Materials derived from both technologies are sometimes accompanied with some levels of structural disorder that in turn may affect the chemical stability and/or performance of the material [88]. To obtain submicron size particles, advanced particle size reduction technologies will likely be involved including bead milling, cavitation milling, or high-pressure homogenization. The latter two involve particle breakage due to cavitation forces after pumping a suspension through a narrow gap at high velocity. Depending on the physical properties requirements, there are plenty of particle engineering technology options to consider.

9.4 Summary

Solid-state properties have a profound impact on the preformulation and formulation development of a drug candidate. The resulting physical forms can drastically alter the quality and performance of drug products including stability, solubility, and bioavailability. The crystal morphology and particle size of a given physical form may affect the processability of the drug product and add significant challenges to formulation development. The early evaluation and optimization of solid-state properties has profound strategic implications for clinical development.

List of Abbreviations

API	active pharmaceutical ingredient
ASD	amorphous solid dispersion
BCS	Biopharmaceutics Classification System
CSD	Cambridge Structural Database
CTA	clinical trial application
EU	European Union
FDA	Food and Drug Administration
HME	Hot Melt Extrusion
IND	investigational new drug
NDA	new drug application
NME	New Molecular Entity

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10

Salt and Cocrystal Screening

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10.1 Introduction

An active pharmaceutical ingredient (API) can exist as different solid forms, as discussed in Chapter 9. Two common forms are salts and cocrystals. Salt formation involves acid–base chemistry and proton transfer between API and a counterion. It is a common strategy to address solubility and bioavailability issues for ionizable API and can be used to change other properties such as melting point, purity, stability, release rate, and hygroscopicity. Crystalline salts can exhibit multiple solid forms including anhydrous, hydrated, and solvated forms, as shown in Figure 10.1. For non-ionizable API or compounds with pK_a values that offer limited possibility of salt formation, cocrystal formation is an alternative means of improving API properties. This involves bonding motifs such as hydrogen bonding instead of proton transfer and thus does not require proton transfer. A third option to improve API properties is a salt cocrystal. This moiety contains both proton transfer and hydrogen bonding between the components. Salt cocrystals are fundamentally more complicated systems but offer another alternative for difficult API. Developing salts or cocrystals are specifically of interest when the parent API cannot satisfy the requirements of the proposed development plan.

While free acids, free bases, and salts have been in marketed drug products for decades, cocrystals are relatively new to the pharmaceutical industry. However, there are now several examples of cocrystals being developed in both early- and late-stage projects. For example, a tablet containing a cocrystal of TAK-020 (Takeda) is currently in phase I clinical trials (https://clinicaltrials.gov/show/NCT02723201). Also, a pharmaceutical cocrystal currently in phase III clinical development is the 1 : 1 ertugliflozin:pyroglutamic acid cocrystal, which was developed through a Pfizer/Merck collaboration [1–3]. In early 2015, Novartis filed Entresto® for European approval to treat chronic heart failure (https://www.novartis.com/news/media-releases/novartis-new-heartfailure-medicine-lcz696-now-called-entrestotm-approved-fda). It is a fixed-dose, combination product developed as

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

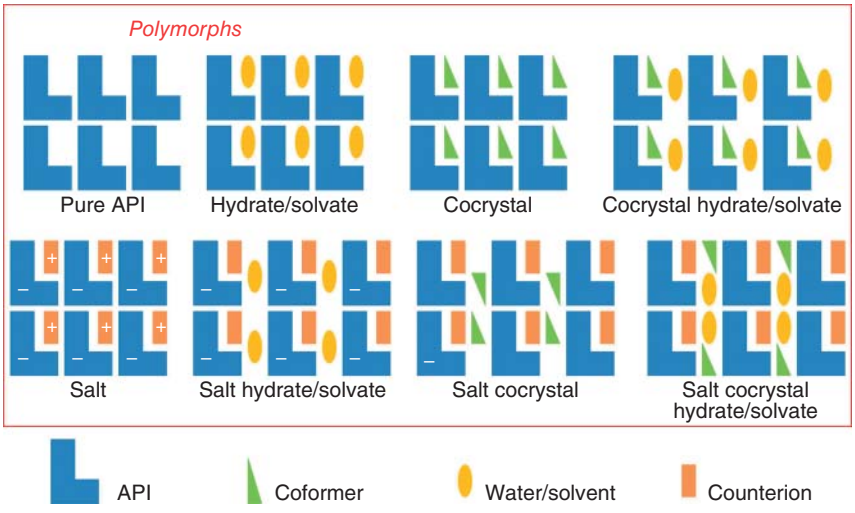


Figure 10.1 Schematic of different crystalline forms. The red box indicates that polymorphs are possible for all the forms listed.

an oral tablet containing a sacubitril and valsartan trisodium hemipentahydrate cocrystal (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR__Public_assessment_report/human/004062/WC500197538.pdf). Recently, an ipragliflozin L-proline cocrystal (Suglat®) was approved in Japan for treatment of type II diabetes [4].

Based on the significant role salts and cocrystals play in the development of pharmaceutical compounds, it is important to understand how to include these materials in both API and formulation development. This chapter covers a number of development areas for these APIs including screening, selection, crystallization, scale-up, and formulation. Case studies are presented to display how salts and cocrystals can be used to improve API and formulation properties throughout development.

10.2 Screening

A salt/cocrystal needs be discovered prior to any detailed evaluation and subsequent development. The scheme shown in Figure 10.2 is an overview of possible screens that can be performed during early- and late-stage development [5]. During the early development stage, the purpose is to find a suitable form that has desirable physical and chemical properties, e.g. crystallinity, hygroscopicity, solubility, and bioavailability, to advance the API in the shortest possible timeframe [6]. Consequently a *fit-for-purpose* study (usually up to ~100 experiments with 3–5 g of material) will be preferred to maximize the design space. Smaller screens focused on finding the most stable, least soluble form (about 25 experiments with 1 g of material) can also be performed at this stage if resources are limited. If the

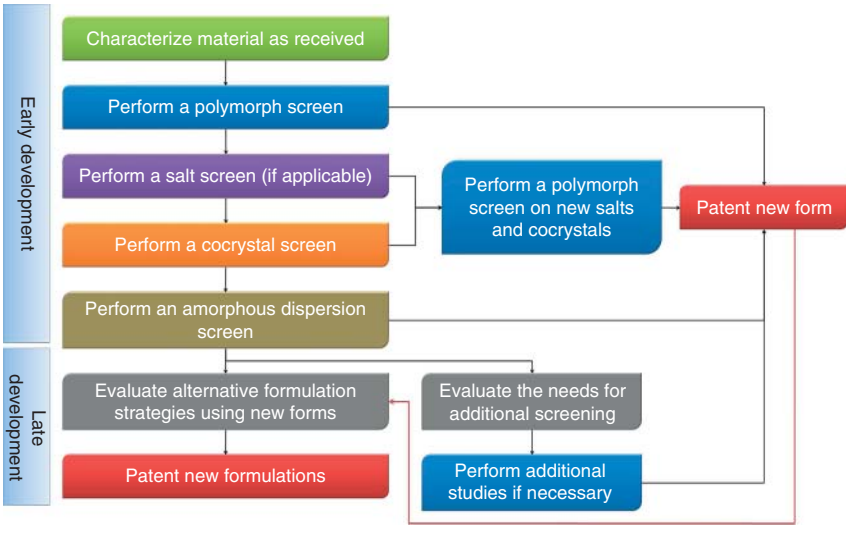


Figure 10.2 Screening strategies in early and late development.

material is very restricted (<500 mg) at discovery stage, then a potential solution is to utilize a high-throughput screening (HTS) platform that can typically work with <1–5 mg of the material. For late phase development, the screening is typically driven by the processes to cover formulation (e.g. whether formulation techniques such as micronization, blending, and granulation will impact the crystal form), crystallization development (any form change in large-scale manufacturing and storage), and intellectual property (IP) protection (to extend the lifetime of the API and maximize the product value), which can encompass >300–400 experiments via both manual and HTS. When focused on crystallization development, the screening experiments are designed to maximize process-related conditions (e.g. temperature, solvent compositions, and crystallization method variation) to investigate robustness and risks, such as polymorph formation, disproportionation of the salt and form conversion of cocrystal. For IP protection purposes, the focus will be on pharmaceutically relevant counterions and any additional polymorphs or solid forms of the salt or cocrystal.

10.2.1 Counterions and Coformers

Counterions used for salts are acids or bases capable of donating or accepting a proton. Coformers used to produce cocrystals can be the same compounds as used for salts, but the proton is not exchanged, and hydrogen bonding or pi-stacking are the typical bonding motifs. Coformers can also be neutral molecules with no proton exchange potential. Counterions/coformers can come from a variety of sources, such as Generally Regarded as Safe (GRAS) (http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/) and Everything Added to Food in the United States (EAFUS) (http://www.accessdata.fda.gov/

scripts/fcn/fcnNavigation.cfm?rpt=eafusListing) lists. Commonly used counterions/coformers and their structures are given in Table 10.1.

A number of criteria have been adopted to select suitable counterions/coformers for salt/cocrystal screening purposes. For instance, the ΔpK_a (pK_a of base − pK_a of acid) guideline has been generally used as a means of selecting formers [8]. If $\Delta pK_a < 0$, a cocrystal will almost always form [8], if $\Delta pK_a > 3$ salt formation is most likely; [8] and if ΔpK_a is between 0 and 3, the product can either be cocrystal or have partial proton transfer. To further improve the success in obtaining cocrystals, there has been a focus on common bonding motifs, which result in a supramolecule within the cocrystal matrix via the formation of "synthons," [9] as shown in Figure 10.3. The synthons can be either homosynthons (the same functional group, such as a carboxylic acid dimer) or heterosynthons (such as sulfonamide–carboxamide in a celecoxib cocrystal [11]). It has been reported that cocrystal formation can be predicted based on structure–activity relationships. A strong correlation was found on the shape and polarity complementarity between pairs of cocrystal-forming molecules [12]. These relationships can often be used in conjunction with computational methods to increase the success rate of finding a new cocrystal. Solubility parameters have also been used to select coformers for cocrystal screening [13]. Other factors such as drug loading (high drug loading warranting a smaller counterion/conformer) and dosage form (acceptable counterions/coformers for various administration routes) [14] should also be included when designing screening experiments.

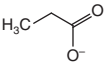
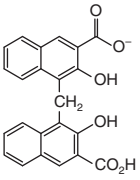
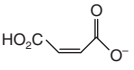
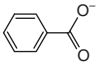
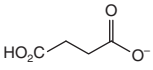
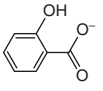
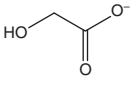
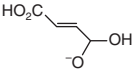
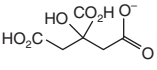
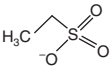
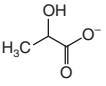
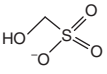
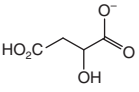
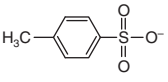
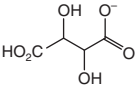
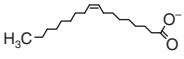
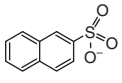
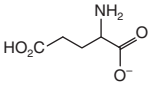
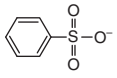
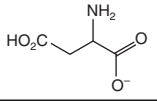
Toxicology considerations of the formers [15, 16] also play an important role in salt/cocrystal selection [15]. A list of maximum daily dose (MDD) for common counterions/coformers is summarized in Table 10.2. Acute versus chronic dosing for the expected dosage forms should also be considered during counterion selection. Short-term effects of counterions are commonly known, but long-term effects may need to be included in the selection process for certain dosage forms [17]. The case of meloxicam aspirin cocrystal has demonstrated a combined approach of computational studies (solid form informatics [18]), toxicology/regulatory considerations, and synthons present in the structure (crystal engineering), which resulted in enhanced solubility and pharmacokinetics (PK) for the cocrystal [19]. To further assist counterion selection, a safety classification system for counterions has been introduced and widely used in pharmaceutical compounds: [20]

Class I. Counterions/coformers can be used without restriction because they form physiologically ubiquitous ions or they occur as intermediate metabolites in biochemical pathways; these compounds are usually preferred for drug development.

Class II. Counterions/coformers are not naturally occurring, but show low toxicity and good tolerability.

Class III. Counterions/coformers can be used in select cases; some have their own pharmacologic activity. It is recommended to review the latest safety records and literature when using these compounds to ensure that it is safe for the intended dosage form.

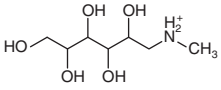
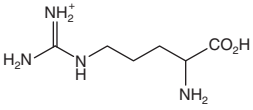
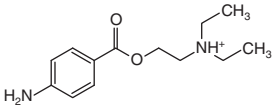
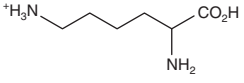
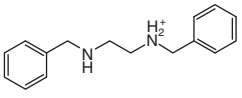
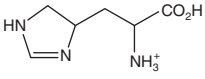
Table 10.1 Partial list of pharmaceutically acceptable counterions; coformers would be neutral counterparts.

Counterion	Structure	Counterion	Structure	Counterion	Structure
Chloride	Cl^-	Acetate	$CH_3CO_2^-$		
Bromide	Br^-	Propionate		Pamoate	
Sulfate	SO_4^- (HSO_4^{2-})	Maleate			
Nitrate	NO_3^-	Benzoate		Succinate	
Phosphate	$H_2PO_4^-$ (HPO_4^{2-})	Salicylate		Glycolate	
Bicarbonate	HCO_3^-	Fumarate		Hexanoate	$CH_3(CH_2)_4CO_2^-$
Mesylate	$CH_3SO_3^-$	Citrate		Octanoate	$CH_3(CH_2)_6CO_2^-$
Esylate		Lactate		Decanoate	$CH_3(CH_2)_8CO_2^-$
Isethionate		Malate		Stearate	$CH_3(CH_2)_{16}CO_2^-$
Tosylate		Tartrate		Oleate	
Napsylate				Aspartate	
Besylate				Glutamate	

Counterion	Structure	Counterion	Structure
Sodium	Na^+	Triethylamine	$(CH_2CH_2)_3NH^+$
Potassium	K^+	Ethanolamine	$HOCH_2CH_2NH_3^+$
Calcium	Ca^{2+}	Triethanolamine	$(HOCH_2CH_2)_3NH^+$

(*continued*)

Table 10.1 (Continued)

Counterion	Structure	Counterion	Structure
Magnesium	Mg^{2+}	Ethylenediamine	$H_2NCH_2CH_2NH_3^+$
Lithium	Li^+	Choline	$HOCH_2CH_2N(CH_3)_3^+$
Zinc	Zn^{2+}	Meglumine	
Aluminium	Al^{3+}		
Arginine		Procaine	
Lysine		Benzathine	
Histidine			

For example, the counterion is acetate and the coformer is acetic acid [7].

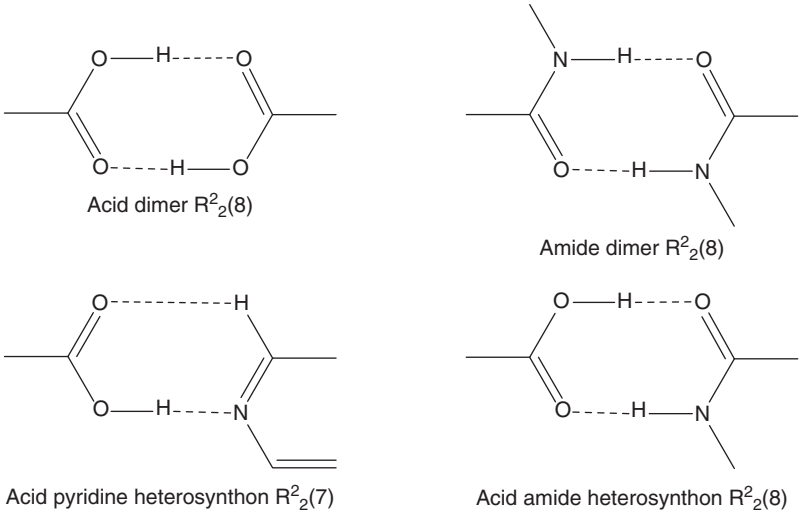


Figure 10.3 Examples of homo- and heterosynthons. *Source*: Thakuria et al. 2013 [10]. Reproduced with permission of Elsevier.

Table 10.2 An example of maximum daily dose identified for counterions.

Counterions	Oral administration		Intravenous administration	
	Maximum daily dose (mg)	API	Maximum daily dose (mg)	API
Acetate	50	Flecainide acetate	<10	—
Benzoate	14	Rizatriptan benzoate	—	—
Besylate	160	Mesoridazine besylate	9	Atracurium dibesylate
Bromide	300	Pyridostigmine bromide	<10	—
Camsylate	—	—	1.8	Trimethaphan camsylate
Carbonate	1500	Lithium carbonate	—	—
Citrate	5250	Piperazine citrate	1400	Caffeine dihydrogen citrate
Edisylate	20	Prochlorperazine edisylate	—	—
Estolate	1450	Erythromycin estolate	—	—
Fumarate	120	Quetiapine fumarate	0.1	Ibutilide fumarate
Gluceptate	—	—	1230	Erythromycin gluceptate
Gluconate	730	Quinidine gluconate	300	Quinidine gluconate
Glucuronate	—	—	45	Trimetrexate glucuronate
Hippurate	1120	Methenamine hippurate	—	—
Iodide	99	Potassium iodide	—	—
Isethionate	—	—	140	Hydroxystilbamidine diisethionate
Lactate	—	—	330	Milrinone lactate
Lactobionate	—	—	1900	Erythromycin lactobionate
Malate	60	Dilitiazem malate	—	—
Maleate	250	Acetophenazine hydrogen maleate	12	Chlorpheniramine hydrogen maleate
Mesylate	420	Nelfinavir mesylate	51	Atrofloxacin-mesylate
Methylsulfate	200	Diphemanil methylsulfate	—	—
Napsylate	170	Propoxyphene napsylate	—	—
Nitrate	—	—	320	Gallium nitrate
Oxalate	5	Escitalopram oxalate	—	—

(*continued*)

Table 10.2 (Continued)

Counterions	Oral administration		Intravenous administration	
Pamoate	325	Hydroxyzine pamoate	—	—
Phosphate	380	Chloroquine dihydrogen phosphate	620	Clindamycin dihydrogenphosphate
Stearate	1500	Erythromycin stearate	—	—
Succinate	90	Loxapine hemisuccinate	—	—
Sulfate	380	Indinavir sulfate	290	Capreomycin sulfate
Tartrate	3900	Cystermine hydrogen tartrate	90	Metaraminol hydrogen tartrate
Tosylate	890	Lapatinib ditosylate	100	Bretylium tosylate

Source: Saal and Becker 2013 [15]. Reproduced with pemission of Elsevier.

10.2.2 Manual Versus Automated Screening

Many variables can impact crystal nucleation and growth including solvent composition, temperature, heating or cooling rate, and addition rate, as summarized in Table 10.3. Walter McCrone stated in 1965 that "the number of forms of a given molecule is proportional to the time, money and experiments spent on that compound." [22] Constraints on API availability, a scientist's expertise in crystallization and data analysis, and limited time for experimentation often result in only a limited number of variables being explored. This can and has already led to unexpected and sometimes undesirable outcomes later on in development (e.g. ritonavir) [23]. For this reason, comprehensive screening employing a variety of methods, via both manual and robotic handling, during the early stage of development is recommended to expose any associated risks. Lately, HTS has been extensively used to provide screening diversity by employing a large range of conditions (multiple 96 well plates with a variety of solvents, temperatures, and stirring functions), using small amounts of material (usually <1 mg per study as compared with >5–10 mg per experiment for the manual method), and ease of handling systems with multiple components (e.g. solvent) [5]. However HTS requires extensive computational programming and processing, which could be very time consuming. Further due to the relatively restricted operation conditions, HTS can only handle cooling, heating, stirring, and evaporation conditions, and the temperature controlling module is often limited. Consequently, experimental design with complex solvent composition, limited temperature conditions, and a large number of experiments (typically >300) are recommended for HTS.

A schematic diagram (Figure 10.4) outlines the stages of HTS, including design of experiment (DOE), execution of experiments via robotics, and data analysis using informatics [21]. The use of HTS to comprehensively identify salt forms (including polymorphs, hydrates, and solvates) was initiated about a decade ago,

Table 10.3 Crystallization composition and processing variables.

Composition type		Process variables (applicable to all types of screens)				
Polymorph/solvates	Salts/cocrystals	Thermal	Antisolvent	Evaporation	Slurry conversion	Other variables
Solvent (combinations)	Counterion type	Heating rate	Antisolvent type	Rate of evaporation	Solvent type	Mixing rate
Degree of supersaturation	Acid/base ratio	Cooling rate	Rate of antisolvent addition	Evaporation time	Incubation temperature	Impeller design
Additive type	Solvent (combinations)	Maximum temperature	Temperature of antisolvent addition	Carrier gas	Incubation time	Crystallization vessel design (inc. capillaries)
Additive concentration	Degree of supersaturation	Incubation temperature (s)	Time of antisolvent addition	Surface–volume ratio	Thermal cycling and gradients	
	Additive type and concentration pH Ionic strength	Incubation time				

Source: Morissette et al. 2004 [21. Reproduced with permission of Elsevier.

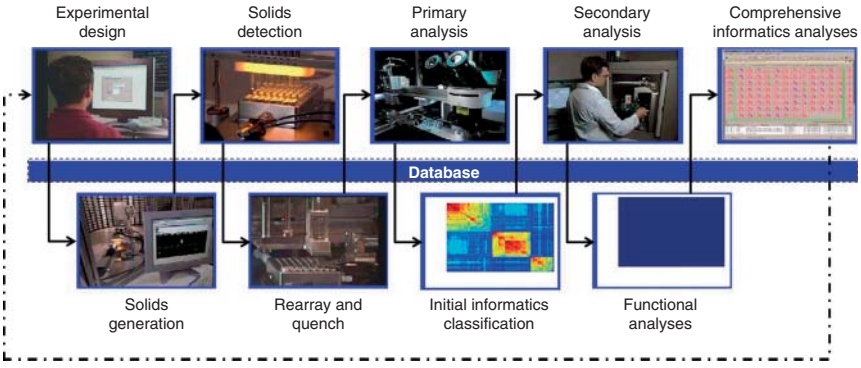


Figure 10.4 Schematic diagram of high-throughput screening. *Source*: Morissette et al. 2004 [21]. Reproduced with permission of Elsevier.

and its impact was demonstrated on a number of pharmaceutically relevant molecules, such as sulfathiazole [24], caffeine [25], and naproxen [21, 25, 26]. Cocrystal formation via HTS has also been reported on *cis*-itraconazole and 1,4-dicarboxylic acids, including succinic acid, fumaric acid, L-malic acid, and tartaric acid (DL-/D-/L-) [21, 27]. Successful cocrystal formation was attributed to the geometric fit of the triazole in itraconazole and the carboxylic acid in the crystal structures [27].

10.2.3 Computational Approaches

Computational approaches are often regarded as a "prescreening" method [28] to identify a number of highly possible "hits" that can then be confirmed by experimental studies [29]. A number of methods used to predict the existence and structure of cocrystals include full structure prediction and surface interactions to predict the H-bond propensity based on Cambridge Structural Database (CSD) statistics [30, 31]. Price and coworkers [32] have used calculated lattice energies to look at the stability of cocrystals compared with that of the parent API forms. The calculations included intermolecular forces, repulsion–dispersion parameters, flexible degrees of freedom, and hydrogen bond geometries. Another approach employed hydrogen bond propensity calculations to investigate the cocrystallization and polymorphic behavior of theophylline and amides. Limitations when using such calculations for predicting cocrystallization are also discussed [33].

Many computational approaches can include up to 2000 coformers, covering both the GRAS and EAFUS lists; other variables that can be included are different conformation of a molecule and stoichiometric possibilities. Bladgen et al. have discussed the details of computational approaches and suggested a cocrystal screening protocol [28]. In addition to prediction, computational approaches can also provide insight into cocrystal formation. Issa et al. have investigated the cocrystallization of succinic acid and 4-aminobenzoic acid with small organic

molecules. The study showed that the computed crystal energy landscapes can be used to predict the stability of cocrystals and to rationalize the observation in terms of hydrogen bonding and close packing [34]. Computational studies can predict the cocrystal structures as well as estimate related properties. Lange et al. have computationally studied the stability and solubility of pharmaceutical cocrystals in aqueous solution, influenced by pH-dependent dissociation and salt formation processes, and demonstrated good agreement with experimental data [35]. Compared with cocrystals, salts are predicted to have additional challenges due to the complexity of long range ionic interactions, charge transfer, and strong polarization effects. Despite the above challenges, current techniques can still satisfy the need for computationally modeling salts and their polymorphs. For instance, two additional anhydrous polymorphs of terazosin hydrochloride (Hytrin®) have been predicted, based on the known structures of the experimental forms [36]. Another example was 1,8-naphthyridinium fumarate, which has been reported in the fifth blind test from Price group, in order to assess the capability of computational prediction/modeling on crystal structures including polymorphs, salts, and cocrystals (CSP2010) [37].

10.2.4 Salt and Cocrystal Screening Strategies

To perform salt/cocrystal screening in an efficient and systematic manner, a workflow that captures all the essential steps and helps organize the activities is generally employed. A representative schematic diagram [38] is shown in

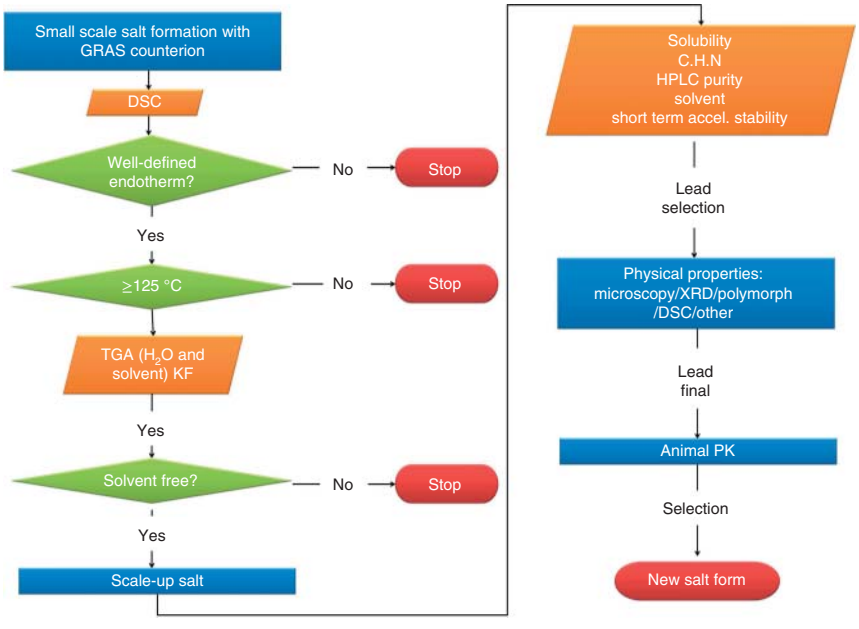


Figure 10.5 Example of a salt screening work flow. *Source*: Gross et al. 2007 [38]. Reproduced with permission of American Chemical Society.

Figure 10.5, which includes both screening and evaluation. Such a workflow is constructed based on several key stages:

1) *Counterion Selection*. Only GRAS compounds and those used in previously Food and Drug Administration (FDA)-approved marketed drugs are allowed in the study. For salt screening, the acids should be sufficient in strength to protonate the drug and predicted by the ΔpK_a guidance previously described. For cocrystals, the strategy is to target coformers with adequate hydrogen bonding acceptors and donors that allow the formation of certain bonding motifs.
2) *Study Design*. Generally a preliminary screen is recommended with a number of salt/cocrystal formers with pH varied across a range of values to pre-evaluate the potential of salt/cocrystal formation and expose any risk (e.g. degradation) with respect to pH conditions. Usually a number of solvents/mixtures with different properties (e.g. polarity, solubility) will also be chosen to investigate any solvent effect. A focused screening can then be performed based on the previous results where counterions/conformers with similar properties can be explored.
3) *Characterization of the Resulting Forms*. A comprehensive data set for any new salt/cocrystal form is needed in order to identify the nature of the form (e.g. stoichiometry, anhydrate, hydrate, solvate). Common techniques include X-ray power diffraction (XRPD), thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), proton or carbon nuclear magnetic resonance (NMR) spectroscopy (or ion chromatography (IC)), and/or high performance liquid chromatography (HPLC). Karl Fisher (KF) titration can be used to confirm the water content for hydrate forms and water sorption/desorption can be used to investigate form changes with relative humidity (RH). Variable-temperature/RH XRPD is an efficient means to characterize systems with complicated form changes, such as hydrates that can reabsorb water upon exposure to ambient conditions.

The order of the above activities or actions could vary depending on the goals of the screening, and steps 2 or 3 may be repeated to maximize the findings in a certain design space. Once the salt/cocrystal hits are obtained and characterized, additional evaluation will follow to select the candidate with desirable properties, such as solubility, hygroscopicity, stability, and bioavailability. The development of APIs that follow this type of strategy include fluoxetine hydrochloride cocrystal [39] and NBI-75043 [38].

Screening experiments for salts and cocrystals need to be performed differently due to unique formation pathways in solution. For salts, molar stoichiometries for the components are used based on the number of ionizable functional groups in the API or counterion. For solution experiments, salt formation is a two-step process. The first step is salt formation in solution based on the pK_a *in a particular solvent*, where a ΔpK_a value greater than 3 is targeted for salt formation [40]. The second step is crystallization of the salt out of solution where varied conditions (see Table 10.3) can result in different crystalline forms of a salt and even unique salt stoichiometries [41]. Ternary phase diagrams (TPDs) can help determine key parameters necessary to produce and crystallize the desired salt. TPDs will also aid scale-up and tech transfer operations [40]. Nonsolvent methods to

produce salts include grinding or liquid-assisted grinding [42]. Crystals obtained from these experiments can then be used as seeds to develop a solution crystallization process.

Cocrystal formation in solution requires supersaturation of at least one of the components. Therefore, stoichiometric ratios of API and coformer are not ideal for these experiments [43, 44]. A variety of methods have been reported for screening cocrystals, such as grinding (neat and liquid-assisted grinding) [45, 46], supercritical fluid technologies [47], solvent-mediated transformation [48], ultrasound-assisted crystallization [49, 50], spray-drying [51], antisolvent addition [52], thermal, [53, 54]and reaction crystallization [43, 55]. While these examples have resulted in successful cocrystal identification, increasing focus has been given to understanding the stable region for the cocrystal scale-up and isolation. In this case, constructing a TPD is critical to determining optimal processing conditions [56].

To distinguish between a salt or cocrystal, several different techniques have been employed, including single crystal structural determination [8] and solid-state nuclear magnetic resonance (ssNMR) [57], infrared (IR) [58], and Raman spectroscopies [59]. Single crystal structure determination is often regarded as a "direct method" that provides an insight into the molecular arrangement, chirality, orientation, and bonding (such as H bonding or complete ionization/proton transfer). Accurate placement of the hydrogen atoms in a routine single crystal structure is not obtained due to the poor scattering of the protons; therefore the length of donor–acceptor bonds is routinely used to determine if proton transfer has occurred. For instance, to distinguish the carboxylic acid group from carboxylate, the C—O bond length is often measured; [60] due to bond resonance, the C—O bonds are often similar in length with a value between C—O and C=O, making salt/cocrystal determination impossible [61]. Since the single crystal structure determination is not always definitive, it is suggested that other characterization data be collected to support the determination of salt or cocrystal, such as ssNMR, Raman, and IR spectroscopies that can exhibit distinct shifts due to hydrogen bonding in a cocrystal or proton transfer in a salt [62, 63].

10.2.5 Polymorph Screen of Salts/Cocrystals

When a crystalline salt or cocrystal with desirable properties is identified from the screening and characterization studies, it is strongly recommended to perform polymorph screening of this lead candidate to:

1) Explore any potential polymorph (anhydrate, hydrate, and/or solvate).
2) Investigate any risk of disproportionation under selected conditions (solvent composition, temperature, moisture, etc.).
3) Identify a robust form that is suitable for further drug development.

Different levels of polymorph screening can be performed on salts and cocrystals. A small, stable form screening [64] can be performed on the top 2–3 salts/cocrystals that exhibit acceptable properties based on the characterization data or preliminary properties (such as estimated solubility). Information from

the polymorph screen will help to determine which salts/cocrystals may have a propensity toward multiple forms, which can be included in the selection process. Once the lead candidate is selected, a larger polymorph screen can be performed to identify possible forms for development or to help avoid unwanted metastable forms during crystallization or formulation process development [65, 66].

Prior to the experimental design for a polymorph screen, it is essential to collect the fundamental data regarding the lead candidate, including the solubility in various solvents with respect to temperature and stability information (light/oxidation/pH/temperature/humidity). Additional information on the drug development stage, including early/late phase, pharmacokinetic/pharmacodynamic (PK/PD) data, dosage form, processing conditions, formulation design, and patent status (if any), is always helpful to provide insight into the screening work and to maximize the design space with the key variables [67]. Using a combination of methods has demonstrated its advantages in exploring a comprehensive range of conditions and thus enhancing the chance of finding as many polymorphs as possible, as demonstrated in the case of phenazine and mesaconic acid cocrystals [68, 69]. Once multiple forms are found, it is important to identify the most thermodynamically stable form and the relationship between the forms (monotropic vs enantiotropic) [70]. Polymorph screening of salts and cocrystals may result in disproportionation, leading to crystallization of the parent API (free acid or base) or the counterion; therefore, specific characterization of solids obtained from the screen should be performed to determine stoichiometry and solvation state. New crystalline forms of the parent API have been reported during polymorph screens of salts and cocrystals [71].

10.3 Salt/Cocrystal Selection

Once salts or cocrystals have been obtained and characterized, selection of a lead candidate and backup are the next steps. The criteria for selection will be specific to the development plan of the API and some variables that need to be considered when choosing a final form include:

- Solubility
- Dissolution
- Melting point
- Dosage form to be developed
- Route of administration
- Loading in dosage form
- Amount of material available
- Previous experience with counterions

There are various ways to assess crystalline forms. General guidelines for common properties are given in Table 10.4. As discussed previously, the counterion should be a good match with the development plan for the API, such as type of dosage form and acute versus chronic dosing. A crystalline material is usually desired for development due to its superior chemical and physical stability, when

Table 10.4 General guidelines for API properties.

Properties	Target	Impact
Counterion acceptability (for salts and cocrystal formers)	Class (I, preferred), GRAS?, dose (daily intake), MW (salt conversion factor) , toxicity, stoichiometry	Toxicity, acceptability
Crystallinity	Crystalline, high mp (>100 °C)	Storage, drug product (DP) process
Hygroscopicity	Not deliquescent at 60–75%RH	Storage conditions, API process, DP process, stability issues, DP physical/chemical stability
Morphology	No needles, prisms, plates preferred	Dissolution, DP process, API handling, and process
pH of aqueous solution	3–10 for parenterals	Biocompatibility
Polymorphism	Polymorphism evaluation, form control (if needed)	Form control
Solid state stability	Pass acceptance criteria. Minimal degradation	Storage, DP development
Solubility/dissolution rate	0.1–10 mg ml^{-1} ODS >10 mg ml^{-1} for parenterals	Bioavailability, stability, formulation

compared with amorphous materials. The water uptake should be investigated to determine handling conditions and possible hydrate formation [72]. The formation of a crystalline hydrate usually results in lower aqueous-based solubility, which may significantly affect formulation performance. Particle morphology and size can be important parameters for drug product dissolution and formulation processing. If a number of crystalline forms are known for a salt or cocrystal, it needs to be determined if the desired form can be made physically pure using a reasonable large-scale crystallization process. Multiple forms can also impact physical stability, and handling/storage conditions need to be investigated that will maintain the desired crystalline form. Dissociation of the salt or cocrystal under storage conditions or during testing (such as dissolution testing) needs to be assessed [73, 74]. Chemical stability can also be related to the solid form; therefore, degradation for different forms should also be assessed [75].

Solubility and dissolution are usually key parameters for selection. They are closely related, but represent different processes that need to be assessed during formulation development. The solubility is the concentration in solution at equilibrium with a solid phase. Many parent APIs are poorly soluble, and the goal of a salt or cocrystal screening is to increase solubility. In some cases, such as extended release or prolonged action formulations, a lower solubility or slower dissolution rate may be desired for the salt/cocrystal [76]. Dissolution rate is the dynamic process during which the solid dissolves to form a solution. The solubility and dissolution rate for APIs containing ionizable groups will be dependent on pH (Figure 10.6); therefore, pH solubility profiles need to be collected and applied

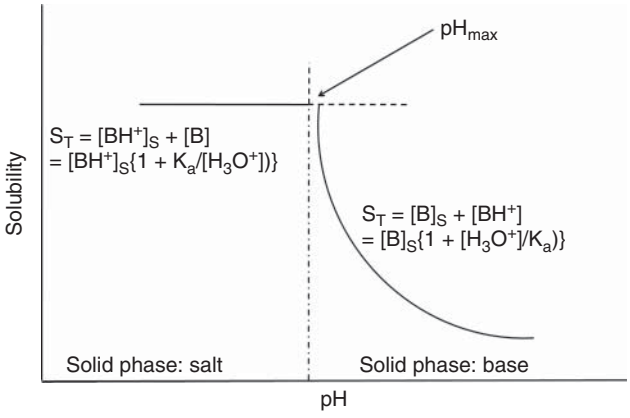


Figure 10.6 Schematic representation of the pH solubility profile of a basic drug indicating that the solubilities may be expressed by two independent curves and that the point where two curves meet is the pH_{max}. S_T is the total solubility; BH^+ and B represent protonated (salt) and free base forms, respectively; the subscript "s" represents the saturation species. *Source*: Adapted from Serajuddin and Pudipeddi 2002 [77].

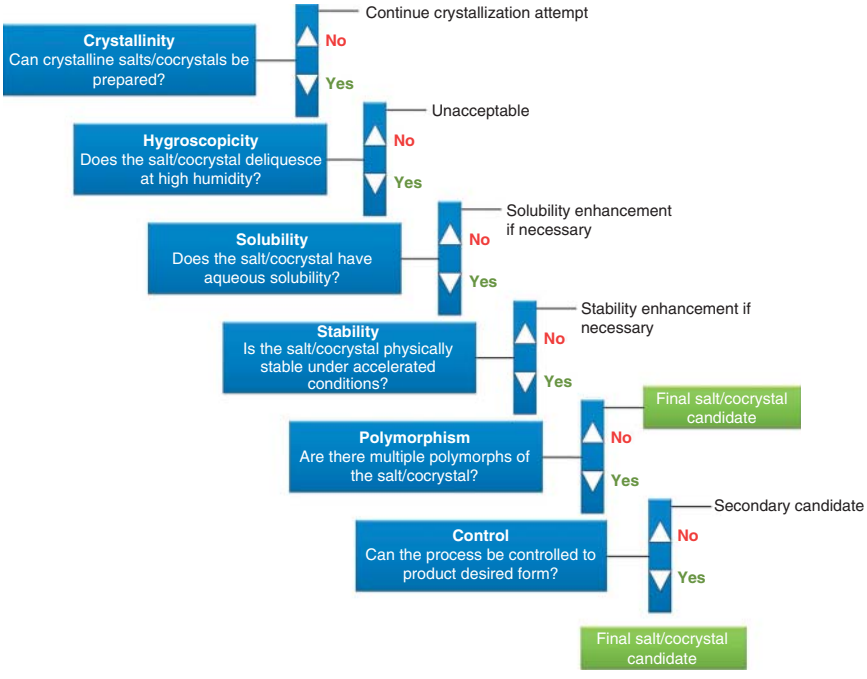


Figure 10.7 Example of a decision tree for salt/cocrystal selection.

to formulation and *in vitro* and *in vivo* studies [78]. There are numerous reports of pH solubility curves [79], such as haloperidol where the curves for methanesulfonate, hydrochloride, and phosphate salts were studied [80]. The pH solubility also needs to be considered for cocrystals, as reported for gabapentin cocrystals and salts [81].

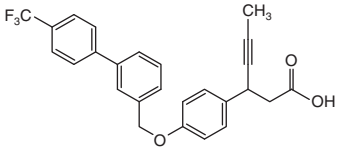
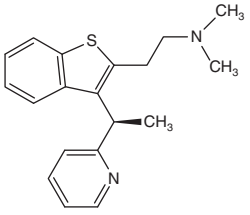
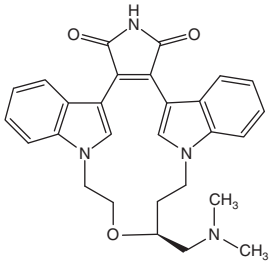
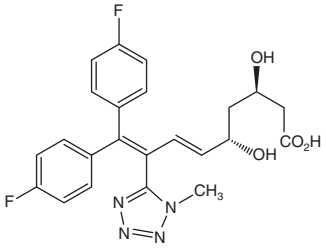
Once relevant data are collected, the properties for each salt/cocrystal need to be assessed in order to select the best form. Two common assessment techniques are flowcharts and form matrices. Flowcharts are used to select the form based on the desired properties for development, and an example is given in Figure 10.7. Every API will have specific requirements and the flowchart needs to be tailored to each project. A form matrix compiles the relevant data in a table, and the acceptable properties are highlighted (Table 10.5); the salt or cocrystal with the best properties will be the lead candidate. A list of reported salt screens and selections are given in Table 10.6.

Table 10.5 Form matrix for salt selection.

Property	L-Lysine salt (N – 1)	Free acid form A	Ca salt
Crystallized directly	Yes	No	No
Crystallized yield	>80%	<40%	>90%
Crystalline	Yes	Yes	Yes
Stoichiometry	1 : 1(elemental, NMR)	N/A	1 : 1(elemental)
Morphology	Thin needles	Thin needles	Thin needles
Neat/solvate/hydrate	Neat	Hydrate (TGA and KF)	Hydrate (TGA and KF)
Single crystal structure	Promising model	Partial structure of slurry form	Partial structure of slurry form
Aqueous solubility at 25 °C	>150 mg ml^{-1}	47 mg ml^{-1}	1.4 mg ml^{-1}
Hygroscopicity at 25 °C	60% RH~1% 90%RH~12%	60% RH~7% 90%RH~8%	60% RH~1.5% 90%RH~2.5%
Physical and chemical stability at 40 °C/75%RH and 50%/ambient RH	Stable up to 4 weeks	Stable up to 4 weeks	Stable up to 4 weeks
[a] Known API crystal form/pattern	NP – 1 (dry and slurry)	P – 1 (dry) P – 2 (slurry)	P – 1 (dry) P – 2 (slurry)

KF Karl Fischer, RH relative humidity, TGA thermal gravimetric analysis.
Properties highlighted in green indicate acceptable properties.
The green box indicates that the L-lysine salt was chosen for further development.
a) N – X indicates that the API crystal form has been identified as the neat form with a known single crystal structure; P – X represents the material whose single crystal structure and possibly the chemical composition are unknown if it is a solvate.
Therefore, P – X symbolized a material that has a unique powder X-ray diffraction pattern.
Source: Yin and Grasso 2008 [82]. Reproduced with permission of Elsevier.

Table 10.6 Examples of salt selections.

Compound	Salts (crystalline)	Salt selected	References
AMG 837 	Free acid, lysine, sodium, ethanolamine, tris(hydroxymethyl) aminomethane (TRIS), choline	Sodium (hemihydrate), good laboratory practice (GLP), good manufacturing practices (GMP) batch	[17]
NBI-75043 	Besylate, fumarate, maleate, tosylate, hydrobromide (HBr)	Besylate, fumarate and HBr (PK studies)	[38]
LY 333531 	Hydrochloride (HCl), sulfate, mesylate, succinate, tartrate, acetate and phosphate	mesylate monohydrate (clinical development)	[83]
BMS 180431 	Sodium, calcium, zinc, magnesium, potassium, lysine, arginine	Arginine	[84, 85]
RPR 111423 (pK_a 4.25)	Free base, hydrochloride, mesylate	Free base	[85]
RPR 127963 (pK_a 4.1)	Free base, hydrochloride, mesylate, citrate, tartrate, sulfate	Mesylate	[85]
RPR 200765 (pK_a 5.3)	Free base, mesylate, camphorsulfonate, hydrochloride, hydrobromide	Mesylate	[85]

10.4 Scale-Up

Crystallization process development is needed after a form is selected. The first step in developing a small-scale (multiple gram) process is to review the solubility data. A general guidance is that the solvent system needs to be process friendly (usually Class 3 [86]) where the parent API and the counterion/coformer have acceptable solubility within a desirable temperature range. These data are then used to investigate other parameters to induce supersaturation and crystallization, such as cooling, antisolvent addition, or solvent evaporation. Seeds of the targeted form, usually <5%, can be employed during the process to optimize nucleation and crystal growth, minimize the risk of undesirable forms, and control the morphology and particle size [87]. A number of appropriate quality attributes may be targeted at this point, including form, purity profile (impurity rejection), acceptable yield (>80%), adequate volume efficiency (10–25 l kg^{-1}), and appropriate particle morphology/size. Undesirable reactions between certain solvents and counterions also need to be considered at this stage such as the reaction of alcohol with methanesulfonic acid to form genotoxic impurities (sulfonate esters with a limit of <10 ppm level) [88].

Prior to defining the operating space, the form stability will need to be explored under the proposed process conditions. As an example, the 2-dimensional phase diagram of caffeine–glutaric acid–acetonitrile in the temperature range of 10–35 °C is plotted in Figure 10.8a [89]. The bold lines are the two eutectic points for caffeine/cocrystal and glutaric acid/cocrystal, respectively, and the equilibrium concentration at each temperature are joined by a dotted line. The stability zone of caffeine–glutaric acid cocrystal is between the two bold

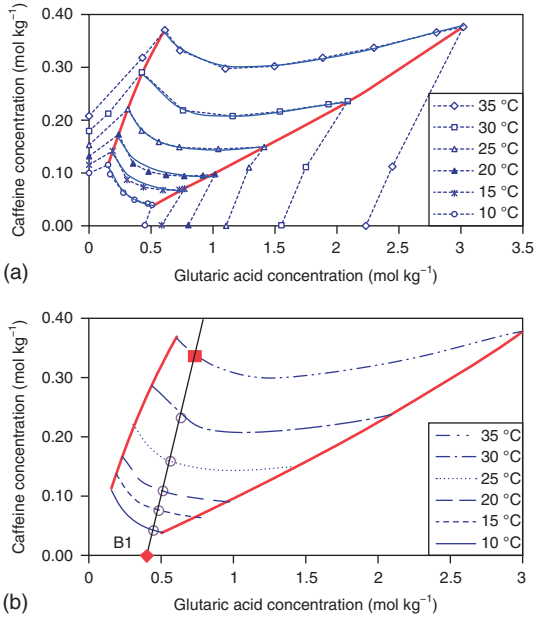


Figure 10.8 (a) Phase diagram of caffeine–glutaric acid–acetonitrile in the temperature range of 10–35 °C; (b) ideal operational region in the phase diagram. *Source*: Adapted from Yu et al. 2010 and 2011 [89, 90].

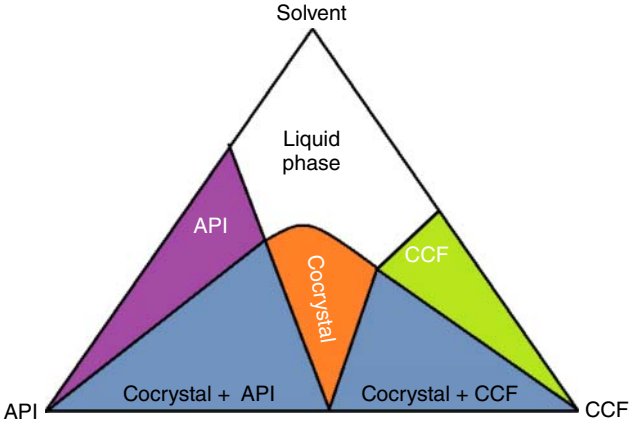


Figure 10.9 Illustration of ternary phase diagram (API, active pharmaceutical ingredient; CCF, cocrystal conformer). *Source*: Aitipamula et al. 2014 [69]. Reproduced with permission of Royal Society of Chemistry.

lines, and ideally the working region should be within this area, as shown in Figure 10.8b. In some cases, a TPD is useful to determine crystallization conditions for a salt or cocrystal, as shown in Figure 10.9 [56, 87, 91], for the ephedrine/pimelic acid/water systems where 1 : 1 and 2 : 1 salt stoichiometries were possible [92].

On large scale (e.g. >10 l reactors), crystallization processes are developed by defining key product attributes including purity, crystal form, morphology, particle size distribution (PSD), and other solid-state properties. To capture and understand these crystallization processes, process analytical technology (PAT), process modeling and optimization, and model regression have assisted the development of crystallization processes. Yu et al. [89, 90, 93] have extended the first principle process modeling to cocrystallization and applied PAT tools to define the design space of seeded cooling crystallization on a bench scale, for instance, in the study of caffeine–glutaric acid cocrystal, where 1 : 1 caffeine and glutaric acid cocrystals have two known polymorphs [94]. Trask et al. [45, 94] observed that Form I showed a tendency for conversion to Form II when exposed to moisture. The needle-like Form I was consistently transformed into a prismatic-like Form II in acetonitrile solutions. Based on these data, polymorphic purity was the target of this process and the important attributes to this process included cooling profile, seed loading, seeding temperature, seed particle PSD, and starting concentration.

Once the operating space for polymorph purity is determined, other key attributes such as supersaturation, composition, cooling rate, and seeding (including loading and size) need to be investigated. In the caffeine–glutaric acid cocrystal example, [89, 90] the authors displayed how many of these properties can be investigated. For example, the effect of starting solution composition on metastable zone width and the polymorph produced was assessed using nucleation temperature and polymorphic outcome over a temperature range of

35–25 °C. During this investigation, Form I was observed under all conditions indicating that this cocrystal form is stable under the operation range solution composition. The study also uncovered that unseeded crystallizations, with cooling rates ranging from 0.1 to 0.4 °C min^{-1}, always resulted in isolation of metastable Form I. Form I then consequently transformed to Form II [89, 93]. In contrast, utilizing a higher seed loading (0.5 g) and lower cooling rate (0.1 °C min^{-1}) effectively inhibited the formation of the metastable Form I and allowed a continuous increase in particle size through slow reduction of supersaturation.

A wide variety of methods can be employed at the laboratory scale to generate salts and cocrystals. These include solution reaction [95], slurry [96], solvent drop grinding [45], antisolvent crystallization [97], slow evaporation [46], and hydrothermal methods; [98] however scale-up of these methods are rather limited. In addition to conventional solvent crystallization processes, large-scale manufacturing of cocrystals has been reported using hot melt extrusion [99, 100] (i.e. carbamazepine, nicotinamide, and Soluplus® as a formulation approach), spray-drying (i.e. theophylline cocrystals [101] and caffeine–glutaric acid cocrystal [51]), and high-shear granulation [102] (i.e. piracetam/L-tartaric acid with excipients).

10.5 Formulation Considerations

Salts and cocrystals can be used in a variety of dosage forms both in early and late-stage development. Early dosage forms may be relatively simple, such as drug in capsule or suspensions. More complex dosage forms, such as tablets, may be developed for late-stage clinical studies or marketed products. Salt formulations are quite routine in early and late development studies, as well as oral and injectable marketed products [16]. Additionally, cocrystals have been used in early animal bioavailability studies, as well as human studies (Table 10.7).

Excipient compatibility studies are often performed to gain insight into chemical stability concerns for formulations. However, these studies can also be used to investigate physical stability of the salt or cocrystal form and the influence of excipients on the properties of the final dosage form. Studies have shown that binary mixtures of basic excipients with miconazole or benzocaine mesylate salts resulted in the formation of the API free base after exposure to moisture [59]. Solubility measurements of a carbamazepine–nicotinamide cocrystal showed that the cocrystal alone would transform to carbamazepine dihydrate, while the addition of low levels of hydroxypropyl methylcellulose (HPMC) (0.5–5 mg ml^{-1}) would prevent transformation and maintain the cocrystal form during the duration of the experiment [120]. High-throughput excipient studies with binary mixtures and varying amounts of water have also been reported to study physical and chemical form changes [121].

As discussed previously, the counterion/coformer needs to be assessed for the desired dosage form, expected dose, and length of use (acute vs. chronic

Table 10.7 Cocrystal formulations reported for early development studies.

API	Coformer(s)	Type of formulation	Type of study	References
(4-(4-chloro-2-fluorophenoxy) phenyl)pyrimidine-4-carboxamide	Glutaric acid	Neat powder in gelatin capsule	Animal bioavailability	[54]
Acetylsalicylic acid	Theanine	Intraveneous aqueous solution	NA	[103]
AMG-517	Sorbic acid	10% Pluronic F1081 in OraPlus1 suspensions	Rat bioavailability	[104]
Carbamazepine	Saccharin	Capsule formulation with lactose monohydrate	Dog bioavailibility	[105]
C-glycoside derivative	L-proline	Aqueous 0.5% methyl cellulose suspension	Rat antihyperglycemic action	[106]
CP-724714	Butanedioic acid	Oral dosing	Human bioavailability and safety	[107]
Danazol	Vanillin	Neat aqueous suspension; 1% vitamin E-TPGS[a)] and 2% Klucel LF Pharm hydroxypropylcellulose (HPC) suspension	Rat bioavailability	[107, 108]
EGCG	Isonicotinamide, nicotimamide, nicotinic acid, isonicotinic acid	Corn oil suspension	Rat bioavailability	[109]
Gatifloxacin	Stearic acid, palmitic acid	Pediatric suspension (xylitol, Avicel RC-591, methylparaben, propylparben, titanium dioxide, sucrose, vanilla flavoring)	NA[b)]	[110]
Indomethacin	Saccharin	Capsule formulation with lactose	Dog bioavailibility	[111]

Itraconazole	Tartaric acid	Melt with hydroxypropylcellulose (HPC) and TPGS	Dog bioavailibility	[112]
L-883555	L-tartaric acid	Oral methocel	Monkey bioavailability	[113]
Lamotrigine	Saccharin	Polyethlene glycol (PEG) 400, 95% methyl cellulose aqueous suspensions	Rat bioavailability	[114]
Lithium chloride	Leucine	Aqueous vehicle		[115]
Meloxicam	Aspirin	PEG 400, 95% methyl cellulose aqueous suspensions	Rat bioavailability, blood–brain barrier penetration	[19]
Meloxicam	Succinic acid, 4-hydroxybenzoic acid, glutaric acid, maleic acid, L-malaic acid, benzoic acid, DL-malic acid, hydrocinnamic acid, glycolic acid, fumaric acid	PEG 400, 95% methyl cellulose aqueous suspensions	Rat bioavailability	[116]
Modafinil	Malonic acid	Capsule formulation with lactose	Dog bioavailibility	[117]
Quercitan	Caffeine, isonicotinamide, theobromine	Vegetable oil suspensions	Rat bioavailability	[118]
Tenefovir	Fumaric acid	Neat sample in capsule	Rat bioavailability	[119]

a) TPGS-alpha tocopheryl polyethylene glycol succinate.
b) NA-not available.

dosing). In addition to solubility and dissolution measurements of the solid forms, other parameters specific to formulation processes need to be considered. These include compaction [28], tabletability [122], drug–excipient interactions [104], and susceptibility to hydration [123]. Properties of the granules, such as flowability, friability, and compactability, also need to be considered for more complex dosage forms [102].

Formulation processes are known to result in process-induced transformations where the crystal form of API is impacted [66]. These form transformations can be induced by temperature [124], pressure, solvents including water [125], or a combination of these factors [126]. Resulting changes can include dissociation of the salt/cocrystal, formation of hydrates of the salt/cocrystal/free API, loss of water to form an anhydrous salt/cocrystal/free API, or transformation to a more or less stable form of the salt/cocrystal. Figure 10.10 outlines possible processes for the production of oral dosage forms [127]. The highlighted boxes contain processes that could potentially change the solid form of the salt/cocrystal. Even capsule filling or drying in a room with variable RH could result in a change in form [128]. Freeze-drying, commonly used to produce cakes for reconstitution into intravenous (IV) formulations, has been shown to produce various solid forms depending on the conditions used [129]. Based on these reports, it is important to understand the various forms of the salt/cocrystal and conditions that could produce them. A large amount of information can be obtained from the initial screen or a specialized screen; [130, 131] however, additional studies covering specific parameters of the intended process are also important [132].

Examples of process-induced transformations are given in Table 10.8. While some entries deal with RH conditions related to handling and storage of both the API and formulation, there are examples of formulation stresses such as wet granulation, grinding, and drying. Many of the form changes are related to the formation of anhydrous or hydrated forms, while others deal with cocrystal formation with excipients [104, 135] or dissociation due to interactions with excipients. This is a small sampling of the types of form changes that can occur upon formulation and should be considered during drug product manufacture.

Form changes may also occur when testing salts, cocrystals, or drug products. For example, AMG 517 cocrystal solubility measurements in fasted simulated intestinal fluid (FaSSIF) resulted in the precipitation of AMG 517 free base hydrate [144]. Amlodipine besylate was also found to dissociate during aqueous solubility measurements, resulting in amlodipine free base anhydrate [136]. A fluoxetine hydrochloride (HCl)–fumaric acid cocrystal was found to dissociate during dissolution testing, resulting in a fluoxetine HCl precipitate [95]. While there was dissociation and precipitation in the dissolution studies, the PK drug study showed a significant increase in blood levels compared with fluoxetine HCl alone, indicating that the initial solubility/dissolution enhancement by the cocrystal was enough to improve bioavailability. Salts have also been shown to dissociate during solubility or dissolution measurements [145].

Changes in solid forms and formulations can produce improved drug products in a process called lifecycle management. Producing better dosage forms to improve patient efficacy, decrease side effects, and increase patient compliance

Table 10.8 Examples of process-induced transformations of salts and cocrystals.

Compound	Salt/cocrystal	Form	Process	Transformation	References
Abbot 232 HCl	Salt	Anhydrous	Wet granulation	Produced amorphous drug that led to chemical instability of the formulation	[132]
Albuterol sulfate	Salt	Anhydrous	Milling and exposure to RH	Milling produced amorphous material which crystallized upon RH exposure causing particle agglomeration	[133]
AMG 517 various coformers	Cocrystals	Various coformers	Solubility measurement in fasted simulated intestinal fluid (FaSIF)	Conversion of cocrystal to AMG 517 free base hydrate	[134]
AMG 517: sorbic acid	Cocrystal	AMG 517 form A	Suspension in 10% Pluronic F108 in OraPlus	AMG 517–sorbic acid cocrystal produced with sorbic acid preservative in formulation vehicle	[135]
Amlodipine besylate	Salt	Anhydrous	Wet granulation and solubility measurements	Partial conversion of anhydrate to dihydrate	[136]
Benzocaine mesylate	Salt	Anhydrous	Presence of basic excipients and moisture	Salt converted to free form	[59]
Caffeine: citric acid	Cocrystal	Caffeine–citric acid cocrystal	Exposure to 98% RH	Conversion of anhydrous cocrystal to caffeine hydrate	[137]
Carbamazepine: saccharin	Cocrystal	Carbamazepine API, saccharin	Milling, RH exposure	Formation of carbamazepine: saccharin cocrystal	[138]
Delavirdine mesylate	Salt	Form XI	Formulated tablets exposed to temperature and RH	Acid/base reaction between salt and excipient to form API free base	[139]
Fluoxetine HCl: succinic acid	Salt cocrystal	Anhydrate	Dissolution studies in water	Conversion of cocrystal to fluoxetine HCl	[95]

(*continued*)

Table 10.8 (Continued)

Compound	Salt/cocrystal	Form	Process	Transformation	References
Imatinib mesylate	Salt	Form alpha	Grinding, aging at RT	Grinding produces amorphous which crystallizes to Form beta upon aging at RT	[140]
Indinavir sulfate	Salt	Ethanol solvate	Exposure to >40% RH	Fast exposure to RH result in amorphous salt, slow exposure to RH results in hydrate salt	[141]
LY334370 HCl	Salt	Dihydrate	Water slurry with Form 1 seeds (anhydrate)	Conversion of dihydrate to anhydrous Form I	[142]
Miconazole mesylate	Salt	Anhydrous	Presence of basic excipients and moisture	Converted to free form	[59]
Naproxen sodium	Salt	Anhydrous	Wet granulation in high-shear mixer granulator	Conversion of anhydrate to tetrahydrate	[143]
Pentamidine isethionate	Salt	Anhydrous forms A,B, C, trihydrate	Heating, freeze drying	Transformation between anhydrous forms upon heating; transformation between all forms during freeze drying under different conditions	[129]
Risedronate sodium	Salt	Hydrate	Fluid bed drying and tablets exposed to temperature and RH	Conversion to low moisture form upon drying, resulted in swelling of tablets upon equilibration due to rehydration of channel water	[126]
Siramesine HCl	Salt	Anhydrous	Simulated wet granulation in water and 60% ethanol	Conversion to hydrate in 60% ethanol	[125]
Theophylline: citric acid	Cocrystal	Theophylline anhydrate and hydrate	Neat grinding, liquid-assisted grinding	Grinding theophylline monohydrate neat or with liquid resulted in cocrystal monohydrate; neat grinding theophylline anhydrate with citric acid resulted in anhydrous cocrystal	[137]

are important aspects of pharmaceutical lifecycle management. Novel crystalline forms, such as salts and cocrystals, incorporated into new formulation approaches or delivery routes are a significant part of this management process for marketed products. Specific counterions or guest molecules need to be considered for certain delivery routes, such as dermal, ophthalmic, IV, or intramuscular formulations [16]. Determining the issues with current products and finding creative solutions to an improved product using form and formulation has been recognized as a true "win–win" in lifecycle management [146]. The change in form could include a polymorph, free acid/base, salt, cocrystal, or amorphous solid dispersion (ASD). Some examples of solid form changes involving salts are presented in Table 10.9.

10.6 Regulatory Aspects

FDA has different regulatory routes for salts and cocrystals. Different salt forms of an API are considered different APIs under current guidelines (see 21 CFR 314.108 and 21 CFR 320.1(c)) and require clinical human data to support the filing. Under the latest revised guidance, cocrystals are treated similarly to polymorphs and classified as a special case of solvates in which the second component is nonvolatile [147]. In these cases, bioequivalence studies are needed to show

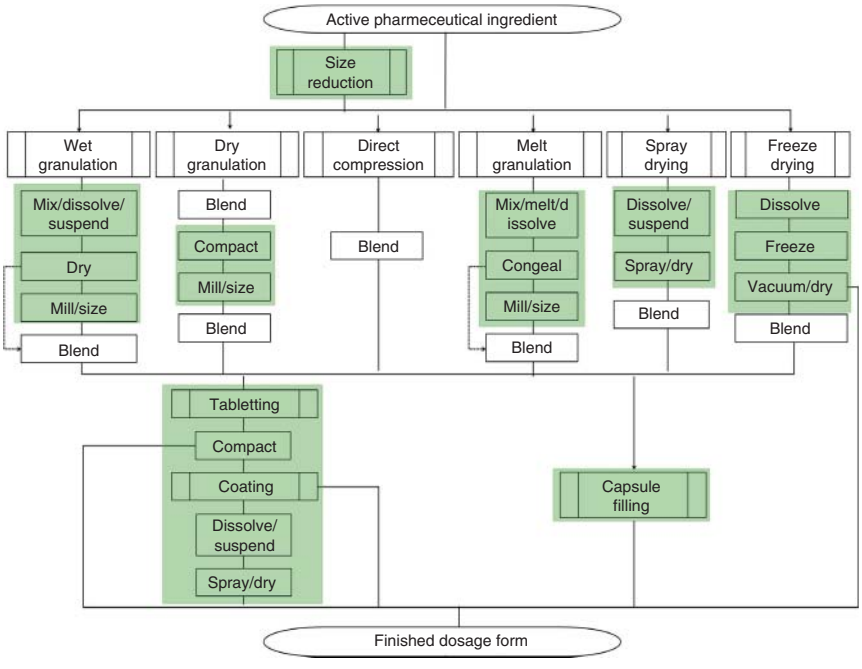


Figure 10.10 Formulation processes used in solid oral dosage forms. Highlighted boxes indicate processes that could result in a form change for API or excipients. *Source*: Zhang et al. 2004 [127]. Reproduced with permission of Elsevier.

that acceptable properties can be attained when compared with the previous solid form. Different cocrystals of a salt API (for example, cocrystals of fluoxetine HCl [95]) will be treated as a polymorph of that salt. As applicable, the pK_a rule and/or orthogonal characterization data outlined in the guidance can be used to provide support for the salt or cocrystal designation.

The European Medicines Agency (EMA) uses the same regulatory path for polymorphs, hydrates, solvates, salts, and cocrystals [148]. These forms are not considered new active substances (NAS) in this regulatory paradigm and require only bioequivalence data for the filing. As outlined in International Conference on Harmonization (ICH) Q11, "commonly available chemicals employed as co-formers in the cocrystal manufacture would be considered as reagents. However, for more complex or novel co-formers, details of the manufacture, characterization and controls, with cross references to supporting safety data, should be provided for them, according to the drug substance format."

Patenting salt and cocrystal forms [149], along with their polymorphs, adds to the IP protection for a new API and is a consideration for generic products. FDA maintains a public and detailed list of drugs and drug products that have been approved for use in the United States. The list was originally called the *Approved Drug Product with Therapeutic Equivalence Evaluations* when it was introduced in 1979 and was bound with an orange cover, and it is now referred to as the *Orange Book*. The Orange Book database is now electronic and is found on the FDA website (http://www.fda.gov/cder/ob/). A new drug application (NDA) requires that the company identify any patents that could serve as a basis for patent infringement. Once the NDA is approved, the patents are listed in the Orange Book, along with the expiration date and exclusivity. When a generic company files an abbreviated new drug application (ANDA), it must identify the

Table 10.9 Examples of commercial products containing different crystalline forms in a variety of dosage forms.

Drug	Crystalline form	Commercial name	Dosage form
Esomeprazole	Magnesium salt Sodium salt	Nexium® Nexium® IV	Oral tablet, oral suspension Injectable
Fentanyl	Citrate salt Free base	Actiq® Duragesic®	Lozenge Transdermal
Metoprolol	Tartrate salt Succinate salt	Lopressor® Toprol XL® extended release	Oral tablet Oral tablet
Olanzapine	Free base Pamoate salt	Zyprexa® ZyprexaRelprevv®	Oral tablet Injectable depot
Oxybutynin	Hydrochloride salt Free base	Ditropan®, Ditropan® XL Oxytrol®	Oral tablet Transdermal patch

Source: U.S. Food and Drug Administration, http://www.fda.gov/cder/ob/.

innovator drug (known as the reference listed drug (RLD)). If there are patents in the Orange Book for the drug, FDA cannot approve the ANDA until the listed patents have expired. Based on the Hatch-Waxman Act, there are four certifications that the generic company must choose:

Paragraph I. There are not patents listed in the Orange Book.
Paragraph II. The patent listed in the Orange Book has expired.
Paragraph III. There is a listed patent in the Orange Book that has not expired, and the generic does not intend to market the product until after the patent has expired.
Paragraph IV. The generic company plans to challenge the listed patents and claim that they are invalid or unenforceable or will not be infringed by the generic product.

Most pharmaceutical litigations deal with Paragraph IV ANDA filings. Examples include ranitidine HCl [150], cefadroxil [150], paroxetine HCl [150], terazosin HCl [151], and aspartame [151].

10.7 Case Studies

10.7.1 Indinavir: Early Salt Form Change

The salt indinavir sulfate, marketed by Merck as Crixivan®, was approved as an HIV-1 protease inhibitor dedicated for treatment of human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS) in adults [141, 152, 153]. Indinavir was initially formulated using the free base monohydrate, but the compound suffered from significant pH-dependent solubility and limited absorption as the free base form (aqueous solubility of 0.02 mg ml^{-1} at native pH 7–7.5) [141]. The pH solubility profile and pK_a of the molecule suggested a highly acidic counterion was necessary to achieve complete dissolution. Unfortunately, indinavir was quite unstable in acidic solutions that presented a stability risk for producing solid salt forms [141]. The crystalline sulfate salt ethanol solvate was chosen as the lead salt form for development. The aqueous solubility for this salt form was >500 mg ml^{-1} with a resulting solution pH of <3 (compared with free base solubility around 70 mg ml^{-1} at pH 3). Issues with the sulfate salt ethanolate were found, including extreme hygroscopicity, degradation, and physical form transformation under elevated humidity conditions. Extensive solid-state stability and excipient compatibility studies were performed and the results showed that a shelf life of >2 years was possible when the RH was kept <30% to prevent degradation; therefore, a dry granulation formulation process was developed for the drug product [154]. Human clinical trials were conducted with both the sulfate salt ethanolate and free base monohydrate [155]. The study showed that the sulfate salt in the fasted state or with a low fat meal yielded the highest exposures. This example clearly demonstrates the utility of identifying the appropriate salt form, studying the impact on the PK profiles, and understanding form stability issues before clinical trials.

10.7.2 Atorvastatin: Crystalline Form Change in Late Development

Atorvastatin (CI-981) is an HMG CoA reductase inhibitor marketed by Pfizer as Lipitor®. As a BCS II drug, it exhibits poor solubility and high permeability [156]. The amorphous form of the hemicalcium salt pure enantiomer was used for early Phase I and II clinical trials [157]. Phase II clinical trials showed an improvement in performance when compared with data from four marketed drugs. The amorphous material exhibited poor filtration and drying characteristics for large-scale batches and required protection from heat, light, oxygen, and moisture [158]. A crystalline trihydrate form was produced at scale during Phase III clinical trials and referred to as Form I [158]. This crystalline form possessed a number of advantages over the amorphous form including higher purity, improved chemical stability, narrow PSD, and better filtration and drying properties. These property improvements were substantial enough for researchers to change the solid form during late development. Development areas that needed to be repeated, included API manufacturing process development, formulation development, stability studies, analytical methods, and human bioequivalence testing. Tablets produced with amorphous and crystalline trihydrate atorvastatin calcium exhibited a difference in the rate of absorption, but resulted in an equivalent extent of absorption in the bioequivalence study [159]. Other crystalline forms (Forms II and IV) were patented along with Form I [158], and additional forms were reported in subsequent patents [160–162]. The FDA Orange Book lists a number of patents for atorvastatin calcium, including the composition of matter patent (expired September 24, 2009), a salt patent covering the calcium salt (expired December 28, 2010), and the crystalline Form I patent (expired July 8, 2016). By using a form other than Form I, generic products were technically allowed on the market in 2010 [157]. This example shows the utility of performing a solid form screen in early development to find a suitable form long before Phase III clinical trials. The patents listed in the Orange Book and the strategy of using patents to maintain market share have also been recognized as an important lesson from this case.

10.8 Summary

Salts and cocrystals fill an important role in pharmaceutical development and can be used to help modify and improve API properties that will result in better formulations and marketed products. Since these are multicomponent systems, additional characterization and solid-state support are often required to ensure that the desired form is produced and maintained during all the processing steps. Salts and cocrystals will continue to be utilized in lifecycle management products to improve performance, efficacy, and compliance. These crystalline forms are an example of how solid forms, both crystalline and amorphous, can be used to streamline development.

List of Abbreviations

AIDS	acquired immune deficiency syndrome
ANDA	abbreviated new drug application
API	active pharmaceutical ingredient
ASD	amorphous solid dispersion
CCF	cocrystal former
CFR	Code of Federal Regulations
CSD	Cambridge Structural Database
DOE	design of experiments
DP	drug product
DSC	differential scanning calorimetry
EAFUS	Everything Added to Food in the United States
EMA	European Medicines Agency
FaSSIF	fasted simulated intestinal fluid
FDA	Food and Drug Administration
GLP	good laboratory practices
GMP	good manufacturing practices
GRAS	generally regarded as safe
HBr	hydrobromide
HCl	hydrochloride
HIV	human immunodeficiency virus
HPC	hydroxypropylcellulose
HPLC	high performance liquid chromatography
HPMC	hydroxypropyl methylcellulose
HTS	high-throughput screening
IC	ion chromatography
ICH	International Conference on Harmonization
IP	intellectual property
IR	infrared
IV	intravenous
KF	Karl Fisher
kg	kilogram
l	liter
MDD	maximum daily dose
MP	melting point
MW	molecular weight
NAS	new active substance
NDA	new drug application
NMR	nuclear magnetic resonance
PAT	process analytical technology
PD	pharmacodynamics
PEG	polyethylene glycol
PK	pharmacokinetics

PSD	particle size distribution
ppm	parts per million
RH	relative humidity
RLD	reference listed drug
RT	room temperature
ssNMR	solid-state nuclear magnetic resonance
TGA	thermogravimetric analysis
TPD	ternary phase diagrams
TPGS	D-α-tocopheryl polyethylene glycol 1000 succinate
TRIS	tris(hydroxymethyl)aminomethane
XRPD	X-ray powder diffraction

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11

Particle Size Reduction: From Microsizing to Nanosizing

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11.1 Strategic Plans and Risk Management of Particle Size

Early drug development is a time to demonstrate whether a new drug substance will achieve the required goals of efficacy and safety as a drug product. In this stage, a risk is that a drug may not achieve its goals due to insufficient bioavailability, which is often related to one of the most important properties of a drug substance that can be controlled: particle size. Particle size is known to be an important parameter for many pharmaceutical compounds [1], which can often be challenging to manage during early drug product development. This challenge is a result of several practical considerations including limited material availability, limited experimental and bioavailability data to accurately develop particle size methods or deduce the best particle size range, or insufficient time and materials to optimize drug substance and drug product processes. Many of these issues are resolved as materials, methods, and processes that are optimized throughout the development program, provided that the first-in-human (FIH) and other proof-of-concept (POC) clinical trials demonstrate adequate safety and effectiveness.

The primary goal of early development is to provide companies with the knowledge to move forward quickly with the right drug for patients and to maximize the opportunity for a drug to be successful with appropriate formulation or process changes. Secondary goals, which are also quite important, augment the foundation used to demonstrate the safety and effectiveness of new drug products through the development of knowledge for regulatory approvals. Ultimately, these will aid in successful full development and transition to manufacturing, with the goal of maintaining safe and effective medications for patients for as long as possible.

With particle size identified as a possible risk, it becomes important to assess that risk. As an important guideline aimed at a single set of global specifications for new drug substances and new drug products, ICH Q6A was established [2]. In addition to the universal tests listed in the guideline, it states that particle size

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

control and analysis may be considered on a case-by-case basis for drug substances and/or drug products. Whether particle size control and measurement is needed depends on historical knowledge of the drug substance and drug product, including particle size relationship to dissolution, bioavailability, drug product processing, and drug product performance. Individual tests/criteria should be included in the specification when particle size has an impact on the quality of the drug substance and drug product for batch control. For some new drug substances intended for use in solid or suspension drug products, particle size can have a significant effect on dissolution rates, bioavailability, and/or stability. In such instances, testing for particle size distribution should be carried out using an appropriate procedure, and acceptance criteria should be provided.

When the development concern is bioavailability, as related to low solubility or slow dissolution rate, then risk mitigation strategies must be identified. There are many ways to perform risk assessments, and one of them includes evaluation of likelihood, velocity, persistence, and impact, with many numbering systems or word descriptors possible for each. Table 11.1 contains a generalized assessment, with the risk parameters and descriptors described as follows. Likelihood, which often uses descriptors including rare, unlikely, possible, likely, and almost certain, describes how sure a development team is that a risk will cause a problem. This table shows that there is a rare likelihood that particle size will affect the bioavailability of a highly soluble drug, while the opposite is true for low solubility drugs. The velocity of the effect of particle size on the bioavailability of highly soluble drugs is low, meaning that it is unlikely that the dependence will be able to be detected in preclinical and early development time frame and that once detected, there may be a significant amount of time to deal with the issue. The velocity of the effect of particle size on bioavailability of poorly soluble drugs is medium to very high, meaning that the problem will be detected immediately or within a few months. Thus, a conventional preclinical formulation of a drug may be sufficiently soluble to show adequate preclinical effect to move the drug into development. However, before it goes into clinical studies, the particle size in the formulation must be well controlled and likely micronized or nanosized, so that exposure and dose response, and possibly effectiveness, can be adequately assessed. Persistence, which evaluates whether an issue is a one-time, unlikely

Table 11.1 Particle size risk assessments for particle size on bioavailability of high and low solubility drugs (may be different based on specific new drug substance and corporate capabilities).

Risk assessment	BCS classes I and III (high solubility)	BCS classes II and IV (low solubility)
Likelihood	Rare	Likely to almost certain
Velocity	Very low	Medium to very high
Persistence	Few	Likely
Impact on early development	Insignificant or minor	Major to catastrophic
Impact on late development and manufacturing	Insignificant or minor	Minor to moderate

event or if it is a continuing event, depends on the company assets but is more often a likely event as solubility of new drug substances has been decreasing for several years.

Table 11.1 also lists potential assessments of impact on early and late development. Impact is the consequence of an issue and ranges from insignificant to catastrophic. Often, risk is related to financial impact, clients' (or staff's) health and safety, business interruption, reputation, or corporate objectives. The impact to late development is relatively small. For highly soluble drugs, there are situations in which the particle size does not even need to be monitored, such as with liquids. For late development, the impact of controlling particle size for insoluble drugs is the long-term cost of controlling and testing, which is often seen as just a part of doing business. The most important issue is related to impact on early development. Whether a company has many drugs in its pipeline or is depending on a new drug as one of the few in its pipeline, insufficient exposure in a preclinical or early clinical trials can be catastrophic, meaning that the monetary costs already expended as well as the cost of unattainable future income can be greater than a large amount of money, e.g. $10 million, if solely evaluating the financial risk. Risk to corporate objectives and, possibly, to the reputation of a company can also be high. The worst impact is on patients who need this medication. For all these reasons, it is imperative that particle size be taken seriously in early development.

Risk mitigation relies on control of particle size distribution during substance preparation, which will significantly impact to the physicochemical and biopharmaceutical properties for drug products with poor solubility in both early stage and commercial product development.

11.2 Particle Size Reduction Techniques

Particle size reduction is one of the oldest techniques for pharmaceutical development. Conventional particle size reduction techniques usually offer approaches to produce particles with a specific size range so that powder property and particle property of the drug substance remain unchanged among various batches during drug product development. This is crucial when the particle size of the drug substance impacts drug product processability, stability, content uniformity, and appearance. In such cases, a robust method for particle size reduction needs to be developed to meet the criteria of particle size for drug substances and drug product [2]. More importantly, particle size reduction must be a safe method to increase the bioavailability of drug substances without altering the chemical nature of the drug candidate. While the conventional particle size micronization still remains a basic particle size reduction procedure, nanosizing crystalline drug substance becomes desirable to achieve suitable biopharmaceutical property of drugs with poor aqueous solubility. In recent years, various nanoparticle techniques to achieve and maintain a nanometer range particles for drug candidates have been developed and applied to formulations for both early stage and commercial product development. [3]

Two principles are utilized in particle size reduction techniques, named as top-down and bottom-up. Top-down techniques involve the mechanical reduction of particle size for coarse drug powders of drug substance to suitable sizes for drug product development. On the other hand, bottom-up techniques involve precipitation from solution to generate crystalline materials with suitably sized particles. Sometimes, both approaches are combined to generate the drug substance with the desired physical and biopharmaceutical properties.

11.2.1 Top-Down Approaches

Pharmaceutical drug substance obtained from small-scale preparation and/or commercial manufacture usually contains crystalline particles of various particle sizes. Sometimes, the larger particle size or the particle size variation causes difficulty in formulation during drug product development. A top-down method of particle size reduction for coarse drug powder is the most direct approach to produce smaller particles with narrow range of size. Top-down mechanical particle size reduction techniques include dry milling (grinding, impact), wet milling techniques (in-line during crystallization and post-crystallization), and wet media milling or high-pressure homogenization (HPH).

A jet mill is the most commonly used dry milling technique as a top-down approach. A jet mill grinds drug substances by using a high-speed jet of compressed air or inert gas to cause particle-to-particle impact to achieve fine or ultrafine pharmaceutical powders with a narrow particle size distribution. Jet mill equipment consists of a cylinder, which allows continuous feed of the drug substance into the mill. Compressed gas is forced into the mill through nozzles tangent to the cylinder wall, creating a vortex. The grinding that occurs inside the vortex and circulating in the smaller, lighter particles moves to the outer edges of the vortex into a fine-powder collection device. Jet mills can be designed to output particles below a certain size while they continue to mill particles above that size, resulting in a narrow size distribution of the resulting product. The jet mill has several advantages of being a dry process, e.g. size reduction of microsized particles with narrow size distributions, absence of contamination, and suitability for heat sensitive drugs [4].

Wet milling is a top-down process in which the particle size of a drug material is reduced with a liquid medium in a milling chamber. As one of the oldest processes to produce ultrafine suspensions, wet media milling was improved to produce and stabilize nanosized crystalline materials. A typical wet media milling process usually uses the drug substance dispersed in a liquid medium (usually water), which is loaded into a milling chamber with the selected milling media. Milling media are small beads or pearls made of ceramic (e.g. yttrium-stabilized zirconium dioxide), highly crosslinked polystyrene resin, stainless steel, or glass. The size reduction in wet media milling process takes place by collisions between milling media and drug particles, between two drug particles, and also between drug particles and the walls of the milling chamber [3]. In order to increase size reduction effectiveness and stabilize nanosized particles, surfactants are usually added into the milling chamber with the drug substance. Surface modifiers play an important role in the formation of stable nanosuspensions [5]. In addition to

being directly used as nanosuspension formulations, the resulting nanosuspensions generated by wet media milling method can be transferred to a solid by freeze-drying or spray-drying technologies and incorporated into solid dosage formulations such as tablets and capsules.

HPH is another top-down technology widely used in preparing nanosuspensions of drugs with poor water solubility. In HPH, the drug substance is first dispersed in a suitable fluid, which is forced through an opening valve of a homogenizer by a high-pressure pump. After being propelled through the valve at a high speed, the particles experience a sudden pressure drop. The reduction of particles is achieved by collisions of particles with each other, as well as collisions between particles and the homogenizer. HPH is compatible for use in both aqueous and nonaqueous fluid media and has been known to overcome the drawbacks of conventional size reduction methods such as amorphization, polymorph transformation, and metal contamination due to high mechanical energy associated with conventional milling processes [6].

11.2.2 Bottom-Up Approaches

Bottom-up approaches use particle size control through crystallization processes. Several traditional crystallization methods, e.g. solution reaction, evaporation, anti-solvent addition, heating and cooling, and slurrying, are used during drug substance preparation and manufacture. These methods usually generate the desired crystalline form; however particle sizes of the crystalline materials may be too large to achieve the desired bioavailability, or the size distribution may be too broad to control physical and biopharmaceutical properties in drug product development. Furthermore, batch-to-batch variation for particle sizes and particle size distribution by conventional crystallization techniques makes it difficult for quality control during drug development. Even with these possible issues, the ease of implementation for bottom-up approaches to optimize crystalline particles by controlling crystallization parameters should be considered prior to top-down approaches during drug substance preparation and manufacture.

The nature of a crystallization process is governed by both thermodynamic and kinetic factors, which can make it highly variable and difficult to control. The size and shape of crystals produced in drug substance crystallization is usually impacted by crystallization conditions, including concentration of solution, temperature, and impurities. Crystallization of drug substance by seeding with crystalline particles is one of the major methods to control batch-to-batch variation and obtain the desired crystalline form with a suitable particle size distribution. It is important to optimize the amount and particle size of seed crystals in order to achieve the highest effectiveness of crystallization [7]. The addition of polymers, other than the drug compound itself, is also used to control crystallization and recrystallization.

In recent years, sonocrystallization (crystallization using ultrasound) is under active investigation as a bottom-up approach in the pharmaceutical field. Ultrasound triggers nucleation at a low supersaturation, and subsequent crystal growth also occurs at lower supersaturation. In this way, particle size and size distribution can be controlled during the crystallization process. Different types

of ultrasound devices are available at the laboratory scale, including ultrasound cleaning baths, probes, and reactors. Sonocrystallization by using various ultrasound devices provides an effective, versatile, and noninvasive way to improve crystal properties, especially smaller crystal size, narrower size distribution, and improved reproducibility of crystallization. It becomes the most popular bottom-up approach during early drug development for crystallization control since the process can be applied to the milligram scale of samples, which are suitable for the discovery phase of pharmaceutical development when only limited amounts of drug candidates are available [8]. Although sonocrystallization is a useful bottom-up approach to produce both microsized and nanosized crystalline particles for early drug development, it is still a challenge to apply it in large-scale manufacturing for commercial drug product development. Other bottom-up approaches, e.g. spray-drying [9], freeze-drying [10], and superfluid crystallization [11], have also been investigated in order to achieve microsized and nanosized crystalline drug substances for both early stage preparation and commercial manufacture.

Top-down, bottom-up, or a combination of both approaches has widely been used to produce crystalline particles with desired particle size and size distributions. In early drug development, top-down wet milling and bottom-up sonocrystallization are widely used methods that can be easily achieved in the laboratory. The technique based on wet media milling method is also a fully developed process to produce commercial scale of nanosized crystalline drug substances and used in the development of several drug products on the market.

11.3 Particle Size Analysis

11.3.1 Regulatory and Quality Considerations

In early development, once a new drug substance sample exists, particle size analysis is a normal requirement. This is not necessarily true for final release of a product to patients, as outlined in ICH Q6A and as depicted clearly in Decision Tree #3: Setting Acceptance Criteria for Drug Substance Particle Size Distribution [2]. This guidance presents criteria for determining whether drug substance release particle size specifications are needed, but it does not discuss methods, validation criteria, or metrics to be reported. Thus it is not completely applicable to early development, when the data for making these determinations and plans are being collected. This does point out, however, the importance of the potential regulatory aspect of collecting particle size and related analytical and bioavailability data in early development as support for decisions in full development and manufacturing. The same guidance also discusses the need to evaluate visible and subvisible particulates in parenteral formulations. Though it is not always traditional to think about evaluating the presence and possible growth of particulates in a particle size discussion, these are important safety and efficacy aspects for liquid products including suspensions and injectables.

Many of the relevant USP-NF, Food and Drug Administration (FDA), and ISO guidelines relating to particle size are listed in Table 11.2. The World

Table 11.2 Relevant guidelines for particle size sample preparation, data analysis, and types of products requiring particle size and/or particulate analysis.

Particle size topic	USP 39-NF 34	ISO standards	US FDA guidances
Particle size and particulates in nasal and inhalation products	<601>, <1601>	27891	Nasal spray and inhalation solution, suspension, and spray drug products -- chemistry, manufacturing, and controls documentation (I)
Globule size distribution in lipid injectable emulsions	<729>		
Particulates in injections	<788>, <790>, <1788>		Q4B Annex 3
Particulates in ophthalmic products	<751>, <771>, <788>, <789>, <1788>		
Particle size for ophthalmic products	<429>, <786>		
Subvisible particulates in protein injections	<787>, <1787>		
Powder fineness, vocabulary	<811>	26824	
Excipient performance	<1059>, <1195>		
Bulk powder sampling	<1097>		
Alternative products			Size of beads in drug products labeled for sprinkle
Particle size data interpretation		9276-1, -2, -3, -4, -5, and -6	

Note that this is not a complete compilation.

Health Organization maintains a list of official pharmacopeias. Since there is extensive ongoing work to harmonize standards worldwide, the United States Pharmacopeia (USP, www.usp.org) and the ISO standards will be referenced in this chapter.

11.3.2 Particle Size Techniques

This chapter will focus on the types of instruments used and especially the challenges and advantages. There are several excellent review articles and text references [12–16] that discuss the instrument design; thus this will be kept to a minimum except where needed. Please note that the term "technique" is used as a general term for instrument types, while the term "method" is used for a

specific instrument with specific setup parameters, sample preparation, sample input, sample testing, data analysis, and data reporting.

Many different types of analytical techniques for particle size analysis are used in early development and beyond. Table 11.3 lists many of the techniques, including on-line, analytical, and particulate analyses, covering all particle size ranges from nanoparticles to granule-sized particles, as well as particle size techniques used for complex systems including nanosuspensions, colloids, proteins, mAbs, and other materials in suspension or as discrete particles. Though this table is not comprehensive, it does contain those methods that are used most often in the biopharmaceutical industry for material evaluation and/or testing by a validated method. Beyond their use in early development, several of these techniques can also be validated as methods for stability testing or release of final product.

11.3.3 Selection of Appropriate Technique or Set of Techniques

It is important to understand that each of these instruments is designed differently using different physics, different configurations when using similar physics, different data analysis (often proprietary), different sources of error, and different aspects of particle size. To make this even more confusing, the term "particle size" has no predefined meaning. Particle size can mean the most obvious length (think of the length of a javelin or the diameter of a child's soccer ball). It can also be as abstract as the length of an equivalent spherical diameter, which is the diameter of a sphere of equivalent volume, often obtained from light scattering intensities on a variety of detectors and calculated via proprietary calculations, such that a typical javelin and a typical child's soccer ball have similar equivalent spherical diameters. Additionally, some techniques directly measure chord length of a particle, which is a random chord length and thus is unlikely to be the longest length. Other techniques measure the aerodynamic particle size that could be affected by particle shape and morphology (i.e. smooth surface vs. craggy surface) and distortion in an airflow and are especially useful for aerosol product testing. All of these measurements are inherently different, and different reported results should be expected. Most texts on particle size analysis include a discussion of sample size and sampling [16, 17], while other texts are specifically geared to sampling and the determination of the appropriate sample size, as well as sampling the bulk appropriately [17].

Even within an instrument class, methods may not be equivalent because sample preparation, dispersion forces, dynamics within the analysis zone, detector arrangement and specifications, and data analysis algorithms differ. Additionally, within a specific instrument model, small differences in instrument manufacturing can bring about differing particle size results. This is most often, and somewhat annoyingly, discovered during method transfer of brittle materials. This issue and related result biases can often be eliminated through instrument prequalification procedures. To make this even more complicated, the definition of particle size within a technique is not always clear. As an example, a model that estimates a targeted particle size will provide discrete numbers that are then interpreted by the development team, which are then transferred to one or more techniques. For instance, a model-derived target of $\leq$20 μm will likely be set as

Table 11.3 Particle size measurement and particulate detection techniques common in the pharmaceutical industry.

Technique	Description	Guidance reference (USP/ISO)[a)]	PSD range[b)] (μm)	Common uses	Method development challenges
On-line techniques					
Phase doppler particle analyzers (PDPA)	Flowing particle scatters light from 2 lasers to multiple detectors – measure phase shift between doppler events	–	0.1–200	Size and velocity of particles or droplets in air or liquid to monitor fluid beds, atomized sprays, and other flowing systems	Detectable particle size range depends on number of detectors, similarity of refractive indices, non-orthogonal velocity, extraneous light, fine particles add noise, and large particles have insufficient flow
Spatial filtering velocimetry (SFV) or focused beam reflectance measurement (FBRM)	Chord length is calculated based on the time it takes for a particle to pass within the laser obscuration or reflectance sensing zone	–	0.5–6000	Particle appearance and growth (number and size) during crystallization, granulation	High concentration, differing particle velocities, non-laminar flow in sensing zone, similarity of refractive indices
Sonic velocity	Size based on time within sonic sensing zone	–	Depends on liquid velocity – (100–10 000 ms^{-1})	Crystallization particle growth and supersaturation	Solvent density changes with temperature, and optical properties have no effect
Real-time optical image analysis	Images taken on time bases – particles are counted and characterized	–	≥0.1	Crystallization, particle growth, morphology, suspensions, granulations	Particles out of view, high concentrations can confound image analysis

(Continued)

Table 11.3 (Continued)

Technique	Description	Guidance reference (USP/ISO)[a)]	PSD range[b)] (μm)	Common uses	Method development challenges
Analytical techniques					
Sieve	Particles pass through screen	USP <786>, ISO 4497 ICH Q4B – Annex 12[c)]	≥75 (smaller if able to validate)	Excipients, highly soluble drugs, aggregates[d)]	Overloading screen, cohesive powders, particle breakage
Optical image analysis (optical microscopy (OM))	Static view through microscope	USP <776> ISO 13322-1	≥0.1	Small sample size, morphology, reference for other techniques	Too few particles (stitching multiple fields improves number count), dispersion technique, objective selection, image analysis criteria
Flow imaging microscopy (FIM)	Optical imaging of particles flowing in microchannels		1–1000	Subvisible particulates – visualize protein aggregates or air bubbles	Sample prep (clean room?), flow, imaging
Dynamic (flow) image analysis (DIA)	Particles flow past camera	USP <776> ISO 13322-2	≥0.1	Size distribution and morphology distribution, (often renamed Brightfield for subvisible particulate characterization)	Particle breakage, insufficient dispersion, image analysis criteria
Scanning electron microscopy (SEM)	Static image via scattered and sample-produced electrons	USP <1181>	≥0.001	Characterization of morphology and size Environmental (ESEM) reduces need for conducting surfaces	Rarely a release method due to poor counting statistics. Sound and temperature variations can reduce sensitivity

Laser light scattering (LLS)	Particles scatter laser light – PSD calculation from angular scattering intensities	USP <429> ISO 13320	0.01 to 3500	Particle size distributions, solids, suspensions, emulsions, droplet size, etc.	Dispersion technique (can result in particle breakage, agglomeration, dissolution, precipitation) Model selection (Fraunhofer, Mie, real and imaginary refractive index), sample size especially for wet methods
Dynamic light scattering (DLS)/photon correlation spectroscopy (PCS)	Temporal measurement of scattered light – detect Brownian motion and diffusion in solution	USP<729> ISO 22412 ISO 13321	0.0003 to 10	Molecules with MW < 1000 Da, evaluate globule size in lipid injectables, colloids, proteins, etc.	Temperature fluctuations, vibration, insufficiently dilute solutions
Cascade impaction (CI)	Particle size and air velocity affect inertial impaction	USP <601> USP <1601>	0.5–5	Inhaled powders, aerosols, sprays, nebulized aerosols. May validate alternate technique (e.g. LD, TOF) via CI	Nozzle exit diameter measurement, gas flow control, heat transfer from CI to aerosol, analytical measurement technique (often HPLC), instrument design (many evaluate several designs per product)
Time of flight (TOF)	Particles accelerate to sensing zone (detected by scattered laser light and/or ID by mass spectrometry)	–	0.3–20	Inhalation products (validate against CI)	Dilution effects, droplet distortion, particle or droplet density, multiple particles in laser measurement zone, ultra-Stokesian diameter affected by density and shape, analytical technique (mass spectra profile)
Matrix-assisted laser desorption/ ionization with TOF mass spectrometer (MALDI-TOF)	A matrix mixed with sample is heated with UV laser to vaporize some of the matrix – this vaporized sample is analyzed by TOF mass spectrometry		Molecular weight	Molecular weight for proteins, peptides, other biomolecules, and polymers	Matrix selection (usually low molecular weight organic acid, volatile, with strong UV/IR absorption) is crucial to optimize ionization and resolution without degrading the protein

(Continued)

Table 11.3 (Continued)

Technique	Description	Guidance reference (USP/ISO)[a]	PSD range[b] (µm)	Common uses	Method development challenges
Electric sensing zone (HIAC)	Particle size in fluid or electrolyte passes through charged orifice (measure resistance change)	USP <1787> ISO 13319	0.4–1600	Particulate counting, particle size distribution of small molecules, contaminant particles such as fibers, proteins, polymers, erythrocytes, etc.	Dilution, dissolving small particles, precipitation, agglomeration in solution, and others. Solvent media selection is critical
PDPA	See phase doppler particle analyzer in on-line techniques section				
Small angle X-ray scattering (SAXS)	XRD deflection at 0.1–10°. The scatter pattern contains info about shape, size, and pore sizes of macromolecules	ISO 17867	0.005–0.025	Macromolecule structure especially when crystalline sample is not available (e.g. multidomain flexible proteins and intrinsically disordered proteins)	Data analysis – Kratky plots help ID folding states and flexibility; *ab initio* and rigid body modeling can build low resolution model without knowing structure, model validation often by orthogonal technique
Differential mobility particle sizer (DMPS)	Measures electrical mobility that is related to particle size and the number of elementary charges	ISO 15900	0.001–1	Aerosols, air contamination	Different challenges occur when the mobility distribution is narrower, the same, or wider than the differential mobility transfer function. In some cases, tandem differential mobility analyzers could be needed
Taylor dispersion analysis (TDA)	Nanoliter sample is injected into laminar flow buffer. Time-evolved concentration profile (UV) measures hydrodynamic radius	–	0.0001–0.1 (optimum for 0.0002–0.05)	Proteins, peptides, monoclonal antibodies (mAb) in solution and product: particle size, self-association, conformational changes	Capillary diameter, buffer matching, poor UV absorbance, temperature instability

Static light scattering (SLS, with specifics for LALS, RALS, and MALS)	Particles in a cuvette or in combo with chromatography, scatter light via Rayleigh theory (large molecules scatter higher angle)		0.001–5 depending on selection of LALS, RALS, or MALS	Molecular weight of proteins, other biomolecules, and polymers	Selection of light scattering detector (LALS – low angle, RALS – right angle, or MALS, multiangle), instrument configuration, solvent, reference
Size-exclusion chromatography/ (SEC-HPLC or SEC-MALS)	Size separation in HPLC column, with one or more detectors – sometimes using MALS detection	–	0.0001–0.1	Protein, biopolymer, synthetic polymer characterization, stable aggregates	Chromatographic specifics (column, solvent, etc.) confirm peak ID for MALS with orthogonal technique
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	SDS denatures and imparts negative charge to proteins – applied electric field causes protein migration, smaller molecules move farther in field than larger molecules		0.0001–0.1	Protein characterization	Membrane proteins and other proteins with a higher hydrophobic content react variably with SDS, making this more difficult to achieve accuracy, selection of standard, buffer, counterion, gel pore size, tracking dye, staining, and other parameters
Asymmetric flow field-flow fractionation with MALS detection (AFFF-MALS)	Sample injected into channel with laminar flow – small molecules are more buoyant and are higher in the channel and thus are moved farther than the heavier particles		0.001–5	Protein and other biomolecule characterization	Setting vertical pressure, selection of channel size and design, laminar flow liquid, sample size and concentration, control of temperature and vibration
Analytical Ultracentrifugation (AUC)	Movement or location of band of particles under ultracentrifugation is monitored optically using UV absorption and/or refractive index interference. The distance moved is related to mass		0.001–0.5	Proteins, mAbs, nanoparticles of any type	Concentrating without changing, optimizing centrifugation, band identification

(Continued)

Table 11.3 (Continued)

Technique	Description	Guidance reference (USP/ISO)[a)]	PSD range[b)] (µm)	Common uses	Method development challenges
Light obscuration	Particle in fluid blocks light in sensing zone	USP <1787>	1–300	Subvisible particle counting/size	Solution concentration, temperature variations, flow rate in sensing zone
Resonant mass measurement (RMM)	Cantilever frequency changes with particle mass flowing through microchannel	–	0.05–5	Subvisible particle counting/size, polydispersity, distinguishes negatively buoyant (e.g. proteins) from positively buoyant (e.g. silicone oil droplets)	Effects of dilution, identifying particle type via signal
Scanning techniques					
Fourier transform infrared (FTIR) imaging	Molecular vibration absorption probes dipole moment changes – imaging with microscope (static or scanning)	–	10–1000	Scanning isolated solids or solid matrices – particle ID and distribution in a matrix	All challenges with FTIR and with microscopy, understand minimum spectroscopic spot size to actual particle size
Dispersive Raman imaging (DR)	Inelastic scattering probes polarizability – imaging with microscope (static or scanning)	–	0.5–1000	Scanning isolated or suspended solids, or solid matrices – particle ID and distribution in a matrix	All challenges with Raman and with microscopy, understand minimum spectroscopic spot size to actual particle size

SEM-EDX (energy dispersive X-ray)	SEM with EDX detector – electron beam excites inner shell e- to escape outer shell e- relax and emit X-rays characteristic of element	–	>0.001	Scanning samples for higher molecular weight atoms – inorganic contamination in organic sample, distribution of drug with an inorganic group (e.g. chloride, metallic complex, organic drug as an inorganic salt, etc.)	All challenges with SEM, accurate identification of X-ray emission pattern, X-ray can diffuse through sample, making particles appear larger than they are, concentration too low to detect (e.g. magnesium in the tablet lubricant magnesium stearate is sometimes challenging to detect in tablets due to low concentration)

Note that this is not a complete compilation.

a) USP = United States Pharmacopeia 39-NF 34, 2016, published by US Pharmacopeial Convention.

b) All ranges are approximate and can be broadened or narrowed by sample and specific instrument design.

c) FDA Guidance for Industry (Finalized), Q4B Evaluation and Recommendation of Pharmacopoeial Texts for Use in the ICH Regions, Annex 12 Analytical Sieving General Chapter.

d) The term aggregates is used to denote aggregates or agglomerates that do not easily break down to the primary particles.

90% ≤ 20 μm, which in microscopy would mean that the 90% of the number of particles is ≤20 μm, while in LLS, this would mean that 90% of the volume of particles is ≤ 20 μm. Within the OM technique itself, the selection of the longest diameter, the Feret diameter, or other measurement can provide different apparent particle size results for the same images.

In all of these cases, it is quite appropriate to use two or more methods in development, evaluate trends, and set specifications for clinical trial materials based on all the data but likely for only one of the techniques – often the technique that is most easily qualified and validated for early development methods, as well as transferrable to late development and marketed product methods.

Technique comparisons are often made and are unavoidable. Two or more subvisible particle techniques are often used to evaluate injectable biologics, while multiple techniques are used to evaluate small molecule particle size distributions. DiMemmo et al. [18] reported a series of experiments designed to determine similarity of data generated by different techniques by transforming data from multiple techniques into equivalent spherical diameters. OM is, for many reasons, the technique to use first, even when planning to validate using a different technique. A correctly taken image can provide critical information to the development team, including the shape and surface characteristics of primary particles, an estimate of the particle size distribution, and the presence of multiple agglomerated or aggregated particles. To assure appropriate microscopy data was obtained, it is important to consider various sources of image analysis (IA) challenges including selection of the most appropriate resolution for the particle size range being analyzed, determination of the required sample size to adequately measure the particle size range, misalignment of particles that cross individual fields of view in the mosaic, particle overlap resulting in some particles appearing larger than the individual particles, threshold selection for optimum measurement of particles, out of focus particles, and air bubbles. The pre- and post-analyzed images were used to identify possible data analysis errors, which aided optimization but not complete elimination of the errors. For spherical beads, the spherical equivalent diameters obtained from two LLS techniques, two DIA techniques with an OM technique, were quite reproducible (Figure 11.1) [18]. Here, the results of the FBRM measurement, which measures chord length, was high compared with the others, possibly due to the algorithm used to transform chord length to equivalent spherical diameter. The comparison of similar measurements for microcrystalline cellulose, which is an irregularly shaped particle, showed something quite different. The three IA techniques, two dynamic and one static, showed similar distributions, with the OM method providing some additional peaks around 200–300 μm attributed to overlapping particles. The spherical equivalent diameters obtained by LLS methods were slightly smaller. The cause of this difference, whether it is from the sample milling during dispersion, the proprietary data analysis algorithms, or other reasons, was not identified.

With so many techniques available for use, technique selection begins with the particle size range of interest to the sample being tested, as well as the goal for the test. The testing ranges listed in Table 11.4 take into account the multiple configurations and sample types possible and thus may not be absolutely valid

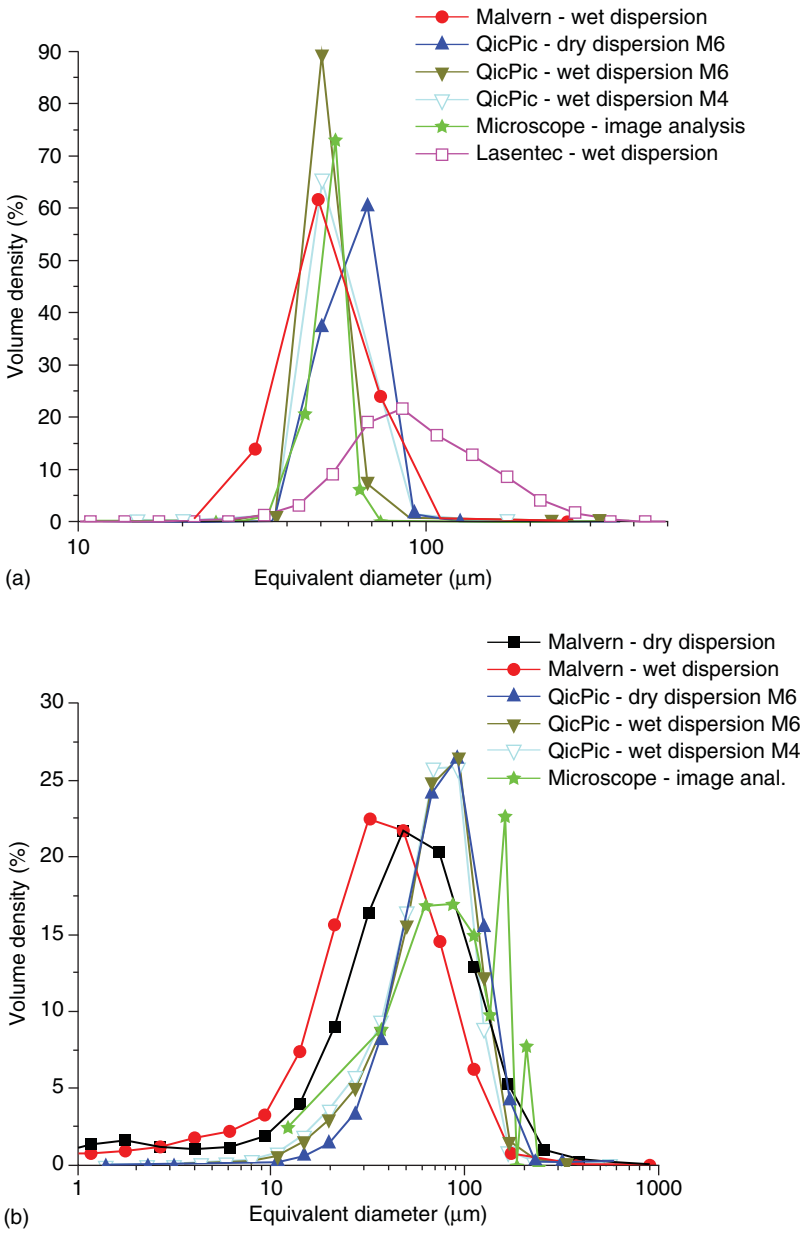


Figure 11.1 Spherical equivalent diameters for spherical polystyrene beads (a) and microcrystalline cellulose (b). *Source*: DiMemmo et al. 2011 [18]. Reproduced with permission of Cambridge University Press.

for the sample being tested. However, even with this caveat, there is always more than one available method for each particle size range. Specific instruments are purchased based on development and compliance needs, and thus a development team may have more limited choices than it first appears.

Table 11.4 Examples of particle size method questions and possible goals.

Particle size question	Possible method goals
Is this milled sample micronized?	Detect particles between 1 and 20 microns, as well as the original particle size to assure that all particles are sufficiently milled
Are the particles in this sample ≤ 20 μm?	Detect particle size distribution between 1 and 100 microns, and show that D90 ≤ 20 μm (i.e. 90% of the volume of particles is ≤20 μm)
Does this nanoparticulate sample have more than 1% of 300 μm aggregates that do not easily break during mixing?	Goal 1: evaluate the size distribution ≤10 μm Goal 2: evaluate distribution of larger particles (≤1000 μm). Test must be gentle enough not to underestimate aggregate size and test sufficient powder volume to detect low percentage of larger particles
Does the particle size in a protein suspension change with time?	Goal 1: evaluate nanoparticle size/molecular weight distribution for single molecules, dimers, trimers, and other small aggregates Goal 2: count and classify (size groupings) subvisible particulates (often by two techniques)

The next step is then to determine the question(s) that need to be answered that enable the definition of particle size method goals. Table 11.4 provides a few typical questions and method goals that will help guide the method development process. Not only do samples have different properties such as particle friability, particle morphology, and other intrinsic properties that cause them to behave differently in particle size techniques, but the samples are also used in different environments, which require different tests and results. Low dose drugs may have content uniformity problems when the drug substance has a small amount of aggregates that do not break up in the normal product process, while a high dose drug may be affected by drug substance particles that are small enough to inhibit product flow while mixing or dispensing into tableting or capsule-filling equipment. Thus content uniformity could be a problem for both the low dose drug and the high dose drug, but for very different reasons. This would lead development teams to ask different questions for the two situations, likely requiring different methods. Table 11.4 provides some examples of typical questions and related method goals. When multiple goals are required, it may be necessary to use multiple techniques or multiple methods within a technique.

In some teams, flowcharts such as the one shown in Figure 11.2 [19] are designed to aid the focus on achieving the particle size method question and goal. This particular flowchart covers the activities from development at the top of the chart to validation and transfer to a production site at the bottom of the chart. No method development occurs until a goal is set, as without goals to define the method requirements, it is impossible to develop anything but a general method, meaning a method adequate to test and report results for many samples but possibly not the one being tested.

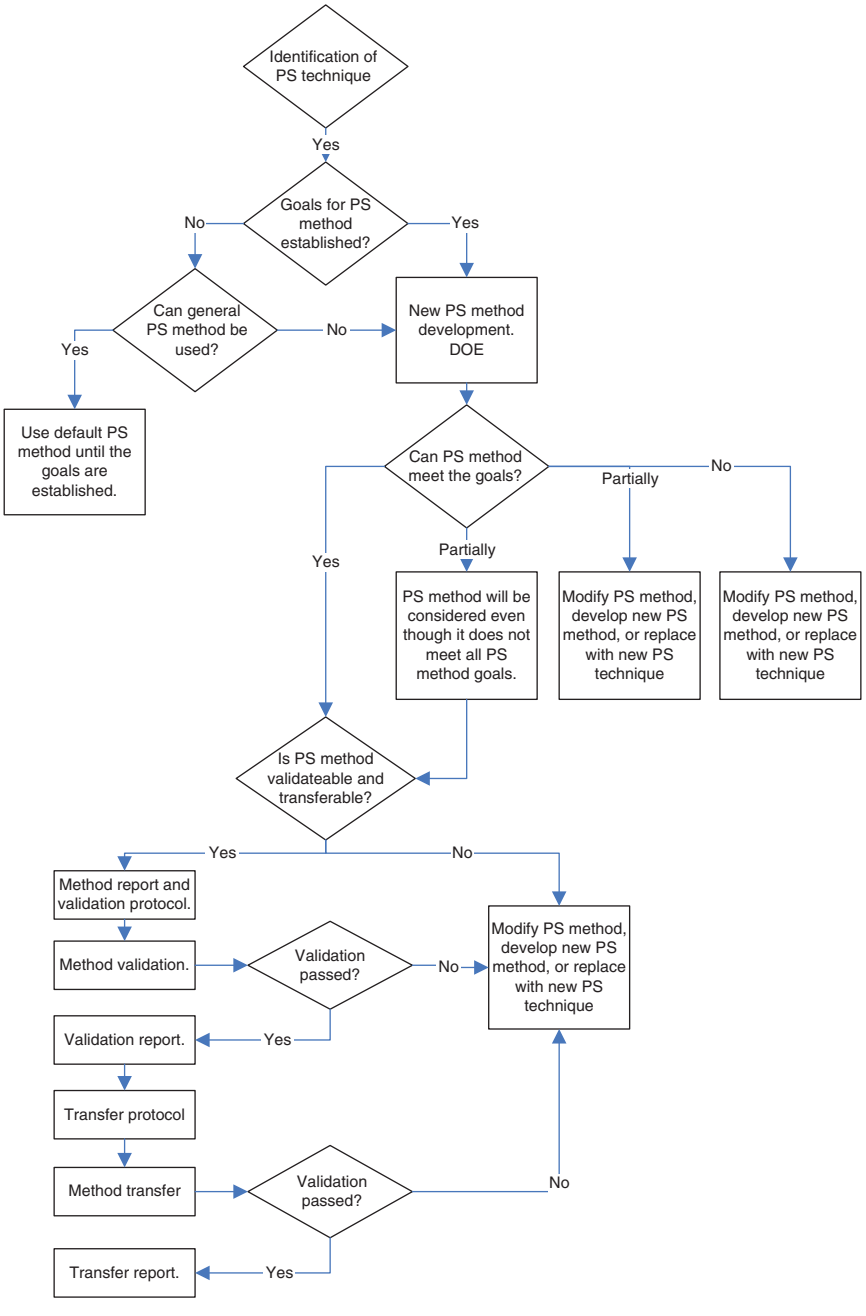


Figure 11.2 Activities and decision points associated with particle size method development and validation. *Source*: Hubert et al. 2008 [19]. Reproduced with permission of American Pharmaceutical Review.

11.4 Bioavailability and the Desired Particle Size

11.4.1 Particle Size and Bioavailability

Improving bioavailability, which can be simply defined as improving dissolution, solubility, and/or permeability, is the arena in which early development scientists work. Formulations can be developed to improve these three important parameters, while also assuring a good base for full development and manufacturing, once POC testing demonstrates adequate efficacy and safety.

The rate of dissolution is directly related to the surface area, as seen in the Noyes–Whitney or the Nernst–Brunner equation (11.1): [20–22]

$$\frac{\mathrm{d}C}{\mathrm{d}t} = \frac{DS}{Vh}(C_\mathrm{s} - C) \tag{11.1}$$

where C is the instantaneous concentration at time t, C_s is the saturation solubility, D is the diffusion coefficient, S is the surface area, V is the volume of the dissolution medium, and h is the thickness of the diffusion layer. This serves as the beginning of the understanding of dissolution, as well as the evolutionary start of the Biopharmaceutics Classification System (BCS) [23], as detailed in Chapter 8. Decreasing particle size, resulting in an increase in drug surface area, results in an increased dissolution rate, which can be more apparent and useful for drugs that have inherently low solubility.

It is also important to understand that the particle size can be related to total solubility. The saturation solubility C_s is normally considered a constant, but this is not always the case. Ostwald ripening [24] is often defined as a spontaneous process during which ultrafine crystals (or liquid droplets in an emulsion) dissolve and the corresponding material redeposits on larger crystals (or droplets) [25]. An extension of this concept, the Ostwald–Freundlich equation (OFE) is shown in equation (11.2),

$$C_\mathrm{s} = C_\infty \exp\left(\frac{2\gamma M}{r\rho RT}\right) \tag{11.2}$$

where C_∞ is the saturation solubility of an infinitely large crystal of a compound, γ is the crystal medium interfacial tension, M is the compound molecular weight, r is the particle radius, ρ is the particle density, R is the gas constant, and T is the temperature, which demonstrates that the saturation solubility increases at very small particle sizes [26]. This effect was made clear by Eslami and Elliott [27], who devised experiments and models to evaluate microdrop concentrating processes used in microfluidic technologies. This increase in solubility is possibly due to the higher surface area of smaller particles as modeled by the Noyes–Whitney or the Nernst–Brunner equation (Eq. (11.1)), higher curvature as described by the Gibbs–Kelvin–Kohler equations [28, 29], increased fractal dimensions as described by Mihranyan and Strømme [30], along with other possible mechanisms.

Improving bioavailability can be more complex than purely focusing on increasing solubility. Improving permeability and direct targeting of the desired site of action, though more difficult, are also important newer technologies. It is unlikely that particle size changes of a drug substance will affect permeability rates or mechanisms, unless the drug does not dissolve early enough in the

gastrointestinal (GI) tract to be absorbed, but that then gets back to solubility limitations. In such cases, increased adhesiveness to surfaces/cell membranes of smaller particles, especially for nanosized particles, will benefit the absorption due to larger contact area and longer contact period on the surfaces [31].

It should be noted that one of the most important applications of nanocrystalline formulation is to reduce the food effect. In most cases, food increases the bioavailability of the drug by increasing bile secretion and increasing the duration of gastric emptying time. Micronized or larger particles that have shown improved absorption in the fed state might benefit from solubility enhancement by the micelle formation related to bile salts in the food. Nanocrystals take an advantage by enhancing the initial dissolution rate because of the larger surface area. The higher rate of dissolution leads to an increased rate of absorption and, eventually, enhancement in the overall bioavailability; therefore, the absorption behavior of nanoparticles is irrespective of the bile salts concentrations in the food and is significantly less effected by the fed or fast state [32].

11.4.2 Initial Desired Particle Size

The traditional approach to particle size selection for preclinical and FIH studies was to make what was possible, measure it, and test it *in vivo*. This approach still works extremely well for drug candidates with high aqueous solubility. When it became evident that an increasing number of drugs in early development are practically insoluble in water, other approaches were added to the repertoire. These approaches included increasing drug solubility in the product decreasing particle size, selecting more soluble crystal forms (e.g. salts and cocrystals, as described in Chapter 10), stabilizing amorphous drugs (e.g. spray-drying, hot melt extrusion, coprecipitation with excipients), and targeting delivery systems.

Many development groups will design strategies that rely on the properties of new drugs to determine the desired particle size. Some of these strategies may apply to many new drug molecules in development and could be as apparently simple as milling all low solubility compounds to smaller than a specified particle diameter, often in the range of 5–25 microns as a maximum particle size. Other groups may develop more involved strategies to assure early development success, such as solubilization or freeze-drying. Each of these strategies has inherent issues. As an example, micronization has inherent questions such as whether the desired particle size is achievable by milling and whether the agglomeration of the micronized particles negatively affects solubility, and thus bioavailability, and which formulation aids can be used to decrease these inherent issues. Alternate attempts such as stabilizing an amorphous phase, which is inherently metastable, have a different set of issues including determining the best way to stabilize the metastable phase. Additionally, the particle size of the amorphous phase may also need to be investigated.

For new drug substances with limited solubility, modeling of the desired particle size is often used, initially based on preclinical study data and subsequently updated based on clinical results. Commonly, one or more modeling tools, possibly including GastroPlus™ (Simulations Plus, Lancaster, CA, USA), Simcyp (Certara, Princeton, NJ, USA), and other *in silico* approaches to absorption modeling, are used, often with additional programs, including MatLab

(MathWorks, Natick, MA, USA), and many statistical packages. With regard to particle size, it may be appropriate to evaluate models that include interfacial tension, curvature, surface roughness, and other particle considerations.

Though the use of computational modeling as a tool to guide formulation strategy and clinical study design is often proprietary, a few examples do exist. Figure 11.3 provides an example of a BCS class II lipophilic drug with solubility in aqueous media that varies with pH. The model was built by incorporating the pH solubility profile and the particle size distribution data with a compartmental pharmacokinetic (PK) analysis using clinical plasma levels [33]. The surface response curves in Figure 11.3a and b demonstrate that as particle size decreases, the effect of pH on C_{max} (maximum concentration) and area under the curve

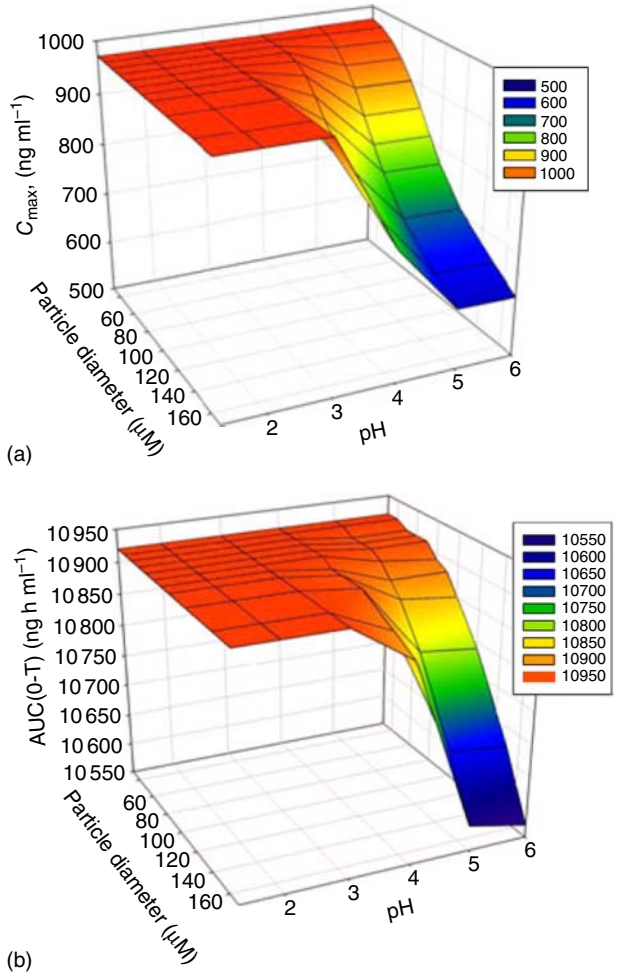


Figure 11.3 Case study for modeling with Bristol-Myers Squibb Company compound A. (a) Surface response plot of simulated C_{max} change with respect to mean particle diameter and pH change. (b) Surface response plot with simulated AUC change with respect to mean particle diameter and pH change. *Source*: Mathias and Crison 2012 [33]. Reproduced with Permission of Springer.

(AUC) is greatly reduced. For this example, maintaining the particle diameter below 100 µm maintains bioavailability even as pH increases, and the drug becomes less soluble. If appropriate preclinical models exist, it is possible to start building the model and provide particle size guidelines based prior to FIH start. It is also appropriate to update the models as more clinical data is available, which may mean that at least one leg of preclinical and/or early clinical studies must test the effect of particle size on exposure.

A different approach to modeling is to use similarity scoring to rank batches in terms of similarity to the reference batch. The introduction in Ferreira's et al. paper [34] provides an anthology of uses in various industries and includes a brief discussion of the various similarity metrics for cluster analysis. To develop similarity scoring, data from many batches is used. For particle size, it is often important to evaluate particle size, morphology, and surface area data. Principle component analysis (PCA) is then applied, and the similarity parameter is determined between the reference batch and all other batches. This makes it possible to mathematically distinguish batches, which can be confirmed experimentally. Figure 11.4 shows the SEM images of the reference batch (a), two batches ranked

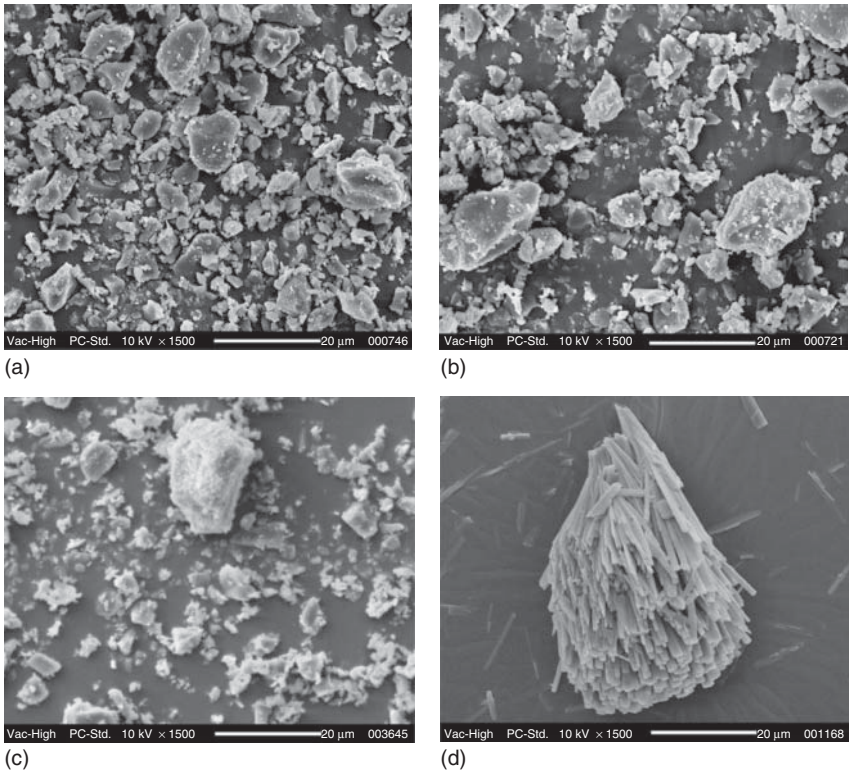


Figure 11.4 SEM images of the reference batch and the most similar and dissimilar materials using particle size, particle morphology, and surface area as the critical factors: (a) Bx-100 (API-07) (reference batch); (b) Bx-091 (API-07) (similar batch); (c) Bx-085 (API-01) (similar batch); (d) Bx-022 (API-15) (least similar batch). *Source*: Ferreira et al. 2016 [34]. Reproduced with Permission of Springer.

to be most similar (b and c), and a batch that is ranked most dissimilar (d). It is a short step mentally to anticipate that particle size, solubility, and bioavailability could be similarly analyzed with a large enough sample size, which could aid a development team to develop guidelines for particle size.

11.5 Enabling Formulation Approach by Particle Size Reduction in Early Drug Development

Many preclinical or early clinical development programs prefer to start with fit-for-purpose formulation options due to limited amount of available samples and short development period. During this phase, new drug candidates are evaluated by both *in vitro* and *in vivo* studies, in which solution formulations are frequently considered first [35]. As a significant proportion of drug candidates are poorly soluble in water, various methods are applied in order to achieve a solution formulation, for example, with pH adjustment if the molecules are ionizable, addition of surfactants, solubilization by cyclodextrins, utilization of cosolvent, or any combination of these methods. Bioavailability can benefit from a higher API concentration achieved by the formation of a supersaturated solution. However, sometimes extreme pH values and/or significant amounts of excipients are necessary in order to obtain a solution formulation. Drug candidates dissolved in solution under such a harsh condition have potential to precipitate *in vivo*. Furthermore, extreme pH values and/or the amounts of excipients required for solubility enhancement may introduce unwanted side effects. In some cases, even with these approaches during early formulation, solutions with the desired concentration could not be achieved within the development time frame. In these situations, suspension formulations are considered to be alternative formulation options. The suspension formulations are usually in a mild pH range and contain limited amounts of excipients, thus minimizing side effects for *in vivo* studies.

When a drug is administered by the oral route, the bioavailability of the specified formulation needs to be assessed. For a solution formulation, dissolution is not a major concern for the drug candidate unless the compound precipitates from the formulation solution or intestinal fluids. However, if the solubility of a drug candidate is low and a suspension formulation is needed, a higher dissolution rate is usually necessary to maintain a sufficient concentration of drug substance, as absorption is related to the amount of substance dissolved in intestinal fluids. Suspensions of reduced particle size drug substances are a classic formulation approach for BCS class II drugs, especially when they have a slow dissolution rate in the GI.

In some cases, microsizing crystalline materials of drug candidates sometime may not lead to sufficient drug exposure *in vivo*, making it necessary to further reduce the particle size of the drug candidates to a nanosized range to achieve a much higher bioavailability. Improvement of bioavailability by particle size reduction also depends on physicochemical properties of the drug substance. Nanosuspension approaches may not be necessary for a poorly soluble compound if the improvement of bioavailability is not significant since nanosuspension formulation is expected to have higher cost and development

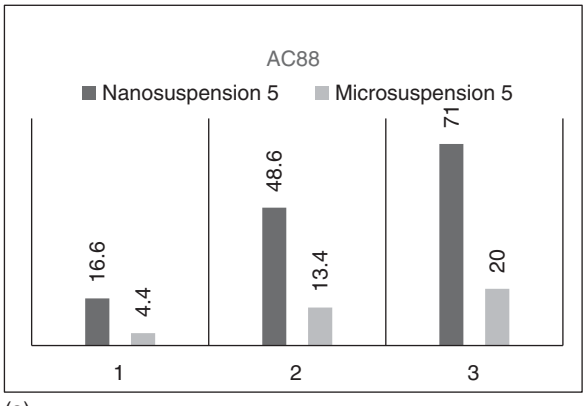
risk compared with that of microsuspension. Understanding the physicochemical properties of the drug substance is crucial to determine nanosizing and microsizing crystalline materials in achieving the desired bioavailability for suspensions. For neutral drugs, a suspension formulation is an attractive approach and maybe the only one to reach sufficient *in vivo* exposure and effect. The nanosuspension approach is also preferably applied on acidic compounds. For a suspension of a basic compound, on the contrary, the substance may dissolve or partially dissolved in the acidic stomach fluid; thus particle size reduction may not result in a bioavailability improvement.

A comparison between the crystalline nano- and microsuspensions of two BCS class II compounds, AC88 and BA99, was published in order to find a suitable formulation to be orally administered to rats in early development of drug candidates [36]. AC88 is an acidic compound, while BA99 is a basic compound. The two compounds have similar solubility in the intestine and can be processed into similar particle sizes for both microsuspensions and nanosuspensions; thus the impact of the physical property of pK_a in bioavailability improvement was assessed against the particle size reduction. A top-down approach was used in preparation of both microsuspension and nanosuspensions. The measured particle size of the nanosuspensions and the microsuspensions of AC88 was about 200 nm and 14 μm, respectively, while the particle size of the nanosuspensions and the microsuspensions of BA99 was about 280 nm and 12 μm, respectively. The PK calculations based on the individual plasma concentration–time data were reported and are shown in Figure 11.5. Each individual per oral exposure was compared with the AUC obtained with the intravenous (i.v.) dose.

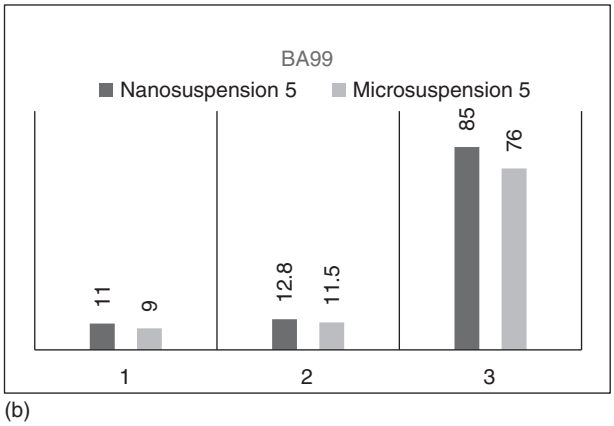
A significant difference was observed between the two suspensions for AC88 at the low dose, 5 μmol kg^{-1}. Compared with the microsuspension, the nanosuspension showed significantly increased C_{max} and AUC. At this dose, C_{max} and AUC for the animals receiving nanosuspensions were about four times larger compared with the exposure from microsuspensions. Moreover, bioavailability, F, of AC88 using nanosuspensions (5 μmol kg^{-1} dose: 70%) was significantly higher than when microsuspensions (5 μmol kg^{-1} dose: 20%) were administered. In contrast with AC88, there were no significant differences in C_{max} or AUC for the animals receiving microsuspensions of BA99 or the nanosuspensions of the compound at the doses administered. Bioavailability of BA99 using nanosuspensions (5 μmol kg^{-1} dose: 85%) was similar to the values obtained after the administration of microsuspensions (5 μmol kg^{-1} dose: 76%) comparing the same dose.

The study demonstrated a clear correlation between particle size and *in vivo* exposures for an acidic compound, the nanosuspensions providing the highest exposure. For a basic compound with the present properties and doses, a microsuspension was sufficient. In the latter case, the basic compound's higher solubility at gastric pH limits the need for particle size reduction.

This study also confirmed that nanosuspensions of AC88 and BA99 could be administered by an i.v. injection without adverse events to rats (at the present dose), demonstrating that neither the substances nor the particles caused negative effects. It should be noted that nanosuspensions may be the only alternative for i.v. solution formulations, as particles larger than 5 μm in i.v. formulation may then cause blockade or embolism since the smallest blood capillaries are about 5 μm in width.



(a)



(b)

Figure 11.5 *In vivo* drug exposure of 5 μmol kg^{-1} AC88 and BA99 nanosuspension ((a), black bar) and microsuspension ((b), gray bar). Three *in vivo* exposure metrics: (1) Maximum plasma concentration C_{max} (μmol l^{-1}). (2) Area under the plasma concentration–time profile AUC(h × kg l^{-1})/dose. (3) Bioavailability (F) was determined by AUCoral/AUCiv F(%). Column charts was drawn based on original data. *Source*: Sigfridsson et al. 2011 [36]. Reproduced with the Permission of Taylor & Francis.

In summary, although a solution is preferred for the fit-for-purpose formulation in the early drug development, it is not an easy task to develop a suitable formulation for poorly soluble drug candidates with a limited amount of available samples in a short time. Furthermore, extreme pH values and/or extra amount of excipients for such solution formulation may introduce side effects in the preclinical or clinical studies. In such cases, microsuspensions and nanosuspensions are fast and practical alternatives for solution formulation for the drug candidates with a poor solubility during early development. With laboratory-milling equipment and ultrasonic-assisted methods being introduced into laboratories, specifically for nanosuspension preparations, particle size reduction to improve the bioavailability of the drug candidates provides a popular nonspecific formulation approach in early drug development.

11.6 Benefits of Commercial Products Using Nanosized Crystalline Particles

Particle size reduction to nanosized range has emerged as a powerful formulation approach to improve the bioavailability of poorly water-soluble drugs. It may decrease systemic side effects in early drug development and may have some additional benefits during late stage and commercial product development.

Among various nanosizing approaches and technology, top-down wet media milling method became the first choice technology for nanosizing crystalline particles and dominated the commercial drug products formulated from nanosized crystalline drug substances [37]. Nanocrystal®, developed by Elan nanosystem at the beginning of the 1990s, is a carrier-free nanocrystalline drug delivery technology that includes the wet media milling technology to reduce the crystalline particles to a nanosized range and the surface modification method to stabilize the nanocrystalline materials for drug product development. Since the introduction of Nanocrystal technology, the scale of nanosizing pharmaceutical crystalline particles has grown to a commercial level and demonstrated significant benefits for commercial products [38]. Rapamune®, developed by Pfizer (formerly by Wyeth), was the first marketed product by using Nanocrystal technology. It was originally approved in 1999 by the US FDA as an immunosuppressive agent to help prevent organ rejection in patients 13 years and older receiving kidney transplants [39]. Rapamune contains rapamycin (sirolimus) as the active drug, which is a macrocyclic immunosuppressive drug derived from *Streptomyces hygroscopicus* (actinomycetes). It is available in two formulations, as an oral solution containing 1 mg ml^{-1} sirolimus and as tablets. In the latter, wet media milling nanosizing technology was applied to the drug substance. In addition to the user-friendly administration as a tablet formulation, the mean bioavailability of sirolimus after administration of the tablet is about 27% higher relative to the oral solution. Another advantage for the tablet formulation based on the nanocrystal technology is the better stability of the solid dosage. Rapamune Oral Solution® bottles should be stored protected from light and refrigerated at 2–8 °C (36–46 °F), while Rapamune Tablets® are recommended to be stored at room temperature up to 20–25 °C (68–77 °F).

Among all the commercial products for nanocrystalline particles, fenofibrate represented one of the best examples for the application of particle size reduction (from microsizing to nanosizing) to product development and drug lifecycle management.

Fenofibrate is a peroxisome proliferator receptor alpha activator used to reduce triglyceride (TG) levels in adult patients with severe hypertriglyceridemia and to reduce elevated total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), TG, and apolipoprotein (Apo) B and to increase high-density lipoprotein cholesterol (HDL-C) in adult patients [40]. Fenofibrate is highly lipophilic, virtually insoluble in water, and poorly absorbed. Various formulation strategies not only increased the bioavailability so that the daily dosage for each new generation was reduced but also improved food effects to benefit patients since coadministration with meals was necessary to maximize bioavailability of early

formulations [41]. The original formulation with non-micronized crystalline particles contains particle size larger than 20 μm, suffering poor and variable absorption of fenofibrate. The administration with food increases absorption to 60%, which is possibly due to availability of lipids and other surfactants in the food for solubilizing fenofibrate, as well as stimulation of gastrointestinal lipophilic absorption mechanisms [42]. Formulations containing micronized crystalline particles (10–20 μm) led to increased dissolution rates and greater bioavailability [43], which significantly reduced the daily dosage to 200 mg from 300 mg of non-micronized formulation and showed better and less variable absorption. Overall oral absorption remained poor, and coadministration with meals was still required. Subsequently, a microparticle drug delivery technology using phospholipid agents to modify surface properties and preserve the expanded drug surface area of the microparticles was developed, resulting in faster dissolution in GI fluids, along with improved and more predictable absorption than conventional micronized formulations [44]. The nanoparticle formulation represents the most prevalent formulation in commercial products of fenofibrate. Wet media milling technology further reduced particle size to 100–300 nm range with surface modification to prevent aggregation of particles, leading to a significantly increased ratio of surface area to volume and greater bioavailability [45]. Moreover the nanocrystalline formulation is the first formulation for which the drug exposure of fenofibrate is not altered when administered with or without food. Thus nanoparticle tablets (formulated as Tricor® by former Abbott Pharmaceuticals) can be taken with or without meals. Due to successful application of nanoparticles in commercial product development of fenofibrate, nanocrystalline formulations became an approach to minimize food interaction effects. Table 11.5 summarizes the various fenofibrate formulation developments.

Other commercial products utilizing nanosized crystalline particles include Emend® (aprepitant) by Merck for prevention of nausea and vomiting, Abraxane® (paclitaxel, taxol) by Celgene (former as Abraxis) for cancer treatment, Megace ES® (megestrol acetate) by Par pharmaceutical as an antianorexic

Table 11.5 Chronology of fenofibrate formulation development.

Formulation	Crystalline particles	Daily dosage (mg)	Food effect on bioavailability
First generation	Non-micronized	300	Administration with food increases absorption to 60%
Second generation	Micronized form	200	Increased 35% if given with food
Third generation	Microcoated-micronized form x	160	Slightly enhanced absorption if given with a meal
Fourth generation	Nanocrystallized form	145	Not altered if given with food or in fasting state

agent, and Invega Sustenna® (paliperidone palmitate) by Jansen, an extended-release injectable suspension for intramuscular use to treat bipolar disorder.

Applications of particle size reduction, especially nanosizing crystalline materials, significantly improved particle surface areas, dissolution, and bioavailability of commercial products. Compared with other formulation technologies, nanoparticle formulations provide several advantages for commercial products to benefit patients by replacing solution and suspension products with solid dosage formulations that are more patient friendly, lengthen chemical stability, and allow more convenient storage conditions. The reduction in food effect eliminates the requirement of coadministration with the possibility of reducing the frequency of dosing and increasing patient compliance.

11.7 Perspectives in Nanosizing Crystalline Particles

11.7.1 Nanoparticles and Targeting Delivery

Particle size reduction is a seemingly simple and direct approach to bioavailability improvement, especially for drugs with low solubility. Particle size also has a direct effect on many processing units such as mixing and on drug product quality attributes including content uniformity and compressibility. In the more recently developed nanoparticle and biopharmaceutical systems, particle size also has an effect on aggregation and activity at the target site [46]. In addition, nanocrystals may provide new treatment options (e.g. targeted intravenous delivery). Completely new products could also be generated.

Complex systems such as drugs intimately coordinated with polymeric or other large molecule carriers (e.g. spray-dried, inclusion complexes), or as functionalized nanoparticles often designed as biomaterial–drug complexes for targeted delivery, are often affected by particle size. In a perspective article by Blanco et al. [46], the size, shape, and surface charge affect distribution within the body. When evaluating distribution to the lungs, liver, spleen, and kidneys, particles less than 5 nm are filtered out by the kidneys, while particles greater than 150 nm in diameter collect in the lungs, liver, and spleen, where particles shaped as discs also predominate when compared with particles shaped as cylinders or spheres.

Often, nanoparticles are administered as i.v. formulations to avoid the rigors of the GI, including digestive enzymes and the first pass effect through the liver. These particles travel through the vascular bed and cross epithelial barriers before reaching the target site [47]. Small particles are eliminated by renal excretion, while larger particles can be taken into cells by phagocytosis, especially in the liver, spleen, and bone marrow. Nanoparticles leave the circulatory system through venous openings called fenestrations. The size of the fenestrations differs by organ. Additionally, some disease states such as cancer and macular degeneration can increase the size of the fenestrations, allowing larger drug molecules and other components of the blood to more easily escape from the circulatory system. Table 11.6 shows examples of some of the measured fenestrations.

One of the key advantages of novel nanosized molecules and/or particles is the potential enhanced permeability and retention (EPR) effect, which translates

Table 11.6 Claimed sizes of fenestrations of the vasculature in different organs and selected pathological states.

Organ or pathological situation	Fenestration size	Animal model
Kidney	20–30 nm	Guinea pig, rabbit, rat
Liver	150 nm	Mice
Spleen	150 nm	Mice
Lung	1–400 nm	Dog
Bone marrow	85–150 nm	Guinea pig, rabbit, rat
Skeletal, cardiac, and smooth muscle	≤6 nm	Mice
Skin, subcutaneous, and mucous membrane	≤6 nm	Mice
Blood–brain barrier	No fenestrations	–
Tumor[a)]	200–780 nm	Mice
Brain tumor[b)]	100–380 nm	Rat
Inflamed organs	80 nm–1.4 μm	Hamster

These values result from indirect measurements and should therefore be used with caution.
a) Implanted tumor.
b) Intravenously inoculated tumor.
Source: Gaumet et al. 2008 [47]. Adapted with permission from Elsevier. Please see this reference for fenestration measurement references.

in a passive drug targeting delivery. EPR is the property by which molecules of certain sizes (typically liposomes, nanoparticles, and macromolecular drugs) tend to accumulate in tumor tissues much more than they do in normal tissues. Most solid tumors possess unique pathophysiological characteristics that are not observed in normal tissues or organs. The vascular endothelium in tumors proliferates rapidly and discontinuously, resulting in extensive leakage and open junctions of blood plasma components. Thus, nanosized components, usually unable to cross the normal tissues, are allowed to cross the tumor-endothelial barrier into the tumor tissue due to these extensive leakage and open junctions. Moreover, the poor lymphatic clearance for nanosized molecules prevents the efficient removal of excess fluid from the solid tumor tissue, thereby allowing the drugs to remain there for substantial periods of time [48]. Taking advantage of the EPR effect has become a gold standard of targeting drug delivery design for cancer treatment. However, the EPR effect does not apply to low molecular weight drugs because of their rapid diffusion into the circulating blood followed by renal clearance [49]. Nanosizing crystalline drug substances makes it possible for smaller molecules to form nanosized crystalline particles, which have potential to take advantages of EPR effect in targeting drug delivery.

11.7.2 Emerging Nanoparticle Techniques

Nanoparticle techniques include both nanosizing of API particles and modifying of nanoparticle surfaces. In order to have a robust manufacturing process for nanosizing crystalline API, the most physically stable crystalline form is usually chosen for the drug substance candidate's nanocrystal formulation. Conventional

nanocrystal technology usually focuses on improving the dissolution rate to enhance bioavailability. The particle size reduction, even to a nanosized range, has a limited impact on the solubility of drug substance, making it difficult to be applied to drug development when a better solubility is needed to reach higher instant concentration for a formulation. Amorphous nanoparticle engineering has also been studied to increase API concentration. However, the use of nanoparticles of the amorphous drug in the formulation needs to overcome the challenge of stabilizing the amorphous form during both manufacture and the shelf life of the drug product. A pharmaceutical cocrystal is a crystalline material composed of two or more molecules within the same crystal lattice. Cocrystals have widely been applied to improve both solubility and dissolution rate for poorly soluble drug candidates, especially neutral molecules [50] and are discussed in details in Chapter 10. Nanococrystal applications can potentially increase API concentration without creating disadvantages in the stability and powder properties of the amorphous form. Special methods have been applied to the preparation of nanococrystals, including sonochemical synthesis [51] and spray flash evaporation [52].

Another emerging application of the nanosizing crystalline particles technique is the layer-by-layer coating technique applied to nanoparticles. Layer-by-layer deposition is a thin film fabrication technique. The films are formed by depositing alternating layers of oppositely charged materials with wash steps in between. This can be accomplished by using various techniques such as immersion, spin, spray, electromagnetism, or fluidics. As we know, surface properties play an important role throughout the development of nanocrystals in preparation and stabilization. Usually a large amount of excipients are needed to stabilize the nanosized crystalline particles, especially for nanosuspensions. The layer-by-layer coating technology offers a method that only coats a very small amount of various excipients (<1% weight) on the surface of crystals, which only increase the size of nanocrystal within a few of nanometers. Furthermore, nanolayer of the excipients self-assembling on the crystal surface to form capsules/nanoshells not only significantly increases the efficacy to stabilize the nanocrystals but also allows better controlling of the surface property of the particles. Such surface modifications offer the possibility of designing nanoparticle surfaces that can achieve controlled release and targeted delivery [53].

11.8 Conclusions

Particle size reduction technology from microsizing to nanosizing crystalline particles provides an extensive approach in pharmaceutical development of drug candidates as well as lifecycle management of existing drug products. Microsizing/nanosizing new drug substances during early drug development offers a fast and reliable formulation tool by modifying the powder and surface properties of the drug substance. As such, it supports the project goals of evaluating efficacy in clinical trials by achieving acceptable bioavailability and reducing the potential side effects introduced by excipients in traditional formulation. To this end, examples of drug products derived from nanosizing technology exhibited improved performances to benefit patients.

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12

Early Drug Development: From a Drug Candidate to the Clinic

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12.1 Preclinical Formulation Selection

Preclinical formulation selection is primarily driven by an assessment of the physicochemical properties of a new chemical entity (NCE). Numerous schemes have been proposed to select an appropriate formulation based on simple physicochemical properties such as pK_a, log D/P, molecular weight, and solubility [1–10]. However, what would appear to be a straightforward formulation exercise is often complicated by the limited availability and variable quality of active pharmaceutical ingredient (API) in the preclinical phases and the need to develop simple robust formulations under tight timelines to support biology/pharmacodynamic (PD) and pharmacokinetic (PK) studies. As a result of these pressures, simple formulations such as solutions or suspensions are preferred if possible. Solid form manipulation (e.g. salt selection) can be considered before more ambitious and resource intensive formulation approaches are evaluated. With the dominance of poorly soluble compounds in pharma's research pipelines, bioenhanced formulation technologies such as wet-milled nanoparticles, amorphous spray-dried dispersions, or supersaturable emulsifying systems are often needed to provide the necessary exposure in discovery biology studies. This is particularly true for formulations developed to support exploratory toxicology studies or later regulatory toxicology studies where the requirement to drive exposure to levels that provide the necessary safety margin for future clinical investigations is a significant formulation challenge.

12.1.1 Guiding Principles and Technology Selection for Preclinical Formulation

During early drug discovery, a range of *in vitro* and *in silico* strategies have been developed to screen a vast number of molecules emerging from high-throughput screening (HTS) and combinatorial chemistry to assess their potential to become successful drug candidates. Compounds emerging from these early screening processes are then tested in a series of *in vivo* studies to determine the critical absorption, distribution, metabolism, and excretion (ADME) properties,

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

Table 12.1 Aims and formulation requirements of different preclinical studies.

	Pharmacodynamics	Pharmacokinetics	Toxicology
Study aim	• Understand mechanism of action • Efficacy	• Define ADME properties	• Determine safety profile • Dose setting for early clinical studies
Dose range	• Low/medium	• Low	• High
Typical route of administration	• Oral • Intravenous • Intraperitoneal • SC	• Oral • IV	• Same as clinical route of administration
Formulation requirements	• Rapid turnaround • Minimal bulk for development	• Does not alter intrinsic PK of compound	• Does not in itself cause toxicity

pharmacological effects, and safety profile of the candidate [11–13]. Despite the different objectives and formulation requirements (Table 12.1), these studies all rely on achieving sufficient and reproducible exposure to enable robust assessment of the parameters tested. In the past, when the majority of NCEs had good solubility and permeability, traditional practices such as the "disperse and dose" approach, where discovery biologists, chemists, or drug metabolism scientists could formulate simple solutions or suspensions for early preclinical studies, were able to achieve the exposure required [3]. However, as the number of NCEs with solubility and/or permeability challenges has increased, this approach is no longer suitable. Solution formulations are still preferred for early PK/PD studies to mitigate the impact of inconsistent exposure due to the quality of the API available at this stage (e.g. crystallinity and particle size). However, the formulation challenge to design and prepare appropriate solution formulations with poorly soluble API has become more complex, and more sophisticated approaches with enabling technologies are often required such as lipid-based drug delivery systems, nanosuspensions, or amorphous spray-dried dispersions.

The limitations imposed on the formulation scientist at this stage – minimal information on API properties, limited material for experimentation, and short formulation development timelines – have resulted in the dominance of empirical formulation development approaches. Typically, formulation design relies on a combination of trial-and-error experimentation using simple pH adjustment or precedented vehicles containing solubilizing agents such as polyethylene glycol (PEG) 200, Tween 80, or sodium lauryl sulfate. Such an empirical approach is not necessarily the most efficient method of formulation development. As a result, significant effort has been invested in developing more structured, efficient approaches for the selection of preclinical formulations to reduce the amount of experimentation required as well as to minimize formulation development cycle time. Examples of these include classical decision trees [1–3, 5, 11, 14], which link physicochemical properties of the API to appropriate formulation technology and solubilization technology application maps (Figure 12.1). More

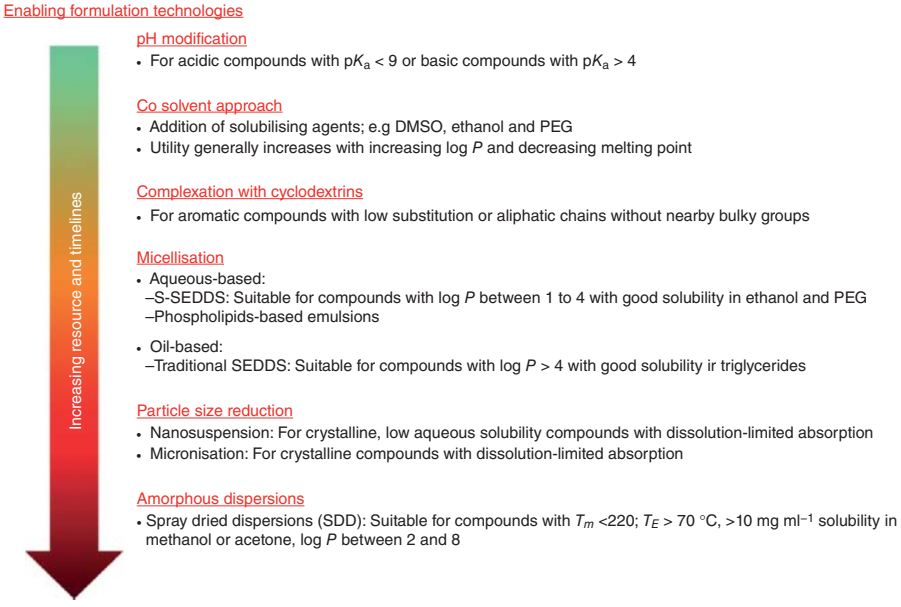

Figure 12.1 An example of a solubilization technology application map linking formulation selection to API properties and resource required for development at the authors' lab.

recently, computational-based approaches using software such as COSMO-RS and SAFT-gamma have been investigated as potential technologies to predict API solubility in solubilizing agents [15, 16].

Another more practical challenge for the formulation scientist is to ensure that the formulation selected can be manufactured reproducibly at a range of scales – from the small volumes (1–5 ml) required to support the PK/PD studies to the larger volumes (greater than 500 ml) to support long-term toxicology studies. An example of a nanomilling setup designed to produce reproducible nanosuspensions over a range of batch sizes is described below (Figure 12.2). The setup involves the use of simple equipment such as conventional magnetic stirrer bars and glass vials for the preparation of small-volume nanosuspensions (1–25 ml), while overhead stirrers in combination with polypropylene impellers are used to support the preparation of larger volume nanosuspensions (50–2000 ml). Both the small and large volume setup follow the same wet milling process to produce nanosuspensions, whereby, the API is added to the vehicle and attrition milled using grinding media such as yttrium oxide or zirconium oxide beads until a suitable API particle size is achieved. This process has been shown to effectively reduce the particle size of the ingoing material over a relatively short period without affecting the crystallinity of the material.

Once an appropriate formulation has been selected, it is recommended that characterization work is conducted on the formulation to confirm the stability of the formulation for the duration of study required. The amount of work conducted should be appropriate for the development stage of the compound. For example, during the early stages of development, this may be limited to visual

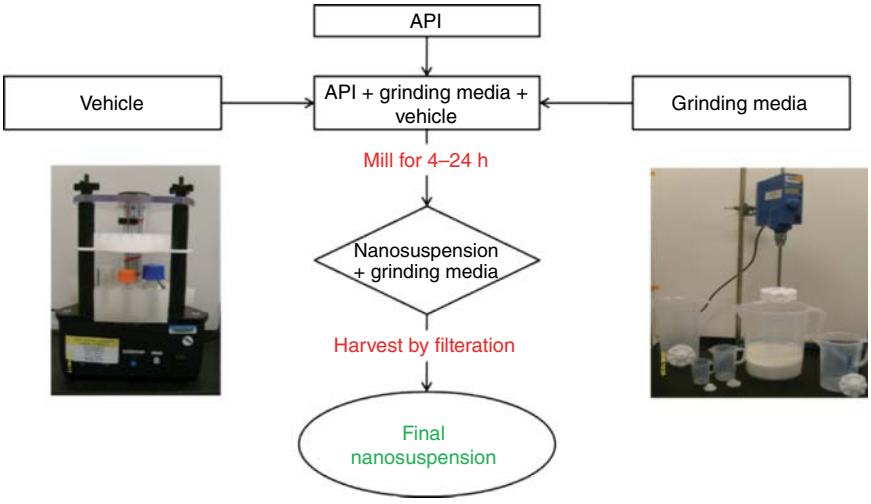

Figure 12.2 Wet milling process for preparation of nanosuspension formulations at different volumes with the setup for the smaller volume (1–25 ml) shown on the left and larger volumes (50–2000 ml) shown on the right.

inspection of solution formulation to ensure no precipitation occurs or the use of light microscopy to assess the particle size and crystallinity of the API in a suspension. At later development stages when larger quantities of material are available, a more thorough series of characterization experiments may be conducted. Table 12.2 provides an example of the characterization work conducted within the authors' labs.

In summary, as the number of NCEs with solubility and/or permeability issues increases, simple solution and suspension formulations can no longer be relied upon to deliver the required exposure for *in vivo* assessment of the NCE. As a result, more complex formulation technologies have been developed. However, the selection of a suitable formulation for the NCE being studied is dependent of a range of factors (Figure 12.3), which the formulation scientist needs to take into consideration prior to the nomination of a final formulation.

12.1.2 Predicting Preclinical Formulation Performance

Currently, preclinical formulation design is typically guided by the physicochemical profiling of an NCE and the availability of *in vivo* exposure data from early PK studies. For simple formulation strategies, this approach is often adequate to guide the critical factors for formulation design. However, with poorly soluble compounds, it is often necessary to triage various technology options, and in this scenario the use of predictive tools to guide selection is not as well established as is the case in later phases of development. Ideally, the process of selecting an optimal bioenhanced delivery approach would be informed by rigorous *in vitro* testing, using scale appropriate dissolution tests. These would characterize formulation performance under conditions that simulate the gastrointestinal environment of the relevant preclinical species. The development of *in vitro*

Table 12.2 Example of the type of formulation characterization work conducted at the different stages of preclinical formulation development.

Non-GLP		GLP
PK/PD	**Non-GLP toxicology studies**	**Regulatory toxicology studies**
All formulation types		
• Chemical stability • Potency (optional)	• Chemical stability • Potency	• Chemical stability • Potency
Solutions		
• Visual (ensure no precipitation over duration required for study) • pH (optional) • Filter compatibility for IV formulations (optional)	• Visual (ensure no precipitation over duration required for study) • pH (optional) • Filter compatibility for IV formulations (optional)	• Visual (ensure no precipitation over duration required for study) • pH • Filter compatibility for IV formulations
Standard suspensions		
• Particle size • Crystallinity (optional) • pH (optional)	• Particle size • Crystallinity • pH • Homogeneity (optional)	• Particle size • Crystallinity • pH • Homogeneity
Nanosuspensions		
• Particle size • Crystallinity • pH (optional)	• Particle size • Crystallinity • pH • Homogeneity (optional)	• Particle size • Crystallinity • pH • Homogeneity
Micellisation (e.g. SEDDS/s-SEDDS)		
• Visual (ensure no precipitation over duration of study)	• Visual (ensure no precipitation over duration of study)	• Visual (ensure no precipitation over duration of study)
Amorphous dispersions (e.g. spray dried dispersions)		
• Crystallinity (bulk and formulated suspension) • Viscosity • pH (optional)	• Crystallinity (bulk and formulated suspension) • Viscosity • pH (optional)	• Crystallinity (bulk and formulated suspension) • Viscosity • pH

dissolution tools that successfully simulate such conditions has not been simple. An important limitation is the ability of *in vitro* test systems to adequately reproduce the gastrointestinal environment of preclinical species. There are a multitude of challenges associated with developing a system, which can be truly deemed biorelevant for preclinical species. One of the most significant hurdles to overcome is to design an *in vitro* test to work with volumes that accurately simulate the low volumes of fluid present in the GI tract of the typical preclinical species used for early biology and safety assessment studies [17]. As an example, in the case of the rat species, this volume is only a few milliliters. Additional challenges are also posed by the requirement for fluid composition and hydrodynamics to be representative of the gastrointestinal environment of commonly used laboratory animals [18]. The compendial dissolution methods that are

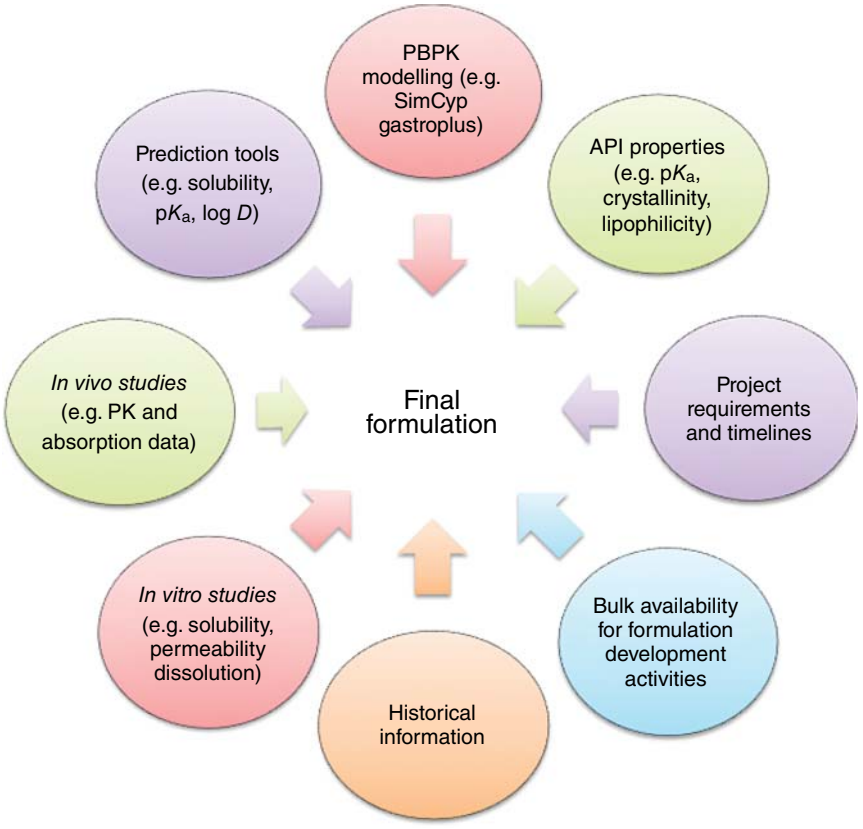

Figure 12.3 Factors to be considered prior to nomination for preclinical formulation work.

widely deployed for formulation testing in later phase clinical development do not adequately simulate the volume and hydrodynamics of preclinical species, and poor correlations have been reported for formulations with poorly soluble compounds [19]. The limitations for the application of compendial approaches to preclinical formulation testing have been widely recognized, and a number of nonconventional dissolution technologies have been developed as alternative approaches [20, 21]. Several systems utilize small-volume vessels that have been devised. These facilitate a relative assessment of *in vitro* performance using a few milligrams of API or formulation intermediates (such as a wet-milled nanosuspension or spray-dried dispersion). Furthermore, they also more closely represent (although still overestimating) *in vivo* fluid volumes for preclinical species. A widely used example of such a system is the μDiss-Profiler™ small-volume system from Pion Inc. (Billerica, MA, USA). This system comprises multiple sample vials each with a typical dissolution volume of between 15 and 20 ml, with controlled temperature and stirring and a multichannel spectrometer providing in-line analysis by UV probes. The Sirius Inform™ equipment (Sirius Analytical, Forest Row, Surrey, UK) has been designed with similar principles in mind but enables the automated change of pH and can provide a second organic

layer, which can operate as a sink for dissolution. The provision of this second compartment can be used to simulate the process of removal of dissolved drug from the GI tract by permeation and maintain sink conditions for dissolution of poorly soluble compounds and formulation intermediates [18, 22]. Previous studies with cell-based dissolution–permeation (D–P) systems have suggested that the correlation to *in vivo* performance can be improved if the dissolution assay can be configured in such a way as to simulate the absorptive permeation step and maintain a sink for dissolution [23]. In a study with bioenhanced formulations (which included solid dispersion, nanoparticulate, and micronized approaches) of fenofibrate, the D–P system was used to correlate *in vitro* dissolution and permeation with *in vivo* data in rats [24]. This study illustrated the potential for combined D–P systems, when used with biorelevant media, to predict formulation performance in rats. The utility of the cell-based D–P approach has been limited by some practical considerations, many of which are related to the resource and specialized equipment/facilities required to grow and maintain the cell-based membranes required for such tests. Additionally, the static compartment in the D–P system does not easily facilitate the simulation of the dynamic processes of the intestinal environment. For example, the gastric to intestinal transfer of weak bases and resulting supersaturation and precipitation profiles that can be critical for understanding bioperformance would not be reproduced under those conditions. Cell-based models can also have significant limitations in terms of using biorelevant media due to toxicity of media components. An alternative to the cell-based D–P approach is offered by the μFlux™ adaption of the μDiss-Profiler system that uses donor and acceptor chambers separated by an artificial membrane. The membrane can be coated with lipids to incorporate a parallel artificial membrane permeability assay (PAMPA) and enable the system to monitor dissolution and permeation simultaneously [25]. It should be noted that the flux/mass transfer rates achievable with simple artificial membrane systems relative to *in vivo* permeation require adaptation of the *in vitro* experimental design to optimize predictive power.

A number of systems have been developed, which seek to address the limitations discussed above [20, 26]. One such widely used example is the artificial stomach duodenal model (ASD), which has been designed to allow the process of gastric emptying to an intestinal compartment to be simulated [27, 28]. In the ASD, API or formulated drug product is dispersed in the stomach chamber, and the gastric contents are transferred at a controlled rate to the duodenum chamber where it is mixed with simulated intestinal fluid (SIF), allowing the dynamic processes of dissolution, supersaturation, precipitation, and recrystallization to be followed. Early studies by Carino et al. [29] reported the successful use of the ASD model to simulate the fasted and fed state for dog physiology for a series of carbamazepine polymorphs. The *in vivo* relevance of ASD dissolution profiles is based on the correlation between the AUC of the concentration–time profiles in the simulated duodenal and *in vivo* bioavailability. Several other studies support the utility of the ASD model to accurately profile the performance of formulations developed for clinical studies [30–33]. However, the use of this model can be compromised for some preclinical studies by the factors previously discussed (e.g. fluid volume or lack of an absorptive surface). In one such

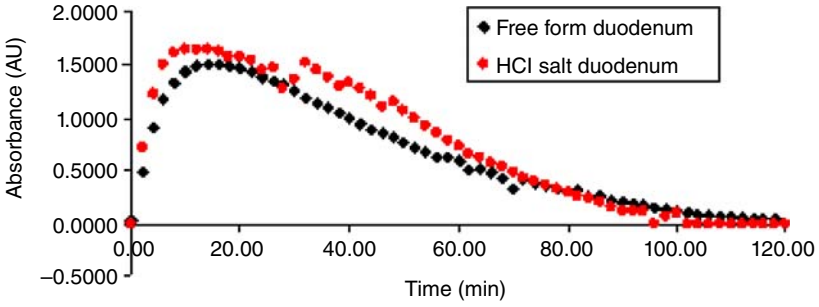

Figure 12.4 ASD profiles for free base and HCl salt forms of a drug candidate molecule.

example, which highlights these issues, we used the ASD model to profile the relative performance of suspensions of a free base and HCl salt for a high-permeability/low-solubility candidate progressing toward toxicology testing. The ASD predicted that the two solid forms would be equivalent in their performance (Figure 12.4), but subsequent rat toxicokinetic studies (at 100 mg kg^{-1}) showed that the HCl salt was far superior with an approximately fourfold increase in AUC. It is difficult to determine the root cause for the lack of correlation with *in vivo* performance in this case, but the relatively high fluid volume employed in the ASD is likely to have minimized the difference between the forms and did not capture the impact of high, supersaturated concentrations of the HCl salt, which are likely to have driven the *in vivo* exposure observed. It is the opinion of the authors that many such studies remain unreported in the literature, and there is a need to reconsider the experimental parameters used in transfer models such as the ASD when they are used to predict formulation performance in small rodent species.

In conclusion, there would still appear to be significant challenges for the successful application of small-scale dissolution tools to predict the performance of preclinical formulations. The additional analytical complexity associated with monitoring dissolution performance from the high doses used in toxicology studies in small volumes of biorelevant fluids remains to be resolved [21]. Further research is required to develop a technology that is specifically tailored to simulate the complex GI physiology of preclinical species. To be successful in this endeavor, it would appear necessary to envisage integration of multiple aspects of the systems outlined earlier in this chapter. A predictive dissolution technology that mimics the physiological aspects of preclinical species in terms of dynamic fluid composition/volume, hydrodynamics, and permeation/absorption would perhaps finally provide formulation scientists with a reliable alternative to *in vivo* PK experimentation.

12.2 Formulation Selection for FiH

The cycle time for pharmaceutical development of NCEs is constantly under review to identify opportunities to reduce the time, resource, and cost of bringing new drugs to market [34, 35]. In response to such pressures, it is

becoming commonplace to limit formulation investment for clinical candidates until key development milestones are reached: e.g. successful readout from Phase II clinical proof-of-concept studies is a typical trigger for full formulation development activities to commence. In this paradigm, simple, fit-for-purpose formulation approaches are being increasingly adopted to advance NCEs to early clinical studies and avoid the considerable pharmaceutical development resource required to design and develop a traditional solid dosage form. Such formulations can range from powder in bottle dispersions, extemporaneously prepared (EP) solution or suspensions, to simple, unformulated powder in capsule (PIC) dosage forms comprising of the API in a hard gelatin capsule shell with no additional excipients [36, 37, 38].

12.2.1 Extemporaneous Preparation

EP formulations offer several advantages over conventionally manufactured dosage forms for early clinical studies. The use of an EP approach can enable the rapid clinical evaluation of an NCE and reduce the resource, development time, and API bulk requirement associated with traditional GMP-manufactured drug product. EP formulations also facilitate a degree of flexibility in dose selection during clinical studies, as doses can be adjusted based on real-time clinical study data. An EP formulation is compounded in an on-site pharmacy at a clinical research unit (CRU). It is prepared individually for each subject under the supervision of a trained pharmacist. In the United States, compounding an EP tablet is a practice of pharmacy, not manufacturing, and requires formulation development with an abbreviated stability program to support the in-use period. As a result of these differences, the lead time for EP formulations is typically much shorter than conventional manufacturing. There are several studies reported in the literature illustrating the use of EP formulations in early clinical studies with examples of solutions [39, 40], suspensions [41, 42], tablets [39], and even controlled release dosage forms [43, 44].

12.2.2 Powder in Capsule (PIC) Formulation

While EP formulations can be a very efficient approach to perform a small-scale clinical evaluation of API or simple formulation variants, they are not applicable to outpatient studies, which require a drug product. A suitable alternative in this context can be provided by using simple "PIC" dosage forms. These have many of the advantages associated with EP formulations in terms of minimal stability and API requirements but have the notable advantage that they can be manufactured using automated equipment to provide the scale of supply required for larger clinical studies. The application of the PIC approach in recent years has been facilitated by the availability of small-scale automated production technology such as the Capsugel Xcelodose® precision powder microdosing systems, which has the capability to fill as little as 100 μg into different size capsules at production rates of between 200 and 600 capsules/hour [45–47]. This approach confers additional benefits such as weight verification, which can eliminate the need for analytical release testing of PICs and provide a route to real-time release

of batches. Stability testing programs can also be simplified through the extrapolation of API drug substance stability to the encapsulated material. Compared with other fit-for-purpose formulation options such as solutions or suspensions, PICs are generally more convenient, particularly in a clinical outpatient setting as a relatively large number of dosage units can be manufactured in a short period of time.

12.2.2.1 Clinical Performance of PIC Dosage Forms: A Retrospective Data Analysis of Pfizer NCEs

A major concern with the PIC approach is the lack of formulation optimization and a potential disregard for the biopharmaceutical properties of the API. Indeed a recent study examining bioequivalence (BE) data has shown a significant failure in conventional BE studies even for class I compounds [48], suggesting that this risk is not small and may lead to formulation-related development challenges at a later stage. There is limited clinical information in the literature on the oral PK performance for PIC dosage form and therefore little understanding of whether the lack of formulation optimization negatively impacts the clinical outcome. PICs contain none of the excipients that are usually incorporated into a conventional tablet or capsule to aid disintegration, wetting, and redispersion of API primary particles to ensure consistent dissolution performance. Given that a significant proportion of NCEs require formulation help to overcome poor or variable oral absorption due to low solubility and/or permeability [49, 50], it is clear that the PIC approach could be viewed as inappropriate for NCEs categorized as low-solubility, BCS class II or IV candidates [14, 51]. When assessed in the context of achieving the key objectives of early clinical studies (such as defining a maximally tolerated oral dose), a PIC dosage form could be considered to bring significant additional risk by virtue of suboptimal *in vivo* performance delivering low or variable exposure. This could lead to subsequent failure of Ph II studies due to low or variable exposure not achieving efficacious concentrations. In addition, Ph II programs may require more subjects and a longer duration in order to statistically power studies to gain sufficient data for compounds exhibiting high variability. However, when the advantages with the associated savings in both time and cost to reach the next clinical milestone are considered, the PIC approach remains an attractive option for early drug development, despite the increase in potential formulation-related risk.

To further understand the most appropriate application of PIC formulations, a review of our extensive in-house databases was undertaken, obtaining clinical data for 21 compounds that used PIC formulations [52]. Information was collated, and studies were undertaken to understand how the biopharmaceutics properties of a drug (solubility, permeability, and dose number) impacted on the oral PK bioperformance of PIC formulations in the clinic. It was the objective of this study to investigate if criteria for physicochemical or biopharmaceutics properties could be established to appropriately select suitable compounds for a PIC approach. To achieve this, clinical data obtained from Phase I and Phase II clinical studies for 21 NCEs were collated and used to assess the impact of NCE solubility, permeability, and dose number on *in vivo* PK performance. Comparability of PIC

to other formulation approaches was assessed by comparing the PK performance (C_{max}, area under the curve (AUC), T_{max}).

For the 21 compounds selected for the study, solubility and permeability values were obtained using standardized methodologies to provide a consistent data set within which cross-compound comparisons could be made. Solubility determinations were performed using a 96-well filter plate automated assay. Measurements were made in pH 1.2 SGF, pH 6.5, 50 mM sodium phosphate buffer, and fasted state simulated intestinal fluid (FaSSiF) as described by Galia et al. [53]. The maximum solubility measurement of the automated assay was 0.3 mg ml^{-1}. In order to calculate an accurate dose number for key compounds with a higher solubility than 0.3 mg ml^{-1}, further manual solubility studies using similar methodology were undertaken with larger amounts of compound.

Physicochemical properties were also collated with the octanol–water partition coefficient (log P_{oct}), the octanol–water distribution coefficient (log $D_{7.4}$), and pK_a, calculated using ACD log *P*, log *D*, and pK_a software (Advanced Chemistry Development Inc., Toronto, Canada). Compounds were assessed for the "rule of 5" compliance. Compounds that were "rule of 5" compliant had no violations of the four rules created by Lipinski [54]: i.e. they had a molecular weight < 500, log *P* < 5, <5 H bond donors, <10 H bond acceptors.

Permeability (P_{app}) data was collated from *in vitro* Caco-2 cell studies conducted in-house using methodology described by Artursson [55]. The permeability models used were validated with high- and low-permeability compounds as described by the FDA's BCS guidance document [56]. As the permeability data was generated over a number of years and collated retrospectively, the permeability classification was confirmed by repeat permeability measurement, for compounds where available, and *in silico* measurements (data not shown).

Dose solubility numbers were generated as described by Amidon et al. [51]. Dose solubility numbers of <1 are consistent with the compound being classified as a high-solubility compound [51]. Dose solubility numbers were calculated for the compounds where confidence in assigning "clinical success" was high, i.e. where comparative reference formulation data was available. The dose number was generated using the dose where the comparative reference formulation data was available rather than using the entire dose range. This is regarded as most relevant as this is the dose where clinical success is actually assessed (rather than the entire dose range).

12.2.2.2 Clinical Data Analysis Methodology

The clinical performance data for this analysis has been compiled from a wide range of Phase I and Phase II studies for 21 NCEs that were designed to specifically investigate the safety and efficacy of the compounds in a small number of volunteers and patients. This analysis is based on a retrospective analysis of the collected PK data from a diversity of study designs and protocols; thus the amount and type of clinical data available varied for each compound and in some cases was limited. However in general, the studies were performed in healthy male subjects aged 18–45 years. All studies were conducted in accordance with the Declaration of Helsinki [57] and in compliance with all International Conference on Harmonisation Good Clinical Practice Guidelines ((ICH) 1997).

Written informed consent was obtained prior to the inclusion of any subject into the studies.

The typical clinical study design employed was a dose escalation study where single increasing doses of the NME were administered in a PIC formulation in the fasting state and serial blood samples ($n = 10–15$) were drawn in order to characterize the pharmacokinetics of the compound. Noncompartmental methods were used to analyze the data – C_{max}, and T_{max} were directly observed from the data. AUC was calculated using the log–linear trapezoidal method [58]. Where more than one formulation was studied, a randomized single-dose crossover study was typically utilized, with an appropriate washout period between doses. Formulation comparisons were made using ANOVA appropriate for the individual study design.

A formulation's oral PK performance and impact on clinic outcomes can be difficult to define at early stages of development. If the formulation delivers sufficient exposure of the NCE to enable oral dose escalation and the safety of the drug in a small number of volunteers is established, then it could be considered to deliver a successful clinical outcome. In this analysis we are focusing on examining whether the formulation performance is optimized. Suboptimum formulation performance could impact pharmacokinetics and could affect the clinical outcome in terms of reaching an accurate no-observed-adverse-effect level (NOAEL) or maximum tolerated dose. In the analysis of the clinical data, we defined an approach that allowed us to categorize the PIC formulations into what we term "successful" or "unsuccessful." This approach was used to classify the utility of the PIC formulation. Success was defined as a PIC formulation showing the same rate and extent of absorption as a reference formulation with no evidence of higher variability than a reference formulation (or where no reference formulation available that would normally be expected). These three criteria were used to assess the clinical performance of the PIC formulations and to allow categorization as "successful," "unsuccessful," or neither.

Further definitions of the criteria that defined successful application of the PIC are shown below:

1) *Extent of Absorption Criteria*. AUC and C_{max} data of PIC formulations were comparable with that obtained for alternative reference formulations, e.g. solution, suspension, or tablet. The conventional 80–125% BE limits recommended in regulatory guidances to compare PK performance of formulations were used. As Phase I studies are rarely powered sufficiently for formal BE comparisons, an additional less stringent criterion to define formulation equivalency using limits of 50–200% for AUC and C_{max} was also used.
2) *Variability Criteria*. A PIC dosage form was defined as equivalent to a reference formulation if variability seen with AUC and C_{max} data did not exceed that observed with the reference formulations. In those cases where a reference formulation was not available, PIC dosage forms were considered nonvariable if the % CV was less than 50% for either AUC or C_{max}.
3) *Rate of Absorption Criteria*. PIC dosage forms were defined as equivalent to reference formulations if T_{max} values were within 2 h of the reported average T_{max} value for the reference formulation.

In order to assess compounds against these criteria, a series of parameters were used to judge clinical success. The parameters were scored in a standardized way to avoid investigator bias and to ensure all compounds were assessed in the same way (Table 12.3). The extent of absorption criteria (as defined above in "1"), where a comparative reference formulation data set was available, was regarded as the strongest evidence to support a classification of clinical success, followed by the variability criteria (as defined above in "2") and lastly by the rate of absorption criteria (as defined above in "3"), which was regarded as the least critical parameter set. The scoring system was devised to reflect this order of importance, and scores in each category were allocated with a suitable weighting to prioritize the

Table 12.3 Criteria used to calculate score for clinical performance of PIC formulations.

Item	Parameters used to judge clinical success	Defined limit	Score assigned for question response			
			Question response (where PIC + reference formulation data available)		Question response (where PIC data only available)	
			Yes	No	Yes	No
1	Is the AUC for PIC and reference formulation similar, i.e. within defined limits?	80–125% 50–200%	12 6	0 −12	– –	– –
	Is the C_{max} PIC and reference formulation similar, i.e. within defined limits?	80–125% 50–200%	12 6	0 −12	– –	– –
2	Is the PIC between subject variability % CV for AUC ≤50%?	–	2	0	1	0
	Is the reference formulation between subject % CV for AUC > 50%?	–	1	−1	–	–
	Is the PIC between subject variability % CV for C_{max} ≤50%?	–	2	0	1	0
	Is the reference formulation between subject % CV for C_{max} > 50%?	–	1	−1	–	–
3	By how much does the T_{max} of the PIC differ from the reference formulation?	≤1 h 1–2 h ≥2	1 0.5 −1	– – –	– – –	– – –
	Score range		31	−27	2	0
	Median of score range		0	2	0	1

three sets of criteria appropriately. The overall score for the clinical performance of the PIC for each compound was obtained, and this established whether the PIC was "clinically successful," "clinically unsuccessful," or whether it could not be determined. If the overall score obtained for a PIC formulation was:

- >median scoring value classified as "clinically successful."
- <median scoring value classified as "clinically unsuccessful."
- =median scoring value classification cannot be determined.

In addition to the above, a scoring system to reflect the confidence in the conclusions was also established. This allows us to represent the degree of confidence we have in the categorization of the compounds as "successful" or "nonsuccessful." The most confidence in the data was where the extent of absorption data was available for comparative reference formulations. Four categories were identified and prioritized in descending order from (a) where there was the greatest confidence through to (d) where there was the least confidence:

a) Statistically relevant formal PK comparison/BE data available in the same subjects.
b) PK comparison data available from same study/subjects though study not statistically designed to demonstrate BE.
c) PK comparison data available from different clinical studies/subjects.
d) No PK comparison data available.

To aid visualization confidence is depicted graphically by the size of the data point. Four different sizes represent the above categories (a)–(d) being the largest and smallest, respectively (Figures 12.5–12.7).

12.2.2.3 Relationship Between Physicochemical Properties and Clinical Performance for PIC Dosage Forms: Results from Clinical Data Analysis

Physicochemical Properties, Solubility, Permeability, and Dose Number The compounds that had been progressed as PICs were from a number of different therapeutic areas/chemical series and thus had high structural diversity and very different physicochemical characteristics (Table 12.4). Molecular weight and calculated log *D* values ranged from 187 to 570 Da and from −1.35 to 3.52, respectively. Acidic, basic, and neutral compounds were represented in the data set. The majority of compounds studied were "rule of 5" (71%) compliant.

The data set was found to include compounds with a broad range of solubility values ranging from <0.0005 to >0.3mg ml^{-1}, which was the limit of the automated assay used to provide a standardized data set for the 21 compounds studied. For compounds with solubilities above the limit of the automated screen, manual studies were performed to more accurately define the solubility profile, and it was seen that some compounds had solubilities as high as 21 mg ml^{-1} (Table 12.5).

A broad range of permeability values were obtained showing the data set contained both high- and low-permeability compounds. P_{app} values ranged from <1to 44.7 ($\times 10^{-6}$ cm s^{-1}). Interestingly, the data set contained compounds that

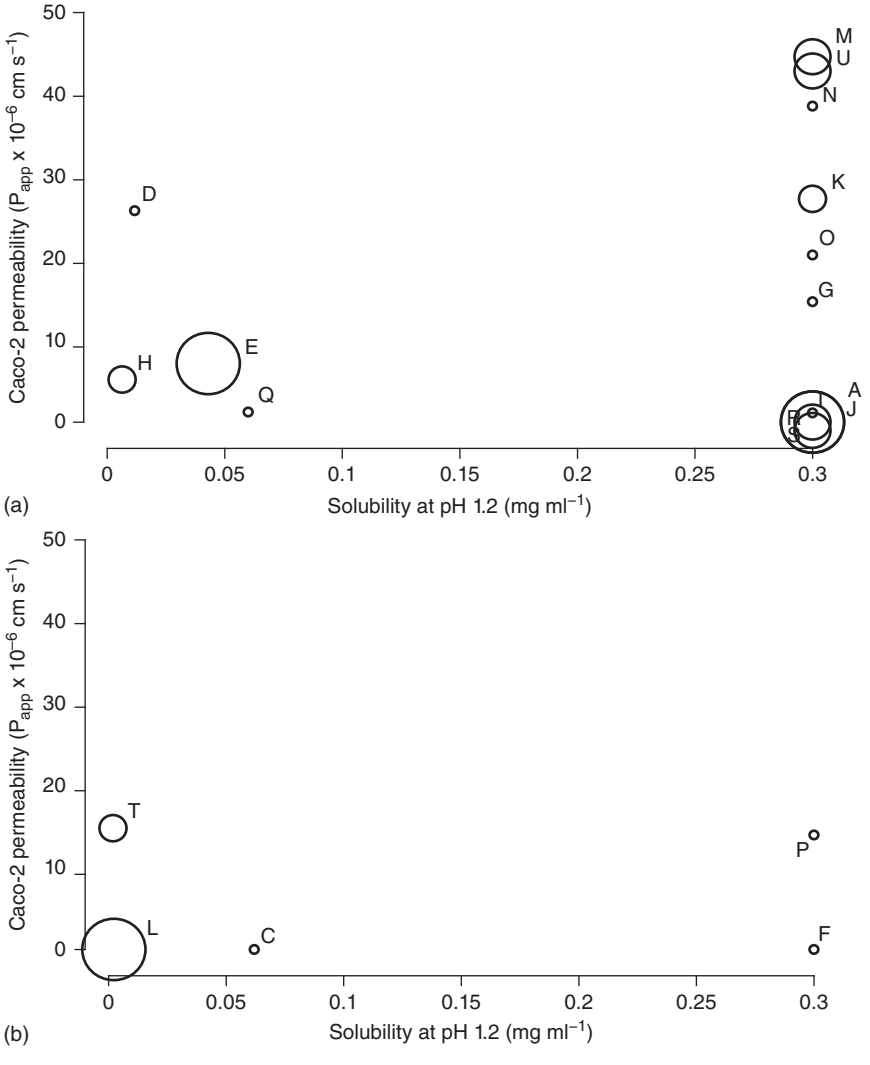

Figure 12.5 Influence of permeability and gastric solubility on (a) clinically successful and (b) clinically unsuccessful PIC formulations.

also appeared to be substrates for efflux mechanisms as can be observed from the high efflux ratios (Table 12.6).

Defining the BCS class and dose number for the compounds in the data set is complicated by the nature of Phase I single-dose escalation studies, which means that the formulation performance is tested over a very wide dosage range (typically a 100- to 1000-fold dose range). Often the clinically efficacious dose is not accurately defined, and it is necessary to use an estimation of this from the Phase I data set. Within this context, dose solubility numbers (D_0) were calculated when subsequent studies had been performed with alternative formulations developed

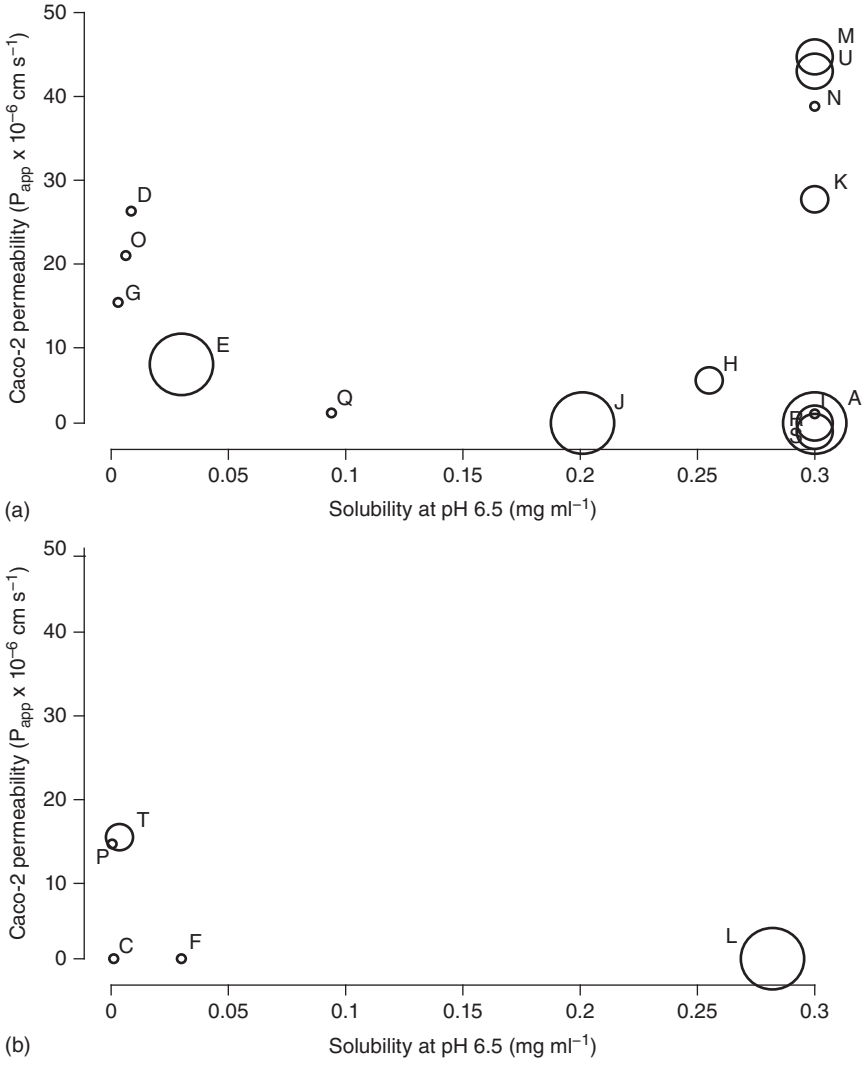

Figure 12.6 Influence of permeability and intestinal solubility on (a) clinically successful and (b) clinically unsuccessful PIC formulations.

specifically for a narrower range of clinical doses. For this set of compounds, dose solubility numbers ranged from <1 to 43.5 (Figure 12.7a, b).

Clinical Success of PIC Formulations: Impact of Solubility, Dose Number, and Permeability
The clinical data for each compound were assessed utilizing the parameters and scoring system detailed in Table 12.3. The overall score was determined, and compounds which had a score above the median were categorized as "successful," whereas compounds with an overall score below the median were categorized as "unsuccessful." Of the 21 PIC formulations studied, 15 (71%) were categorized as

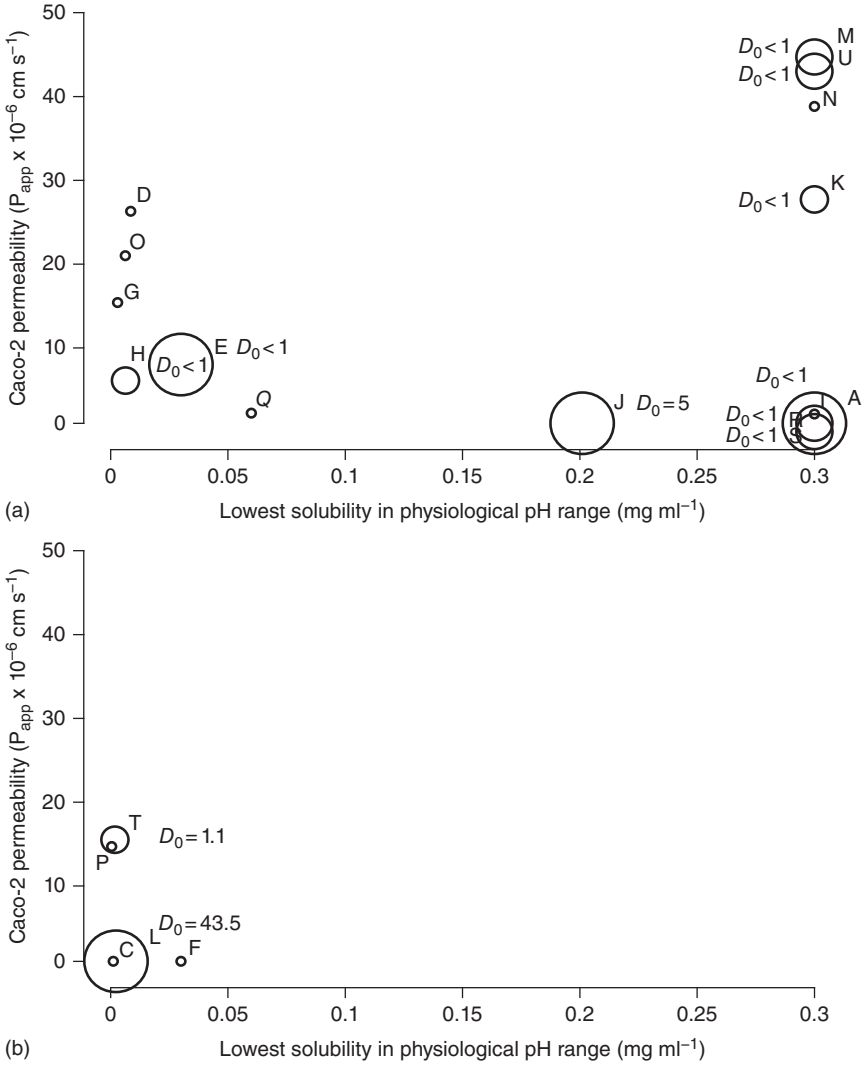

Figure 12.7 Depiction of dose numbers for PIC formulations – influence of permeability and the lowest gastrointestinal solubility measurement on (a) clinically successful and (b) clinically unsuccessful PIC formulations.

"successful," 5 (24%) were categorized as "unsuccessful," and 1 (5%) was neither "unsuccessful" nor "successful" (Figures 12.5 and 12.6).

Of the 21 PIC formulations studied, only 11 (52%) of the compounds had PK data available for comparative reference formulations allowing C_{max} and AUC comparisons. The data show that for these compounds, for which we have relatively high confidence in the assignment of clinical success (depicted by the use of large circles in Figures 12.5 and 12.6), delivery using a PIC dosage form resulted in 9 out of 11 (82%) compounds being classed as successful.

Table 12.4 Compound properties.

Compound	Molecular weight	*c* log *D*	*c* log *P*	Rule of five violations	pK_a	Acid–base assignment
A	187	−0.72	2.37	0	3.81[a], 10.46[b]	Both
B	455	2.29	4.41	1	8.25[b]	Base
C	469	3.52	5.68	1	8.25[b]	Base
D	468	0.073	1.3	0	12[a]	Acid
E	431	2.45	5.1	1	12.8[a], 7.74[b]	Both
F	497	−0.879	2.25	0	4.3[a], 3.4[b]	Both
G	462	3.16	3.59	0	6.25[b]	Base
H	463	0.352	5.38	1	3.15[a], 2.09[b]	Both
I	303	−0.639	0.39	0	Neutral	Neutral
J	450	1.06	4.73	0	9.81[b]	Base
K	377	−0.425	1.64	0	10.1[b]	Base
L	570	0.174	4.63	1	3.91[a], 1.01[b]	Both
M	322	−0.477	1.77	0	9.16[b]	Base
N	350	0.386	2.65	0	9.16[b]	Base
O	372	1.67	0.052	0	4.66[b]	Base
P	510	−1.35	−0.744	2	3.1[b]	Base
Q	396	0.879	3.47	0	4.72[a], 2.76[b]	Both
R	392	0.782	4.45	0	8.95[a], 10.6[b]	Both
S	392	0.782	4.45	0	8.95[a], 10.6[b]	Both
T	477	2.38	3.36	0	Neutral	Neutral
U	274	2.92	3.42	0	7.39[b]	Base

a) Acid functional group.
b) Basic functional group.

There does appear to be a relationship between solubility and clinical success for PIC dosage forms (Figures 12.5 and 12.6). In general, compounds that were unsuccessful appear to have low intestinal solubility of $<0.03 mg\,ml^{-1}$ (Figure 12.6b). In contrast, there does not appear to be any relationship between permeability and clinical success. Clinically successful compounds are observed for both high- and low-permeability compounds.

The dose was also considered in addition to solubility. The dose numbers were generated for the compounds for which there was confidence in the clinical success assignment status due to the availability of reference data. Dose numbers were generated at the dose where comparative reference formulation data was available. It is interesting to note that for 10 of the 11 compounds, which were assigned as clinically successful, the dose number was calculated to be not greater than 5 and typically was <1(Figure 12.7a, b).

Dissolution or kinetic solubility measurements may be more important parameters to use in this analysis. This is exemplified by compound T, which may not be appropriate for development using a PIC dosage form despite having a low dose

Table 12.5 Physiologically relevant solubility.

Compound ID	Solubility mg ml^{-1}		
	pH 1.2 SGF	pH 6.5 (50 mM sodium phosphate buffer)	pH 6.5 FaSSIF
A	ND[a)]	**21***	ND[a)]
B	0.005	0.0008	0.002
C	0.062	0.0007	0.0012
D	0.0117	0.0091	0.0086
E	0.043	0.0118	0.03
F	>0.3	0.02	0.03
G	0.3	0.003	ND[a)]
H	0.0064	0.155	0.255
I	>0.3	>0.3	>0.3
J	>0.3	0.128	0.201
K	>0.3	>0.3	>0.3
L	0.0023	>0.3	0.282
M	>0.3	>0.3	>0.3
N	>0.3	>0.3	>0.3
O	>0.3	>0.0032	0.0063
P	>0.3	0.0006	0.0005
Q	0.06	0.073	0.094
R	**>10***	**1.49***	ND[a)]
S	**1.95***	ND[a)]	**5.12***
T	0.0019	0.0018	0.0036
U	>0.3	>0.3	>0.3

Bold text indicates data generated by manual assay. The lowest solubility obtained in either pH 1.2 or pH 6.5 FaSSIF was used to generate Figure 12.5. Where pH 6.5 FaSSIF data was not available (see * table), then solubility data for pH 6.5 media was used.
a) Not determined.

number. There are considerable differences in human PK when the PIC dosage form is compared with a simple tablet formulation (containing excipient lactose, crospovidone, sodium lauryl sulfate, and magnesium stearate) (Figure 12.8). It should be noted that the PIC and tablet data were generated in separate clinical studies: i.e. the comparison is being made between studies. However, formulation comparison is strengthened by the fact that the same batch of API was used for both PIC and tablet dosage forms, so differences due to particle size can be excluded. It was observed that in healthy male subjects, the 1mg tablet showed a fivefold increase in C_{max} and twofold increase in AUC_{inf} compared with a 1 mg PIC. The PIC formulation appears to have slow dissolution, which impacts both the rate and extent of absorption, whereas the tablet formulation appears to have better dissolution *in vivo*, resulting in a significantly improved profile. This particular compound has very low solubility (Table 12.5) across the physiological pH

Table 12.6 Summary of Caco-2 permeability data.

Compound ID	Apical to basolateral permeability (P_{app} – 10^{-6} cm s^{-1})	Basolateral to apical permeability (P_{app} – 10^{-6} cm s^{-1})	Efflux ratio
A	0.6	2.6	4.3
B	5.7	11.1	2.0
C	1.0	4.3	4.3
D	26.3	7.6	0.3
E	8.0	12.6	1.6
F	<1	8.4	>8.1
G	15.4	26.7	1.7
H	6.1	23.1	3.8
I	2.1	23.1	11.0
J	<1	15.9	>15.9
K	27.7	28.7	1.0
L	<1	6.8	>6.8
M	44.7	34.9	0.8
N	38.8	30.0	0.8
O	21.0	27.9	1.3
P	14.7	ND[a)]	N/A[b)]
Q	2.2	7.0	3.2
R	<1	40.3	>40.3
S	<1	9.9	>9.9
T	15.5	38.8	2.5
U	43.0	37.0	0.9

a) Not determined.
b) Not applicable.

range, and the superior performance of the tablet is likely to be attributable to the impact of excipients, particularly the use of sodium lauryl sulfate as a wetting agent, which will have improved the wettability and subsequent dissolution of compound T.

In summary, this retrospective analysis of clinical study data shows that PIC formulations can be a useful tool in early development. Evaluation of historical data has shown that where we have confidence in our assignment of clinical success (due to the availability of data for alternative formulations), then >82% of such compounds selected for a PIC dosage form appear to be successful. All compounds that were not found to be successful had low solubility (<0.03 mg ml^{-1}) at either gastric or intestinal pH. In this data set, a large number of clinically successful compounds had a dose number less than 5 with the majority being below a dose number D_0 of 1. Further expansion of the data set would be required to understand in additional details where PIC formulations can be successfully applied beyond comfortable BCS Class I-type physicochemical boundaries. This

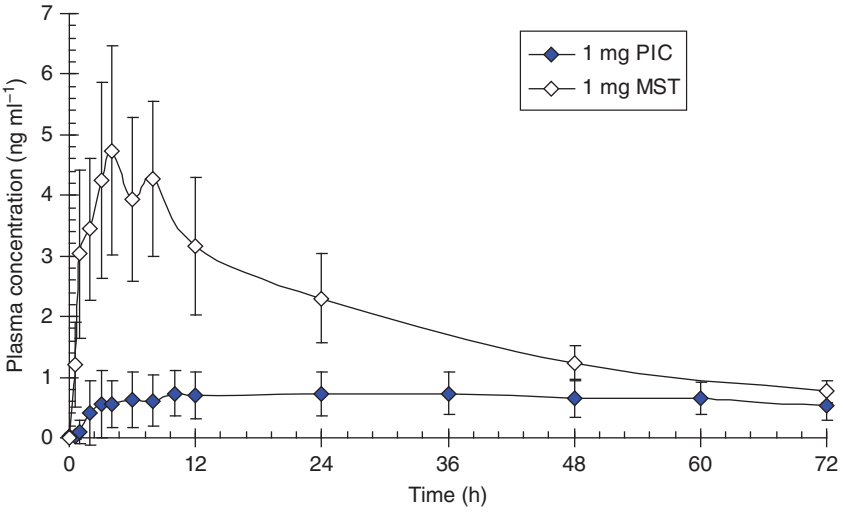

Figure 12.8 Comparison of compound T pharmacokinetic profiles for a tablet and PIC formulation.

is particularly relevant given the continued low-solubility APIs being produced from discovery pipelines across the pharma industry [59].

There are a number of additional limitations in this analysis and data set, which should be recognized and addressed to fully understand the "design space" for PIC application. The data set is relatively limited especially in the number of confidently assigned "unsuccessful compounds." There is only one compound (L) in the data set to which we have confidently assigned a high dose number (D_0 44). We therefore do not yet have sufficient evidence to define the edge of failure. In addition the second compound assigned as confidently unsuccessful (compound T) has a low dose number (1.1), which exemplifies the importance of broadening this evaluation to consider other parameters such as dissolution, wettability, and particle size. Thermodynamic solubility measures may not be the optimum parameter to assess *in vivo* performance, and there may be more value in using intrinsic dissolution rate (IDR) measurements or an IDR/solubility ratio to more accurately correlate to *in vivo* dissolution performance for poorly soluble APIs.

In this analysis permeability values do not appear to show any relationship with clinical success. Permeability is complex, and there are a number of different transport mechanisms involved in absorption: e.g. paracellular/active uptake/efflux mechanisms that are not necessarily reproduced appropriately with *in vitro* models. For further analysis and future work, it would be reasonable however to consider the interplay of permeability and kinetic solubility with regard to the presence of sink conditions for dissolution within the lumen of the GI tract. This might help determine if compounds could be selected for PIC or other simple formulation platforms based on a composite analysis of their permeability/dissolution rate or kinetic solubility characteristics, perhaps through an extension of the developability classification system (DCS) approach proposed by Butler and Dressman [60].

The clinical studies included in this analysis show that PIC dosage forms have been used to deliver wide dose ranges in early phase studies. However, the analysis performed has not allowed us to establish whether the PICs were successful over the entire dose range. This is because the judgment of clinical success used a narrower dose range as subsequently defined in later studies with formulated drug product. As this data set continues to be expanded, it would be useful to consider if additional analysis of PK parameters could prove useful in this respect. For instance, one could assess trends for dose nonlinearity that are not anticipated through preclinical absorption modeling and can be directly attributed to drug product performance and absorption rather than distribution, metabolism, or excretion factors. Further work is required to understand the utility of the PIC approach, for the ever-increasing number of low-solubility compounds, which are progressing into first-in-human (FiH) studies.

While further work will facilitate the more rigorous application of PIC dosage forms beyond high-solubility compounds as defined by BCS or DCS, in reality the practical application of PIC dosage forms is likely to be for those compounds that have reasonable solubility and a single dose that could be considered as midrange (very broadly defined as in the region of 10–200 mg). More development work is required to robustly manufacture low submilligram doses. The impact of API isolation/crystallization needs to be carefully assessed, as changes in physical properties (which are often seen with early small-scale API batches) can have a significant impact on the filling process. High doses can also be problematic for PIC dosage forms due to the time required for filling unit dosage forms. Resolving such concerns simply through the use of multiple unit dosage forms to achieve the target dose needs to be aligned with the clinical study protocol in terms of supply chain practicalities and clinical acceptability. We anticipate that, at least to some extent, the design space for PIC will be dictated by practical considerations in addition to the design space as defined by clinical performance. Further expansion of the design space for PIC dosage forms would benefit from a holistic analysis of kinetic solubility measures, API intrinsic dissolution, wettability, and biorelevant dissolution of PIC drug product.

12.3 Conclusion

It is widely recognized that industrial drug development pipelines contain an ever-increasing number of NCEs, which possess challenging pharmaceutical properties in terms of dissolution and solubility. As a result, it is often necessary to adopt enabling formulation technologies during the early preclinical development phase to ensure consistent exposure in toxicology/safety evaluation trials. The selection process varies widely across industrial preformulation groups and in some cases is driven by the availability of enabling technologies. However, it is apparent from the divergence in approaches with regard to technology selection that there is often not just a single, unique delivery solution, and the different decision trees published by industrial research formulation groups reflect this position. Different technologies that address dissolution and/or

solubility rate limitations can be used interchangeably in many instances if the fundamental limitation of oral absorption is well characterized/understood and the technology is selected on this basis.

Once a NCE has successfully passed through safety toxicology testing, the onward need for enabling technologies for FiH or first-in-patient (FiP) studies can be assessed. Often, a simpler formulation approach can be adopted for these studies as the challenge for delivering the dose range for single and multiple ascending dose studies is comparatively less relative to that posed by preclinical toxicology studies. At this stage in development, speed and flexibility for clinical dosing become key considerations, and the use of "fit-for-purpose" formulations is commonplace. In this context, EP formulations provide much needed flexibility and offer an accelerated path to the clinic through reduced development time and API requirements. Encapsulation of API within a hard gelatin or HPMC capsule to provide a PIC dosage form provides similar benefits to EP in terms of speed and API requirements and facilitates FiP use. However, it is apparent from the analysis of bioperformance of these simple dosage forms that the use of the PIC approach should be limited to NCEs with appropriate biopharmaceutic properties. Ultimately, the decision to use a simple EP or PIC dosage form for early clinical studies will need to take into account a number of factors including how the utilization of these approaches will position a project for onward development. In the context of accelerated development programs, which are now widely precedented in therapeutic areas such as oncology, the decision to use a PIC to move quickly into FiP or FiH clinical studies needs to be balanced against the prospect of rapid progression to subsequent Phase II studies. In this scenario, bridging studies to establish relative bioavailability between FiH and Phase II dosage form will be needed. In this context, the impact of any potential delays due to lack of formulation equivalence will need to be considered. In conclusion, the use of simple enabling formulation approaches for early clinical studies may have several inherent risks as we have reviewed, but the savings in resources required and shorter development timelines that may be achieved will continue to drive the use of such approaches during early drug development.

Acknowledgments

The authors would like to acknowledge Jake Morris and Michael Cram for the provision and analysis of data for the Pfizer compound data set.

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13

A Practical Guide for the Preparation of Drug Nanosuspensions for Preclinical Studies: Including *In Vivo* Case Studies

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13.1 Introduction

The tolerability of vehicles for sparingly soluble drugs is, and has always been, a frequent challenge for the pharmaceutical industry. Considering vehicle side effects and excipient interferences in sensitive pharmacokinetic and/or pharmacodynamic (PK/PD) models, a formulation with low excipient content is desirable. Furthermore, there is currently a trend toward fewer compounds that are neither pH adjustable nor soluble in the strongest cosolvent vehicles approved for *in vivo* studies. Because of time and resource limitations during early drug development, suspensions are a common and practical formulation approach for a large fraction of drug hunting projects.

The most common problem with poorly water-soluble drugs is the low exposure *in vivo*. If a compound showing low exposure has a medium to high permeability and no other physiologically related problems (e.g. first pass metabolism, efflux), the major cause to insufficient exposure is mainly limitations in dissolution (and/or solubility) at therapeutic doses. The biopharmaceutical classification system (BCS) is commonly used to emphasize two central compound properties for formulation development: solubility and permeability [1], as described in Chapter 8. A common design goal for the medicinal chemist is to achieve a certain level of lipophilicity to guarantee sufficient cellular permeability. As a result, more BCS class II (low solubility, high permeability) compounds are being assessed during drug development today, as compared with BCS class IV (low solubility, low permeability) compounds. This means that the largest challenge is achieving adequate solubility (and therefore also dissolution) at a cellular permeability level compatible with oral absorption. For compounds that fall into the BCS class II category, absorption and bioavailability are improved, up to a certain dose level, by maximizing the dissolution rate. This can be achieved, as also described from a more theoretical point of view in Chapter 11, with smaller particles that present a larger surface area and reduced diffusion layer thickness to the dissolution media [2]. Therefore, for a compound with intermediate solubility, a microsuspension may give adequate exposure at therapeutic doses, although at a certain compound-specific dose, there will be saturation in exposure. For more

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

sparingly soluble compounds, or at "medium/high" doses of intermediate-soluble drugs, absorption may be hampered [3]. In these cases, a drug nanosuspension, characterized by a significantly reduced particle size (particle diameter ≪1 μm), will dissolve and solubilize faster, thus providing the sparingly soluble drug with a much better opportunity for absorption.

Another possible way to increase exposure is to achieve a supersaturated state, like, e.g. the amorphous state [4, 5] or a metastable crystalline state [6–8]. The higher apparent solubility will provide not only a higher dissolution rate but also a higher driving force and compound concentration gradient for absorption, as compared with the most stable crystalline state. On the other hand, a higher supersaturation will also result in faster crystallization. Some compounds have very stable amorphous states that can be utilized for development of amorphous drug nanosuspensions for long-term toxicological studies. Other compounds have amorphous states just stable enough to benefit from during early preclinical research. For some compounds the supersaturation is so high that crystallization takes place instantly.

A typical example of a metastable crystalline state, often used, is a simple salt of a weak basic drug. Depending on the solubility of the possible species (salt, amorphous or crystalline free base), quite high supersaturations can be achieved over a range of pH, as illustrated by a hypothetical example in Figure 13.1. However, amorphous and/or crystalline free base will eventually precipitate, and the supersaturation benefits will finally be lost. When preparing suspensions of such

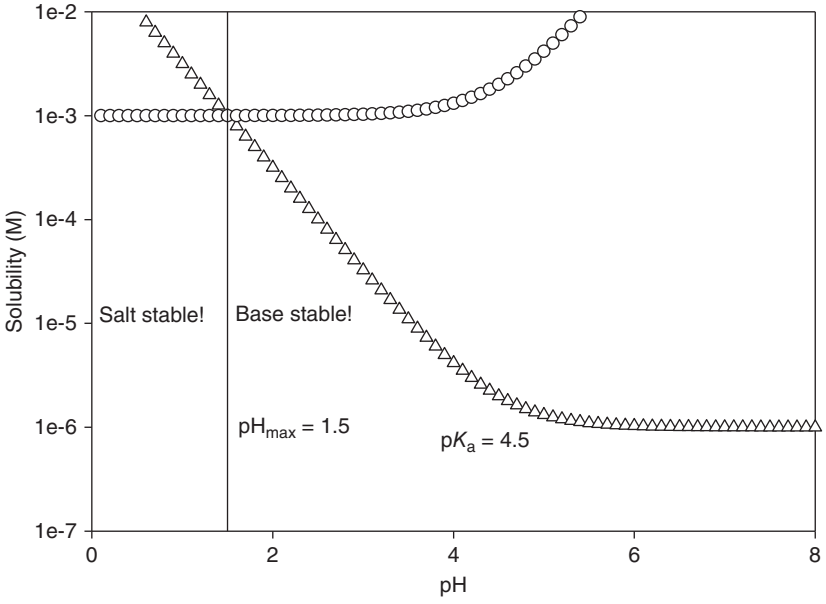

Figure 13.1 pH-solubility profile for a simple salt (circles) of a weak basic (triangles) drug with an intrinsic solubility of 1 μM and a pK_a of 4.5 and where the salt form has a solubility of 1 mM. The line indicates the pH$_{max}$, where the thermodynamic solubility of the drug is at its maximum. Below this pH the salt is the stable form and at higher pH the free base is stable.

salts, it is important to keep the pH of the suspension in the region where the salt is thermodynamically stable, i.e. below pH_{max} as indicated in Figure 13.1 to avoid conversion to the free base.

Crystalline drug nanosuspensions (nanocrystals), using the most stable form, can almost always be stored frozen and reconstituted to the initial particle size [9]. Amorphous drug nanoparticles are generally not suitable for storing, but for a few compounds, it has been possible to freeze and thaw such suspensions with consistent particle size and without any occurring crystallization. Today, both crystalline and amorphous drug nanoparticles are used on a frequent basis in routine preclinical work at AstraZeneca R&D Gothenburg. Working with a few prototype nanosuspensions, as dictated by the compound properties, it has been possible to support early *in vivo* studies in a rational and safe way with minimal usage of precious material. Nanosuspensions are also suitable for parenteral routes of administration. For some drugs, nanoformulations are the only option available for intravenous (i.v.) administration, thus filling an important need during animal PK studies, where the compounds are often administered both orally and i.v., preferably by using the same stock preparation. Moreover, both amorphous and crystalline drug nanosuspensions have been used for intraperitoneal (i.p.) [10], subcutaneous (s.c.) [11, 12], and intracerebroventricular (i.cv.) [13] injections (and data on file, AstraZeneca). The parenteral opportunities have enabled more sensitive PD models to be used during early development and also to evaluate new challenging biological targets in the lead identification phase. As mentioned, a general benefit is that the same formulation can be used for different routes of administration; thus a minimum amount of vehicle screening together with a minimum amount of vehicle controls needs to be run *in vivo*. A nanosuspension offers a further advantage since the drug does not need to be solubilized and small volumes with high concentrations are readily prepared. For i.v. injection, the administered dose can be considered as a "controlled" precipitation. However, when a poorly water-soluble compound is administered by the i.v. route, a slow infusion is preferred over a bolus injection.

This chapter focuses on approaches to the development of preclinical drug nanoparticle formulations that has been developed at AstraZeneca R&D Gothenburg based on work with more than 2000 different compounds. Briefly, as a mean of introduction, alternative formulation approaches are discussed. At the end of the chapter, three case studies using nanoparticles are presented to show the versatility, advantages, and limitations of the approaches.

13.2 Selecting the Appropriate Type of Formulation Based on Compound Properties and Type of Study

To develop an early formulation, one needs to collect all available data for the compound and relevant information about the *in vivo* model. When the compound is received for the first time, calculated values for pK_a and log P and experimental values for solubility albeit typically without conclusive information about the solid nature of the dissolved material (for instance, purity, amorphous form,

crystalline material, or mixtures thereof) are available. For a solution, the nature of the solid state is not so important, but for the development of a suspension, it is certainly a critical factor. It is important for formulators to be aware of the type of animal study envisaged (e.g. PK, PD, or toxicological study), its purpose, and readouts. This will help to define any formulation restrictions based on the animal species, the administration route, and the dose levels (including administration volume). The evident aim is to select the simplest formulation in terms of preparation that can ensure the required compound exposure is maintained throughout the course of the study. Solutions and drug suspensions are relatively simple to prepare. For more complex formulations, containing different kinds of particles not so convenient for early preclinical work, the reader is referred to excellent reviews on the subject [14–17].

13.2.1 Solutions

Solutions are always to be preferred, especially in preclinical formulation work, where knowledge of the solid state of a compound is limited. It is also easier to verify the homogeneity of a solution, when compared with a suspension, which will make it easier to use for people not versed in formulation preparation and characterization. This is an important consideration when studies are externalized to small companies that have expertise in specific *in vivo* models but are not so familiar with formulation properties. In these situations, an easy "ready to use" formulation is desired. However, one has to consider the risk for compound precipitation with all solutions. Below we discuss four groups (13.2.1.1–13.2.1.4) of solution and/or liquid formulations in general terms, before we focus on suspensions.

13.2.1.1 pH Adjustment

Is it possible to achieve the desired concentration within an acceptable pH for the route of administration? The acceptable pH for different routes of administration is affected not only by several conditions, such as body location (e.g. central or peripheral blood vessel), dose volume, animal species, and the occurrence of animal sedation, but also by the content and purity of the drug batch. A recommended interval for oral and i.v. administration, used by Novartis and Sanofi-Aventis during typical *in vivo* studies, is pH 2–9 [18]. The pH-dependent solubility is readily calculated using the pK_a (measured or calculated) of the substance and the intrinsic solubility, S_0. A solubility vs. pH curve can then be plotted. For this purpose, several software is available [19]. Here, it seems appropriate to mention that a few compounds are surface active and could form micelles. Typically, for such compounds, much higher concentrations than predicted by the pH-solubility curve could be prepared by pH adjustment. This, of course, facilitates the formulation work early on in the project, but the surface activity could have negative implications during further formulation development. When solid formulations of such compounds are considered later on, the dissolution properties could be different compared with ordinary compounds. Further, surface active compounds could also interact unfavorably with common excipients, like polymers, as well as with *in vivo* components, e.g. cell membranes and proteins.

13.2.1.2 Cosolvents

Selecting a suitable cosolvent formulation without consuming large amounts of compound in vehicle screening activities is not straightforward. It should be kept in mind that the solubility of a compound is not a linear function of cosolvent concentration. Therefore, upon dilution with water or the relevant *in vivo* fluid, the compound will at some point precipitate (which in some cases can be delayed by addition of small amounts of different additives [20, 21]). Despite this, the amount of cosolvent in a formulation should be minimized, not only because of possible side effects but also to maximize exposure. For instance, in the intestine, the drug will partition between the cell membranes and the surrounding intestinal fluid. If the intestinal fluid contains an excess of cosolvent, the partitioning will be unnecessarily shifted to the intestinal fluid side, thereby reducing cellular absorption.

Another way to benefit from cosolvent solubilization is to combine the solubilizing effect of a cosolvent with a pH adjustment [22]. The total solubilizing effect is not straightforward to estimate since the two effects counteract each other. A pH adjustment will increase solubility by ionization, while cosolvents will normally solubilize uncharged species. Nevertheless, a synergistic effect is often observed, and this might be a practical advantage provided that the *in vivo* animal model tolerates the resulting formulation mixtures.

13.2.1.3 Solubilization in Cyclodextrins

Some compounds are easily solubilized in cyclodextrins (here we mainly refer to hydroxypropyl-β-cyclodextrin and sulfobutylether-β-cyclodextrin), forming inclusion complexes. Unlike in the cosolvent vehicles, the solubilization of a compound is typically a linear function of the cyclodextrin concentration, and a solution could therefore be diluted in water without precipitation. There are, however, exceptions where drugs associate in other complex ratios than 1 : 1 or as noninclusion complexes [23–25]. The linear dependence can be applied to minimize the amount of compound needed in a vehicle screen by simply determining the solubility in one low cyclodextrin concentration solution. This is also a first good approximation toward the optimization of the cyclodextrin/drug ratio, which should be kept constant and as low as possible. When using cyclodextrin-based formulations, one needs to consider an additional free drug – bound drug equilibrium [26]. Thus, when changing the cyclodextrin/drug ratio, the concentration of free drug will also be affected. Likewise, using a higher cyclodextrin/drug ratio could result in lower exposure as a consequence of less free drug available for absorption. Improved solubility, obtained with a combination of cyclodextrin solubilization and pH adjustment, is sometimes possible, as it is for cosolvent vehicles. Moreover, small additions of polymers have been used to further enhance drug solubility with cyclodextrin-type formulations [27, 28].

13.2.1.4 Solubilization in Surfactants

Surfactants (as well as surface active drugs; see 13.2.1.1 above) generally suffer from the drawback that they interact with most cellular membranes *in vivo*.

This also includes a tendency to induce, for instance, hemolysis of red blood cells [29, 30], which might pose an unacceptable toxicity burden on the intended formulation. Further, the solubilizing capacity of surfactants is generally rather low, and considering the aforementioned problems, we essentially just use surfactants for wetting and particle stabilization purposes.

Even if solutions are the preferred option in most cases, there are drawbacks and pitfalls to be aware of. In the described formulations (13.2.1.2–13.2.1.4), poorly soluble compounds are typically delivered in solutions that contain high amounts of excipients, which could result in different kinds of adverse events and/or complicate the *in vivo* model readout, depending on the administration route [9, 31, 32]. Upon dilution, there is a risk for precipitation (although this is minimized for pure 1 : 1 inclusion complex cyclodextrin formulations) that also could cause adverse events and/or affect the planned PK/PD evaluation [33]. To reduce the risk of precipitation and increase the solubility of a compound, mixtures of cosolvents and surfactants are commonly used. In a recently performed study, Liu et al. investigated seven structurally diverse discovery compounds after i.v. administration of Cremophor EL-based formulations [34]. A higher percentage of Cremophor EL in the vehicle resulted in progressively increased alterations of the plasma clearance (CL) and volume of distribution (V_{ss}). These findings indicated that Cremophor EL altered the intrinsic PK properties of the tested compounds. The impact of Cremophor EL on the PK parameters in clinic has been reported for, e.g. paclitaxel [35, 36], doxorubicin [37], etoposide [38], and cyclosporine A [39]. In fact, there are examples of effects on most PK parameters; besides the already mentioned CL and V_{ss}, the area under the plasma concentration time curve (AUC), the peak plasma concentration (C_{max}), and the bioavailability were also significantly affected. Effects on PK parameters have also been observed for cyclodextrin complexes, but in these cases mainly reduced exposures have been reported due to high complex constants and/or unoptimized formulations [40].

There is a large volume of literature discussing the benefits of different particle formulations including emulsions, liposomes, and different types of nanoapproaches [14, 41, 42]. All these delivery systems are characterized by a drug release mechanism that must be clearly understood. This makes such systems less suitable during preclinical studies, as time-consuming formulation development activities are typically required. In the present chapter we focus on drug nanosuspensions, i.e. nanoparticles primarily composed of drug, water, and small amounts of particle stabilizers. The sought attributes for the described drug nanosuspensions are that they dissolve quickly *in vivo* and behave as a solution, i.e. they will not affect the intrinsic PK parameters. In addition, since the formulation mainly contains drug and water, the risk for confounding effects originated from formulation additives is minimized. However, at high doses or for compounds with aqueous solubilities significantly below 1 µM, stabilizer rearrangement and liver uptake may affect the PK and PD readout [43]. Before we describe the nanosuspensions in more detail, we want to say some words about microsuspensions and how we select between micro- and nanosuspensions.

13.3 Microsuspensions

For oral administration a microsuspension can be a good choice of formulation due to the low amount of additives (normally 0.5% HPMC 10000–15000 cPs, possibly in conjunction with a wetting agent like Tween 80, if necessary, based on the compound's characteristics). If the most stable crystalline form of the compound is available, the preparation using a suitable homogenizer is relatively straightforward. However, if the starting material is amorphous, it can be difficult to prepare a good microsuspension (<10 μm). Amorphous materials are often adhesive, which renders particle size reduction and stabilization, particularly challenging. Furthermore, there is always a risk for crystallization that will result in a heterogeneous suspension and alter the *in vivo* behavior. This may also occur for suspensions of metastable crystalline forms. Even if a good crystalline starting material is provided, conversion to another polymorph during formulation preparation and storage can make development of the formulation rather time-consuming. Clearly, solubility has to be high enough to afford adequate exposure at the intended dose. No strict solubility guidelines exist, because exposure is dependent not only on solubility and particle size but also on the dose, cellular permeability, and different *in vivo* properties such as, for instance, metabolic stability. Nevertheless, as a rule of thumb, for a highly permeable drug with solubility less than 10 μM, we recommend to use a nanosuspension whenever possible. For low permeability compounds, it could be beneficial to use nanoparticles even for solubilities up to around 50 μM, especially for high doses (where particle size begins to affect the exposure).

13.4 Nanosuspensions

A nanosuspension is the formulation of choice when it is not possible to make a solution, and a microsuspension has suboptimal characteristics for the intended route. It should be pointed out that when relatively small volumes and low concentrations of compounds are used and solid-state compound information (e.g. purity, solubility, and crystallinity) is limited, we routinely make nanosuspensions instead of microsuspensions. The reasoning is threefold: (i) in small volumes at low concentrations, it is normally much more practical to prepare a nanosuspension than a microsuspension, (ii) we give the drug the best chance to expose (nanoparticles will never give lower exposure compared with a microsuspension prepared of the same compound batch, but similar or improved), and (iii) smaller compound amounts are required. In the following sections of the chapter, two kinds of drug nanosuspensions with four different preparation approaches and some central characterization parameters/methods are described.

13.4.1 Amorphous or Crystalline Nanosuspension?

For compounds with very high melting temperatures and, thus, very stable crystalline states, crystalline nanosuspensions are the obvious first choice. Compounds with high amorphous to crystalline solubility ratio will also be

prone to crystallize. The ratio can be conveniently determined experimentally using nanosuspensions and turbidimetric determination of the solubilities, as described [44] and discussed below. The definition of a high or low ratio is arbitrary, but a ratio of 20 is worth testing, and a ratio of 100 is considered too high to result in a stable amorphous nanosuspension possible to use for *in vivo* studies. Furthermore, if the intended study will continue for several days or requires large volumes or high concentrations, crystalline nanosuspensions are preferred. In fact, crystalline nanosuspensions can be prepared on one occasion, are physically more stable during storage, and are normally easier to prepare at high concentration as compared with amorphous nanosuspensions. Nevertheless, because of the ease of preparation of amorphous nanosuspensions at concentrations $\leq$10 mM, sometimes the least time-consuming choice is to make such a formulation every morning for several days. For compounds with extremely low solubility, there is also an opportunity to increase the bioavailability by using amorphous nanosuspensions, if at all possible to prepare. Both properties, amorphous material and small particles, will increase the dissolution rate and the apparent solubility, properties that will improve *in vivo* exposure.

In early projects, differential scanning calorimetry (DSC) data are not normally at hand, and only small amounts of the compound are available. In order to test which type of nanosuspension is the most suitable, a good starting point is to prepare a 120 mM drug stock solution in dimethylacetamide (DMA). This solution gives full flexibility to try either amorphous or crystalline nanoparticles with different stabilizers, using the low concentration manufacturing methods described below. For amorphous nanoparticles a portion of the drug stock solution is mixed with a stock solution of Ostwald ripening inhibitor in DMA, to give a drug concentration of 100 mM and a drug/inhibitor ratio 4 : 1 (w/w). For crystalline nanoparticles a portion of the drug stock solution is diluted to 100 mM with pure DMA. A typical test preparation is normally performed at 1 ml by precipitation from the 100 mM solution to 1 mM in an aqueous stabilizer solution. The prepared nanosuspensions are characterized by visual examination, eventually by microscopy, followed by particle size measurements. If the diameter of the particles are small enough, <350 nm, and no growth occurs within at least 2 h, the formulation is regarded as fit for purpose (if the animal administration is performed at a close proximity lab). For amorphous particles, it is important to check carefully that no crystals have formed. With this approach it is possible to test most of the low concentration prototypes using only approximately 5 mg of the compound. More details regarding preparation follow below in the Section 13.5.

13.4.2 Selection of Stabilizers

A thorough selection of stabilizers is crucial for preventing particle aggregation and thus for the stability of the suspension. There are two principles of stabilization: steric hindrance, usually attained with polymers, and electrostatic stabilization, usually attained with surfactants. The two principles are often used simultaneously, so-called electrosteric stabilization, e.g. for highly charged

surfaces a polymer alone can be enough for stabilization. For surfaces with a low charge, a mixture of a polymer and an anionic surfactant could be used.

For a stabilizer to be effective, it should adhere to the surface of the particle. Two properties, log P and pK_a, can be used to describe the surface and select stabilizers. The log P value gives indication about the hydrophilicity of the surface and the pK_a, whether there is a positively or negatively charged surface on the particle. These properties (used below) can be either calculated or measured, depending what data is available at the time of manufacturing. Based on these two properties, we have found three different stabilizer approaches that have worked for most of the compounds we have formulated as amorphous or crystalline drug nanosuspensions over the years [3, 9–13, 43–48]. There are exceptions, but we suggest the following framework as a good starting point for further experimentation. Three general rules are recommended with two exceptions specific for the high concentration manufacturing methods (point 3):

1) For all compounds with a log P $\leq$ 3, *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG2000) or Pluronic F127 will be the first choice.
2) For bases with pK_a > 6, hydroxymethylpropylcellulose (HPMC), 6 cPs is the first choice.
3) For all other compounds, polyvinylpyrrolidone (PVP) and sodium dodecyl sulfate (SDS) is the first choice, with exceptions for both wet milling to crystalline nanosuspension and melt emulsion for amorphous nanosuspension where Aerosol OT (or dioctyl sodium sulfosuccinate, AOT, Cytec Industries Inc.) replaces SDS. In the case of milling, AOT replaces SDS due to a significantly reduced tendency of foaming. In the case of melt emulsion, AOT replaces SDS since AOT is a much better stabilizer at elevated temperatures.

Nanosuspensions for the parenteral route must be isotonic. To use 0.9% (w/w) of sodium chloride or any other salt solution is not recommended since it will decrease the electrostatic stabilization (see Section 13.6). Instead we recommend 2.6% (v/v) DMA, 5% (w/w) mannitol, 2.2% (w/w) glycerol, or 10% (w/w) sucrose or trehalose. Moreover, 5% mannitol and 10% sucrose or trehalose will also act as cryoprotectants and maintain the particle size during freezing and thawing. They will also work as matrix-forming excipients during further processing of the nanosuspensions through spray-drying or freeze-drying.

13.4.3 Manufacturing Method Selection

The desired concentration and volume, together with the compound properties, inform the choice of which manufacturing method to use. This has important implications for downstream formulation activities toward clinical studies.

13.4.3.1 Low API Concentrations (Up to Approximately 10 mM)

There are two similar methods for preparing either amorphous or crystalline drug nanosuspensions. The *precipitation method* is applied for amorphous nanosuspensions, and the *ultrasonic crystallization method* is applied for the

crystalline nanosuspensions [44–46]. Both methods involve dissolution of the compound at a high concentration in an organic, water-miscible solvent (typically DMA, with dimethyl sulfoxide (DMSO) and ethanol as alternatives) and are independent of the solid state of the compound. The two methods are carried out in almost the same way by a rapid injection of a drug solution into an aqueous stabilizer solution during ultrasonication. The two different characteristics distinguishing the methods are that the amorphous nanosuspension usually contains an Ostwald ripening inhibitor and is only sonicated a few seconds to obtain an instant mixing, while the crystalline nanosuspension does not need an inhibitor and is sonicated for several minutes. Which of the two methods to use depends on the ability of the compound to crystallize.

Common to both methods is that typically 1–10% (v/v) of organic solvent is retained in the formulation (depending on the desired final concentration of the compound). This is usually not a problem, but residues above 5% are generally not recommended for i.v. administration, especially not for studies utilizing conscious dogs, for which solvents should be kept as low as possible. If the residue content is an issue, e.g. affecting results in sensitive animal models, one can either try to increase the drug concentration in the organic solution or dialyze (or ultrafilter) the final nanosuspension against water or an isotonic solution (depending on administration route). Practical recommendations toward achieving nanosuspensions' isotonicity are provided under Section 13.4.2.

13.4.3.2 High API Concentrations (Above 10 mM)

There are two methods for nanoparticle preparation at high concentration (around 10% w/w). Briefly, crystalline nanosuspensions are afforded by first preparing a microsuspension/slurry of the drug in an aqueous stabilizer solution followed by wet milling in a planetary mill using small beads of zirconium oxide. Amorphous nanosuspensions are prepared by the melt emulsion method, which involves mixing a microsuspension/slurry of the drug with a premade emulsion and heating above the drug melting temperature for a short period of time (in the order of 2–10 min, based on physicochemical properties of the drug and particle characteristics). At high temperature the drug will diffuse to the oil phase, and upon cooling nanoparticles will form. With a good crystalline starting material of the most stable polymorph, wet milling is the method of choice, unless there is a great demand to improve bioavailability due to low solubility. In such a case the melt emulsion method could be used provided the compound is chemically stable at high temperature and not too prone to crystallization. The melt emulsion method could also be applied to amorphous starting materials. A problematic case is when a crystalline material of a metastable polymorph is considered. Sometimes wet milling will work anyway, typically if there is only a small difference in solubility between the most stable and the metastable polymorph. In other cases, a conversion to the stable polymorph could take place during the milling process resulting in a poor preparation. For such materials, we recommend that the compound is recrystallized to the stable polymorph (in some cases, during the slurry preparation, there can be a transition to a more stable form when standing overnight or over a longer period of time). A similar situation can appear due to changed amount of drug in the starting material

because of residual solvent from syntheses and/or different impurity profiles. Batch-to-batch variation can have a significant impact on the preparation protocol and should be carefully monitored.

13.5 Manufacturing Methods

13.5.1 Amorphous Nanoparticles at Low Compound Concentrations: The Precipitation Method

This method is suitable for drug concentrations ≤10 mM and for preparation volumes ≤10 ml. The method is very practical and allows for consecutive preparations to be prepared in a relatively short time.

First, a drug stock solution in DMA (typically 100 mM) that also contains an Ostwald ripening inhibitor is prepared. The ratio of drug/inhibitor should be 4 : 1 (w/w). The drug/inhibitor stock solution is conveniently prepared by using a 100 mg ml^{-1} inhibitor stock solution in DMA and pure DMA. The inhibitor should be completely miscible with the amorphous drug and should be characterized by lower aqueous solubility than the drug. Based on our experience, we have drafted a number of guidelines that are helpful in selecting the inhibitor:

Miglyol 812	Works for the majority of compounds.
Miglyol 812/1-decanol (1 : 1 w/w)	log P < 3 compounds if Miglyol alone does not work.
Miglyol 812/ Pluronic L121 (1 : 2 w/w)	log P > 3 compounds if Miglyol alone does not work.

The mechanism of Ostwald ripening and its inhibition was thoroughly described by Lindfors et al. [45]. Briefly, the Ostwald ripening is determined by recording the particle size with dynamic light scattering (DLS) during at least 1 h. If the volume (the diameter of the particles expressed as diameter^3) is increasing linearly with time, there is an ongoing Ostwald ripening [45] (Figure 13.2).

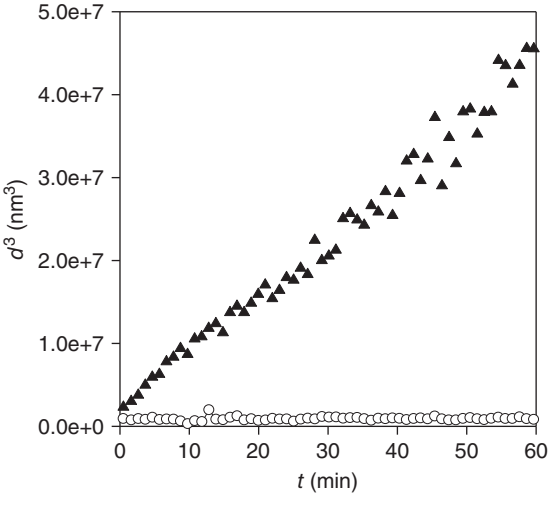

Figure 13.2 Amorphous nanoparticles of felodipine show Ostwald ripening (filled triangles). With Miglyol present, the increase in particle size was inhibited (open circles).

The aqueous stabilizer solution is selected according to the general rules described above. For this method the stabilizer solutions used for the different prototype formulations identified are:

0.2% (w/w) PVP K30 and 0.25 mM SDS	All compounds with log P > 3, base pK_a < 6.
0.2% (w/w) of HPMC 6 cPs	All compounds with log P > 3, base pK_a > 6.
0.2% (w/w) DPPE-PEG2000 or Pluronic F127	All compounds with log P < 3.

A test formulation giving 1 ml of the desired concentration is made as follows:

1) A 4 ml glass vial containing approximately 1 ml stabilizer solution is placed in a sonication bath using a stand and clamp (the exact volume is determined by the amount of drug stock solution to be added).
2) Sonication is applied, and the desired volume of the drug stock solution (100 mM, drug/inhibitor 4 : 1 w/w, DMA) is rapidly injected into the stabilizer solution by a Hamilton® syringe.
3) The Hamilton® syringe is removed, and after a few seconds, sonication is turned off, and the preparation is finished. It is important to note that an amorphous nanosuspension should never be shaken, stirred, or sonicated due to the risk of crystallization. Right before administration, the vial can be gently tilted upside down a few times to make sure the formulation is homogeneous.

The size of the nanoparticles is measured by DLS and followed for at least 2 h. The nanosuspension is also examined visually and by a polarization microscope in order to observe the presence of possible crystals. If the particle size is constant and no crystals are observed after 2 h, the formulation is stable enough to be prepared close to the *in vivo* study (in case the same substance batch is used). Particle distributions <300 nm are usually achieved, but distributions up to 400 nm are considered good enough for their purpose. It should be remembered that the formulation contains a certain amount of DMA (see above) and compatibility with the intended *in vivo* study should be evaluated.

13.5.2 Amorphous Nanoparticles at High Compound Concentrations: The Melt Emulsion Method

The melt emulsion method could be performed at volumes ranging from 1 to 20 ml using small-scale equipment and for drug concentrations ≤10% (w/w). The melting temperature of the substance must not exceed 200 °C.

As for the *precipitation method*, it is required that the Ostwald ripening inhibitor is miscible with the amorphous drug. The inhibitor is basically selected according to the same criteria previously discussed. However, for drugs with melting temperatures >160 °C, we have found that Miglyol/L121 1 : 2 (w/w) often works better than Miglyol alone. The drug/inhibitor ratio normally used with this method is 1 : 1 (w/w) rather than 4 : 1. This is because the inhibitor also reduces the driving force for crystallization, and, based on our experience, this problem seems to be more pronounced at high concentrations. The

recommendations are thus to use:

Miglyol 812	Compounds with $T_m < 160\,^{\circ}C$.
Miglyol 812/L121 (1 : 2 w/w)	Compounds with $T_m > 160\,^{\circ}C$.

It is highly advisable that the drug/inhibitor miscibility is evaluated before preparation by an Ostwald ripening test. This is done by first making a 1 mM amorphous nanosuspension *without* inhibitor using the *precipitation method,* monitoring the particle size as a function of time (Figure 13.2). If growth is observed, a new preparation *with inhibitor* is made, and the size is again measured as a function of time. If the growth is inhibited, the drug is miscible with the inhibitor. If it is not inhibited, another inhibitor is assessed. For some compounds with very poor solubility, no growth is seen for the preparation without an inhibitor. If this is the case, both experiments should be repeated at 10% (v/v) DMA in order to increase the solubility of the API [45].

When this method was developed, it was found that it was very difficult to stabilize the emulsions/suspensions at high temperature. The stability of the systems tested was found to decrease with droplet/particle concentration, surfactant concentration, polymer concentration, polymer molecular weight, and temperature. Furthermore, the only surfactant that worked at high temperature was AOT. The stabilizer solutions recommended are thus carefully chosen in order to give the best success rate, and any deviation from those is likely to fail:

0.6% (w/w) AOT, 0.5% (w/w) PVP K30	Compounds with $T_m < 160\,^{\circ}C$.
0.6% (w/w) AOT, optionally 0.1% (w/w) PVP K12	Compounds with $T_m > 160\,^{\circ}C$.

When a drug miscible inhibitor and a stabilizer solution have been selected, the preparation is carried out as follows:

1) A 20% (w/w) oil-in-water emulsion of the inhibitor is made, using a 0.7% (w/w) AOT (aq) stabilizer solution. The quality of this emulsion is critical: the droplet size should be minimized as it will determine the size of the final nanosuspension. At small scale, it is possible to make such emulsions by using vortex mixing, followed by sonication, to achieve a droplet size of 150–200 nm. Larger volumes could be prepared using conventional equipment for emulsion preparation e.g. a Polytron followed by high-pressure homogenization.
   If Miglyol/L121 is selected, it is sometimes challenging to afford a good emulsion. In small scale, a mixture of Miglyol and L121 is first made, and the emulsion is formed as described above. However, in order to get a good homogeneous emulsion with small droplet size, the emulsion needs to be cooled below 10 °C and then sonicated at room temperature. This process may need to be repeated a few times. Alternatively, after the vortex-mixing step, the coarse emulsion can be stirred for 20 h in the fridge, followed by sonication at room temperature. For larger volumes, an emulsion of 20% (w/w) Miglyol and 1.7% (w/w) AOT is first prepared using standard equipment.

The appropriate amounts of L121 and water are then added to the Miglyol emulsion, followed by stirring in the fridge for 20 h, and continued stirring for another 20 h at room temperature. The droplet size of the Miglyol/L121 emulsion should ultimately not exceed 150 nm.

2) A 20% (w/w) drug slurry in the appropriate stabilizer solution is prepared by stirring and sonication. It is important to make this drug slurry as good as possible in order to facilitate the following process steps. Normally it is possible to get particle sizes <10 μm.
3) Equal volumes of 20% emulsion, 0.6% AOT and 20% drug slurry, 0.6% AOT, and optionally 1% PVP K30 or 0.2% PVP K12 are mixed in a high-pressure vial using a pipette. A magnetic follower is added to the vial and the vial is capped. If drug concentrations lower than 10% (w/w) are desired, the dilution is conveniently made at this stage by first adding water and then equal appropriate volumes of the emulsion and drug slurry.
4) The high-pressure vial is locked in a holder and transferred to a preheated silicon oil bath with a magnetic stirrer at 250 rpm. The temperature is set to 10 °C above the drug melting temperature, and the mixture is normally incubated for 10 min. After that, the heating and stirring are switched off, and the vial (with holder) is removed from the heat and allowed to cool at room temperature without stirring.
5) When the nanosuspension has cooled, the cap is removed, and the nanosuspension is transferred to another vial using a pipette. There is sometimes a small fraction of material at the bottom of the vial or at the top of the suspension. Care should be taken to avoid this residue, and for a 1 ml preparation, 0.8 ml is normally taken out.

The nanosuspension is characterized and handled in the same way as described for the *precipitation method*. In addition, the concentration and purity should be determined by, e.g. liquid chromatography (LC).

13.5.3 Crystalline Drug Nanoparticles at Low Compound Concentrations: The Ultrasonic Crystallization Method

Ultrasonic crystallization is easily carried out with instruments that generate very powerful high acoustic energy, such as a Covaris S220X instrument (from the 220 series, Covaris Inc.). A common ultrasonication bath can be utilized too. However, we strongly recommend the use of a Covaris instrument since it is much more user-friendly, the solutions do not need to be cooled, the instrument can be used consecutive times without loss of energy transfer, and the very powerful high-energy provided yields superior reproducibility, as compared with a common ultrasonic bath.

Essentially this method is almost the same as the precipitation method but with two major exceptions: an Ostwald ripening inhibitor is never used and ultrasonication is always carried out for at least 20 min. This is because we have found that the Ostwald ripening process, fortunately, is not as pronounced for crystalline

samples as it is for amorphous material of similar solubility. The method is suitable for drug concentrations ≤10 mM and for preparation volumes ≤10 ml. In a few cases concentrations up to 15 mM or larger volumes up to 20 ml have been prepared.

Two prototype stabilizer solutions are used:

0.2% (w/w) PVP K30 and 0.25 mM SDS	All compounds with log P > 3, base pK_a < 6.
0.2% (w/w) DPPE-PEG2000 or Pluronic F127	All compounds with log P < 3.

The HPMC 6 cPs stabilizer solution is not identified as a prototype for ultrasonic crystallization since it was found to always give rise to large particles. Nevertheless, it might still be worth testing it, since for a few drugs preparations with 0.2% HPMC (6 cPs) have actually been the best alternative.

There are several options for different vials to be used in the Covaris instrument. We find it very convenient to use the disposable microwave vials from Biotage (Biotage, LLC). These vials are available in the following sizes: 0.5–2 ml, 2–5 ml, 10–20 ml, and the size ranges are also in accordance with the process volumes to be used for each size. For the best performance it is recommended to choose a vial that keeps at least a 1.5 cm level in the vial, i.e. the minimum amount to process is approximately 1 ml in the smallest vial. The focus of the acoustic energy is about 1 cm below the level of the water bath, and the 2–5 ml vial has been optimized for a process volume of 2.67 ml. In case different, larger vials are used, it is recommended to choose vials where the level will not exceed the surface of the water bath by more than 1 cm.

A test formulation giving 1 ml of the desired concentration is made as follows (preferably using a Covaris equipment):

1) Prepare a drug stock solution of 100 mM in DMA (some drugs may require DMSO as a solvent).
2) A 0.5–2 ml microwave vial containing approximately 1 ml stabilizer solution is placed in a sonication bath using a stand and clamp (the exact volume is determined by the amount of drug stock solution to be added).
3) Sonication is applied, and the desired volume of the drug stock solution (100 mM in DMA) is rapidly injected into the stabilizer solution using a Hamilton syringe.
4) The Hamilton syringe is removed, sonication is turned off, and the microwave vial is capped and immediately put in the Covaris instrument for a 20-min process time.

The process procedure used for ultrasonic crystallization use the following settings:

power tracking mode, number of cycles = 20, and total process time = 20 min. Treatment is set to duty cycle, 20%; intensity, 10; cycles/burst, 1000; and time 60 s. The particle size distribution is finally measured by DLS since individual particles usually measure <300 nm.

13.5.4 Crystalline Drug Nanoparticles at High Compound Concentrations: The Wet Milling Method

Wet milling of a compound is all about defragmentation and deagglomeration of the solid state, and the method is dependent on a good quality crystalline starting material. A Fritsch Planetary micro mill Pulverisette 7 classic line is used for milling amounts from 25 mg up to 2 g. For amounts below 500 mg, we have designed our own milling bowls. A common material used for the beads is zirconium oxide (Glen Creston Ltd.), which is also the material in our milling bowls. The immediate problem with milling beads is the risk of contamination from the beads. To minimize the contamination, we rinse the beads with 1 M NaOH followed by 1% SDS and plenty of purified water before we dry the beads and finally consider them fit for purpose. However, solid residuals from the beads as well as fractions of larger particles can be removed by overnight sedimentation or centrifugation.

To achieve an effective milling process, there are two things to consider: one is to run the milling at a minimum of 5% (w/w) drug and the other is to make sure that the bowl is filled to the top in order to avoid extensive foaming. Milling at drug concentrations 5–15% (w/w) is usually straightforward, and several compounds are also feasible to handle up to 20% (w/w). Wet milling is generally more efficient at higher concentrations, but sometimes an increase in viscosity makes it difficult to handle.

In the wet milling method, we typically use a 10% (w/w) suspension as a standard for both different milling scales loadings and for the composition of the stabilizer solutions. Table 13.1 shows the different scales with recommendations of how to load each scale. As an example, for 57 mg of substance, add stabilizer to a total of 570 mg to achieve 10% (w/w). 0.51 ml of this slurry and 2.4 g of 0.6–0.8 mm beads are needed to fill this 1.2 ml bowl properly. It is recommended to choose a scale that follows the advice to mill at least 5% (w/w) drug. For an unknown compound, we generally choose a scale of milling between 5% and 10% (w/w). We normally prepare an excess of 10% to compensate for the loss of drug left in the milling bowl, and we use *ultrasonic crystallization* to find appropriate stabilizer(s). This is typically carried out at a drug concentration of 1 mM with a stabilizer/drug ratio equivalent to the prototype (see above).

Table 13.1 Scales of milling in Fritsch Planetary micro mill Pulverisette 7 classic line using beads of zirconium oxide.

V_{bowl}	V_{slurry}	$m_{substance}$	m_{beads}	$Ø_{beads}$ (mm)
45 ml	19.5 ml	2.17 g	80 g	0.8–1.0
25 ml	10.2 ml	1.13 g	50 g	0.8–1.0
12 ml	5.1 ml	570 mg	24 g	0.8–1.0
3.6 ml	1.53 ml	172 mg	7.2 g	0.6–0.8
1.2 ml	0.51 ml	57 mg	2.4 g	0.6–0.8
75 µl	31 µl	3.41 mg	125 mg	0.3–0.4

Three prototype stabilizer solutions are used for a 10% (w/w) wet milling with the following compound properties:

1.33% (w/w) PVP K30 and 0.067% (w/w) AOT	All compounds with log P > 3, base pK_a < 6
2% (w/w) of HPMC 6 cPs	All compounds with log P > 3, base pK_a > 6
2% (w/w) DPPE-PEG2000 or Pluronic F127	All compounds with log P < 3

In order to achieve similar stabilization for different weight fractions, we scale the stabilizer solution accordingly, i.e. for a 5% (w/w) suspension, we dilute the prototype stabilizer solution by a factor 2.

When a stabilizer solution has been selected, the preparation is carried out as follows:

1) The suspension is prepared in a vial compatible with ultrasonication. Making sure that the resulting slurry is visually homogeneous before milling is absolutely critical. An inferior milling is sometimes a result of a suboptimal slurry. Magnetic stirring until the material is wet and ultrasonication for at least 10 min using a bath will be enough for most compounds. Some compounds may need switching between stirring and sonication several times in order to obtain a usable slurry. When using HPMC as the stabilizer, a homogeneous slurry is sometimes hard to achieve due to poor wetting. This problem can be solved by adding AOT to a final concentration of 0.067% AOT, resulting in 1.33% HPMC based on 10% drug.
2) Loading of the milling bowl is most efficiently done by first adding the slurry and then the beads. The default milling process is 4 × 30 min at 700 rpm with 15 min intermission to dissipate the heat generated.
3) The nanosuspension is collected using a syringe and a needle with a gauge smaller than the beads. After collecting the first concentrated suspension, it needs to be washed out from the bowl. It is convenient to wash with a suitable sugar solution since this works both as an isotonicity agent and a cryoprotectant component, e.g. a mannitol solution (5–10%w/w) in order to obtain a final 5% mannitol concentration. Washing is done until no more substance is seen to come out or after having repeated washing for at least three times with half of the initially added volume, if a concentrated suspension is needed. On a few occasions the mannitol has been seen to destabilize the nanosuspension. Thus, it might be wise to collect the concentrated nanosuspension separately before mixing with the washed suspension until this feature is known. In accordance, milling with, e.g. 5% mannitol from the start, is not recommended until its behavior has been evaluated.
4) Size analysis of milled nanosuspensions should be assessed with laser diffraction and not with DLS, as sometimes published in the literature. This is because residues of larger particles after the milling process will not be detected by DLS if these particles are above the sedimentation limit (i.e. approximately 1 μm in diameter). We use a MasterSizer2000 equipped with a Hydro 2000 cell requiring only 5–10 μl of a 10% nanosuspension for analysis. A typical particle distribution has a volume mean between 150 and 250 nm, a particle distribution with 90% of the material below 400 nm, and no significant amount of material above 1 μm is considered acceptable for

parenteral administrations. The washed-out and collected nanosuspension will need to be analyzed for its concentration before use.

13.6 Additional Characterizations and Considerations Before *In Vivo* Dose Decisions and Administration Route Selection

The measurements of particle sizes of the freshly prepared nanosuspensions and stored preparations have been discussed. Selection of stabilizers and Ostwald ripening inhibitors as well as concentration measurement with LC has been detailed as integral components of the formulation preparation and analysis. Before *in vivo* studies, said characterization data should generally be complemented with information regarding solubility and dissolution rate from the particles in liquid environment as well as colloidal stability (for parenteral administration) in *in vivo* relevant milieu, at least for selected candidate drugs.

13.6.1 Solubility Measurements

The method used is based on the scattering of light from particles or undissolved material. The scattered light intensity depends strongly on the particle size. Although the scattering from a molecular dispersed solution in general is very small, a colloidal dispersion of the same concentration may have a significant turbidity. Hence, if colloidal particles are gradually added to a solvent, they will dissolve at concentrations below the effective solubility, and the solution will essentially not scatter light. As the concentration is increased above the solubility, the solution is saturated, and the added particles no longer dissolved. The scattered intensity then increases to a much greater extent than before, and the solubility is determined from the onset of the intensity increase. The method is suitable for both crystalline and amorphous solid material and is carefully described and evaluated for six different compounds with measured solubilities in the range from 1 µM to 1 mM [44]. Indeed, this method has been used for compounds with solubilities as low as 10 nM [43]. Examples of measured scattering intensity versus drug concentration, for crystalline and amorphous drug nanosuspensions, will be discussed in Case Study 2.

Alternatively, with an experimental value for the crystalline solubility (S_0), a reasonable value for the *pure* amorphous solubility (S_{am}) can be calculated using Eq. (13.1):

$$\frac{S_{am}}{S_0} = e^{[(\Delta S_m/R)\ \ln(T_m/T)]} \tag{13.1}$$

S_0 is the intrinsic solubility, i.e. the crystalline equilibrium solubility of an uncharged compound. ΔS_m, ΔH_m, and T_m are the entropy, the enthalpy, and the temperature of melting, respectively, where the two latter are easily obtained from DSC and $\Delta S_m = \Delta H_m / T_m$. R is the gas constant and T is the absolute temperature. A good agreement was found between calculated and experimentally

measured amorphous solubilities [44]. The ratio of S_{am}/S_0 is a valuable parameter in predicting how prone a compound is to crystallize (see Section 13.4.1). As an additional step, the *actual* solubility in the amorphous nanoparticle suspension used in experiments can be calculated using derived approaches [44].

13.6.2 Measurements of Dissolution Rate

The fluorescence intensity of a compound is often significantly higher in the crystalline state than the amorphous state, which is in turn higher than the one in solution. Dissolution experiments are performed by diluting rapidly a particle suspension to a final concentration, which is typically an order of magnitude, or more, below the intrinsic solubility. The dissolution process involves the diffusion of molecules away from the particles, and the steady-state dissolution rate depends on S_0. This kind of measurement is best suited for crystalline particles, since the aqueous amorphous solubility in general is significantly higher than the crystalline solubility, and often the dissolution rate becomes too fast to be measured accurately. The method is best suited for drugs with solubility around 5 μM or lower. The approach is carefully described and evaluated with felodipine as a model compound [46]. The excitation wavelength was set to 370 nm and the emission recorded at 430 nm. Figure 13.3 shows the dissolution curves of felodipine formulated as nanocrystal formulations. The dissolution was followed at two different conditions. The solid lines are theoretical predictions describing the dissolution of the actual compound [46]. If the predicted and experimental curves do not fit, suboptimal nanosuspension performance due to dissolution is to be expected *in vivo*. Examples of significant *in vivo* PK and PD variation due to compound dissolution issues have been observed. These can be caused, for example, by API quality variability across batches and can easily result in significant delays and higher costs for a development program. Dissolution measurements "early"

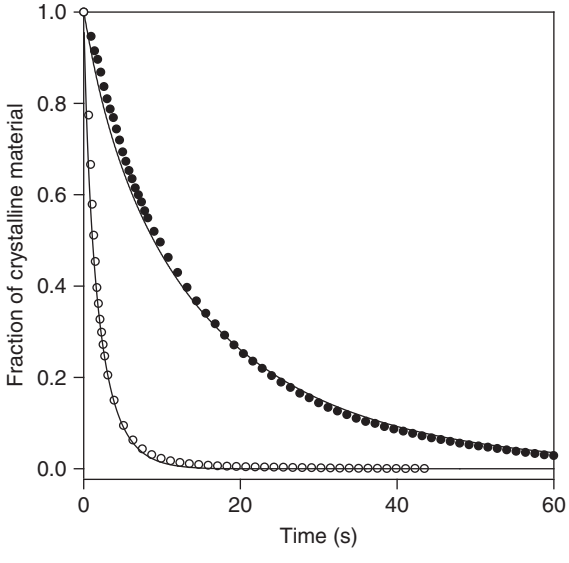

Figure 13.3 Experimental results for the fraction of crystalline drug nanoparticles of felodipine versus time in dissolution experiments performed at 25 °C where milled crystalline nanoparticles are dissolved in pure water (filled circles) and in water/DMA 9/1 (v/v) (open circles). The total concentration of felodipine was 0.2 μM (water) and 2 μM (water/DMA). The solid lines are the results of theoretical predictions. *Source*: Adapted from Lindfors et al. 2007 [46]

in a project and associated follow-up routines are an effective method to minimize these risks. Where i.v. injection is the selected route of administration, dissolution measurements should also be performed in the presence of relevant amount of albumin dissolved in a suitable solution [43].

13.6.3 Colloidal Stability

Before nanosuspensions are administered i.v., colloidal stability is evaluated (typically during stressed conditions like 500 mM sodium chloride for 5 min [43]), to confirm that there is no significant aggregation taking place, e.g. due to desorption of the stabilizers [49]. In some cases it can be of value to measure the colloidal stability in the presence of 4% (w/v) albumin dissolved in PBS [43]. During oral administration, precipitation does not have a crucial impact on the welfare of the animal, as opposed to the i.v. route of administration. Nevertheless, especially when the obtained PK data appear suspicious, the colloidal stability should be followed across the whole physiological pH interval in relevant media.

13.6.4 Chemical Stability of the Compound

From a chemical point of view, a nanosuspension, with most part of the drug in solid phase, is less prone to chemical degradation, compared with a solution of the same compound. The chemical stability can be further improved by freeze-drying nanosuspensions or processing the particles to a solid formulation [50, 51].

13.6.5 Sterilization Before Parenteral Administration

Parenteral administration of nanosuspensions is typically discussed within the context of preclinical studies and nonsterilized formulations. When sterilized formulations are required, it is noteworthy that sterile filtration is not advisable due to significant material loss on the filter. Heating and gamma radiation are useful alternatives provided that the drug tolerates these conditions. Frank and Boeck have showed that milling in a ball-mill process decreased the presence of living microorganisms [52]. It was suggested that the physical energy applied during the milling step generates enough shearing force to destroy living organisms. In a similar way, one can anticipate ultrasonication to have a similar influence on the organisms. However, frozen formulations or freshly prepared formulations in a sterile environment are normally the most practical approach.

13.7 Case Studies

13.7.1 Case Study 1: Milled Nanocrystals of a Compound for Toxicological Studies

The investigated compound was active in preclinical gastroesophageal reflux disease (GERD) models and was considered as a promising candidate for further development. The current version of ACD/Labs indicated a low basic pK_a. It was

not possible to obtain a pK_a value using a conventional titration approach (only applicable for determinations of pK_a values above 2). No solubility improvement was obtained at pH 1 compared with higher pH values. The compound is thus a very weak base and can be considered as a neutral compound at all physiological conditions. Log P was determined to 3 in octanol-water (pH 7.4, in 0.1 M phosphate buffer) by a microscale shake flask method. The compound is a non-hygroscopic, crystalline drug (melting point of about 200 °C) and has solubility of 6–7 μM to 13 μM at 37 °C in all tested media, with the aqueous solubility at the lower limit (Table 13.2). Simulations using GastroPlus™ indicated that the absorption was dissolution rate limited. The *in vitro* intestinal permeability of the compound was investigated using the Caco-2 cell model. The high permeability in the apical to basolateral direction (a–b, 33×10^{-6} cm s^{-1} for the used cell line) corresponded to a predictive fraction absorbed *in vivo* in humans to 100%. No study was performed to investigate the permeability in the reverse (b–a) direction. The drug is classified as a tentative BCS Class II substance within the expected therapeutic dose range 10–80 mg. The calculations were based on solubility in phosphate buffer at pH 7.4. The slightly higher solubility in human intestinal fluid (HIF) and human gastric fluid (HGF) does not change the classification of the compound in the predicted dose range.

The possibilities to find another polymorph with improved solubility or a metastable amorphous form with increased solubility and/or dissolution rate were unsuccessful.

Due to the very low pK_a of the compound, only a few counter ions were suitable to salt formation with the compound. Of the ions tested, only the bromide salt

Table 13.2 Solubility in different media. All measurements after 24 h, at 37 °C. The measurements were not performed with the fluorescence method described, but as described elsewhere [53, 54].

Medium	Concentration (μM)	pH
Phosphate buffer, 0.1 M, pH 7.4	6.68	7.56
Water	7.26	9.13
Saline	6.06	8.31
0.1 M HCL	7.30	1.08
HIF[a)]	9.87	6.88
HGF[a)]	10.60	2.12[b)]
FaSSIF[a)]	8.56	6.47
FeSSIF[a)]	13.40	4.96[c)]

HIF (human intestinal fluid), HGF (human gastric fluid), FaSSIF (fasted simulated small intestinal fluid), FeSSIF (fed simulated small intestinal fluid).

a) Measured also after 1 and 5 h, with unchanged solubility.
b) pH adjusted to 6.77, no change in solubility.
c) pH adjusted to 6.39, solubility decreased to 11.7 μM.

Table 13.3 Solubility in different vehicles. The measurements were not performed with the fluorescence method described, but with a common LC approach [54].

Vehicle	Concentration (mg ml^{-1})	Concentration (mM)
PEG400/DMA/water (20/20/60)	0.025	0.088
TEG/DMA/water (20/20/60)	0.033	0.12
PEG400/DMA/water (80/5/15)	0.71	2.50
20% HPβCD	0.045	0.16
30% HPβCD	0.072	0.25
Labrasol	3.09	10.9
Labrafil M1944CS	0.89	3.1
Peceol	0.67	2.4

PEG = polyethylene glycol 400, DMA = dimethyl acetamide, TEG = tetra ethylene glycol, HPβCD = hydroxyl propyl-β cyclodextrin, Labrasol = caprylocaproyl macrogol-8 glycerides, Labrafil M1944 CS = glycolysedethoxylated glycerides, Peceol = glyceryl monooleate.

was possible to isolate. The salt was, however, found to be too unstable for further development. It was thus decided to evaluate the free base form of the drug.

Solubility in different formulations was assessed and the results are summarized in Table 13.3. The solubility in all tested vehicles was very low, and no acceptable solution with sufficient solubility for *in vivo* studies was identified. The highest concentration in a physiologically acceptable formulation (>2.5 mM), for single administration to rats (the first toxicological species to be tested in, according to the development plan), was found in solutions of PEG400 or TEG/DMA/water solutions (where caution must be applied regarding the volume of DMA administered). Tested surfactants showed improved solubility, but were not compatible with the envisioned animal models.

The next step was to investigate the possibility to prepare different suspensions. For low doses conventional micronization was sufficient to assure complete absorption. However, for high doses (for instance, in the planned toxicological studies) a particle size reduction technique was necessary. The initial milled nanocrystals were stabilized with 1.0% w/w PVP and 0.2% w/w SDS, and 2.6% (v/v) glycerol was added as cryoprotectant and tonicity modifier. Useful nanosuspensions were produced from slurries containing 5.5–10% (w/w) of the drug. However, there was a fraction of the nanosuspension measured in the micrometer region. Both sedimentation and centrifugation were used to obtain a single, more homogeneous fraction, mainly targeting an i.v. injection as the administration route. The compound could be stored frozen in the presence of glycerol (2.6% v/v). Thawing and sonication (to reduce possible aggregates before administration) resulted in a suitable formulation. However, when glycerol was excluded, it was not possible to afford the sought nanometer fraction. A first oral

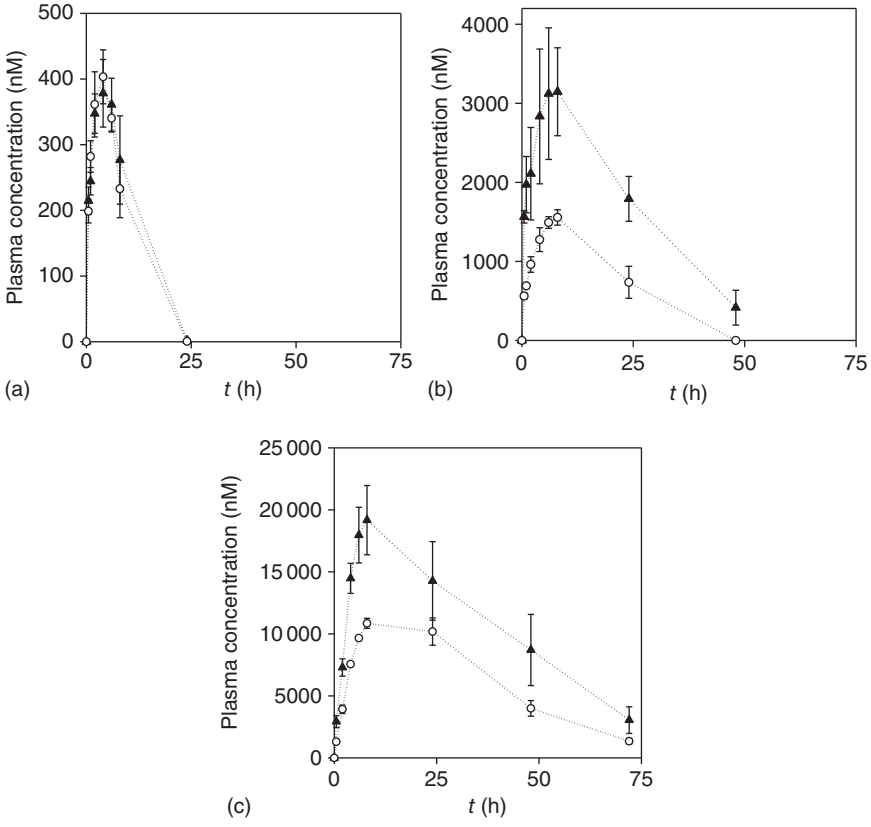

Figure 13.4 The mean plasma concentration (±SEM) of the compound versus time after oral administration of the drug as crystalline microsuspensions (open circles) and as nanocrystals (filled triangles) to rats at 3 μmol kg^{-1} (a), 30 μmol kg^{-1} (b), and 300 μmol kg^{-1} (c). $N = 3$ for each formulation.

PK study in rats, comparing two different suspensions, indicated that the use of nanocrystals (350 nm) resulted in equal exposure compared with microsuspensions (11 μm) at 3 μmol kg^{-1}, i.e. a dose comparable with an anticipated clinical relevant dose (Figure 13.4). At 30 and 300 μmol kg^{-1}, the exposure increased significantly when using nanosuspension compared with a microsuspension. As a consequence of further formulation development activities, the stabilization mixture was replaced with PVP/AOT and 5% mannitol, and a single fraction was obtained without the need for sedimentation or centrifugation as a subsequent optimization step. Nanocrystal formulations of the compound for both oral (administered to rats, dogs, and ferrets) and i.v. (to rats) administrations have been prepared and characterized. No Ostwald ripening was observed after 6 months storage at refrigerated temperatures. The formulations were also chemically and physically stable during this time period.

13.7.2 Case Study 2: Amorphous Nanosuspensions Selected for Preclinical and Toxicological Studies Due to Improved Exposure Versus Crystalline Suspensions with Different Particle Sizes

The investigated compound is a weak acid (sulfonamide on pyridyl ring) with pK_a of 10 and log$D_{pH7.4}$ 4.0, measured with a capillary electrophoresis (CE) and mass spectrometry (MS) set up and a LC-MS approach, respectively [55]. The substance is crystalline white and with an onset melting point around 144 °C, without any significant property variability across the investigated batches of the API. None of the batches are hygroscopic (water uptake < 0.2% (w/w) between 0% and 80% relative humidity). During initial polymorphism investigation, only one crystal modification was found. The aqueous solubility of crystallized nanoparticles of the compound is 1.8 μM. This can be improved about 25 times when nanoparticles of the amorphous form are prepared, as shown in Figure 13.5. The solubility, in both cases, was measured from nanoparticles. The compound is a tentative Class II drug according to the BCS, based on the low crystalline solubility and high permeability in the gastrointestinal tract. Permeability measurements in the *in vitro* Caco-2 cell assay predicted the fraction absorbed to be high (Papp 46×10^{-6} cm s^{-1} with the present cell line) at the point estimate of a 12 mg daily dose. *In silico* simulations predict that the absorption of the compound is solubility limited and particle size dependent. These data indicate a significant risk that solubility limitations could result in failure of achieving high enough exposure in toxicological and clinical phase 1 studies using a conventional crystalline microsuspension. For these reasons, we started evaluating different nanosuspension formulations.

The compound was formulated as a crystalline microsuspension (3 μm), crystalline nanosuspension (220 nm), and amorphous nanosuspension (159 nm). The particle size increased both when no Ostwald ripening inhibitor was present, as well as with Miglyol as the only inhibitor included (Figure 13.6). By also including

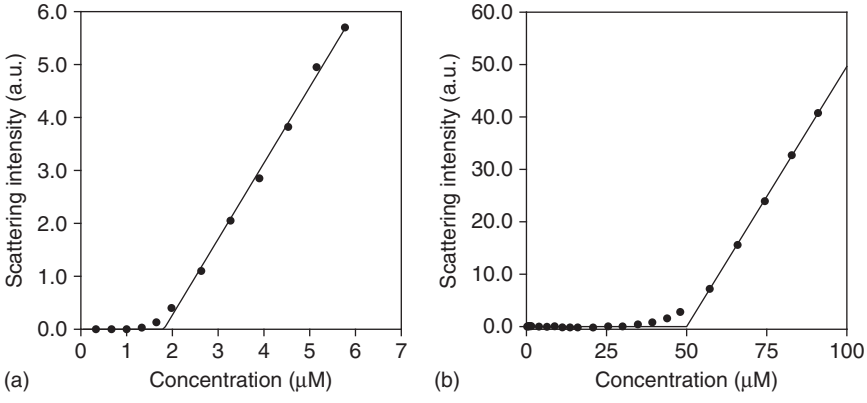

Figure 13.5 (a) Aqueous solubility of ultrasonically crystallized nanoparticles of the compound, stabilized by DPPE-PEG2000, (b) Aqueous solubility of amorphous nanoparticles of the drug in 1% DMA (v/v) at room temperature (drug/Miglyol/Pluronic L121 3 : 1 : 2, w/w/w). Pluronic was included to inhibit Ostwald ripening. Miglyol as the only additive did not inhibit particle growth. In this experiment the presented light scattering of the nanoparticles was background corrected for the light scattering from the inhibitors.

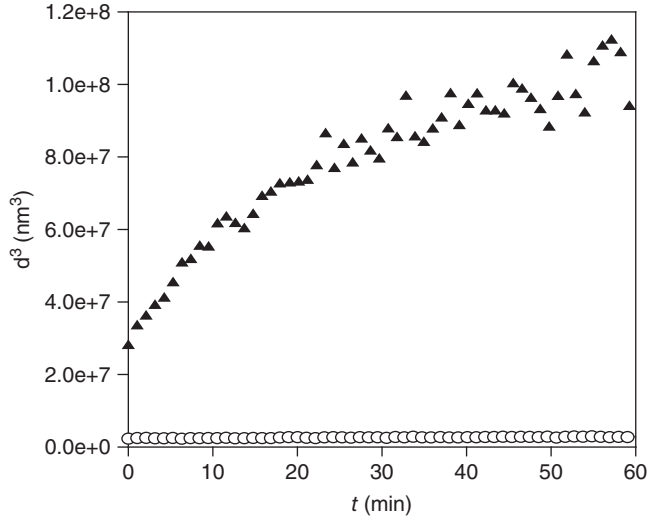

Figure 13.6 The amorphous particles of the compound increased in particle size without an Ostwald ripening inhibitor present (filled triangles). By including Miglyol/Pluronic L121, the particle growth was avoided (open circles).

Pluronic L121 to Miglyol, particle growth was avoided. In PK studies in rats, only amorphous nanosuspension provided high enough exposures (Figure 13.7), and therefore this formulation was used for safety pharmacology (i.v.) and repeated rat and dog (oral administration) toxicological studies. To bring this drug further along the development chain, a large-scale method for the amorphous drug

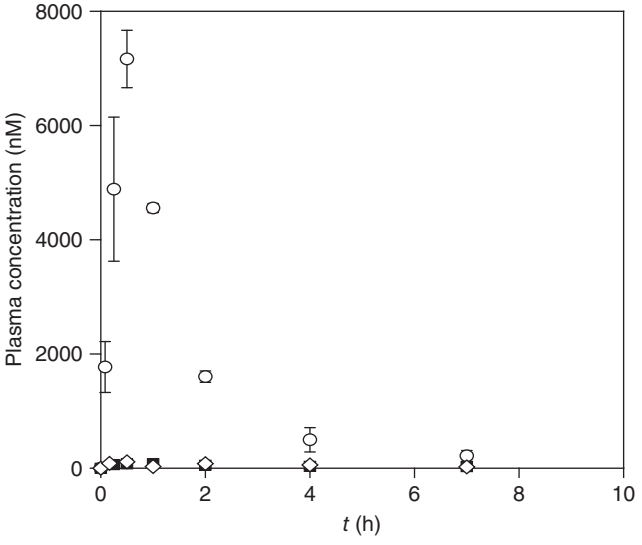

Figure 13.7 The mean plasma concentration (±SEM) of the compound versus time after oral administration of 50 μmol kg^{-1} drug as crystalline microsuspensions (filled squares), nanocrystals (open squares), and amorphous nanoparticles (open circles) to rats, $n = 2$. The two crystalline suspensions are superimposed.

was needed. For this purpose, an emulsion method was successfully employed to produce the selected amorphous nanoparticle formulation. In order to facilitate the formulation process for the repeated dosing studies, the standard amorphous precipitation method was simplified by using a preformed emulsion of Miglyol and Pluronic L121 with AOT as the emulsion stabilizer. This emulsion was further diluted with PVP and SDS solution, and the actual precipitation step was made by addition of this mixture to premade solutions of the drug in DMSO. The mixing scheme was set up to give the correct drug concentration and ratio of drug to inhibitor as well as a sufficient amount of PVP and SDS in order to stabilize the amorphous nanoparticles. The benefit of this procedure was that the mixing could be performed without sonication and still give small particles (160 nm), since the size of the particles now was determined by the size of the premade emulsion droplets (similar to the *melt emulsion method* discussed earlier). The present preparation method could be considered as a mix of the two earlier described amorphous approaches, suitable for the present drug and current animal setups.

13.7.3 Case Study 3: Amorphous Nanoparticles as a Screening Approach During Lead Optimization and in Repeated Toxicological Studies

During a lead optimization project, >100 compounds were evaluated in a single dose PK (oral and i.v.) study followed by a 14-day repeated (oral) study in rats with daily administration. The compounds were poorly soluble in water (in the order of 0.1–5 μM, characterized by high lipophilicity (log $D_{pH6.8}$ 5–7) [55], high molecular weight (ca. 600 g mol^{-1}), and no pK_a in the physiological interval [55]), so there were very few options available to develop a solution as a formulation of choice for early clinical development. The few alternatives that were identified *in vitro* were not compatible with the animal models used in the project due to the risk of affecting the PD component(s) of interest. The option with just compound and water appeared to be the more attractive path forward. Some of the earliest synthesized compounds from the series were evaluated for formulation development, and nanocrystals versus amorphous drug suspensions were compared. After these initial studies, amorphous drug nanosuspensions were selected as default formulation for forthcoming compounds from the same series in the project due a significantly improved exposure after oral administration to rats. The administered dose was manufactured 1 h before administration every day for the duration of the 14-day efficacy study. This was not due to Ostwald ripening, because the selected inhibitor Miglyol effectively precluded that, but for the risk of precipitation caused by crystallization of the drug.

When one compound was selected for further development, there was a need for highly concentrated nanoparticles to be available for the enabling toxicological studies. The natural choice was to investigate preparation of the nanoparticles with the melt emulsion method. To make sure that the drug was miscible with the oil phase, particle growth of an amorphous nanosuspensions (in small scale, described above) with and without inhibitor was monitored as a function of time

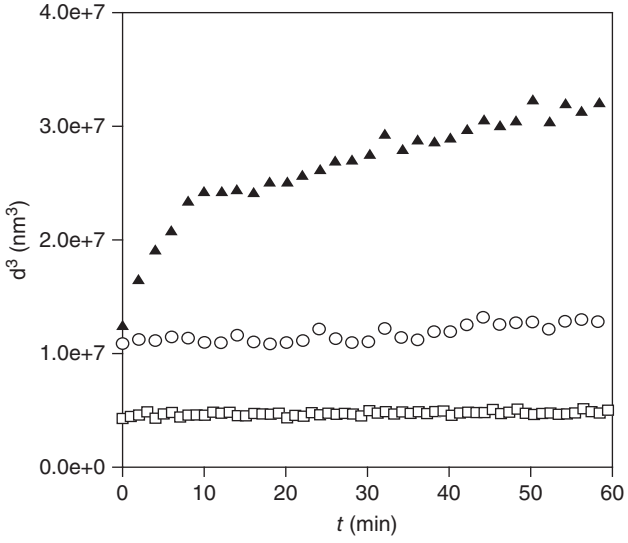

Figure 13.8 The amorphous particles of the compound increased in particle size without an Ostwald ripening inhibitor present (filled triangles). By including Miglyol (open circles) or Miglyol/Pluronic L121 (open squares), the particle growth was avoided.

by DLS (Figure 13.8). Ten percent of DMA was added to the system to increase solubility and accelerate the Ostwald ripening.

The actual preparation was performed as follows. An oil-in-water emulsion containing 20% (w/w) Miglyol 812N and 0.57% (w/w) AOT was prepared using a Polytron homogenizer followed by high-pressure homogenization. The emulsion droplet size was measured using DLS to 155 nm. For a total of 3.5 ml of nanosuspension, 0.57 ml of the 20% (w/w) emulsion was mixed with 1.75 ml of the 6.5% (w/w) suspension and 1.18 ml of water in high-pressure vials. When adding PVPK30 to the slurry and using Miglyol as Ostwald inhibitor, the nanosuspension prepared by melt emulsion method was acceptable (250 nm). Particle size of the nanoparticles was stable for at least 2 h after preparation, and no crystal growth was seen. The drug was also stable during a freezing–thawing cycle in 10% (w/w) sucrose. Two hours after thawing neither particle growth nor crystals were observed.

Finally, the amorphous drug nanosuspensions prepared with the melt emulsion method were tested *in vivo* in rats. Female Sprague Dawley (SD) rats, age about 12–15 weeks, were used. Four rats were used to evaluate the amorphous particles, and four animals received milled nanocrystals orally, of similar particle size (250 *versus* 210 nm) and dose, 100 μmol kg^{-1}. The nanosuspensions of the drug prepared by the melt emulsion method resulted in higher plasma concentrations than nanosuspensions prepared by the wet milling method (Figure 13.9). Bioavailability was 4–5 times higher for amorphous nanoparticles than for crystalline nanoparticles. For these reasons, the described nanosuspension was selected as the compound formulation for all early clinical development studies.

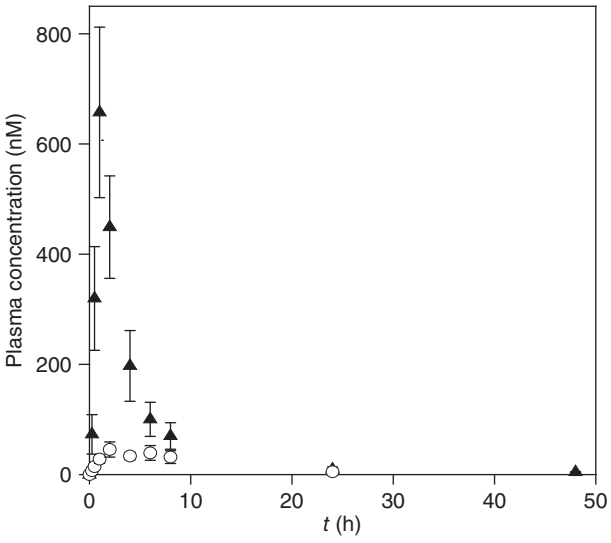

Figure 13.9 The mean plasma concentration (±SEM) of the compound versus time after oral administration of the drug as nanocrystals (open circles) and amorphous nanoparticles (filled triangles) to rats, $n = 3$. The administered dose was 100 μmol kg^{-1}.

13.8 Conclusions

Nanosizing has become a well-established and frequently used formulation approach for poorly soluble compounds during preclinical work. Extensive research has generated many different approaches to produce drug nanoparticles. Up to now two standard approaches, wet milling and high-pressure homogenization [56], have been used and also delivered drug nanocrystal products to the market. In the present chapter, the former, wet milling is described as one of four approaches in a preclinical toolbox. Regarding crystalline drug nanoparticles, the milling approach is mainly used for high doses and large quantities, while a precipitation approach is used for smaller amounts (approximately some milliliters) up to about 10 mM. Two different main approaches are described for amorphous drug nanoparticles: one appropriate for low-dose animal studies and another for larger-scale toxicological studies. With this toolbox, almost all poorly soluble drugs can be administered, using every route and animal species. For example, for bioavailability studies, the same formulation and preparation can be used for both routes. Nanocrystals used for oral administration can result in improved *in vivo* exposure compared to larger particles suffering from dissolution rate limitations. Amorphous drug nanoparticles can add one more dimension to the *in vivo* exposure, supersaturation, due to amorphous material. In two of the case studies presented, amorphous drug nanoparticles resulted in superior oral bioavailability compared with nanocrystals of the same compound. Drug nanosuspensions add tremendous dosing flexibility, rapid manufacturing possibilities, and the opportunity to use all conceivable administration routes.

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Part III

Pharmacokinetics and Pharmacodynamics

14

Integration of Pharmacokinetic and Pharmacodynamic Reasoning and Its Importance in Drug Discovery

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14.1 Introduction

Pharmacokinetic/pharmacodynamic (PK/PD) integration is vital to any attempt to link preclinical results to the acute and long-term drug treatment consequences in humans. There is an ongoing debate among scientists across the industry, academia, and regulatory bodies on whether, and if so how, preclinical findings can be integrated and translated to the human situation. There is also controversy as to how complex metabolic systems can be understood by gauging a single-time point only. Moreover, in our experience, the crucial integration of pharmacokinetic (PK) and pharmacodynamic (PD) information is often suboptimally addressed and exploited in preclinical drug evaluation contexts. To this end we have pointed to some of the challenges typically seen in integrative pharmacology within drug discovery in a series of recent articles [1–4].

In the current chapter we will highlight some of the basic concepts in PK/PD reasoning and anchor them in real-life case studies. These specifically chosen examples are aimed at demonstrating areas of recognized improvement between the fundamental understanding of traditional drug metabolism and pharmacokinetics (DMPK) and pharmacology and how they are applied in pharmacological research throughout discovery phases. Some of the topics discussed include, for example, how to understand target biology, prune data prior to drawing any conclusions, understand concentration– and response–time courses, concentration–response relationships, translational context, and also how to improve communication across disciplines.

Many medically treatable conditions are long lasting in nature, and chronic indications typically require chronic dosing. The basic premise is that there exists an exposure (dose)–response relationship upon repeated dosing, and it should thus also be possible to establish a dose–concentration relationship. If both dose–response and dose–concentration relations exist for the studied test compound (drug) and pharmacological response, then some kind of

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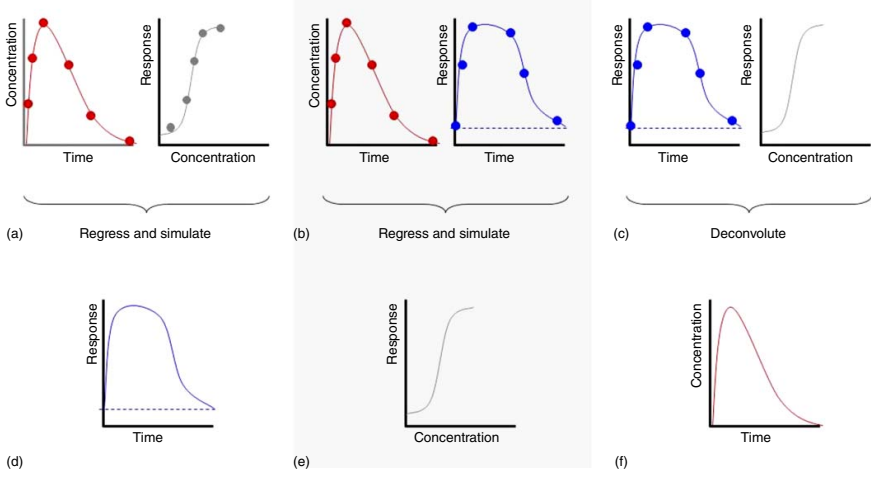


Figure 14.1 Concentration–response–time relationships and how they may be utilized. (a, d) Available information about the *in vivo* pharmacokinetic parameters and dose are combined with, e.g. *in vitro* concentration–response (binding) parameters to simulate the *in vivo* response–time course prior to the execution of an *in vivo* study. (b, e) Acute concentration–time data are used to "drive" the response–time data in a kinetic/dynamic model. From the regression of acute *in vivo* response–time data, the equilibrium concentration–response is predicted. (c, f) *In vivo* response–time data are deconvoluted with an *in vivo* concentration–response model (or *in vitro* binding data) in order to obtain the exposure–time profile behind the response–time course.

concentration (exposure)–response (effect) relationship also prevails at steady state. It may not always be immediately apparent, but there is one, and one that also may be useful when selecting the appropriate dose, translating preclinical data for humans, or assessing the safety margin.

Three situations where concentration–time, concentration–response, and response–time data are available or sought are shown in Figure 14.1. Sometimes the *in vivo* response–time course is simulated based on already available information on the concentration–time and *in vitro* response (or binding)–concentration data. On the reverse, concentration– and *in vivo* response–time data are available, and the equilibrium concentration *in vivo* response is sought. Thirdly but less frequently, information about the *in vivo* response–time course is deconvoluted by, e.g. *in vitro* concentration–response data, in order to predict the required concentration–time profile. In all three situations the unknown is sought from the two available pieces of data.

We will center the current chapter on the five central themes in project PK/PD reasoning, namely, to understand:

- *Your target biology*
- *Your concentration–response relationship*
- *Your pharmacological response–time course*
- *Your translational options*
- *Interdisciplinary communication.*

In our experience, a lack of target and biology understanding, inadequate application of PD and PK knowledge, and deficient interaction between disciplines may seriously delay or, in the worst-case scenario, even kill an otherwise perfectly sound project poised for clinical development. Thus, for example, substandard insights into target biology and concentration–response and concentration–time relations, lead to flawed design of experiments and interpretations of drug action. This will also have repercussions on how to further optimize a promising lead molecule. Failure to address translational options, including species biology and readout particulars, may result in wildly erroneous dose/exposure–response predictions toward early drug development and tests in man – in turn resulting in safety/tolerability issues or in under-dosing. Needless to say, an important key to efficient, safe, and speedy project delivery into late lead optimization/early development is also high quality communication among all disciplines involved.

14.2 Understand Your Target Biology

A complete understanding of the target biology and mechanism(s) of action might be difficult to establish in a very early drug discovery phase. Needless to say, insufficient information on the target biology in question makes the process decidedly more complex, time-consuming, and challenging at several levels [5]. Whereas of course still not impossible, such drug discovery and development ventures may then have to involve various deconvolution approaches [6–8]. At the same time it should be recalled that whereas early work in most small molecule drug discovery is carried out by screening chemical libraries against

single targets in a true "reductionist" manner, the required pivotal assessment of selected agents has to await well-designed *in vivo* studies to define drug action in the broader organism context. Consequently, a detailed understanding of the location, function, and situational impact of a target across basic and perturbed physiology conditions will greatly facilitate identification of links between drug exposure and effect. In the present chapter, we have endeavored to include pointers to pertinent biological principles across the case studies discussed.

Nevertheless, even with limited knowledge of the underlying biology, modeling approaches based on available pharmacological response–time data may be possible to utilize for ranking of compounds, support study design, safety assessment, and provisional human dose predictions. When applying a "pattern recognition" tactic [9] with relatively few assumptions, it is possible to develop rational mechanistic theories of how a key component may be modulated by a drug to produce the actual response readout. Thus, Figure 14.2 shows six data patterns, the first three of which represent examples where the target biology is relatively well understood (*(i) reversible inhibition of response biomarker synthesis, (ii) combined reversible inhibition and stimulation of biomarker response, (iii) irreversible enzyme binding*), while the latter three demonstrate instances where either less is known about the target biology or the response

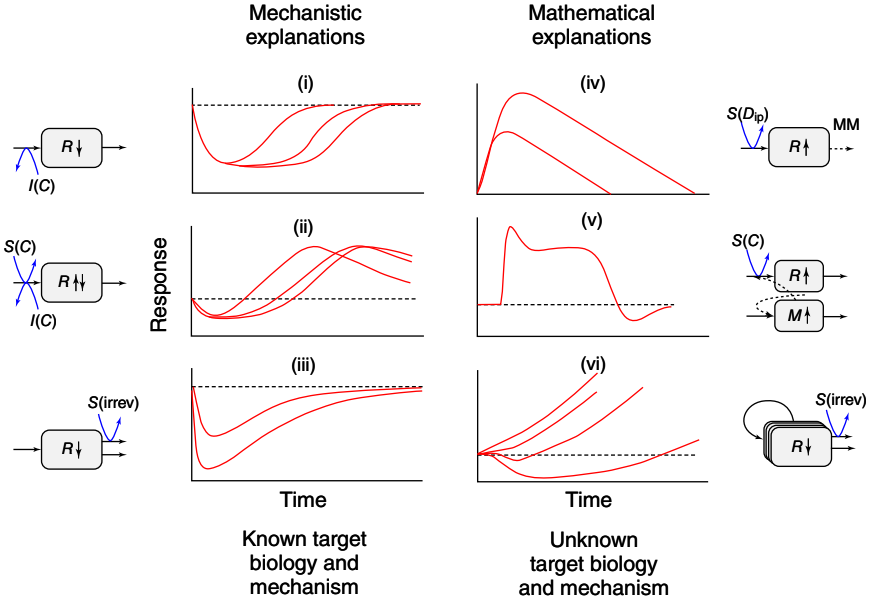


Figure 14.2 Schematic presentation of data patterns from three data sets (Examples 1–3) of which the underlying mechanism of action is known (*known target biology*), and three (Examples 4–6) where the exact relation to target mechanism of action is less easy to pinpoint (*unknown target biology*) and therefore a mathematical description may suffice [4, 9]. *C, R, S,* and *I* denote the drug concentration, the pharmacological response, and the symbols for stimulatory and inhibitory drug action, respectively. MM, D_{ip}, and irrev denote Michaelis–Menten loss, intraperitoneal dose, and irreversible action, respectively.

readout is a conglomerate of several mutually interacting components thus complicating simple target-derived mechanistic interpretation (*(iv) locomotion, (v) EEG response, (vi) cell killing*) [4, 9]. In examples (i)–(iii) we start out using the background biology knowledge and use the identified target mechanism to "drive" the model building process. Conversely, in examples (iv)–(vi) we start with the actual observed data, and let that guide us in setting up equations that mimic the time–response data.

A couple of additional comments may be worthwhile here also: In Case example (iii) the baseline is defined by the natural turnover rate and loss of an enzyme. By adding a drug that irreversibly binds to the enzyme, the biomarker response is suppressed due to an accelerated loss process. Recovery of the response in this case, on the other hand, is governed by the basal natural turnover – primarily the regeneration rate of the enzyme. If the model is constructed around the mechanism of action, it is also easier to make predictions outside the realm of the data. A purely mathematical explanation of data suits its purpose for interpolations between doses and assessment of pharmacologically efficacious concentrations, but does not always help furthering biological insights related to background target processes.

Behavioral readouts in an intact animal represent a special situation in the pharmacological testing context. Any such model used needs to be particularly carefully controlled for any confounders of the data. In Case example (iv) the response readout is locomotor activity in rats after two intraperitoneal doses of amphetamine [10]. The drug is a well-known indirectly acting CNS dopamine (DA) stimulant, and the hyperactivity response observed is likely a reflection of this. However, the *exact* mechanism of amphetamine likely comprises several different DA transmission-promoting (including possibly other direct and indirect) events, the relative influence of which may also vary across doses (cf. e.g. Ref. [11]). Moreover, the baseline, nondrug motor activity level varies across the light–dark cycle and is dependent on whether the environment is familiar or not and a number of other related factors. Additionally, it is known that at high exposures of DA stimulants, behavioral stereotypies may interfere with (more or less) coordinated locomotion. This notwithstanding, a mathematical model capturing the apparently linear rise and decline in experimental data readout could be successfully fitted to the data set [12].

Below are listed some additional basic biology considerations that may be worthwhile taking into account in the design of a drug evaluation study or developing a PD model.

14.2.1 Physiological Context

An important strategic decision for a drug project is to know where to strike in a chain of events involved in the response to be modulated. For example, in the early days, histamine H_2 or muscarinic ACh receptor blockers were used to treat conditions with increased gastric acid secretion – hitting upstream of the proton pump generating HCl. Nowadays the same effect is achieved in a more selective and efficacious manner by the use of direct irreversible proton pump inhibitors

(PPIs), skipping receptor transduction steps, and instead acting straight on the H^+/K^+ ATPase enzyme pump on the luminal side of gastric parietal cells, thereby blocking the generation of acid (until new proton pump molecules have been produced). The PPIs are prodrugs that are activated by the only acidic milieu in the body – the stomach – and will therefore also selectively accumulate in parietal cell secretory canals, thereby limiting the exposure of nontargeted tissue (see Ref. [13]). An integrated assessment of drug mechanistic PK and PD properties relative to the physiology in the intended clinical treatment indication is thus an essential factor for a successful drug development venture [5].

14.2.2 Which Biomarker to Monitor

In choosing target-related response readouts, it needs to be decided which would be the most appropriate biomarker to address. Do easily accessible, validated, and direct target-responsive markers exist? Alternatively, would a surrogate marker – that is, a physiological, behavioral, hormonal, or other signal biomarker reflecting the same target interaction, but outside the primary context function to be modulated by the drug (see Ref. [14]) – or other approach be satisfactory? Ideally, the biomarker(s) to be monitored for kinetic/dynamic purposes are continuous variables, possible to follow over time in relation to drug exposure, stable over time even in the face of progressing disease, and homogenous and translatable across species. With regard to dichotomous (e.g. all-or-none) data, there are also several studies in which successful analysis has been performed by means of logistic PD models [15]. The outcome variable is then the logit, which means, for example, the probability of 50% response.

14.2.3 Buffering, Tolerance, and Redundancy in Targeted Functions

Few if any physiological systems work in isolation. The presence of backup/buffering mechanisms is the rule, so as to shield and uphold adequate system function even if one key mechanism has been affected – this is relevant both to disease or condition to be treated and to the drug–target action. Consequently, analysis and interpretation of any experimental response data should always be carried out against background knowledge and theories on the putative involvement of redundancy and compensatory processes that may influence the net readout – particularly with chronic dosing.

14.2.4 Target vs Off-Target or Confounding Factors

These "non-specific" aspects should always be considered. For example, assessing whether an anorexigenic drug results in the desired target-derived reduction of food intake, special care needs to be taken to exclude any nonspecific action that may confound the readout (e.g. sedation, stress, nausea, motor effects, etc.). If at high doses of a drug a qualitatively distinct effect or direction of response is observed, there may be reason to suspect that the drug exposure has entered a range where secondary target actions begin to show. This may display as bell- or U-shaped concentration–response curves (cf. example case study further below).

14.2.5 Experimental Model and Protocol

The optimal design should take into account how the disease progress might affect the target organ/tissue/system. *In vivo* models are clearly always advantageous if possible, in particular by incorporating disease-like perturbation of the function of the affected organ system. The experimental paradigms also need to consider possible disease progress during the course of treatment – if different responses result from treatment at an early stage as compared with that at a later stage – and if so how to include matching baseline controls, adjusting testing conditions, etc. Responses that are objectively monitorable and quantitative, rather than subjectively and/or qualitatively appraised, are clearly preferable, not least from the integrative PK/PD modeling perspective. Drug testing to determine preclinical target activity toward a disease or other clinical condition may be misleading if performed in normal, healthy state models and should thus be avoided even if the target is present and measurable (this should be self-evident, but is not always the case in our experience).

14.2.6 Experimental Design Aiming at Target Biology

In the overwhelming majority of models employed in drug discovery research, there is an underlying system dynamic that needs to be considered. Experimental design is however far too common addressing responses from a static-only viewpoint. Take, for example, food intake: with certain genetic mutation-derived exceptions, food is typically consumed in a few larger meals, interspersed with snacking bouts. This thus brings the question regarding which strategy approach to be taken for novel anti-obesity treatments. Should we aim for short-acting agents to be administered just before every main meal, to take the edge off hunger urges and help achieving early satiety – or, alternatively, go for longer-acting agents aiming to cover the 24 h cycle in its entirety, to also dampen, e.g. between-meals snacking? While either tactic may prove useful, they logically bring distinct challenges from the drug development perspective, with regard to selecting both the optimal obesity patient population and preferred target and how to work with chemistry, PK and PD properties of novel agents, etc.

A related real-life example is observed in the area of lipid control. Thus, it is known that due to tolerance development, the strategy of constant around-the-clock nicotinic acid (NiAc) exposure fails to deliver durable FFA lowering [16]. By contrast, an intermittent NiAc dosing strategy produced sustained reduction in FFA and acute insulin-sensitizing effects. This approach however did not reverse lipid excess, unless achieved through timing NiAc exposure to feeding periods. This synergy between pharmacology and physiology reversed peripheral lipid accumulation and profoundly improved lipid and glucose control. Anti-lipolysis applied in conjunction with feeding can be an effective means of reversing lipid overload-induced insulin resistance and dyslipidemia [16]. Pharmacological principles for treating metabolic disease (and probably other diseases in general) may therefore require careful fine-tuning (timing and shape) of drug exposures. In turn, such reasoning has important bearings on the preclinical model chosen, as well as on how experimental modeling and simulation for target and response must be tackled.

14.2.7 Species and Translation

Much of species and translation deliberations come down to *comparative* species *physiology*. Thus, as part of designing study protocols, it is important to ascertain (i) that the target is present and subserves a corresponding function in animal as in man, (ii) that the mechanistic environment (systems, circuits, targets) that may modulate this function is either identical across species or can be satisfactorily controlled for, and (iii) that the affinity and selectivity of the drug for the primary target vs off-target interactions are maintained in relation to exposure levels encountered when translating dose from preclinical to human clinical studies.

14.2.8 Treatment vs Cure

Finally, it should be acknowledged that with few exceptions (e.g. antibiotics/chemotherapeutics), new drugs only rarely aim for the root cause of a disease or ailment. Thus, most will end up being symptomatic treatments, but not cures of the indication intended. It is also worth remembering that despite our efforts and aims toward rational and directed drug design, discovery, and development, the exact (or even main) mechanism of action of many commonly used efficient therapeutics still remains controversial – or sometimes unknown. This therefore remains a factor to keep in mind, and one that may complicate simple, straightforward PK/PD reasoning. An open mind, and sometimes a "black box" deconvolution approach, might be considered to facilitate interpretations and onward progress in cases like these.

14.3 Understand Your Concentration–Response Relationship and Time Delays

One of the major challenges during the discovery process is to establish a reasonable plasma concentration range to guide later human dose predictions. Human clinical effects are often projected by means of nonhuman biomarker data, an issue that in itself may pose a significant challenge in many therapeutic areas. Additionally, across species there are differences in plasma protein binding, active metabolites, and other drug handling matters, as well as variation in the physiological and target biology setup (e.g. presence/absence of endogenous ligand and tone); temporal differences between plasma concentration and biomarker responses further muddle interpretation. Below, we will specifically address the concentration–response relationship and temporal (time) differences and how these are combined in order to understand what governs the onset, intensity, and duration of a pharmacological response (Figure 14.3).

Figure 14.4 shows schematically the equilibrium concentration–response relationship of an agonist. It also shows an example of variation in the ranges for a low (red) and a high (blue) dose/concentration relative to the potency value (EC_{50}). These ranges are represented as a concentration–time course (Figure 14.4, a) and the corresponding two response–time courses (Figure 14.4, b and c). It is clear that a response–time course obtained from plasma exposure

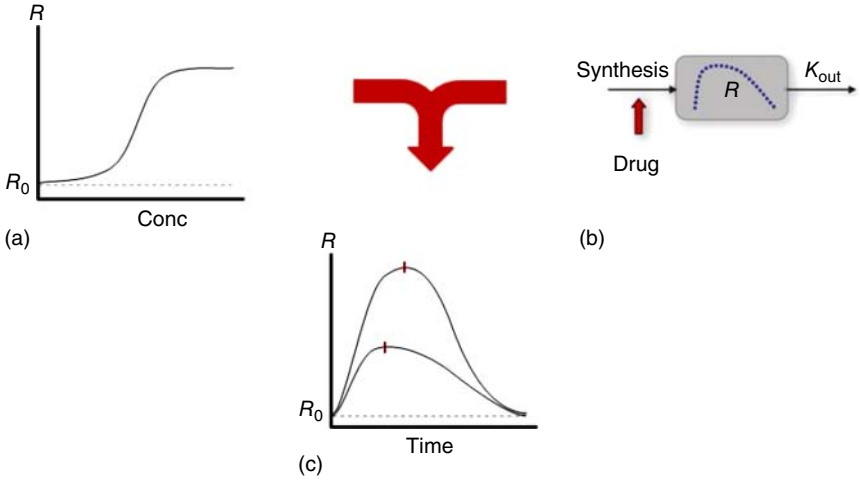


Figure 14.3 (a) Schematic illustration of the concentration–response relationship at equilibrium. (b) The pharmacodynamic model of drug action as stimulation of the production (turnover rate) or buildup of response. (c) Response–time courses that then show the onset, intensity, and duration of response as well as the peak shift with increasing doses. The time delay between concentration (red bar indicates time of maximum concentration in plasma) and response–time courses will manifest itself as a counterclockwise hysteresis plot (not shown). A peak shift is also seen in the response–time course with increasing doses.

below the EC_{50} value mimics the concentration–time course to a large extent independently of rapid or slow plasma kinetics. When plasma – and thus, by proxy, target biophase exposure – exceeds the EC_{50} value, the corresponding response–time course is less dependent on the concentration–time course. In the latter case the plasma half-life of drug will only determine the terminal portion of the duration of response.

14.3.1 Nonmonotonous Concentration–Response Curves

Bell- or U-shaped, biphasic, concentration–response relationships are well-known, frequently encountered phenomena in pharmacology [17]. (For a review and discussion of biphasicity/hormesis in physiology and toxicology, see Ref. [18] and references cited therein.) They may be due to a loss of drug-to-PD target specificity when entering a supramaximal exposure range (relative to the primary target). They may also reflect the gradual recruitment of target populations in other locations and with different sensitivities as exposure is increased. Additionally, they may involve adaptational mechanisms, the engagement of which unveil and give rise to contrasting response(s) from the expected.

Figure 14.5 shows the effect of a novel atherosclerosis target test compound treatment on a corresponding PD biomarker readout. As seen in the graph, low to intermediate drug (0.1–4 μM) exposure leads to a reduction in atherosclerosis. However, when the concentration is raised above 4 μM, there is a sudden and sharp loss of the anti-atherosclerotic action, and no difference relative to control levels is seen even at ~20 μM exposure. The first phase reflects the drug effect

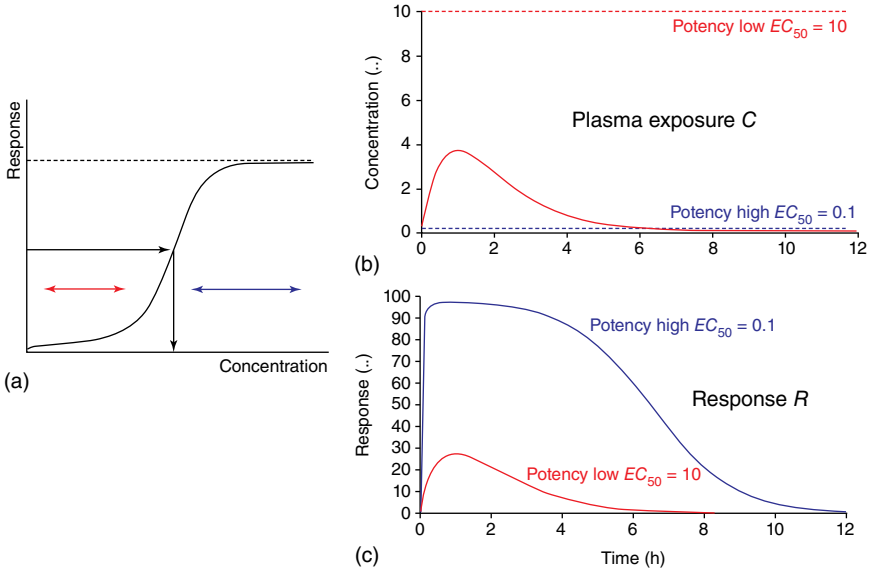


Figure 14.4 (a) Schematic presentation of the *in vivo* concentration–response relationship. The red and blue double arrows depict the concentration intervals covered (graph b) and the corresponding response–time courses (graph c). (b) The red line curve demonstrates the concentration–time course and its relative position to the potency values (a low numerical value of EC_{50} equals high potency; a high numerical value equals low potency). (c) The response–time courses corresponding to plasma exposure and potency is indicated in graph b. When potency (EC_{50}) is 10, plasma exposure is insufficient to elicit a full response; in this case the response–time course by and large mimics the concentration–time course. For comparison, when plasma exposure exceeds the target potency (EC_{50} = 0.1), the response–time course substantially differs from the shape of the concentration–time course [3].

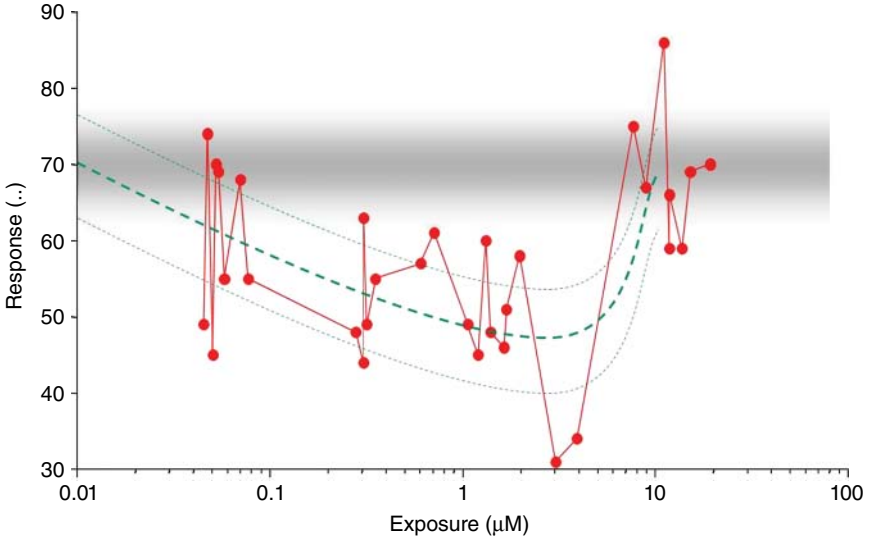


Figure 14.5 Anti-atherosclerotic effect of compound X across an exposure range covering more than three orders of magnitude. PD response readout dots represent single observations. The suggested anti-atherosclerotic concentration falls within the 0.1–2 μM range.

at its primary intended target. The return to baseline control response at high concentrations is considered to derive from affinity of the drug for a secondary target; the interaction of which nullifies the therapeutic PD readout in this model.

The abovementioned example serves to illustrate the importance of understanding (i) the specificity of the drug understudy over its *entire* intended exposure range, (ii) the particular mechanisms and confounders influencing readout in the experimental model and conditions used, and (iii) whether an analogous exposure–response relation can be expected also in men, that is, how well the biomarker biology can be used for translation into the clinical context. Adequate insight into factors that underlie potential nonlinearities in drug PD action is a prerequisite not only for defining pharmacological properties of the drug in question but also with regard to identifying safety threshold exposures and ratios toward therapeutic use (e.g. no-observed-adverse-effect levels, (NOAEL) and similar).

A further, more general but essential aspect to keep in mind is the impact of a well-defined baseline and window for the PD response readout. Thus, while a high baseline will make it difficult to discern stimulatory effects of a drug, a low baseline might preclude detection of inhibitory actions; a too narrow response window (irrespective of baseline) is also suboptimal for high-resolution concentration (dose)–response studies.

14.3.2 Dose Scheduling ("Dose Fractioning")

Schedule dependence is a dose–concentration–response phenomenon that can be predicted with models and observed in clinical practice. Essentially, for any drug dose that is given over a defined period of time, the total drug effect will depend on the dosing schedule. Schedule dependence is the result of a nonlinear concentration–response relationship and occurs for drugs that demonstrate reversible binding properties at the site of action. It is therefore an important determinant of the dosing regimen for most drugs. An example is highlighted in Figure 14.6 using the diuretic furosemide [19]. In this case a single 120 mg dose causes 50% smaller area under the natriuresis–time curve than does 40 mg *t.i.d.* (Figure 14.6). The total drug response is therefore highly dependent on the dosing schedule.

A lowering of the metabolic load of a drug and simultaneous improvement of its therapeutic potential are possible by utilizing the nonlinear concentration–response relationship. A primarily response-centric approach was applied in this case, i.e. to increase either the area under the response–time curve (AUC_R) or lessen the response–time fluctuations by splitting the daily total dose into three doses. Dose scheduling is an old and well-defined approach, but unfortunately one that has become less utilized compared to the widespread once-a-day dosing paradigm.

As evident from the diuretic example above, switching from a once-daily to trice-daily administration protocol has several potentially beneficial corollaries. Dispensing the 12-h dosage (120 mg) in three split portions once every 4 h instead of in a single-bolus dose results in (i) ~threefold lower C_{max} levels, (ii) smaller peak-trough oscillations, and (iii) extended duration of exposure

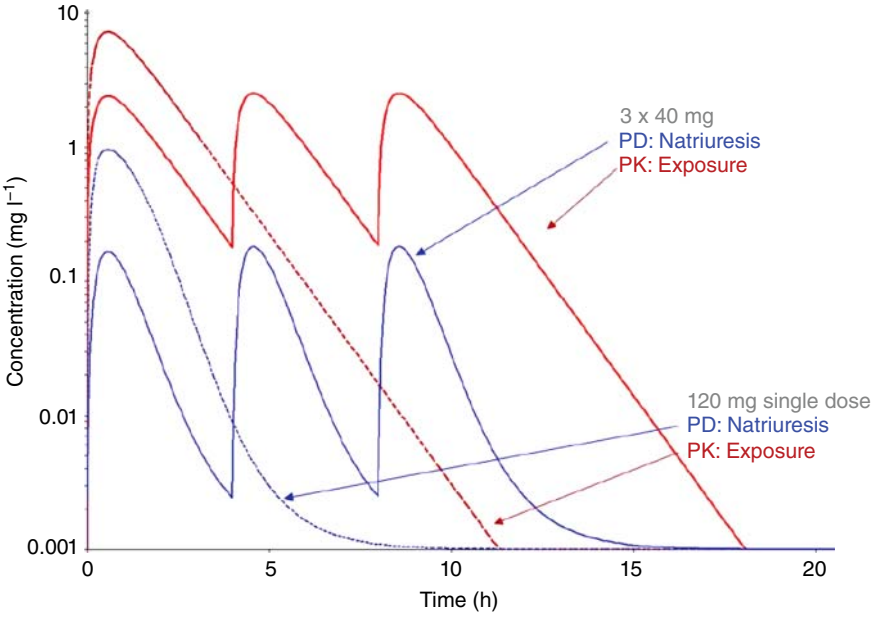


Figure 14.6 Furosemide and schedule dependence. A single 120-mg dose of furosemide results in less total (Na^+-)diuresis over 12 h than does three 40-mg doses. The 40-mg area under the effect curve $AUC_e = 600$ mmol Na^+/12 h; the 120-mg $AUC_e = 430$ mmol Na^+/12 h. *Source*: Adapted from Wright et al. (2011) [19].

within the therapeutic concentration range. From a PD perspective, firstly, dividing a 12 h dosage into three separate fractions will avoid unnecessarily high-peak concentrations of the compound while still delivering the desired clinical therapeutic effect (diuresis) at an even better margin vs off-target and safety levels. Secondly, the 12 h drug response efficacy (AUC_R) increases nearly 40% in the divided compared to the single-bolus schedule. Finally, the split-dose response lasts at least as long as the single administration, with little exposure fluctuation over this period. For conditions where sustained target coverage is of particular value, a dose-scheduling approach – if feasible – is thus generally much more appealing compared to single-dose pulses. In these cases a stable concentration–time exposure profile is more important than a (single) high C_{max} level. An alternative approach to escape high plasma concentration fluctuations would be to consider an extended release formulation, aiming to keep the same average concentration but avoiding the high peaks and low troughs. The dose-scheduling strategy thus in our opinion deserves serious consideration in relation to the intended indication and risk–benefit ratios of new drugs.

14.3.3 What Matters Is Steady State for Chronic Indications

What matters in compound optimization for chronic indications is steady state and not necessarily the "free drug hypothesis" [20]. For low molecular

compounds with a rapid equilibration time between bound and free drug in plasma, it is commonly assumed that:

- At steady state, the free drug concentration is the same on both sides of any biomembrane.
- Free drug concentration at the site of action, the therapeutic target biophase is the molecular species that drives pharmacological activity.

Unbound plasma drug concentrations are widely applied in drug discovery and development to establish PK/PD relationships, to predict the therapeutically relevant dose, and to monitor drug concentration in clinical studies [21, 22]. It should be kept in mind however that, whereas total plasma concentrations are analytically determined in samples from *in vivo* studies, corresponding unbound concentrations are the result of multiplying these by a factor derived from separate *in vitro* protein binding experiments. Generally, provided plasma protein binding is accurately and precisely determined *ex vivo*; less variability is to be expected when total plasma concentrations are converted to unbound. However, unbound plasma concentrations do not necessarily equal unbound concentrations at the *target site*, therefore deviating from the free drug hypothesis as stated above. Reasons for this include membrane transporters, compound bulk flow, metabolic capacity, irreversible binding to target, and other properties of the tissue that may result in unbound concentration gradients. Hence, unbound concentrations in plasma may differ from those in the target tissue. Nonetheless, from the modeling perspective, the unbound plasma concentration may still be used as a substitute driver of the pharmacological response, as it can be assumed that at steady state, there is a constant ratio between the unbound concentration in plasma and the unbound concentration at the target. At the same time the relation between unbound plasma concentration and the target *in vitro* potency value can be quite different from the "true" ratio in the biophase matrix *in vivo* (i.e. drug concentration relative to target affinity in the biophase). This may be particularly relevant for drugs with high target specificity and for *in vitro*/*in vivo* systems with large differences in target expression [5].

Sometimes plasma protein binding determined in a single species is used in studies across several species. Figure 14.7 is aimed to highlight differences and issues that may occur by using such an approach. The data demonstrate the large interspecies differences at pharmacological concentrations (<0.1 μM). Further, there is also nonlinear binding in all species as the unbound concentrations increase of a compound that binds primarily to alpha$_1$-acid glycoprotein. It is evident from Figure 14.7 that prediction of an effective exposure in man would be markedly overestimated had the f_u determined in the rabbit been used. Further, in this example it can be seen that whereas the f_u in the mouse and rabbit remains relatively stable across a wide concentration span, the change is 10- or even 20-fold in the dog and guinea pig, respectively. This illustration attests to the view that it is wrong to assume *a priori* that protein binding and free fraction are similar across species and thus that interspecies differences have to be accounted for when comparing nonhuman with human data. Such findings not only have implications for the interpretation and extrapolation of

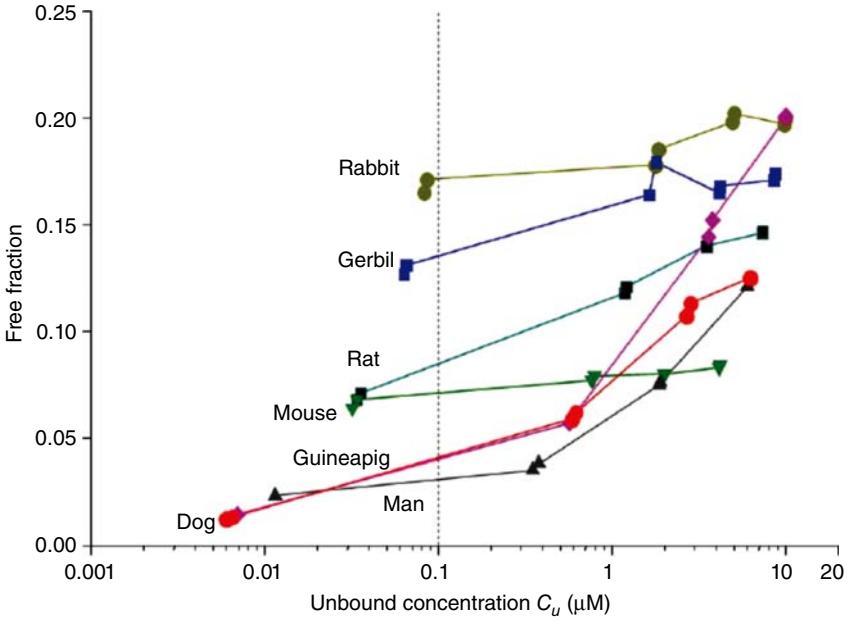


Figure 14.7 Unbound concentration vs free fraction of test compound X in mouse, gerbil, rat, guinea pig, rabbit, dog, and human plasma. Note the large interspecies differences at pharmacological concentrations ($C_u < 0.1$ μM), and the species-dependent nonlinear increase in free fraction f_u with increasing unbound concentrations [3].

pharmacological data but also influence the assessment of safety margins and benefit-risk ratio.

Figure 14.8 shows a schematic picture of three steady-state situations where unbound concentration in plasma and tissue are the same (a), plasma concentration is higher (b), and plasma concentration is lower than tissue concentration (c). It is not primarily necessary that the plasma and tissue unbound concentrations are the same at equilibrium, but form a *constant ratio*. If that ratio is established at pharmacodynamic steady state, the unbound concentration in plasma may then serve as a substitute for the unbound concentration at the target driving the pharmacological response. Recall though that the plasma unbound concentration in cases (a) and (c) will however over and underestimate, respectively, the *absolute biophase exposure* needed for pharmacodynamically relevant target occupancy [23]. This will therefore also affect any *in vitro/in vivo* correlation exercises. Also, the relation between drug concentration and pharmacological response may change over time, should the disease condition entail progressive physiological alterations in processes that affect biophase access [24].

For an orally administered drug, the total and unbound drug concentrations in plasma become

$$\begin{cases} C = \dfrac{C_u}{f_u} = \dfrac{\frac{\text{Dose rate}}{Cl_u}}{f_u} \\ C_u = f_u \cdot C = f_u \cdot \dfrac{\text{Dose rate}}{f_u \cdot Cl_u} = \dfrac{\text{Dose rate}}{Cl_u}. \end{cases} \tag{14.1}$$

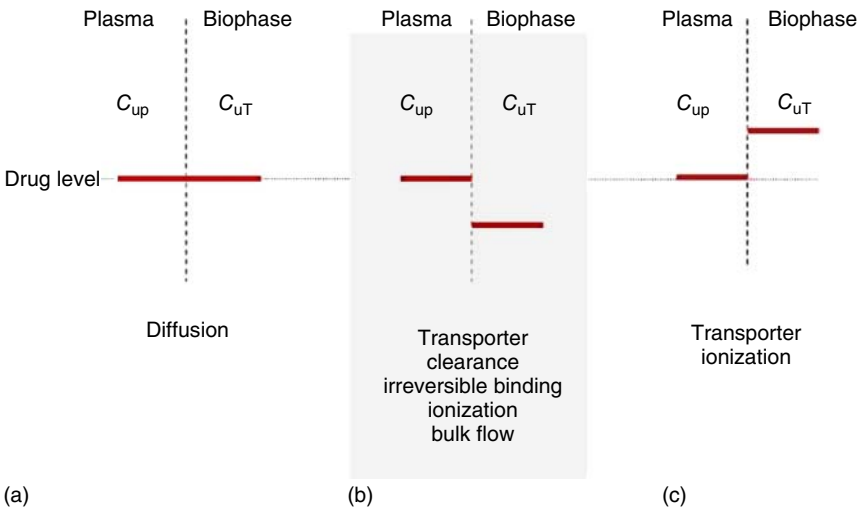


Figure 14.8 (a) Unbound (red line) concentration is equal in plasma, C_{up}, and tissue (biophase) C_{uT} at steady state due to simple diffusion. (b) Unbound concentration in plasma C_{up} is higher than in tissue C_{uT} due to different *sink* conditions in tissue such as transporters, clearance, irreversible binding, ionization (ion trapping), or bulk flows (CSF). (c) Unbound concentration in plasma C_{up} is lower than in the tissue biophase due to transporters or ionization.

If the effective plasma concentration is $C_{u,e}$ (or EC_{u50} or IC_{u50}), then the parameters to optimize in order to lower the *dose rate* become

$$\text{Dose rate} = C_{u,e} \cdot Cl_u. \tag{14.2}$$

In other words, the preferred parameters to adjust are (i) the target plasma concentration (or potency) and (ii) the primary parameter determining removal of test compounds, that is, the unbound clearance, Cl_u. In situations where plasma protein binding is very high ($f_u < 0.05$), the precision of the free fraction becomes low (and conversion of C to C_u may further leverage the imprecision), or even unknown. Under such conditions, conversion of total concentrations to unbound concentrations should be avoided. Still the product of the total target concentration and total clearance can be a good second choice.

$$\text{Dose rate} = C_e \cdot Cl \tag{14.3}$$

We do not endorse optimizing volume of distribution V_{ss} for low molecular compounds with a rapid equilibration time between bound and free drugs in plasma because it is primarily a storage term that is not involved in the removal of drug. The volume term is also a conglomerate of unspecific and target-specific binding processes. Extending or reducing the volume term affects not only the tissue-to-plasma partitioning but also the specific binding. Nonspecific tissue partitioning and target binding do not necessarily correlate, neither does binding to specific tissues. Equation (14.4) illustrates this by a mathematical description of what makes up the apparent volume term in terms of nonspecific and specific binding parameters, extraction, and anatomic volumes:

$$V_{ss} = V_B + \sum_{i=1}^{n} V_{Ti} \cdot K_{Pi} \cdot (1 - E_{Ti}) + \frac{a_{trg}}{\varepsilon_{trg} + C_{u,trg}} \cdot V_{trg} \tag{14.4}$$

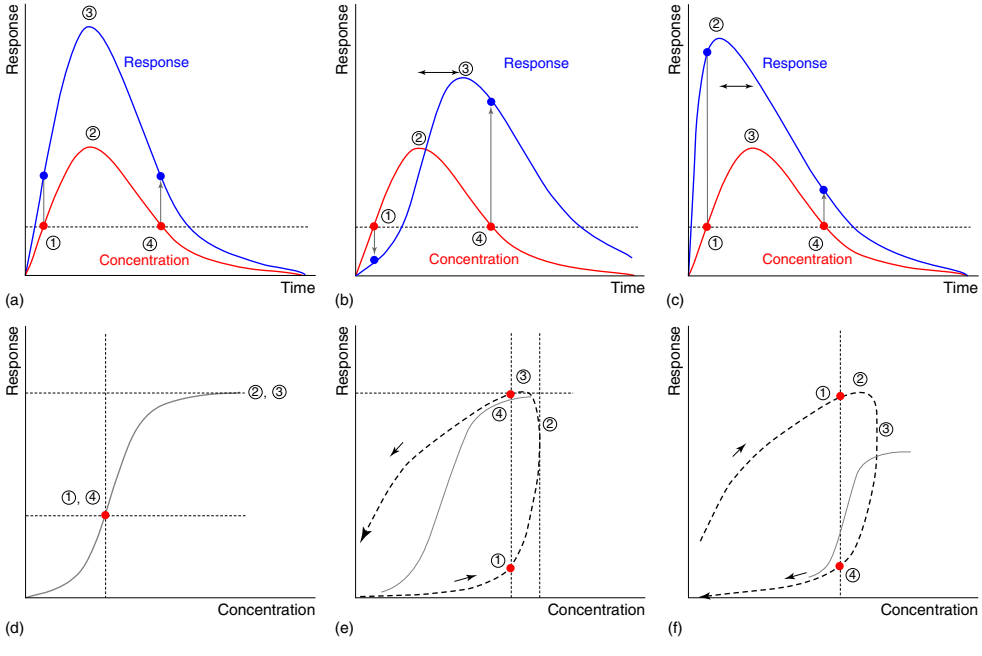
where V_B, V_{Ti}, K_{Pi}, E_{Ti}, a_{trg}, ε_{trg}, $C_{u,trg}$, and V_{trg} are the blood volume, tissue volume, tissue-to-blood partition coefficient, extraction ratio across eliminating organs, maximum target concentration, concentration at half-maximal saturation of specific target binding, and the unbound concentration at the pharmacological target site and target mass, respectively.

14.4 Understand Temporal Differences Between Concentration and Response

Temporal differences between plasma concentration and the pharmacological response manifest as hysteresis curves when the plasma concentration is plotted against the pharmacological response *in time order* (Figure 14.9). When a rapid equilibrium is established between plasma concentrations and the pharmacological response, the rise and fall of the concentration–response curve do not display any hysteresis (Figure 14.9a, d).

The data in Figure 14.10 and Table 14.1 exemplify drug concentration–response patterns in which the response expression and duration are primarily governed by exposure (Case Study PD21 in Ref. [4]) or basic biology factors (omeprazole) – specifically in the latter case relating to the production of response. Figure 14.10 shows experimental plasma concentration–time relationship and suppression of response–time data obtained after a 3 h constant rate infusion of drug X to a rabbit; the concentration–response relation displays hysteresis (Case Study PD21) [4]. The predicted half-life of response is about 22 min (as compared to a plasma half-life of ~120 min) and the potency 30–40 nM. This suggests that the terminal plasma half-life of about 120 min is the rate-limiting step and determines much of the duration of response. In other words, to elicit and maintain a pharmacological response, the critical concentration needs to be exceeded across time. When the plasma exposure drops below the target concentration (e.g. 50–100 nM), the response is also weakened.

Figure 14.9 (a, d) Schematic illustration of a situation with an instantaneous equilibrium between plasma concentration (red curve) and the pharmacological response (blue curve). The upper graph shows the concentration– and response–time courses which peak at the same time (② and ③; t_{max}) together with two time points with the same exposure and response values, respectively (① and ④). The corresponding lower graph shows the concentration–response relationship derived from the upper two time courses. Note that the rise in response when concentrations increase superimposes the decline in response when concentrations decrease. (b, e) This illustrates a small delay between concentration– and response–time curves, with a shift in their t_{max} values. Plotting the matching concentration–response relationship now results in a loop (hysteresis) for increasing and decreasing concentration–response values. (c, f) A substantial time delay is found between concentration– and response–time courses, carrying noticeably different t_{max} values and terminal slopes. The resultant lower plot also shows a large loop (large hysteresis) with the equilibrium concentration–response relationship occurring within the loop. The two time points with equal exposure (similar C_p; ① and ②) demonstrate very different response values due to the time it takes for the pharmacological response to develop [3].


Response
Time
Concentration
(a)
(b)
(c)
(d)
(e)
(f)

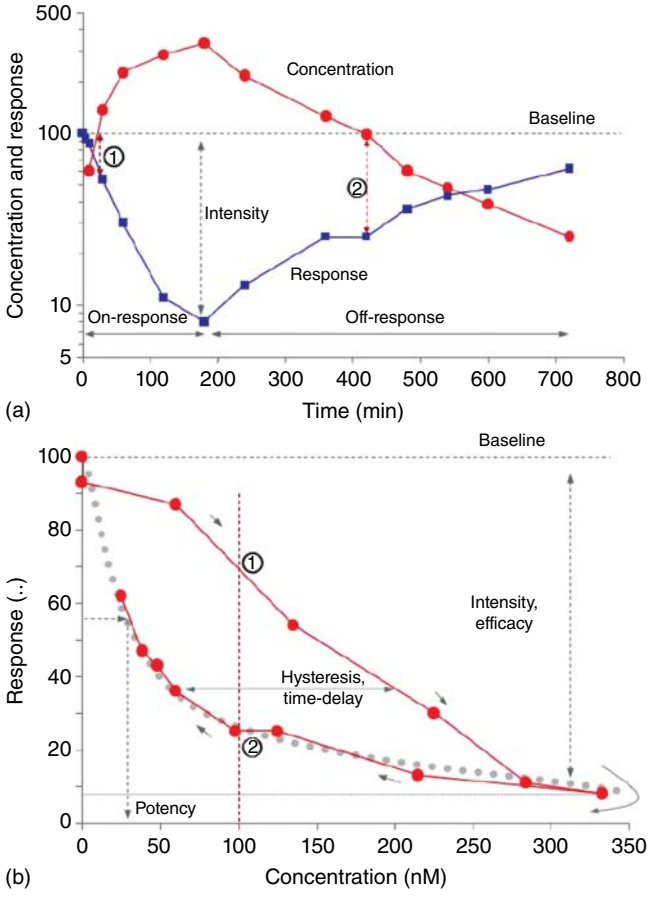


Figure 14.10 (a) Semilogarithmic plot of the concentration–time (red symbols) and suppression of response–time (blue symbols) courses after a 3-h constant rate drug (Case Study PD21) [4] infusion. Drug action is by inhibition of the turnover rate (buildup) of response. (b) Clockwise concentration–response data from the left graph plotted in time order. Note the differences in the down and upswing of the hysteresis plot. Small gray arrows show the time order. The dotted gray line shows the equilibrium concentration–response relationship. The potency (concentration resulting in half-maximal response, IC_{50}, is approximately 30 nM) and efficacy (intensity, ~90 response units) are also shown. Note that an initial (~20–30 min) plasma concentration of 100 nM gives a mild suppression of the response (①) because the onset of response lags after the rapid rise in the plasma concentration. At the same concentration but in the decay part of the exposure curve (②), the response is much more suppressed. With a half-life of 0.4 h for the response, 17 half-lives (400 min) during which the response has developed will have elapsed at this stage. The response is at each time point a consequence of the prior history of drug exposure, not only total exposure but also the rise, fall, and duration of exposure.

This example is summarized in Table 14.1 (Case Study PD21) [4] to illustrate where exposure and drug plasma half-life (2.3 h) will determine the duration of response. A contrasting case is the irreversible PPI omeprazole, which has a 45 min half-life in plasma, whereas the proton pump turnover half-life (response) is in the range of 15–20 h (Table 14.1, omeprazole). In the latter

Table 14.1 Comparison of half-lives and rate-limiting processes for two drug case studies.

	Case study PD21 [4]	Omeprazole
Plasma $t_{1/2}$	2.3 h	45 min
Response $t_{1/2}$	0.4 h	15–20 h
Rate-limiting process	Exposure	Biology

case the duration of response is governed by the drug mechanism of action linked to slow target turnover. Thus, irreversible removal of the target by (the metabolite of) omeprazole means that until the proton pump protein level has been replenished by *de novo* synthesis, the action of the irreversible inhibitor will linger on. A consequence of the longer biological response half-life in comparison with plasma half-life is that once-a-day dosing of omeprazole will suffice for an adequate therapeutic effect.

14.4.1 Models of Time Delays

There are numerous reasons as to why the PD response does not track the plasma concentration–time course of a drug. The drug molecule may take time to reach the pharmacological target due to perfusion, diffusion, and transporter barriers. This is what typically constitutes the so-called distributional delays. In other instances the drug acts directly on factors responsible for the buildup or loss of response. In the case of omeprazole, a metabolite to the drug irreversibly binds to active proton pumps, resulting in an increase in the loss (increased removal of proton pumps) of response and thereby a lower acid secretion in the stomach. Models that mimic the buildup (turnover rate) and loss (fractional turnover rate) are called turnover models. The binding process (on/off) between a ligand and the pool of free receptors may be slow and thereby becomes the rate-limiting step for a pharmacological response. Table 14.2 contains a compilation of distributional, turnover, and binding models that capture apparent time delays between plasma drug concentrations and biomarker responses. For elucidating the intrinsic behavior of these models based on PD patterns, see Ref. [9]. A large number of real-life case studies are also compiled in Ref. [4].

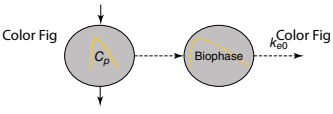
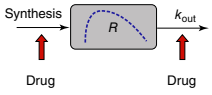
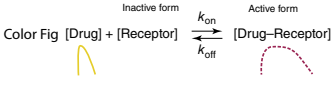
14.5 Understand Your Translational Context and Options

The translational framework in drug discovery projects obviously encompasses multiple aspects. Under this heading, we will discuss an example with significant translational impact.

14.5.1 Matching Drug Delivery to Target Biology

The project case study summarized below incorporates many of the elements discussed above. In an aim to discover and develop novel anti-obesity agents, the

Table 14.2 Comparisons of the basic distributional, turnover, and on/off-binding response models with respect to the conceptual structure, parameters, auxiliary parameters, equations, baseline value, and determinants of pharmacodynamic steady state.

	Distributional model	Turnover	On/off binding
Model			
Parameters	E_0, E_{max}, EC_{50}, k_{e0}	E_{max}, EC_{50}, n, k_{in}, k_{out}	B_{max}, k_{on}, k_{off}
Auxiliary parameters	$t_{1/2} = \dfrac{\ln(2)}{k_{e0}}$	$t_{1/2} = \dfrac{\ln(2)}{k_{out}}$	$\begin{cases} t_{1/2} = \dfrac{\ln(2)}{k_{off}} \\ K_d = \dfrac{k_{off}}{k_{on}} \end{cases}$
Equations	$\begin{cases} C = f(\text{Dose}, t, \theta) \\ \dfrac{dC_e}{dt} = k_{e0} \cdot C - k_{e0} \cdot C_e \\ E = E_0 \pm \dfrac{E_{max} \cdot C_e^n}{EC_{50}^n + C_e^n} \end{cases}$	$\begin{cases} \dfrac{dR}{dt} = k_{in} \cdot \left(1 - \dfrac{I_{max} \cdot C^n}{IC_{50} + C^n}\right) - k_{out} \cdot R \\ \dfrac{dR}{dt} = k_{in} - k_{out} \cdot \left(1 - \dfrac{I_{max} \cdot C^n}{IC_{50} + C^n}\right) \cdot R \\ \dfrac{dR}{dt} = k_{in} \cdot \left(1 + \dfrac{S_{max} \cdot C^n}{SC_{50} + C^n}\right) - k_{out} \cdot R \\ \dfrac{dR}{dt} = k_{in} - k_{out} \cdot \left(1 + \dfrac{S_{max} \cdot C^n}{SC_{50} + C^n}\right) \cdot R \end{cases}$	$\begin{cases} \dfrac{dRC}{dt} = k_{on} \cdot C \cdot (R_T - [RC]) - k_{off} \cdot [RC] \\ \dfrac{dRC}{dt} = k_{on} \cdot C \cdot (B_{max} - [RC]) - k_{off} \cdot [RC] \\ E = \dfrac{k_{on} \cdot C \cdot B_{max}}{k_{on} \cdot C + k_{off}} = \dfrac{C \cdot B_{max}}{C + \dfrac{k_{off}}{k_{on}}} = \dfrac{B_{max} \cdot C}{K_d + C} \end{cases}$
Baseline/ steady-state response	$\begin{cases} E = E_0 \\ E = E_0 \pm \dfrac{E_{max} \cdot C_e^n}{EC_{50}^n + C_e^n} \end{cases}$	$\begin{cases} R_0 = \dfrac{k_{in}}{k_{out}} \\ R_{ss} = \dfrac{k_{in}}{k_{out}} \cdot f(I \text{ or } S) \end{cases}$	$\begin{cases} E_0 = \dfrac{B_{max} \cdot C_{endo}}{K_d + C_{endo}} \\ E = \dfrac{B_{max} \cdot (C + C_{endo})}{K_d + (C + C_{endo})} \end{cases}$
Typical features	• First-order buildup and loss of response • No peak shift with increasing doses	• Zero-order buildup and first-order loss of response • Peak shift with increasing doses provided nonlinear inhibition I or stimulation S • Separates drug and system parameters	• Second-order onset and first-order offset of response • Peak shift with increasing doses

Source: Adapted from Gabrielsson and Weiner, 2016 [4].

path taken was to target appetite/food intake to achieve body weight loss. First, an *in vivo* experimental model was established, suitable for drug screening and for prediction and translation of efficacy toward the human condition targeted. To this end, it is imperative to understand the physiological context in which the drug is supposed to act. Thus, there is a continuum from normal food intake/body weight control that is of clear fundamental importance to health and survival to the excess caloric overeating/body weight gain associated with severe metabolic deterioration and wide-ranging negative consequences across bodily functions in the obesity condition. It should further be appreciated that whenever interacting with vital functions (e.g. energy intake), one or more homeostatic processes are likely implicated. Examples of such mechanisms that buffer the acute action of a drug include the concomitant triggering of redundancy pathways and systems, adaptation upon chronic dosing, and of course pathophysiological alterations in the target and circuits involved. It follows that the more insight into the physiological setting and potential factors that may influence drug action, the more precise design may be applied to create a model to faithfully mimic the human condition to be addressed [5].

14.5.2 Designing Experiments for Discrimination of Drug Candidates

Accordingly, in this example two new agents toward the anorexigenic target chosen were studied and compared to a benchmark compound that had already shown the desired primary clinical efficacy outcome – body weight loss – in human obesity. In the model used, mice had prolonged *ad libitum* access to a calorie-dense "cafeteria diet" to promote excessive eating and obesity, with the end result being a markedly adipose phenotype suitable for drug screening (Figure 14.11). As indicated, the overall goal was to find agents with body weight-reducing efficacy, but the path taken to achieve this was to suppress appetite. The link between these two readouts in the acute single-dosing situation is evident, but becomes clearly more complex upon chronic drug treatment and withdrawal (cf. Ref. [25]).

As seen from Figure 14.11, there is a delay of about 3 weeks until maximum drug efficacy is attained with the benchmark and the new anorexigenic drugs. Modeling of the data was based on assumptions derived from empirical observations: a maximum drug-induced body weight loss 0–18 d of 35% from baseline (approaching approximately the body weight of lean mice of similar age, on standard chow diet only) and a maximum body weight gain of 30% in nondrug controls over the full-time course of the study – from baseline to end of the weight regain phase in the treated groups. In a preamble to the body weight studies, the effect on food intake of several candidate agents was assessed in order to validate their intended anorexigenic properties. In addition to serving as a selection screen, such studies revealed – unsurprisingly – a marked tolerance to the significant initial food intake-suppressing action, developing over 1–2 weeks of repeated drug administration. This pattern is typical of centrally acting anorexants and believed to reflect the basic biology of the system with increased engagement of other competing targets and circuits to counter a budding state of perceived (in this case, drug-induced) starvation. The leveling

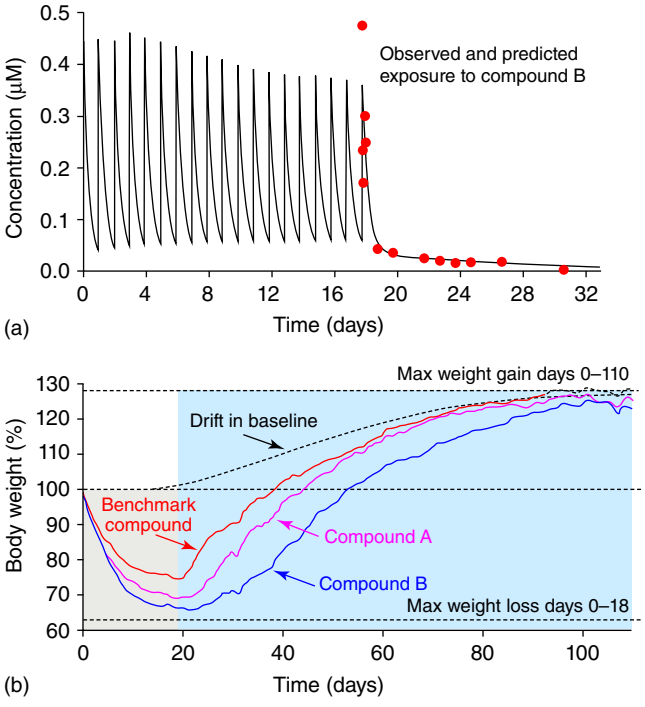


Figure 14.11 (a) Body weight change in control (baseline), benchmark (red), and test compound A-(magenta) and test compound B (blue)-treated animals. Note the drift in baseline, the maximal weight gain (upper limit), and the maximum weight loss (lower limit). Drug treatment occurred between days 0 and 18. (b) Exposure vs time data of test compound B. Test compounds were given as a μmol kg^{-1} body weight dose.

off in body weight loss seen from the 7th – 10th day onward (cf. Figure 14.11) is thus likely a token of recruitment of redundancy/defense mechanisms, including central and peripheral adjustments in transmitters and hormones operating as hunger/satiety signals, as well as in energy utilization and metabolic rearrangements to the new body weight. An analogous pattern of body weight loss, metabolic alterations, and weight regain after treatment withdrawal is observed in clinical studies with the benchmark compound – supporting translational significance of the rodent studies referred.

To extract even more information about the dynamics of the systems involved, the body weight regain of the mice was followed for an extended period, up to about 90 days after cessation of the 18-day repeat drug treatment (Figure 14.11). This revealed important and likely clinically relevant differences among the compounds studied – particularly a target off-rate of one of the agents that greatly outlasted the basic PK duration of exposure. The response–time data in Figure 14.11 indicate time ranges that contain specific information about system parameters (e.g. *turnover rate,* k_{in}*, or fractional turnover rate,* k_{out}) and drug parameters (e.g. *potency*, EC_{50}) and may therefore be of special interest for experimental design [5].

14.5.3 A Model for Differentiation of System and Drug Properties

A PD turnover model was applied to accommodate the data. Thus, the proposed drug action on turnover of body weight was modeled as a stimulatory "drug mechanism" function acting on the loss of response [25]. The physiological interpretation of this is that the drug target interaction results in a net readjustment of energy balance mechanisms (increased dependence on body energy stores in the face of a reduced caloric intake and hence a loss of adipose tissue), thereby promoting reduction of body weight.

This real-life project example features, *i.a.*, a broader understanding of the target in the integrated biology perspective. This in turn helped forming strategies concerning the design and use of experimental models for *in vivo* drug assessment and (backward and forward) translational and comparison aspects (*i.a.*, through access to a clinically tested forerunner compound). In addition, the studies provided recognition of PK, PD, and basic biology (target)-related factors in the concentration-to-response pattern observed (time delays, drug target off-rate, compensatory adjustments, adaptation, etc.), thereby enhancing utilization of the data for modeling purposes.

Taken together, we hope that this serves to illustrate the significance of understanding the biological underpinnings of the function to be targeted. Although admittedly all of the details that may influence readouts in this example are not known, a high-precision modeling could be performed, and important information was fed back to the project for further advancement of the candidate compounds toward human testing.

14.6 Communication Across Discovery Disciplines

It is of utmost importance to any successful project that communication across discipline borders is seamless and effective. Regrettably, from our experience, this is not always the case. In the discussion below, we have tried to capture and pinpoint some common aspects. We hope that our lessons and conclusions may help resolving such challenges and facilitating coherent communication.

14.6.1 Misconceptions in Cross-Functional Communication

First, experts from key disciplines in a drug discovery project come from varying background education, laboratory "traditions," and scientific fostering (e.g. medicinal chemists, pharmacologists, and pharmacokineticists). Consequently, they may have distinct approaches and views on how to interpret experimental data, as well as to what the next step should be; also terminology/"lingo" differences may sometimes create confusion. Therefore, probing and clarifying between project team members what the common ground is and where modes of expression and views may diverge help in creating a good communication platform. For example, the term "potency" (i.e. which depends both on target affinity of a drug and its ability to elicit a response) is sometimes – incorrectly – referred to as equivalent to the "maximum effect" attainable or binding affinity;

"tolerance" in drug treatment contexts depicts adaptational changes and should not be mistaken for "tolerability," which signifies whether a particular drug dose/exposure is not tolerated – i.e. more of an adverse action/safety event descriptor. Also the free fraction, f_u, is sometimes – and erroneously – used as a substitute for unbound concentration C_u. For an authoritative summary of terminologies in quantitative pharmacology, see Ref. [26].

Even within one and the same domain of discipline expertise, opinions may diverge on the usage of drug-related parameters in a project context. One example of a grave simplification of what drives a pharmacological response is the notion that the measured pharmacological response is driven *either* by means of the peak plasma concentration, C_{max}, *or* by the total area under the plasma concentration–time curve, AUC. While both parameters are important exposure measures, a pharmacological response is always a consequence of the *entire* prior history of drug exposure up to the time point when the response is observed. Thus, when there is an instantaneous equilibrium between plasma concentration and the pharmacological response measured, the rapid association between the two may appear to eliminate any need to integrate (sum up or calculate the AUC of) the full concentration–time course preceding the response readout. Hence, this situation is an example of a direct relationship between plasma concentration and response (commonly denoted "C_{max} driven"). However, such an interpretation disregards that there are in fact several steps between exposure across time and a resulting response related to the temporally shifting drug property display at different concentrations in the targeted, likewise variable biological system. The illusion that the response is only "C_{max}-*driven*" implies that the post-peak time courses will not impact the response, which then creates a biologically implausible scenario (Figure 14.12, bottom right ④).

Conversely, when equilibrium between plasma concentration and a pharmacological response is slow, or the drug effect is apparently irreversible, the response may seem to better correlate with the integrated concentration–time course AUC rather than an individual concentration–time point, such as C_{max} or C_{min}. The onset, intensity, and duration of response are then more easily associated consequences of the prior drug history (exposure history) leading up to the measured pharmacological response at time t. This said, unless the exposure time course exceeds a minimum concentration level, adequate drug–target interaction – and hence pharmacological response – may never be attained, thus demonstrating the simultaneous relevance of C_{max} as well. Typical illustrations of this may be found, e.g. in the antibiotic drug discovery project area, where both the C_{max} and AUC parameters are clearly important to the treatment of infections [27, 28].

Figure 14.12 endeavors to demonstrate why responses are never only C_{max} or AUC "driven". It is hopefully evident from this graph and the reasoning above that a more integrated and response-centric view – describing, *i.a.*, the rate of onset, intensity, duration, and decay of response in relation to drug exposure – is essential for accurate pharmacodynamic data analysis.

In safety and toxicology evaluation situations, the no-observed-adverse-effect level (NOAEL) of drug exposure needs to be established. This represents a certain maximum (therapeutic) exposure level that should not be exceeded. Under these conditions, C_{max} is often used as a substitute for a particular adverse response

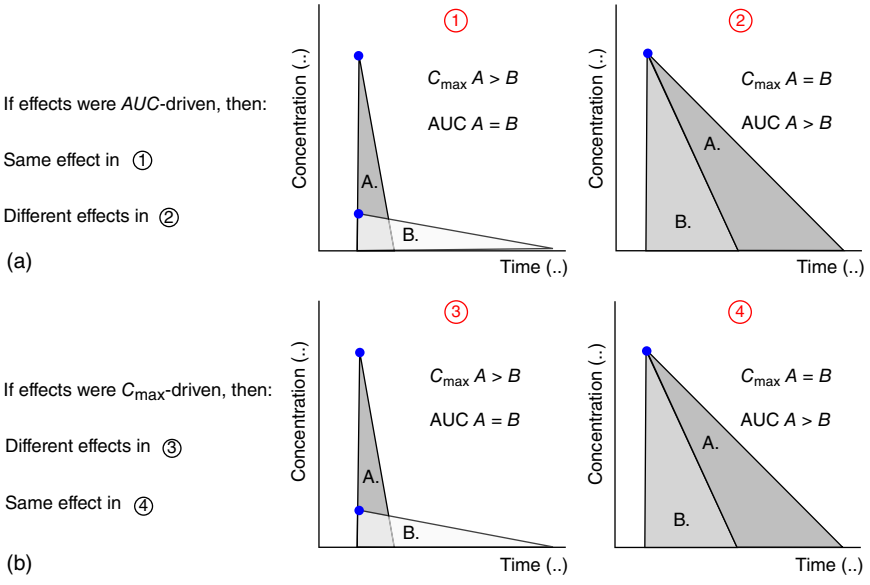


Figure 14.12 Schematic illustration of concentration vs time data. (a) Assuming data are only AUC *driven* gives the same effects in graph ① and different in graph ②. Situation ① may work for strictly irreversible effects, but is questionable for reversible systems displaying saturation. Slowly developing pharmacological responses are often erroneously portrayed as being AUC *driven*. (b) Assuming data are only C_{max} *driven* agrees with the differentiation in ③ but creates a biologically implausible situation in graph ④. Instances with a rapid equilibrium between plasma concentration and pharmacological response are often erroneously portrayed as being C_{max} *driven*.

level, provided a rapid equilibrium exists between plasma concentration and the pharmacological (or safety) response. However, AUC is also employed as an at least equally useful parameter for safety/toxicological biomarkers, especially because the development of safety/toxicology-related processes may be slower in progress (e.g. carcinogenicity, reproductive toxicity) compared to the intended therapeutic drug responses. Proper attention should thus be paid to desired and adverse drug responses across the full exposure–time curve, including during expected drug washout and recovery phases. In this context, a comprehensive discussion of "therapeutic index" aspects relevant to the aforementioned is found in Muller and Milton [29]. The plasma concentration, C_{max} or C_{min}, is often used as a substitute for the therapeutic response level and range, possibly because an exposure figure is more easily communicated across disciplines than components of a pharmacological effect.

Since a response is never *either* C_{max} *or* AUC driven, we advocate refraining from the use of these dogmas. We recommend a response-centric approach instead of one defined by simple exposure measures. Thus, the actual reason(s) should be established for what underlies the onset, intensity, and duration of the drug response – put within the corresponding (patho)physiological perspective. A similar reasoning also holds for the concept of plasma threshold concentration levels relative to drug responses. It may appear as if we need a certain plasma

threshold concentration, but biology seldom works that way. It is a gradual change that leads up to a response. Again a more response-centric approach is advocated.

14.6.2 The *In Vitro* vs *In Vivo* Perspective

In the very early days of a (small molecule) drug discovery project toward a novel target, medicinal chemists primarily focus on finding suitable hit/lead structures that may be elaborated upon, typically from a potency and relevant pharmacokinetics point of view. Much of this work is naturally carried out *in vitro*, usually via high-throughput assays. However, as soon as a lead with adequate target potency has been identified, it should be considered for *in vivo* testing – parenteral administration modes are perfectly fine in this regard. Access to a benchmark "tool" agent (even an imperfect one might suffice) might be equally helpful in this context. Early *in vivo* assessment will yield valuable information on PK and PD properties in relation to the target, as well as to the biology of the response and the sensitivity of the model employed. Such information is of key significance to guide continued chemistry efforts and to enable timely and efficient drug project progress. Close collaborative interaction among chemists and biologists are imperative to this end.

14.6.3 Integrated Thinking

Pharmacokineticists are typically focused on drug disposition and its determinants and may as a consequence have a less holistic view of exposure and target biology. They are also encouraged to translate a sometimes abstract modeling jargon into a concrete PD and/or biological/physiological tractable language. Equally, pharmacologists should aspire to put interpretations of absolute PD response readouts and underlying mechanisms of action into the context of target biology and PK and PD relations as much and early as possible. To avoid overlooking valuable information, we strongly encourage a joint *pattern analysis* approach (cf. Ref. [9]), involving expertise from both the aforementioned disciplines against the backdrop of medicinal chemist knowledge of drug properties. Constituents of such an exploration are exemplified in Table 14.3 and include reflections related to the drug properties *per se*, the biological system studied (the mechanism(s) of action), doses used (dose, rate, and route of administration), the nature of the biomarker readout used, its maximum efficacy, any potential confounders to its expression, baseline behavior, number of phases in data (e.g. convex or concave bending), time lags, peak shifts, shape of onset of action, intensity level, saturation, route-dependent response–time courses, total duration of response, shape of decline of response, functional adaptation/buffering, synergistic effects, etc.

The new-generation drug discovery scientists need a combination of thorough quantitative thinking and an in-depth understanding of the target biology. This inevitably requires insight also on how drug properties interact with PK handling processes. We therefore strongly support joint knowledge building and

Table 14.3 Some typical features in response–time data interpreted by medicinal chemists, pharmacologists, and kineticists.

Features in data	Med chem's view	Pharmacologist's view	Pharmacokineticist's view
Baseline	Given the target baseline, what drug profile is preferable (e.g. reversible/irreversible inhibition, full/partial agonist/antagonist, allosteric modulator properties)? Is systemic exposure required, or is there a specific intended site of action (e.g. local, GI, CNS, other)?	Is the baseline response suitably defined and validated? If not, how to control for variation, drift, confounders, and to maximize the response window?	Is the baseline constant, oscillating, and handling sensitive? What model captures features of rest/sleep, handling, and disease progression?
Onset of action	Chemical modification to accelerate or delay absorption pro-drug approaches? Contribution from active metabolite(s)?	If delayed, to what extent are administration route-, drug formulation-, PK- and/or PD response-related (or methodology) factors involved?	Is the response delayed or does it precede C_{max} initial rate of rise, steepness? How can this be formalized in terms of equations?
Intensity/efficacy	What molecular properties and decorations confer activity, high potency, and selectivity at the target in question; can a clear-cut *in vitro* (Q)SAR be established, and how does it compare to *in vivo* studies within a compound series?	How does the observed response compare to what is expected based on the target biology, including empirical findings with other agents targeting the system? What is the relation to dose/exposure? Has maximum efficacy been reached, and to what extent does this reflect desired target action vs nonspecific effects?	Is the response delayed or does it precede C_{max} high, low, dose proportional, saturated, waning, synergistic? Is there a physiological limit or is the limit drug dependent? Do not confound *in vivo* with *in vitro* data
Duration	Analyzing molecular properties relative to metabolic and/or elimination handling in the body; how to best modify compound toward desired PK profile? Is the drug target interaction dynamics (e.g. off-rate) important to the overall response?	How does the duration relate to drug properties, underlying physiology/biology vs target characteristics and level of drug exposure? What is the $t_{1/2}$ of the PD response?	Dose proportionality, offset, decline, steepness, regimen dependent, monotonic decline, rebound? Hysteresis? Is it caused by drug and/or system properties?

(Continued)

Table 14.3 (Continued)

Features in data	Med chem's view	Pharmacologist's view	Pharmacokineticist's view
Acute findings	Identification of any putative nontarget-related drug properties that may interfere with the response (e.g. local irritant action, other)	To what degree does recruitment of counterregulatory mechanisms limit expression of the acute PD response? If present, how may such mechanisms are circumvented?	Can data be used for prediction of chronic dosing? Tolerance, adaptation, synergy?
Chronic findings	Do the drug properties predispose to accumulation *in vivo*; if so, is this beneficial or not?	Is the PD response size and/or profile altered upon chronic vs acute dosing? If so, what biology (tolerance, adaptation) vs PK-related factors may be involved?	Does model mimic chronic data? Tolerance, adaptation, synergy aspects?
Rebound	Possible contributions of active metabolite(s) to an observed rebound response? If so, how may this be avoided?	Is there an over- or under-shoot in the PD action upon drug withdrawal? What may be the cause of this, and can it be avoided by altering drug, formulation and/or treatment scheduling properties?	What is the area under the rebound *vis-á-vis* area under response? Is the system dampened? Can modeling teach us something about avoiding the rebound effect?
Utilization of information	Generation of new molecules that challenge and/or validate initial interpretations of PK/PD data relative to drug properties	Creating a preliminary qualitative view on how drug properties, exposure, and biological system variables may interact to generate the pattern observed	Creating the input to model-based (quantitative) assessment of data. Are data appropriate for translation across species?

Source: Gabrielsson and Hjorth, 2016 [9]. Modified with the permission of Springer.

reasoning around the combined features (such as basic compound and biology characteristics relative to effective plasma concentrations and clearance, the concentration–response curve, temporal differences, etc.) – a holistic method culture. We are convinced that intimate and amalgamated collaborative communication between these three main subject domains in early drug discovery phases – medicinal chemistry, pharmacology/bioscience, and pharmacokinetics (schematically illustrated in Figure 14.13) – is absolutely pivotal to efficient and successful continued project progress. To that end, we consider all efforts to break down "silo" mode thinking and working highly recommendable.

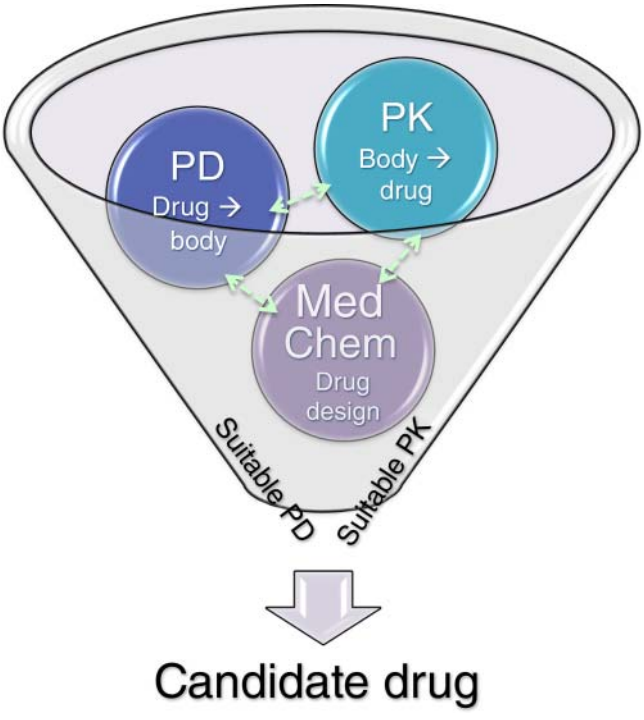


Figure 14.13 Illustration schematic depicting the iterative interdisciplinary collaboration work mode toward optimization and integration of drug pharmacokinetic and pharmacodynamic properties and a desired candidate drug profile.

14.7 Final Remarks

This chapter has reviewed some of the pivotal aspects of kinetic/dynamic reasoning and how they fit together. As evident from the account, there is often much "more to the picture than meets the eye". Thus, several biologically derived elements may, in addition to PK (exposure) causes, obscure analysis and interpretation of relations between drug concentration and PD response. This is highlighted with different examples on the impact of:

- *Target Biology*. Including system-dependent time delays (visualized by, e.g. hysteresis plots), biphasicity/multiphasicity in concentration–response curves, choosing target-related PD markers, redundancy/adaptational mechanisms, confounders, etc.
- *Concentration–Response Relationship*. Stressing, *i.a.*, the primacy of unbound drug concentrations at steady state, ideally in the biophase matrix, effects of dose scheduling ("fractioning"), the shortcomings of a C_{max} vs AUC reasoning, etc.
- *Pharmacological Response–Time Course*. Discussing, *i.a.*, reasons for time delays, rate-limiting process (exposure or response biology), hysteresis,

characteristics of distributional, turnover or target on/off-rate patterns and models, etc.

- *Translational Options*. Considered, *i.a*., within the context of overall target strategy and experimental design, choice of species and model(s) in a project case study, as well as data exploration, elucidation and modeling/prediction efforts, etc.

We hope that the examples given illustrate the intricate interdependent nature of pharmacokinetics and pharmacodynamics in project work, and the consequent need for integrated approaches to target understanding and data analysis. With enhanced understanding of background biology and (patho)physiology, the elucidation should be greatly facilitated of whether and how system vs drug properties can be modified to obtain optimal therapeutic benefit. Finally, from the *interdisciplinary communication* point of view, we have also elaborated on how smooth communication across scientific discipline borders is a prerequisite for successful, resource-efficient, and timely project progress and delivery. In this regard, we hope that the notes reviewed in Table 14.3 may trigger in the presumptive reader reflections to be successfully applied in the context of his/her own drug project.

While omnipresent throughout the life of a project, the abovementioned integrated technical and communication aspects become all the more significant as progress toward human testing evolves. Choosing a suboptimal candidate drug against the backdrop of an inferior understanding of biology, target and/or PK/PD relations may easily lead a project astray, examples of which we have illustrated in the present account. A thorough insight thus not only has impact upon efficiency and smoothness of project work, but – not least – will minimize patient risk and simultaneously save money and manpower in a stage where development costs start to rise considerably. Finally, a robust and coherent appreciation of candidate drug properties vs target and system biology will also facilitate presentation of any project to internal governance and enable the writing of transparent and well-defined Investigational Medical Product Dossier (IMPD) and Investigator's Brochure (IB) documentation required in contact with regulatory bodies in the late lead optimization/early development phase.

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15

Prediction of Human Pharmacokinetics and Pharmacodynamics

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15.1 General Introduction

Predicting human pharmacokinetics (PK) and the relationship between the PK and the efficacy or pharmacodynamics (PKPD) is an essential part of the evaluation and selection of a new chemical entity before deciding to advance into clinical development. The predicted human PK and PKPD properties provide early clinical development with a hypothesis for the dose schedule and exposure levels that are likely to generate the desired efficacy. The predicted therapeutic exposure also provides useful input for dose selection and exposure targets in good laboratory practice (GLP) toxicity studies that precede first in human trials. Prediction of human PK and PKPD is a process that should begin in the discovery phase and should be continuously refined to guide the lead optimization stage and help to select the best drug candidate [1, 2]. A quantitative integration of PK and PKPD properties is essential and determines the dose and the frequency of dosing needed to achieve the sought effect. The integration also helps to understand the most critical factors involved and to focus efforts on the most important parameters for further optimization [3]. The size of the dose has direct implications for usage feasibility in humans (drug load), formulation development, risk for drug–drug interactions, and cost of goods. The predicted human dose is thus a good indicator of the status of lead optimization efforts and how much further improvement is needed in order to have a molecule qualifying for clinical development. *In vivo* models for PK and PKPD can capture and integrate the complexity involved, but differences between animals and humans make it necessary to use *in vitro* information to address species differences in biology and to add mechanistic insight. There has been tremendous development over the last decades with respect to *in vitro* methodologies to study the underlying processes involved in PK (e.g. cryopreserved human hepatocytes, recombinant systems and cell lines for metabolism, active transport, and permeability) [4]. How to effectively integrate all this information constitutes an area of active development but still represents a challenge to the drug discovery scientist. As an example, despite numerous attempts to evaluate the relative performance of different scaling methods for PK, there is yet no single PK scaling methodology that works in a

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

satisfactory manner for all molecules [5]. A solid understanding of PK principles and appreciation of the factors involved for the specific molecule are essential to avoid pitfalls, to appreciate when more information is needed, and to select methods that are most appropriate in the individual case when predicting human PK. Similarly for PKPD, the level of understanding of the pharmacology, the application of system pharmacology, and the awareness of species differences and experimental tools have rapidly grown in the past [6, 7]. Due to the complexity involved, we have to rely on a combination of empirical models and mechanistic information when translating from animals and from in *vitro* system to humans.

The objective of this chapter is *not* to yield a complete review of all available methodologies or define a single recipe, but to provide researchers with scientifically sound principles and concepts and describe some of the more basic but still useful methods including their limitations. This chapter is written in the context of drug discovery/development given the experimental shortcomings, time, and resource constraints one typically faces in this environment. An appreciation of these principles will guide the PKPD scientist to select the most appropriate strategy and method(s) while also recognizing sources of uncertainty for the particular drug molecule. To this end and in keeping with the focus of the book, the discussion is entirely devoted to small molecule drugs intended for oral administration.

15.1.1 PK, PKPD, and Dose Prediction: Overview

Understanding the PKPD relationship provides information about the human plasma exposure target required to achieve a certain level of response. Although the underlying PKPD relationship can be complex, the goal is to define an exposure target that can be used to predict the actual dose and its scheduling. The exposure target could be, for example, the area under the concentration–time curve (AUC), time over a minimally effective concentration, $C_{e,min}$, or an average plasma concentration depending on the nature of the PKPD relationship. A good PKPD understanding also includes an appreciation of the temporal aspects involved in the specific pharmacology of interest, which can impact the dose scheduling and design aspects of future clinical trials. Prediction of therapeutic plasma concentrations is typically derived from an integration (modeling) of exposure–response data for biomarkers and other efficacy endpoints, *in vitro* potency, and the translational aspects associated with these relationships. Prediction of the key parameters governing the plasma concentration–time profile involves their integration using either compartment models or physiologically based models (PBPK models) [8]. The therapeutic dose predictions are then derived from the integration of the PK and PKPD predictions with due considerations based on the intended patient population, which can impact several of the underlying parameters.

All individual components involved in PK, PKPD, and dose predictions are associated with uncertainty coming from variability in experimental data, the scaling methods, and other translational aspects of both the PK and the PKPD. It is often useful to integrate this uncertainty and not only predict point estimates but also provide ranges of likely outcomes (e.g. degree of efficacy, exposure

target, dose, etc.), as detailed in Section 15.5. Dose predictions help to manage expectations, assess risk in the project, and provide valuable input to other drug development activities like, for example, large-scale synthesis, formulation development, clinical pharmacology, and preclinical safety.

15.2 Predicting Human Pharmacokinetics

Predicting PK for new drug candidates should ideally not be a one-off exercise that begins with the clinical candidate molecule, but is a process that should start well before selecting the final clinical drug candidate. A successful prediction of PK requires a solid understanding of fundamental PK principles and limitations associated with prediction methods. In the discovery phase, one should try to learn about the disposition mechanisms that are involved in the PK of the particular chemical series so that appropriate *in vitro* assays and *in vivo* models are selected to screen compounds and to quantify these key processes for the final drug candidate. In the end, the dose and the half-life are key end products of the PK prediction. In order to afford these endpoints, the human PK prediction focuses on the key parameters governing the PK, i.e. plasma clearance (CL), volume of distribution (V_{ss}), rate of absorption (k_a), and oral bioavailability (F), which are integrated with the PKPD (see Section 15.3) into a PK model to predict the relationship between dose and the plasma concentration–time profile. The underlying experimental data, some of the preferred methods and considerations, are discussed in the following sections.

15.2.1 Experimental Data

Many sources of information are needed for prediction of human PK, and these include both *in vivo* and *in vitro* data. *In vivo* PK is usually derived from rat, dog, mouse, and sometimes other preclinical animal species like cynomolgus monkey. With respect to *in vitro* methods, the prediction of hepatic clearance is best derived using human hepatocytes since they express the enzymes and transporters important for hepatic drug elimination. Liver microsomes can also be useful if the compound is mainly metabolized by cytochrome P450 enzymes (CYPs). Recombinant systems for CYPs, conjugating enzymes, and drug transporters provide useful quantitative data about what enzymes and transporters are critical in the absorption and elimination processes. The permeability across the intestinal epithelium is typically assessed in the Caco-2 cell assay [9], although other cell lines like the Madin–Darby canine kidney (MDCK) cell line can also be used in a similar manner [10]. The points below provide a recommended *minimum* amount of data that are needed for human PK predictions:

- *In vivo* PK in rat and dog species following both intravenous and oral administration at "therapeutic" doses providing information about CL, V_{ss}, bioavailability, and absorption properties.
- The intrinsic clearance of unbound drug ($CL_{int,u}$) in human, rat, and dog hepatocytes. $CL_{int,u}$ is estimated from depletion curves run at "therapeutic"

concentrations (e.g. 1 μM). The fraction unbound in the *in vitro* systems should also be measured in order to correct for unspecific binding to cellular components [11, 12].
- An estimate of the fraction of parent compound excreted unchanged in urine (f_e) following intravenous administration, in at least one animal species.
- Whole blood/plasma partitioning ratio and plasma protein binding in rat, dog, and human plasma at "therapeutic" concentrations.
- Intestinal permeability from apical to basolateral side (a–b), ideally in Caco-2 cells.
- Physicochemical characterization of the molecule: pK_a, dissolution rate and solubility in water (over a range of physiologically relevant pHs), and intestinal fluid.

The following additional data are *desirable* and will help in selecting the appropriate scaling method(s) and will provide more confidence in the scaling results:

- The correlation between *in vitro* $CL_{int,u}$ from hepatocytes and *in vivo* $CL_{int,u}$ for at least one *in vivo* PK species (e.g. rat or dog) for the chemical series used in lead optimization. At least 10 compounds with a good spread in $CL_{int,u}$ values are needed to assess the correlation.
- Metabolic stability ($CL_{int,u}$) estimated in human hepatocytes or liver microsomes at different starting compound concentrations to assess whether nonlinear kinetics will play a role [13].
- Data about transporter interactions with e.g. P-glycoprotein (P-gp) and organic anion transporting polypeptide 1B1 (OATP1B1) from appropriate recombinant systems or cell systems (e.g. Caco-2 or hepatocytes).
- *In vitro* comparison of metabolic pathways between human and animal species used for *in vivo* PK, as derived from hepatocytes.

15.2.2 Predicting Clearance

Small drug molecules are typically cleared via four mechanisms: hepatic and intestinal metabolism and renal and biliary secretion. Although there is metabolic capacity in other highly perfused organs like kidneys and lungs, these routes of elimination are usually insignificant compared with total clearance and can often be ignored from a quantitative standpoint. The exception is for molecules that are sensitive to hydrolysis by e.g. esterases, which are extensively expressed in many tissues including blood cells and plasma. However, these molecules often have a very high CL and are rarely useful for oral delivery unless they are administered as prodrugs. The prediction of prodrugs PK has unique challenges that will not be covered in this chapter, and the reader is referred to a recent review on the topic [14].

Total clearance, CL, is the sum of hepatic (CL_H) and renal clearance (CL_R), which are two independent parameters and should be predicted separately. The route of elimination (hepatic vs. renal) is mainly driven by the lipophilicity of the compound. Here, compounds with octanol:water partition coefficient at pH 7.4 ($\log D_{7.4}$) > 2 tend to be primarily metabolized in the liver, while less lipophilic molecules can have significant contribution from renal eliminaton [15].

The majority of compounds intended for oral administration have physicochemical properties belonging to the first category, and thus hepatic metabolic CL is often the dominating clearance mechanism. Nevertheless, transporters can also contribute to the CL and should be accounted for. Uptake and efflux transporters are located in the liver and kidneys, and there are today numerous *in vitro* tests based on recombinant systems, which can be used to evaluate their involvement, also with a view to assess species differences [16]. Although there are examples showing how transporter interactions can be integrated and accounted for when predicting CL [17], the methods for scaling transporter activity from *in vitro* to *in vivo* in a quantitative manner are still under development, and more experience in how to prospectively apply them is needed. In addition, although data detailing the magnitude of transporter expression and activity at tissue levels across species is starting to become available [18], data are still sparse. While animal PK studies provide useful information about the interplay and the net result of all these contributing factors, species differences in metabolism and transporters activity can sometimes be significant, and *in vivo* data in isolation should be interpreted with caution. Therefore, the basis for predicting human CL is often best derived from animal *in vivo* PK studies combined with *in vitro* studies that can address and quantify species differences in transporters and metabolism.

15.2.2.1 Hepatic Metabolic Clearance

Hepatic CL is for the majority of orally administered compounds of the dominating elimination pathway, and it affects their oral bioavailability (F), average plasma concentration, and half-life. Therefore, it has a significant impact on both the therapeutic dose and the dosing frequency and is the single most important parameter for human PK prediction.

In Vitro *Based Prediction of Hepatic Metabolic Clearance* Hepatocytes have become the most widely used *in vitro* systems for measuring hepatic metabolic clearance [12] since they contain all the main drug-metabolizing enzymes and also express liver uptake transporters. The elimination capacity of the liver is best quantified by measuring intrinsic clearance, CL_{int}. It has become a practice to estimate CL_{int} from the monoexponential decline in drug concentration in a suspension of hepatocytes [12].

- A low starting compound concentration (typically 1 μM) is used to minimize risk of saturated kinetics (compound concentration assumed to be $\ll K_m$, where K_m is the concentration resulting in half the maximum rate of metabolism, V_{max}). Incubations at higher compound concentrations are useful to confirm that the assumption of linear kinetics holds over a therapeutically relevant concentration range [13].
- CL_{int} is estimated from the monoexponential decline (half-life) of parent compound in the hepatocyte incubation. The unspecific binding of the compound to the incubation system should be accounted for (e.g. by dividing CL_{int} by the fraction unbound of compound in hepatocyte, f_u) to generate the intrinsic CL for unbound drug, $CL_{int,u}$.

- The $CL_{int,u}$ is then scaled to a whole human liver using scaling factors for hepatocellularity (cells/g_{liver}), and liver weight [19].

The hepatic plasma clearance is then predicted using a physiologically based liver model. Here the well-stirred model is most widely used [20–22]. Other liver clearance models have been proposed but these generally provide similar CL predictions [23].

According to the well-stirred model, hepatic blood clearance is calculated as

$$CL_{H,Blood} = \frac{CL_{H,Plasma}}{(B/P)} = Q_H \cdot \frac{CL_{int,u} \cdot f_{u,P}/(B/P)}{Q_H + CL_{int,u} \cdot f_{u,P}/(B/P)}$$

where $f_{u,P}$ is the unbound fraction in plasma, B/P is the whole blood : plasma partitioning, Q_H is the liver blood flow (LBF), and $CL_{int,u}$ is the intrinsic clearance corrected for unspecific binding from the *in vitro* system [12]. The well-stirred model is based on whole blood and is converted to plasma clearance as shown in the equation above. This scaling method has been widely used, and the experience is that *in vitro* $CL_{int,u}$ on average underestimates the *in vivo* $CL_{int,u}$ (back calculated from *in vivo* plasma CL by rearranging the equation above). This is possibly due to downregulation of transporters and metabolic enzymes during the preparation process of hepatocytes. However, a "regression offset" approach can be used to account for this systematic underestimation [24]. Briefly, a regression line is generated from the correlation between *in vivo* and *in vitro* $CL_{int,u}$ for a number of drugs where the human hepatic clearance has been determined (Figure 15.1).

It is recommended that the correlation is generated in each laboratory since the *in vitro* to *in vivo* correlation can vary depending on slight differences in

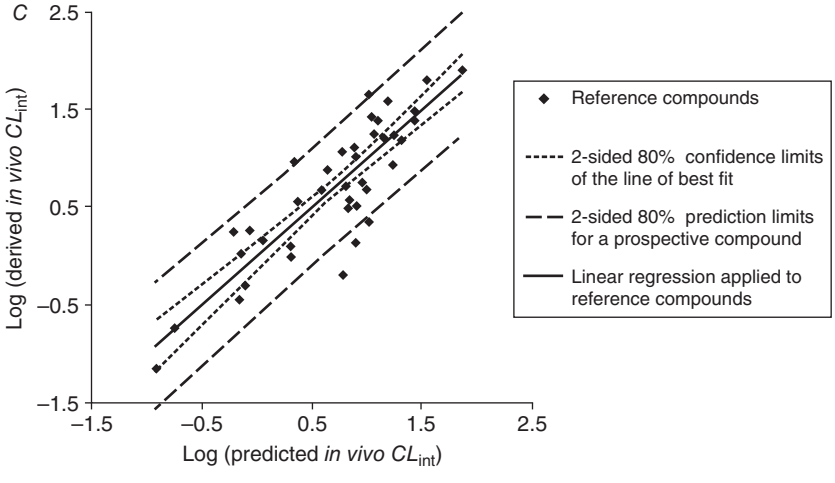

Figure 15.1 Illustration of the "regression-offset" method for correcting $CL_{int,u}$ generated in human hepatocytes due to its systematic underprediction of *in vivo* CL. A regression correlates the measured $CL_{int,u}$ in hepatocytes to the observed $CL_{int,u}$ estimated from PK data in humans. *Source*: Sohlenius-Sternbeck et al. 2012 [24]. Reproduced with permission of Taylor & Francis.

preparation of hepatocytes as well as minor differences in assay conditions (e.g. temperature, stirring) [24].

Most drug discovery programs focus on reducing the rate of metabolism and minimizing $CL_{int,u}$. A clear limitation with the hepatocyte assay is that for compounds with a slow rate of metabolism, reliable estimate of the decay (i.e. half-life) cannot be made thus precluding an accurate estimate of the $CL_{int,u}$. Alternative methods have been evaluated using longer incubation periods with hepatocytes and other cell lines [25]. Although these approaches show some promise, more experience and evaluation are needed before they can be used with confidence.

Using* In Vivo *Data to Predict Hepatic Clearance *In vivo* PK models are useful since all the processes impacting the hepatic CL are integrated, i.e. passive diffusion, transporter activities, and all routes of metabolism. Although the relative contribution of pathways can differ between species, *in vivo* kinetic studies in e.g. rat and dog species can provide insight into elimination pathways that could warrant more detailed studies using various *in vitro* tools. Studying the *in vivo* to *in vitro* correlation in animals is also a useful validation of how well *in vitro* $CL_{int,u}$ in hepatocytes predicts *in vivo* CL_H. When estimating CL_H in animal species, it is important to estimate CL_R and subtract it from total CL (see Section 15.2.2.3). Allometric scaling of CL_H has been used widely over the years and is based on the fact that many processes (e.g. liver blood flow) scale well with body weight [26]. The original allometric approach uses CL from several species in a regression method to predict human clearance:

$$CL = a \cdot W^b,$$

where W is the body weight and a and b are the intercept and the allometric exponent, respectively. This method can also be applied for CL_H. The predicted human CL_H is based on the extrapolation of this regression to the body weight of a human. Allometry assumes that CL scales according to size, and similarly to blood flow, but it does not account for species differences in the intrinsic rate of metabolism ($CL_{int,u}$), plasma protein binding, and transporter activity. Although multispecies allometry using this regression approach has certainly been shown to be an accurate method for many compounds, it can also lead to large errors in predictions [5, 27]. *Allometry based on this regression principle is therefore not recommended as the default method*. A more robust approach is to use single species allometry assuming an allometric exponent of 0.7 and then averaging the single species-derived values across multiple species. This reduces the risk of significant under- or overestimation. Even better is to combine allometric principles with measurable species differences in rate of metabolism and plasma protein binding. The free fraction correction intercept method (FCIM), which is based on an allometric regression paradigm that accounts for the difference in protein binding between rat and humans, provides a useful alternative [28]. Here, CL_H is predicted as

$$CL_H = 33.35 \cdot \left(\frac{\alpha}{R_{fu}} \right) \cdot 0.77$$

where α is the intercept from the allometric regression and R_{fu} is the ratio of unbound fraction in plasma, $f_{u,P}$, between rat and humans. The numbers 33.35

and 0.77 are fixed values derived from the analysis of 61 cmpds [28]. Although no correction is done for metabolic differences, this is an empirical approach that has shown to perform well in a number of different analyses [5, 29]. Single species-based on scaling can also be used to account for differences in protein binding (f_u) and LBF according to

$$CL_{H,plasma} = \frac{CL_{H,animal} \cdot f_{u,human}}{f_{u,animal}} \cdot \frac{LBF_{human}}{LBF_{animal}}$$

If there are species differences in the *in vitro* rate of metabolism ($CL_{int,u}$), the difference can be accounted for by normalizing the observed CL_H in the animal for the species difference in $CL_{int,u}$ giving $CL_{H,animal,norm}$ [30]:

$$CL_{H,animal,norm} = CL_{H,animal} \frac{CL_{int,u,human}}{CL_{int,u,animal}}$$

The calculated $CL_{H,\ animal,\ norm}$ can then be used in a single species allometric prediction as described above. Ideally this is done using several species and then averaged to provide a mean and a range of predicted CL_H. Note that the protein binding correction should be avoided for compounds with moderate to high clearance since protein binding has little or no influence on CL, according to the well-stirred liver model [22]. In these cases, the protein binding correction can result in a predicted human hepatic CL greater than the LBF if the $f_{u,human}$ is higher than $f_{u,animal}$ (see equation above).

15.2.2.2 Hepatic Biliary Clearance

Significant active transport of the compound into the bile, typically by uptake into hepatocytes followed by active secretion at the canalicular membrane into the bile, is required to generate a significant biliary clearance (CL_{Bile}). The CL_{Bile} of parent compounds is often low and insignificant in humans, but if there is a strong indication that the observed CL_H in animals is markedly higher than what is predicted from hepatocyte incubations, bile-cannulated animals (e.g. rats) can provide an estimate of biliary elimination based on the following equation:

$$CL_{Bile} = \frac{Ae_{0-t}}{AUC_{0-t}}$$

where Ae_{0-t} is the total amount of parent compound excreted in bile and AUC_{0-t} is the area under the plasma concentration–time profile during the bile collection interval. It is however important to recognize that a significant portion of the secreted compound can be reabsorbed in the intestine in the intact animal, leading to enterohepatic recirculation (EHC). The extent of EHC can be assessed by estimating CL_H in an intact sham-operated animal and then comparing it with CL_H in a bile-cannulated animal:

- If total CL values are similar, EHC is likely to be low, and the estimated CL_{Bile} is truly an elimination pathway and contributes to the total hepatic CL.
- If total CL is significantly larger in the bile-cannulated animal, EHC is likely significant, and biliary secretion is not contributing to CL. Biliary secretion (and the associated EHC) is then not in strict terms a clearance pathway, but

can be viewed as a reversible distribution of drug into the intestinal compartment, which will impact the volume of distribution estimation and therefore the half-life [31].

There are unfortunately no well-validated and robust methods for predicting biliary clearance in humans. Sandwich-cultured human hepatocytes have been used to predict CL_{Bile} of angiotensin blockers quite successfully [32], and plated hepatocytes have also been shown to be a potentially useful method [33]. The experience with these methods is still limited and needs substantially more evaluation before they can be used routinely for prospective predictions. In this context, allometric approaches have also been evaluated and showed some promise [34]. In summary, biliary clearance predictions are associated with a great deal of uncertainty due to significant species differences, lack of reliable experimental methods, and lack of human biliary clearance data to allow for a thorough evaluation of prediction models. A rule of thumb is that biliary clearance is usually markedly lower in humans than in rats on a simple body weight basis [34]. An empirical approach according to the following workflow, derived based on unpublished data, can thus be taken:

- Measure CL_{Bile} in rat as described above.
- Divide the CL_{Bile} from rats (ml kg^{-1}) by 10 to get a rough estimate of human CL_{Bile} per kg.
- If the predicted value is >20% of the total predicted CL, additional detailed experiments using the techniques described above may be warranted.
- If the predicted value is <20% of the total predicted CL, CL_{bile} and its contribution to CL can be ignored.

15.2.2.3 Renal Clearance

Renal plasma clearance is the net result of filtration, active secretion, and reabsorption of a given compound in the kidney. Metabolic activity in the kidney can usually be ignored. Renal plasma clearance is defined as the net rate of excretion in relation to the compound's plasma concentration C_p, according to the following equation:

$$CL_R = \frac{\text{Rate of filtration} + \text{Rate of secretion} - \text{Rate of reabsorption}}{C_p}$$

These three processes are to a large extent determined by the physicochemical properties of the compound and active transport processes mainly localized in the proximal tubule.

- *Filtration.* In healthy human males, the glomerular filtration rate (GFR) is *ca.* 120 ml min^{-1}. Only the unbound compound in plasma is filtered in the glomerulus. The degree of plasma protein binding has thus a direct impact on the rate of filtration of the compound. The filtration portion, CL_{filtr}, of the renal clearance, is thus a function of the compound's free fraction in plasma, $f_{u,p}$, and the GFR:

$$CL_{filtr} = f_{u,p} \cdot GFR$$

- *Secretion*. The unfiltered compound in the blood can be actively secreted by transporters located on the basolateral and apical side of the tubular cells into the urine compartment. The activity is driven by the capacity of the transporters and the free concentration of the compound in plasma.
- *Reabsorption*. Once the compound is in the primary urine, drug can be reabsorbed as the concentration gradient versus free compound in plasma increases when water is being reabsorbed from the urine back to the blood stream. Although active transporters can play an important role also for reabsorption, passive diffusion can by default assume to be the dominating process. The lipophilicity, ionic character, and molecular size of the compound, alongside the urinary pH, impact the rate of passive diffusion, i.e. the permeability, and thus reabsorption [35].

Highly permeable compounds with high degree of protein binding can be assumed to have a very low CL_R and therefore have an insignificant contribution to total CL.

CL_R is easily estimated in animal PK experiments by collecting urine (similar to biliary CL) after oral or intravenous administration according to

$$CL_R = \frac{Ae_{0-t}}{AUC_{0-t}}$$

where Ae_{0-t} is the amount of parent drug excreted during the collection interval and AUC_{0-t} is the area under the plasma concentration–time profile during the same interval. To understand whether active transporters are involved, one can apply the following guidelines:

- If the observed $CL_R < GFR * f_{u,p}$, then passive processes (filtration and passive reabsorption) are dominating. In this case, a kidney blood flow (KBF)-based method, adjusted for species difference in plasma free fractions, can be used with confidence:

$$CL_{R,human} = CL_{R,animal} \cdot \frac{fu_{human}}{fu_{animal}} \cdot \frac{KBF_{human}}{KBF_{animal}}$$

- If the observed $CL_R > GFR * f_{u,p}$, then active secretion significantly contributes to CL_R. This introduces more uncertainty due to potential species differences in active transportation.

Predictive *in vitro* methods for CL_R are essentially lacking or at best only when sparsely evaluated. Therefore, in a drug development context, one has to rely to a large extent on CL_R estimates from *in vivo* PK studies in animals to predict human CL_R. Some systematic evaluations have been made with respect to scaling of compounds with and without involvement of active transport. Both rat and dog appear to predict human CL_R fairly well using the KBF-based method, even for compounds with active secretion [36] (Figure 15.2). While recognizing the limitations of allometric approaches to predict renal CL, these often provide sufficient accuracy for the purpose of predicting human PK.

15.2.2.4 Scaling of Clearance: Points to Consider

Many attempts have been made to evaluate different scaling methodologies for predicting CL. No single method has been demonstrated to be superior. A common issue with many of these evaluations is that data sets are often

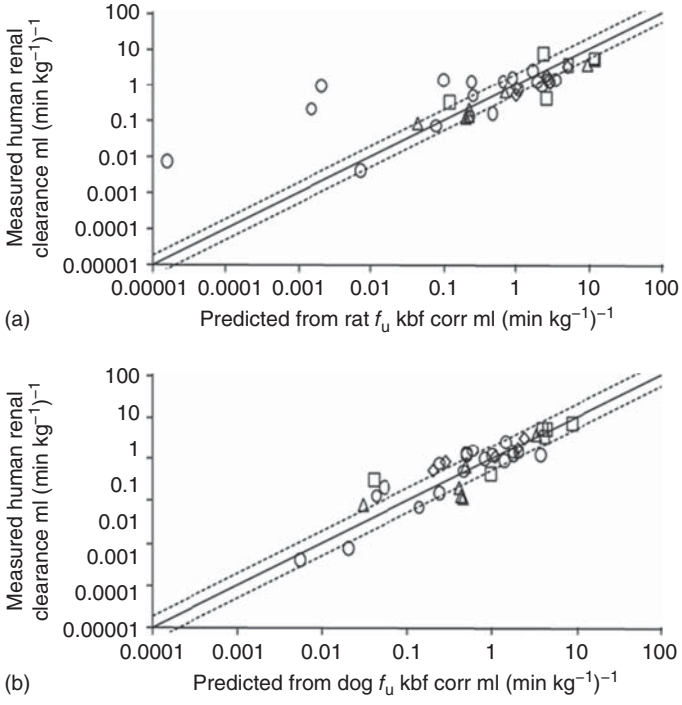

Figure 15.2 Prediction of renal clearance (CL_r) using the KBF method, corrected for species differences in plasma protein binding (f_u) in rat (a) and dog (b). *Source*: Reproduced with the permission of Paine et al. 2011 [36].

small, and the data used are heterogeneous in that they come from different laboratories using different assay and study conditions. Even with large, more homogenous data sets, no single method stands out [5]. The advice to drug developers working with novel compounds is to consider a number of factors when selecting scaling method(s) and when assessing the uncertainty in the prediction. Here, the main and far too common drawback is that the amount of data available for quantitative prediction will often be limited, because of time and resource constraints. Specifically, detailed studies of potentially important transporter interactions in the gut, liver, and kidneys and of enzymes involved in the metabolism are time- and resource-intensive activities. These need to be balanced against the potential development risks associated with uncertainty in the predictions. A list of questions and points for consideration is provided below. This can be used to give certain methods more or less weight and provide guidance as to when, and which, additional studies may be needed to provide more insight and improve confidence in the predictions.

1) Is the predicted dose low or high? If the predicted dose is high and close to what is considered acceptable, the margin of error for the prediction will be small. To this end, accurate predictions are critical, and more efforts can be devoted to improve their accuracy. On the other hand, if the predicted dose is very low and the margin to what is acceptable is large, one can tolerate less

accurate predictions without significant consequences. The same reasoning can be applied to the predicted half-life if this is considered critical for dose scheduling.

2) What clearance mechanisms appear to be dominating? If renal and biliary CL are predicted to have a minor contribution, it could be safe to accept uncertainty in these parameters since relatively large prediction errors will still have minor impact on total CL.
3) How well can $CL_{int,u}$ be determined *in vitro*? If a compound has a slow rate of metabolism (i.e. long half-life) in the hepatocyte system, a precise value may be difficult to determine. In such instances *in vivo* based methods could be given more weight.
4) How well does *in vitro* $CL_{int,u}$ predict *in vivo* hepatic clearance within a given animal species for the drug candidate and for compounds from the same chemical series? A number of compounds should be evaluated during lead optimization both *in vivo* and *in vitro* in at least one animal species. A good *in vitro* to *in vivo correlation* (with or without a correction factor) increases confidence to use *in vitro* $CL_{int,u}$ from, e.g. human hepatocytes as a predictor for CL_H. Systematic underestimation (overestimation is unusual according to this author's experience) of *in vivo* hepatic clearance should trigger the evaluation of other factors contributing to CL.
5) Is there evidence for extra hepatic metabolism, e.g. hydrolysis by esterases or oxidation by aldehyde oxidases? In this case, metabolic clearance can be significantly underestimated based on hepatocyte data alone, and further investigations of extrahepatic metabolism are warranted.
6) Is there evidence for EHC of the parent compound? EHC is often due to formation of a glucuronidated metabolite that is secreted via the bile into the intestine, hydrolyzed back to the parent compound by bacterial flora in the lower part of the intestine, and then partially or completely reabsorbed back into the blood. Species differences in glucuronidation and active secretion into bile can also make animal-based predictions more uncertain [37]. Quantification of the extent of biliary secretion of the parent compound and/or glucuronidated metabolites in bile-cannulated animals (as described under Section 15.2.2.2) combined with *in vitro* based (i.e., hepatocytes) species comparison of the extent of glucuronidation versus other metabolic routes can provide some quantitative insight with respect to what degree of biliary secretion, and EHC can be expected in humans. It is often safe to assume that the degree of biliary secretion (leading to EHC) is lower in humans than in rat [37].
7) How do metabolite patterns compare between animals and humans? If major metabolites observed in humans are not observed in the standard preclinical species (e.g. rat, mouse, and dog), or vice versa, it can be useful to screen other species *in vitro* to identify a species with more humanlike metabolism (e.g. cynomolgus monkey or other primates) and run *in vivo* PK studies in this species.

15.2.3 Volume of Distribution

Although multiple volumes of distribution terms are commonly used to account for multiphasic elimination, the volume of distribution at steady state, V_{ss}, is

generally the most important parameter. It impacts the effective half-life and time to reach steady-state, alongside fluctuations in plasma exposure following repeated dosing.

For simplicity, the reasoning below focuses on the V_{ss} because this term truly reflects the distribution and binding properties of the molecule. V_{ss} is governed by the extent of binding to tissue (mainly unspecific) relative to the binding to plasma proteins and is mainly determined by physicochemical properties of the compound such as its ionic character and lipophilicity. Typically, lipophilic acids have a small V_{ss} (<0.6 l kg^{-1}) due to their high degree of plasma protein binding and low degree of tissue binding (low affinity to negatively charged phospholipids). Lipophilic bases tend to have higher V_{ss} (>4 l kg^{-1}) due to high affinity for phospholipids and occasionally trapping in the acidic lysosome environment, while less lipophilic bases and neutral compounds typically fall in between 0.6 and 4 l kg^{-1} [38]. Since affinity of a compound to tissues is very similar across species, the difference in V_{ss} between species is to a large extent driven by species differences in plasma protein binding (mainly albumin and alpha-1-acid glycoprotein). Prediction of human V_{ss} for clinical drug candidates is most robustly estimated using observed V_{ss} from animal PK studies, adjusted for differences in plasma protein binding between human and the animal species used in the preclinical PK studies.

The preferred method that has repeatedly proven to be accurate across several compound classes is the Øie–Tozer method [39]. It incorporates physiological factors such as the distribution of drug binding proteins between plasma and extracellular water, physiological volumes of extracellular water, and the assumption that the affinity to tissues is similar across species. This has proven to be a reliable method in several independent evaluations. [5, 40, 41] The Øie–Tozer equation uses the relationship between a number of species-dependent physiological factors and plasma protein binding to predict V_{ss}:

$$V_{ss} = V_P(1 + R_{E:I}) + f_{u,p} V_P \left(\frac{V_E}{V_P} - R_{E:I} \right) + \frac{V_R f_{u,p}}{f_{u,t}}$$

where V_P represents the plasma volume, V_E is the extracellular fluid volume, $R_{E:I}$ is the ratio of extravascular to intravascular protein level, V_R is the physical volume into which the compound distributes (minus the extracellular space), $f_{u,p}$ is the fraction of unbound compound in plasma, and $f_{u,t}$ is a composite measure of the fraction of unbound compound in tissues. The factor describing the tissue affinity for the compound, $f_{u,t}$, is assumed to be constant across species. Typical values for V_P, V_E, $R_{E:I}$, and V_R in various species are listed in Table 15.1.

The observed V_{ss} in the animal species from an *in vivo* PK experiment and the physiological parameter values are used to calculate $f_{u,t}$ for each preclinical PK species. The average of the $f_{u,t}$ from the different species provides a point estimate of the human $f_{u,t}$, and the range of predictions from the individual species gives an approximate indication of the uncertainty in the point estimate. Allometric methods can also be applied in this context, as V_{ss} often scales proportionally to body weight, i.e. with an allometric exponent very close to unity. Various methods correcting for the differences in plasma protein binding have also been proposed and evaluated [5]. The Øie–Tozer equation and allometry method using protein binding corrections are basically very similar. Both methods rely on accurate V_{ss}

Table 15.1 Physiological values from various animal species and humans to be used in the Øie–Tozer equation to predict the volume of distribution (V_{ss}).

	Rat	Monkey	Dog	Human
V_P ($l\,kg^{-1}$)	0.031	0.045	0.051	0.044
V_E ($l\,kg^{-1}$)	0.265	0.208	0.216	0.154
$R_{E/I}$	1.4	1.4	1.4	1.4
V_R($l\,kg^{-1}$)	0.364	0.485	0.450	0.380

The ratio of extravascular to intravascular protein level, $R_{E:I}$, is assumed to be 1.4 for all drug binding proteins, regardless of species.
Source: Reproduced with the permission of Obach et al. 1997 [42].

estimates from *in vivo* PK as well as accurate measurement of plasma protein binding. The following important points are worth to consider when estimating V_{ss} in animal PK studies:

- Accurate estimates of V_{ss} in animal PK studies require a precise measure of the total area under the plasma concentration–time curve (AUC) after single intravenous bolus dosing. Ensure a sufficient number of appropriately spaced sampling times so that the initial decay and terminal half-life are well captured. As a rule of thumb, the extrapolated AUC beyond the last observed data point should be <10% of total AUC.
- If a compound undergoes EHC in an animal species, this can increase the apparent terminal half-life and thus increase the estimated V_{ss} [31]. The extent of EHC depends on active transport of the parent compound and/or its metabolites into the bile and can differ significantly between species. Humans typically show less biliary secretion than rats [37], which can lead to an overestimation of human $V_{ss.}$ Biliary secretion studies in, e.g. rat, can be used to estimate the contribution of EHC to V_{ss} [31].
- Does the Øie–Tozer equation based on one species (e.g. rat) data accurately predict V_{ss} in other animal species? Discrepancies between predicted and observed values would warrant further investigation about the potential mechanisms involved (e.g. EHC and active transportation).
- For compounds exhibiting very high plasma protein binding ($f_{u,p} < 1\%$), estimation of $f_{u,p}$ can be challenging using standard methods. However, it has been shown that alternative experimental methods (diluted plasma) can be used to estimate $f_{u,p}$ with good precision, even for very highly protein bound drugs [43].

15.2.4 Oral Bioavailability, Rate, and Extent of Absorption

The oral bioavailability, F, is the fraction of the dose reaching the systemic circulation as intact parent compound. F is the product of the extent of absorption (F_{abs}) and the fraction of the absorbed dose surviving the first-pass effect in the gut (F_G) and liver (F_H):

$$F = F_{abs} \cdot F_G \cdot F_H$$

F_{abs} is determined by compound properties such as permeability, water solubility, dissolution rate, and metabolic and chemical stability in the gastrointestinal (GI) tract. F_G and F_H are determined by the affinity and the capacity of metabolic enzymes and transporters in the erythrocytes and in the hepatocytes. The rate of absorption (k_a) describes the relative rate by which the compound enters the systemic circulation and has impact on the peak concentrations (C_{max}) and the time to reach C_{max} (T_{max}). Absorption and bioavailability are thus multifactorial processes, and predicting these for humans requires an integration of *in vitro* and *in vivo* derived information.

15.2.4.1 Extent of Absorption and Gut First-Pass Metabolism

F_{abs} is mainly driven by the compound's intrinsic passive permeability, solubility in the intestinal fluid, and interactions with transporters in the intestinal tissue. The complexity involved in drug absorption and the numerous consideration and methods used to predict absorption are thoroughly reviewed elsewhere [44]. From an *in vivo* PK study in preclinical species, the fraction of the dose surviving the liver first pass (F_H) is estimated from the hepatic blood clearance, $CL_{H(blood)}$:

$$F_H = 1 - \frac{CL_{H(blood)}}{Q_H}$$

and the product

$$F_{abs} \cdot F_G = \frac{F}{F_H}$$

F_{abs} in humans has been shown to be well correlated to the one observed from rat PK studies [45, 46]. Permeability measured *in situ* in jejunal segments also correlates well between rats and humans [47]. F_G is difficult to estimate directly with good precision in animal experiments since portal vein measurements are required. There are nevertheless studies showing a reasonable correlation between rats and humans for CYP3A substrates [48, 49]. The scarcity of accurate *in vivo* estimates of F_G in humans also contributes to the difficulty in developing and evaluating the accuracy of new methods. PBPK models using bottom-up approaches are available [50, 51] that integrate multiple factors such as rate of metabolism, permeability, and transport interactions. These methods are conceptually attractive but experience in applying them is still limited. Since F_G is challenging to estimate experimentally, one often has to assume that species differences in F_G have little overall impact on the overall prediction of F.

In addition to the *in vivo* based prediction of F_{abs}, it is very informative to measure the effective permeability from the apical to basolateral (A–B) side using, for example, Caco-2 cells. A good correlation has been demonstrated between Caco-2 A–B permeability and F_{abs} in humans [9], and this method has nowadays become routine in many laboratories. Caco-2 is the preferred cell line because of its human origin, but other cell lines and fresh human intestinal segments can be used in a similar fashion [52]. When *in vivo* and *in vitro* based predictions of absorption differ markedly, limited solubility of the tested compound and/or its gastrointestinal metabolism are likely contributing factors. This generates significantly more uncertainty in the prediction of F_{abs} and thus F, and more

experimental data related to solubility, transporter interactions, and intestinal metabolism combined with PBPK modeling is then recommended [50, 51].

15.2.4.2 Rate of Absorption

The absorption rate, $k_{a,}$ is impacted by a combination of compound properties (e.g. size, ionic character), the size of the dose (if solubility is limiting), the formulation used, and physiological factors like pH and volume of the intestinal fluid. The most accurate methods of estimating k_a from animal data are to simultaneously fit a PK model to intravenous and oral plasma concentration–time data. From a practical point of view, the mean of the estimated k_a from preclinical species PK data (typically rat and dog) can be used directly as a rough estimate of the k_a for humans, assuming appropriate formulations (comparable with the formulation envisaged for humans) are used preclinically.

15.2.4.3 Prediction of Bioavailability and Absorption: Points to Consider

- It is important that oral doses in animal species PK studies reflect *therapeutic* levels similar (on per kg basis) to what is expected in humans in order to avoid irrelevant solubility issues in the GI tract and saturation of metabolic processes during the compound's first pass through the intestinal wall and liver.
- Is there consistency between *in vitro* and *in vivo* F_{abs} predictions? If both *in vitro* and *in vivo* data predict high values, it is very likely that the human F_{abs} will be high.
- The accuracy of F and F_{abs} predictions usually deteriorate for low permeability compounds (Biopharmaceutical Classification System (BCS) class III and IV; see Chapter 8), particularly when combined with poor solubility.
- PBPK modeling can be applied to study the sensitivity of the predictions to changes in solubility and permeability and when assessing the impact of formulations [50, 51].
- If the compound shows limited bioavailability *in vivo* in one or more animal species that cannot be explained by the product of the estimated F_H and F_{abs}, intestinal metabolism is likely contributing to the observed, decreased bioavailability. Further investigation of cross-species comparisons of intestinal metabolism is thus required to enable better predictions. A thorough review of theoretical and experimental considerations regarding intestinal first-pass effect was recently published [53].
- Carefully consider the formulation used in the animal *in vivo* studies. Aggressive solubilizers that are sometimes used in the formulation of compounds with limited solubility can lead to a higher degree and rate of absorption than what can be expected using the intended human formulation. Additional experiments with, e.g. a suspension, can provide further insight and a more realistic prediction of absorption rates.

15.2.5 Predicting PK Profiles

Ultimately, the objective is to predict the plasma concentration–time profiles of a given compound. This allows the estimation of the dose and dose schedule that will result in the desired range and duration of exposure. In this context,

predictions of plasma concentration–time profiles are typically made using compartment models or more advanced PBPK models. One-compartment models are often sufficient, but when more complex PK properties are apparent, or where animal PK suggest very distinct multicompartment behavior, PBPK models can be very useful [8].

15.2.5.1 One-compartment Model Predictions

Once the key parameters CL, V_{ss}, F, and k_a have been estimated, these are integrated in a PK model to simulate the concentration–time profile following either single or multiple dosing. For oral administration, a one-compartment model can often be used to simulate the plasma concentration–time profile as

$$C_p = \frac{F \cdot k_a \cdot \text{Dose}}{(k_a - k_e)} \cdot (e^{-k_e \cdot t} - e^{-k_a \cdot t})$$

where $k_e = \text{CL}/V_{ss}$. Multiple dosing kinetics can be derived using the same model, where the plasma concentration at any time point is the sum of concentrations from each individual dose. A useful exercise when simulating PK profiles is to vary the input parameters within the estimated range of uncertainty for each parameter. This is done to assess the sensitivity of the predictions to the individual parameters and provide a range of possible concentration–time profiles. A Monte Carlo (MC) simulation approach is useful since it can provide both point estimates and a range of potential outcomes with the associated probability [54].

15.2.5.2 PBPK Models

Although one-compartment models are useful, they are empirical and do not incorporate detailed information about the compound's disposition and the interplay between different factors. Furthermore, one-compartment methods cannot capture multiphasic PK profiles. A mechanistically more appealing approach is to use PBPK modeling. PBPK models offer the opportunity to integrate the many factors that impact PK, for example, transporter interactions, metabolism, physicochemical properties, food effects, and physiological changes [8].

A PBPK model uses a combination of physiological constants (e.g. blood flow to tissues, tissue volumes) and compound-related properties such as $\text{CL}_{\text{int,u}}$, permeability, tissue partitioning, protein binding, and other disposition properties. The fundamental assumption when translating PBPK models from animal to humans is that the drug affinity to tissue is similar across species according to

$$Kp_{\text{u,animal}} = Kp_{\text{u,human}}$$

where Kp_u is the tissue concentration of a compound divided by the unbound plasma concentration at equilibrium. Transporter kinetics data could also be integrated in the final model if such data are available. PBPK models require expert handling of the underlying assumptions, input data, and resulting predictions. Nevertheless, commercially available software packages are nowadays available, including standard physiological parameters and predefined models for humans as well as various animal species [51]. It is however worth pointing out that a pure *bottom-up* approach without appropriate animal PK data can sometimes be associated with large errors in PK predictions [55]. A sound approach

is to *calibrate* the PBPK model before applying it to humans, for example, by fine-tuning Kp_u values so that the predicted plasma concentration–time profiles accurately describe the observed animal PK [8]. The calibrated model can then be used for human predictions.

15.2.5.3 PK Predictions for the Target Patient Population

The methods discussed above and in literature are normally developed to predict the PK profile of a compound in healthy young (typically male) subjects. Nevertheless, the final PK predictions should consider the target patient population and the specific PK properties of the compound in question. Age, gender, body weight, and disease state can alter many of the physiological functions and impact all the kinetic parameters discussed earlier in this chapter. Also comedication can impact PK parameters through drug–drug interactions, as described in Chapter 18 from a toxicological point of view. PBPK models are best suited to integrate these various factors when predicting PK for a given patient population or condition [51].

15.3 Predicting Human PKPD

15.3.1 Introduction

One important goal of human PKPD predictions is to define the plasma exposure target level that has a high likelihood of generating sufficient target engagement/modulation, which will result in the desired magnitude of therapeutic response. The predicted PK properties are used to estimate what dose and dose schedule are likely to achieve this exposure target. The exposure target is typically an average plasma concentration, a concentration range, the area under the plasma concentration–time curve (AUC), or a minimal efficacious concentration ($C_{e,min}$). The exposure target should be based on a good understanding of the quantitative pharmacology (QP) hypothesis that link, as an example, the compound's interaction with the pharmacological target, the involved biological transduction processes, and how this translates to the therapeutic effect or a surrogate biomarker. As discussed in more details in Chapter 14, a good understanding of QP hypotheses and their refinement through experiments has been shown to be associated with greater probability of success in clinical development [56, 57]. Each pharmacological target class and disease poses unique challenges, and there is no single recipe on how to predict PKPD in humans. There are however fundamental principles that are common irrespective of the pharmacology involved. This section describes some of these key principles and addresses points to consider when predicting human PKPD. For a more thorough discussion on the integration of PK and PD, the reader is referred to Chapter 14.

15.3.2 Fundamental Principles for Successful PKPD Prediction and Translation

Predicting PKPD is a process that should be an integrated part of any drug discovery effort [1–3]. Successful prediction of PKPD requires a solid understanding

of quantitative aspects of the pharmacology related to the target, the disease pathology in the experimental system, and the associated translational aspects. The term *quantitative understanding* refers to both steady-state (equilibrium) and temporal relationships between, for example, the PK of the compound and the kinetics of target modulation or between target modulation and changes in downstream events linking the target to the pathology and the ultimate therapeutic response. It is important to distinguish between compound-related properties and system properties (Figure 15.3). Compound-related properties include the kinetics of the compound in plasma, its distribution kinetics to the site of action, and its interaction with the target. System properties refer to the intrinsic biology involved, alongside target modulation, and transduction processes that are independent of the compound properties. The more that is understood about these events, the better is the likelihood of identifying key parameters and relationships that need to be accounted for when translating preclinical PKPD to the patient. Biomarkers and PD measurements reflecting target engagement or modulation, disease process, and the therapeutic response (or a surrogate) are needed in order to quantify these processes. For highly studied disease areas and target classes, system properties may be well understood. At the other end of the spectrum, for unexplored targets where the biology may be less known, biomarkers are either lacking or translational aspects of the target are not fully understood.

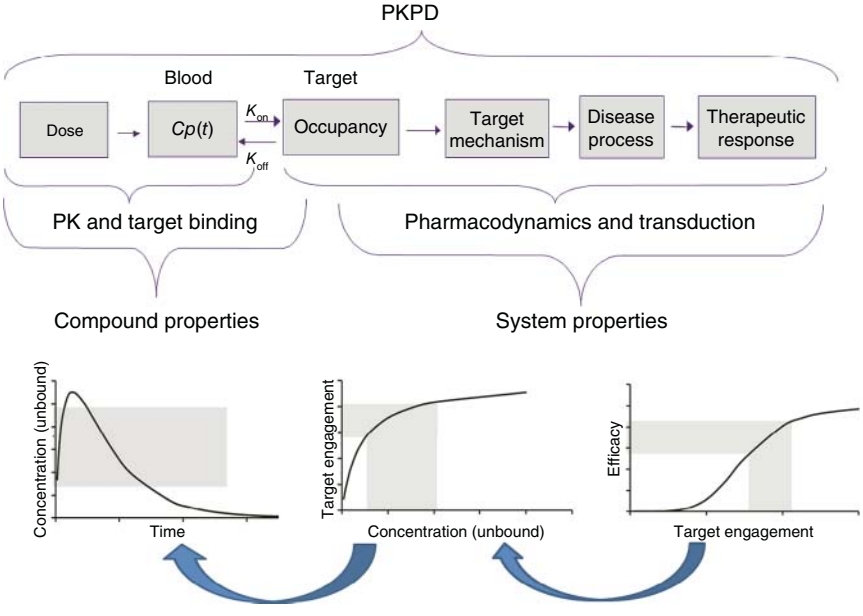

Figure 15.3 Schematic illustration of a PKPD prediction model-building framework. This captures both compound and system properties (upper panel) and the key quantitative relationships one should strive to establish, relating the level of efficacy to target engagement to drug concentration time profile (lower panel). The shaded areas in this hypothetical example illustrate the desired level of efficacy (lower panel, right) and how that translates to level of target engagement (lower panel, middle) and drug exposure (lower panel, left).

Irrespective of the target and disease area, it is valuable to develop and constantly refine a strategy for QP during the discovery process by considering the following questions:

- What is the desired magnitude of therapeutic response in patients, and what degree of target engagement/modulation is needed to achieve a meaningful effect?
- What critical parameters and quantitative relationships are involved in the causal pathway that could impact the PKPD relationship and its quantitative translation to humans?
- What quantitative and translational information is available about the system properties, either from the public domain or from previous compounds with a similar mode of action?
- What quantitative information can be generated from *tool* compounds with a similar mode of action or from genotype/phenotype information from the public domain?
- What biomarkers are needed to quantify the degree of target engagement/modulation, downstream events, and efficacy? Can these be used to quantify their relationships preclinically? Which of these biomarkers can be used clinically?

This mapping will help to identify critical gaps, lay out a strategy, and clarify what areas require further investments. An early understanding of QP can streamline the lead optimization phase since it helps to specify the property criteria for a clinical drug candidate regarding, e.g. potency, selectivity, and PK properties.

For translational purposes, it is *not always* necessary to define all the intermediate steps between target engagement and the therapeutic response. The level of effort depends on the availability of suitable biomarkers, previous knowledge about the pharmacological pathway, and level of risk the organization is willing to accept. The default position should however be to define two fundamental relationships illustrated in Figure 15.3:

1) The relationship between level and duration of target modulation/engagement and the therapeutic response.
2) The relationship between plasma PK and the extent and kinetics of target engagement/modulation.

These two relationships will provide a clear hypothesis that can be used for therapeutic dose prediction, design, and interpretation of preclinical/clinical proof of mechanism (PoM) and/or proof of concept (PoC) studies. Eventually this will support to the definition of clinical go/no go decisions.

15.3.2.1 Target Modulation/Engagement and the Pharmacologic Effect

Understanding the level of target modulation/engagement required to elicit a meaningful pharmacologic response is a key component of the PKPD relationship. This relationship is different for different types of target classes and modalities. For instance, it is known from receptor pharmacology that due to the receptor reserve, G protein-coupled receptor (GPCR) agonists usually require a

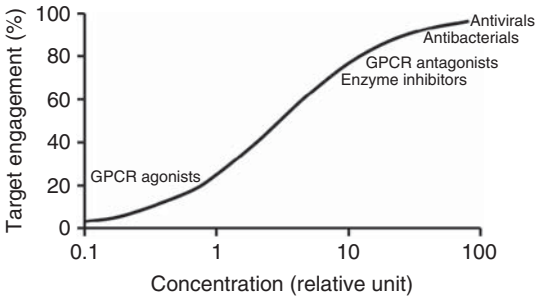

Figure 15.4 Schematic illustration of the wide range of target engagement required to elicit a meaningful pharmacological effect for different pharmacological target classes.

low degree of binding (*ca*. 10 %) to generate a clear response [58], while GPCR antagonists and enzyme inhibitors usually require a higher degree of binding (>70–80%) [59, 60] and for antivirals and antibacterial agents, even higher target engagement is required (>95–99%) [61] to result in a meaningful clinical response (Figure 15.4).

The desired level of target engagement has thus a very significant impact on the overall properties required for the drug candidate with respect to potency, PK, and selectivity. The relationship between target engagement and efficacy is a system property and is by definition the same for different compounds, assuming the compounds have the same binding mode and interaction with the target. An initial quantitative relationship of this system property can be derived using tool compounds early in lead optimization. The relationship should then be refined and confirmed with more mature compounds and finally with the clinical drug candidate. A target engagement biomarker could be, for example, receptor occupancy or a proximal measure of target modulation like, e.g. the product of an enzymatic reaction that is either inhibited or stimulated by the compound. If such markers are not available, more downstream markers could be used as surrogates for target engagement, although this usually introduces more noise and uncertainty. The duration of target engagement also needs to be considered since transduction processes often introduce time delays in the system and therapeutic response or surrogate markers often have a different temporal behavior compared to the PK of the molecule and the degree of target engagement. To this end, specifically designed experiments (e.g. dose fractionation) where efficacy variation from the same total daily exposure or the same average target engagement, as obtained with different dosing frequencies (say once daily vs. three times daily) and different study durations, can provide useful insights. Dose fractionation studies have been frequently applied to understand the underlying PKPD of antibiotics [62] but could certainly be extended to other types of pharmacology as well.

15.3.2.2 Relationship Between Pharmacokinetics and Target Engagement/Modulation

The relationship between the compound's PK and its degree of target engagement is the other cornerstone of the PKPD model. The goal is to derive a quantitative relationship that can describe the steady state relationship as well as the temporal relationship (Figure 15.5).

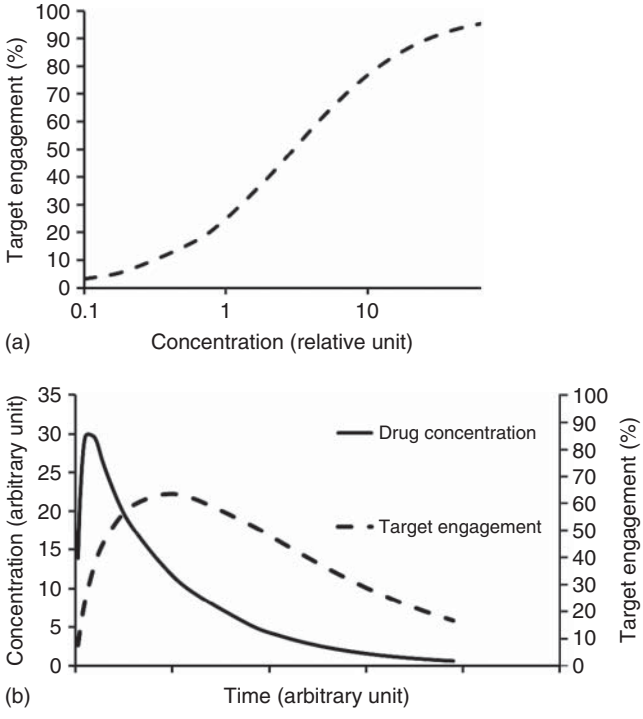

Figure 15.5 Illustration of the steady state (a) and temporal relationship (b) between PK and the target engagement that the PKPD model should quantify.

There are typically three key factors contributing to these relationships including:

- Kinetics and extent of distribution of the compound to the site of action. These depend on blood perfusion to the site of actions, diffusion barriers, and transporter interactions that may affect the rate and extent of distribution of drug between the plasma and the site of action, e.g. from plasma to CNS (blood–brain barrier) or from the interstitial fluid across cell membranes if the target is located intracellularly.
- The compound's affinity and binding kinetics to the pharmacological target.
- Turnover of the pharmacological target itself and/or the proximal biomarker used to quantify target engagement/modulation.

Specifically designed experiments are needed to elucidate these relationships, and the following guiding principles might support their implementation:

- Evaluate the intended pharmacological effect over a wide dose range for a compound to allow for a good description of the entire exposure–response relationship.
- Study the time course of both the PK and the target engagement to identify potential temporal aspects of the relationship.
- Comparison of *in vivo* and *in vitro* potency (based on unbound concentrations) can provide insights about barriers limiting the access of drug to the target *in vivo*.

15.3.2.3 Analyses of PKPD Data

Appropriate PKPD modeling using nonlinear regression applied to data from well-designed experiments can estimate, e.g. the maximum effect, E_{max}, the compound concentration required to elicit the half maximal effect (EC_{50}), and temporal aspects (delays to the onset of effect) related to either distribution of drug to the target and/or kinetics related to the target modulation and transduction. There are numerous PKPD models that can be used to capture the underlying equilibrium relationships (steady state) and temporal phenomena involved in kinetics of the drug and the response. E_{max} models, or variants of it, combined with indirect response models [63] or even more mechanistic approaches are very useful methods to capture both the steady-state relationships and temporal relationships. These and several other options are extensively described elsewhere [64]. Importantly, the establishment of a PKPD model in preclinical species including the two key relationships (exposure to target engagement and target engagement to efficacy) described above allows translation of PKPD to humans using known or measured differences in compound and system properties between the animal and humans, as described in Section 15.3.2.5.

15.3.2.4 Predicting Efficacious Concentration Without a Target Engagement or Mechanistic Biomarker

In the absence of proper target engagement or proximal biomarkers, the preclinical PKPD model can be derived from plasma concentrations of the compound and the efficacy endpoints as measured in an animal pharmacology or disease models. This approach introduces more uncertainty, since lacking information about target engagement and transduction processes reduces the possibility for a mechanism-based translation, and a more empirical approach is needed. However, if there is a PKPD relationship between animals and humans for other compounds with identical mode of action, these models can be very informative in deriving empirical *scaling factors* between the animal model and humans. In other instances, the compound might be selective for the human version of the target and have no or very low affinity for the target in animal models. Furthermore, there might not be an animal model available, or their translational ability might be inappropriate, and the PKPD predictions may have to be done based on *in vitro* assays alone. This approach requires *in vitro* methods, typically human cell assays, where the intracellular signaling and transduction processes are intact and a clinically relevant functional readout can be measured. When using this approach, it is critical to have a good understanding of what magnitude of effect in the *in vitro* model is needed to translate to a meaningful therapeutic effect. This could be derived, for example, from other compounds binding to the same target with known clinical efficacy or from genotype–phenotype data that link the degree of target modulation to severity of disease [65]. Simple cell models can also be used to study temporal relationships with properly designed experiments (e.g. the use of compound washout techniques, varying the compound concentration over time, and assessing the kinetics of the functional readout). The exposure target for the compound can then be defined based on its (unbound) concentration–response relationship and potential temporal relationships as measured in the *in vitro* assay and the insofar defined target efficacy.

15.3.2.5 Translating from Preclinical PKPD to Human PKPD: Points to Consider

When translating the PKPD model from preclinical species to patients, the goal is to define the target exposure range, level of target engagement, and temporal aspects that are involved in the pharmacology of interest. The default approaches when translating preclinical PKPD to human are listed in the following:

- Maintain the parameters in the PKPD model that are assumed or predicted to be very similar when translating from animals or from *in vitro* models to humans. Replace the system- and compound-related parameters that reflect potential differences between the animal species and human (e.g. the intrinsic affinity/potency based on a binding assay or a functional cell assay for the postulated functional potency).
- Adjust parameters related to differences in temporal aspects involved in the causal chain of events from plasma kinetics of the drug to the therapeutic response. These system-dependent properties can include, e.g. turnover of cells, protein production and degradation, and other cellular or physiological processes that differ when translating from small animals to humans.
- Target exposure should be based on unbound concentrations when translating from either *in vivo* models or *in vitro* pharmacology models to humans.

While potency at the target level is easily measured *in vitro*, transduction processes are more challenging to account for and require a good understanding of the underlying biological mechanisms involved. Rate processes are usually more rapid in smaller animals than in humans, and these differences need to be considered. As an illustrative example, following administration of rHuEpo to humans, the increase in reticulocytes (RET), red blood cells (RBC), and hemoglobin (HB) over time was very well predicted using a semi-mechanistic PKPD model accounting for species differences in various system processes (Figure 15.6) [66]. In the absence of known species differences in, e.g. transduction processes for translating across species, allometry (as discussed in the PK scaling Section 15.2.2.1) can also be used to scale physiological, biochemical, and cellular events [26], according to

$$\phi = a \cdot W^b$$

where ϕ is the parameter of interest, W is the bodyweight, a and b are the intercept and the allometric exponent, respectively. It has been shown that physiological events related to rate, like breathing, heartbeat, and even cell life span, are highly correlated with size and scale with an exponent b of approximately -0.25, while organ sizes and physiological volumes scale with an exponent close to unity [26]. Allometric scaling should be used with caution, but there are several examples showing reasonable accuracy using this approach [66].

15.4 Dose Predictions

Most discovery projects aim to develop a molecule that can deliver the desired pharmacology with as low dose as possible. Low doses are preferred as they are less likely to cause formulation issues, have less solubility problems, cause less

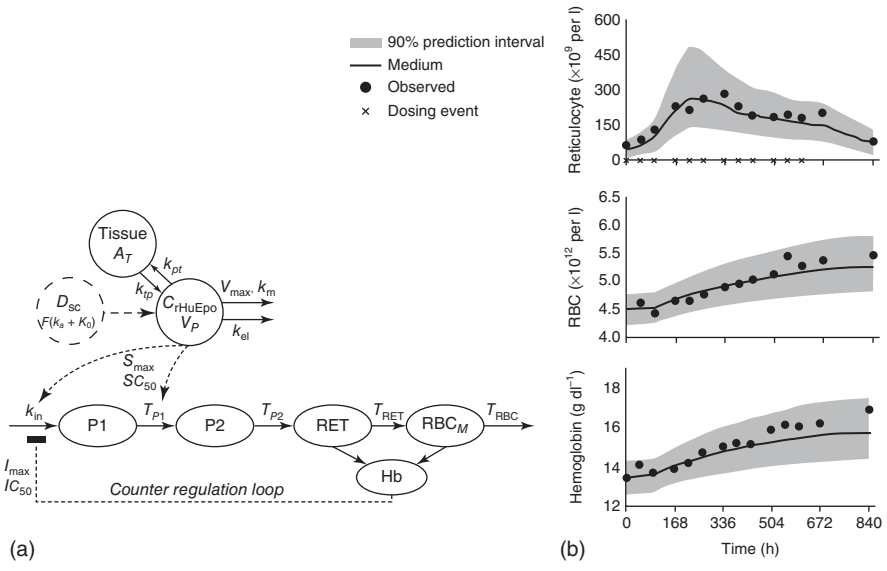

Figure 15.6 PKPD model diagram for the absorption and disposition of rHuEpo and its effects on reticulocytes (RET), red blood cells (RBC), and hemoglobin concentrations (Hb) (a). The model was developed in rodents and then translated to humans using a combination of allometry and known species differences in, e.g. cell life span and baseline values for blood cells. The humanized model was used to simulate the pharmacodynamic response to rHuEpo for reticulocytes (RET), red blood cells (RBC), and hemoglobin (HB) (b). Median (solid line) and 90% CI (shaded area) of predictions recapitulate well the observed data (filled circles). *Source*: Mager et al. 2009 [66]. Reproduced with permission of Elsevier.

drug–drug interactions or local/systemic toxicity, and reduce the cost of manufacturing. Estimating the dose and the dose schedule is therefore an important part of assessing the overall progress during the optimization phase and selection of clinical drug candidates, since all the critical individual parameters are integrated into one or two highly relevant metrics.

When predicting the therapeutic dose, the full PKPD model is combined with the PK model, and simulations are used to predict the concentration range, dose, and dose schedule that are likely to translate to sufficient efficacy while minimizing safety risks. It is useful to run simulations using the predicted PK profile and the full PKPD model to explore what PK metrics best correlate with the response. Parameters like AUC, the time within a concentration range or above a predefined concentration, are easy to understand and communicate and will also simplify estimation of efficacious dose and dose schedules. If, for example, the PKPD model, when translated to a human setting, suggests that the compound response is well predicted by the average concentration, Ce_{av}, the therapeutic dose can be predicted using the following steady state relationship:

$$\text{Dose} = \frac{Ce_{av} \cdot \text{CL} \cdot \tau}{F}$$

where CL is the total clearance, τ is the dose interval, and F is the bioavailability. In such a scenario, the dose is linearly correlated with each of these parameters.

On the other hand, if the PKPD model simulations predict that a minimum concentration is required, the half-life also becomes an important determinant of the dose and dose schedule. The sensitivity to the input parameters can be assessed using a PBPK model or, if appropriate, the simpler one-compartment model at steady state:

$$\text{Dose} = \frac{Ce_{\min} \cdot (k_a - k_e) \cdot V_{ss}}{k_a \cdot F \cdot (e^{-k_e \cdot \tau} - e^{-k_a \cdot \tau})}$$

where $Ce_{\min}$ is the target threshold concentration, k_a is the absorption rate constant, and k_e is the elimination rate constant (which is equal to the ratio between clearance (CL) and volume of distribution V_{ss}).

Sensitivity analysis is a good way to assess the importance of the individual parameters for the predicted dose. As an example, the shorter the predicted

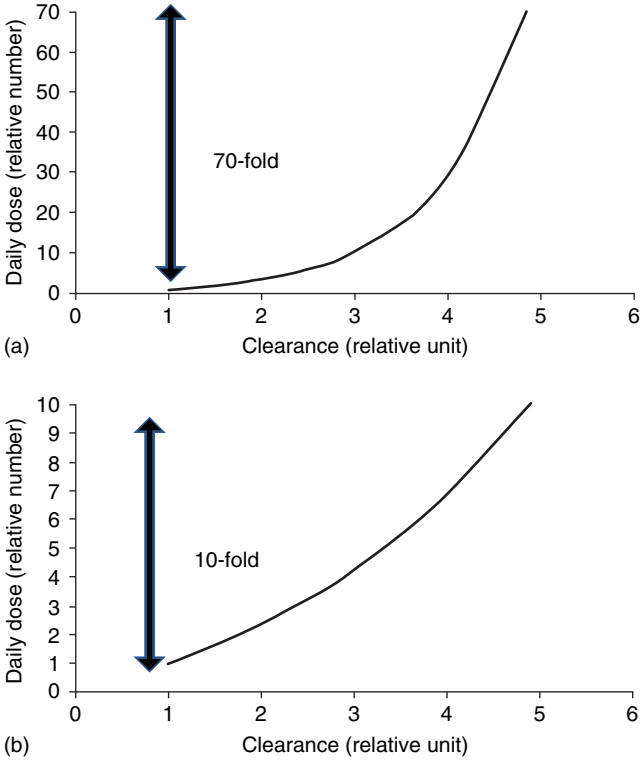

Figure 15.7 Simulations showing the relationship between predicted daily dose (relative fold change) and the predicted CL in a case where the PKPD relationship suggests that the plasma concentration needs to be maintained over a minimal concentration, $C_{e,min}$, during the entire dosing interval. Two cases are presented here for an approximate fivefold range of predicted CL: A short half-life compound ((a): midpoint half-life estimate *ca*. 7 h) and a long half-life compound ((b): midpoint estimate for the half-life *ca*. 30 h). These simulations illustrate how the predicted daily dose can be very sensitive (almost exponentially related) to the predicted CL for the short half-life compound but less so (close to linearly related) for a compound with a longer half-life.

half-life, the more sensitive the predicted dose is to the uncertainty in the CL estimate and the half-life (Figure 15.7).

15.5 Estimating and Conveying Uncertainty in PK and PKPD Predictions

Predicting PK and PKPD is always associated with a degree of uncertainty, and this can vary significantly between compounds and different pharmacological targets. When assessing and selecting a drug candidate for clinical development, it can be valuable to integrate the uncertainty in the predictions. This will help to inform decision-makers and other parts of the organization about the range of potential outcomes and manage expectations. As an example, the predicted daily exposure levels and dose have implications for the preclinical toxicology program, for planning of large-scale synthesis, and for drug formulation activities. However, simply providing a range of potential outcomes without any weighting or probability distribution is not very useful since the total range of theoretical outcomes can be very wide. A better way to approach this situation is to utilize Monte Carlo simulations that can integrate the estimated uncertainty in the individual parameters in the PK or the PKPD model and generate an overall probability distribution of outcomes [54]. These probability distributions are by no means exact since estimating the uncertainty in predicted parameters is *per se* imprecise. Nevertheless, it is often better to incorporate the best estimate of the uncertainty than completely neglecting it. The MC approach can be applied to any parameter or combination of parameter of interest (e.g. CL, V_{ss}, total dose, level of target engagement, plasma exposure levels). The principle is presented in more detail elsewhere [54] but is here illustrated with respect to dose prediction in Figure 15.8.

15.6 Future Perspectives

Predicting PK and PKPD is a science undergoing rapid development, and significant advancement can be expected in the coming 5–10 years. We are currently at a transition stage where we still have to rely heavily on animal data combined with *in vitro* models that can capture species differences or mechanistic details involved in the PK disposition or the pharmacology. The tools and the biological understanding of the essential mechanisms involved in PK and pharmacology are however developing rapidly. We can expect that PBPK models that allow integration of physicochemical properties, drug metabolism, and transport will be used more frequently as data regarding transporter activity in various tissues and the interplay between transporters and metabolism is better understood and becomes available for broad use and also incorporated into commercial software. The same is true for PKPD, where fundamental biological understanding is increasing and systems pharmacology (in the form of mechanistic PKPD models) is being increasingly used in drug discovery. We can therefore expect these types

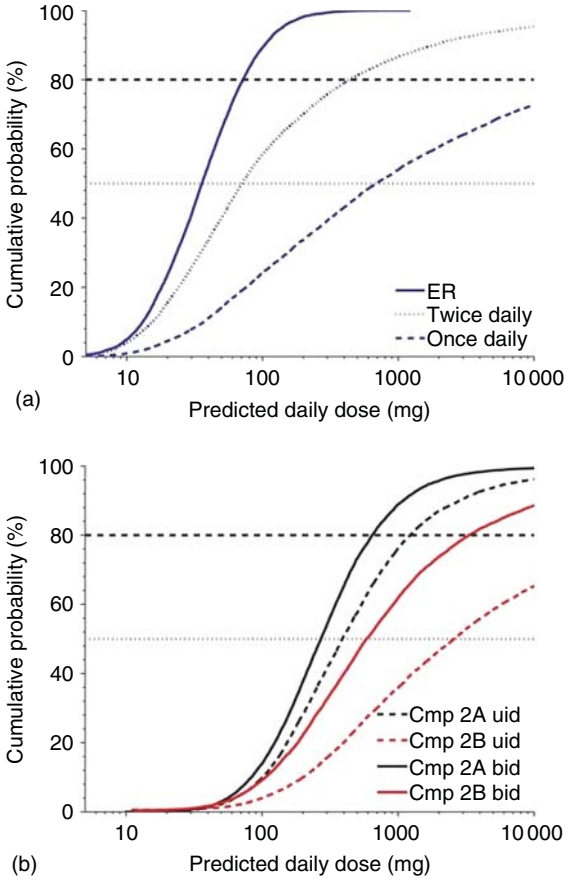

Figure 15.8 Examples of cumulative probability distributions of predicted daily doses from Monte Carlo simulations integrating uncertainty in the predicted individual PK and PKPD parameters. The panel (a) compares the predicted total daily dose for once daily, twice daily dosing, and an extended release (ER) formulation. The panel (b) illustrates the comparison between two competing molecules for once daily (UID) versus twice daily (BID) dosing. In both examples, the PKPD model suggested that concentration in plasma needed to be above a certain threshold. *Source*: Sundqvist et al. 2015 [54]. Reproduced with permission of John Wiley and Sons.

of mechanistically and physiologically/biologically based methods for both PK and PKPD prediction to slowly, but steadily, replace the empirical and allometric approaches in the coming years.

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16

Translational Modeling and Simulation for Molecularly Targeted Small Molecule Anticancer Agents: Case Studies of Multiple Tyrosine Kinase Inhibitors, Crizotinib and Lorlatinib

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16.1 Introduction

In clinical drug development from first in human to registration, the overall average success rate of new molecular entities (NMEs) was approximately 10% in 10 large pharma companies in the United States and Europe during a 10-year period (1991–2000) [1]. As would be expected, the success rates varied considerably among the different therapeutic areas. For instance, the success rates were as much as fourfold lower in oncology (~5%) than in other therapeutic areas such as cardiovascular, arthritis/pain, and infectious disease (15–20%). Regarding the transition probability in each clinical development stage (i.e. Phase I, II, III, and registration), the probability to successfully achieve the transitions from Phase II to III trials was the lowest across all therapeutic areas including oncology. The trend remains similar in more recent analyses [2–4]. Thus, despite an increased understanding of translational pharmacology from nonclinical studies to the clinic, the major reason for clinical failures of NMEs is the attrition risk to demonstrate their efficacy in Phase II trials [5, 6].

Historically, most NMEs in oncology have been small molecule cytotoxic agents, which generally show narrow therapeutic windows due to their anticancer mechanism, e.g. cytotoxic and antiproliferative activities. However, the recent prominent advances in molecular biology have enabled novel personalized targeted therapies with molecularly targeted agents (MTAs), which interfere with specific molecules involved in tumor cell growths and survivals [7–10]. In the targeted cancer therapies, tyrosine kinase inhibitors (TKIs) have recently emerged as one of the promising cancer therapies among small molecule MTAs. As a subclass of protein kinases, tyrosine kinases have been recognized as especially important targets for their roles in cancer because they are essential mediators in many basic biological processes such as cell growth, proliferation, and differentiation [11]. In the analysis of clinical trials with nearly 1000 anticancer agents from 1995 to 2007, the success rate of TKIs from first in human to registration was reported to be 50–60%, which was remarkably higher

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

than 20–30% for all anticancer agents [2]. Apparently, TKIs have a promising future as targeted cancer therapies.

Mathematical modeling and simulation (M&S) is a powerful dynamic approach linking drug exposures to pharmacological responses, e.g. pharmacokinetics (PK) to pharmacodynamics (PD) and diseases (DZ); therefore, M&S approach can quantitatively provide mechanistic understanding of pharmacokinetic–pharmacodynamic–disease (PK–PDDZ) relationships together with mechanistic insights of drug action [12–16]. Dynamic M&S approaches are increasingly being applied to virtually all phases of drug discovery and development such as (i) drug candidate selection with the most favorable PK–PDDZ properties, (ii) extrapolation of PK–PDDZ relationships from nonclinical studies to the clinic, and (iii) PK–PDDZ evaluation in patients with the aim to optimize clinical trial design. Consequently, a growing emphasis is being placed upon mechanistic M&S approaches to quantitatively evaluate drug exposure–response (ER) relationships of NMEs, particularly MTAs, as the Food and Drug Administration (FDA) has encouraged study sponsors to use M&S to determine the best dosing strategy in patients [17–21]. One of the main goals in targeted cancer therapies with MTAs is to tailor the right drug at the right dosage to the right patient with the right target. To achieve this goal, quantitative M&S approaches are essentially required to estimate ER relationships. This review illustrates an overview of the mathematical M&S frameworks used to understand ER relationships of small molecule TKIs, crizotinib (previously known as PF02341066, marketed as Xalkori®) and lorlatinib (previously known as PF06463922), in nonclinical tumor models, with the ultimate goal to predict their pharmacologically active concentrations (PAC) in patients.

16.2 Translational Pharmacology in Oncology

Over the past several decades, human tumor xenografts subcutaneously implanted in immunocompromised mice have played significant roles in nonclinical studies with anticancer agents. The *pros* and *cons* of human tumor xenograft models used for *in vivo* evaluation of anticancer drug potency in nonclinical studies have been discussed extensively in excellent review papers [22–27]. Although human tumor xenograft models were historically established to mainly evaluate an *in vivo* antitumor efficacy of cytotoxic agents, they have also recently been used to evaluate an *in vivo* antitumor efficacy of MTAs such as TKIs [22, 25, 26], often in conjunction with dynamic M&S approaches to translate PK–PDDZ relationships from nonclinical models to the clinic [28–35]. Since TKIs are designed to interfere with specific molecular pathways, their pharmacological responses (e.g. PD endpoints) should be correlated either directly or indirectly with drug exposure levels, i.e. unbound drug concentration at the target site and ultimately with antitumor efficacy (e.g. DZ endpoints). Therefore, there has been a growing interest and increased emphasis upon quantitative

extrapolation of observed PD and/or DZ endpoints from nonclinical models to patients via mechanistic M&S approaches.

For a reliable translation of TKI-mediated *in vivo* PK–PDDZ relationships from nonclinical models to the clinic, it is critical to choose an appropriate *in vivo* nonclinical model including properties of human tumors. For example, selecting human tumor cell lines for nonclinical models requires cautious consideration of molecular pathways and relevant genetic events, which may possibly and potentially take place in an intended population of cancer patients such as mutations, amplification, overexpression, or translocation of oncogenic proteins. In addition, it is crucial to perform nonclinical studies under conditions that are the most relevant, such as appropriate dosing route, dosage range/regimen, number of animals/groups, frequency of data collection, and assay performance [26]. Lastly, it is important to understand underlying assumptions required to extrapolate the PK–PDDZ relationships from nonclinical models to the clinic. One of the primary assumptions most often used is the *free drug hypothesis* that is practically applied to the extrapolation of PK–PDDZ relationships from nonclinical tumor models to patients by taking account of interspecies differences in plasma protein binding. That is, an unbound concentration of TKIs at a target site is assumed to be equivalent to that in the systemic circulation (e.g. plasma) and responsible to elicit the *in vivo* pharmacological activity, as shown by the target modulation. This assumption also presumes that microenvironments in tumor cells are functionally and physiologically comparable between nonclinical tumor models and patients' tumors; therefore, the distribution of unbound concentrations of TKIs to their target sites in tumors would be equivalent between nonclinical models and the clinical setting. Collectively, two of the most important questions in translational pharmacology for TKIs are whether (i) unbound drug exposures in systemic circulation observed in nonclinical models can be achieved sufficiently in cancer patients, so as to elicit their desired PKPD responses (e.g. target occupancy/modulation or their surrogate biomarkers, if reliable and measureable), and (ii) the required PKPD responses can be achieved successfully in cancer patients then elicit their desired PDDZ responses (e.g. antitumor efficacy). Figure 16.1 graphically summarizes these two key aspects in translational pharmacology of MTAs, which are required to be systemically and comprehensively examined in the clinic when considering the translational value of nonclinical models for the clinical setting.

If TKIs met these two criteria in their clinical trials, the understanding of translational PK–PDDZ relationships could reasonably support the prediction of their successes through quantitative M&S approaches. The quantitative M&S approach should maximize their success rates or minimize attrition rates related to efficacy such as a proof of mechanism (POM) and a proof of concept (POC) in clinical development. Accordingly, the pharmaceutical industry proactively and extensively utilizes quantitative M&S approaches to gain a more integrated understanding of translational pharmacology for successful drug discovery and development of MTAs such as TKIs [36, 37].

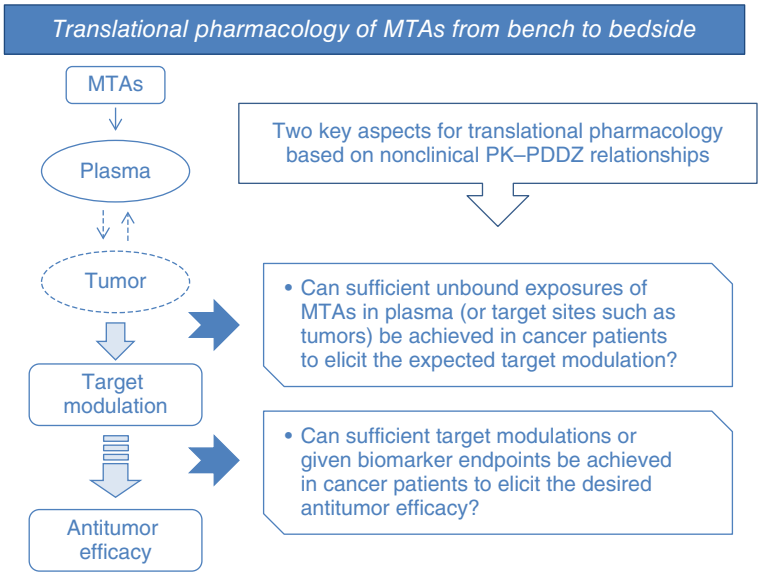

Figure 16.1 Two key aspects for translational understanding of pharmacokinetic–pharmacodynamic-disease relationships from nonclinical models to the clinic. MTAs, molecularly targeted agent; PK–PDDZ, pharmacokinetics–pharmacodynamics-disease.

16.3 Quantitative M&S Approach

To quantitatively determine PK–PDDZ relationships of MTAs in nonclinical tumor models, the application of M&S approaches for translational pharmacology is typically divided into three main tiers, as summarized in Figure 16.2: (i) modeled dose-dependent relationships of drug exposures to pharmacodynamic biomarker responses such as target modulation, i.e. PKPD relationships; (ii) modeled dose-dependent relationships of drug exposures to antitumor efficacy such as tumor growth inhibition/regression (TGI), i.e. PKDZ relationships; and lastly, (iii) modeled dose-dependent relationships between biomarker responses and antitumor efficacy executed in parallel followed by comparison of the corresponding PKPD and PKDZ relationships.

In vivo drug potency of MTAs against pharmacological responses such as target modulation and antitumor efficacy is typically characterized as a function of plasma concentrations, i.e. PKPD and PKDZ relationships. That is, a two-step approach is extensively used to characterize dose-dependent ER relationships, i.e. PKPD and PKDZ in parallel [30–35, 39–41]. Subsequently, systemic exposures of MTAs (e.g. *PAC*) required for some degrees of PD responses (e.g. ≥50% inhibition) are quantified in relation to a certain degree of DZ responses (e.g. ≥50% TGI). Overall, the PK–PDDZ relationships of MTAs in nonclinical tumor models could depend upon several key factors such as the MTAs *per se*, their targets, and tumor cell lines used in studies. Therefore, it is critical to quantitatively characterize dose-dependent PK–PDDZ relationships of each MTA in each nonclinical tumor model by appropriate mathematical M&S frameworks.

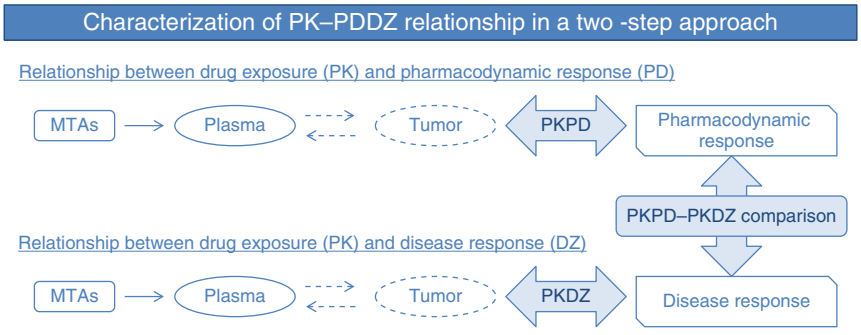

Figure 16.2 Main work stream of quantitative modeling and simulation approaches to characterize *in vivo* pharmacokinetic–pharmacodynamic–disease relationships of molecularly targeted agents. MTAs, molecularly targeted agents; PKPD, pharmacokinetic–pharmacodynamic response; PKDZ, pharmacokinetic–disease response. *Source*: Adapted with permission from Yamazaki et al. (2016) [38].

An overview of M&S frameworks used to characterize PK–PDDZ relationships for the TKIs, crizotinib and lorlatinib, in nonclinical tumor models is summarized in the following sections.

16.3.1 PK Modeling

Drug exposure–time profiles in the systemic circulation (and at the target site if available) are the first variable to be quantified in mathematical M&S approach to evaluate PK–PDDZ relationships. In general, drug concentration–time profiles are characterized by a one-, two-, or three-compartment PK model [42]. The PK parameter estimates obtained by a compartmental PK model are then used to describe drug concentrations as a function of time to drive time-dependent PKPD and PKDZ models as described later. Thus, it is critical to characterize drug concentration–time profiles in each animal or group as accurately as possible. Any errors or noises on the obtained PK parameters could in turn make significant impacts on estimations for PKPD and PKDZ parameters. One of the advantages to use the compartmental PK models is its ability to simulate novel dosing regimens. In the nonclinical studies performed with crizotinib and lorlatinib, a one-compartment PK model was applied to characterize their plasma concentration–time profiles after oral drug administration:

$$C_p = \left[\frac{D \cdot F \cdot k_a}{V \cdot (k_a - k)}\right] \cdot (e^{-k \cdot t} - e^{-k_a \cdot t})$$

where C_p is drug concentration in plasma, D is dose amount, F is oral bioavailability, k_a is the first-order absorption rate constant, V is volume of distribution, k is the first-order elimination rate constant, and t is time after dosing.

To ensure a robust M&S approach, it would be desirable to characterize full PK profiles of MTAs in each animal with an assessment of both intra- and interindividual variabilities. However, a full PK profile in each animal may not be available in many cases since a subset of animals per group at each time point is often

euthanized to collect blood and tumor samples. In addition, the evaluation of intra- and interanimal variabilities is of limited value to quantitatively extrapolate PK–PDDZ relationships from nonclinical models to patients although it is important to understand the model's performance and goodness-of-fit. In such cases, all individual plasma concentrations per group are pooled together as if they came from a single individual to estimate PK parameters [43]. This approach is called a *naïve-pooled* PK analysis, which is extensively being used in both nonclinical and clinical studies. Naïve-pooled PK analyses were utilized in both crizotinib and lorlatinib nonclinical studies to estimate their one-compartment PK parameters as described later [34, 35].

16.3.2 PKPD Modeling

A variety of different mathematical models have extensively been applied to characterize the relationship between drug exposures and resulting PD responses [42]. To estimate *in vivo* ER relationships, an appropriate "fit-for-purpose" model can be selected based upon the mechanism of action (MOA) by each MTA. One of the most popular nonlinear PKPD models is the sigmoidal E_{max} model [42, 44–46]. The sigmoidal E_{max} model represents the ER relationship as the modulation of a baseline effect:

$$E = E_0 \pm \frac{E_{max} \cdot C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}$$

where E is the PD response, E_0 is the PD response baseline, E_{max} is the maximal effect, C is the drug concentration, EC_{50} is the drug concentration at one-half of E_{max}, and γ is a Hill coefficient determining the steepness of the ER curve.

The sigmoidal E_{max} model is often referred to as the Hill equation [47] and is called the *ordinary* E_{max} *model* when γ is fixed at unity. In general, the sigmoidal E_{max} model is only applicable to direct PKPD relationships, where PD responses are seen to occur simultaneously with drug exposures without any time-delay. However, a lag time in PD responses behind drug exposures is observed in many cases, i.e. the so-called *hysteresis* phenomenon. In order to estimate *in vivo* PKPD parameters by taking account of hysteresis, when this is present, two types of potential PKPD models, a link (or effect compartment) model and an indirect response model, have been applied extensively, but not exclusively, to nonclinical and clinical data [45, 48–50]. In both models, drug concentrations in the systemic circulation are generally characterized by an appropriate PK model such as a one- or two-compartment PK model. The link model then assumes that the on- and offset rates of PD responses are governed by the rate of drug distribution to and from the systemic circulation and a hypothetical effect compartment (which is also called the *biophase*):

$$\frac{dC_e}{dt} = k_{e0} \cdot (C_p - C_e)$$

where k_{e0} represents the first-order rate constant for equilibration of drug concentrations between plasma (C_p) and biophase (C_e).

Subsequently, PD parameters such as E_{max} and EC_{50} are estimated by a sigmoidal E_{max} model using drug concentrations in the biophase compartment in

relation to the PD response in the target organ. Since a rate-limiting step in PD responses is assumed to be the drug distribution from systemic circulation to the biophase, k_{e0} estimates represent the differences in time to reach maximal PD responses as well as the time required for the responses to return to baseline. Therefore, the time to reach maximal responses is *dose-independently* constrained by k_{e0}. The link model was applied to characterize PKPD relationships of crizotinib-mediated target modulation in ALK- and MET-studies. [34, 35]

In contrast to the link model, hysteresis is often caused by other reasons, e.g. related to an indirect MOA such as stimulation or inhibition of formation (k_{in}) or degradation (k_{out}) of substance-regulating PD responses. In order to take account of hysteresis in such cases, the concept of biological turnover has been introduced through the so-called "indirect response model", which assumes that hysteresis is caused by the time required for changes in k_{in} or k_{out} to be fully reflected in PD responses [48, 51]. Four basic indirect response models have been proposed based upon potential MOAs, i.e. either inhibition or stimulation on k_{in} or k_{out}:

$$\frac{dE}{dt} = k_{in} \cdot \left(1 \pm \frac{E_{max} \cdot C_p^{\gamma}}{EC_{50}^{\gamma} + C_p^{\gamma}}\right) - k_{out} \cdot E$$

$$\frac{dE}{dt} = k_{in} - k_{out} \cdot \left(1 \pm \frac{E_{max} \cdot C_p^{\gamma}}{EC_{50}^{\gamma} + C_p^{\gamma}}\right) \cdot E$$

where k_{in} and k_{out} represent the zero-order formation rate constant and the first-order degradation rate constant, respectively.

In contrast with the link model, the delay to reach maximal PD responses *dose dependently* increases with increase in doses, particularly when drug concentrations exceed EC_{50}. That is, k_{out} *dose-dependently* represents the differences in time to reach maximal PD responses as well as time required for the responses to return to the baseline. The indirect response model has extensively been applied to characterize the dose-dependent ER relationships of MTAs in nonclinical models [31, 33, 40, 41]. In many cases, MTAs such as TKIs are assumed to inhibit k_{in} because of their MOA, e.g. ATP-competitive inhibition. Moreover, the indirect response model can be further extended by integrating a modulator when PD responses are counterbalanced by some pharmacological mechanisms, which often result from depletion or downregulation of physiological substances such as cofactors, precursor, and receptor [43, 46, 52]. These phenomena are often described as, e.g. desensitization, feedback, rebound, or tolerance. In some of these cases, PD responses may further increase or decrease relative to the levels of predose or baseline during or after repeated dose administration; therefore, PKPD models require more detailed and expanded mathematical functions to characterize ER relationships [47, 50, 52]. Since a rebound of lorlatinib-mediated ALK phosphorylation was observed at 24–36 h postdose (i.e. the response ratios of greater than unity in the treatment groups relative to the vehicle-control group), the introduction of a modulator was required for an indirect response model to take account of the observed rebound on ALK responses [30]. The PKPD model used for lorlatinib assumed that the formation and degradation rates of a modulator (M) included

as a precursor were at zero-order (k_{in}) and first-order (k_{md}), respectively, and an ALK phosphorylation level (E) was maintained by the balance of the first-order formation rate provided by the modulator degradation rate (i.e. k_{md}) and the ALK degradation rate (k_{out}). Lorlatinib was assumed to inhibit the formation rate (k_{md}) because of its competitive ATP-binding mechanism:

$$\frac{dM}{dt} = k_{in} - k_{md} \cdot \left(1 - \frac{E_{max} \times C_p^{\gamma}}{EC_{50}^{\gamma} + C_p^{\gamma}}\right) \cdot M$$

$$\frac{dE}{dt} = k_{md} \cdot \left(1 - \frac{E_{max} \times C_p^{\gamma}}{EC_{50}^{\gamma} + C_p^{\gamma}}\right) \cdot M - k_{out} \cdot E$$

where k_{in} is the zero-order formation rate constant (h^{-1}), k_{md} is the first-order formation rate for ALK phosphorylation provided by the modulator degradation rate (h^{-1}), E_{max} is maximum effect, C_p is the plasma concentration of lorlatinib ($ng\,ml^{-1}$), EC_{50} is the plasma concentration of lorlatinib ($ng\,ml^{-1}$) causing one-half E_{max}, γ is the Hill coefficient, and k_{out} is the first-order degradation rate constant (h^{-1}) of ALK phosphorylation.

16.3.3 PKDZ Modeling

As mentioned earlier, the systemic exposures of MTAs can be linked to either pharmacodynamic response (e.g. PKPD) or disease modulation (e.g. PKDZ). In order to estimate *in vivo* antitumor efficacy of anticancer agents by PKDZ modeling, one of the primary objectives is to appropriately characterize temporal tumor growth trajectories (e.g. DZ) in each animal or each group in addition to characterizing the PK profiles of MTAs. Time-dependent tumor growth curves in nonclinical tumor models without drug treatment are typically described by an early exponential growth phase followed by a linear growth phase and then a plateau phase [53, 54]. The spontaneous slowdown in tumor growth rates is commonly attributed to insufficient supplies of oxygen and nutrient, mainly due to the large tumor mass. In these cases, temporal tumor growth curves can be modeled by incorporating a factor (e.g. logistic function) constraining unbound exponential growth. For example, the exponential tumor growth models without and with a logistic function (i.e. the exponential tumor growth model and the logistic tumor growth model, respectively) are extensively being utilized to provide a tumor growth function at baseline (in the absence of drug treatment). The exponential and logistic tumor growth models are, respectively, defined as

$$\frac{dT}{dt} = k_{ng} \cdot T$$

$$\frac{dT}{dt} = k_{ng} \cdot T \cdot \left(\frac{1 - T}{T_{SS}}\right)$$

where k_{ng}, T, and T_{ss} represent the first-order net-growth rate constant, tumor volume, and the maximal sustainable tumor volume, respectively.

In the logistic model, when T is relatively small, the net-growth rate is roughly first-order (i.e. near-exponential growth) because the logistic function

$(1-T/T_{ss})$ approximates unity away from steady state. Thereafter, the net-growth rate approaches zero when T reaches T_{ss} (i.e. $1-T/T_{ss} \approx 0$). These two models were used in nonclinical studies with crizotinib and lorlatinib to characterize tumor growth curves as a baseline tumor growth function [30, 34, 35].

To estimate PKDZ relationships of MTAs by linking drug exposures to antitumor efficacy, the sigmoidal E_{max} model or modified E_{max} model (K_{max} model) can be incorporated into the exponential or logistic growth models. Assuming that MTAs stimulate the tumor killing rate, a representative example of TGI models can be defined as

$$\frac{dT}{dt} = g(T) - \left(\frac{K_{max} \cdot C_p^{\gamma}}{KC_{50}^{\gamma} + C_p^{\gamma}} \right) \cdot T$$

where $g(T)$ is the tumor growth function characterized in vehicle-control group (such as, for example, the exponential tumor growth model), K_{max} is the drug-mediated maximal tumor killing rate constant, KC_{50} is the drug concentration at one-half K_{max}, and γ is the Hill coefficients.

This PKDZ model can be viewed as a modified indirect response model because the formation rate constant (k_{in}) in the indirect response model is replaced by a growth function (i.e. tumor growth rate). In addition, K_{max} is analogous to E_{max} in the indirect response model although K_{max} can be expressed in the same unit as k_{ng} (e.g. h^{-1}) instead of being unitless. For this reason, a direct comparison between K_{max} and k_{ng} becomes feasible, particularly when the exponential tumor growth model is used as the tumor growth function. More in detail, the model-predicted maximal antitumor efficacy is less than the tumor stasis (<100% TGI) when the model-estimated K_{max} is smaller than k_{ng} (which corresponds to $E_{max} < 1$). By contrast, the model-predicted maximal antitumor efficacy is greater than 100% TGI, i.e. tumor regression, when K_{max} is greater than k_{ng} (which corresponds to $E_{max} > 1$). Furthermore, the plasma concentration of anticancer agents required to maintain 100% TGI, i.e. tumor stasis concentration (T_{sc}), can be calculated by the above equations with the obtained PKPD parameter estimates, assuming zero net tumor growth rate, i.e. $dT/dt = 0$ as $C_p = T_{sc}$. The modified indirect response models were applied to characterize the PKDZ relationship of crizotinib and lorlatinib in nonclinical studies [30, 34, 35].

16.4 Case Study: Crizotinib (PF02341066)

Crizotinib was identified as an orally available, potent ATP-competitive small molecule inhibitor of multiple tyrosine kinases including anaplastic lymphoma kinase (ALK), mesenchymal–epithelial transition (MET) factor, and c-Ros oncogene 1 (ROS1) [55, 56]. Crizotinib entered clinical Phase I dose-escalation studies in patients with solid tumors in 2006 primarily as a MET inhibitor [57, 58]. Following the discovery of oncogenic ALK rearrangements such as echinoderm microtubule-associated protein-like 4 (EML4)-ALK in non-small cell lung cancer (NSCLC) patients in 2007, the first patient with ALK-positive NSCLC enrolled in the dose-escalation trials in 2007 followed by the second

patient in 2008 [59, 60]. In parallel, a companion diagnostic assay kit to detect ALK rearrangements (i.e. a breakapart fluorescence *in situ* hybridization assay) was being developed simultaneously with the crizotinib clinical trials to select a specific population of ALK-positive NSCLC patients, i.e. the right patients with the right targets [58, 61]. Because of promising clinical responses, an expanded cohort of crizotinib for ALK-positive NSCLC patients was promptly added to the ongoing clinical studies in 2008 in parallel with the screening for MET-positive patients, e.g. MET mutation and amplification [57, 58]. Subsequently, an overall response rate of 61% (confirmed complete and partial responses) with a median progression-free survival (PFS) of 8–10 months was reported in an expanded cohort of 143 patients with NSCLC harboring ALK rearrangements [57, 62, 63]. Crizotinib was approved by the FDA for the treatment of patients with metastatic ALK-positive NSCLC, followed by worldwide approvals. Approval of crizotinib by the FDA in August 2011 was less than 4 years after the discovery of its molecular targets [59, 60]. For personalized targeted cancer therapies, crizotinib prescribing information in the United States states that "Xalkori is a kinase inhibitor indicated for the treatment of patients with metastatic NSCLC whose tumors are ALK positive as detected by an FDA-approved test." [64] Thus, the FDA approval was accompanied by the simultaneous approval of the companion diagnostic kit, Vysis (Abbott Molecular, Abbott Park, IL). Investigations into the clinical responses derived from crizotinib-mediated MET inhibition appear to be still ongoing with case reports describing promising clinical responses in some patients with MET-amplified NSCLC, gastroesophageal carcinoma, and glioblastoma [58]. Very recently, crizotinib has also been approved by the FDA for the treatment of patients with metastatic NSCLC whose tumors are ROS1 positive [64].

16.4.1 Nonclinical Study Outlines

The detailed experimental designs, methods, and results of crizotinib studies *in vivo* with human tumor xenograft models were previously reported [34, 35, 65, 66]. In this review, we focused on crizotinib multiple oral-dose studies in athymic nu/nu mice implanted with H3122 NSCLC cells harboring wild-type EML4-ALK (henceforth referred to as ALK^{WT}– models) or MET-amplified GTL16 gastric carcinomas (GC) (henceforth referred to as MET– models). In the original reports [34, 35], four separate studies of crizotinib in ALK^{WT}– and MET– models were reported: two studies for each xenograft model to determine target modulation (ALK and MET inhibition) as PD responses, and antitumor efficacy as DZ responses. In order to avoid confusion in this review, two studies for each tumor model were combined and indicated as ALK^{WT}– and MET– studies, respectively. The outlines of these studies with ALK^{WT}– and MET– models are summarized in Table 16.1.

Briefly, mice were orally treated with crizotinib at the doses of 25–200 mg kg^{-1} once daily in ALK^{WT}– models and at the doses of 6.25–50 mg kg^{-1} once daily in MET– models. A subset of mice ($n = 3$/time point) was humanely euthanized at 1, 4, 7, and 24 h after the last dose to collect blood and tumor samples. The protein levels of phosphorylated ALK (ALK phosphorylation) or MET

Table 16.1 Outlines of *in vivo* nonclinical tumor studies with crizotinib.

Study	Tumor cells	Dose mg kg^{-1}	Endpoint
ALK^{WT}	H3122 NSCLC-EML4-ALK^{WT}	25–200	PD (ALK) & DZ (TGI)
MET	GTL16 GC-MET	6.25–50	PD (MET) & DZ (TGI)

ALK, anaplastic lymphoma kinase; NSCLC, non-small cell lung carcinoma; PD; pharmacodynamic response; DZ, disease response; TGI, tumor growth inhibition (as antitumor efficacy); MET, mesenchymal–epithelial transition factor; GC, gastric carcinoma. Study outlines are cited from the previous reports.
Source: Adapted from Yamazaki et al. 2008 and Yamazaki et al. 2012 [34, 35].

(MET phosphorylation) in tumor samples were determined using a capture enzyme-linked immunosorbent assay (ELISA). The levels of ALK and MET phosphorylation were expressed as the ratios to their baseline (i.e. unity) following baseline normalization with the mean values of the vehicle-control group. During the treatment period, individual tumor volumes in animals were measured by electronic vernier calipers. The tumor volumes were then calculated as the product of length $\times$ width^2 $\times$ 0.4.

16.4.2 PK Analysis

Since a subset of mice was humanely euthanized at each time point to collect blood and tumor samples in both ALK^{WT}– and MET– models, all individual plasma concentrations of crizotinib (one sample per animal) at each dose were pooled together to estimate the PK parameters by a naïve-pooled one-compartment PK analysis as indicated before [34, 35]. Representative examples of the observed and model-fitted plasma concentrations of crizotinib in ALK^{WT}– and MET– models are shown in Figure 16.3. Overall, the plasma concentration–time profiles of crizotinib in all studies were reasonably described by the one-compartment PK model. Crizotinib PK parameters determined by the one-compartment PK model are summarized in Table 16.2.

There was a trend in all studies that the estimated *CL/F* values decreased as the doses increased. That is, crizotinib exposures increased in a supra-proportional manner with the increases in doses. For this reason, all PK parameters (i.e. *CL/F, V/F*, and k_a) were estimated at each dose. The observed dose-dependent PKs may possibly be associated with crizotinib-mediated auto-inhibition of hepatic/intestinal metabolism since crizotinib is a substrate and inhibitor of CYP3A [67, 68]. It might be worth noting that the approach to determine crizotinib PK parameter at each dose provided better fittings compared with the compartmental PK models with nonlinear functions such as Michaelis–Menten elimination, which could estimate PK parameters at all doses together by taking account of a dose-dependent saturation of clearance [34, 35]. The obtained typical PK parameters were used to simulate plasma concentrations as a function of time following oral administration to drive the time-dependent PKPD and PKDZ models as described later.

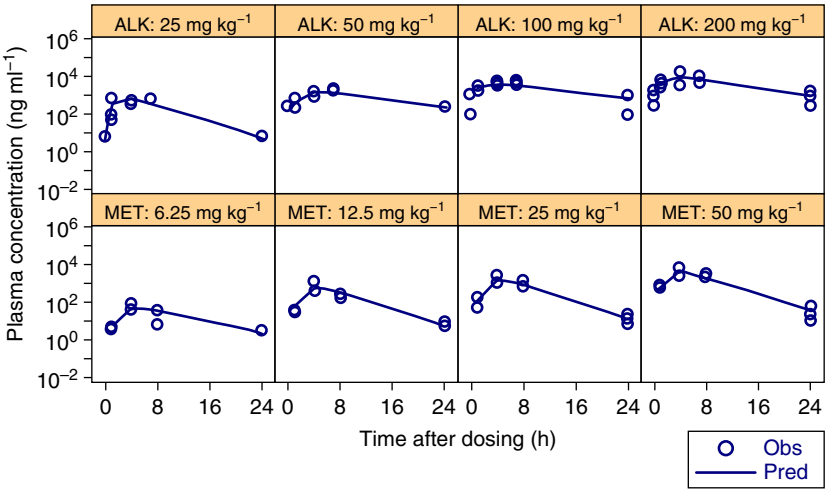

Figure 16.3 Observed and one-compartment PK model-fitted plasma concentrations of crizotinib in ALK^{WT}– and MET– Models. The x-axis represents the time after dosing in hours, and the y-axis represents the observed crizotinib plasma concentrations (Obs) with the model-fitted typical crizotinib plasma concentration–time courses (Pred) in nanograms per milliliter on a logarithmic scale in ALK– and MET– studies. *Source*: Adapted with permission from Yamazaki et al. (2008) [34] and Yamazaki et al. (2012) [35].

Table 16.2 PK parameter estimates of crizotinib in ALK^{WT}– and MET– models.

Study	Dose mg kg^{-1}	k_a h^{-1}	CL/F l (h kg^{-1}) $^{-1}$	V/F l kg^{-1}
ALK^{WT}	25–200	0.094–0.33	1.9–5.3	1.7–5.2
MET	6.25–50	0.24–0.34	1.5–14	3.2–56

ALK, anaplastic lymphoma kinase; NSCLC, non-small cell lung carcinoma; MET, mesenchymal–epithelial transition factor; GC, gastric carcinoma.
Results are cited from the previous reports.
Source: Adapted from Yamazaki et al. 2008 and Yamazaki et al. 2012 [34, 35].

16.4.3 PKPD Relationships

In both ALK^{WT}– and MET– models, temporal disconnects (i.e. *hysteresis*) were clearly observed between crizotinib concentrations in plasma and target modulations in tumor, i.e. measured ALK and MET inhibition. That is, maximal plasma concentrations of crizotinib were generally observed earlier than maximal ALK and MET inhibition, which were further sustained relative to declines in crizotinib plasma concentrations. The link model described before reasonably fit the time courses of PD responses in all groups of ALK^{WT}– and MET–models (Figure 16.4). In contrast, the indirect response model could not fit the time courses of ALK and MET inhibition well in both studies [34, 35].

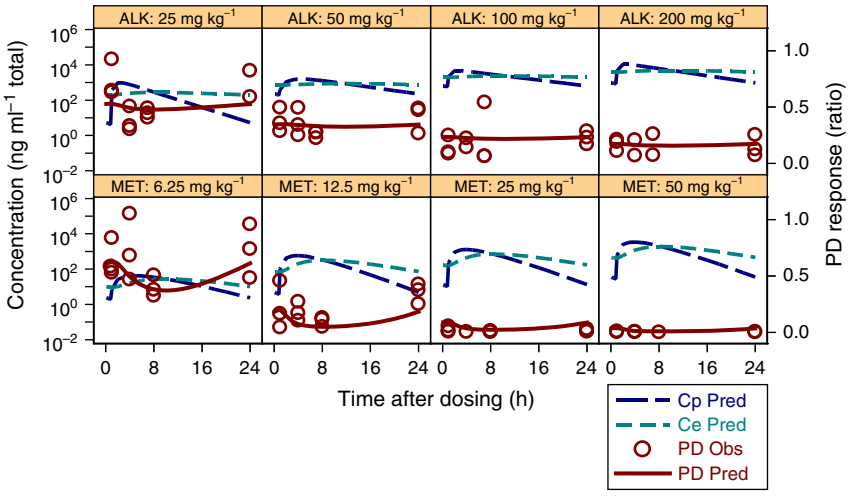

Figure 16.4 Observed and model-fitted ALK and MET inhibition by crizotinib in ALK^{WT}– and MET–models. The x-axis represents the time after dosing in hours, the left side of the y-axis represents the model-fitted typical crizotinib concentrations in plasma (Cp Pred) and the effect compartment (Ce Pred) in nanograms per milliliter on a logarithmic scale, and the right side of y-axis represents the observed and model-fitted typical PD responses (PD Obs and PD Pred, respectively) in the ratio to the mean value of control animal data in ALK– and MET– studies. *Source*: Adapted with permission from Yamazaki et al. (2008) [34] and Yamazaki et al. (2012) [35].

Table 16.3 PKPD parameter estimates of crizotinib in ALK^{WT}– and MET– models.

Study	EC_{50} ng ml^{-1}	E_{max}	K_{e0} h^{-1}	γ
ALK^{WT}	233 (153)	1 (fixed)	0.030 (0.013)	0.56 (0.11)
MET	18.5 (2.65)	1 (fixed)	0.135 (0.020)	1 (fixed)

Precision of the estimates is expressed as SE in the parentheses.
ALK, anaplastic lymphoma kinase; NSCLC, non-small cell lung carcinoma; MET, mesenchymal–
epithelial transition factor; GC, gastric carcinoma.
Results are cited from the previous reports.
Source: Adapted from Yamazaki et al. 2008 and Yamazaki et al. 2012 [34, 35].

The estimated EC_{50} values by the link model were 233 and 18.5 ng ml^{-1} total (bound plus unbound) in ALK^{WT}– and MET– models, respectively (Table 16.3). These total EC_{50} estimates corresponded to the unbound concentrations of 19 nM in ALK^{WT}– models and 1.5 nM in MET– models by accounting for mouse plasma protein binding ($f_{u,mice} = 0.036$), resulting in that the *in vivo* EC_{50} estimates were, respectively, three- and sevenfold lower than the *in vitro* EC_{50} estimates (i.e. 60 and 10 nM, respectively). Crizotinib showed relatively high nonspecific binding (approximately 90%) in liver microsomes and hepatocytes, along with high plasma protein binding (91–96%) across species [69]. Therefore, the correction for nonspecific binding in the *in vitro* EC_{50} estimates in the

cell-based assay might be required to further evaluate the *in vitro/in vivo* correlation for EC_{50} estimates. Additionally, the *in vivo* expression levels of drug-metabolizing enzymes and transporters could be altered after tumor cell inoculation to animals [70]. Since crizotinib is a substrate of CYP3A and P-glycoprotein, the *in vitro/in vivo* differences in EC_{50} might be possibly due in part to the changes of the expression levels of drug-metabolizing enzymes and transporters in ALK^{WT}– and MET– models.

16.4.4 PKDZ Relationships

The observed crizotinib antitumor efficacy on the last dosing day (quantified as TGI%) was 17%, 29%, 86%, and 100% at the doses of 25, 50, 100, and 200 mg kg^{-1}, respectively, in ALK^{WT}–models (day 18) and 34%, 60%, 89%, and 100% at the doses of 6.25, 12.5, 25, and 50 mg kg^{-1}, respectively, in MET– models (day 11). Thus, antitumor efficacy of crizotinib in both studies was clearly dose dependent at the doses tested, while crizotinib exposures dose dependently increased. For the PKDZ modeling, the logistic and exponential growth models were utilized as baseline tumor growth functions in ALK^{WT}– and MET– models, respectively, largely due to their goodness-of-fit properties. The observed difference in tumor growth curves in vehicle-control groups between these studies might be simply due to the specific baseline kinetics of tumor growth. These are known to vary among a variety of xenograft models under certain experimental conditions. The PKDZ models adequately fit the dose-dependent tumor growth curves in both ALK^{WT}– and MET– models (Figure 16.5). The estimated PKDZ parameters in both studies are summarized in Table 16.4.

In both studies, K_{max} was fixed at unity to determine PD parameters: therefore, the model-predicted maximal antitumor efficacy of crizotinib was assumed to be 100% TGI, i.e. tumor stasis, which was consistent with the observed maximal TGI at the highest doses. The net tumor growth rate (k_{ng}) in vehicle-control groups of ALK^{WT}– models was 0.011 h^{-1} with an estimated T_{ss} of 1410 mm^3 by the logistic growth model, whereas that of MET– models was 0.0063 h^{-1} with the exponential growth model. The estimated crizotinib KC_{50} values were roughly comparable between ALK^{WT}– and MET– models (255 and 213 ng ml^{-1}, respectively). These KC_{50} estimates were equivalent to the crizotinib plasma concentrations required for 50% TGI since the model-predicted maximal antitumor efficacy was fixed at 100%.

16.4.5 PK–PDDZ Understanding

To quantitatively understand PK–PDDZ relationships of crizotinib in ALK^{WT}– and MET– models, crizotinib ER curves in each xenograft model were compared with their respective target modulation. Each ER curve was simulated at the plasma concentration range of 1–10 000 ng ml^{-1} using the parameters obtained by PKPD and PKDZ modeling in ALK^{WT}– and MET– models (Figure 16.6). Following the conversion of total plasma concentrations of crizotinib to unbound concentrations with an unbound fraction in mouse plasma ($f_{u,\,plasma} = 0.036$), the

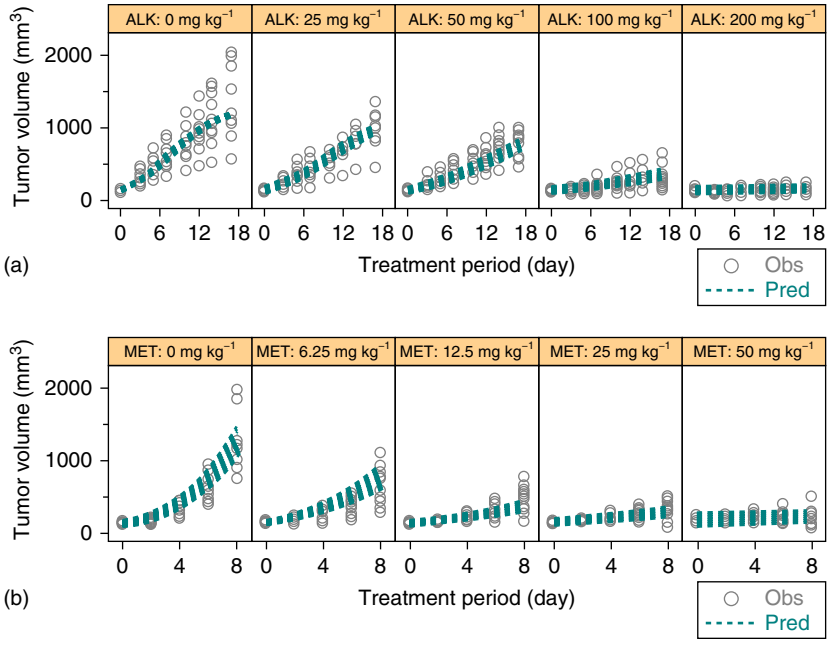

Figure 16.5 Observed tumor volumes and model-fitted TGI curves by crizotinib in ALK^{WT}– and MET– models. The x-axis represents the treatment period in days, and the y-axis represents the observed individual tumor volumes (Obs) with the model-fitted typical tumor growth curves (Pred) in cubic millimeters in ALK (A)– and MET (B)– studies. *Source*: Adapted with permission from Yamazaki et al. (2008) [34] and Yamazaki et al. (2012) [35].

Table 16.4 PKDZ parameter estimates of crizotinib in ALK^{WT}– and MET– models.

Study	KC_{50} ng ml^{-1}	K_{max}	k_{tg} h^{-1}	k_{td} h^{-1}	T_{ss} mm^3
ALK^{WT}	255(22)	1 (fixed)	0.0126 (0.0008)	0.00115 (0.000003)	1410 (155)
MET	213 (123)	1 (fixed)	0.0130 (0.0021)	0.00672 (0.00243)	–

Precision of the estimates is expressed as SE in the parentheses.
ALK, anaplastic lymphoma kinase; NSCLC, non-small cell lung carcinoma; MET, mesenchymal–epithelial transition factor; GC, gastric carcinoma.
Hill coefficients (γ) were fixed at unity in both studies.
Results are cited from the previous reports.
Source: Adapted from Yamazaki et al. 2008 and Yamazaki et al. 2012 [34, 35].

crizotinib PK–PDDZ parameter estimates in ALK– and MET– studies are summarized in Table 16.5.

Based upon the comparisons of crizotinib ER curves, the unbound EC_{50} estimate for ALK inhibition (19 nM) was comparable with that for TGI (20 nM) in ALK^{WT}– models, whereas the EC_{50} estimate for MET inhibition (1.5 nM) was approximately 10-fold lower than that for TGI (17 nM) in MEK– models. Thus, the calculated EC_{90} for MET inhibition (13 nM) by the Hill equation

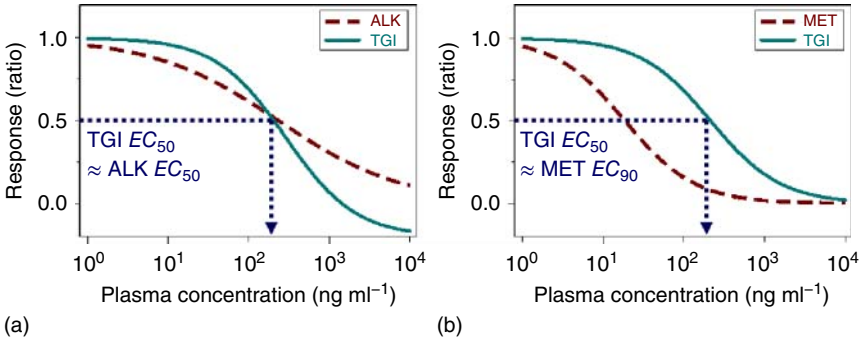

Figure 16.6 Comparison of crizotinib exposure–response curves for target modulation and TGI in ALK^{WT}– and MET– models. Crizotinib exposure–response curves for target modulation (ALK and MET) and tumor growth inhibition (TGI) were simulated at the concentration range of 1–1000 ng ml^{-1} with sigmoidal E_{max} model using the estimated PK–PDDZ parameters (EC_{50}, E_{max}, and γ) obtained from ALK (a)– and MET (b)– studies. The *x*-axis represents the plasma concentration of crizotinib in nanograms per milliliter on a logarithmic scale, and the *y*-axis represents the ratios of PD responses from 0 to 1, i.e. target modulation (ALK and MET inhibition) and tumor growth inhibition *Source*: Yamazaki (2013) [55]. Reproduced with the permission of Springer.

Table 16.5 Summary of crizotinib PK–PDDZ parameter estimates in ALK^{WT}– and MET– models.

Study	Parameter	$EC_{50,vitro}$ nM free	$EC_{50,vivo}$ nM free	$EC_{90,vivo}$ nM free
ALK^{WT}	ALK	60	19	–
	TGI	–	20	–
MET	MET	10	1.5	13
	TGI	–	17	–

–, not calculated.
Results are cited from the previous reports.
Source: Adapted from Yamazaki et al. 2008 and Yamazaki et al. 2012 [34, 35].

was roughly comparable with the EC_{50} estimate for TGI. These PK–PDDZ relationships, when taken together, suggested that target modulation required for significant antitumor efficacy (>50% TGI) could be >50% and >90% in ALK^{WT}– and MET– models, respectively. Accordingly, ALK could be a more effective target than MET to achieve a similar degree of antitumor efficacy in cancer patients if the PK–PDDZ relationships were comparable between the nonclinical tumor models and patients. In support of this, as we mentioned earlier, crizotinib has been approved for the treatment of ALK-positive NSCLC patients as a "single agent" globally, whereas clinical trials with MET-positive patients appear to be still ongoing with some case reports indicating promising clinical responses in patients with MET-amplified NSCLC, gastroesophageal carcinoma, and glioblastoma [58, 62, 71]. Overall, the PK–PDDZ relationships among crizotinib systemic exposure, ALK or MET inhibition, and TGI in

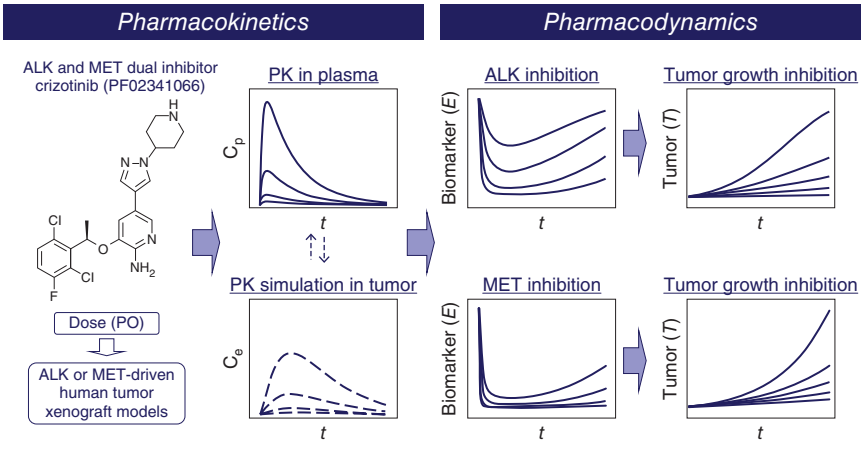

Figure 16.7 Summary of quantitative characterization of crizotinib PK–PDDZ modeling for target modulation and antitumor efficacy in ALK^{WT}– and MET– models. C_p, plasma concentration; *t*, time after dosing; C_e, effect-site concentration; *T,* tumor volume. *Source*: Adapted with permission from Yamazaki et al. (2012) [35].

nonclinical tumor models were characterized well in a quantitative manner using mathematical M&S approaches to understand nonclinical PKPD (ER) relationships (Figure 16.7).

16.4.6 Translational Pharmacology

Phase I dose-escalation studies of anticancer agents are generally conducted in patients with cancer to investigate their safety profiles including dose-limiting toxicities (DLTs), maximal tolerated dose (MTD), and the recommended Phase II dose (RP2D) as primary endpoints along with PK and efficacy profiles as secondary endpoints. Making Go/No-Go decisions on POM could be possible if the PKPD relationships between drug exposures and target modulation and/or its surrogate biomarker responses in patients were established in Phase I studies, e.g. through an expanded cohort with selected patients at MTD. However, it is difficult and challenging to obtain tumor biopsy samples from patients, especially in a serial manner, although it is critical to identify a pharmacologically active dose (PAD) in Phase I studies by determining MTA-mediated PD responses. It is worth noting that no clinical data regarding crizotinib-mediated ALK- and MET-related biomarker responses are available to date. Under these circumstances, an extrapolation of quantitative PK–PDDZ understanding from nonclinical models to the clinic would become critical for successful translational pharmacology. In this context, clinical crizotinib PKPD relationships in Phase I dose-escalation study at a starting dose of 50 mg once daily to the highest dose of 300 mg twice daily were simulated based upon the nonclinical PKPD understanding summarized above. Particularly, the projection of crizotinib PKPD relationship in patients at the RP2D, 250 mg twice daily (500 mg per day), was crucial to make the Go/No-Go decision to move forward.

For the PKPD simulation, crizotinib plasma concentration–time profiles were first simulated in patients following the twice-daily doses of 250 mg for 14 days using one-compartment PK parameters of *CL/F* of 70 l h^{-1}, *V/F* of 1500 l, and k_a of 0.75 h^{-1} [35]. These one-compartment PK parameters were adjusted from the clinically observed single-dose PK parameters to simulate the required steady-state plasma concentrations [72]. Adjustments from the single- to multiple-dose administration were required because of the observed nonstationary PKs. The differences in the maximal plasma concentration (C_{max}) and the area under the plasma concentration–time curve during the 12-h dosing interval ($AUC_{0-\tau}$) at steady state between the observed (368 ng ml^{-1} and 3641 (ng h) ml^{-1}, respectively) and simulated (342 ng ml^{-1} and 3570 (ng h) ml^{-1}, respectively) values were minimal (<10%). Crizotinib-mediated ALK and MET inhibition in patients' tumors were then predicted using the PKPD parameters estimated in nonclinical ALK– and MET– studies (Table 16.3). These PD parameter estimates (e.g. k_{e0}) were used for the simulation without any correction since human tumor cells were subcutaneously inoculated into animals to establish nonclinical xenograft models. In contrast, the "free drug hypothesis" was made that the unbound EC_{50} values were assumed to be comparable between nonclinical models and patients; therefore, the EC_{50} values obtained from nonclinical models were corrected for the difference in plasma protein binding between mice and humans ($f_{u,plasma} = 0.036$ and 0.093, respectively). Collectively, crizotinib EC_{50} values for ALK and MET inhibition in patients were assumed to be 90 and 7.2 ng ml^{-1} total, respectively, calculated from the EC_{50} estimates of 233 and 18.5 ng ml^{-1} total in the nonclinical models. The predicted crizotinib-mediated ALK and MET inhibition in patients following multiple-dose oral administration of crizotinib at the clinically recommended twice-daily doses of 250 mg is graphically presented in Figure 16.8.

The PKPD simulation in patients predicted that the crizotinib-mediated ALK and MET inhibition reached approximately 75% and near-complete inhibition (~98%), respectively. Thus, the predicted ALK and MET inhibition in patients'

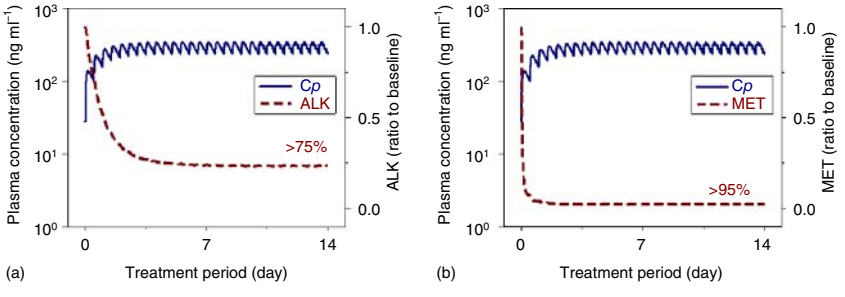

Figure 16.8 Prediction of crizotinib-mediated ALK and MET inhibition in patients following oral administration of crizotinib at the doses of 250 mg twice daily. Crizotinib-mediated ALK (a) and MET (b) inhibition in patients following 14-day multiple-dose oral administration of crizotinib at the clinically recommended doses of 250 mg twice daily were simulated by the link model with the estimated PD parameters obtained from nonclinical ALK– and MET– studies *Source*: Yamazaki (2013) [55]. Reproduced with permission of Springer.

tumors at the RP2D was higher than the predicted minimal required target modulation (i.e. >50% ALK and>90% MET) to achieve the expected antitumor efficacy. Furthermore, crizotinib-mediated ALK inhibition was predicted to be 65% and 60% in patients at the doses of 200 mg twice daily (400 mg per day) and 250 mg once daily (250 mg per day), respectively, since the reduction from the clinically recommended dose to these dosing regimens has been recommended in the United States prescribing information of Xalkori based upon individual safety and tolerability if necessary. Thus, the predicted ALK inhibition at these dosing regimens appears to be still sufficient to achieve antitumor efficacy in patients.

16.5 Case Study: Lorlatinib (PF06463922)

One of the successful MTAs for personalized cancer therapies can be TKIs of activating epidermal growth factor receptor (EGFR), gefitinib, and erlotinib, in NSCLC patients [73–75]. Unfortunately, clinical efficacy responses to these first-generation inhibitors are not durable in most patients due to drug resistance mechanisms such as the secondary mutations in EGFR (e.g. T790M), MET amplification, and increased activation of the receptor tyrosine kinase, AXL, in tumors [73–77]. In order to overcome acquired and/or adaptive drug resistances, next-generation EGFR inhibitors have rapidly been identified and entered the clinical development [78, 79]. The principles and practices of targeted cancer therapy with the EGFR inhibitors immensely influenced clinical development of crizotinib in ALK-positive NSCLC patients [62, 71, 80]. However, the secondary ALK mutations in tumors from crizotinib-resistant NSCLC patients were reported even before its approval [81]. Crizotinib-resistant mechanisms in 69 reported cases were secondary mutations in the ALK kinase domain in 20 patients (~30%), most commonly the "gatekeeper" mutation L1196M, followed by amplification of the rearranged ALK locus in six patients (9%) and activation of alternative receptor tyrosine kinases (e.g. EGFR) in a few patients [82]. These resistance mechanisms to crizotinib have rapidly led to clinical development of next-generation ALK inhibitors in patients with NSCLC [82–85].

Lorlatinib (PF06463922) has recently been identified as an orally available, ATP-competitive ALK and ROS1 inhibitor [86]. Lorlatinib is highly potent against ALK inhibition in H3122 NSCLC cells expressing the EML4-ALK fusion protein with an IC_{50} of ~2 nM against the wild-type EML4-ALK (without ALK mutations) and ~20 nM against one of the most frequently detected crizotinib-resistant EML4-ALK mutations, EML4-ALK^{L1196M}. Hence, lorlatinib is ~40-fold potent against EML4-ALK^{L1196M} compared with crizotinib, which has an approximate IC_{50} of ~800 nM. Lorlatinib is also exceptionally potent against ROS1 inhibition with an IC_{50} of ~0.2 nM. Furthermore, lorlatinib demonstrated significant *in vivo* ALK inhibition and antitumor efficacy in H3122 NSCLC xenograft models with EML4-ALK^{L1196M}. In contrast, antitumor efficacy by crizotinib was negligible to minimal in this xenograft model at twice-daily oral doses of 75 mg kg^{-1} per dose, which yielded unbound plasma concentrations higher in mice than those in patients at clinically recommended twice-daily oral

doses of 250 mg [87]. Lorlatinib is currently being evaluated in patients with ALK- or ROS1-positive NSCLC in a Phase I/II trial [84].

16.5.1 Nonclinical Study Outlines

The detailed experimental designs, methods, and results of lorlatinib studies with *in vivo* nonclinical tumor models were previously reported [30, 88]. Three separate studies of lorlatinib were conducted in female athymic nu/nu mice implanted subcutaneously with H3122 NSCLC cells expressing the EML4-ALK^{L1196M} (henceforth referred to as ALK^{MT}– models) or NIH3T3 cells expressing CD74-ROS1 (henceforth referred to as ROS1– models) in the original report [30]. Two studies in ALK^{MT}– models were combined and referred to as an ALK^{MT}– study in this review. The outlines of these studies with ALK^{MT}– and ROS1– models are summarized in Table 16.6.

Lorlatinib was orally administered to animals twice daily, 7-h apart, in ALK^{MT}– models at the doses of 0.3–10 mg kg^{-1} per dose for 4 days or 0.3–20 mg kg^{-1} per dose for 13 days and in ROS1– models at the doses of 0.01–3 mg kg^{-1} per dose for 9 days. The protein levels of phosphorylated ALK (ALK phosphorylation) in tumor samples were determined using a capture ELISA and expressed as the ratios to their baseline (i.e. unity) following baseline normalization with the mean values of the vehicle-control group. Phosphorylated ROS1 *in vivo* was not determined due to a lack of specificity of the ROS1 antibody. Individual tumor volumes in animals during the treatment period were measured by electronic vernier calipers and calculated as the product of length × width^2 × 0.4.

16.5.2 PK Analysis

A subset of mice ($n = 3$/time point) was humanely euthanized at each time point to collect blood and tumor samples in both ALK^{MT}– and ROS1– models. All individual plasma concentrations of lorlatinib at each dose were pooled together to estimate the PK parameters by a naïve-pooled one-compartment PK analysis as was done in crizotinib PK analyses [30]. The observed and model-fitted plasma concentrations of lorlatinib in ALK^{MT}– models are shown in Figure 16.9 as representative examples.

Table 16.6 Outlines of *in vivo* nonclinical tumor studies with lorlatinib.

Study	Tumor cells	Dose mg kg per dose	Endpoint
ALK^{MT}	H3122 NSCLC-EML4-ALK^{L1196M}	0.3–20	PD (ALK) & DZ (TGI)
ROS1	NIH3T3-CD74-ROS1	0.01–3	DZ (TGI)

ALK, anaplastic lymphoma kinase; ROS1, c-ROS1 oncongene; NSCLC, non-small cell lung carcinoma; PD; pharmacodynamic response; DZ, disease response; TGI, tumor growth inhibition (as antitumor efficacy).
Study outlines are cited from the previous reports.
Source: Adapted from Yamazaki et al. 2014 [30].

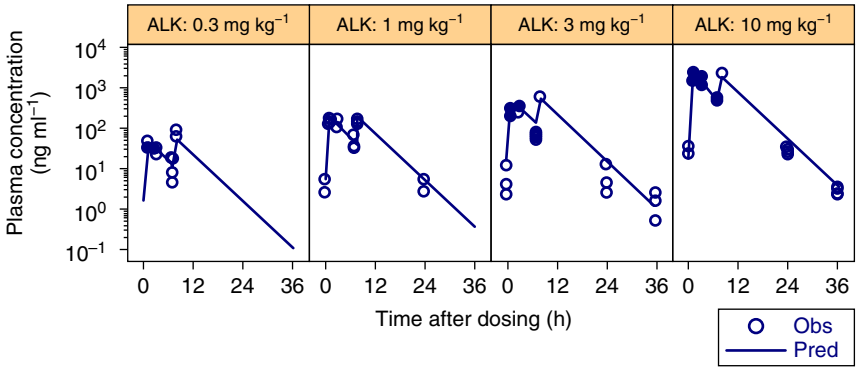

Figure 16.9 Observed and one-compartment PK model-fitted plasma concentrations of lorlatinib in ALK^{MT}– models. The *x*-axis represents the time after dosing in hours, and the *y*-axis represents the observed lorlatinib plasma concentrations (Obs) with the model-fitted typical plasma concentration–time courses (Pred) in nanograms per milliliter on a logarithmic scale. *Source*: Adapted with permission from Yamazaki et al. (2014) [30].

Table 16.7 PK parameter estimates of lorlatinib in ALK^{MT}– and ROS1– models.

Study	Dose mg kg^{-1}	k_a h^{-1}	*CL/F* l (h kg^{-1})$^{-1}$	*V/F* l kg^{-1}
ALK^{MT}	0.3–20	1.3–2.0	1.1–1.2	5.3–7.0
ROS1	0.01–3	4.0	1.7	11

ALK, anaplastic lymphoma kinase; ROS1, c-ROS1 oncogene.
Results are cited from the previous report.
Source: Adapted from Yamazaki et al. 2014 [30].

The plasma concentrations of lorlatinib in both ALK^{MT}– and ROS1– models were adequately described by the one-compartment model. The increase in oral exposures of lorlatinib was roughly dose proportional at the doses tested in both the ALK^{MT}– and ROS1– models. Typical PK parameter estimates for k_a, *CL/F* and *V/F* were, respectively, 1.3–2.0 h^{-1}, 1.1–1.2 l h^{-1} kg^{-2}, 5.3–7.0 l kg^{-1} in ALK^{MT}– models and 4.0 h^{-1}, 1.7 l h^{-1} kg^{-2}, and 11 l kg^{-1} in ROS1– models (Table 16.7). Accordingly, these PK parameter estimates were used to simulate plasma concentrations of lorlatinib as a function of time following oral administration for driving PKPD and PKDZ models.

16.5.3 PKPD Relationships

Plasma concentrations of lorlatinib reached their maximal levels at 1 h postdose and then rapidly declined in both models. In contrast, the inhibition of ALK phosphorylation in tumors was relatively sustained after the first- and second-daily doses. Unexpectedly, the responses of ALK phosphorylation in tumor (expressed as ratios to the vehicle-control group) were partially back to near or above the baseline around 24 h postdose (i.e. near or greater than unity) following the significant inhibition (e.g. near-complete inhibition). The observed

rebounds of ALK responses were dose-dependent in ALK^{MT}– models not only with lorlatinib but also other *in-house* ALK inhibitors [30, 39]. Furthermore, the ALK rebound was more pronounced on day 13 than day 4, suggesting that the ALK rebound time dependently increased with the increases in the treatment days. To adequately describe the dose- and time-dependent lorlatinib-mediated ALK responses including rebound, a modulator was incorporated into an indirect response model as a precursor as described before [48, 51, 52]. The PKPD model with a modulator sufficiently fit the time courses of ALK phosphorylation including the rebound at 24–36 h postdose (Figure 16.10).

The estimated *in vivo* EC_{50} for ALK inhibition was 58 ng ml^{-1} total (bound plus unbound), whereas the other PKPD parameter estimates, k_{out}, k_{md}, and γ, were 1.8 h^{-1}, 0.021 h^{-1}, and 1.1, respectively (Table 16.8). The *in vivo* EC_{50} estimate of 58 ng ml^{-1} total corresponded to an unbound concentration of 36 nM when accounting for mouse plasma protein binding ($f_{u,mice} = 0.25$), resulting in the twofold difference in the EC_{50} estimates between *in vivo* and *in vitro* (15 nM free). Biological feedback mechanisms including the observed *in vivo* ALK rebounds could be one of the potential reasons for the twofold difference in the estimated EC_{50}, whereas the difference might be within the expected variability derived from *in vitro* and *in vivo* experiments.

16.5.4 PKDZ Relationships

Lorlatinib exhibited dose-dependent robust antitumor efficacy in both ALK^{MT}– and ROS1– models. The observed antitumor efficacy (quantified as TGI%) on the last dosing day was 57, 87, 101, 121 (63% regression), and 120% (66% regression) in ALK^{MT}– models at the doses of 0.3, 1, 3, 10, and 20 mg kg^{-1} per dose, respectively, whereas it was 26, 38, 84, 104 (20% regression), 116 (73% regression), and 120% (85% regression) in ROS1-model at the doses of 0.01,

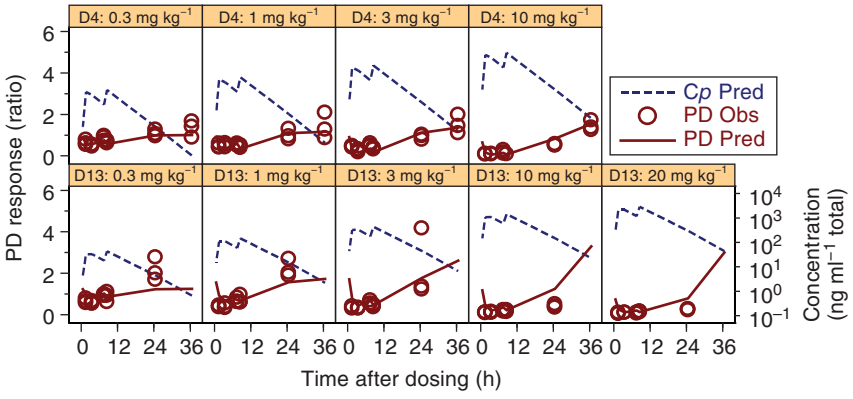

Figure 16.10 Observed and model-fitted ALK inhibition by lorlatinib in ALK^{MT}– models. The x-axis represents the time after dosing in hours, the left side of the *y*-axis represents the observed and model-fitted typical PD responses (PD Obs and PD Pred, respectively) in the ratio to the mean value of control animal data, and the right side of *y*-axis represents the model-fitted typical plasma concentrations of lorlatinib (Cp Pred) in nanograms per milliliter on a logarithmic scale. *Source*: Adapted with permission from Yamazaki et al. (2014) [30].

Table 16.8 PKPD parameter estimates of lorlatinib in ALK^{MT}– models.

Study	EC_{50} ng ml^{-1}	E_0	E_{max}	k_{out} h^{-1}	k_{md} h^{-1}	γ
ALK^{MT}	58	1	1	1.8	0.021	1.1
	(14)	(fixed)	(fixed)	(0.4)	(0.003)	(0.1)

Precision of the estimates is expressed as SE in parentheses.
Results are cited from the previous report.
Source: Reproduced with the permission of Yamazaki et al. 2014 [30].

0.03, 0.1, 0.3, 1, and 3 mg kg^{-1} per dose, respectively. Thus, antitumor efficacy of lorlatinib including tumor regression was clearly dose dependent in both studies at the doses tested while lorlatinib exposures dose dependently increased. To characterize *in vivo* antitumor efficacy of lorlatinib, the PKDZ models (i.e. modified indirect response models) were applied to ALK^{MT}– and ROS1– studies as described before. The PKDZ models reasonably fit the observed dose-dependent tumor growth curves (i.e. DZ) in all groups of both studies as a function of lorlatinib plasma concentration (i.e. PK) (Figure 16.11). In ALK^{MT}– models, the

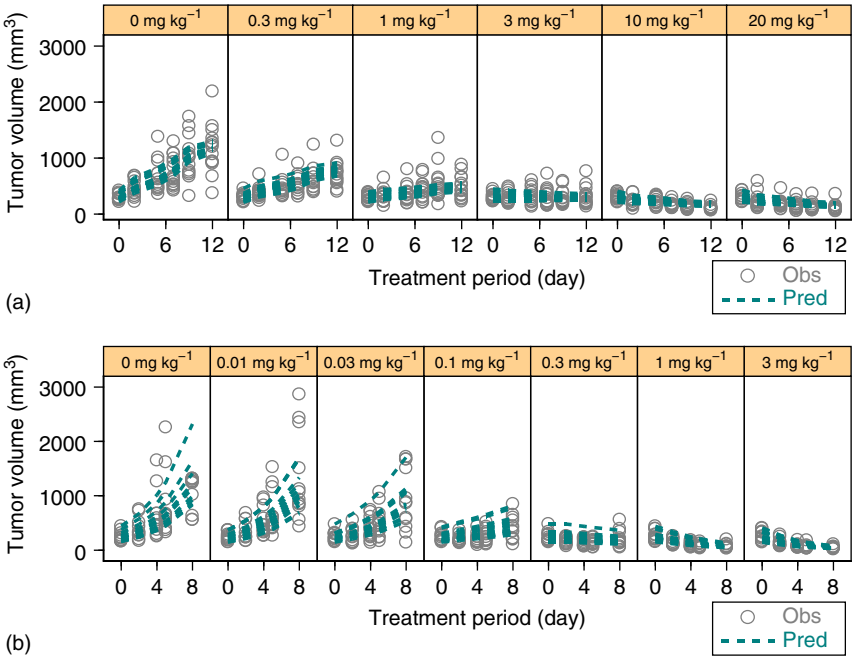

Figure 16.11 Observed tumor volumes and model-fitted TGI curves by lorlatinib in ALK^{M}– and ROS1– models. The x-axis represents the treatment period in days, and the *y*-axis represents the observed individual tumor volumes (Obs) with the model-fitted typical tumor growth curves (Pred) in cubic millimeters in ALK (a)- and ROS1 (b)-studies. *Source*: Adapted with permission from Yamazaki et al. (2014) [30].

Table 16.9 PKDZ parameter estimates of lorlatinib in ALK^{MT}– and ROS1– models.

Study	KC_{50} ng ml^{-1}	K_{max} h^{-1}	k_{ng} h^{-1}	T_{ss} mm^3	γ
ALK^{MT}	33	0.011	0.0094	1530	1
	(14)	(0.001)	(0.0012)	(201)	(fixed)
ROS1	13	0.020	0.0086	–	1
	(3)	(0.001)	(0.0008)		(fixed)

Precision of the estimates is expressed as S.E. in parentheses.
–, not applicable.
Results are cited from the previous report.
Source: Reproduced with the permission of Yamazaki et al. 2014 [30].

PKDZ model-predicted maximal antitumor efficacy of lorlatinib was greater than tumor stasis, i.e. tumor regression, since the estimated k_{max} (0.011 h^{-1}) was 1.1-fold higher than the estimated k_{ng} (0.0094 h^{-1}) (Table 16.9). The estimated KC_{50} was 33 ng ml^{-1} total, whereas the calculated T_{sc} from the obtained PKPD parameters was 83 ng ml^{-1} total, which corresponded to the unbound concentrations of 51 nM. In ROS1– models, the estimated k_{max} (0.020 h^{-1}) was approximately twofold higher than the estimated k_{ng} (0.0086 h^{-1}), indicating that the PKDZ model-predicted maximal antitumor efficacy of lorlatinib was a robust tumor regression. The estimates of KC_{50} and T_{sc} were 13 and 10 ng ml^{-1} total, respectively (Table 16.9). Therefore, the estimated T_{sc} was approximately eightfold lower in ROS1– models (6.2 nM unbound) than ALK– models (51 nM unbound), demonstrating that antitumor efficacy of lorlatinib was more effective in ROS1– models than ALK^{MT}– models.

16.5.5 PK–PDDZ Understanding

For PK–PDDZ understanding of lorlatinib in ALK^{MT}– models, the ER relationships between target modulation (i.e. PKPD) and antitumor efficacy (i.e. PKDZ) were graphically compared in Figure 16.12. The ER curves were simulated at the plasma concentration range of 0.01–10 000 ng ml^{-1} using the parameters obtained with PKPD and PKDZ modeling in ALK^{MT}–models. Furthermore, the ER curve for antitumor efficacy (i.e. PKDZ) of lorlatinib in ROS1– model was also simulated at the same range of plasma concentrations (Figure 16.12), whereas the PKPD relationship was not available as mentioned before. It may be worth pointing out that the TGI range in y-axes is 0 –120% (including tumor regression), while the range of ALK inhibition is –100%.

In ALK^{MT}– models, the EC_{50} (36 nM unbound) for ALK inhibition was 1.4-fold lower than the T_{sc} (51 nM unbound); thus, the EC_{60} (52 nM) for ALK inhibition was roughly comparable to the T_{sc} estimate. Thus, the PK–PDDZ relationships of lorlatinib suggest that 60% ALK inhibition would be required for tumor stasis. Consequently, the PK–PDDZ relationship of lorlatinib in ALK^{MT}– models was different from that of crizotinib in ALK^{WT}– models. As

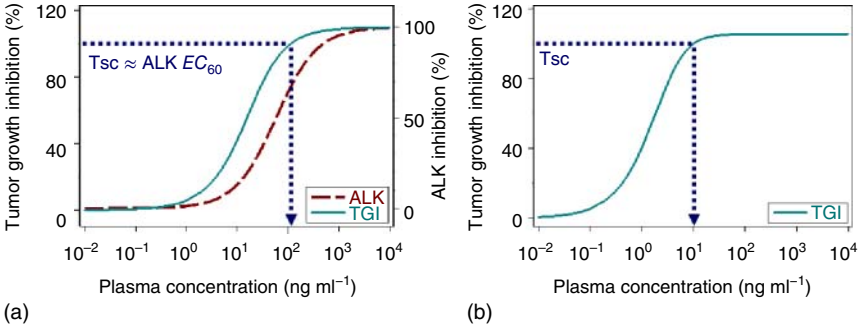

Figure 16.12 Comparison of lorlatinib exposure–response curves for target modulation and TGI in ALK^{MT}– and ROS1– models. Lorlatinib exposure–response curves for ALK inhibition and tumor growth inhibition (TGI) were simulated at the concentration range of 0.01–10 000 ng ml^{-1} with sigmoidal E_{max} model using the estimated PK–PDDZ parameters (EC_{50}, E_{max}. and γ) obtained from ALK (a)- and MET (b)-studies. The *x*-axis represents the plasma concentration in nanograms per milliliter on a logarithmic scale, the left side of the *y*-axis represents the tumor growth inhibition from 0% to 120%, and the right side of y-axis in represents the ALK inhibition from 0% to 100% in ALK-models. *Source*: Adapted with permission from Yamazaki et al. (2014) [30].

described before, 50% ALK inhibition by crizotinib was associated with 50% TGI in ALK^{WT}– models expressing wild-type EML4-ALK (without ALK mutation). Accordingly, lorlatinib-mediated ALK inhibition in ALK^{MT}– models (with ALK mutation) led to more pronounced antitumor efficacy than crizotinib-mediated ALK inhibition in ALK^{WT}– models. In ROS1–tumor model, the estimated T_{sc} of lorlatinib (6.2 nM unbound) was approximately eightfold lower than that (51 nM) in ALK^{MT}– models, demonstrating that lorlatinib-mediated antitumor efficacy was much more potent in ROS1– models compared with ALK^{MT}– models. Although the measurement of *in vivo* target modulation by lorlatinib in ROS1– model was not technically possible due to a lack of specificity of ROS1 antibody, the difference in antitumor efficacy *in vivo* was consistent with the difference in the *in vitro* EC_{50} estimates of 0.2 and 15 nM for ROS1 and ALK inhibition, respectively. Overall, the PK–PDDZ relationships of lorlatinib between systemic exposure, target modulation, and antitumor efficacy in nonclinical tumor models were quantitatively characterized by mathematical M&S approaches (Figure 16.13). The present results suggest that >60% ALK inhibition by lorlatinib would be required to achieve significant antitumor efficacy (>100%) in the clinic.

16.5.6 Translational Pharmacology

Since lorlatinib is a next-generation ALK inhibitor following the first-generation ALK inhibitor, crizotinib, 100% TGI (i.e. tumor stasis) was targeted as the minimum required antitumor efficacy in nonclinical ALK^{MT}– models compared with the original 50% TGI for crizotinib in ALK^{WT}– models. Based upon the PK–PDDZ relationships of lorlatinib, the estimated unbound EC_{60} of ~50 nM was proposed as a *PAC* in NSCLC patients with EML4-ALK mutations.

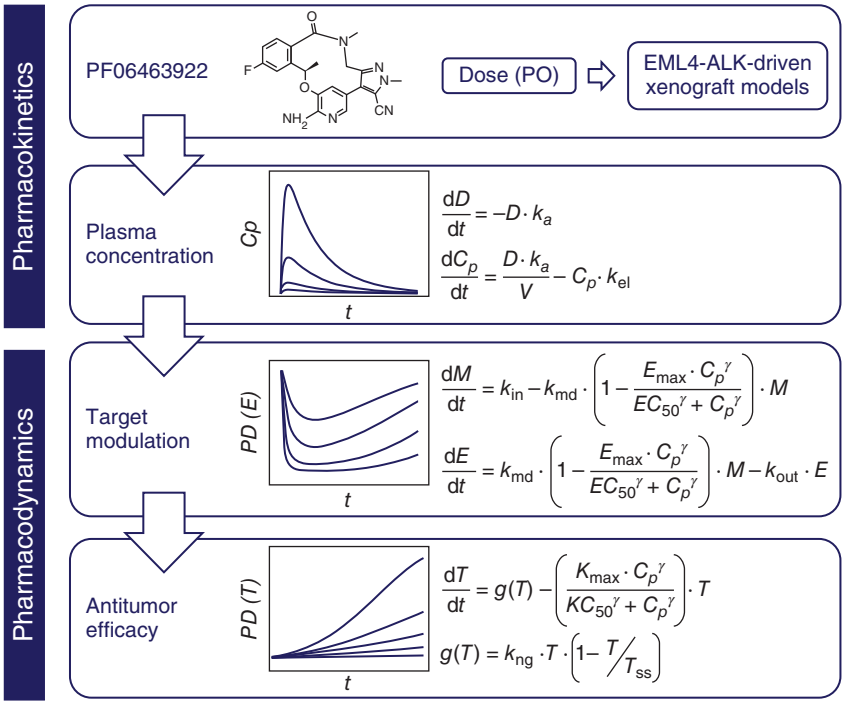

Figure 16.13 Summary of quantitative characterization of lorlatinib PK–PDDZ modeling for target modulation and antitumor efficacy in ALK^{MT}–models. *Source*: Adapted with permission from Yamazaki et al. (2014) [30].

Additionally, the unbound EC_{75} estimate (100 nM) for lorlatinib-mediated ALK inhibition could be considered another target plasma concentration of lorlatinib in crizotinib-resistant NSCLC patients. This would be required to achieve equivalent antitumor efficacy as that observed in crizotinib-sensitive NSCLC patients because the crizotinib PKPD simulation suggested that crizotinib-mediated ALK inhibition could reach >75% at steady state in patients at the dose of 250 mg twice daily [55].

Lorlatinib is an exceptionally potent ROS1 inhibitor with an *in vitro* unbound EC_{50} estimate of 0.2 nM, which was >50-fold lower than that for *in vitro* ALK inhibition (15 nM). In contrast, the *in vivo* T_{sc} estimate (6.2 nM) of lorlatinib in ROS1– models was approximately eightfold lower than that (51 nM) in ALK^{L1196A}– models. Thus, while one might expect more pronounced antitumor efficacy of lorlatinib in ROS1– models relative to ALK^{MT}– models, actual antitumor efficacy could apparently depend upon a variety of different *in vivo* factors through complex biological mechanisms such as extensive and intricate signaling cross talk and scaffold networks in tumor cells [59, 89, 90]. In ROS1– models, the observed *in vivo* average unbound plasma concentration of lorlatinib (~0.5 nM) at the lowest dose of 0.01 mg kg^{-1} was roughly threefold higher than *in vitro* unbound EC_{50} (0.2 nM) for ROS1 inhibition. However, the observed antitumor efficacy at this dose was minimal with TGI of 26% on the

last dosing day. These findings suggest that lorlatinib *in vivo* EC_{50} for ROS1 inhibition required to exhibit a significant antitumor efficacy in ROS1– models may be likely higher than the *in vitro* EC_{50}. Nonetheless, based upon the comparison of lorlatinib-mediated antitumor efficacy between ALK^{MT}– and ROS1– models, it is highly likely that antitumor efficacy of lorlatinib can be achieved more readily in NSCLC patients with ROS1 rearrangements than ALK-positive patients. Therefore, a robust antitumor efficacy by lorlatinib would be expected in patients with ROS1 rearrangements when systemic exposure of lorlatinib reached a proposed *PAC* based upon nonclinical PK–PDDZ understanding in ALK^{WT}– models.

16.6 Closing Remarks

Cancer treatments have historically been largely limited to chemotherapies with cytotoxic medicines, which are typically not only unselective to certain cancers but also often limited by severe adverse effects. The recent improved understanding of MOA for cancer therapeutics accompanied by rapid advances in molecular biology has enabled the development of personalized, targeted cancer therapies with MTAs. Clinical therapeutic strategy with MTAs should differ from traditional chemotherapies because of the profound differences in MOA. A common practice in traditional chemotherapies is to identify a MTD as a RP2D in Phase I dose-escalation studies, whereas a PAD should be identified in Phase I studies with MTAs by monitoring MTA-mediated PD responses. Clinical applications to identify PADs of MTAs based upon PD responses are relatively rare in clinical trials, mainly because of the difficulty in obtaining biopsy samples, especially serial samples. To make this practice successful, the quantitative *PAC* estimation of MTA by nonclinical PK–PDDZ modeling could potentially fill the gap since the estimated *PAC*s could be used to target systemic exposures needed to achieve

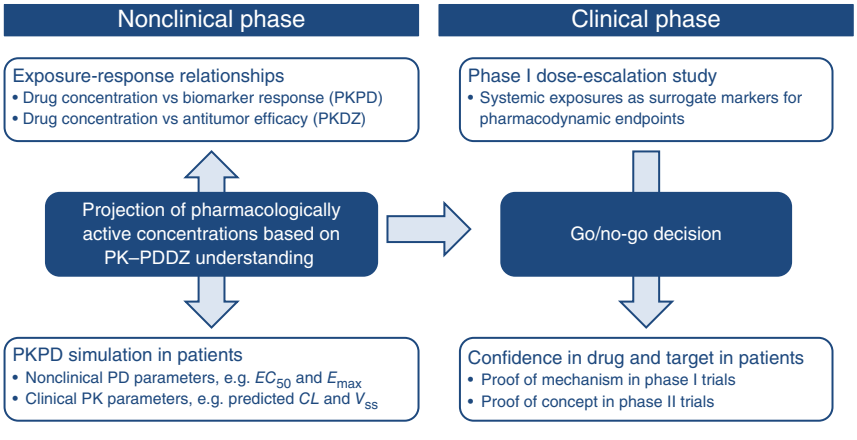

Figure 16.14 Main work stream to make a Go/No-Go decision based upon PK–PDDZ understanding in nonclinical studies to increase confidence in drug and target in the clinic. *Source*: Yamazaki et al. (2016) [38]. Adapted with permission of Taylor & Francis.

promising target modulation in patients, as summarized with the case studies of crizotinib and lorlatinib in this review. In this context, the *PACs* of MTAs could serve as a surrogate marker of target modulation to project their PADs in patients, particularly when no biomarker responses are available in clinical trials. This approach could also possibly contribute to make a Go/No-Go decision based upon whether clinical systemic exposures would or would not be expected to reach the *PACs*. Accordingly, understanding quantitative PK–PDDZ relationships of MTAs in nonclinical studies can ultimately increase confidence levels in both drug and target to achieve POM and POC during clinical development, as summarized in Figure 16.14.

Acknowledgment

The author would like to thank Drs. Bhasker Shetty (Pfizer, San Diego, CA, USA), Bill J. Smith (Gilead, Foster City, California, USA), and Paolo Vicini (MedImmune, Granta Park, Cambridge, UK) for their critical insights and helpful discussions throughout the development of this chapter.

List of Abbreviations

C	drug concentration
C_e	drug concentration in an effect site
C_p	drug concentration in plasma
E_0	biomarker response baseline
EC_{50}	drug concentration at one-half of maximal effect
E_{max}	maximal effect
$g(T)$	the tumor growth function
k_{e0}	first-order rate constant for equilibration between plasma and effect site
KC_{50}	drug concentration at one-half maximal effect
K_{max}	drug-mediated maximal tumor killing rate constant
k_{ng}	first-order net tumor growth rate constant
MTA	molecularly targeted agent
M&S	modeling and simulation
NME	new molecular entities
NSCLC	non-small-cell lung cancer
PD	pharmacodynamics
PK	pharmacokinetics
PKDZ	pharmacokinetic-disease
PKPD	pharmacokinetic–pharmacodynamic
PK–PDDZ	pharmacokinetic–pharmacodynamic-disease
RP2D	recommended Phase II dose
TKI	tyrosine kinase inhibitor
T_{ss}	maximal sustainable tumor volume
γ	Hill coefficient.

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17

Informing Decisions in Discovery and Early Development Research Through Quantitative and Translational Modeling

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17.1 Introduction

Clinical success rates in the pharmaceutical industry have been consistently low (~10%) for close to two decades [1]. Companies have adopted a number of strategies in an effort to reduce the high rate of clinical failure, one of the most significant being the implementation of translational and modeling approaches to quantitatively inform decisions in early clinical development and, more recently, in the discovery phase. Increasing use of these approaches has been driven by a number of factors:

1) Multiple analyses of the root causes of program attrition in Phase II clinical testing revealed that the probability of delivering positive results greatly decreased in the absence of a quantitative understanding of drug exposure (pharmacokinetics, PK), target engagement (TE), and transduction of TE to efficacy and safety (pharmacodynamics) [2–4].
2) Regulatory agencies are increasingly advocating for model-based approaches to inform decision making and optimize trial design, both during early-stage and late-stage development [5–9].
3) Given the high monetary and societal costs of clinical failures, there is a strong impetus to shift the traditional empirical, linear discovery research paradigm toward a more flexible and efficient paradigm in which expectations are prospectively predicted; experimental results are generated to confirm, refute, or refine the biological hypothesis and/or quantitative interpretation, and the prosecution of research plans is adapted accordingly.

The adoption of translational pharmacology and modeling by discovery project teams has enabled the integration and interpretation of all available preclinical information to guide decision making. In addition, translatable assays and study

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

designs have been put in place to close the gap between preclinical and clinical research. This is expected to increase the probability of clinical success by leading to more well-designed and information-rich preclinical and early clinical studies, improved communication between discovery and early development teams on data sets and assumptions, improvements in the quality of compounds and targets entering clinical investigation, and more clearly defined Go/No-Go criteria that quantitatively and objectively guide decisions [10–15]. Translational pharmacology is agnostic of therapeutic area, although it is recognized that certain areas such as diabetes, infectious diseases, and hypercholesterolemia are currently more amenable because of greater insight into the disease-relevant biology and/or availability of meaningful biomarkers. Integral to improving the success of translational pharmacology efforts, however, is the critical need to develop *in vitro* and animal models and biomarkers across all therapeutic areas that are more predictive of therapeutic efficacy in humans and/or that assess pharmacodynamic endpoints that are similar or identical to those that will be measured in human proof-of-concept (POC) trials (see below).

While we appreciate that the problem of declining R&D efficiency is complex and multifactorial [3, 16], there is considerable optimism that emphasizing translational and quantitative approaches from early discovery to clinical development will offer opportunities to disruptively improve clinical success rates. Drug discovery and development is a long process, and companies continue to incrementally move toward increased consistency in the application of translational and quantitative thinking into multidisciplinary drug discovery and development teams. Regardless of the final verdict about its ultimate impact on Phase II success rate, implementation of translational pharmacokinetic/pharmacodynamic (PK/PD) analysis in discovery and early development has already proven its value. This value is evidenced by the case studies presented below and the additional wealth of case studies in the public domain [17]. Due to its integrative nature, i.e. the combination of all relevant information from different sources on absorption, distribution, metabolism, and excretion (ADME) properties and the efficacy and safety properties of the drug, translational PK/PD has the potential to clarify the complexity of the different processes between drug exposure and therapeutic effect. As such, it has been recognized as an analytical method that promotes "truth-seeking" behavior when searching for answers to critical key questions that inform internal decisions [3, 12].

Key application areas of quantitative approaches have been grouped in themes and summarized in Figure 17.1. The utility of translational PK/PD analysis to objectively inform decisions around the selection of the best drug candidate and prediction of a realistic human dose and regimen is a more intuitive and well-established application in drug discovery. Translational PK/PD analysis can also be used to quantitatively integrate clinical information produced with related molecules directed to the same target or pharmacologic pathway with available preclinical knowledge. Clinical data are likely to provide the most accurate PK/PD relationship with fewest assumptions and can be used favorably in discovery to improve the confidence in the projections of human PK, efficacy, safety, and appropriate dose, thereby shortening the development timelines. Furthermore, combining knowledge from clinical competitors or standard of

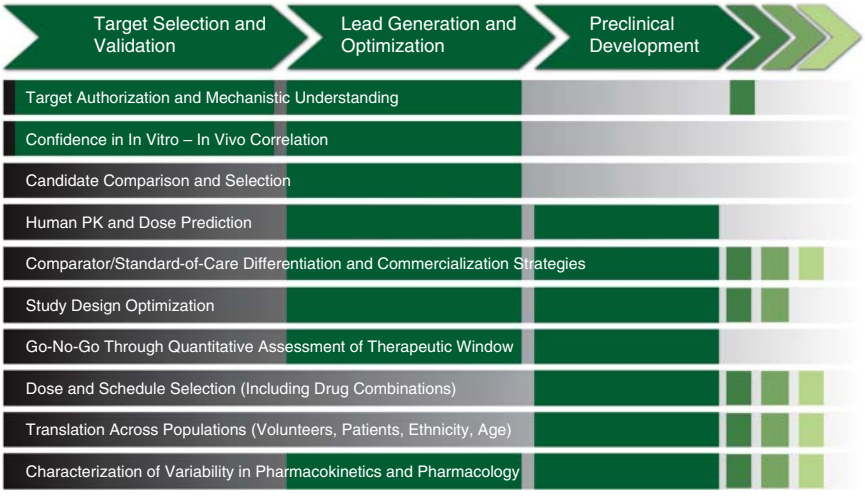

Figure 17.1 Key application areas in which translational PK/PD can be applied in discovery and clinical development. The colored blocks indicate the different phases in which addressing these key questions is most likely to be impactful. *Source*: From Marshall et al. 2016 [17]. Reproduced with permission of John Wiley and Sons.

care drugs with internal molecules, even if they are different modalities, may lead to the identification of critical parameters and realistic targets that a new molecule must meet to provide unambiguous therapeutic benefit for patients. Understanding therapeutic benefit, and as such the differentiation potential, prevents progression of molecules through the later stages of preclinical characterization that never have a realistic chance of making a clinical difference, despite good compound properties. Highlighting other efficiencies that PK/PD can introduce into screening paradigms is also important. For instance, once a quantitative relationship between preclinical *in vitro* and *in vivo* potency is identified, novel compounds can be more selectively triaged for evaluation of *in vivo* potency, which would diminish the overall need for expensive and laborious animal experimentation [13]. Hence, the screening cascade for evaluating newly synthesized molecules can be simplified, making more efficient use of resources during the discovery process without compromising the ability to make informed decisions. Thus, the ultimate goal and benefit of translational PK/PD is not only to develop mathematical models to describe observed data but rather to gain insight into biological mechanisms and the properties of molecules targeting those mechanisms, thereby informing decisions and accelerating drug discovery through more adaptive, efficient, and flexible research operating plans.

Based on the specific key questions to be addressed, different quantitative approaches can be adopted. For comparison of drug candidates on a specific target in a well-described biological pathway, relatively mathematically simple PK/PD models can be very informative even in the early stages of a discovery program. Depending on its intended purpose, mechanistic features can be added to enhance its predictive nature. To help assess the best approach to interfere with a specific pathway of interest (e.g. in target selection), quantitative

systems pharmacology or system biology models can be used. These models are frequently mathematically complex and highly mechanistic in nature in order to adequately represent the biology and enable hypothesis generation. These models may also be required to examine favorable combinations of drugs probing multiple pharmacological targets in the pathway of interest, which could lead to an additive or synergistic response. Finally, to address questions concerning different populations or variability within a population, application of physiologically based pharmacokinetic (PBPK) models may be appropriate. PBPK models could be considered as a subcategory to the previously mentioned systems pharmacology models. By their very nature, these models are built to translate drug pharmacokinetics or pharmacodynamics to a new target population or species provided that relevant physiological characteristics of the target population are available. Developments are occurring at a tremendous pace, and modified hybrid versions of these aforementioned generic model approaches are being published continuously [18–22].

17.1.1 Translational Plan

Biomarkers are taking an essential place in translational PK/PD. At Merck & Co., Inc., Kenilworth, NJ, USA, we have adapted the translational biomarker scheme, originally proposed by Danhof et al. [23] and further developed by Visser et al. [10] (Figure 17.2). The original scheme represents the causal cascade of events for drug effect from dose to clinical outcome. We have adapted this scheme to illustrate the *in vitro* and *in vivo* assays and biomarkers that query

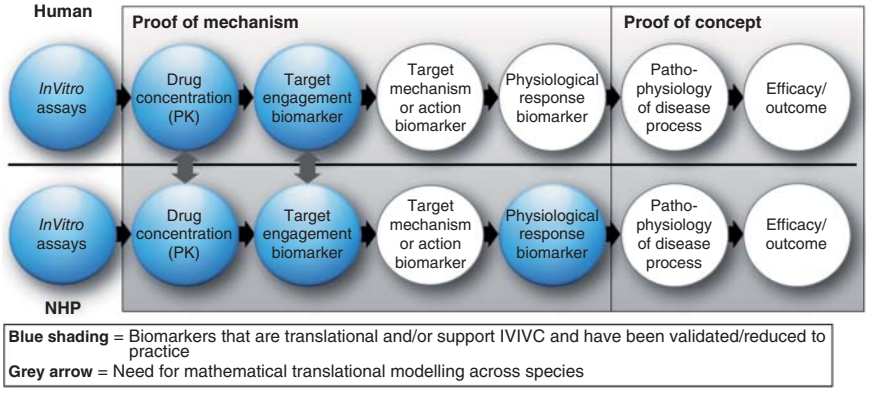

Figure 17.2 The translational biomarker scheme representing an overview and mapping of potential biomarkers and assays that can be used to establish PK/PD relationships of a target of interest within a single species and the opportunities for translation across species. This particular scheme shows the availability of assays and data relevant for translational modeling for MK-1. In the nonhuman primate (NHP), data was generated for MK-1 and standards of care; data in human was collected for standards of care alone. More generally, this translational biomarker scheme facilitates transparent communication of the perceived translational opportunities and knowledge gaps within a discovery program and promotes alignment on objectives when developing translational modeling plans. *Source*: Adapted from Visser et al. 2013 [10] and Danhof et al. 2005 [23].

the pharmacology of a specific target within a specific species and in humans. These assays and biomarkers should ideally have a wide enough dynamic range to allow detection of a graded response, allow repeated measures over time, be reproducible and specific, and be relevant to the clinical or disease endpoints of interest. For each species of interest, a similar schematic summary can be constructed. Assays and biomarkers that have been reduced to practice in discovery teams and for which data are being collected are represented with blue-colored solid circles, whereas assays and biomarkers that need to be developed remain as open circles, thus permitting easy visualization and communication of data gaps. The bidirectional, gray arrows are the nodes over which quantitative translation across species is achieved through the use of mathematical modeling, i.e. to extrapolate the time course of biomarkers and efficacy of a novel molecule from the preclinical arena to the clinical setting or *vice versa*. The translational biomarker scheme is readily adaptable to specific needs: e.g. similar principles can be used to map out and translate assays and biomarkers capturing target-mediated toxicity or to extrapolate data from healthy volunteers to patients. The translational biomarker scheme is a tool to transparently communicate between scientists from different functions as well as to senior management and governance bodies during the planning stages of the translational and quantitative strategies for preclinical and clinical activities. As mentioned above, the use of this tool also exposes knowledge gaps, which may lead to strategic investment in translational and modeling efforts within the program.

The translatable value of an assay or biomarker characterized in a preclinical model is determined by its relevance to the clinical readouts. When the biological target in the disease context is distinct from what can be studied in preclinical animal models or *in vitro* systems, a strategy needs to be developed to acquire and use the most appropriate preclinical information that can best inform the required properties of new molecules. Particularly for the physiological or pathophysiological pharmacodynamic responses (Figure 17.2), experimental paradigms and measurements are emphasized wherever possible that show high similarity between animals and human. With that strategy, we move away from more classical animal behavioral or disease models that have been the subject of much recent debate due to increasing concerns over their inability to predict clinical efficacy [24, 25]. For example, for a particular target in a pain program, imaging techniques that interrogate an aspect of the biology relevant to the target were used for determination of the anticipated clinically relevant concentration rather than a battery of preclinical behavioral pain models (data not shown). Also for *in vitro* assays, much effort is put into studying the biology in relevant native or patient-derived tissues or, at a minimum, in cell systems with relevant expression levels of the target.

In the following, four case studies are presented that illustrate how quantitative modeling strategies are used in drug discovery and development within our company to inform decisions. They cover different therapeutic areas, i.e. neuroscience, diabetes, antibacterial, and inflammation, demonstrating that these translational concepts are indeed broadly applicable. Each example is selected to highlight different key questions (Figure 17.1) that were interrogated with

quantitative translational approaches. The first example shows how modeling was used to predict the human PK, TE, and anticipated therapeutic dose to inform the selection of a clinical candidate and first-in-human (FIH) study design. The second example describes how the experimental pharmacology evolved as clinical information provided additional insights into human biology, how this information allowed predictions of human pharmacodynamic responses, and how these learnings were leveraged preclinically to inform selection of next-generation clinical candidates. The third example shows how *in vitro* and *in vivo* preclinical information were used to inform optimal dose selection in human clinical trials for the increasingly common case of a drug combination. The last case study exemplifies how a quantitative analysis of a competitor candidate molecule led to the decision to terminate a clinical compound after a small but informative Phase Ib study.

17.2 Neuroscience: Prediction of the Clinically Efficacious Exposure and Dose Regimen for a Novel Target

MK-1 is being developed as a novel reversible inhibitor of a target expressed in the central nervous system (CNS) with limited prior information on the relationship between plasma concentration, CNS TE, and desired clinical response. Translational PK/PD modeling and simulation based on relevant *in vitro* and *in vivo* preclinical data describing the ADME properties and pharmacology of MK-1 was applied extensively to guide team decisions from lead optimization to early clinical development, including candidate nomination, dose selection for FIH-enabling preclinical toxicity studies, human dose prediction, and early clinical planning.

Figure 17.2 presents the translational biomarker diagram showing the availability of data and assays relevant for translational modeling of MK-1 in blue. The diagram illustrates that there are gaps in the causal link between target binding, downstream response, and clinical efficacy. In particular, no information was available to study the primary pharmacology directly in animals or humans. In addition, preclinical studies conducted across a number of animal efficacy models, validated with existing standards of care, helped to establish a quantitative TE level that was hypothesized to be required for efficacy in humans (data not shown). However, these animal models have a tenuous relationship to the human disease and therefore have uncertain utility in predicting the clinical efficacy of compounds such as MK-1 that act via a novel, unprecedented mechanism. Not having a prior knowledge on relationship between TE and efficacy is a typical situation for many programs, particularly in neuroscience. Also, having information available for each category of the translational biomarker classification plan is rare and certainly is not a prerequisite for the development of a successful translational model. However, it does highlight a key risk inherent in the translation of preclinical data to clinical efficacy (see below). Using the available information, a translational PK/PD model framework was developed that allowed the prediction of human dose, plasma concentration–time profile, and TE-time profile in

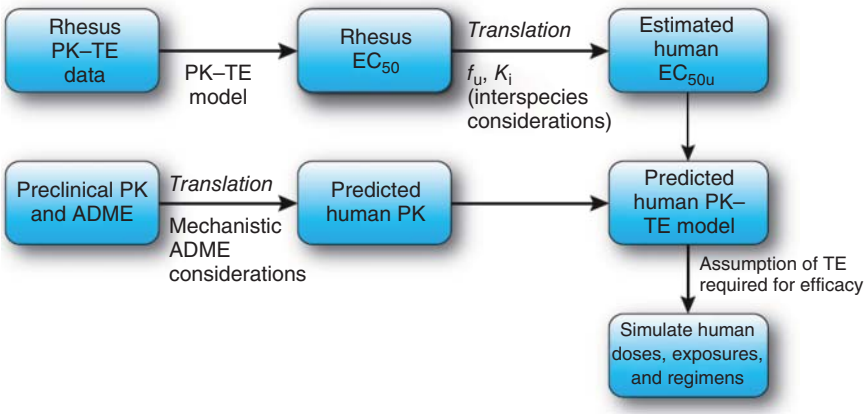

Figure 17.3 Schematic representation of the translational PK/PD framework for MK-1.

humans that would achieve the desired level of target occupancy hypothesized to be efficacious as shown in Figure 17.3.

The pharmacokinetic–target engagement (PK–TE) relationship of MK-1 in the CNS was evaluated *in vivo* in rhesus monkeys using positron emission tomography (PET) following intravenous bolus and infusion administration of MK-1 at different doses. Blood samples were collected at several time points to determine the concentration of MK-1. The TE of MK-1 in rhesus was measured at various time points, allowing evaluation of any potential hysteresis. Graphical analysis of the plasma concentration–TE data collected in rhesus at different time points and doses suggested the data conformed to a direct effect relationship (i.e. no hysteresis observable) and accordingly an E_{max} model (Equation (17.1)) was fit to the data:

$$\text{Target engagement(TE)} = \frac{E_{max} * C_p}{EC_{50} + C_p}, \tag{17.1}$$

where the E_{max} was fixed to 100% because complete occupancy of the target is expected under saturating conditions, EC_{50} is the total plasma concentration of MK-1 that produces half-maximal target occupancy, and C_p is the total plasma concentration of MK-1.

Although preclinical PK–TE data was also available from *ex vivo* experiments in rat, the translational modeling framework used for human predictions was centered on the data collected from rhesus monkeys. Both rat and monkey express the biological target of MK-1 in the relevant brain region, and both *in vitro* and *in vivo* data from these species provided key data in terms of efficacy and potential adverse effects of MK-1. However, the discovery team selected the rhesus monkeys as the most translationally relevant species based on experience across a number of other internal neuroscience programs demonstrating that rhesus monkey PET data was similar to human [26]. Additionally, the *in vivo* PET imaging approach used in rhesus monkey provided direct clinical line of sight for a human PET study. The mathematical translation of the PK–TE relationship from rhesus to humans (Equation (17.2)) focused first on the prediction of the

potency (EC_{50}) in humans where f_u represents the unbound fraction in plasma and K_i the intrinsic binding potency measured in cell culture models:

$$\frac{EC_{50_{human}} {}^{*}f_{u_{human}}}{K_{i_{human}}} = \frac{EC_{50_{rhesus}} {}^{*}f_{u_{rhesus}}}{K_{i_{rhesus}}}. \tag{17.2}$$

Several factors were assumed when performing the model-based potency translation to account for known drug- and species-specific differences between rhesus and humans. It was assumed that unbound MK-1 plasma concentrations were in rapid equilibrium with the unbound, pharmacologically relevant concentrations of drug at the active site in the CNS following oral administration. Importantly, MK-1 was not a substrate of any known uptake or efflux transporters that were evaluated, including P-glycoprotein (P-gp), and had excellent passive permeability in cell culture. *In vitro* pharmacology studies suggested that there was no meaningful difference in the unbound intrinsic potency (K_i) of MK-1 on its biological target between rhesus and humans. Accordingly, Equation (17.2) was simplified to include only a term for the difference in the unbound fraction between rhesus and human. The resulting predicted human PK–TE relationship is shown in Figure 17.4.

To ultimately project the clinical dose and dose regimen of MK-1 that would be anticipated to be efficacious, the predicted human PK–TE relationship needed to be integrated with a translational PK model allowing the prediction of a plasma concentration–time profile of MK-1. Several preclinical ADME studies were used to build the translational PK model for MK-1, including radiolabeled MK-1 studies in bile-duct-cannulated animal models, which, when used along with knowledge gained from other *in vitro* and *in vivo* metabolism studies, enabled hypotheses around the possible mechanisms of clearance of MK-1 in humans. Key human PK parameters such as total plasma clearance, volume of distribution, bioavailability, and absorption rate were all individually predicted according to well-established methods described elsewhere [27] and integrated into a translational PK model.

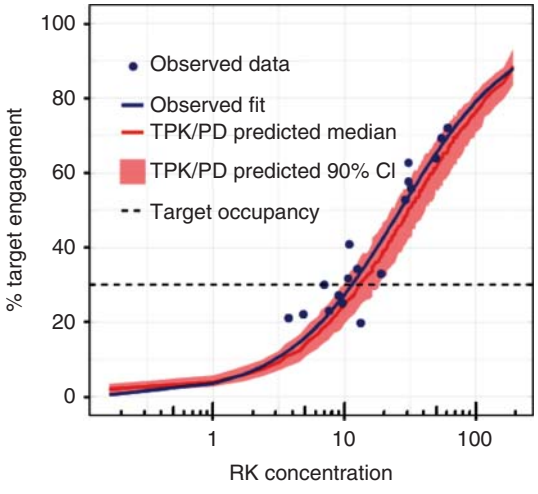

Figure 17.4 Concentration versus receptor occupancy relationship for MK-1. The red solid line represents the predicted PK–TE profile of MK-1 in humans using the developed translational modeling framework. The shaded area indicates the 90% confidence interval of the predicted curve calculated from the experimental uncertainty. The blue circles are the actual observed TE data in human, and the blue solid line is the modeled PK–TE profile using Equation (17.1). The dashed line identifies the target occupancy of MK-1 that is hypothesized to be clinically effective.

Overall, when leveraging translational PK and PK/PD models for predictive simulations, it is important to consider experimental and methodological sources of uncertainty. Standard errors derived from each of the source experimental data or modeled parameter estimates were used to generate uncertainty distributions for the human-predicted PK/PD parameters by Monte Carlo simulations. Recently, our company has implemented a standardized process and tools to incorporate uncertainty in its predictions [28]. Other companies have developed similar approaches [29].

Simulations using the integrated PK/PD model allowed the team to address the key question of "what dose and dose regimen would be needed for efficacy in humans" (Figure 17.5). This simulation informed on the likelihood of MK-1 to achieve the desired TE-time profile at reasonable doses and dose regimens and was a key component of the decision to select MK-1 for clinical development. The modeling framework was continuously updated in a "learn-and-confirm" paradigm from discovery to early clinical development, where clinical information, when available, replaced parameters in the model to continuously reduce translational uncertainty in the predictions. Figure 17.4 shows an overlay of the observed human PK–TE relationship of MK-1 that was conducted in early clinical development, demonstrating the predictive value of the model. Additional uses of the developed translational PK/PD model were the estimation of the plasma exposure in humans following efficacious doses that enabled the rational selection of doses for GLP toxicity testing to maximize safety margins, guiding formulation strategies for early clinical development and providing the clinical development team a framework to propose what doses and dose regimens could be tested in early-stage clinical studies, including the human PET study. A quantitative translational model as described here also supports setting objective criteria for Go/No-Go decisions in early clinical studies and calculations to determine the appropriate group size to be studied in early clinical testing.

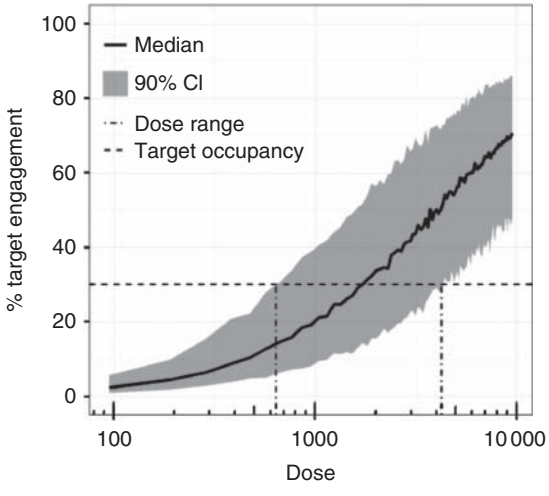

Figure 17.5 Predicted human MK-1 dose versus TE relationship including an explicit expression of modeling uncertainties (shaded area). These simulations graphically show the likelihood of a specific dose of MK-1 to achieve the desired level of target occupancy hypothesized to be clinically effective.

17.3 Diabetes: Leveraging a Platform Approach for Two-way Translation and Integration of Knowledge Between Clinical Lead and Backup Discovery Compounds

The second example encompasses work at our company to develop GPR40 receptor agonists. GPR40 is a family type A G-protein-coupled receptor expressed primarily in the β cells of the pancreas [30]. In 2003, long-chain free fatty acids were shown to bind to GPR40 and mediate glucose-dependent insulin secretion (GDIS) to control circulating glucose postprandially under normal circumstances [31]. Once the connection between GPR40 and GDIS was made, several companies sought to discover GPR40 agonists as a new treatment for type 2 diabetes. An important feature of GDIS is that glucose-lowering mechanisms (such as insulin secretion) are only activated in a hyperglycemic state and will not be activated in a euglycemic or hypoglycemic state, thus reducing the risk of hypoglycemia. Indeed this principle was demonstrated in diabetic/insulin-resistant rodents [32], enabling the progression of GPR40 agonists toward clinical validation in humans.

Takeda Pharmaceuticals was the first company to show clinical validation of the GPR40 target. The GPR40 agonist TAK-875 demonstrated significant and sustained reduction of glucose levels in patients with type 2 diabetes in both fasted and glucose tolerance test (GTT) paradigms, the latter accompanied by a moderate increase in insulin secretion [33, 34]. Given the intense competitive landscape for type 2 diabetes treatments, several approaches to streamline progression of novel GPR40 agonists from discovery to clinical development were undertaken, including targeted investments in building quantitative translational approaches. Implementing a quantitative translational approach led to changes in the *in vivo* models employed, how pharmacological properties were evaluated during lead optimization, and enabled decisions in the discovery and development efforts. One critical question interrogated by quantitative PK/PD modeling included the backward translation of clinical learnings to the GPR40 discovery team in order to select new clinical candidates with a high confidence in achieving a clinical dose less than 50 mg. A low clinical dose was considered critical to reduce overall bioburden and thus mitigate potential risk for liver toxicity [35], which was observed with the clinical compound TAK-875 [36]. This case study describes the evolution of the GPR40 agonist discovery program and the application of learn-and-confirm cycles in the preclinical and clinical arenas.

17.3.1 Evolution of Pharmacology Experiments

Much of the early discovery work on GPR40 agonists was focused on identification of compounds that demonstrated robust acute glucose lowering during a GTT in healthy mice. This pharmacodynamic assay provided a rapid screen for evaluating *in vivo* performance based on lowering of the area under the glucose concentration versus time curve after a glucose challenge is given to fasted animals. The glucose response was correlated to the average plasma concentration

through a single representative plasma sample taken from a subset of animals at 3.5 h after administration, a time by which the glucose excursion had already returned to baseline. Although the GTT in healthy mice was a convenient screening model at the time, the value of this animal model as a translational pharmacological tool needed additional consideration. As mentioned earlier, there is a significant emphasis at our company to develop pharmacology assays that are translationally relevant. Ideally, such translationally relevant preclinical models should reproduce the human disease symptoms as closely as possible (face validity) through the relevant biological mechanism of action (construct validity) while also allowing the assessment of endpoints similar to those used in human clinical trials (predictive validity). Clinical results with TAK-875 demonstrated that glucose lowering in a GTT was not apparent in healthy human subjects [37]. Furthermore, a maximal reduction in fasted glucose (and a modest reduction in glucose during a GTT at higher doses) in type 2 diabetes patients required 2 weeks of chronic dosing [29]. Thus, this new clinical insight revealed several shortcomings of the acute GTT model in the healthy mouse as a translationally relevant pharmacology assay. To more exactly mimic the patient population in which efficacy was observed with TAK-875, the GTT assay was migrated from healthy mice to the Goto-Kakizaki (GK) rat, which is considered to be a model of type 2 diabetes displaying robust glucose intolerance in a GTT and mild fasting hyperglycemia. The GK model provided a platform that allowed rapid screening of newly synthesized GPR40 agonists in an acute GTT paradigm as well as assessment of the most interesting leads in a chronic 2-week dosing paradigm in which compounds were administered in feed and fasted blood glucose was used as a clinically relevant readout similar to what was described by Araki et al. [34] The GK rat therefore more closely resembled clinical paradigms in terms of pharmacodynamic readouts, i.e. change in fasted glucose levels, while enabling both a rapid screening paradigm (the GTT) and a more clinically relevant chronic dosing paradigm for selected lead compounds.

Given the importance of identifying a compound with a low human dose, human dose predictions were conducted on an ongoing basis as compounds progressed through *in vitro* and *in vivo* evaluation. The targeted human trough plasma concentration for maximal fasted blood glucose lowering used for the human dose prediction of lead compounds was tentatively identified in the acute GK rat GTT paradigm by measuring the drug concentration in plasma samples taken immediately post GTT in animals administered the maximal efficacious dose. This trough PK target was then further solidified in a chronic 2-week GK rat study for the most promising compounds where changes in fasted blood glucose were the key assay endpoint. The trough PK target for potential clinical candidates was translated to human by adjusting for differences in plasma protein binding. To arrive at a predicted human dose, the anticipated human PK target was then combined with a translational PK model for each new GPR40 agonists based on preclinical ADME studies as described in the previous case study. This approach assumes a rapid, direct drug response and assumes no difference in affinity of compounds for rat and human GPR40. Initial compounds progressing into the clinic using this discovery paradigm demonstrated submaximal or no

efficacy due to underprediction of the human dose. The inaccuracy in predicting an efficacious human dose stemmed from multiple sources. The assumption that ambient drug concentrations directly elicited the observed response at that time point (direct response) was false. Moreover, the *in vitro* assays did not appear to correlate well to *in vivo* preclinical or human response across compounds, thereby masking potential species differences that should have been accounted for. Several *in vitro* assays had been utilized to select compounds for further evaluation in animal models. These assays included an inositol phosphatase 1 (IP1) accumulation assay, a fluorescence imaging plate reader (FLIPR) assay, and a serum shift assessment in the FLIPR assay to experimentally evaluate the impact of protein binding on the potency determination. However, none of the *in vitro* assays had demonstrated an obvious correlation to *in vivo* preclinical or clinical response in the earlier data sets. The underlying reason for this is that the *in vivo* potency and efficacy are influenced by time-dependent phenomena and by baseline glucose levels, neither of which are accounted for in simplistic correlations with the *in vitro* assays used at that time.

To improve the accuracy of the human dose prediction for reference compounds such as TAK-875 and internal early clinical candidates and to understand the optimal PK/PD profile needed to maximize efficacy in the clinic, the design of the GK rat assay was further evolved to better characterize the PK/PD relationship of GPR40 agonists. As a result a single-dose study in fasted GK rats was designed, in which a full dose range could be efficiently studied. In this design, serial samples of the plasma concentration and concomitant changes in fasted blood glucose and plasma insulin were collected up to 48 h post-dose, which is needed to characterize the hysteresis that was not studied previously. A specific feature of this study design is its focus on collecting as much information as possible describing the concentration–response relationship of the test compound rather than trying to demonstrate statistically significant differences between treatment groups. Thus, the group size of each treatment arm was large enough to adequately capture the PK/PD relationship, but not necessarily sufficient to perform robust pairwise comparisons. This philosophy was especially important when measuring changes in plasma insulin, which proved invaluable information when building the translational PK/PD model described below even though treatment effects were never statistically significant between groups. This also allowed us to reduce the group sizes from 8 to 10 animals to approximately 5 animals, a change in experimental design that provided not only scientific advantages but ethical and cost benefits as well. Lastly, the PK and pharmacodynamics were measured in each animal for every time point rather than in a representative subset at the end of the assay. The individual animal data more accurately captured the PK/PD relationship, as some of the variability in the pharmacodynamics could be explained by individual differences in PK. Moreover, the random variability could be characterized and used in prospective simulations as a measure of uncertainty. A number of GPR40 agonists were investigated in this modified model, including those that had been evaluated in humans with fasting glucose levels as the readout. This proved an important preclinical data set that was used to develop and calibrate a mathematical translational PK/PD model, which is described in the next section.

17.3.2 Development of a Translational PK/PD Model

In order to integrate the *in vitro*, preclinical, and clinical data into a modeling framework that could be predictive, several assumptions are required, many of which are made by project teams regardless of whether a quantitative model is employed or not. We have named these "program assumptions." These program assumptions include that all GPR40 agonists bind to and act similarly on the receptor (supported by *in vitro* data), that these compounds have no other mechanisms of action or unknown off-targets, and that the GK rat model is a translationally relevant model for the target patient population. With these assumptions as stated, a translational, semi-mechanistic PK/PD model was developed from a human integrated glucose–insulin (IGI) model [38] by incorporating GPR40 pharmacology and extended to allow back and forward translation across multiple species using the same model construct (Figure 17.6).

The model includes the following sub-elements:

- A population PK model relating dose to plasma concentration.
- A link model connecting the drug PK to stimulation of insulin secretion through GPR40 receptor pharmacology.
- A biological system model relating glucose and insulin homeostasis, which has been extensively studied in human.

The majority of the mathematical parameters describing the dynamics between insulin and glucose in the model were obtained from the literature for human

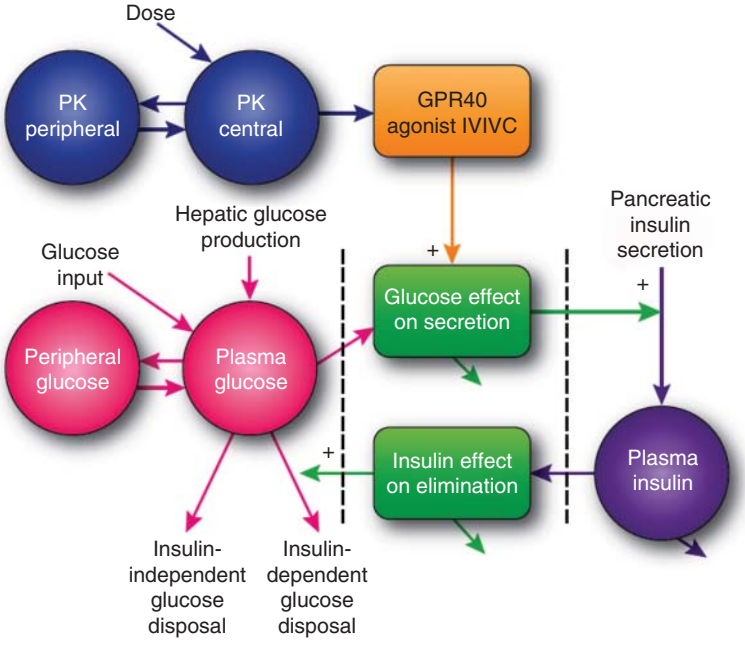

Figure 17.6 Schematic representation of the translational integrated glucose–insulin model for GPR40 agonists.

and were allometrically scaled to the rat. This model was used to characterize the PK/PD relationship of GPR40 agonists in the GK rat, to establish an IVIVC, and to predict glucose-lowering effects in human. The IGI model describes the dynamic response of glucose and insulin levels following meals, glucose administration, and drug administration. It also adequately captured data from the GK rat and relevant clinical data of TAK-875 under multiple experimental conditions, including the fasted state, after a meal, and after an oral GTT (Figure 17.7).

17.3.3 Application of the GPR40 Agonist Translational PK/PD Model to Establish IVIVC

The most translationally relevant *in vitro* assay to predict the *in vivo* potency was examined by exploring the IVIVC relationships between *in vitro* EC_{50} values determined with the different assay formats described earlier and the estimated *in vivo* EC_{50} value for drug action in the single-dose fasted GK rat PK/PD model, described above. The *in vivo* EC_{50} value is defined as the drug concentration at which 50% of the additional maximal insulin secretion is attained; the actual magnitude of insulin secretion is dependent on both drug and glucose concentration (Figure 17.7). The goal was to identify a quantitative relationship, such as a linear or exponential equation, that would allow prediction of the *in vivo* potency on the basis of *in vitro* data. The data set used to drive this decision was a combination of human and rodent *in vitro* and *in vivo* data in order to assure that the relationship was built for translation across species. Where simple correlations failed to identify a convincing IVIVC, the use of a more mechanistic modeling approach established a correlation between the potencies measured in the IP1 assay, with subsequent adjustment for plasma protein binding and estimated *in vivo* potency values (Figure 17.7). The establishment of this relationship was an important accomplishment. It enabled simplification and cycle time reductions in the *in vitro* pharmacology screening by focusing efforts on a single, translationally relevant IP1 assay, and it improved the identification and selection of compounds with high potential for more detailed downstream pharmacological characterization.

17.3.4 Application of the GPR40 Agonist Translational PK/PD Model to Predict Clinical Outcomes

The translational PK/PD model described above was employed to prospectively predict the expected reduction of fasted plasma glucose during a POC Phase Ib study in type 2 diabetic subjects following the administration of different doses of the GPR40 agonist MK-8666 for 2 weeks. The comparison of the prediction with the actual study results is shown in Figure 17.8 and illustrates that the translational platform accurately predicted the level of efficacy achieved with MK-8666. Given this successful prospective evaluation, the platform was then further used to give guidance to dose selection during the planning of later-stage clinical studies with MK-8666.

The PK/PD model was subsequently utilized to guide the selection of the next-generation GPR40 agonist clinical candidate. Given the concerns around

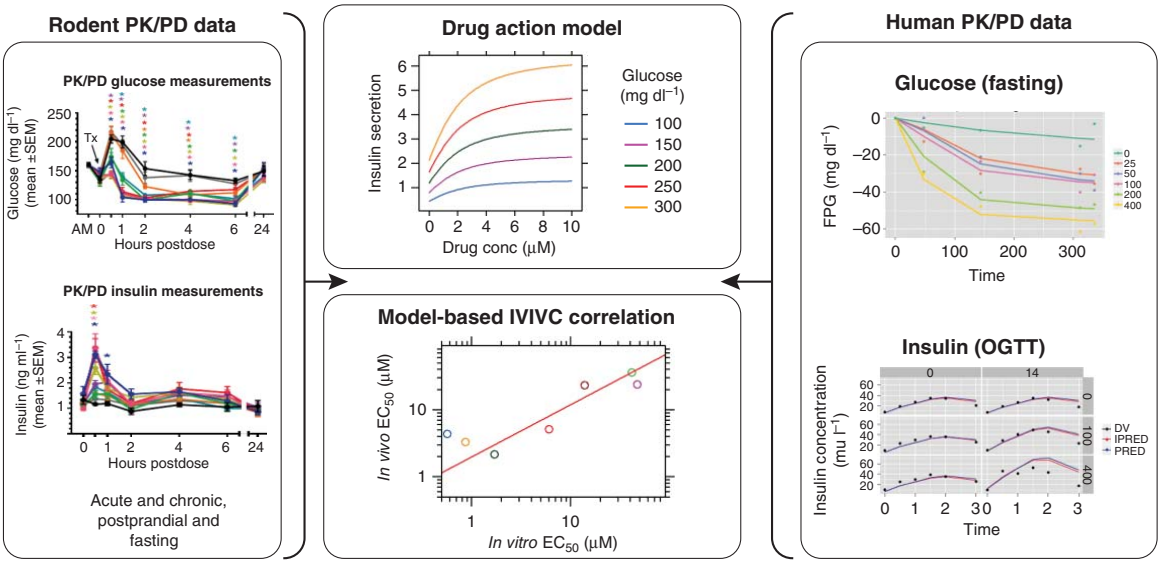

Figure 17.7 Illustration of data incorporated in the translational analysis, including PK and temporal GK rat glucose and insulin data from a typical PK/PD study (a), human glucose and insulin data for TAK-875 (b) [34], and the IVIVC developed to predict *in vivo* EC_{50} driving insulin secretion from the *in vitro* IP1 assay EC_{50} values across different compounds (middle bottom). The panel at middle top illustrates graphically how the IVIVC is used in the model to relate how increasing plasma drug concentration further enhances glucose-dependent insulin secretion.

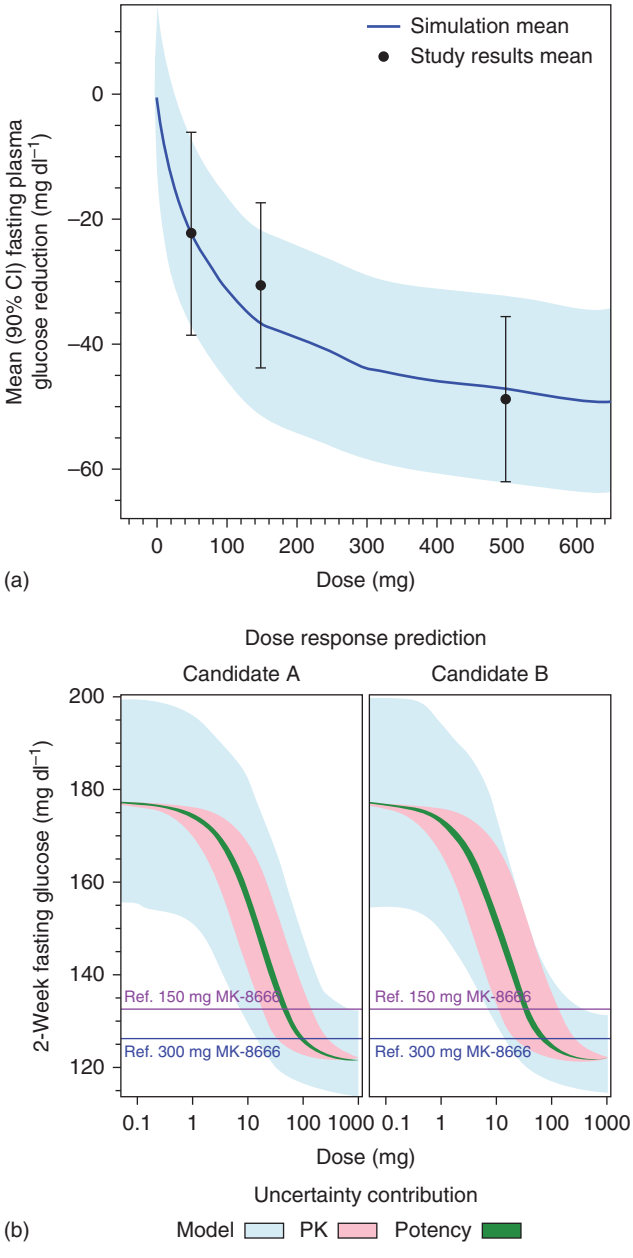

Figure 17.8 (a) Simulation by the modified IGI model of the relationship between the dose of GPR40 agonist MK-8666 and the reduction in fasting glucose after two weeks of dosing in diabetes type 2 patients. The solid line represents the mean predicted response and the shaded area the 90% confidence intervals. The black circles show the observed clinical results. (b) Simulations of potential candidates in a similar two-week proof-of-concept study, with reference simulated glucose levels for 150 and 300 mg MK-8666 shown in light purple and dark blue, respectively, to capture the corresponding projected clinical dose range for the potential candidate molecules.

hepatotoxicity observed with TAK-875 and the relatively high dose of MK-8666 required to achieve maximal reductions of fasting plasma glucose (Figure 17.8), it was important that the next clinical candidates have a high probability of reducing fasted plasma glucose levels to similar levels as 150–300 mg MK-8666 but at a projected clinical dose below 50 mg. Clinical trial simulations of treatment for 2 weeks in patients with type 2 diabetes were conducted using the translational PK/PD model to explore the expected dose range of novel preclinical candidates. In these simulations, uncertainty was initially incorporated on just the potency parameter, then on potency and the predicted PK profile, and finally also including the random model uncertainty. Fasting plasma glucose results from simulations of two preclinical drug candidates are shown in Figure 17.8b. This allowed for objective comparisons of new molecules in an integrated manner with MK-8666. By evaluating the simulations with different uncertainty on explicit parameters, it can be seen that the relative uncertainty in PK translation was much greater than that for the potency parameter (Figure 17.8b). This allowed for decisions on which type of data to collect in additional experiments that would have the greatest impact on improving the accuracy of the prediction. For instance, additional experimentation on *in vitro* potency would be futile as its contribution to the overall spread of predicted doses was limited. On the other hand, the decision on the selection between the two final candidate molecules could be further influenced by attempting to improve human PK prediction through further preclinical experimentation or by taking both candidates into the clinic for a limited single-dose evaluation. While model uncertainty also arises from the system parameters, i.e. related to glucose and insulin homeostasis, these are shared parameters across all compounds.

In summary, this example showcased the use of integrative and translational pharmacology in the discovery and early development of novel GPR40 agonists. It highlights the impact that this scientific approach can have in guiding the design of preclinical and clinical studies. With proper planning and analysis, complex data sets from multiple experiments were integrated into a single picture, which allowed for simplification of the screening strategy, extrapolation into clinical dose selection, and objective determination of whether next-generation molecules really provided a reduction in expected therapeutic dose. The forward and back translation shown here ensured that decisions were made with the most up-to-date knowledge.

17.4 Antibacterials: Semi-mechanistic Translational PK/PD Approach to Inform Optimal Dose Selection in Human Clinical Trials for Drug Combinations

The PK/PD relationship for β-lactam-containing (BL) antibacterials such as carbapenems and cephalosporins against Gram-negative bacteria is driven by the percentage of time during the dosing interval that the antibacterial concentration is above the minimal inhibitory concentration (MIC; %T > MIC) [39, 40]. The steady rise of drug-resistant bacterial strains has renewed the

efforts to discover new antibiotic drugs or develop new combinations that offer effective treatment against resistant strains. Combination therapy is typically viewed through the lens of two (or more) agents used together such that each drug has activity against a specific and distinct target protein that is relevant for the disease of interest. The pharmacology of such combinations is then further characterized by terms such as whether their combined activity is additive or synergistic. However, for antibacterials, particularly those used against drug-resistant strains, the strategy of combination therapy can be employed in a different way. β-Lactamase inhibitors (BLIs) are compounds that themselves do not possess any inherent bactericidal or bacteriostatic properties. Instead, BLIs are utilized to inhibit the activity of the β-lactamase enzyme, which is produced by drug-resistant bacteria to hydrolyze the BL antibiotic, thus restoring the activity of the underlying antibiotic. In this paradigm, the interdependence between the exposure–response relationship of both the BL antibiotic and the BLI makes it truly challenging to characterize the exposure–response relationship for BL/BLI combinations in Phase II/III clinical studies given the rare occurrence of resistant organisms and the many confounding factors that are present in the clinic (including surgical intervention, use of non-BL standard of care antibiotics, and many others). Thus, there is a need to rely on data generated in *in vitro* and animal models to understand and project the clinical efficacy of BL/BLI combinations. The most appropriate approach to integrate and summarize the various preclinical and clinical data is through a translational semi-mechanistic framework. In this case example, it will be illustrated how such a semi-mechanistic model using preclinical and clinical information was leveraged to inform important Go/No-Go drug development decisions and to select the optimal dose for the combination of MK-7655 (BLI) and imipenem/cilastatin (IPM/CIL). Imipenem (IPM), a carbapenem antibiotic, is administered with the dehydropeptidase inhibitor cilastatin to prevent its metabolism and ensure adequate levels of drug are achieved in the urine for the treatment of urinary tract infections.

17.4.1 Development of the Semi-mechanistic Translational PK/PD Model

MK-7655 (relebactam) is a novel BLI currently under late clinical development in combination with IPM/CIL, a previously approved BL antibiotic, for the treatment of drug-resistant Gram-negative bacteria. To fully synthesize the wealth of PK and PK/PD information gathered via susceptibility testing, *in vivo* infection models in rodent, *in vitro* hollow fiber PK/PD studies, and Phase I human PK data, a semi-mechanistic PK/PD model has been developed (Figure 17.9) [41, 42]. A summary of the data used in the semi-mechanistic model is detailed in Table 17.1.

Similar to the previously explained IGI model structure, this mechanistic model consists of several modules. The first module is a PK sub-model, describing the PK of MK-7655 and IPM. Furthermore, the second module is a mathematical model describing the growth of *Pseudomonas aeruginosa* in a rodent or human, as *P. aeruginosa* is a bacterial strain that is a key contributor

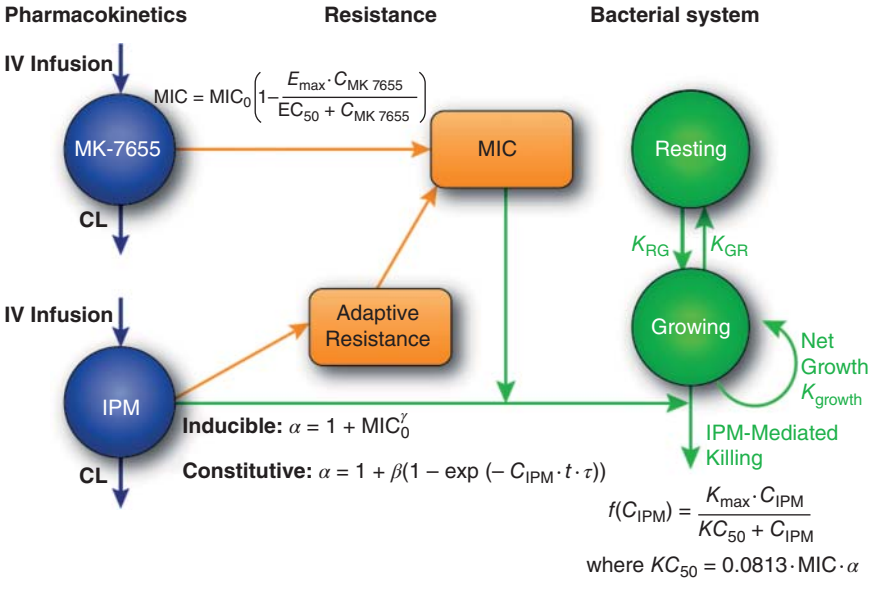

Figure 17.9 Schematic representation of the semi-mechanistic PK/PD model structure describing antibacterial effects of the MK-7655 and imipenem (IPM) combination. *Source*: Adapted from Rizk et al. 2012 [41] and Ahmed et al. 2012 [42].

Table 17.1 Summary of *in vitro* and *in vivo* data utilized in the semi-mechanistic translational PK/PD model of MK-7655.

Data source	Description	Utility
In vitro static exposure (checkerboard) studies	IPM MIC versus MK-7655 concentration profiles (~500 data points) from 93 strains of *Pseudomonas aeruginosa*	Characterize relationship between MK-7655 concentration and IPM MIC
In vitro hollow fiber time–kill studies	Time-varying PK and bacterial CFU values when treated with IPM alone or IPM/MK-7655	Link PK data to bacterial killing and adaptive resistance to fit model parameters
In vivo rodent infection model	Time-varying PK, with bacterial CFU values at study endpoints	Simulated to demonstrate that model translates from *in vitro* to *in vivo*
In vivo human pharmacokinetic data	Single- and multiple-dose MK-7655 and IPM PK data from Phase I to II studies	Project efficacy of IPM/MK-7655 based on PK of Phase II doses

to drug-resistant Gram-negative bacterial infections. Such a module could be readily extended to other bacterial strains following the generation of appropriate *in vitro* data including bacterial growth curves and *in vitro* MIC data. This mechanistic model is based on a published PK/PD model that was originally developed for *in vitro* time–kill data and then adapted for murine infections treated with carbapenems, including IPM [43]. The bacterial system parameters

describing the bacterial proliferation, i.e. rate constants K_{growth}, K_{RG}, and K_{GR}, were all fixed to literature values, KC_{50} expression used MK-7655-dependent MIC values, and K_{max} was fit using hollow fiber data (see below). Finally, it contains a "resistance" module. This module describes mathematically the behavior of resistant pathogens, which will decrease the effectiveness of IPM (via increase of the MIC), and the impact of MK-7655, which will increase the effectiveness of IPM against these resistant organisms (via decrease of the MIC). Resistant pathogens may be either constitutive expressers of β-lactamase, synthesizing high levels of the β-lactamase enzyme in the presence or absence of IPM, or inducible expressers, whose β-lactamase expression is directly related to the presence or absence of IPM. This property of resistant pathogens can be measured *in vitro*. The quantitative relationship between MK-7655 concentration and MIC was studied in *in vitro* static exposure (checkerboard) experiments using 93 strains of *P. aeruginosa*. Checkerboard experiments are static *in vitro* experiments where fixed dilutions of antibiotic concentrations are added to bacterial cultures (often in a high-throughput manner such as in 96-well plates) to generate MIC data in a large number of strains. When considering multidrug combinations such as MK-7655/IPM, a 2-D checkerboard approach can be taken where concentrations of both drugs are varied to understand how the MIC of each strain varies as a function of MK-7655 concentration. A population approach was used to estimate the different parameters characterizing the interdependency between MK-7655 and IPM. This also generated a random effect error capturing the interstrain susceptibility for MK-7655, which can be used prospectively in simulations. Experiments were conducted in a dynamic *in vitro* hollow fiber system evaluating different combinations of MK-7655 and IPM. In this system pathogens grow in fibers, and variable drug concentrations, which mimic the clinical PK profile, can be led through these fibers to investigate the drug's antibacterial effect. The *in vitro* hollow fiber time–kill data was used to fit the remaining parameters, including adaptive resistance. As can be seen in Figure 17.10, the model describes the hollow fiber data well, with 94% of data points falling within the 95% prediction interval.

17.4.2 Application of the Translational PK/PD Model to Predict Preclinical Efficacy

The translational PK/PD model was used to predict the efficacy of the MK-7655/IPM combination in a mouse model of lung infection to demonstrate that the model, predominantly developed and calibrated on *in vitro* experiments, translated to *in vivo* experiments. The PK of MK-7655 and IPM were determined in mouse, and population PK models were developed, which were subsequently used to simulate the expected outcome of the lung infection study. The only parameter changed to simulate *in vivo* efficacy was the maximum bacterial growth, which was set to the observed value in the no treatment control arm of the *in vivo* experiment in mice. As can be seen in Figure 17.11, the model showed good performance in mice, indicating that it successfully translated the information obtained in *in vitro* models to an intact animal model and thus supported further simulation in humans. Although not explicitly shown in this

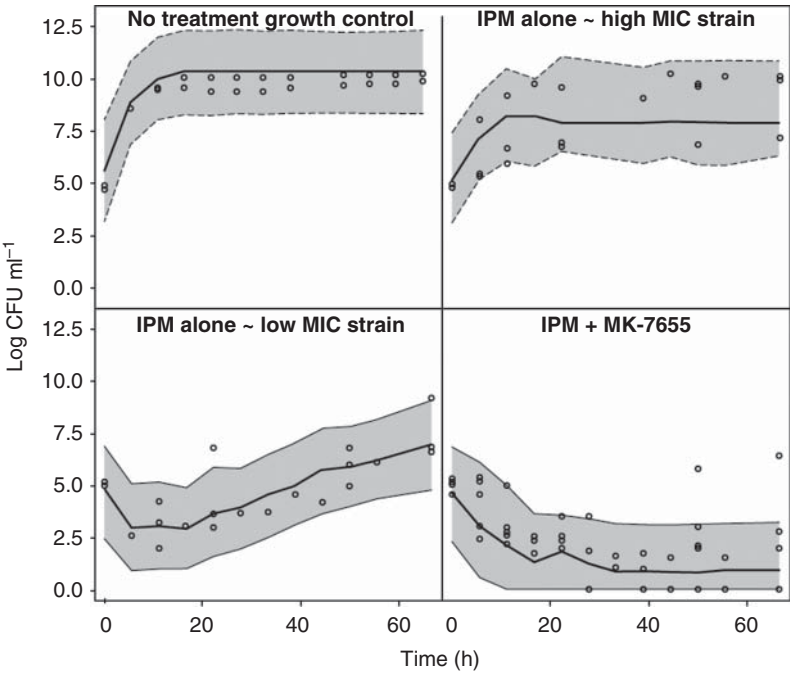

Figure 17.10 Visual predictive check upon fitting the translational model to *in vitro* hollow fiber time–kill data of the MK-7655 and imipenem (IPM) combination. The open circles are the observed data, the solid line represents the median predicted response, and the shaded area represents the 95% prediction intervals. CFU = colony forming unit.

example, this model could now be employed to predict *in vivo* efficacy of other BLIs and use such predictions to rationally select and prioritize new compounds as soon as the BLI–MIC relationship is characterized in checkerboard studies and some PK information is collected or predicted.

17.4.3 Application of the Translational PK/PD Model to Predict Clinical Efficacy

The model was subsequently used to simulate efficacy in humans (Figure 17.12). Clinical population PK models were developed for both IPM and MK-7655 using Phase I and Phase II PK data, and all other parameters remained fixed to the values used in mouse simulations. The simulations presented were based on the approved human dose of 500 mg IPM/CIL in combination with varying doses of MK-7655. A variety of *P. aeruginosa* strains were simulated including constitutive strains with MICs of 1–64 mg l^{-1} and inducible strains with MICs of 1–64 mg l^{-1}. From the dose–response relationship of MK-7655, a rational decision about the clinical dose can be made based on the desired efficacy relative to the expected MIC values of the pathogens.

In summary, a semi-mechanistic PK/PD model that describes the combined PK profile of IPM + MK-7655 as well as PD profiles of resistant bacteria in the *in vitro*

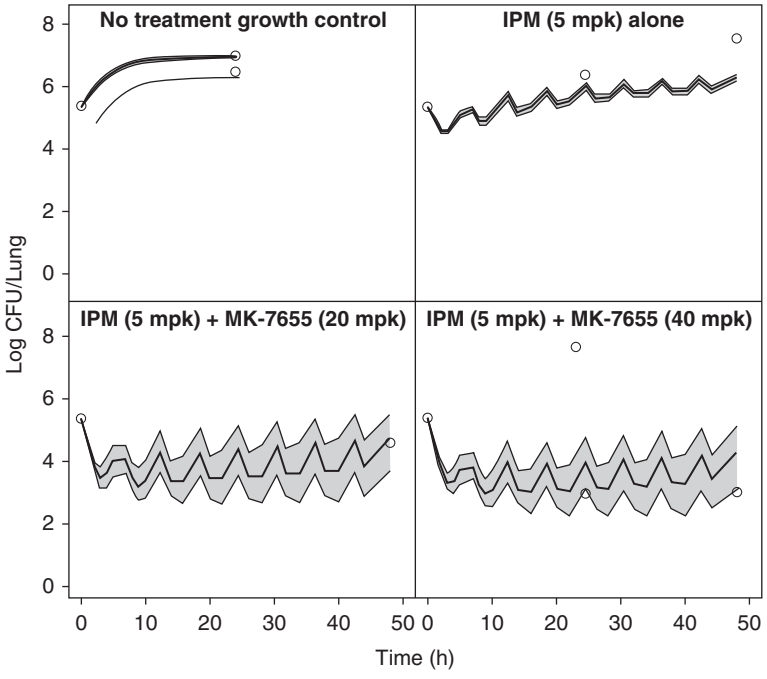

Figure 17.11 *In vivo* model-based simulations of the bacterial growth–kill response by the MK-7655 and IPM combination in mouse. The open circles are the observed data, the solid line represents the median predicted response, and the shaded area represents the 95% prediction intervals.

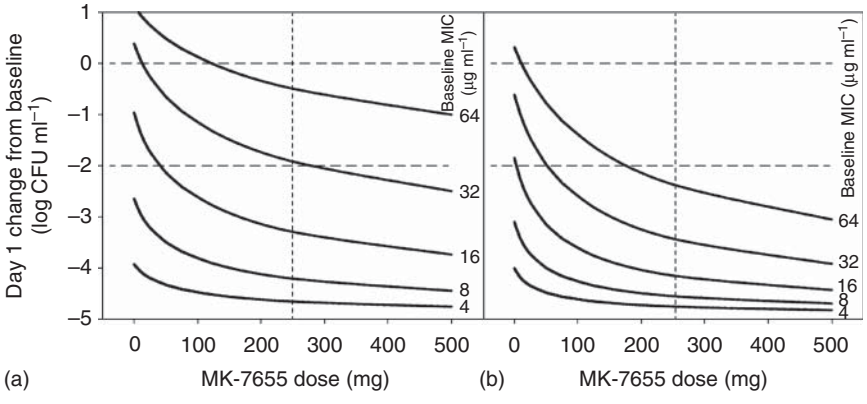

Figure 17.12 Clinical model-based simulations of the dose–response relationship of MK-7655 24 h after administration. The solid reference line indicates static growth, and the dashed reference line indicates a one hundred-fold reduction in CFU. A variety of *P. aeruginosa* strains were simulated including constitutive strains with MICs of 4–64 μg ml^{-1} (a) and inducible strains with MICs of 4–64 μg ml^{-1} (b).

system was developed and validated in mice, accounting for the interdependencies in the pharmacology of the BL antibiotic and the BLI. Following successful *in vitro* to *in vivo* translation utilizing mouse data, this model enabled transition from discovery to clinical development and design of FIH trials. Subsequent clinical translation using human data and simulations in humans projected that the MK-7655 (250 mg) + IPM (500 mg) combination would be an effective regimen for the treatment of IPM-resistant *P. aeruginosa* infections by strains with MIC up to 16–32 mg l^{-1}, which comprise ~95% of the *P. aeruginosa* isolates from the SMART 2009 surveillance study [44]. The translational PK/PD model was further applied to aid transition from early to late clinical development and support dosing regimen selection, including dose adjustments where clinically warranted. Further simulations in humans revealed an essentially flat dose–response relationship above the Phase II dose of 250 mg MK-7655 in combination with 500 mg IPM given every 6 h, indicating that a plateau in the dose–response is achieved at this dose. Hence, this was projected to be an effective dosing regimen for use in pivotal Phase III trials for the treatment of IPM-resistant *P. aeruginosa* infections.

Integrated mechanistic or semi-mechanistic models thus provide a powerful tool to synthesize information from various data sources (in this case *in vitro* static and dynamic experiments combined with preclinical *in vivo* pharmacology studies and human PK data) to project the efficacy of drug combinations that may act through unique, interdependent mechanisms.

17.5 Anti-inflammation: Early Go/No-Go Based on Differentiation Potential Compared with Competitors

The lack of differentiation is a primary cause of commercial failure once a drug has been approved. For the pharmaceutical industry, Phase III success and regulatory approval are obviously important, but differentiation from existing treatments is equally important to gain market access, especially as payors cast an increasingly critical eye on the added value of new medicines. The case study here shows how a modeling approach can be used to focus on "differentiation value" early in the development process through early data integration, thereby informing decisions that take economic value into account.

For a particular inflammatory disease, multiple biopharmaceutical companies were developing novel approaches for a range of targets involved in the underlying inflammatory response. Our company developed compound MK-2 with the intention to become first in class to treat this inflammatory disease. Given the heavy competition in this area, an accelerated clinical development program was proposed that involved a single ascending dose study in healthy subjects, followed by a combined Phase Ib multiple ascending dose (MAD) and POC study in patients, which would allow MK-2 to directly move into a Phase IIb dose-finding study.

Conducting the Phase Ib study in the patient population of interest had several benefits, the most important being the establishment of safety and tolerability in the target population and getting an early readout of clinical PK/PD and efficacy

before enrolling a large number of subjects in late-stage clinical trials. In this particular case, the pharmacodynamic endpoint of the Phase Ib MAD/POC study was similar to the Phase IIb endpoint and was judged to be a reasonable predictor of Phase III success. The first part of the Phase Ib study was a randomized, double-blind, placebo-controlled MAD study designed to evaluate safety/tolerability and preliminary PK/PD relationship of 4 dose levels of MK-2 in the target population. Upon successful completion of the MAD study, a second part was planned to expand the data set on the pharmacodynamic endpoint already investigated in the MAD study by evaluating a definite POC under robust statistical assumptions for the maximally feasible dose.

Around the time of completion of the MAD part of the Phase Ib study in which eight subjects had been studied per dose level, a competitor company published Phase II data on a compound with a related mechanism of action and similar indication. These Phase II data comprised efficacy results in approximately 100 patients. When comparing the emerging results with MK-2 with published data from the competitor, two issues were identified: (i) the onset of action of MK-2 was slower and (ii) the effect size of MK-2 on the pharmacodynamic endpoint was somewhat smaller for the clinically viable dose (i.e. the maximal dose that could be formulated) compared with the competitor compound. The key question for the team and governance bodies was whether it was appropriate to continue with the second part of the Phase Ib study, i.e. enrolling more subjects at the dose level of interest to demonstrate a definite POC.

A model-based analysis of the multiple rising dose data was conducted to address this key question. This analysis indicated that MK-2 was effective and that adding more subjects would indeed increase the confidence around definitive estimates of the effect size for the clinically viable dose. However, the analysis also showed that the onset of action and the effect size of this dose of MK-2 were likely inferior to the competitor compound despite the fact that it would offer improvement over current standard of care (Figure 17.13).

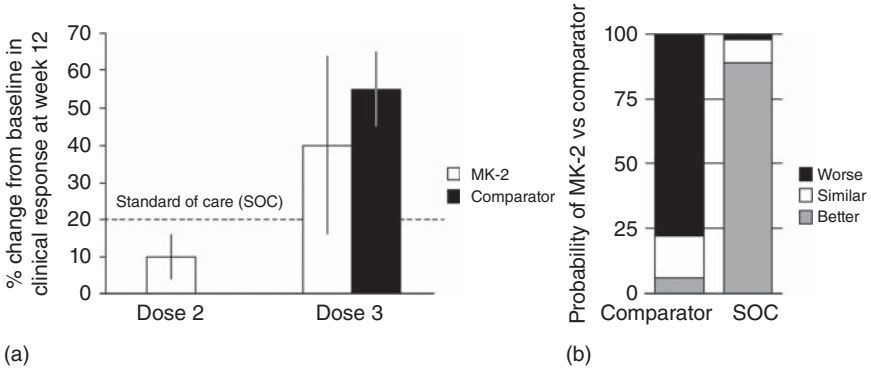

Figure 17.13 (a) Effect sizes for doses 2 and 3 of MK-2 from the MAD study, compared with the competitor Phase II data and the current SOC (dotted line). (b) The calculated likelihood that MK-2 performs worse, similar, or better than either the comparator drug (left) or the SOC (right).

Initially, the team was hesitant to make a decision based on data from 8 patients and wanted to initiate the second part of the study by enrolling additional 20 patients. However, simulations demonstrated that the probability of MK-2 being comparable with that of the competitor was low without changes in clinical strategy, such as finding ways to increase the maximal viable dose. Given the limited options that were available to revise the clinical strategy and the competitor being substantially ahead in the development process, the decision was made to stop the program.

Despite the fact that this small number of subjects studied implied high uncertainty around the exact predictions of whether MK-2 would differentiate from the competition, the team felt confident that the simulations provided sufficient rationale that the probability of outperforming the competitor molecule would be very small (Figure 17.13) and hence enabled the early No-Go decision.

17.6 Summary

Quantitative and translational pharmacology comes in different forms as these different case studies illustrate. The case studies were deliberately drawn from different therapeutic areas not only to show that translational PK/PD concepts are generically applicable but also to exemplify that each disease presents different opportunities and limitations. The neuroscience example shows that programs in this therapeutic area often rely on TE determined in animal behavioral models, which will require confirmation in the clinical setting in a dose-ranging study due to lack of rich biomarker data directly downstream of the target. In addition, the translational value of certain behavioral models used to establish the expected efficacious concentration has been questioned in the literature [24, 25], which highlights another key risk in the translation of preclinical data to clinical efficacy. Unfortunately, for certain neurological diseases, there are currently no viable alternatives. While this issue may be more prevalent in the neuroscience therapeutic area, there is a critical need across all therapeutic areas to better understand the biology of human disease and then to use that knowledge to develop animal models that more accurately reflect the biology of human disease, more accurately predict human therapeutic response, and/or utilize pharmacodynamic endpoints similar or identical to those that will be utilized in human studies. On the one hand, such advances would increase the utility and applications of quantitative and translational pharmacology, while on the other hand quantitative and translational pharmacology can also contribute to an increased understanding of human disease biology. In contrast to the neuroscience case study, in the antibacterial case study efficacy is comparably easy to measure as the actual microbial pathogen is used in robust and relevant *in vitro* and *in vivo* models. The increasingly common challenge illustrated by the antibacterial case study is how to explore the optimal dose and dose regimen in cases where combination therapy is required to address resistant variants. As shown here, antimicrobial therapy greatly benefits from optimized antibiotic dosage regimens that are supported by mechanistic PK/PD

analysis. The prospective prediction of the efficacy seen in the mouse lung infection model builds confidence in its translational value, although a confirmatory trial in patients will ultimately give the final verdict on this approach. Even in the infectious disease space, more complex models may be needed to address more complex therapeutic strategies that are emerging, e.g. prodrug and "flush-and-kill" approaches to treat HIV [45]. Thus, each therapeutic area will need to adopt the translational PK/PD modeling strategy in its discovery programs in concordance with the current knowledge of its biology, the practical limitations in clinical and/or preclinical models, and available information on related compounds. Of particular importance in implementing a successful translational strategy is to give enough attention to the utilized experimental *in vitro* and *in vivo* models and the study design of these preclinical studies to optimize the information obtained from each experiment as discussed in the diabetes example. Data generated in test systems and animal models that have questionable clinical relevance and that presumably poorly translate target functions and disease conditions should preferably be avoided. Mathematical models will only be as good as the quality of the data that is used to build and calibrate them. Ideally, these translational models are subsequently qualified with clinical data obtained with agents directed at the same target or the same pathway, although in many cases such data is unavailable, e.g. for unprecedented targets. Because of these experimental considerations, it is important to have line of sight to the clinic when developing an idea into a discovery project. To effectively execute on a translational PK/PD strategy, the right assays and models need to be put in place early in a discovery program to enable the generation of the appropriate data required to inform clinical development. Thus, discovery projects benefit from translational PK/PD thinking even before experimental data is generated. Within the discovery project team, alignment should be sought regarding the translational utility of the preclinical experimental *and* mathematical models in terms of the limitations and assumptions. The aforementioned translational biomarker plan (Figure 17.2) can help to frame these discussions.

Finally, a common feature of all the presented examples is that the translational PK/PD models were built to address one or more key questions that were important in order to make a well-informed decision. PK/PD models are not developed to just describe the experimental observations in order to summarize the estimated parameters in an organized table. We believe in fit-for-purpose approaches; the PK/PD models developed in support of drug discovery and development programs should be robust enough to adequately predict new scenarios with enough flexibility to aid decisions around study planning, compound and dose selection, or compound progression. It also includes an understanding of the uncertainty that is associated with the predictions as described previously. However, a certain level of pragmatism is warranted as developing the presented quantitative frameworks demands time and resources. In the current drug discovery environment, both time and resources are under constant constraints and scrutiny. Therefore, models are typically not over-engineered even though larger, more mechanistic models may more accurately reflect the actual biology.

Application of translational PK/PD is strongly promoted within our company, and we have noticed many associated benefits in our discovery and early development projects, such as increased rigor and efficiencies in screening routines and more precise guidance on the selection of the most promising clinical candidate or dose. PK/PD facilitates collaboration across different functions as scientists with different skill sets and access to different assays and resources are called upon. The overall impact of translational PK/PD on collaboration may be the greatest if the PK/PD effort is started early in a project's life to allow model development to grow simultaneously with the project and to allow multiple scientists to organically develop a shared ownership. PK/PD analysis often hones in on important discussions within a project team, e.g. to fill in a perceived gap, to explain deviating, unexpected data, to identify properties to improve upon in the next molecule, or to avoid unnecessary focus on parameters or scientific aspects that are of limited impact to the overall program. Thus, translational PK/PD modeling provides a powerful tool to synthesize all relevant information from various data sources in one common place, which informs complex decisions based on the most current integrated insights.

List of Abbreviations

ADME	absorption, distribution, metabolism, and excretion
AUC	area under the curve
BL	β-lactam
BLI	β-lactamase inhibitors
CFU	colony forming unit
CNS	central nervous system
FIH	first in human
FLIPR	fluorescence imaging plate reader
GDIS	glucose-dependent insulin secretion
GK	Goto-Kakizaki
GTT	glucose tolerance test
IGI	integrated glucose–insulin
IPM	imipenem
IP1	inositol phosphatase 1
IVIVC	*in vitro* to *in vivo* correlation
LC–MS	liquid chromatography–mass spectrometry
MAD	multiple ascending dose
MIC	minimal inhibitory concentration
NHP	nonhuman primate
PBPK	physiologically based pharmacokinetics
PD	pharmacodynamics
PET	positron emission tomography
PK	pharmacokinetics
PK/PD	pharmacokinetic/pharmacodynamic
POC	proof of concept
SOC	standard of care

TE	target engagement
%T > MIC	time over the minimal inhibitory concentration

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Part IV

Toxicology

18

Preclinical Toxicology Evaluation

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18.1 Introduction

Toxicities can be broadly ascribed to target-based mechanisms or related to the chemical structure of the compound of interest. Target-related toxicity includes effects from exaggerated pharmacology or interactions with associated pathways, receptors, and ion channels and for these reasons is sometimes referred to as specific toxicity. For example, compounds that act by lowering blood glucose in diabetes can cause intolerable hypoglycemia at higher doses. These effects are often specific and related to the intended primary pharmacology. In those cases where the primary pharmacology is expressed in an animal toxicity study, the pharmacological relevance of that animal species is confirmed. If the exaggerated pharmacology causes dose-limiting intolerability, there is however a concern that other toxicities remain undetected.

Toxicities that are not related to the primary pharmacology of the compound are sometimes referred to as unspecific toxicity. These types of toxicity emanate from the chemical structure of the drug candidate and include genotoxicity (e.g. mutagenicity, clastogenicity), reactivity (e.g. as from metabolites of the compound), irritability (e.g. as from its chemical or physical properties), and non-selectivity (i.e. as from pharmacological effects due to the action of the compound on biological targets other than the primary pharmacological targets) [1].

In the early discovery phase, the discovery toxicologists support the identification and design out of toxicology risk factors. This is performed in lead identification (LI) and lead optimization (LO) via the so-called design–make–test cycles with a view to short-list potential candidate drugs [2]. Once the short list of compounds has been identified, the candidate drugs may be subject to a more elaborate testing, including *in vivo* experiments.

In order to assess risk from a toxicology data set, toxicity findings need to be related to the intended patient population and to the therapeutic exposures as a way to define the expected patient benefit. To this end, predictions of clinical therapeutic exposures can be used as derived from preclinical pharmacokinetic (PK)

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

information. The ratio between the exposure without adverse effects from either detailed *in vivo* studies or approximated by half-maximal activity concentration (AC_{50}, either inhibitory or activating) in a relevant *in vitro* assay and the predicted therapeutic exposure is usually referred to as safety margin, or more correctly the therapeutic index (TI or exposure window) from preclinical data. In most cases, designing studies to achieve the highest possible TI serves to identify hazards, and this will also allow the exploration of a wider range of doses/exposures in a clinical setting compared with studies having a narrower TI. However, it is important to remember that in order to reduce the potential risk to humans in clinical trials, the shape of the curve for toxicity (e.g. steep dose–response for adverse effects vs. gradual dose response), the severity of the finding(s), and the ability to understand the underlying mechanisms of toxicity are as important as the TI in defining the way forward.

In this chapter, the main components of nonclinical toxicology evaluation (summarized in Table 18.1) to support the risk assessment of a clinical candidate and enable human clinical trials will be discussed.

18.1.1 Target Safety Assessment

The scientific rationale providing confidence that a particular drug target has the potential to modulate or reverse disease should be complemented with an equally thorough scientific review of the potential safety liabilities that could be associated with the same target. At an early stage this may not require any specific experimental approaches, but building a strategy that effectively mitigates perceived and actual safety risk relating to the target is an effective means to create confidence in progressing the project toward clinical development. There is no exact number on how often primary pharmacology is considered to drive compound attrition, but one estimate suggests that 28% of clinical or preclinical toxicity failures are associated with primary target-mediated toxicities [10].

It is possible to distinguish between two different kinds of target-related safety issues based on the tissue/cellular distribution of the target: (i) unwanted pathophysiology due to modulation of the biological target when expressed in a non-pharmacologically relevant compartment or (ii) side effects directly associated with the pharmacology of the primary target in the intended compartment, so that while the drug is achieving its primary endpoints, it also escalates preclinical and clinical adverse events. The first kind of target-related toxicity has a plethora of examples. For instance, the development of most kinase inhibitors outside the oncology space has been thwarted by the ubiquitous expression pattern and pleiotropic responses of kinases and the risks associated with their widespread inhibition [11]. The second kind of target-related safety concerns is particularly common in anti-inflammatory and immunosuppressive treatments where excessive modulation of the target leads to host defense issues, e.g. the development of opportunistic infections or the spreading of cancer [12–14]. Novel approaches in regenerative medicines similarly come with increased risk of developing malignant transformation of the target cells [15, 16].

When approaching the task of generating a target safety assessment for a novel target, the first and possibly the most important activity is to investigate whether

Table 18.1 A generic drug toxicity development program to support first-time-in-man Phase I clinical studies.

Safety area of concern	Regulatory guidelines	Types of safety signal detection	Assessments	Examples of studies	Examples of impact on clinical trial/ human use if positive findings established	Typical compound requirement
Secondary and safety pharmacology	[3, 4]	Pharmacological effects toward "unwanted" systems	Interactions with receptors, enzymes, and ion channels Effects on vital systems	• *In vitro* screen for binding to secondary pharmacology targets with functional follow-up studies as needed • Single-dose *in vivo* studies to assess effects on CV, CNS, respiration, GI, and renal function	Include specific monitoring of clinical study subjects Setting of exposure limits Setting of exclusion criteria	<100 g
Phototoxic potential	[5]	Photo activation or formation of reactive degradation products	Absorption spectrum in visible light range *In vitro* testing *In vivo* testing	• 3T3 neutral red phototoxicity test	Requirements for shielding from light during drug exposure Consider follow-up studies in animals and/or humans	<1 g
Genotoxic potential [6, 7][a)]	[6, 7]	Prediction of genotoxic carcinogenicity	Mutations chromosomal damage	• Bacterial Ames test • Mouse lymphoma assay *in vitro* • Micronucleus test *in vivo* (may be optional for Phase I trials)	In most cases, a clearly defined genotoxic signal is preventing human studies Consider initiating early carcinogenicity testing to allow further development	<1 g (2 g if an *in vivo* test is included)

(*continued*)

Table 18.1 (Continued)

Safety area of concern	Regulatory guidelines	Types of safety signal detection	Assessments	Examples of studies	Examples of impact on clinical trial/ human use if positive findings established	Typical compound requirement
General toxicology[a)]	[7–9]	Pharmacological and pharmacokinetic relevance. Target organ(s) for toxicity Reversibility of lesions	Clinical signs, food and water consumption, and body weight Serum and urine analysis Histopathology Systemic exposure levels Establish no observed adverse effect level in most sensitive species	• Single escalating dose to identify maximum tolerated dose (MTD) • Repeat dose range finding (DRF) study to determine suitable dose levels • Pivotal (GLP) repeat dose studies	Provide rational for a safe starting dose Include specific monitoring for potential toxicities Limit dose and exposures	100–1000 g, depending on MTD, duration of repeat–dose studies (14–28 days and inclusion of recovery group
Local toxicity	[9]	If other route of administration to be investigated, then assess tolerability at administration site	Determine local effects related to the administration route	• Single species, single-dose intravenous study, including perivascular administration • Subcutaneous/topical administration • Formulation and dose rate should be the same as that intended for the clinical study	Specific monitoring and control of dose rate	1 g

a) *According to ICH S9* [7] *Genotoxicity studies are not considered essential to support clinical trials for therapeutics intended to treat patients with advanced cancer. Furthermore, toxicology studies to determine a no-observed-adverse-effect level (NOAEL) or no effect level (NOEL) are not considered essential to support clinical use of an anticancer pharmaceutical. Nevertheless, assessment of the potential to recover from toxicity should be provided to understand whether serious adverse effects are reversible or irreversible.*

there are documented human genetic polymorphisms (e.g. loss of function or gain of function) that could help understand what modulation of this target could lead to. As an example, the healthy appearance of humans harboring proprotein convertase subtilisin/kexin type 9 (PCSK9) loss-of-function mutations would support PCSK9 inhibition as a safe and effective therapy for hypercholesterolemia [17]. In lieu of such information, one could explore preclinical genetic models where the target has been either mutated or deleted. However, genetic animal models should be interpreted with caution: the expression pattern of a given target or its relevance and regulation in a biological pathway may differ across species, as exemplified by the different phenotypes of loss of function of the retinoid-related orphan receptor gamma in human and mouse [18, 19]. A target that is closely associated with a pathology in man may have different expression patterns in mice that suppress the resulting phenotype, which may explain the poor translatability of preclinical models. This can be exemplified by differences in lipoprotein profiles in rodents and man [20]; in mouse, plasma cholesterol is primarily carried in HDL particles that result in a natural atheroprotective effect. But when the lack of translation is properly understood, this can be mitigated by generating humanized models system that reconstitute the human phenotype [20], although in most cases such in-depth understanding is lacking, and the preclinical models can fall short. Most importantly, genetic models in most cases do not accurately reflect pharmacological modulation of the target [21, 22], likely due to compensating mechanisms that arise from genetic manipulation. Another aspect to take into consideration when exploring the phenotype of transgenic models is not to completely rely on published observations. In most cases, these genetic models are investigated, while the animals are relatively young and adverse events occurring beyond the age of 6 months are seldom reported.

It is normally difficult to confidently attribute a given toxicity to the target of interest. Nevertheless based on experience and as data on a particular target are generated, the picture might become increasingly clear. A good example is that of the peroxisome proliferator-activated receptor (PPAR) agonists [23]. The preclinical toxicology profile of most PPAR-alpha and -gamma agonists is extremely complex [24, 25]. It is characterized by different toxicity signals of varying severity across multiple organs. Without the accumulated clinical experience with this type of drugs and the sharing of preclinical data [26], their regulatory acceptance would not have been possible – and is still the matter of debate in the case of PPAR-gamma agonists [27, 28].

The key question is "how can understanding of target-related safety issues be incorporated into chemical design toward preclinical candidate selection?" One obvious way to mitigate target-related safety issues is to tailor the distribution properties of the compound. In a simplistic approximation, a target whose modulation in the central nervous system (CNS) results in toxicological liabilities could be avoided by designing compounds that will not be able to cross the blood–brain barrier. Alternatively, for a drug that is pharmacologically acting on a target on the luminal side of the gastrointestinal (GI) tract, one may wish to completely avoid systemic bioavailability due to toxicity associated with widespread modulation of the same target in systemic compartments.

Selectively directing the drug to the target organ may reduce the systemic exposure and toxicity associated with a certain treatment. Different targeting linkers, for instance *N*-acetylgalactosamine (GalNAc) for liver targeting [29], have been fused to an active cargo, and there are several examples of such approaches currently being tested in clinical trials [30, 31]. This approach has been mostly exploited in the antisense oligonucleotide space, where toxicity associated with this modality, in particular severe thrombocytopenia, has recently caught considerable attention [32]. Furthermore, several novel targeting approaches are being evaluated in early clinical development [33–36].

Optimization of the excretion route could also hypothetically be explored: if modulation of a particular target was expected to have adverse effects on kidney function, one could consider optimizing the compound toward biliary clearance (e.g. by increasing plasma protein binding or altering the affinity for different metabolic pathways) [37, 38]. Modification of the route or frequency of administration could also be considered, appreciating of course that this may not be favorable for a given indication. For instance, it may be possible to mitigate side effects in the liver that are driven by maximum compound concentrations (C_{max}) by switching to a parenteral route of administration and thus reducing high compound concentrations in the GI tract and the liver first-pass metabolism. However, this may not always be feasible for a chronic, once-daily dosing regimen when patient compliance or commercial attractiveness is considered.

There are also examples where target-related toxicology has been mitigated by modulating the biochemical/pharmacological properties of the compounds. For example, many companies have endeavored to develop glucocorticoid receptor (GR) agonists that interfere with specific receptor– co-receptor interactions in order to maintain the anti-inflammatory effects while avoiding common side effects observed with glucocorticosteroid treatment [39], such as impaired growth in children, decreased bone mineral density, skin thinning and bruising, and cataracts, all which are related to the GR target [40].

In summary, target-related liabilities are inherent in many drug projects, and while it is sometimes possible to optimize compounds toward a favorable TI, there could also be options to completely avoid future adverse effects in the clinic. These opportunities need to be evaluated as part of a preclinical safety risk assessment.

18.1.2 Compound Safety Assessment

In simple terms, in the absence of target-related safety liabilities, highly potent, selective molecules are unlikely to cause toxicity, as demonstrated by the fact that considerably fewer drugs with a human efficacious concentration (C_{eff}) of $\leq$250 nM (total drug) and $\leq$40 nM (free drug) have regulatory safety warnings than abovementioned exposure levels [41]. Nevertheless, it is likely that despite careful optimization of potency, selectivity, and PK properties, the best compounds will still reside above those exposure benchmarks. Therefore, effective means to identify which compounds will have the largest therapeutic window are required. In the following, standard toxicological characterizations of lead compounds aimed at assessing the risk associated with their clinical development path will be discussed.

18.1.2.1 Cytotoxicity

Cytotoxicity endpoints are normally incorporated in the drug discovery screening cascade at an early stage mainly with the purpose to ensure that *in vitro* pharmacodynamic (PD) models are not confounded by cytotoxicity. While the inherent property of a molecule to cause cell death would be expected to be an important parameter in identifying a safe drug, the translatability of *in vitro* cytotoxicity to toxicity is far from consistent [42]. It was shown that molecules that are highly cytotoxic (half-maximal cytotoxic concentration – CC_{50} <10 μM) are more likely to fail in early preclinical safety studies [43]. For this reason, many pharmaceutical companies now include *in vitro* cytotoxicity assessment as part of their standard safety screen. There is a need to balance the requirements for high-throughput *in vitro* readouts and the wish to utilize models that mimic the *in vivo* situation. As these are not currently possible to reconcile, it is preferable to use both in a tiered approach.

Standard cytotoxicity assays would typically utilize a nonadherent tumor cell line over a series of increasing compound concentrations, incubated for 24–48 h, and record cellular viability as the endpoint. The most commonly used viability assays measure total adenosine triphosphate (ATP) levels, which correlates well with total cell number [44], or mitochondrial dehydrogenase activity (MTT assay or equivalent) [45]. The primary objective is not to ensure that the compounds are safe, but to identify molecules that have physicochemical properties that cause disruption of cellular integrity and/or have a promiscuous secondary pharmacology profile that results in apoptosis or necrosis [43]. In addition, drugs like the nucleoside analogs used in oncology (e.g. gemcitabine, cytarabine) need to be metabolized inside the cells in order to fulfill their pharmacological action [46], and this needs to be taken into account when analyzing cytotoxicity data.

In this context it is noteworthy that the predictivity of the hepatoma cell line (HepG2) is just marginally worse over complex three-dimensional (3D) cellular models and human stem cell-derived hepatocytes in identifying compounds that cause liver toxicity in subacute toxicology studies [47]. However, in order to be able to evaluate cytotoxicity originating not only from the parent compound but also from its metabolites, as described above, it is necessary to employ a cellular model that ensures that the relevant metabolic enzymes are expressed. For instance, it is well established that the most commonly utilized hepatoma cells express very low levels of these enzymes [47].

18.1.2.2 Mitochondrial Toxicity

Standard cytotoxicity assays are normally run on tumor cell lines that due to the Warburg effect rely very little on mitochondrially produced ATP [48]. For this reason, molecules that specifically interfere with the respiratory chain or oxidative phosphorylation are not typically identified as cytotoxic in these assays [49, 50]. To circumvent this limitation, it is possible to cultivate and adapt tumor cell lines, normally the hepatoma cell line HepG2, to grow in galactose rather than glucose. As there is no net production of ATP via anaerobic metabolism of galactose, the tumor cells are forced to utilize their mitochondrial oxidative phosphorylation for ATP production. This causes a dramatic increase in sensitivity to mitochondrial toxicants. By comparing the ratio of the cytotoxic CC_{50} values in cells growing in galactose with those growing under normal culture

conditions, it is possible to identify potential mitochondrial toxicants for further characterization and risk assessment. This assay setup is now used frequently by many pharmaceutical companies [51].

18.1.2.3 Biotransformation and Reactive Metabolites

Drug metabolism increases the compounds' hydrophilicity to facilitate their excretion from the body and represents a major clearance pathway for drugs. When assessing the safety of small molecules, understanding their metabolic activation, deactivation, and detoxification is of great importance, since this process may result in reactive intermediates with increased toxicity [52–54]. The potential risk with the formation of reactive or electrophilic metabolites is that these could give rise to, for example, genotoxicity, target organ toxicity, or idiosyncratic (unpredictable) toxicity. Assessing the potential risk of idiosyncratic adverse reactions from drug compounds or their reactive metabolites is still a major challenge for the pharmaceutical industry, and most pharmaceutical companies have developed their own approach to address this risk. Thompson et al. have recently reviewed different reactive metabolite risks and hazard assessment approaches [55]. Metabolic systems are highly complex and adaptable, and the enzymes' expression patterns and substrate specificities can vary greatly among different species. This implies that there is a significant risk of underestimating or neglecting toxic metabolites that are formed in humans when extrapolating from *in vitro* and *in vivo* animal testing results. Conversely, metabolites that are exclusively formed in animal systems can result in spurious toxicology with no human relevance.

In addition, the expression patterns of metabolic enzymes also differ across tissues and organs and are significantly affected by age and sex [56–58]. There are indications that metabolic enzymes and transporters result in synergistic functions (for example, cytochrome P450 3A4 (CYP3A4) and P-glycoprotein (P-gp)), adding an additional level of complexity to the use and interpretation of *in vitro* data for risk assessment purposes [59–61]. To this end, several *in vitro* systems, as well as new *in vivo* approaches, are available and can be specifically used depending on the issue or problem to be addressed [62]. It should however be realized that a full human metabolite pattern will not be available until a human PK study with radiolabeled compound has been conducted, usually not earlier than Phase II of clinical drug development. These studies will allow the detection of human disproportionate metabolites, for which further *ad hoc* toxicity testing may be required [63].

Optimization of metabolic liabilities can be supported by using known structural alerts (e.g. aromatic systems generating chemically reactive electrophiles). Structural features that could form reactive metabolites or "offending" motifs in drug candidates will not be covered in this section as several excellent recent reviews on the subject have been published [64–66].

Primary hepatocytes, recapitulating all the major Phase I and Phase II metabolic transformation activities, are considered a "gold standard" approach for metabolite identification [67], although cell line-derived hepatocytes and/or liver microsomes are also commonly used. In order to address potential metabolism that occur in the gut wall, blood plasma, or lungs, complementary

metabolic studies using these cells/tissues types may be required for some projects. In addition, the metabolic activation used in mutagenicity test (see below) is usually the S9 fraction, which consists of both the cytosolic and microsomal fractions of livers. Here, the most common is the Aroclor 1254-induced S9 fraction from rat livers [68].

A trapping agent (e.g. glutathione (GSH), methyloxamine, cyanide) can be added to the incubation in any of the systems described above to investigate if a compound is bioactivated to a reactive metabolite. Hard or soft electrophiles exhibit specific chemical preference for different trapping agents, and using more than one trapping agent may help to understand the potential reactive nature of a compound. Further assessment may include the use of a labeled compound in covalent binding experiments in microsomes or hepatocytes [69]. This data can be extrapolated to predict the daily body burden of reactive metabolites [70, 71].

With these data in hand, the risk for idiosyncratic toxicity will not be entirely removed, even in the most favorable cases. Therefore, regulatory authorities expect that critical information on the formation of metabolites including, where appropriate, both Phase I and Phase II metabolism and comparison of drug metabolism routes and exposures in men and the species used for toxicology studies are provided before the onset of larger scale clinical trials, as detailed in Section 3 of the International Conference on Harmonization (ICH) M3 guideline [9].

18.1.2.4 Secondary Pharmacology

Secondary pharmacology studies aim to detect any off-target or unintentional effects of a compound. These studies are initially conducted in multiwell *in vitro* assay format against a broad range of targets (e.g. receptors, ion channels, enzymes, and transporters) that are distinct from the intended therapeutic target (or targets) in order to identify specific molecular interactions that may cause adverse drug reactions in humans. For those targets where binding occurs, a follow-up test is used to determine the nature of the biological effect originating from binding to the receptor (e.g. agonistic, antagonistic), and the concentration at which this occurs. This concentration can then be compared with the therapeutic concentration at the primary pharmacology target and used to predict margins to untoward effects. This type of data can inform the design of *in vivo* safety pharmacology studies to verify and monitor specific detrimental effects, as detailed in Chapter 19.

The most characterized drug-related adverse event due to off-target pharmacology is the inhibition of the human ether-a-go-go-related gene (hERG) potassium ion channel. hERG inhibition may result in cardiovascular effects due to changes in cardiac action potential. These changes could lead to QT interval prolongation in a clinical setting, a condition also known as "torsades de pointes," which has been associated with many sudden death cases and consequent black box warnings as well as drug discontinuations [72, 73].

Several *in silico* [74, 75] and *in vitro* models are available to assess hERG inhibition. Voltage clamp techniques represent the "gold standard" in the field, as it provides real-time mechanistic information on ion channel functions and its perturbation. As a follow-up to an *in vitro* hERG inhibition signal, *in*

vivo studies using anesthetized guinea pigs [76] and nonrodent telemetrized conscious animals are normally performed. Assessment of hERG inhibition for drug candidates and its metabolites is covered in the ICH S7B guideline for the nonclinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals [4].

The rationale, strategies, and methodologies for *in vitro* pharmacological off-target profiling in discovery phase at four major pharmaceutical companies were published in 2012, including rational for inclusion of the targets in the proposed 44-target panel [77]. In addition, the authors highlight different approaches in assessing the therapeutic window comparing AC_{50} data with (i) primary target *in vitro* biochemical data, (ii) the predicted therapeutic free plasma concentration (free C_{max}) in humans, and (iii) experimental exposures in preclinical models for a more integrated risk assessment. Early off-target screening may support design and selection of lead series and/or identify the most promising candidate with reduced risk for off-target toxicities. Prior to clinical trials, additional and broader off-target screens are employed to characterize the candidate drug.

18.1.2.5 Phototoxicity

For human drugs with distribution into light-exposed tissues (skin, eye), phototoxicity may arise if compounds are activated to a reactive species by light within the range of natural sunlight (290–700 nm). Assessment of potential phototoxicity is described in relevant ICH guidelines [5]. The initial step is to determine the absorption spectrum. If the molar extinction coefficient (MEC) does not exceed 1000 l $mole^{-1}$ cm^{-2} at any wavelength in the natural sunlight range, no further testing is needed. However, when higher levels of MEC are identified, there is a concern for generation of reactive oxygen species with phototoxic potential. In such cases, the tissue concentration of the compound at the time of light exposure is an important parameter used to predict the phototoxicity risk. Here, tissue distribution studies provide important information for drug presence and retention in light-exposed tissues.

The most widely used *in vitro* test to predict phototoxicity is the 3T3 neutral red phototoxicity test, for which an Organisation for Economic Co-operation and Development (OECD) guideline exists [78]. Although this is a well-established assay and negative test results are considered reliable, experience within the pharmaceutical industry suggests false–positive results are possible. In the latter case, a positive result from the test should be taken as a flag for follow-up studies and/or clinical precautions (i.e. shielding subjects form daylight during the trial).

In vivo study protocols to evaluate the phototoxicity potential require a good understanding of the PK profile of the compound to ensure that irradiation of the animals occurs at the time point associated with maximum compound exposure (T_{max}) and to determine study duration. The compounds ability to bind to melanin also dictates whether pigmented or nonpigmented animal models should be used. A positive readout from an animal phototoxicity study can be managed by a no observed adverse effect level (NOAEL) approach, where

human exposure limits are applied to mitigate the risk for clinical phototoxicity.

There are options to determine phototoxicity potential in clinical trials. These should be considered on a case-by-case basis and may include standard reporting of adverse events (e.g. erythema, skin irritation) or dedicated photosafety trials [5].

18.1.2.6 Genetic Toxicology

Genetic toxicology studies focus on the identification and analysis of agents that display toxicity directed toward the hereditary components of a living organism. The term genotoxic is a general descriptor used to distinguish chemicals that have an intrinsic affinity for DNA from those that do not. Genotoxic substances have several common chemical or physical properties for interaction with nucleic acids (e.g. electrophilicity) [79]. Genotoxicants are classified by their ability to induce specific stable changes in the nucleotide sequence of genes, the chromosome structure, or the chromosome numbers. Changes in the nucleotide sequence are classified as mutations; chromosomal damage is referred to as clastogenicity and changes in chromosome number as aneuploidy. Since genotoxicity is often confused with mutagenicity, it is important to note that not all genotoxic substances are mutagenic as they may not cause retained alterations in DNA sequence; however, all mutagens are by definition genotoxic.

In silico assessment of the genotoxic potential of the compounds of interest as from chemical substructure-based alert systems, such as MCASE, MC4PC, and Derek [80] (see Chapter 20), may be used as a first filter, although follow-up *in vitro* assessments are required to substantiate the findings [81].

Genotoxic tests assess the compound's effects on DNA in order to verify their potential to damage the genetic structure of living organism. Many contract research organizations (CROs) provide non-good laboratory practice (GLP) that scaled down high-throughput versions of the Ames test (see below), which need a limited amount of compounds (milligram) to support early decision making and chemical design. This limited Ames test serves as a good predictor of genotoxicity during screening of drug candidates. During LO, many pharmaceutical companies include a non-GLP version of the 5-strain Ames test for mutagenicity and an *in vitro* chromosomal aberration assay, such as the micronucleus assay [82].These assays identify mechanisms of DNA interaction or damage that predict mutagenicity and therefore potential carcinogenicity. Based on these results, follow-up mechanistic studies can be planned to evaluate the mode of genotoxic action (i.e. direct or indirect) as a further risk assessment step. Toxicogenomic approaches have also been developed and used to support discrimination of indirect from direct acting genotoxins [83].

To enable initial clinical trials, the standard test battery GLP tests for genotoxicity include an assessment of mutagenicity in a bacterial reverse mutation test (5-strain Ames test ± metabolic activation). Moreover, genotoxicity should also be evaluated in mammalian cells *in vitro* or *in vivo* [6]. The following two options are described for the standard battery.

Option 1 Comprises

1) A test for gene mutation in bacteria (5-strain Ames test) [84].
2) A cytogenetic test for chromosomal damage (*in vitro* metaphase chromosome aberration test [85] or *in vitro* micronucleus test [86]) or an *in vitro* mouse lymphoma Tk gene mutation assay [87].
3) An *in vivo* test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells [88].

Option 2 Consists of

1) A test for gene mutation in bacteria (5-strain Ames test).
2) An *in vivo* assessment of genotoxicity with two different tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second *in vivo* assay. Typically this would be a DNA strand breakage assay in liver, unless otherwise justified.

During the early stages of clinical development, negative genotoxicity test results are normally used as a surrogate for long-term carcinogenicity risk, and further testing before marketing application of the drug is not warranted. A compound identified as genotoxic *in vitro* with an indirect mode of action would require additional *in vivo* data to determine the biological significance of these *in vitro* signals and assess its intrinsic genotoxic properties. A positive response in a genetic toxicology assay may be a liability and potential showstopper for repeated dose clinical studies unless or until it can be demonstrated that human exposures do not present a risk to trial participants. In addition, understanding the mechanism behind an *in vitro* positive compound supports the definition of further risk assessment activities: an *in vitro* clastogenic response would require additional testing to determine its *in vivo* relevance, while an aneugenic compound may directly progress to further development if a safety margin is provided [89]. It is recommended to seek additional scientific advice and consult relevant guidelines [6] to devise a strategy for these follow-up studies. However, if the *in vitro* genotoxicity tests are negative, there is no further testing requirement in order to support early clinical studies. In the case of drugs intended for the treatment of advanced cancer, genotoxicity studies are not considered essential to support clinical trials in patients [7]. This guidance (ICH S9) applies to "patients with advanced cancer whose disease is refractory or resistant to current therapy, or where current therapy is not considered to be providing benefit" [7] . However genotoxicity studies should be performed to support trials in healthy volunteers or patients with extended life expectancy.

18.1.2.7 Genotoxic Impurities

An additional area of genotoxic assessment of new drug candidates is the assessment of impurities that are likely to arise during the synthesis, manufacturing, and storage of the active ingredient. The new ICH M7 guidance provides a practical framework and emphasizes considerations of both safety and quality risk management in establishing levels of mutagenic impurities

that are expected to limit the potential carcinogenic risk of the impurity [90]. It focuses on DNA reactive impurities, which are generally identified using the Ames test described in the previous section. ICH M7 proposes that a computational toxicology assessment should be performed using two complementary quantitative structure–activity relationship (QSAR) methodologies that predict the outcome of a bacterial mutagenicity assay as part of a regulatory submission [90]. Specifically, one methodology should be expert rule based, while the other methodology should be statistical based. QSAR models utilizing these prediction methodologies should also follow the validation principles set forth by the OECD. The M7 guideline is intended to complement ICH Q3A(R2), Q3B(R2), and ICH M3(R2) [9, 91]. It does not apply to drug substances and drug products intended for advanced cancer indications as defined in the scope of ICH S9 [7, 90, 92].

Briefly, the impurity assessment is a two-stage process:

- Actual impurities that have been identified should be considered for their mutagenic potential.
- An assessment of potential impurities likely to be present in the final drug substance is carried out to determine whether further evaluation of their mutagenic potential is warranted.

A consensus view of M7 from a number of pharmaceutical companies has been published in 2016. The proposal details on how a supplemental expert review of a given prediction may be generated. It also provides suggestions detailing the contents of an expert analysis and delineate its inclusion in a regulatory submission [92].

The threshold of toxicological concern (TTC) concept defines an acceptable intake for any unstudied chemical that poses a negligible risk of carcinogenicity or other toxic effects. When applying the TTC concept in the assessment of acceptable limits of mutagenic impurities in drug substances and drug products, a value of 1.5 μg per day corresponding to a theoretical 1 in 10^6 excess lifetime risk of cancer can be justified. For less-than-lifetime (LTL) daily intakes, a staged TTC for clinical development has been established based on the approximate relationship between concentration (c) of toxicant and time (t) of exposure to toxicant, as from Haber's law [93]. Based on this, an acceptable daily intake of a potentially genotoxic impurity may be 120 μg per day in a treatment scenario of less than 1 month. However, it is stressed that the application of the staged TTC for impurities must be considered on a case-by-case basis.

Some structural groups were identified to be of such high genotoxic potency that intakes even below the TTC would theoretically be associated with a potential for a significant carcinogenic risk. This group of highly potent mutagenic carcinogens, referred to as the cohort of concern, comprises aflatoxin-like-, N-nitroso-, and alkyl-azoxy compounds [90].

18.1.2.8 Incorporation of Safety Endpoints in Preclinical Efficacy and PK Studies

The early identification of potential safety liabilities is important since the data will guide the design of better compounds. Bespoke toxicological studies to

address specific questions are a valid option, but these usually require dedicated planning, resources, and budget and may therefore be impractical. An important alternative for investigating toxicology-related liabilities is the modification of standard *in vivo* PD and PK studies. Additional endpoints or an additional group at a higher dose could be added to a PD experiment to verify a risk flagged from *in vitro* data. Routine measurements such as general plasma biochemistry for liver enzymes or hematology and histopathology of core organs (e.g. liver, heart, and kidney) could aptly complement the PD study to reveal potential side effects. When exploiting PD studies for safety investigations, background knowledge of the PD models is of critical importance before drawing conclusions from a toxicological point of view. For instance, a fatty liver and/or necrosis may be simply background in an obesity model and not necessarily compound induced.

In PK studies, inclusion of higher doses (e.g. reaching 10 or 30 times predicted human C_{max}) could confirm whether higher exposure can be achieved without clinical symptoms as well as testing the hypothesis of dose/exposure linearity with a view to assess the potential risk of compound accumulation.

As such, the ability to monitor potential clinical symptoms observed in PD or PK studies will support the design of *ad hoc* toxicological studies and selection of the best candidates for progression.

18.1.2.9 Safety Pharmacology

Safety pharmacology studies evaluate the potential impact of new drugs on vital organ systems before first-in-human drug testing and are described in detail in Chapter 19. In a core battery of *in vivo* studies, usually acute single-dose studies, assessments are made on CNS, cardiovascular (CV), and respiratory functions. A rodent species is typically used for the CNS and respiratory assessment, while a nonrodent species is employed for evaluating CV-based endpoints. An opportunity to reduce animal usage exists by combining specific safety pharmacology parameters in GLP-based toxicological studies, and this approach is gaining an increasing demand also from regulatory authorities. The reader is referred to Chapter 19 for an in-depth description of the core studies and any appropriate required follow-up experiments.

18.1.2.10 Maximum Tolerated Dose (MTD) / Dose Range Finding (DRF) Studies

In order to derisk future pivotal GLP studies, it is advisable to generate exploratory information on the absorption and toxicological potential of the compound. This can be achieved by the studies of simpler design with a small number of animals and a limited set of endpoints, which may include histopathological evaluation of core organs. Since bridging of dose levels across these studies is of critical importance, it is advisable to make as few changes as possible to the choice of animal supplier, the properties of the compound (e.g. salt form, crystal form), and the formulation and to ensure that the impurity profile in the batch used for toxicity testing is representative of the batch intended for the clinical study.

The initial study is often a single escalating dose given to a small set of animals (typically one male and one female for nonrodents and three males plus three females for rodents) up to the maximum tolerated dose (MTD). It is important

to allow for appropriate compound washout (often approximated by five or more plasma half-lives of the compound) between dose escalations to minimize drug accumulation. For nonrodents, blood sampling to assess compound exposure can be obtained from the same animals from which signs of intolerability are monitored. For rodents, microsampling methods [94] should be considered wherever possible, but satellite groups may be needed, depending on the blood sampling volume required for the bioanalysis of compound concentration.

Once an MTD has been determined based on clinical signs, the animals may be observed for an additional time (e.g. 14 days) to capture any latent, delayed toxicity, before being necropsied for gross pathology examinations. It is important to escalate doses up to an intolerable dose limit in order to define the MTD. However, there is no expectation to dose escalation beyond 1 $g\,kg^{-1}$ for drugs where the predicted clinical dose does not exceed 1 g, in the presence of systemic exposure saturation or due to galenic limitations (e.g. maximal feasible dose). The MTD study and possibly specific, associated safety pharmacology studies do fulfill requirements for single-acute toxicity testing and therefore should be reported in a suitable format, although GLP compliance is not required.

The MTD study is usually followed by a dose range finding (DRF) study. This consists of repeated compound dosing over a period of 7–14 days. A typical design would include a control group receiving vehicle alone and 2 or 3 dose groups. The high-dose group in a DRF study normally uses a dose comparable to the MTD, depending on the effects identified in the MTD study. The lower doses should normally be well separated from the high dose and from each other in terms of systemic exposure (area under the curve (AUC) and C_{max}). It is worth emphasizing that exposure differences smaller than threefold between two dose levels seldom add value to the study. Group sizes typically range from two males plus two females for nonrodents to five males plus five females for rodents. In some cases, it may be possible to reuse the nonrodent animals from the MTD study, following the principles of the 3Rs (replacement, reduction, and refinement) [95], but that should be taken into consideration when interpreting the results. The main purpose of the DRF study is to guide the design and dose levels for the subsequent GLP studies. A DRF study will not (and should not) define safety margins as the study design and a small number of animals are unlikely to provide a fully resolved dose response, and the necropsy evaluations may be limited. Nevertheless, assessment of systemic exposures (toxicokinetics (TK)) and their relationships to the administered doses provide useful information for dose setting in GLP toxicology studies.

18.1.3 GLP Toxicology

When a suitable candidate drug compound has been selected for clinical development, the working principles of safety assessments change. In the discovery phase and particularly in the design–make–test cycles of lead optimization, the focus is on testing a large number of compounds of limited availability (mg scale) in order to maximize wanted effects and minimize unwanted effects. During preclinical development, the focus becomes to enable large-scale (kg) manufacturing of the selected compound to support documenting studies. In

particular, toxicity studies with the compound should be designed to detect any unexpected toxicities, which significantly increases demands on spending, animals, and other resources.

To ensure acceptability of these studies toward enabling clinical studies in humans, guidelines are available to provide a uniform standard for quality (OECD GLP) and scope (ICH) for major regions (i.e. North America, Europe, and Japan). The OECD principles of GLP ensure the generation of high quality and reliable test data related to the safety of industrial chemical substances and preparations. The principles have been created in the context of harmonizing testing procedures for the mutual acceptance of data (MAD). The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has issued guidance documents that apply to the situations usually encountered during the conventional development of pharmaceuticals and should be viewed as general guidance for drug development. For small molecules, the M3 guidance document provides a starting point for most clinical development programs [9].

To support the first dose to humans, a comprehensive package of nonclinical studies is required. These include *in vitro* and *in vivo* studies of secondary and safety pharmacology, general toxicity after single (MTD) and repeat dosing in two animal species (rodent and nonrodent), genotoxicity, and phototoxicity (see Table 18.1). In general, the route of administration in nonclinical *in vivo* studies should be the same as the one intended for the clinical studies. If, for example, a program is designed to support oral administration of the drug to humans, a complementary intravenous toxicity study may be needed to support an eventual intravenous PK study in humans.

In order to avoid delays in the development program once a drug candidate has been selected and larger-scale manufacturing resources are secured, suitable animal species for nonclinical safety testing should be identified. Factors that should be considered in the selection of the animal species for GLP toxicology studies include availability of relevant metabolic patterns recapitulating all human metabolites, potential to achieve sufficient systemic exposures in relation to the anticipated human exposures, feasibility of route of administration to afford intended dosing regimen, and pharmacological relevance. This information is normally obtained from the nonclinical PD, PK, and safety studies that led to the drug candidate selection, although some of these studies were conducted in disease models. When required, complementary studies should be designed to ensure the suitability and relevance of a given animal species.

In most small molecule drug development programs, a rodent and a nonrodent species are required for toxicity testing. It is important that at least one of the selected species displays pharmacological responsiveness similar to the intended human mode of action and that at least one species provides a similar metabolic pattern to that predicted for humans and is able to provide TK coverage in excess of that expected for human therapeutic effect. In many cases the rat (e.g. Han Wistar or Sprague Dawley) and beagle dog (e.g. Harlan or Marshall) are the default species for safety pharmacology and general toxicology studies, given their health and calm temperament. Importantly, both rat and

dog strains are readily available at toxicity test sites of relevant CRO's and have well-characterized background pathology. Furthermore, several assays directly suitable for analyses of clinical parameters in these species have been developed. Other species often encountered in GLP toxicology studies are the mouse, hamster, mini pig, and nonhuman primates (e.g. cynomolgus monkey).

18.1.3.1 General Toxicology

The general design of these studies includes control groups and dosing groups at low (i.e. near therapeutic), middle, and high (i.e. near MTD), to ensure a relevant exposure range is evaluated with respect to the detection of unexpected toxicities. The route of administration should be that of the intended clinical trial. The dosing intervals should provide for an exposure profile over time that covers or extend over the predicted clinical exposure. The duration of the pivotal studies should match the duration of the clinical trials they are intended to support, with a minimum of 14 days even for short single-dose human trials. For later stage clinical development (Phase III and or registration), the duration of the subsequent toxicology studies will be longer, for example, 3 or 6 months to support trial durations of up to 3 or 6 months, respectively. For longer clinical use and for registration, the duration of the general toxicology studies is limited to 6 months for rodents and 9 months for nonrodents.

The high-dose group compensates for animals that are potentially less sensitive to the toxicity effects compared to humans and helps to obtain confident exposure margins of safety for the human trial. The middle dose group serves as providing a dose–response relationship to the toxicities and is often the geometric mean between the low and high doses. The dose levels should most often provide at least a threefold separation of systemic exposure ranges. If the ranges of dose levels are very broad (e.g. steps significantly larger than 3×), additional groups at intermediate dose levels may be considered, depending on circumstances determined in the MTD and DRF studies. In all cases, a justification based on all available data is needed to support the selected dose levels. Although not mandatory, it may be useful to also include recovery groups in the initial pivotal study in order to determine the reversibility of any finding.

The endpoints in a typical pivotal study include assessment of systemic drug exposure, clinical signs (including behaviors, food and water consumption, body weight, ophthalmoscopy, electrocardiograms on nonrodents, survival), clinical chemistry (including electrolytes, liver enzymes, plasma glucose, hematology), urine analysis, gross necropsy (weights and appearance of typically up to 20 different organs), and histopathology (>40 different tissues form each animal [96]). As appropriate, other endpoints may be included as well, depending on the drug class and any known concerns.

The purpose of the pivotal study is to establish target organ(s) of toxicity, types of toxicity (e.g. pharmacological vs unspecific), dose dependency, time dependency, specie specificity, and safety ranges. All these readouts form the basis for determining the NOAEL and the associated exposure (C_{max} and AUC) to the drug.

18.1.4 Enabling the First Clinical Trial with Toxicological Data

Before the first human dose, a safe clinical starting dose is determined based on all toxicological and pharmacological data. According to FDA guidelines [97], there are two options for this, based on either the NOAEL or the minimum anticipated biological effect level (MABEL), and usually the most conservative of these is applicable. In the NOAEL option, the dose with no adverse effect in the most sensitive species is converted into the human equivalent dose (HED) based on body surface conversion [97]. The safe starting dose is usually 1/10 or less of the NOAEL HED, to compensate for the difference in species sensitivity. In the MABEL option, PK scaling, target affinity, and plasma protein binding are used to estimate the lowest plasma concentration at which a pharmacological effect occurs. With the intention that the starting dose to be evaluated in humans should not elicit significant pharmacology effects, the starting dose is then set to 1/10 of the MABEL. Modification of the 1/10 safety factor is possible, depending on the types of toxicity identified and steepness of the dose–toxicity–response curve.

In addition, the maximum allowed dose or exposure to humans is determined from the toxicity studies. Often the toxicities are classified as:

- Those that are not significant if they were to occur in man (e.g. vomiting, as frequently observed in the dog species during the described studies). In this case, the NOAEL in the most sensitive species is often the relevant parameter used.
- Those that can be monitored are reversible and nonserious or manageable in the context of the clinical trial (e.g. blood pressure changes, hypoglycemia). Here, premonitory biomarkers are used to define stopping criteria during the dose escalation in the tolerability studies.
- Toxicities that cannot be monitored are not reversible and/or would be serious if they were to occur in man (e.g. seizures, arrhythmia, and organ toxicities). Since humans may be more sensitive than animals in the toxicology program, the exposure limit may herein be reduced to a fraction of NOAEL.

Once all information on the safe starting dose, the exposure limits, and the toxicity profile are collected, the toxicologist can contribute to the nonclinical modules 2 and 4 of the common technical document (CTD), where study reports are referenced, and a summary of all data is included to provide guidance to clinical investigators. The CTD format is accepted in all ICH regions, including the United States (IND) and Europe (IMPD). Further information on the content of the CTD including templates can be found on the ICH website [98].

When there is a need to generate early human data on a compound in a very limited clinical trial, alternative approaches to the program outlined above can be supported. These exploratory clinical studies include instances where there is neither therapeutic intent nor evaluation of human tolerability of the compound, e.g. PK studies, biomarker studies, and micro dosing studies [42]. For example, a nonclinical safety program to support a single dose with a positron emission tomography (PET) ligand in men usually consists of *in vitro* target profiling, animal pharmacology, a single-dose rat toxicology study at a dose

of 10 mg kg^{-1} with extended post dose observation according to GLP, and *in vitro* genotoxicity testing (Ames test and mouse lymphoma test according to GLP and/or structure–activity relationship (SAR) analysis) [42]. These studies can be performed on the cold compound without the need to use radioactive material for toxicity testing. This testing paradigm allows for a human study with a cumulative dose of not more than 100 μg, where any such doses are nonpharmacological. Other safety testing programs may be designed to support different exploratory clinical trials, such as single sub-therapeutic or therapeutic doses, and the reader is recommended to consult the current ICH guidelines [42] for further examples and principles.

18.1.5 Toxicology Studies Beyond Phase I Clinical Trials

As a program progresses through clinical development, later stages of clinical studies may require additional nonclinical toxicological studies, as exemplified in Figure 18.1. Enabling the duration of long pivotal studies is often a critical consideration, as is the patient population to be treated. As an example, in order to include women of childbearing potential (WOCB) or pediatric patients, relevant toxicity studies on fertility (often in rodent), reproduction, embryo/fetal and postnatal development (in rodent and rabbit), and juvenile animals will be required [9], as described in relevant OECD guidelines [99]. Furthermore, before registration of a new drug or when specific carcinogenicity concerns exist, as from the intended mode of action (e.g. PPAR-alpha agonist) or results from preclinical studies, carcinogenicity studies in two rodent species are required. These typically entail evaluation of the drug for 2 years in rats and 6 months in a transgenic mouse model [100, 101]. These types of study are conducted in a stepwise fashion to enable proper advancement of the clinical development program and associated decision making. There is therefore an opportunity to ensure that both human metabolites identified from clinical trials and compound/product batch impurities are evaluated in the said toxicology studies, as appropriate.

18.2 Conclusions

In this chapter, general safety assessment elements have been described with reference to a typical safety package from a regulatory point of view. Relevant guidelines have been cited as an aid to navigation during early clinical development. It is important to realize that safety assessments to support Phase I clinical trials are only the initial part of the toxicology evaluation in a drug development program. The continuous investigation and monitoring of toxicity signals, as from specific nonclinical studies or as observed in clinical experimentation, is of fundamental importance to the effective management and success of a program.

It cannot be overemphasized that the toxicity evaluation of a new drug is dependent on its experimental profile and mode of action, the context of its intended therapeutic use, and the associated competitive landscape. In most cases, the evaluation will be different across drug projects and will always reflect different

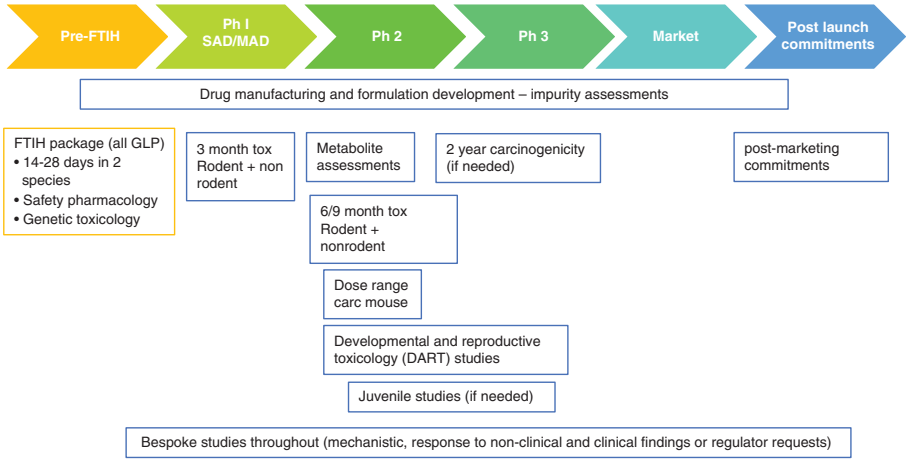

Figure 18.1 *The relation between clinical development stages and nonclinical safety activities*. To enable First Time In Man (FTIM) clinical trials, repeat dose, safety pharmacology and genetic toxicity studies are needed. The duration and timing of the toxicology studies should consider the clinical phases. Human metabolite assessments are needed to ensure the appropriateness of toxicology species, carcinogenicity assessment and reprotoxicity potential. Juvenile toxicity studies may be needed depending on the indication, the toxicological profile and the target ages of paediatric patients. Approval of the drug product may be contingent on post-approval studies. Throughout development and the product life cycle, additional toxicological support is needed to address emerging impurities.

risk perceptions and strategies at various institutions. It is therefore paramount to include consultations with other drug development disciplines (e.g. clinicians, drug substance manufacturers, drug product formulators), external experts, and regulatory agencies as an integral element of safety assessment. This will always inform the best possible decisions based on available investments, resources, and results in order to ensure that the toxicology program is fit for purpose.

List of Abbreviations

AC_{50}	Half-maximal activity concentration (either inhibitory or activating)
AUC	Area under the curve
CC_{50}	Half-maximal cytotoxic concentration
C_{eff}	Effective plasma concentration
C_{max}	Maximal plasma concentration
CNS	Central nervous system
CRO	Contract research organization
CV	Cardiovascular
CYP3A4	Cytochrome P450 family 3 subfamily A member 4
DRF	Dose range finding
GalNAc	*N*-Acetylgalactosamine
GI	Gastrointestinal
GLP	Good laboratory practice
GR	Glucocorticoid
HED	Human equivalent dose
ICH	International Conference on Harmonization
IMPD	Investigational Medicinal Product Dossier
LO	Lead optimization
MABEL	minimum anticipated biological effect level
MTD	Maximal tolerated dose
NOAEL	No observed adverse effect level
OECD	Organization for Economic Co-operation and Development
PCSK9	Proprotein convertase subtilisin/kexin type 9
PPAR	Peroxisome proliferator-activated receptor
QSAR	Quantitative structure–activity relationship
TI	Therapeutic index
TTC	Threshold of toxicological concern

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19

Nonclinical Safety Pharmacology

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19.1 Introduction

Primum non nocere, "first, do no harm," is one of the fundamental tenets of medicine and equally embraced in clinical research. In drug development, the iterative evaluation of risk–benefit is in constant play. The transition from laboratory research to the clinic is particularly challenging since prior to clinical testing, benefit is purely hypothetical. Therefore, the assessment of risk becomes paramount in this early stage of developing new drugs. With a few exceptions, all new chemical entities (NCEs) will need to be tested in humans, and to "do no harm," we must mitigate the risk in the transition from lab to human. The Nuremberg Code [1] and Declaration of Helsinki [2] provide ethical considerations and constraints to clinical research studies that center around evaluation and management of the risk to the human subject.

Toxicology studies are performed to assess the effect of an NCE on pathology and the physical–structural changes to the organism (see Chapter 18). The risk of serious injury and/or death in early clinical research can often involve acute failure of major organs like cardiovascular (CV), respiratory, and central nervous systems (CNS). Safety pharmacology focuses on adverse effects on organ function that are not easily detected by standard toxicity testing.

The general field of pharmacology can be subdivided into two designations: primary pharmacology, related to a drug's intended target and indication, and secondary pharmacology, which is unrelated to a drug's intended indication. Systematic integration of these secondary pharmacology studies in drug development was recognized and resulted in the formation of safety pharmacology as a distinct discipline. Safety pharmacology is multidisciplinary and pulls from physiology, biochemistry, anatomy, pathology as well as genetics, and cell and molecular biology.

The definition of safety pharmacology is to investigate potential undesirable pharmacodynamic effects on physiological functions in relation to exposure

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Edited by Fabrizio Giordanetto.

in therapeutic range and above (doses at or higher than the therapeutics dose/exposure). The objectives for safety pharmacology studies are threefold:

1) Identify undesirable pharmacodynamics relevant to human safety.
2) Evaluate adverse pharmacodynamics.
3) Investigate mechanism of adverse pharmacodynamics.

Prior to the formation of the safety pharmacology, the *ad hoc* evaluation of safety by various pharmacology studies was nonuniformed and taken on a case-by-case basis. Formalization of safety pharmacology as a distinct scientific discipline helped build a community to harmonize the research approach and data interpretation.

19.2 Historical Background

Safety pharmacology focuses on the development of predictive models to assess the risk of new drug candidates. The first regulatory document to specifically request adverse pharmacological evaluation of new drugs was published in 1975 by the Japanese Ministry of Health and Welfare, "Notes on Application for Approval to Manufacture (Import) New Drugs," which focused on the major organ systems. In 1995, the Japanese formalized the "Japanese Guidelines for Nonclinical Studies of Drugs Manual" that grouped general pharmacology studies into two lists [3]. List A became the "core" list and list B being the follow-up studies to be conducted depending on the results from list A. These lists of general pharmacology studies became the basis for the formalized international safety pharmacology guidance, ICH S7A, "Guidance for Industry: S7A Safety Pharmacology Studies for Human Pharmaceuticals," published in July 2001.

Somewhat in parallel, a growing discussion focused on CV safety arose from the high-profile drug withdrawals in the 1990s. Sudden deaths from Propulsid (cisapride), Seldane (terfenadine), Hismanal (astemizole), and Serdolect (sertindole), drugs of distinct pharmacological classes, mechanisms, and therapeutic use, were linked to prolongation of the QT interval resulting in torsades de pointes (TdP), a form of ventricular tachycardia. In 1996, the European Medicines Agency's Committee for Proprietary Medicinal Products (CPMP) published a draft "points to consider" document on QT prolongation, and the following year, the formal document, "The assessment of QT interval prolongation by noncardiovascular medicinal products," CPMP/986/96 established a guideline for testing. The fallout of these developments focused on the pharmaceutical industry to develop, validate, and standardize safety pharmacology models to identify and predict this cardiac risk, the evolution of which resulted in the nonclinical guidance document, ICH S7B, and clinical guidance, ICH E14.

Safety pharmacology as a formal drug development discipline grew out from general pharmacology in the 1990s. Initially, discovery scientists and researchers working in the area of secondary pharmacology would meet at the general pharmacology meetings as a specialized discussion group (see Ref. [4]). In 2001, the international Safety Pharmacology Society (www.safetypharmcology.org) was founded with the following mission statement:

Safety Pharmacology Society is a nonprofit organization that promotes knowledge, development, application, and training in Safety Pharmacology—a distinct scientific discipline that integrates the best practices of pharmacology, physiology and toxicology. The objective of Safety Pharmacology studies is to further the discovery, development and safe use of biologically active chemical entities by the identification, monitoring and characterization of potentially undesirable pharmacodynamic activities in nonclinical studies. The Safety Pharmacology Society also supports the human safety of drugs and biologicals by fostering scientific research, education, and dissemination of scientific information through meetings and other scientific interactions.

19.3 Regulatory Framework

Safety pharmacology now represents a structured and mature discipline. As with most drug development areas that evaluate safety, there are a number of international guidance documents that provide the regulatory framework for these studies. It is important to remember that the operative word is "guidance" and that these documents are not intended to be prescribed as checklists of studies, requirements in order to gain regulatory approval to conduct clinical trials, or for drug approval. Solid science and a mechanistic understanding must prevail in order to properly and appropriately assess risk of new drug candidates.

The practice of safety pharmacology is governed by guidance documents developed and published by the International Council on Harmonisation (ICH). The need to harmonize regulatory requirements became apparent in the early 1980s with the formation of the European Community, now called the European Union. The ICH began in April 1990 with representatives from Europe, Japan, and the United States and has formal processes and procedures for generating and adopting various guidance documents (http://www.ich.org/about/articles-procedures.html).

Key international guidance documents that govern safety pharmacology can be found at http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html, accessed February 14, 2017 and include:

1) ICH S7A. Safety Pharmacology Studies for Human Pharmaceuticals. The final form of the guidance was published in November 2000. ICH S7A addresses the definition, objectives, and scope of safety pharmacology studies as well as the need to conduct such studies before the initiation of Phase I clinical studies (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S7A/Step4/S7A_Guideline.pdf, accessed February 14, 2017).
2) ICH S7B. The Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals. The final form of this guidance was published in May 2005. ICH S7B describes the nonclinical testing strategy to assess the potential of new human pharmaceuticals to delay ventricular repolarization and possibly resulting in a fatal cardiac arrhythmia, such as TdP (http://www.ich.org/fileadmin/Public_

Web_Site/ICH_Products/Guidelines/Safety/S7B/Step4/S7B_Guideline.pdf, accessed February 14, 2017).
3) ICH M3(R2). Guidance on Nonclinical Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals. Published in 2009, ICH M3(R2) provides international harmonized standard for the nonclinical safety studies to support human clinical trials of a given scope and duration (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M3_R2/Step4/M3_R2__Guideline.pdf, accessed February 14, 2017).
4) ICH S6. Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals, published in June 2011. This guidance covers the preclinical testing requirements for biologics. It addresses the use of animal models of disease in the evaluation of safety (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S6_R1/Step4/S6_R1_Guideline.pdf, accessed February 14, 2017).
5) ICH S9. Nonclinical Evaluation of Anticancer Pharmaceuticals. This guidance provides information for pharmaceuticals that are only intended to treat cancer in patients with late-stage or advanced disease. ICH 9 was published in October 2009 (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S9/Step4/S9_Step4_Guideline.pdf, accessed February 14, 2017).

19.4 Role in Discovery and Candidate Selection

Safety pharmacology is integral in the discovery and development of new drugs. In drug discovery, it is critical to define a compound's pharmacological properties and select compounds with optimal properties. The therapeutic and safety profile of a compound is defined by its primary pharmacology as well as secondary and safety pharmacology. Other considerations include regulatory constraints, intended patient population, and competitive landscape.

New drug candidates are often identified by their binding to a target receptor or enzyme. This becomes the basis for the compound's primary pharmacology. Binding to a target can result in the activation, inhibition, or modulation of a pharmacological response, and the primary pharmacological responses can be measured *in vitro* or using cell-based assays. The observed responses might include activation of a second messenger pathway, regulation of gene transcription, or changes in cellular metabolism. The primary pharmacology response propagates in increasing order of complexity, starting with biochemical changes at the cellular level to changes in the organ or tissue function to physiological or behavioral changes in the whole organism. Importantly, during the drug discovery phase, the amount of compound required also increases with assay complexity. Receptor binding and cell-based assays require milligrams of compound, whereas *in situ* organ preparations like the isolated Langendorff heart can use hundreds of milligrams. *In vivo* or whole animal studies can frequently require gram quantities of drug.

Compound selectivity is critical in developing new therapeutics. Once the primary pharmacology is defined, the off-target interactions of a compound can be assessed by screening the putative ligand against a wide variety of molecular targets (i.e. receptors, ion channels, enzymes, and transporters). These ligand displacement assays are described elsewhere in the book (see Chapter 18 for detailed review). Contract service providers, such as Eurofins Pharma Discovery Services (http://www.eurofins.com/biopharma-services/discovery/, accessed February 14, 2017), formerly Panlabs and Cerep, offer binding assays for numerous molecular targets with over 1500 validated *in vitro* pharmacological assays that cover a broad range of targets. Usually, ~1 mg of compound is sufficient to screen against 120 or more potential targets using these *in vitro* displacement assays. "Hits" are initially identified by >50% target occupation using a 0.1–1 μM concentration of the test compound. The initial hits are generally further characterized with the quantitative precision of determining the IC_{50} and assessing potency. As the initial screening procedure using a binding assay cannot distinguish between agonists and antagonists, further *in situ* or cell-based assays need to be conducted.

Selectivity of drug interaction can be classified as either on-target or off-target. Off-target interactions are when a compound interacts with an unintended target. Although compounds are intended to activate or inhibit a particular target, seldom is the interaction 100% selective for that intended target. This lack of selectivity is more likely for small molecules than for biological drugs including monoclonal antibodies. Within the realm of safety pharmacology, if an unexpected pharmacological response for a lead molecule is observed in a toxicity study or clinical trial and is related to off-target activities, it may be possible to switch to a backup compound that happens to have less of the off-target interactions. Efavirenz is an antiviral drug of the non-nucleoside reverse transcriptase inhibitor class. It has off-target interactions with serotonin 5-$HT_{2A/C}$ receptors, serotonin and dopamine reuptake transporters, monoamine transporter, and $GABA_A$ receptor. The off-target properties of this drug are responsible for some of the key side effects, namely, mood changes, anxiety, dizziness, sleep disturbance (e.g. insomnia, nightmares), and even psychosis. One strategy for "me-too" drugs is to conduct medicinal chemistry on the compound so as to "engineer out" the off-target activity with the objective to minimize the side effects associated with a "next-in-class" drug. An improved safety profile can be an important differentiator.

Another major source for adverse pharmacological action is from on-target interactions (as from the intended receptor engagement) in unintended tissues or organs often referred to as right target, wrong tissue. The adverse on-target pharmacological property of some drug candidates can be managed by designing a partial agonist/antagonist or designing some sort of allosteric regulator of the target [5]. Pharmacokinetic variations or changing the route of administration, such as subcutaneous or intramuscular routes of administration for an IV drug, can avoid the associated concentration-related adverse events (AEs) without sacrificing exposure. Localized administration, direct to the target organ, or the identification of a backup molecule with better tissue selectivity can also minimize on-target toxicity. An example of right target, wrong tissue is the histamine H_2

antihistamines. The first-generation antihistamines had sedation as a side effect. This is because these drugs could cross the blood–brain barrier and the interaction with CNS histamine receptors resulted in drowsiness and sedation. The second-generation, non-sedating antihistamines were engineered to keep them out of the CNS. By avoiding the CNS histamine receptor, these next-generation drugs were found to lack the side effect. All these can be confirmed in safety pharmacology studies. However, if the undesired pharmacology is an adverse on-target effect, then serious consideration must be given to whether that target is appropriate for therapeutic intervention at all.

19.5 Preparation for First-in-human Studies

Safety pharmacology studies are key in aiding the transition of a compound from preclinical to clinical research. As the key to early clinical research is minimizing and managing risk, the detailed understanding of the adverse pharmacological properties and its potential mechanism is needed prior to the first-in-human study. Good translatability and predictability of the nonclinical models to human response is also paramount. The specific safety pharmacology guidance documents were developed to make use of models with good translatability and to standardize the testing paradigm in order to be able to compare study-to-study results. As mentioned previously, it is important to remember that these are not prescribed checklists. Good scientific practice and experiments set forth to prospectively test a hypothesis is central to all research and critical in research that will be used to assess the risk to human safety.

19.5.1 Introduction

ICH S7A: Safety pharmacology studies for human pharmaceutics was published in November 2000. The objective of the guideline was to protect human safety in early clinical trials by focusing on the effect of NCEs and biotechnology-derived products on vital organ function (Table 19.1). The general principle of ICH S7A was to adopt a rational and consistent approach to evaluate pharmacological risk. As safety pharmacology investigates potential adverse pharmacodynamic effects on critical physiological functions, its scope has been focused on vital organ systems, namely, the central nervous, CV, and respiratory systems. Although the guideline focuses on the key organ systems, the effect of new drugs on other organ systems (e.g. renal, gastrointestinal (GI)) should not be ignored, especially if a pharmacological rationale can be made.

Table 19.1 Key elements of the core battery assessment (ICH S7A).

CNS	Cardiovascular	Respiratory
Neurological–muscular	Blood pressure	Respiratory rate
Autonomic	Heart rate	Tidal volume
Behavior	Electrocardiogram (ECG)	Gas exchange

Table 19.2 Ventricular repolarization assessments (ICH S7B).

Nonclinical testing categories	Guidance
Chemical/ pharmacological class	Consider whether the compound belongs to a chemical or pharmacological class in which previously member has been shown to pose a risk for QT prolongation
In vitro I_{kr} assay	Typically, an electrophysiological assessment of a compound on the hERG potassium channel that is primarily responsible for the I_{kr} current
In vivo QT assay	Evaluation of ECG waveforms in an animal. ECGs can be collected by surface electrodes (via jacket) or implanted telemetry devices

The core battery concentrates on the following vital organ functions:

1) *CNS*. Motor, behavior, coordination, sensory, body temperature.
2) *CV*. Blood pressure, heart rate, electrocardiogram (ECG).
3) *Respiratory*. Respiratory rate, tidal volume, oxygen saturation.

ICH S7B, the Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals, extends CV evaluation delineated in ICH S7A to include specific strategies for assessing the potential for fatal arrhythmia (Table 19.2). The primary focus of ICH S7B is on changes in electrical conduction; however, it is important to reiterate that other factors can contribute to delayed ventricular repolarization such as autonomic nervous system activity and metabolic state.

19.5.2 Objectives

In the selection and design of safety pharmacology studies, it is important to consider various properties of the test article, including:

1) Intended therapeutic function.
2) Chemical class.
3) Pharmacological (both on-target and off-target).
4) Pharmaceutical properties.

The selection of the core battery test system(s) and the study design of safety pharmacology studies needs to be carefully considered. Off-target receptor interactions can help inform on potential pharmacological responses, and they need to be evaluated in the core battery tests.

The intended patient population may not tolerate even mild changes in a particular organ function. For example, Parkinson's patients are prone to falls, and therefore, a potential therapeutic that causes dizziness or results in hypotension may be especially problematic in that patient population, exacerbating instability and falls. Other organ systems can be equally important depending on the comorbidities of the intended patient population. For example, a particular drug

may be well tolerated in a young, generally healthy population; however, in a diabetic patient with renal dysfunction, the reduction of drug clearance by the kidney could lead to supra-therapeutic levels of drug.

19.5.3 General Principles

Safety pharmacology studies are expected to be conducted under good laboratory practices (GLP), and as with toxicity studies, when feasible, safety pharmacology studies need to employ the clinical route of administration. The sample size for safety pharmacology studies should take into consideration the variability of the outcome measures from both an analytical and biological point of view. There needs to be sufficient confidence to draw a correct scientific interpretation of the data. Because of this, safety pharmacology studies often include both positive and negative controls. Prior validation of the model system is also helpful in data interpretation from new compounds.

Safety pharmacology studies should define a dose–response relationship; therefore, a minimum of three doses or concentrations need to be tested. More detailed dose–response curves are often needed to compare the adverse (secondary) pharmacology with the efficacy (primary) pharmacology profiles. Doses and concentrations should exceed the primary pharmacological response. In some cases, the maximal feasible dose will need to be used and justified.

19.5.4 Central Nervous System

CNS is a critical organ system and one in which there is significant difference between animals and human. As such, the translation or prediction potential from the observations of animals to human is imperfect. CNS evaluation should include changes in motor activity, behavior, coordination, sensory and motor reflexes, and body temperature (Table 19.3). This is often accomplished with a functional observation battery (FOB) or modified Irwin test [6, 7]. Rodent is a commonly used species for these tests; however, as with all *in vivo* pharmacology studies, it is important to justify the use of a particular species, considering any difference in pharmacology between species including the prevalence of the target in the test species compared with human. The primary objective of all safety pharmacology studies is to help assess human risk.

A survey of industry best practices was recently conducted [8]. For CNS safety pharmacology, the FOB/Irwin tests were most often conducted in rat with about an equal number of studies done in mouse, monkey, and dog. Only a very small percentage of FOB studies were conducted in mini pig. Almost three-quarters of the CNS safety pharmacology studies were done as stand-alone studies; however, adding FOB to a toxicity study was fairly common. Key factors in conducting the FOB/Irwin study include ensuring animal evaluation is conducted in a dedicated and quiet room and by blinded and trained personnel. Of the parameters collected in FOB/Irwin studies, a majority included rectal temperature, open-field evaluation, grip strength, and pupillary light response. Additional CNS safety pharmacology studies to be considered in early drug development include seizure liability [9] as well as drug abuse liability studies [10].

Table 19.3 Test parameters and domains for cerebral function evaluation.

Neurological
- Muscle tone
  - Forelimb grip strength
  - Hypotonia
- Gait and equilibrium
  - Righting reflex
  - Ataxia
  - Gait
  - Posture
- CNS excitation
  - Tremors
  - Twitches
  - Clonic convulsions
  - Tonic convulsions
  - Stereotypic behavior

Autonomic
- Lacrimation
- Exophthalmia
- Pupil reflex
- Pupil size
- Palpebral closure
- Salivation
- Piloerection
- Breathing
- Rectal temperature

Behavioral
- Spontaneous activity
  - Hypoactivity
  - Hyperactivity
- Affective response
  - Reactivity to catching
  - Reactivity to handling
  - Behavior
  - Fur appearance
  - Grooming
  - Defecation
  - Urination
- Sensory
  - Auditory startle reflex
  - Visual stimulus
  - Touch response
  - Tail-pinch response

Common drug-induced CNS issues encountered included seizure, tremors, gait–coordination abnormalities, emesis, salivation, and sedation. Interestingly, in Phase I clinical trials, the most common CNS AEs were dizziness, headache, fatigue, and emesis/nausea [11]. Of the common clinical adverse findings, headache, dizziness, and fatigue do not have a preclinical correlate, a challenge for future preclinical CNS testing models.

19.5.5 Cardiovascular System

CV system evaluation as specified in ICH S7A should include effect on blood pressure, heart rate, and ECG. Additional guidance on proarrhythmic assessment is included in the ICH S7B guidance document, and best practice in the industry does not separate tests to satisfy ICH S7A from ICH S7B. The combined objective is to gain insight into CV and cardiac risk. In order to do so, an initial evaluation starts with assessing the interaction of a test compound with the primary cardiac ion channels. This is often done during drug discovery when looking for off-target interactions, specifically evaluating interaction with the hERG channel protein. However, most often in drug discovery, hERG channel screening is not done to the standards and controls necessary for regulatory submission (e.g. GLP compliance, use of controls, measurement of drug concentrations).

Nonclinical CV safety testing is best categorized by the various elements and functions of the CV system. Heart function and blood pressure are the most common elements of the CV system, and they are profoundly interconnected. There are several physiological factors that regulate blood pressure including blood volume, cardiac output, and peripheral resistance. Drug-induced changes in blood pressure can result from a pharmacological change in any of these factors. For example, diuretics that change kidney function with the removal of sodium and water lower blood pressure. A drug that can alter either heart rate or stroke volume, two key parameters of cardiac output, will result in blood pressure changes. Peripheral resistance that includes compliance or the elasticity of the blood vessels will change the amount of force necessary to push blood through the vasculature in which an increase in resistance will result in an increase in blood pressure.

In order to pump blood throughout the body, the heart muscle itself must function properly. Additionally, the coordinated contraction–relaxation of the heart ventricles is of special importance. The coordinated contraction is regulated by electrical conduction that can be measured by electrodes placed either on the surface of the animal or in the internal cavity. The resulting change in electrical conduction measured as a function of time is the ECG.

The surface ECG represents a three-dimensional composite of the electrical activity of the heart and corresponds to a summation of the cardiac action potential (Figure 19.1). The resting membrane potential of a cardiac muscle cell is typically −70 mV. An action potential is triggered by the initial opening of sodium channels. The flow of positively charged sodium ions results in membrane depolarization in which the membrane potential increases and becomes slightly positive. This sodium current or I_{Na} produces the characteristic upstroke of the action potential (Figure 19.1a). The sodium channels are sensitive to membrane potential and will inactivate or close. When the membrane is depolarized (zero or slightly positive), calcium channels open and allow calcium to flow into the cell, which gives rise to the slight increase in membrane potential as seen in the cardiac action potential. Membrane depolarization also opens potassium channels, and potassium ions will flow from inside to the outside of the cell, resulting in repolarization or resetting the membrane potential to −70 mV.

The initiation or upstroke velocity of the cardiac action potential corresponds to the start of the QRS complex on the ECG. Repolarization of the cardiac action

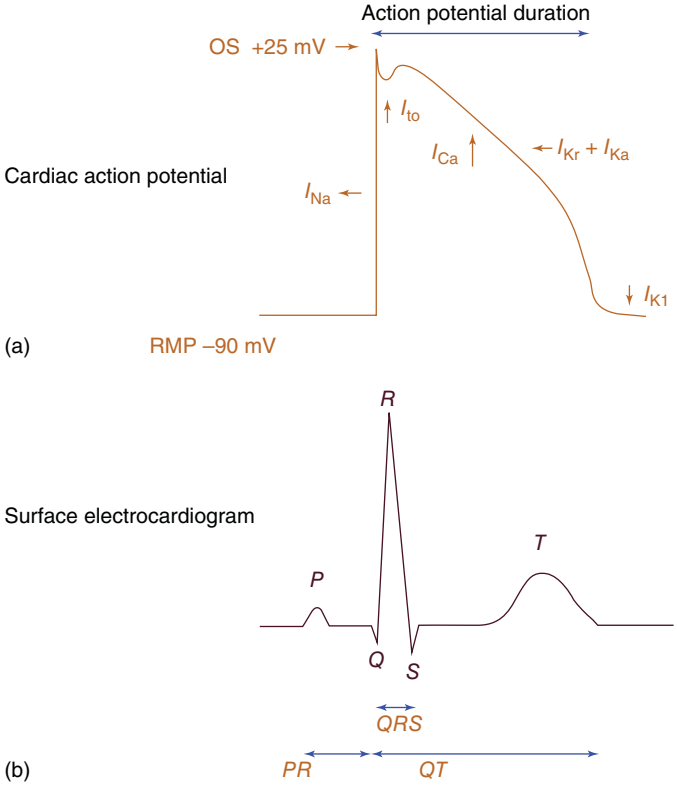

Figure 19.1 Temporal relationship between surface ECG and cardiac action potential. Reducing repolarization of the cardiac action potential by inhibiting the I_{kr} current results in an increase in the action potential duration (APD), which, in turn, increases the surface ECG QT interval. *Source:* From: Ref. [12]. http://www.dddmag.com/Article-Guarding-the-Heart-060109.aspx.

potential then represents the end of the T wave in the ECG. Inhibiting potassium efflux will result in a time lag or delay in repolarization which in turn lengthens the QT interval. The inward potassium ion flux that contributes to repolarization is predominantly mediated by two channels, the rapid delayed rectifier, I_{kr}, and the slow delayed rectifier, I_{ks}, and inhibition of the inward potassium current results in an increase in the action potential duration (APD). Drugs that block I_{kr} (hERG) or I_{ks} can prolong the QT interval. Delayed repolarization and potentially QT prolongation can also result from sodium- or calcium-mediated inward currents, and their contribution to QT prolongation should also be considered.

QT prolongation increases the risk to develop a ventricular arrhythmia; however, other factors or triggers are needed to create atrial fibrillation and cardiac arrest. One such situation is when cardiac depolarization occurs before repolarization from the previous beat is complete ("R on T" phenomenon) and can be observed as early after depolarizations in the cardiac action potential.

19.5.5.1 Chemical or Pharmacological Class

Certain classes of compounds are known to be associated with QT prolongation such as the macrolide (erythromycin) and quinolone (ciprofloxacin, moxifloxacin) antibiotics. Certain antipsychotics like haloperidol, chlorpromazine, and thioridazine are also known to be associated with QT prolongation and proarrhythmic risk. NCEs of these chemical or pharmacological class should be carefully evaluated. A database of potential drugs with QT risk can be found on the Arizona Center for Education and Research on Therapeutics (AZCERT) website (http://www.crediblemeds.org; accessed January 12, 2017).

19.5.5.2 *In Vitro* Evaluation of I_{kr} (hERG) Inhibition

Although delayed repolarization and QT prolongation can be mediated by multiple mechanisms and ion currents (sodium, potassium, or calcium), the I_{kr} current is primarily responsible for repolarization and potassium efflux mediated by the hERG (human *Ether-à-go-go*) channel, also known as KCNH2 or $K_v11.1$. The hERG channel is known to be promiscuous, allowing for many classes of small molecule drugs to bind and inhibit. This is due to the structure of the channel, which includes aromatic amino acids lining the central pore [13].

The quantitative assessment of hERG-mediated inhibition of potassium current can be evaluated *in vitro* using heterologous cell systems expressing the cloned hERG channel. Single-cell electrophysiological recordings are made with voltage- or patch-clamp techniques. The concentration effect of various drugs on current through this channel can be measured, and the ability of a compound to block or inhibit the current is expressed as the concentration required for 50% inhibition or IC_{50}.

Inhibition of the hERG channel is not perfectly predictive for clinical arrhythmogenic risk. Although some drugs like dofetilide (3.9 nM), cisapride (6.5 nM), and astemizole (0.9 nM) are potent hERG inhibitors ($IC_{50} < 10$ nM) and known to be associated with TdP risk, there are examples of other drugs associated with TdP risk in which the hERG IC_{50} is very high (e.g. 0.1–0.6 mM for D-sotalol). Likewise, potent hERG inhibitors like verapamil are not known to cause TdP.

A retrospective study of 100 drugs found that a 30-fold margin between the IC_{50} for hERG to the potency for the intended target may provide reasonable risk for early drug development [14]. The 30 : 1 ratio of hERG IC_{50} to target provides a concentration margin for off-target interaction with hERG, with the larger the ratio, the lower the risk.

19.5.5.3 Cardiac Action Potential

Generation of the cardiac action potential is mediated by sodium, potassium, and calcium ion currents; therefore, evaluation of the potential drug effect on the cardiac action potential provides an integrated assessment. The action potential can be studied in isolated Purkinje fibers (rabbit or dog), ventricular myocytes, or intact papillary muscles. The excised tissue or cellular preparation is electrically stimulated, and the resulting action potential electrophysiologically recorded. Several concentrations of drug are tested in the same preparation by means

of perfusion or superfusion in order to produce a dose–response curve. The parameters measured include the time required for the action potential to reach 50% repolarization (APD_{50}) or 90% (APD_{90}) as they are generally correlated with the ECG QT interval (see Figure 19.1). Inhibition of repolarization mediated by the I_{kr} or I_{ks} currents would increase both the APD_{50} and APD_{90}. Additionally, delayed repolarization due to changes in sodium or calcium currents could also be detected by a change in APD_{50} and APD_{90}. The drug effect on *in vitro* APD measurements is known to be influenced by the frequency of electrical stimulation; most drugs that delay repolarization have greater effect at slower stimulation rates.

One advantage of the cardiac action potential model is that cardiac liability other than delayed repolarization can be evaluated. Drug-induced inhibition of sodium channels will reduce the initial depolarization or upstroke velocity (V_{max}), and calcium channel blockers will shorten the APD_{50}. Moreover, effects on the resting membrane potential as well as changes in the upstroke velocity and amplitude can slow ventricular conduction and also have the potential to promote arrhythmia.

19.5.5.4 *In Vivo* Assessments

A variety of species (rat, guinea pig, dog, pig, monkey) can be used to evaluate the effect of a new drug on heart rate, blood pressure, and ECG. Although there are many advantages to anesthetized animals like being able to pace or control the heart rate, in safety pharmacology evaluations, conscious animals with either surface electrodes or implanted telemetry transmitters are most common.

There are limitations to all animal models. For example, the rat lacks I_{kr} completely, the architecture of the pig Purkinje system is unlike that of man [15], and the dog has a large heart (~1% of body weight), whereas the human heart is ~0.3–0.5%. Although the dog Purkinje architecture is similar to man, the dog has a short QT interval and is prone to respiratory sinus arrhythmia. Species differences need to be considered when interpreting results from these animal models.

Many physiological factors can affect the QT interval; therefore, these factors such as stress and activity need to be well controlled. It is common practice to accustom the animal to dosing and/or restraint. For conscious animal studies, often 3–4 animals per group are evaluated. Telemetry transmitters can be surgically implanted, which measure blood pressure, heart rate, and the lead II ECG. Surface electrodes are also used in restrained (sling) animals or, more recently, with a jacket that allows the animal greater mobility. Interesting to note, the vagal tone of a relaxed dog, as common in a sling restraint, dramatically changes the QT interval [16].

Heart rate is a major factor influencing the QT interval. The QT interval becomes longer as the heart rate slows. The inverse relationship between heart rate and QT interval is not simple as it takes a period of one to two minutes for the QT interval to reach a steady state after a period of heart rate change. If at all possible, it is therefore prudent to ensure that the complexes used in the analysis of QT interval be preceded by a period of stable heart rate.

19.5.6 Respiratory System

Many drugs from all drug classes are known to have an adverse effect on the respiratory system. The onset of drug-induced bronchoconstriction or respiratory depression can be rapid and life threatening. Aspirin and beta-blockers are two common drugs that cause bronchoconstriction, and the opioid analgesics are well known to depress respiratory rate. Safety pharmacology studies of the respiratory system should therefore evaluate respiratory rate and measures of respiratory function like tidal volume or oxygen saturation (see Ref. [17]). The clinical observation of animals in a toxicity study is usually not sufficient. Rodent models using plethysmography (head out or whole body) can capture many clinically relevant respiratory parameters, and more recently, animals instrumented with pressure inducers have been used to monitor the effect of drugs on respiratory function [18].

The acute effects of drugs on the respiratory system can be divided into two main areas: (i) alteration of the pumping mechanism and (ii) changes to gas exchange. Adverse effects to either of these areas will result in compromised lung function (see Ref. [19]).

The pumping mechanism is responsible for the inflation and deflation of the lung, which is controlled by changes in the pleural pressure mediated by the diaphragm. When the diaphragm contracts, the chest cavity enlarges, which reduces the pressure inside the lungs. This in turns results in air entering the lung. When the diaphragm relaxes, the change in pleural pressure pushes air out of the lung. Since respiratory muscle contraction is regulated by the respiratory center in the brain stem, drugs can have an adverse effect on the respiratory pumping mechanism by affecting the respiratory center in the brain and/or affecting the respiratory muscles themselves.

The key function of the lungs is its ability to exchange oxygen and carbon dioxide between air in the environment and blood. Adverse drug effects could result in a reduction of gas exchange efficiency by decreasing airflow into the lung. The two main drug-induced causes for this are bronchoconstriction and reduction in lung elasticity. The most common drug-induced bronchoconstriction is the result from smooth muscle contraction.

Plethysmography is a common technique used to assess respiratory function. Essentially, a pneumotachometer (pneumotach) measures airflow and the corresponding change in volume. For recording in animals, the pneumotach can be connected to a face mask (large animal) or chamber (rodents). Plethysmograph chambers measure either direct lung volume changes or changes in thoracic movements. Ventilatory parameters such as tidal volume, respiratory rate, and minute volume are derived from these measurements.

19.5.7 Supplemental Safety Pharmacology Studies

The core battery represents evaluation of the major organ systems; however, as the mammalian (and human) body is an integration of all organs, malfunction of one organ system can have a profound effect on the others. For example, the autonomic nervous system controls arterial muscle tone. Constriction mediated by α-adrenergic stimulation results in an increase in blood pressure (hypertension),

which, in turn, can affect the function of both the heart and kidney. The kidney can have an impact on cardiac function since electrolyte balance is controlled by the kidney, and hypokalemia (low blood potassium) can result in ECG changes, namely, ST segment depression, inverted T waves, large U waves, and prolonged PR intervals. Therefore, careful and critical evaluation of all data is necessary to provide an assessment of risk.

Supplemental safety pharmacology studies are meant to evaluate potential adverse effects on organ system function not specifically addressed by the core battery. The word "supplemental" is unfortunate in that it suggests studies falling in this category are optional. Supplemental safety pharmacology studies can and should investigate unexplained observations from other nonclinical studies or even provide a mechanistic understanding of adverse pharmacology observed in clinical studies. Two examples of supplemental safety pharmacology studies are listed below; however, this is not meant to be an extensive or exclusive review. Scientific exploration needs to be taken on a case-by-case basis.

19.5.7.1 Renal Safety Pharmacology

The kidney is a major organ for the elimination of drugs and/or metabolites, and much focus in drug development is the effect of renal impairment on pharmacokinetics (see Ref. [20]). However, the kidney is critical in the regulation and maintenance of a variety of physiological functions like fluid and electrolyte balance and control of blood pressure.

Assessment of drug-induced changes in renal function is either accomplished as a stand-alone, single-dose safety pharmacology study or, more often, incorporated into a repeat–dose toxicity study. Renal parameters that are measured include urinary volume and urine properties like specific gravity, osmolality, pH, electrolyte composition, and presence of protein or cells in the urine. Blood chemistry measurements such as blood urea nitrogen, creatinine, and plasma protein can also be an indicator of renal function. Various animal and *in vitro* models have been used to evaluate renal function (see Ref. [21]).

19.5.7.2 Gastrointestinal System

Serious adverse effects and adverse drug reactions (ADRs) resulting from GI injury are rare, representing less than 2–3% of drug withdrawals [22]; however, GI disturbances are one of the most common adverse effects reported in early clinical studies. Adverse GI effects can range from nausea, diarrhea, and constipation to ulceration and inflammation. Certain therapeutic classes of drugs are known to have GI side effects like opioids resulting in constipation and NSAIDs causing GI ulcers. Common parameters measured to evaluate renal function include gastric secretion, bile secretion, intestinal transit time, and ileal contraction.

19.6 Translation from Nonclinical Safety Pharmacology to the Clinic

A key objective to the conduct of nonclinical safety pharmacology studies is to understand the potential risk of NCEs to clinical AEs. There have

been several published reviews mapping nonclinical findings to ADRs (see Refs [3, 23, 24]). Although imperfect, continual refinement of our models has improved predictability.

A publication from Japan explored the ability to predict clinical ADRs from nonclinical safety pharmacology studies [3]. They found some intuitive findings like a correlation of decreased locomotor activity in rodents to dizziness and sleepiness in humans and constipation in humans correlated with a decreased intestinal transit in animals. There were additional findings that were more difficult to explain with face validity, such as analgesia, decreased body temperature, and anticonvulsive activity in animals mapping to thirst in humans.

The ability to predict human ADRs from animal studies was published in 2000 [24]. The results from 150 compounds found an overall concordance rate of 71% and a 63% prediction based on non-rodent/dog data and 43% for rodent.

Recently, the predictive value of rodent CNS safety pharmacology studies to ADRs observed in Phase I studies analyzed unpublished data from 141 small molecule compounds at 5 pharmaceutical companies [23]. In the data analyzed, the most frequent clinical AEs were headache, nausea, dizziness, fatigue/somnolence, and pain. Prior to data analysis, the investigators postulated that the most common clinical AEs would map to specific nonclinical parameters, termed "plausible correlates." For example, nausea would correlate to a decrease in body weight gain or decreased food consumption in animals, and dizziness in human would map to a decrease in horizontal locomotion and decreased rearing in animals. Unfortunately, there was a lack of translation for the plausible correlates, which may be the result of a difference in the physiological response between species.

The validity and predictability of nonclinical QT assessment is key to the nonclinical-to-clinical translation. Two major initiatives were undertaken by the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI-HESI) and the Japan Pharmaceutical Manufacturers Association (JPMA) to validate the assays outlined in the ICH S7B guidance document. Multiple laboratories using drugs that are known to prolong QT as well as negative controls replicated the hERG, cardiac action potential, and ECG assays.

In vivo assessment of QT prolongation was undertaken by the QT PRODACT (QT Interval Prolongation: Project for Database Construction), a JPMA initiative. A total of 21 compounds, 11 of which are known to prolong the QT interval, and 10 QT negative compounds were studied in an APD assay to look at the drug effect on the cardiac action potential. The same set of drugs were also evaluated in both conscious and anesthetized dog and the *Cynomolgus* monkey [25].

The APD assay was conducted in guinea pig papillary muscle. Seven of the eleven positive control compounds tested resulted in an increase in APD_{90} of more than 10%. Nine of the eleven compounds increased the APD_{30-90}, an index of I_k or "triangulation." The triangulation parameter, APD_{30-90}, had better predictability for inhibition of potassium-mediated changes in repolarization. This basic observation was confirmed by the ILSI-HESI initiative in dog Purkinje fiber, in which the APD_{90} was an imperfect predictor of delayed repolarization, whereas triangulation, APD_{90-40} in the case of the ILSI-HESI study, was more reliable. For the *in vivo* studies, a good correlation ($R^2 = 0.947$) was

observed between the results obtained in conscious dog and conscious monkey, suggesting both species were equally robust with monkey being more sensitive than dog to ventricular arrhythmias with astemizole, cisapride, and sotalol at equivalent plasma exposures. A good correlation ($R^2 = 0.816$) was observed between conscious and anesthetized dogs with the anesthetized dog expressing haloperidol-induced ventricular arrhythmia but not in conscious animals.

The ILSI-HESI initiative complemented QT PRODACT and focused on three nonclinical assays: hERG current inhibition, Purkinje fiber repolarization, and *in vivo* QT evaluation in conscious dog [26]. All positive control compounds in the conscious dog study increased QTc by approximately 20 ms during periods of high drug plasma concentrations, and none of the negative control compounds produced a signal of QT prolongation when QT was corrected for heart rate using Fredericia or individual formulas [26]. QT correction by Bazett misidentified some negative control compounds as QT prolonging, possibly because Bazett's formula overcorrects QT at higher heart rates [26]. The conclusion of the ILSI-HESI study is that QT evaluation in dog is a good predictor of potential risk.

The ILSI-HESI and QT PRODACT projects confirmed that the nonclinical safety pharmacology studies outlined in ICH S7B are sufficient to evaluate potential for QT prolongation and delayed repolarization. However, to adequately evaluate human risk, an integrated risk assessment looking at a body of evidence is necessary since single, individual tests are always imperfect and not 100% predictive.

19.7 Future Directions and Current Discussions

As new classes of therapeutics are developed, the biological, pharmacological, and biochemical interactions on the major organ systems need to be understood. Additionally, validation of the predictive value of these nonclinical studies will be key in the translation of human risk. These will remain a constant challenge in safety pharmacology.

The most significant future changes in safety pharmacology may involve how CV safety testing is conducted. ICH E14, the clinical QT prolongation and proarrhythmic guidance document, was published in final form at the same time as the nonclinical guidance, ICH S7B. ICH E14 introduced the "thorough QT study (TQT)" into the drug development lexicon and specified that NCEs needed to be clinically tested for the potential to prolong the QT interval regardless of nonclinical findings. This regulatory framework has been in place for more than ten years, and reevaluation of the testing paradigm is currently under discussion.

Recently, several joint meetings between the FDA and the Cardiac Safety Research Consortium (CSRC) have occurred to discuss various topics on the future of both nonclinical and clinical cardiac safety evaluation in drug development (see Ref. [27]). Much of the discussion was spurred with the publication of the December 2015 E14 Q&A(R3) document elaborating on the role of pharmacokinetic–pharmacodynamic (PK–PD) modeling in early-phase clinical studies as an alternative to a dedicated TQT trial [28, 29].

In parallel to the discussion regarding changes in the clinical evaluation of QT prolongation, comprehensive *in vitro* proarrhythmia assay (CiPA), a nonclinical initiative, began following a 2013 FDA workshop. The objective of CiPA was to develop an assay for assessment of the proarrhythmic potential of new drugs with improved specificity, an alternative or possibly complement to the current hERG plus TQT study. Current information on the progress of and publications from the CiPA initiative can be found at www.cipaproject.org.

The CiPA initiative is a comprehensive mechanistic approach to characterize a compound's proarrhythmic liability, which is designed to potentially supplant the current ICH S7B preclinical model. This is a quadripartite effort involving the following components:

- Explore the impact of seven ion channels on the QT interval rather than focus just on the hERG potassium channel.
- Use *in silico* computer modeling to determine the proarrhythmic potential of the compound on ventricular tissue.
- Incorporate human-derived stem cell cardiac myocytes in the testing of arrhythmogenicity of the compound.
- Integrate the data from the above studies and benchmark them against 28 known compounds with predefined proarrhythmic risk.

19.8 Summary

The core battery for safety pharmacology as specified in the ICH S7A and S7B guidance documents provides a solid, robust, and validated paradigm for testing all NCEs. A wealth of data has been accumulated, and an appreciation and understanding for the limits of these experimental test systems used. That said, it is important to consider the body of evidence and understand the basic pharmacological and biochemical mechanisms in order to prepare an integrated risk assessment.

There are many published reviews on the specific conduct of safety pharmacology studies: for example, Current Protocols in Pharmacology (Wiley), volume 10, is solely focused on methods for safety pharmacology studies. Additionally, the first complete textbook on safety pharmacology was recently published (see Ref. [30]). These and other resources are available for the conduct of safety pharmacology studies [31–40].

List of Abbreviations

AE	adverse event
CiPA	comprehensive *in vitro* proarrhythmia assay
CNS	central nervous system
CSRC	Cardiac Safety Research Consortium
CV	cardiovascular system
FDA	US Food and Drug Administration
ICH	International Council on Harmonisation

NCE new chemical entity
TdP torsades de pointes

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Early Drug Development

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20.1 Introduction

Methods and design of predictive technologies are currently undergoing intense development and refinement to identify and anticipate drug toxicities prior to further downstream investments in a drug development program. Over the past decade, the field of computational toxicology has seen an explosion of research especially in the development of prediction tools. These tools include toxicogenomic applications but in recent years have especially focused on chemical informatics (aka cheminformatics) approaches [1, 2]. Cheminformatics approaches generally aim to test and evaluate drug and chemical substances *in silico* (assisted by computer analysis). In terms of drug development, the value of cheminformatics approaches goes beyond compound library screening for lead identification of a defined therapeutic target. Cheminformatics is now frequently utilized to provide an evidentiary base of information that, when combined with human expert knowledge, generates scientific evidence on the potential of a compound to possess liabilities to the safety profile of an eventual drug product. Furthermore, the cheminformatics techniques of computational toxicology are utilized for hazard identification screening, mechanism-of-action studies to discover off-target toxic effects, classification of compounds, and exposure assessment and when integrated with toxicogenomic applications help to define genetic susceptibility. From both ethical and financial standpoint, the obvious expectation for any predictive computational toxicology method is to reduce the use of animal-based experimental testing [3, 4].

At the US Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), drug safety evaluators have access to predictive data from computational toxicology methods, in particular, generated through cheminformatics analysis. Electronic databases of drug molecular structures house relational data sets from public and private sources [5]. The FDA has launched landmark regulatory science initiatives and strategies that embrace the development of predictive computational toxicology models. The purpose is

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Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

to modernize toxicology and enable risk assessments so as to make better and more informed decisions on the safety of regulated products [5, 6]. Predictive computational toxicology is now globally recognized for regulatory use through the finalization and implementation of internationally harmonized guidance. This is exemplified by the recommendation of structure-based computational toxicology assessments in the absence of mutagenicity testing data to qualify the safety of impurities found in human pharmaceuticals [7]. Several white papers in regulatory science have recently appeared describing the use and place of predictive computational toxicology methods for chemical safety assessment [8–13].

Given the intensity of investigation and scientific stature that computational toxicology methods are achieving in applied toxicology, there is tremendous interest and necessity to describe the opportunities, practicalities, and limitations of these enabling methods. Predictive computational toxicology methods can effectively strengthen the evidentiary base of information and help translate nonclinical data into useful science-based information informing safety and risk assessments. As with all technological methods, computational toxicology methods are evolving, and there are shortcomings that should be taken into account when considering their use and application. This chapter will provide the reader valuable and up-to-date information on the opportunities and challenges of predictive computational toxicology. Case examples and investigative studies will be described to demonstrate the present and future role of predictive computational toxicology methods in early drug development.

20.2 Predictive Toxicology

Predictive toxicology aims to identify the hazard of substances to humans and environmental species. The role of computer models in this context has been very prominent as it will become apparent in the following. However, it is important to keep in mind that in the United States, the National Environmental Policy Act (NEPA) of 1969 requires federal agencies to evaluate all major agency actions in order to determine if they will have a significant impact on the human environment (e.g. endocrine disruption effects on aquatic species). Under this law, the US FDA is required to consider the environmental impact of approving certain drug product applications as an integral part of its regulatory process. Therefore, sponsors of drug products including animal drugs need to submit an environmental assessment or environmental impact statement to the FDA if it is determined that no categorical exclusion applies 10. An environmental assessment includes aquatic toxicity and metabolic fate testing to predict the potential of impact of the proposed new drug on the aquatic environment [10, 11, 13]. The use of computerized models (Pharmaceutical Assessment and Transport Evaluation (PhATE) and geography-referenced regional exposure assessment tool for European rivers (GREAT-ER)) to estimate exposure levels of drugs in surface waters has been developed and recently has been focusing on pharmaceutical estrogens such as ethinyl estradiol [14, 15]. These computer models have been in use and noted by regulatory authorities [10]. Therefore, predictive toxicology and computational modeling in drug development are not limited only to humans.

In the broadest sense, predictive toxicology assessments use classical toxicology methods. Classical toxicology usually relies upon investigative toxicity testing starting with *in vitro* models to develop a testing strategy for future *in vivo* studies using animals, the most common of which are rodent species. *In vitro* testing can help identify target organs of toxicity for a drug or help characterize biochemical interactions such as inhibition of critical enzymes involved in metabolism (e.g. cytochrome P450 (CYP) 3A4) or off-target safety liabilities (CYP2B1). In addition, metabolic fate considerations are taken into account in guiding toxicology testing strategies. Consequently, assessment of absorption, enzymatic metabolism, distribution, and elimination of substances is undertaken through the use of a wide spectrum of approaches and models [14–16]. Quite logically, the key concept of exposure driving toxicity is appreciated, and therefore, exposure modeling is used to elucidate a dose–response for particular toxicity endpoints [17]. The elucidation of a dose–response is critical to hazard characterization and can be explored through risk assessment practices in predictive toxicology such as establishment of a no-observed-adverse effect level (NOAEL) for substances with a threshold of toxicity. The benchmark dose (BMD) approach models a response from the complete dose–response data set. The BMD approach estimates the dose associated with the specified effect by fitting a set of dose–response models and estimating the BMD confidence intervals for each model resulting in a set of confidence intervals and deriving a single BMD confidence interval for the effect by averaging the models. The lowest benchmark dose level (BMDL) is the dose where the change in response is likely to be small (e.g. 5%) and is the point of departure for deriving exposure limits. The BMD modeling approach is transparent, computer assisted, and becoming more popular for use by risk assessment practitioners because of reported limitations in the use of NOAELs [17]. BMD modeling software are available and continue to offer valuable information for applying the BMD approach to toxicology data on substances being assessed for risk of harm to human and animal health [1, 18, 19].

In the context of *in silico* toxicology, a number of approaches have been developed for drug development programs to data mining for nonclinical and clinical adverse effects [20, 21] and predict toxicity through qualitative and quantitative modeling and construction of electronic knowledge bases [22–28]. In addition, the application of genomic technologies in guiding toxicological risk assessments is also a modern method for enriching classical toxicology data with newly discovered response patterns as a result of drug and chemical exposures [2]. The key concept behind the use of computational tools is to produce evidence grounded in good science using techniques fundamentally accepted in biostatistics, computer science, toxicology, pharmacology, chemistry, and clinical science. The overarching purpose of *in silico* toxicology is to produce scientifically defensible data for evaluation as decision support in risk assessment, prioritization and ranking of chemicals for testing, and the mitigation of toxicological risks in safety evaluations. In order to achieve this ambitious goal, computational scientists are interested in harnessing the efficiency of computers for maintaining high-level data architectures, their deep capacity for data storage and organization, and the speed by which computers carry out mathematical processes and analysis under carefully designed strategies. Clearly

predictive science is nothing new, and these features of computer power have been exploited in several industries for many years. The financial sector and engineering fields have used computationally driven predictions for decades using mathematical modeling approaches as a key science [29, 30]. The main question is how best to employ these technologies toward drug development especially in the early stages to quickly transform experimental data into useful evidence for medical product development. Application of predictive modeling early on also helps to reduce late stage attrition and costs and helps discover therapeutic breakthroughs [12].

20.3 Predictive Modeling

There are a number of important factors to consider in undertaking predictive modeling. The first step is to understand what kind of modeling approach is needed given the situation and data being modeled. For example, it may be that what is called for is to fit a well-defined model to a large data set through automatic selection of the parameters using appropriate learning algorithms so that the model is not overfitted. Another use case scenario could be the need for a lesser transparent model to predict as accurately as possible the dependent variable but one that can operate to automatically identify the structure, interactions, and relationships of the data. In this case, decision trees such as random forest (RF) may fare better than a generalized simple linear regression model. In early drug development, it is advantageous to learn about the genotoxic liability of a new compound because if the substance is found to be mutagenic to bacteria, it will be regulated as a genotoxic substance for regulatory purposes. Such a regulatory determination leads to labeling implications that could compromise the drug's uptake in medical practice and its marketing. Therefore, predictive models for genetic toxicity provide important contributions toward the development of a potential drug. The types of models that are suitable for genetic toxicity vary. However, if one considers that there are only four possible outcomes from a genetic toxicity test such as the Ames assay [7] (i.e. negative, positive, equivocal, or study inadequate), one can appreciate that a categorical binary model would fit this prediction evaluation type. There have been many cheminformatics-based models described for predicting genetic toxicity [1, 8, 11, 12, 23, 31–38]. Examination of these predictive computational toxicology models indicates that the majority rely upon the use of machine learning algorithms, while others employ computerized knowledge bases as detailed below.

20.3.1 Machine Learning Algorithms

Developments in technology have led to the use of systematic data analysis techniques that have become very important in computational prediction. These techniques known as machine learning algorithms are both fascinating and complex yet could be effective and highly accurate. Computational prediction models driven by computers frequently utilize machine learning algorithms [39]. Consequently, the field of computational toxicology has employed machine

learning algorithms to facilitate approaches taken in toxicological predictive science. Machine learning algorithms have become an important part of producing evidence on chemical risk and toxicity.

There are several machine learning procedures that are well described. The most common are decision trees, ensembles, *k*-nearest neighbors (*k*-NN), naïve Bayes classifiers, artificial neural networks (ANN), and support vector machines (SVM). The basic anatomy of these procedures with references to their application in predictive toxicology will be briefly described.

20.3.1.1 Decision Trees

There are two general types of decision trees: classification trees and regression trees. Classification trees use quantitative and categorical (i.e. binary) data to model categorical outcomes, such as the toxicity of a compound as being toxic (positive) or of no toxic concern (negative). Categorical decision tree models have been developed to predict the mutagenicity of robust compound data sets [18, 40]. Regression trees use quantitative and categorical data to model quantitative outcomes. For example, a regression decision tree classifier algorithm has been developed and used to quantitatively predict toxicity of endocrine and nonendocrine disrupter chemicals [41]. Some generalities can be made regarding the advantages and disadvantages of decision trees. Decision trees are flexible: they work very well with large data sets and can handle a variety of data set types with minimal preparation. Decision trees are also simple to interpret, being highly transparent in understanding the process of how a particular prediction ended up in the categorical assignment. The transparency of decision trees means they bode well with the desire to explain a prediction. This is particularly of interest when predictive data are needed to inform critical decision-making in a drug development program or, for example, in settings such as applied regulatory science purposes (i.e. to help make a regulatory decision). Thus, decision trees can be thought of as *white box* models. However, decision trees generally rely on heuristics and local optima, leading to overfitting of the available data, as they are designed to match the candidate algorithm to the type of data and situation for the specific question at hand. Therefore, human expertise and decision-making are crucial for the appropriate application of decision trees.

20.3.1.2 Ensembles of Models

When a series of models is combined to provide the estimate or prediction, the resulting machine learning technique is referred to as ensembles of models. Ensembles of models have become very common and can be seen in everyday life. For example, when we watch the weather forecast, the weather prediction is actually the result of an ensemble of models. Ensemble modeling is the average of many estimates for a particular outcome. For drug development safety assessment efforts, the application of ensemble modeling can be applied to genetic toxicity outcomes including DNA reactive mutagens [40]. This approach follows the theory that the combined estimates of individual techniques, each with its own weaknesses, are more accurate than any one individual prediction. There are three major approaches to combining the individual estimates: bagging, boosting, and blending.

Bagging is also called boosted aggregating. With bagging, randomly drawn data sets are generated and the model is built on those data sets, predictions are made, and then the predictions are combined via a voting process from each of the model. Probably one of the most widely reported methods of bagging algorithm is RF. RF was invented by the late Dr Leo Breiman of the University of California, Berkeley [42]. RF is an ensemble method using decision tree predictors such that each tree depends upon the values of the randomly sampled vector. A recent example of the use of RF in toxicological predictive sciences includes a study that modeled drug vehicle relationships to select a vehicle with the lowest possible toxicity for a particular drug [43]. In this study, a large data set of drug vehicle data encompassing more than 2.7 million records from the US National Institute of Health's Developmental Therapeutics Program (DTP) of more than 225 000 potential antitumor drug candidates was modeled using RF and decision trees. The RF method was compared with a decision tree classifier algorithm called C4.5 (C = classifier, ver. 4.5) originally developed by Quinlan Schafer [44]. C4.5 decision tree classifiers use the divide and conquer algorithm and heuristic criteria to rank possible tests while maximizing information gain and minimizing total entropy. Although C4.5 has been superseded by C5.0, because it is significantly faster with similar performance output yet smaller decision trees and less memory making it more efficient, it is still a useful algorithm for research [45]. In the drug data mining study [43], the number of trees in the RF model was 100, and the study employed 10-fold cross-validation to assess vehicle prediction. The authors reported that prediction accuracies of 80% were achieved using RF models compared with decision tree models with accuracies much lower in the 70% range. Another study applied RF to a QSAR analysis of 644 chemicals tested for aquatic toxicity to *Tetrahymena pyriformis* using two external test sets of 339 and 110 chemicals. This approach resulted in better statistical characteristics compared with partial least squares (PLS) and *k*-NN models [46]. As mentioned earlier, aquatic toxicity can be important in drug development due to regulatory requirements for environmental assessments. Therefore, having an early screening tool that is predictive of these events for appropriate species is important. Modeling the mutagenic potential of chemicals for the purpose of qualification of pharmaceutical impurities has been one of the most active areas for toxicologists in applying machine learning algorithms, and the use of RF for this critical endpoint is no exception. A recent study used two different mutagenicity benchmark data sets (4000+ compound/each) and evaluated models using RF, naïve Bayes, J48, and SMO classification methods with PowerMV (6122) descriptors. It was found that RF clearly outperformed the other classification methods with an accuracy of 90% based on the testing with two different external validation data sets [18].

In boosting, each classifier puts greater weight on the previous classifier's errors. This ensemble learning approach has been described in chemical toxicology but only to a limited extent. For example, one study employed a decision tree boost implementing stochastic gradient boosting to predict toxicity to algae (*Pseudokirchneriella subcapitata*), *Daphnia*, and bacteria [47]. A high classification accuracy (>95%) was reported with the model predicting toxicity to algae.

In blending (also known as stacking), a second-order model is used to combine the results of the first-order models. No predictive toxicology studies have been reported using this ensembling method so far.

The aforementioned studies using ensemble models collectively suggest that the use of many estimates for a predictor, in this case toxicity, often outperforms the estimate of just one model. Moreover, it is also apparent that the diversity of the data stemming from randomly selected data leads to more diverse models, and this attribute also produces more accurate estimates. Therefore, randomness in data selection and model generation is an important attribute when deciding which predictive toxicology model to employ.

20.3.1.3 *k*-Nearest Neighbors

A common machine learning technique used in predictive toxicology is called *k*-NN. The *k* stands for the number of neighbors, where the neighbors represent the number of instances surrounding a particular case. Therefore, *k*-NN can be thought of as instance-based learning and is sometimes called a *lazy learner* algorithm because of the simplicity of the learning concept. One of the first considerations in performing the technique is to determine the distance between neighbors (i.e. how far away are the instances from each other). If the variables used to predict are quantitative such as continuous variables (IC_{50} values), then a distance metric that can be used to measure the distance between neighbors could include, for example, the Euclidean distance. However, if the modeler has categorical variables used to make the prediction, then a more appropriate distance metric could be the Hamming distance [48], which measures the number of dimensions where two vectors have different values. If there are a large number of variables, such as that that might derive from large genotoxicity data set, then principal component analysis could be used to reduce the number of dimensions before performing the *k*-NN. A central question to ask is how many neighbors to work with when analyzing your data set. Logic might dictate that the higher number of neighbors (*k*) increases the predictivity of the model. However, a higher *k* elevates the risk of random noise being modeled and consequently misclassification. To help mitigate the risk of noise fitting, there are variations of *k*-NN that weigh the neighbors that are closest to the case and condensed *k*-NN that decreases the weight of neighbors further away from the case being predicted. The *k*-NN technique is referred to as a nonparametric classification method because it does not use any other parameters other than the closest neighbors around the case as its data. Thus, the advantages of *k*-NN are that it is not difficult to understand in terms of its concept, or mathematically, it is easy to implement and can actually be effective with data sets where dimensions have been reduced so that neighbors can be identified. A recent example for the use of *k*-NN in predictive toxicology was reported in a computational toxicology model designed and built to predict the bacterial mutagenicity of drug impurities [23]. This computational model used the Symmetry software platform (Prous Institute for Biomedical Research, Spain). A large number of descriptors were used to analyze the data set of over 7300 different chemical structures. For model validation, external testing was performed using four data sets including testing of applicability domain, for suitability with drug molecule space, and a test set that represented a

randomly generated 10% holdout set. Prediction accuracy based on the external validation testing ranged from 80% to 83%. Sensitivity and negative predictivity values were also in a similar, high confidence range at 83% and 84%, respectively. As will be discussed in a subsequent section, models with high sensitivity and high negative predictivity are desirable for safety qualification of drug impurities due to regulatory objectives in protecting public health. This example of computational toxicology predictive model using *k*-NN learning with a large data set of descriptors and exceptional performance in validation tests clearly demonstrates the value of this technique.

20.3.1.4 Naïve Bayes Classifiers

Naïve Bayes classification algorithm uses the probability of a given data set to belong to a particular group or class as derived from the available data during training. Naïve Bayes classifiers works well when data preparation steps are performed such as balancing of training set data (i.e. the class size). Other data preparation techniques work well with naïve Bayes such as transformation of the data to emulate a power-law distribution. Naïve Bayes works with both quantitative and categorical predictors, and its results are not as complex to interpret as with other machine learning techniques. A recent investigation built a naïve Bayes computational prediction model for the Ames test [49] results of 8300 compounds using their 2D structural fingerprints as descriptors [50]. An external validation test set, although limited in its size (731 compounds, ca. 10% of the training set), provides a reasonable judgment for predictive performance. Although naïve Bayes classification was not among the top models built in the study, it did provide an acceptable degree of specificity at 75%, which would be a desirable performance attribute for early drug development purposes, as models performing well in specificity lead to higher confidence in positive predictions. This bodes well for early drug development as in order to expend resources and time on a lead compound; a drug development program would want to be confident that a compound is predictive positive as there are several regulatory consequences of having a genotoxic active pharmaceutical ingredient (API). Of course all models are context dependent in relation to the data the model is working with, but it can be concluded that naïve Bayes is one of the dominant machine learning classification techniques and is being used in investigative settings and in the applied sense as a predictive toxicology method.

20.3.1.5 Artificial Neural Networks

ANN are a modeling approach intended to resemble a "brain-like" system of interconnected processes (models). Networks of models are built and many can be built in layers with inputs and outputs. In addition, there are layers between inputs and outputs but these are hidden. The resulting architecture depends upon the number of attributes being inputted and the data being predicted (i.e. the output). There are weights assigned to data points, and once built the network of models functions efficiently. ANN is thought of as a technique that can be especially powerful for nonlinear functions and in situations when knowledge-based methods and simple *k*-NN techniques cannot resolve the signal. ANN models can learn from experience and also infer complicated rules. One of the major

downsides of ANN models is explaining the prediction through the entire network. Because of this low degree of interpretability, sometimes ANN models are referred to as black box models. In building ANN models, one has to specify the weights between neurons and the number of models to build. A recent example of the use of ANN modeling to develop an *in silico* tool to help resolve a drug development safety liability issue was the construction of ANN models to predict drug-induced phospholipidosis [51]. Drug-induced phospholipidosis is a phenomenon characterized by accumulation of drugs and phospholipids in lysosomes. Histopathologically, drug-induced phospholipidosis presents itself as foamy macrophages or cytoplasmic vacuoles in various tissues of both animals and humans [52]. There is a chemical feature component to drug-induced phospholipidosis in which many (but not all) drugs that possess cationic and amphiphilic moiety induce phospholipidosis. The physiological consequences of drug-induced phospholipidosis or its mechanism of induction are still unclear. Because drug-induced phospholipidosis may raise regulatory questions, this phenomenon has become important for industry from a predictive standpoint. A number of *in vitro* and *in silico* models have been developed to predict *in vivo* drug-induced phospholipidosis in order to increase the throughput of prediction compared with the low-throughput, gold standard method of transmission electronic microscopy [53–56]. An *in silico* ANN model was constructed by the FDA using an internal database of drugs found in regulatory applications and the public literature. The FDA ANN *in silico* model used physicochemical properties as the principal descriptors of prediction. This ANN model used higher quality drug data and an external validation test set. When sensitivity and concordance are considered, it outperformed other FDA *in silico* models previously built using different algorithmic techniques involving structural fragment descriptors, thus demonstrating the predictive value of ANN [57].

20.3.2 Knowledge-based Methods

Knowledge-based methods in toxicology involve systems and techniques to support learning and human decision-making. The implementation of knowledge-based methods comes in various forms including design, processes and workflows, models, and software tools. By their virtue, knowledge-based methods are data driven and can facilitate data exploration.

In terms of computational models for toxicology, the knowledge-based system known as Derek Nexus (formally known as Derek for Windows) is an iconic software program designed to evaluate the potential toxicity of existing and prospective chemicals and does so based on chemical structure [31, 58]. Derek Nexus's approach is often referred to as expert rule based because it involves a set of rules created by human expert knowledge regarding structure–activity relationships (SAR) of toxicity. Derek Nexus generates predictions in the form of an overall likelihood for toxicity based on the analysis of the query molecular structure after analyzing its knowledge base. The toxicity endpoints available for prediction include carcinogenicity, genotoxicity, skin sensitization, teratogenicity, respiratory sensitization, reproductive toxicity, and irritation. One of the unique features of Derek Nexus is its knowledge editor, which enables one to create a unique

knowledge base. In other words, one can add specific knowledge that is directly relevant to the research program of interest to the system in order to maximize its predictive performance. The knowledge can include chemical structures, rules, alerts, and example compounds with literature references. The Derek system has been heavily tested for accuracy in predicting bacterial mutagenicity with varying results. There seems to be consensus on the value of this system to help portend mutagenicity concerns for new molecules under development as described in a series of publications [8, 9, 32–35]. Derek is a commercial system, has web service capability, and can be integrated with Pipeline Pilot [59]. Another valuable feature of Derek is its integration with Meteor, a software also available from the same developer (Lhasa Ltd.). Like Derek, Meteor is a knowledge-based system but does not predict toxicity; it predicts metabolism of compounds using their 2D molecular structure as input. Meteor was evaluated for its prediction power of human drug metabolites, and the software performed well predicting over 60% of major drug metabolites that are known to be hepatotoxic [36].

Toxtree is another commonly used chemical structure analyzing knowledge-based system for computational prediction of toxicity [37, 38]. Toxtree is an open-source software tool developed by Idea Consult Ltd. (Sofia, Bulgaria). Toxtree is informed with a robust carcinogenicity and genetic toxicity knowledge base of SAR rules. The source of this knowledge derives from a published rule-based system described by Benigni and colleagues in a report of the Joint Research Centre of the European Commission [60]. The actual SAR rules derive from several sources of scientifically vetted structural alerts [61–65]. A set of rules for nongenotoxic carcinogens is also included in Toxtree's knowledge base as well as the Cramer decision tree, which predicts CYP inhibition among other endpoints [66]. The reliability of the Toxtree knowledge base for carcinogenicity and mutagenicity has been evaluated [38, 67]. In a recent validation study of Toxtree for predicting mutagenicity of chemicals, a robust test set of 6489 chemicals from the Hansen benchmark database was used to externally validate the mutagenicity module. The results showed 80% sensitivity, 66% specificity, and 74% concordance for predicting bacterial mutagenicity, implying a 20% false negative rate. These results are highly promising given that the predictions were based solely on a computer-encoded knowledge base of structural alerts from a diverse set of publications and population of chemicals reflecting a broad chemical space (e.g. drugs, food ingredients, pesticides, nonfood industrial compounds). Toxtree was also recently evaluated for its ability to predict ocular irritation [68]. However, the prediction test results showed it performed poorly as standalone but significantly improved when physicochemical properties and electrophilic reactivity mechanisms such as Schiff base formation and acylation reactions were incorporated. According to the report, Derek Nexus also suffered from an inability to predict this highly difficult endpoint.

20.4 Industry Perspectives

Predictive computational toxicology plays a key role in industrial assessments of safety liabilities, and this role begins in early drug discovery [12]. It is nevertheless

an integral component of decision-making in later stages of drug development. The development of predictive computational tools, their application, and interpretation of data represents an integrative science where a number of specialists are needed to fully exploit the strengths of these assessments while minimizing the risk of a misprediction. The use of predictive computational toxicology tools can be very powerful in that it can enable analysis of chemical liabilities while uncovering patterns that would be unknown from a heuristic human assessment of the data. From an industry perspective, a key question is what would be the value of the computational assessment? Will it actually contribute critical knowledge to forward a compound in its stage of development and really inform the many decisions that have to be made to support the development from a safety and efficacy standpoint? As with the saying "the devil is in the details," the answer might depend on the type of data used by computational assessments. High quality data should in fact contribute to high quality predictions. However, this is not the only caveat. One might have the highest quality data, for example, an OECD 452 guideline-compliant chronic rodent study conducted with good laboratory practice (GLP) that has been reviewed and given regulatory approval, yet if the wrong type of analysis is performed using an algorithm that is not appropriate to the type of data, then the value of the assessment is diminished. In fact, the assessment can be completely inappropriate and of no value. So the goal should be not only to use the highest quality data but also to use expert knowledge and apply the most appropriate type of data analysis technique that is most suitable for the problem at hand. To further focus on the value of the computational assessment to help resolve a scientific issue, let us assume that the highest quality data were used and the most appropriate data analysis technique employed. One might think then that the results will lend to a valuable predictive computational assessment. However, what is still missing as an additional critical component is the interpretability of the assessment (i.e. a narrative of the computational evaluation). By maximizing interpretability, the value of the assessment is also maximized. Because predictive computational modeling in the pharmaceutical arena is an integrative science that relies upon a series of multiple disciplines (e.g. safety science, computer science, clinical science, pharmacology/toxicology, chemistry, and statistics), proper communication of the interpretability of the modeling effort is essential. False consensus often plagues discussions around predictive toxicology results. Here, modelers tend not to share important details of model development and performance (e.g. how the data are transformed for analysis, the difference between sensitivity and specificity) with the intended audience based on the assumption that these are by default clear and understood. This leads to confusion on the interpretability, reliability, and applicability of the overall method, and even interpretable models tend to be considered by the end users as black box approaches, therefore reducing their acceptance and impact. The users are encouraged to seek guidance from computational toxicology model developers on the overall usability and limitations of a given predictive tool.

The use of computational toxicology predictive modeling is grounded scientifically to a significant extent, and it is now accepted as a screening strategy in drug discovery programs [69]. It is also in use for later stages of drug development including Phase IV such as signal detection of human adverse effects using

bioinformatic techniques and data mining [70]. However, due to inherent limitations with certain data types (post-market spontaneous observational data), one is advised to proceed with caution [71].

The adoption of predictive computational toxicology tools is now formalized in international guidelines for the assessment and control of mutagenic impurities in drug product [7]. Many authors have concluded that the application of computational predictive toxicology models for genotoxicity has sufficient accuracy. Nevertheless, when the evaluation of predictive performance metrics and chemical applicability domain assessments are considered, the obtained results would seem to question the performance and reliability. As illustrated in Table 20.1, when applied to genotoxicity data sets, the external validation performance metrics for computational toxicology models to predict bacterial mutagenicity varies widely. Model versions, software versions, imbalanced data sets, and low sampling are among the many factors that contribute to this variability. Values of sensitivity, the percentage of correctly predicted true positives, and specificity, the percentage of correctly predicted true negatives, are often looked at together with concordance (percent agreement between true negative and true positive predictions) when assessing a computational model. Sensitivity and specificity quantify false negatives and false positives avoidance, respectively. Ideally, one would want to maximize both sensitivity and specificity, but there is an intrinsic trade-off between the two parameters, and reliance upon these two values alone to judge the predictive performance can be misleading.

Several statistical metrics that are useful for assessing the predictive value and uncertainty of models have been derived, and some of these are listed in Table 20.2. In the context of drug discovery and development and the use of computational toxicology assessment for control of genotoxic impurities in a drug product, the performance metrics of sensitivity, negative predictive value, concordance, and false negative rate have become especially important. The following confusion matrix (Table 20.3) is classically used to evaluate the relationship between predictions and observations.

The performance survey outlined in Table 20.1 illustrates the predictive performance for the most commonly used commercial software and noncommercial models. It is not intended to benchmark all available software but to highlight the most prominent computational toxicology software in the pharmaceutical industry and in chemical risk assessment. The most commonly used commercial software for chemical structure-based predictions is Derek (Lhasa Ltd.), a human expert rule-based system, also known as a knowledge-based approach [58]. According to the data in Table 20.1, Derek displays an excellent, average negative predictive value (85%), providing high confidence in negative predictions. In terms of specificity and negative predictive value, MC4PC (MultiCASE, Inc.) shows an average value of 85% across several validation studies. This value is directly comparable to the interlaboratory reproducibility of the Ames assay [84, 85]. It is noteworthy that the software ADME Works [86] shows very good concordance (78%) for predicting mutagenic and nonmutagenic chemicals in external validation studies. Furthermore, the statistical QSAR system, Symmetry (Prous Institute for Biomedical Research), has high average specificity, negative predictive value, and concordance (81%) and also shows very good sensitivity

Table 20.1 Summary of representative performance metrics from testing computational toxicology predictive models applied to bacterial (*Salmonella typhimurium*) mutagenicity data sets to predict DNA-reactive chemicals and drugs.

Predictive model	Description of the external validation test set with reference	Sensitivity	Specificity	Negative predictive value	Concordance
Derek	Imbalanced test set. 224 negatives, 48 positives [72]	68%	97%	94%	92%
Derek	95 positives, 178 negatives [73]	82%	47%	83%	59%
Derek Nexus	8541 public chemicals [34]	77%	82%	79%	66%
Derek	409 pharmaceuticals, 82 positives, 327 negatives [74]	46%	69%	84%	65%
Derek	Imbalanced test set of 159 positives, 495 negatives using a data set of proprietary drug synthesis intermediates [73]	70%	73%	88%	72%
Derek Nexus	801 chemicals, 253 positives, 548 negatives [32]	68%	72%	83%	71%
Derek Nexus	3970 private chemicals [34]	78%	88%	84%	84%
Derek Nexus	3863 private chemicals, 5-strain Ames [34]	77%	88%	80%	82%
Derek Nexus	438 private pharmaceuticals [34]	68%	79%	90%	77%
Derek Nexus	Imbalanced data set of 249 positives, 93 negatives [75]	87%	84%	71%	86%
Derek Nexus	Imbalanced data set of 197 proprietary pharmaceutical-related chemicals, 57 positives, 140 negatives [8]	65%	50%	78%	54%
Derek Nexus	Imbalanced data set of 256 proprietary pharmaceutical-related chemicals, 72 positives, 184 negatives [8]	85%	81%	93%	82%
Derek	4971 chemicals. 2300 positives, 2671 negatives [76]	67%	79%	74%	74%
Derek	688 chemicals, 357 positives, 331 negatives [77]	82%	80%	79%	81%
Derek	Imbalanced data set of 355 flavor chemicals, 24 positives, 331 negatives [78]	39%	93%	96%	88%
Derek	Imbalanced data set of 206 chemicals, 40 positives, 166 negatives [77]	73%	88%	88%	66%
Sarah Nexus	235 positives, 523 negatives [32]	51%	79%	78%	71%

(Continued)

Table 20.1 (Continued)

Predictive model	Description of the external validation test set with reference	Sensitivity	Specificity	Negative predictive value	Concordance
Derek	Imbalanced data set of 608 proprietary pharmaceutical-related chemicals, 153 positives, 455 negatives [8]	44%	78%	80%	69%
Derek	Imbalanced data set of 269 proprietary pharmaceutical-related chemicals, 39 positives, 230 negatives [8]	72%	70%	94%	70%
Derek	119 proprietary pharmaceutical-related chemicals, 37 positives, 82 negatives [8]	97%	6%	83%	34%
Derek	Imbalanced data set of 394 marketed drugs, 27 positives, 275 negatives [79]	52%	75%	95%	74%
Derek	Imbalanced data set of 480 marketed drugs, 38 positives, 442 negatives [80]	62%	88%	96%	86%
Derek	400 chemicals and drugs, 239 positives, 161 negatives [40]	93%	83%	88%	82%
Derek	4633 chemicals, 2038 positives, 2595 negatives [33]	75%	78%	80%	75%
Derek	2630 chemicals, 1350 positives, 1350 negatives [33]	81%	59%	76%	74%
Derek	2327 proprietary pharmaceuticals, 232 positives, 2095 negatives [33]	43%	92%	94%	86%
$n = 26$	$\bar{x}$	69% SEM 3.0	75% SEM 3.6	85% SEM 1.5	74% SEM 2.4
MC4PC	95 positives, 178 negatives [73]	48%	75%	73%	66%
MC4PC	984 chemicals [81]	84%	73%	Unknown	79%
MCASE	522 chemicals, 241 positives, 281 negatives [77]	88%	98%	98%	74%
CASE Ultra	207 positives, 498 negatives [32]	48%	77%	78%	68%
MCASE	Imbalanced data set of 357 marketed drugs, 27 positives, 330 negatives [79]	48%	93%	96%	90%
MCASE	Imbalanced data set of 355 flavor chemicals, 24 positives, 331 negatives [78]	25%	94%	95%	88%

MCASE	Imbalanced data set of 166 chemicals, 26 positives, 140 negatives [77]	65%	91%	91%	88%
MCASE	Imbalanced data set of 429 marketed drugs, 38 positives, 391 negatives [80]	45%	97%	95%	93%
MC4PC	2018 chemicals, 888 positives, 1130 negatives [33]	65%	83%	75%	72%
MC4PC	1099 chemicals, 736 positives, 363 negatives [33]	75%	74%	59%	74%
MC4PC	1444 chemicals, 633 positives, 770 negatives [82]	70%	90%	79%	81%
MC4PC	2284 proprietary pharmaceuticals, 228 positives, 2056 negatives [33]	31%	86%	92%	79%
$n = 12$	$\bar{x}$	58% SEM 5.8	85% SEM 2.9	85% SEM 3.8	79% SEM 2.6
ADME Works	Imbalanced data set of 249 positives, 93 negatives [75]	93%	80%	78%	90%
ADME Works	692 chemicals, 416 positives, 276 negatives [77]	75%	56%	56%	66%
ADME Works	Imbalanced data set of 355 flavor chemicals, 24 positives, 331 negatives [78]	14%	92%	94%	84%
ADME Works	Imbalanced data set of 204 chemicals, 73 positives, 131 negatives [77]	73%	70%	70%	70%
$n = 4$	$\bar{x}$	64% SEM 17.1	75% SEM 7.6	75% SEM 7.9	78% SEM 5.6
SVM	438 pharmaceuticals [34]	61%	88%	91%	83%
SVM	3970 private chemicals [34]	92%	98%	95%	95%
SVM	8541 public chemicals [34]	79%	84%	81%	82%
SVM	3863 private chemicals, 5-strain Ames [34]	77%	90%	80%	84%
SVM	Imbalanced data set of 731 chemicals, 614 positives, 117 negatives [50]	99%	73%	93%	95%
SVM	Imbalanced data set of 837 chemicals, 403 positives, 88 negatives [83]	84%	78%	52%	81%

(Continued)

Table 20.1 (Continued)

Predictive model	Description of the external validation test set with reference	Sensitivity	Specificity	Negative predictive value	Concordance
$n=6$	$\bar{\bar{x}}$	82% SEM 13.1	85% SEM 3.6	82% SEM 6.5	88% SEM 6.6
Symmetry	730 chemicals [23]	83%	77%	78%	80%
Symmetry	361 active pharmaceutical ingredients, 361 negatives [23]	N/A	81%	N/A	81%
Symmetry	1535 chemicals, 568 positives, 967 negatives [23]	73%	86%	84%	81%
$n=3$	$\bar{\bar{x}}$	78% SEM 7.0	81% SEM 2.6	81% SEM 4.2	81% SEM 0.3
Toxtree	4971 chemicals. 2300 positives, 2671 negatives [76]	76%	70%	77%	73%
Toxtree	6391 chemicals, 3454 positives, 2937 negatives [38]	80%	66%	74%	74%
Toxtree	4698 chemicals, 3147 positives, 1551 negatives [33]	78%	70%	61%	74%
Toxtree	2647 chemicals, 1773 positives, 874 negatives [33]	85%	53%	64%	75%
Toxtree	2335 proprietary pharmaceuticals, 233 positives, 2102 negatives [33]	43%	78%	93%	73%
Toxtree	Imbalanced data set of 731 chemicals, 614 positives, 117 negatives [50]	94%	68%	68%	84%
$n=6$	$\bar{\bar{x}}$	76% SEM 7.1	68% SEM 3.3	73% SEM 4.7	76% SEM 1.7
k-NN	4971 chemicals. 2300 positives, 2671 negatives [76]	87%	87%	89%	87%
k-NN	400 chemicals and drugs, 239 positives, 161 negatives [40]	92%	91%	89%	84%
k-NN	Imbalanced data set of 731 chemicals, 614 positives, 117 negatives [50]	99%	91%	95%	98%
$n=3$	$\bar{\bar{x}}$	93% SEM 3.5	90% SEM 1.3	91% SEM 2.0	90% SEM 4.2
ANN	400 chemicals and drugs, 239 positives, 161 negatives [40]	89%	95%	85%	84%
Naïve Bayesian	4971 chemicals. 2300 positives, 2671 negatives [76]	87%	91%	89%	90%

Table 20.2 Common statistical measures of performance for predictive computational toxicology classification models.

Statistical parameter	Equation	Definition
Concordance (accuracy)	$= \frac{(TP + TN)}{(TP + TN + FP + FN)}$	Percent of chemicals in the training set of a model that were correctly predicted by the model
Sensitivity	$= \frac{(TP)}{(TP + FN)}$	Percent known positives that are correctly predicted
Specificity	$= \frac{(TN)}{(TN + FP)}$	Percent known negatives that are correctly predicted
False positive rate	$= 1 - \text{specificity} = \frac{(FP)}{(TN + FP)}$	Percent known negatives that are incorrectly predicted as positive
False negative rate	$= 1 - \text{sensitivity} = \frac{(FN)}{(TP + FN)}$	Percent known positives that are incorrectly predicted as negative
Positive predictivity	$= \frac{(TP)}{(TP + FP)}$	Positive predictions that are true positives (probability of a positive prediction being correct)
Negative predictivity	$= \frac{(TN)}{(TN + FN)}$	Negative predictions that are true negatives (probability of a negative prediction being correct)
Matthews correlation coefficient (MCC)	$= \frac{(TP * TN - FP * FN)}{\sqrt{(TP + FN) * (TP + FP) * (TN + FN) * (TN + FP)}}$	Correlation between observed and predicted binary classifications. Ranges between −1 and +1. A value of +1 represents a perfect prediction, 0 represents a random prediction, and −1 indicates total disagreement
Cohen's kappa (k)	$= \frac{p_o - p_c}{1 - P_c}$	Measure of concordance in categorical classification. Compensates for classifications that may be due to chance. Ranges from −1 (total disagreement) through 0 (random classification) to 1 (total agreement)[a)]

a) Scale for interpreting the Kappa value.

TN, true negative; FN, false negative; FP, false positive; TP, true positive; p_o, observed agreement probability; p_c, hypothetical probability of chance agreement.

Table 20.3 Simple confusion matrix to describe the performance of a classification model.

	Predicted negative	Predicted positive	Total
True negative (nontoxic)	TN = 50	FP = 20	70
True positive (toxic)	FN = 7	TP = 75	82
Total	57	95	152

TN, true negative; FN, false negative; FP, false positive; TP, true positive.

(78%). By comparison, when the performance characteristics of the noncommercial systems and models are considered, there is a robust amount of validation studies reported for Toxtree (Idea Consult). Toxtree has very good sensitivity and concordance values (76%). What is impressive is that machine modeling techniques implemented in open-source software (e.g. R) have been reported by different laboratories and do indicate exemplary sensitivity, specificity, negative predictive value, and concordance [76, 83]. In fact, they show better performance than commercial off-the-shelf systems, thus offering a valid, cost-effective alternative. However, the extent of validation testing is not as robust as for some of the commercial software and could represent interesting avenue for further research. It is worth noting here that large pharmaceutical companies have their own in-house computational toxicology software that is seldom documented in the literature due to its proprietary nature [8], thus making direct comparison with public models challenging.

Clearly, judging predictive performance across different studies such as those illustrated in Table 20.1 has its own caveats. The first consideration is that each software has different training and external validation sets. Here the size (e.g. number of observations) and context (e.g. the chemical space covered) of a given data set is of special importance to a final statistical performance assessment. For example, examining the sensitivity of a prediction for data sets with fewer than 100 data points should be taken with extreme caution as the statistical power of such assessments is very low. An erroneous prediction on 1–2 compounds out of 50 has a much greater impact (10-fold) on the statistical value of performance compared with an erroneous prediction of only 1–2 compounds out of 500. In addition, many external validation test sets reported in the literature are imbalanced in that they contain a far greater fraction of negative than positive records. This observation is especially true for pharmaceuticals. The public domain has reportedly fewer instances of positive Ames test molecules because drug development programs typically enrich in nonmutagenic drugs due to regulatory implications on drug labeling and of course consideration of patient safety. As there are yet no common standards for training and external validation sets that would allow for a more objective performance assessment, evaluating average model performance over time seems to be a realistic and practical approach.

More recently, assessments of predictive metrics have been performed judging the predictive value of the models alone and in combination with human expert intervention. The incorporation of human expert judgment alongside the computational assessment has been found to significantly enhance the predictive

performance. For example, in the study by Dobo et al. [31], in order to assess how expert judgment increased the confidence of *in silico* predictions to identifying nonmutagenic drug impurities, a survey of eight pharmaceutical companies was conducted. When human expert interpretation of the *in silico* predictions was added in to the assessment, the negative predictive value increased from 94% to 99%, illustrating the importance of human expert interpretation of computer model generated predictive data. The notion of human expert judgment combined with computerized predictive data has been recommended in the ICH M7 guideline. Sometimes the human expert judgment is referred to as expert knowledge. Although the incorporation of human expert interpretation and knowledge is crucial, caution should be exercised because expert opinions depend on individual experience and skills, which are factors that can vary dramatically [87]. Some authors have described a "best practice" approach to address practical application of the human expert interpretation of predictive models [9]. Although a well-intended concept, the standardization of human knowledge and its interpretation is a challenging task.

Predictive toxicology models need to be considered into the wider context of experimental toxicological profiling (detailed in Chapters 18 and 19). For instance, most pharmaceutical companies use genotoxicity screening predictions in the early stages of development. During this stage there is an emphasis to filter out as many genotoxic substances as possible while ensuring a low false positive rate. The types of *in vitro* screens commonly used are the BiolumAmes assay and mini-Ames assay [88]. In fact, these tests are the first genotoxicity assessments performed for a potential drug candidate, and a positive result from these tests will severely impact the development of the drug molecule [89]. Newer alternative mutagenicity tests such as the GADD45a-Gluc BlueScreen assay have been developed and being used for screening this liability [90]. Consequently, false negatives from a computational mutagenicity model will likely be picked up during later stages of *in vitro* genetic toxicity screening. *In silico* computational mutagenicity evaluation is conducted before selection of the drug candidate. For many pharmaceutical companies, the use of *in silico* computational mutagenicity prediction based on SAR/QSAR methods is used as complementary data to strengthen the evidence of mini-Ames screening before continued drug development. Although the emphasis at the earliest stages of development are on the API, in later development stages, the emphasis switches to the evaluation of genotoxic impurities in drug substance and drug product. Genotoxicity impurity (GTI) assessment with the use of computational toxicology software is conducted under the guidelines of ICH M7. Therefore, it is reasonable to anticipate that different types of computational models will be needed on the basis of different intended applications.

20.5 Regulatory Perspectives

There is an important role for the use of predictive computational toxicology in a regulatory context to evaluate the potential toxicity of substances present in

pharmaceutical products [5]. In 2010, the US FDA launched its landmark report on "Advancing Regulatory Science Initiative" that builds upon existing agency programs such as the Critical Path Initiative to transform product development through groundbreaking efforts to provide the public innovative medical products [6]. The FDA's Advancing Regulatory Science Initiative positions the agency to foster innovation through applied research and testing of novel methods. One of the science priority areas is to modernize toxicology through the use and development of computational methods and *in silico* modeling. Specifically, the development of computer models of cells, organs, and systems to better predict product safety and efficacy is identified in the agency's report. Furthermore, knowledge development systems and data visualization tools to inform computer model development, clinical risk prediction, and regulatory decision-making are a priority. Therefore, the FDA has made clear its interest in the development and use of computational toxicology methods and models for prediction and knowledge development to improve product safety. In line with FDA's vision, the accomplishment of these broad goals will help assure patient safety and reduce the probability to withdraw approved products.

Similarly the FDA's Critical Path Initiative was established to more effectively advance medical products through the development and use of transformative technologies to drive innovation [5]. This regulatory initiative was launched in 2004 and has been a successful program. In fact, the FDA lists 76 tangible examples in its annual reports where collaborators have been able to move forward new methods and technologies including computational predictive methods. Some specific examples funded by the FDA's Critical Path Initiative include a training set of carefully selected drugs from internal FDA reviews and approved drugs that have been studied in human thorough QT (TQT) studies to predict the potentially fatal cardiac arrhythmia, torsade de pointes [25]. This innovative and truly translational work used human data only to inform a computerized model to generate predictions to help regulatory reviewers. The limitation, however, is that the model contained proprietary drugs, and thus it could not be utilized outside of the FDA context. This same limitation applies to proprietary knowledge-based computerized toxicology models generated by the pharmaceutical industry. Despite the advances in computer technology, there remain challenges in predictive computational toxicology with regard to safeguarding intellectual property in data sharing through informatics systems, at least from a regulatory science perspective. Other examples of useful FDA's Critical Path Initiative regulatory science research in the context of computational predictive tools include modeling and simulation approaches in predicting the *in vivo* performance of drug products. To this end, the major modeling approaches are physiologically based pharmacokinetic (PBPK) models. Since the bioavailability of a drug can affect efficacy and the consequence of a failed efficacy can lead to progression of human disease and toxicity, the use of computational PBPK models is rather important as it can better define formulation strategies to improve drug design and ensure the manufacture of safer and efficacious drugs. At the FDA, PBPK models having physiologically based heuristics for absorption connected with empirical distribution and clearance predictors have been built to support formulation development under FDA's "Critical Path Opportunities

for Generic Drugs" [91]. Such efforts help guide regulatory drug applicants in the implementation of the agency's quality by design (QbD) paradigm. The purpose of QbD is to intentionally build a predefined quality into the final drug product by understanding and controlling manufacturing variables through the implementation of systematic approaches and processes [92]. QbD has been implemented not only for small molecule drugs but also for biopharmaceuticals [93]. Understanding manufacturing variables has become paramount given the recent reports of drug shortages connected to product quality manufacturing issues. Product quality concerns have included serious outbreaks of morbidity due to drug compounding issues in the recent past that are of public health importance [94, 95]. Some quality manufacturing issues include toxicology problems such as toxic heavy metal impurities or appearance of degradants, leading to less effective drug products [96]. Although QbD is not intended as a direct predictive system for toxicology, the lack of appropriate quality control can lead to medical toxicology problems. Moreover, it is interesting and worth pointing out in the context of this chapter that there exists computational software to help fulfill FDA QbD requirements [97]. However, a more direct impact is the role that *in silico* PBPK models play on defining relevant exposures to toxicity and ensuring higher quality pharmaceutical products for protection of public health [98, 99]. The estimation of ADME parameters through *in silico* PBPK models is essential in drug discovery prior to the conduct of extensive *in vivo* toxicokinetic studies in animals.

An increasing number of drug discovery frameworks appear to support drug discovery through the use of computational formulation prediction. With such tools, rapid identification of a solubility profile enables the prediction of drug absorption and selection of appropriate vehicles for delivery of a new drug [100]. By using computational models to develop a formulation strategy, necessary excipients and molecular dynamics between the therapeutic target and API can be predicted. These meaningful predictions provide contemporary decision support for toxicology considerations (e.g. potential target organs) and decision-making in later stages of drug development.

One of the approaches that has been central to many regulatory initiatives is the use of (Q)SAR technologies to predict the mutagenic potential of pharmaceutical impurities [101]. In addition, there has been substantial investigation from regulatory researchers on the use of knowledge databases for critical endpoints of interest such as endocrine disruption [102]. Significant strides have been made by regulatory investigators in modeling drug-induced cardiac safety endpoints such as the potentially fatal torsade des pointes and its surrogate marker, drug-induced QT prolongation [25]. Of great interest as well have been translational techniques to enhance knowledge of critical endpoints in drug safety such as liver toxicity [103]. Thus, it is clear that computational predictive toxicology modeling is an important part of advancing regulatory science in the drug safety arena. This will in turn modernize toxicology assessments to reduce drug attrition and help protect public health.

As discussed in the previous section, the modeling strategies that have been the most widely described as successful are those that support the application of predictive technologies to assess the mutagenic potential of pharmaceutical

impurities for the purposes of safety qualification under ICH M7 guideline. Namely, the use of global chemical training sets using binary classification model output has been the most commonly described and widely accepted [8]. According to the Organisation for Economic Co-operation and Development (OECD) principles for regulatory use of (Q)SAR models, an unambiguous algorithm should be a characteristic of a computational model [104]. Unfortunately, most of the commercial off-the-shelf and proprietary software fail to comply with this principle. As for other types of predictive computational technologies, there are practically no regulatory standards in place for their use and application, and consequently, there is little regulatory "acceptance," however, for many types of regulatory drug approval submissions, submission of corroborative scientific evidence can be voluntary, but this leaves the possibility of actual regulatory consideration of computational toxicology data questionable and relatively unknown as a practice.

The regulatory perspective for predicting the toxicity of a substance can be understood from the regulatory mission and obligation of authorities to protect public health. For regulatory considerations, the computational model's predictive performance is recognized to be imperfect, and the desire, in the context of a prediction error, is to side on protecting public health. Thus, models that are highly sensitive are preferred from a regulatory perspective because they minimize false negatives (e.g. toxic compounds predicted as nontoxic) and therefore reduce patient exposure to toxic substances.

As regulatory agencies do not normally have access to supercomputers or substantial IT infrastructure due to the nature of the regulatory work in review of drug applications, it is imperative that computational predictive models be as transparent as possible with a full narrative to the prediction and how the model was constructed. The ability to explain the prediction whether it portends to toxicity or whether it produces evidence that mitigates concern of the substance in question is crucial for regulatory consideration of computational model predictions. In the case of structure-based predictions, the ability to assess the confidence in the prediction is paramount. For example, are there irrelevant structural alerts present on the molecule that should be ignored as part of the assessment, are there mitigating features present on the molecule that might lead to inactivation (i.e. deactivating groups), or are there strong activating features present on the molecule that could reinforce a positive prediction? Furthermore, the analysis of the number, characteristics, and toxicity profiles of the training set molecules that are similar to the query substance could help in refining a prediction. These questions illustrate the importance of delving further into a prediction rather than simply relying upon computer output as regulatory scrutiny of the data will arise on various fronts.

20.6 Conclusion

In conclusion, predictive computational toxicology involves several integrated systems and specialties for predicting possible effects of toxicity, metabolism, fate, and physicochemical properties of substances of interest, as enabled by

computer databases and analysis. Developments in predictive computational toxicology result from multidisciplinary efforts drawing from specialized areas including:

- Computational model development and validation testing.
- Application of machine learning techniques.
- Software evaluation.
- Translational science.
- Big data science.
- Discrete and nondiscrete toxicity endpoint prediction.
- Read-across approaches to meet data gap analysis needs.
- Mechanistic pathway prediction.
- ADME–Tox prediction.
- Structure-based PBPK, *in vitro–in vivo* extrapolation models.
- Knowledge-based systems.
- Systems toxicology pathway analysis.
- Toxicology scientific data management and analysis.

As a result, the predictive performance for the genetic toxicology endpoint of mutagenicity has risen to an acceptable level of quality, reliability, and interpretability, which is of direct utility to early drug development. These results have built confidence in the use and application of predictive computational toxicology systems in general. Nevertheless, scientific challenges remain when predicting carcinogenicity and reproductive toxicology endpoints, while target organ toxicities (e.g. lung and other major organs) are still in need of investigative development with computational predictive techniques. In these cases, it is anticipated that further development and integration of physiology-based models will be required.

It is noteworthy that regulatory science initiatives have recognized the opportunities within predictive toxicology by funding the associated computational science research and development. As new prediction models are developed, special emphasis should be placed in making them as accessible and transparent as possible, and that their predictions are supported and contextualized by an understandable narrative. This will significantly help wider dissemination in the scientific community and facilitate the review practices of regulatory authorities. Here, collaborative noncompetitive research between technology developers, drug development programs, and regulatory scientists could help resolve intellectual property protection concerns associated with model development. With the typical drug entering Phase I trials having only an 8% chance of reaching the market, the grand challenge for predictive toxicology methods lies in accurately identifying idiosyncratic adverse drug reactions, such as drug-induced liver injury and torsade des pointes. Formalizing the knowledge of human experts in these areas still poses a significant challenge given the variability of unintended and intended bias in human expert opinions and approaches. Therefore, this author believes that the future will be in appropriate selection and use of advanced machine learning techniques guided by human expert decision-making on the most appropriate data to model for predictions. This will result in computational toxicology effectively "de-risking" early drug development programs.

List of Abbreviations

CYP	Cytochrome P450
US	United States
FDA	Food and Drug Administration
GLP	good laboratory practice
OECD	Organization for Economic Co-operation and Development
QSAR	quantitative structure–activity relationship
QbD	quality by design
SAR	structure–activity relationship
(Q)SAR	quantitative structure–activity relationship and structure–activity relationship systems

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21

Addressing Genotoxicity Risk in Lead Optimization: A PDE10A Inhibitor Case Study

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21.1 Introduction

In the pharmaceutical industry, the development of efficacious and safe drugs remains a continuous challenge. It is an increasingly long process, with an estimated cost of up to 2.6 billion US dollars to bring a new drug to the market [1]. John Arrowsmith and Philip Miller investigated the reasons for the high attrition rates of Phase II and III studies between 2011 and 2012. They came to the conclusion that, besides efficacy, safety issues are still a significant hurdle even in late stages of development [2]. To improve on this, various approaches have been proposed, which focus largely on more and better predictive toxicity assays as well as improved models especially during the lead optimization phases of the drug discovery process [3].

In recent years, toxicity testing is undergoing a major paradigm shift toward the use of *in vitro* approaches for assessing chemical risk. Besides several global initiatives that demonstrated the utility of high-throughput screening to prioritize compounds for further testing, the next step is to move from prioritization to prediction, aiming at replacing animal-based risk assessment strategies with safety assessments based on human biology. Quantitative high-throughput screening (qHTS) techniques are now used in combination with computational methodologies to probe how chemicals interact with biological systems. Progress is being made in recognizing the patterns of response in genes and pathways induced by a broad range of chemicals or chemical classes that might be predictive of adverse outcome pathways (AOPs) in humans.

The toxicology in the twenty-first century (Tox21) movement [4] aims to identify chemical structure–activity signatures derived through *in vitro* testing that could act as predictive surrogates for *in vivo* toxicity. The use of relevant cell-based assays will allow the identification of key pathways and proteins linked with toxicity, an approach that finally will lead to a better understanding of the underlying mechanisms of toxicity [5]. A comprehensive analysis of the

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

available Tox21 data resulted in compound clustering by structure similarity and activity profile similarity that revealed structure–activity relationships useful for the generation of mechanistic hypotheses [6]. Predictive models for *in vivo* toxicity endpoints were build using a cluster-based approach. It was shown that models based on *in vitro* assay data perform better in predicting human toxicities than animal toxicity, while a combination of structural and activity data results in better models than using structure or activity data alone. These results suggest that *in vitro* activity profiles can be applied as signatures of compound mechanism of toxicity and used in prioritization for more in-depth toxicological testing.

In order to find connections between chemicals, genes, and adverse events, connectivity mapping [7] is a method used in the pharmaceutical industry to improve predictive toxicology and allows grouping of chemicals based on their mode of action [8]. Within the field of genotoxicity testing, new and innovative test platforms are being developed. The ToxTracker assay, a mammalian reporter stem cell-based genotoxicity assay, uses a panel of six green fluorescent protein reporters to discriminate between different primary reactivity of chemicals and their ability to react with DNA and block DNA replication, induce oxidative stress, activate the unfolded protein response, or cause a general P53-dependent cellular stress response [9]. Another example of focused data integration was reported by Bryce and coworkers [10] who showed that the genotoxic mode of action could be predicted from a multiplexed flow cytometric assay and a machine learning approach. Univariate analyses identified biomarkers and time points that were valuable for classifying test compounds as clastogenic, aneugenic, or nongenotoxic. In general, understanding the underlying mechanisms of the observed hazard will allow for a more efficient translation to human risk assessment.

The development of integrated testing strategies connects discovery technologies and know-how with preclinical safety assessment to deliver innovative and optimized drug candidate selection procedures. The molecular properties of a drug candidate are finalized at the interface between discovery and development. This means that properties causing mechanism (target)-related toxicity, off-target side effects, and compound-chemistry-related toxicity are all fixed at that point.

During a large collaborative project between Janssen and several academic institutions, we explored the use of transcriptional profiling for the prioritization of compounds during lead optimization. One main hypothesis that we explored was to what extent the gene expression signatures of compounds can be used to capture the range of polypharmacological effects of compounds. Furthermore, we were interested in assessing to what extent the technology could be used to monitor the impact of medicinal chemistry optimization of a lead series and ideally to explore whether the data may even be used to generate hypotheses as to what compound substructures are responsible for a given effect to support the medicinal chemist in designing new structures to make. This project named quantitative structure–transcription assay relationships (QSTAR) explored eight drug discovery projects across multiple therapeutic areas, biological targets, and chemical scaffolds [11]. This project integrated

high-throughput gene expression profiling data, chemical information, and bioassay data within the lead optimization phase. Building on these findings, it was shown that for one particular project (PDE10A inhibitors) a subset of close analogs could be identified commonly downregulating multiple tubulin genes across cellular contexts, suggesting possible spindle poison effects [12]. Positive responses in the *in vitro* micronucleus test (MNT) and the identification of a characteristic aggregate-formation phenotype via exploratory high content imaging validated these initial findings. This case study, described in this chapter, illustrates the potential to flag toxicity issues by utilizing data from exploratory experiments that are typically generated for target evaluation purposes during lead optimization.

21.2 Lead Optimization Project: Searching for PDE10A Inhibitors

The case study presented in this chapter had been an active drug discovery project within the Neuroscience Disease Area at Janssen. The aim of the project team was to identify and optimize a small molecule inhibitor against phosphodiesterase PDE10A [13, 14]. Characteristic for this protein is the almost exclusive expression in one particular region of the brain, the striatum, which promises fewer side effects in other parts of the body. The modulation of the activity of this enzyme was explored as a new therapeutic approach for the treatment of schizophrenia: inhibiting phosphodiesterase results in increased cAMP/cGMP levels that are essential for signal transduction processes. In this context chemical compounds from a novel series of 3-alkoxy pyrrolidine derivatives (Figure 21.1) were synthesized. During the optimization process, adverse effects linked to extrapyramidal symptoms emerged as a point of concern. Therefore, a subset of 58 compounds was transcriptionally profiled to investigate potential polypharmacological effects.

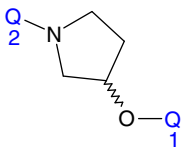


Figure 21.1 1,3-Alkoxy-pyrrolidines substituted with different heteroaromatic ring systems: Q_1 is (substituted) benzofuran, phenyl, quinoxaline, pyridine, quinoline, or pyrazine. Q_2 is (substituted) quinazoline, quinoline, imidazopyridazine, or triazolopyridine.

21.3 Transcriptional Profiling to Capture Polypharmacology

A subset of compounds within the novel series were profiled with respect to their induced gene expression on human embryonal kidney cells (HEK293, ATCC CRL-1573) transfected with the mouse homologue of PDE10A. The cells were treated with the compounds at a concentration of 10 μM in 0.1% DMSO for 8 h. Exploratory analysis of the induced transcriptional effects of all

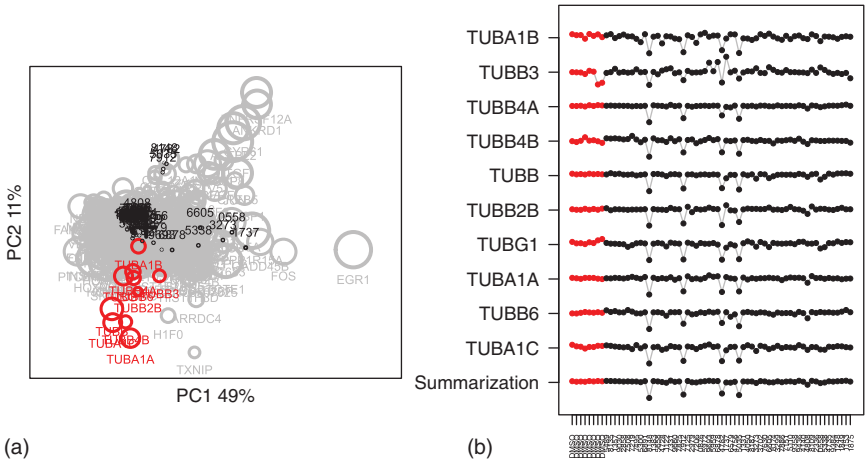


Figure 21.2 Exploratory analysis of transcriptional effects of all quality filtered informative genes induced by 58 compounds. (a) Spectral map analysis showing on the *y*-axis a clustering of four compounds based on a subset of tubulin genes indicated in red. (b) Gene profile plot of the tubulin genes together with a summarization of the tubulin genes at the bottom. The summarization reduces the random noise and clearly shows a subgroup of four compounds downregulating the tubulin genes. DMSO samples are indicated in red.

quality filtered informative genes [15] revealed two subgroups of compounds differentiated from the others (Figure 21.2a) [16]. The second component, displayed on the *y*-axis, distinguishes a subgroup containing four compounds (8148, 4782, 5035, and 7912) based on a set of tubulin genes. Investigation of individual expression levels for all informative-called tubulin genes across all compounds revealed that this subset of four compounds downregulate the tubulin genes (Figure 21.2b). A summarization score of the tubulin genes, indicated at the bottom of the plot, reduces the random variation and clearly reveals the four compounds [17]. Downregulation of tubulin genes suggests a possible genotoxic effect on the microtubule-based chromosome segregation, during which the duplicated chromosomes of a cell are separated into two identical sets before cleavage of the cell separated into two daughter cells. Interfering with microtubule dynamics causes mitotic arrest and cell death in different tumor cells [18].

Compound 8148 was subsequently tested in three other human cellular backgrounds available at the time, namely, LNCaP (human prostate cancer cells), HepG2 (hepatocarcinoma cells), and SK-N-BE (neuroblastoma cells), to explore the biological reproducibility of the tubulin signature. Figure 21.3 shows the distribution of the fold changes for all informative genes for compound 8148 in four cellular backgrounds with the tubulin genes colored in red. In general, the tubulin genes are among the most downregulated genes, suggesting that the observed effects are largely cell-line independent.

Hence, a subset of four compounds from our initial chemical series is identified using high-throughput techniques already during lead optimization with potential genotoxicity effects.

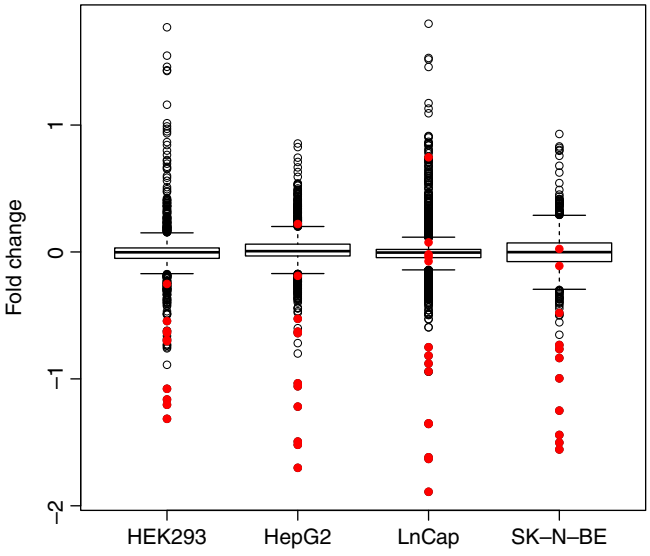


Figure 21.3 Boxplot of the fold changes of all quality filtered informative genes in four different cell lines for compound 8148 with tubulin genes colored in red. The tubulin genes are in general among the most downregulated genes.

21.4 High Content Imaging as an Independent Confirmation

High content imaging combines automated microscopy with image analysis approaches to simultaneously quantify multiple phenotypic and/or functional parameters in biological systems. The technology has become an important tool in the fields of safety sciences and drug discovery, because it can be used for mode of action identification, determination of hazard potency, and the discovery of toxicity targets and biomarkers. It allows the identification of signaling pathways underlying cell defense, adaptation, toxicity, and death. Therefore, high content imaging is considered a promising technology to address the challenges for the "toxicity testing in the twenty-first-century" approach [19].

Within the current context, high content imaging was used to further explore the possible genotoxic effects in a subset of the leading chemical series. Eleven compounds, both positive and negative compounds with respect to tubulin downregulation, together with some known genotoxic compounds, were added to osteosarcoma cells (U2OS) expressing an endogenous green fluorescent tubulin (TUBA1B) at different doses (from 1 nM to 25 µM) to cover a broad concentration range. Cells were monitored over a period of 24 h and imaged at each hour, which allowed us to follow the tubulin proteins (Figure 21.4).

Nocodazole, a genotoxic reference compound, shows a particular phenotype with microtubule aggregates at the edges of the cell and around the nucleus (Figure 21.4a) in contrast to the DMSO controls (Figure 21.4b). The four compounds that downregulate tubulin genes show, at certain concentrations and time points, similar profiles to the reference compound. One of which is shown

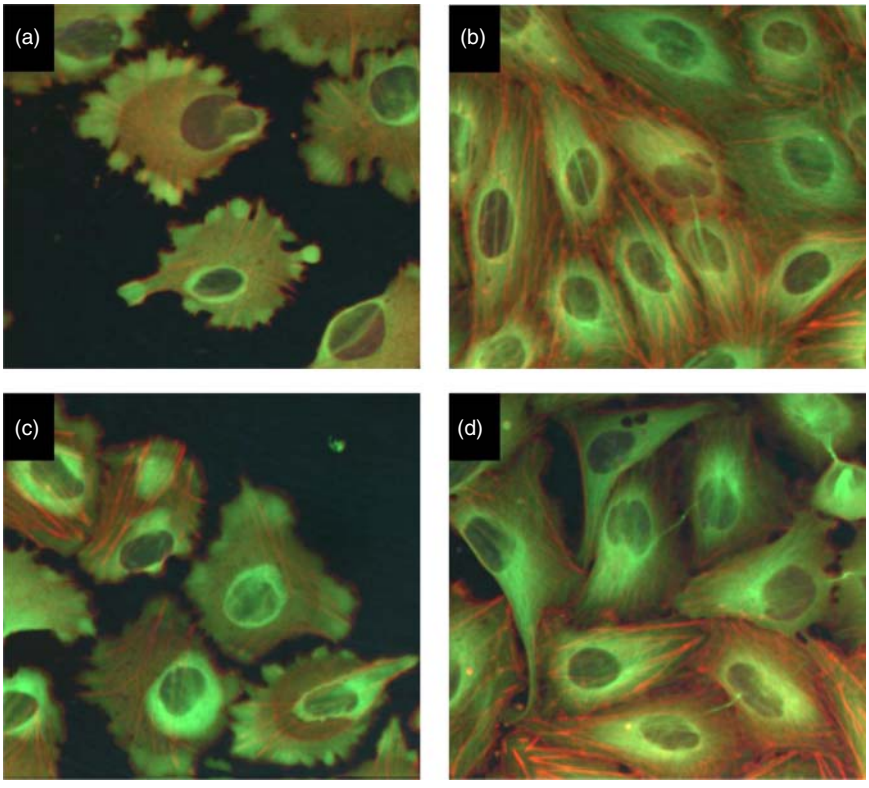


Figure 21.4 High content images of U2OS cells expressing an endogenous green fluorescent TUBA1B at 8 h. (a) Nocodazole, an aneugenic MNT positive compound, showing microtubule aggregate formation at a concentration of 25 μM. (b) DMSO controls do not show the typical microtubule aggregates. (c) Compound 8148, showing tubulin downregulation, shows aggregate formation at 10 μM. (d) Compound 0558, within the same chemotype as 8148 showing neither tubulin downregulation nor aggregate formation at none of the concentrations (picture at 30 nM).

in Figure 21.4c. Interestingly, structural analogs within the same chemotype that did not show the tubulin downregulation display phenotypes similar to DMSO controls across the different concentrations and time points (Figure 21.4d). Hence, the presence of the particular phenotype seems to be correlated with genotoxicity and tubulin downregulation.

To quantify this phenotype, 661 features related to cell intensity, shape, texture, geometry, etc. were extracted from the images. Following typical feature selection procedures, three features that discriminated images showing microtubuline aggregates from images showing other phenotypes were retained. All three are texture features derived from the cellular regions of the tubulin–GFP channel and thus directly represent tubulin characteristics. A microtubule aggregate score, summarizing the three features, was derived through linear discriminant analysis. A positive score corresponds to the phenotype of interest. The microtubule aggregate score was calculated as the maximum score observed across

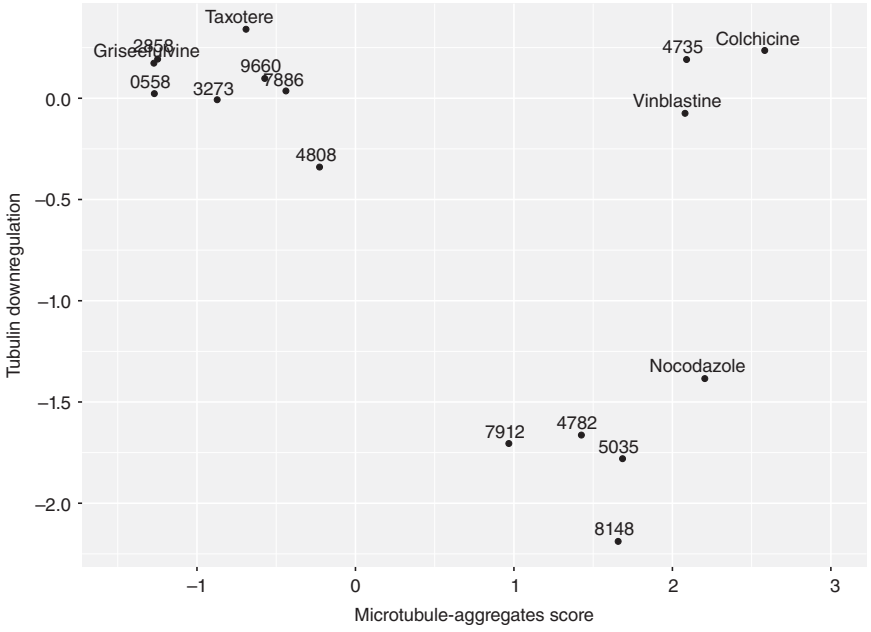


Figure 21.5 Scatterplot of the maximum microtubule aggregate score, summarizing three features, observed across the different concentrations at time point 8 h versus the summarization of the quality filtered informative tubulin genes. Only a subset of compounds, the ones profiled in high content imaging, was used to calculate the summarization score of the tubulin genes. A positive microtubule aggregate score corresponds with microtubule aggregate formation. The four compounds downregulating tubulins also show microtubule aggregate formation.

different concentrations at 8 h. Only the time point at 8 h was used to ensure correspondence with the gene expression fold changes, which were also measured at 8 h. The maximum microtubule aggregate score is plotted against the summarization score of the fold changes [17] in the tubulin genes (Figure 21.5). The reference compounds were transcriptionally profiled under similar circumstances as the original PDE10 series (HEK293 cells transfected with the mouse homologue PDE10A at 10 μM for 8 h).

All four compounds identified as potentially genotoxic through transcriptional profiling have a positive microtubule aggregate score together with the reference compound nocodazole. For compounds in the upper left corner, the two high-throughput profiles agree as well and do not suggest any potential genotoxicity. On the other hand, the three compounds vinblastine, colchicine, and 4735 have a positive microtubule aggregate score observed in the concentration range at 8 h but did not show the tubulin downregulation. However, the expression profiling was only performed for a single concentration (10 μM) in contrast to the high content imaging.

Therefore, we investigate in more detail the microtubule aggregate scores observed at 8 h as a function of the concentration of the three compounds but also for two positive and one negative control of the correlation (Figure 21.6).

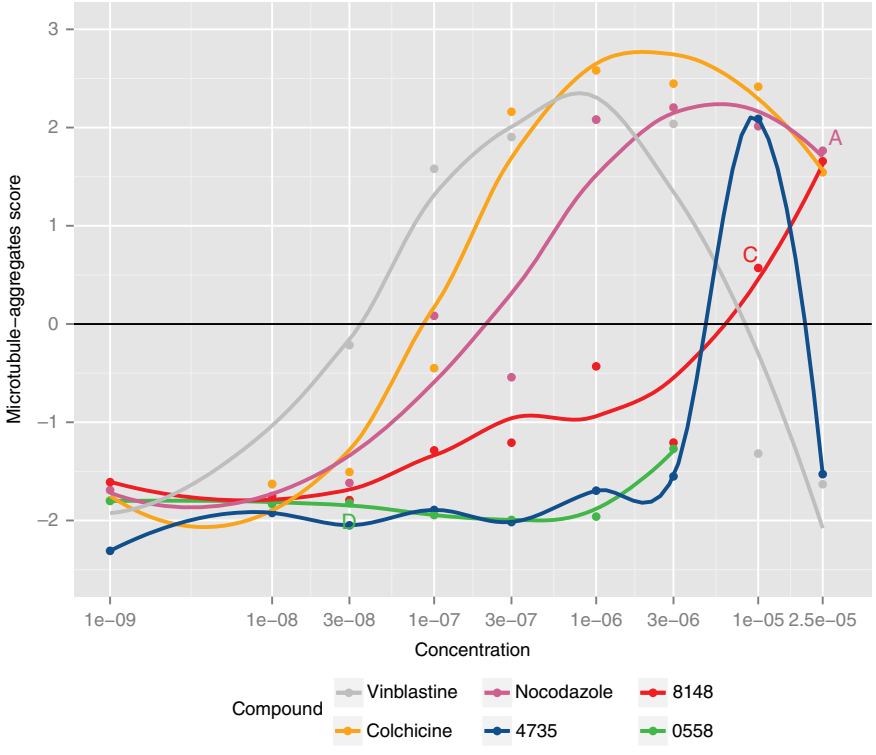


Figure 21.6 Concentration profile plot of the microtubule aggregate score at 8 h for six compounds (vinblastine, colchicine, nocodazole, 4735, 8148, and 0558) indicated with different colors. The concentration at which the image is shown in Figure 21.4 is indicated with the corresponding letter.

The negative control, compound 0558, does not show the formation of the microtubule aggregates over the full concentration range. All the other compounds show the formation of the microtubule aggregates from a certain concentration onward, except for compound 4735, which shows it only at one concentration, namely, 10 µM. So either a technical issue (e.g. only one plate well shows the aggregates) occurred or the compound has a small window of genotoxicity.

Vinblastine shows a bell-shaped curve: microtubule aggregates start to form from 0.1 µM but disappear around 10 µM where investigation of the images suggests the formation of another phenotype, tubulin paracrystals, which is supported by literature precedents [20]. Therefore, at the concentration profiled in gene expression, no microtubule aggregates were present, which might correspond to the absence of tubulin downregulation.

Colchicine would downregulate tubulin genes based on the concentration profile plot of the microtubule aggregate score. A retesting in the transcriptional screen showed indeed a tubulin downregulation of onefold (cf. nocodazole: 1.4-fold decrease).

Both transcriptional data and high content imaging data are able to flag potential toxicity issues. However, having no signal in these data does not imply that

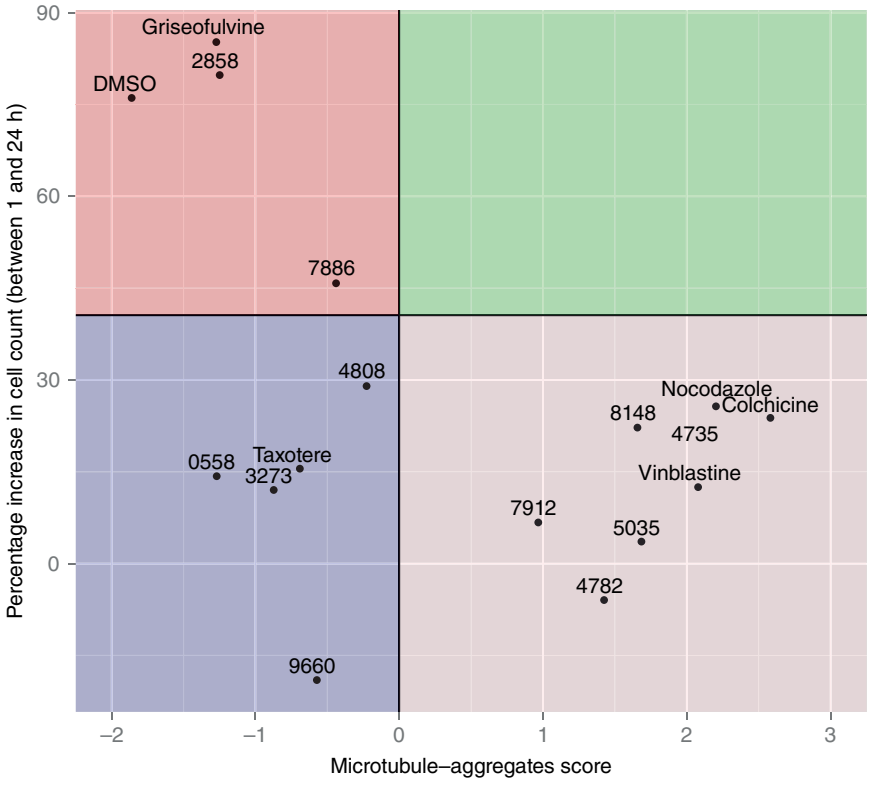


Figure 21.7 Scatterplot of the maximum microtubule aggregate score observed across the different concentrations at time point 8 h versus the percentage increase in cell count over 23 h at the corresponding concentration. A positive microtubule aggregate score suggests formation of microtubule aggregates. The horizontal line indicates the minimum increase in cell count over the replicates of the DMSO controls, whereas the average increase in cell count is plotted for DMSO.

compounds will be safe. One reason for the lack of any signal could be just too low exposure to the compound of interest. Therefore, the imaging data were used to assess if the cells were sufficiently exposed to compounds by quantifying the relative increase in cell count over 24 h (Figure 21.7). This revealed that griseofulvin, compound 2858, and 7886 have similar cell count increases compared with the DMSO controls, indicating that these compounds were not dosed high enough to induce the toxicity.

21.5 *In Vitro* Micronucleus Testing to Validate Transcriptional Signature

Genetic toxicity testing has traditionally been used for hazard identification, with dichotomous classification of test results serving to identify genotoxic agents. The *in vitro* MNT has become an attractive tool for genotoxicity testing because of

its simplicity of scoring and wide applicability in different cell types. Since the two basic mechanisms leading to the formation of micronuclei are chromosome breakage and disturbance of the chromosome segregation machinery, micronucleus expression requires a mitotic or meiotic division and assessment of cell division in the presence of the test substance [21].

Here, three compounds (8148, 4782, and 0558) were profiled in the *in vitro* MNT to confirm that the signals observed both in transcriptional profiling and in high content imaging would flag indeed potential genotoxicity effects. The *in vitro* MNT is a qualified assay and part of the genotoxicity test battery (see Chapter 18) to detect micronuclei in the cytoplasm of interphase cells. A dose–response assay was performed in TK6 cells, and a positive response was obtained when the compound induced a twofold or higher and concentration-dependent increase in the frequency of micronucleated cells above the control value. For two tested compounds, 8148 and 4782, showing clear tubulin downregulation, more than 10-fold increase in the frequency of micronucleated cells were observed. On the other hand, the negative control, compound 0558, did not show the formation of micronuclei.

The MNT identifies compounds that can either cause chromosomal breaks (clastogen) or affect the formation of the mitotic spindle or microtubule (aneugen). The observation of large-sized micronuclei in the positive compounds and an increased number of bi- and polynucleated cells is typical for spindle poisons and suggests aneuploidy [22, 23]. Figure 21.8 shows micronucleated TK6 cells which were treated with (a) mitomycin C (clastogen) and (b) compound 8148 which clearly show large sized micronuclei.

Also within the spindle poisons, several binding regions of compounds with microtubules are known [24]. Taxanes, including paclitaxel and griseofulvin, interfere with microtubule dynamics through the promotion of tubulin stabilization. Vinblastine, a vinca alkaloid, promotes depolymerization of microtubules

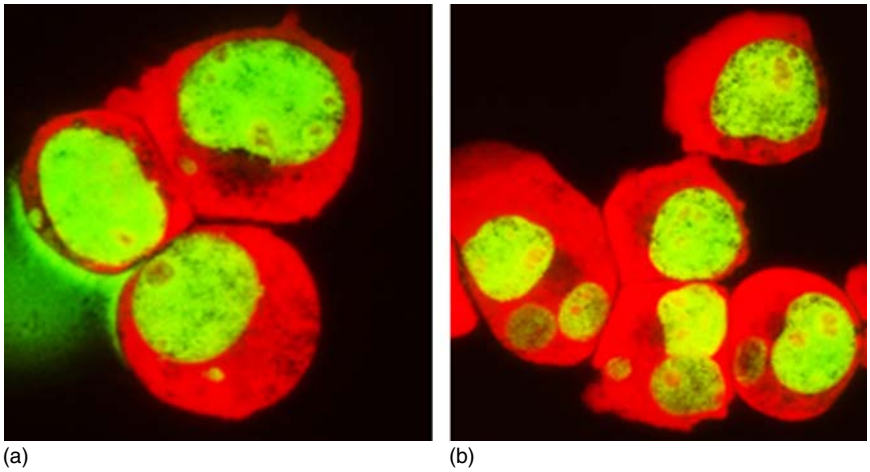


Figure 21.8 Micronucleated TK6 cells induced by (a) clastogen mitomycin C and (b) compound 8148 that clearly show formation of large-sized micronuclei, suggesting an aneugenic mode of action.

by preventing self-association of tubulin through interaction at the interface between two αβ-tubulin heterodimers. A third group of spindle poisons with a colchicine-like binding site inhibits microtubule assembly by introducing a steric clash between colchicine and α-tubulin. Hence, the intracellular pool of tubulin monomers is increased, which triggers the degradation of microtubulin mRNAs [25].

Reference compounds, representing the three different mode of action, were profiled with the high-throughput techniques. However, not all of them showed tubulin mRNA downregulation and/or microtubule aggregate formation. Vinblastine did not show tubulin mRNA downregulation, possibly because of the formation of paracrystals at the concentration at which it was transcriptionally profiled. However, Cleveland et al. [26] also failed to show a reduction in tubulin synthesis for vinblastine, possibly indicating another working mechanism of this microtubule-depolymerizing compound. Taxanes did not show the tubulin mRNA downregulation neither the microtubule aggregates, while colchicine and nocodazole showed the two phenotypes. This would suggest that PDE10A compounds, showing tubulin mRNA downregulation and microtubule aggregate formation, act according to a similar mechanism as colchicine [26].

The identification of an aneugenic mode of action for this compound class is important. It allows the application of threshold-based risk assessment to define a safety window and thus de-risk flagged drug candidates [27]. Recently, the utility of genotoxicity data is augmented by employing dose–response analysis and point of departure determination. Via interpolation from a fitted dose–response model, the benchmark dose (BMD) approach showed that potency rankings can be employed to support mechanism of action evaluations to expedite chemical evaluations and regulatory decision making [28].

21.6 Data Integration

The different behaviors of aneugenic reference compounds in the high-throughput techniques with only one corresponding to the subset of the PDE10 compounds suggest that we are able to identify a particular mechanism of action. However, to further increase our confidence in this finding, we explored the integration with public data. We investigated the connectivity map (CMAP) data [7] for more gene expression profiles of the reference compounds. Three of the five reference compounds, vinblastine, colchicine, and nocodazole, were profiled in CMAP at different concentrations and in different cellular backgrounds representing two different aneugenic mechanisms of action.

At first we queried the whole CMAP database using tubulin downregulation as a signature using the method of Zhang and Gant [29], which checks for compounds having a similar ranking of the tubulin genes (Figure 21.9). The query was based on the ranks of tubulin genes for compound 8148. The top-scoring CMAP compound was found to be vinblastine, with a high score of 0.98 meaning that our compounds have very similar transcriptional tubulin effects. The top three compounds showing the highest transcriptional similarity are fenbendazole (scores 0.97, 096), (+)-chelidonine (score 0.93), and mebendazole (score 0.93 and 0.92).

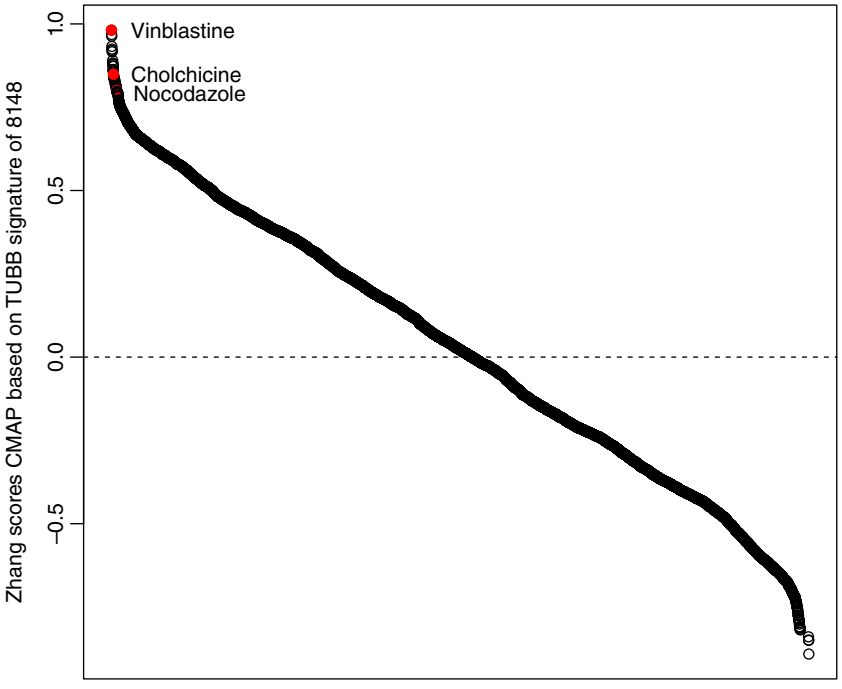


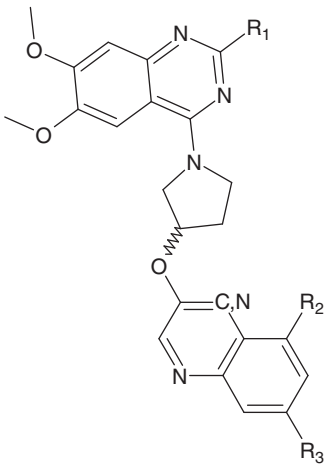
Figure 21.9 Ordered Zhang scores of all instances in CMAP based on the tubulin signature of compound 8148. A positive score indicates a similar ranking of the tubulin genes. The top scores of the three reference compounds present in CMAP (vinblastine, nocodazole, and colchicine) are indicated with red dots and annotated.

All ordered Zhang scores are shown in Figure 21.9 and the highest scores of the reference compounds profiled both in our high-throughput experiments as well as in CMAP are also annotated. Indeed, the fact that the tubulin signature is able to detect the known reference compounds indicates that the tubulin downregulation and microtubule aggregates are linked to spindle poisons, but we cannot make a distinction between the respective mechanisms of action for vinblastine and colchicine.

21.7 Hypothesis for a Potential Structure–Activity Relationship

Since only a few compounds within the same chemotype showed strong tubulin downregulation, namely, those where Q_2 is a quinazoline and those where Q_1 is either a quinoline or a quinoxaline, a structure–activity relationship was performed (cf. Figures 21.1 and 21.10). It appeared that only a specific substitution pattern on the heteroaromatic ring systems is associated with the tubulin downregulation. Compounds with electron-donating groups as R_1 do not show tubulin downregulation, and substitutions in R_2 and R_3 are not permitted.

Figure 21.10 New compounds were synthesized in the quinazoline series, without substitution at R_2 and R_3 and small or electron-with-drawing groups at R_1.



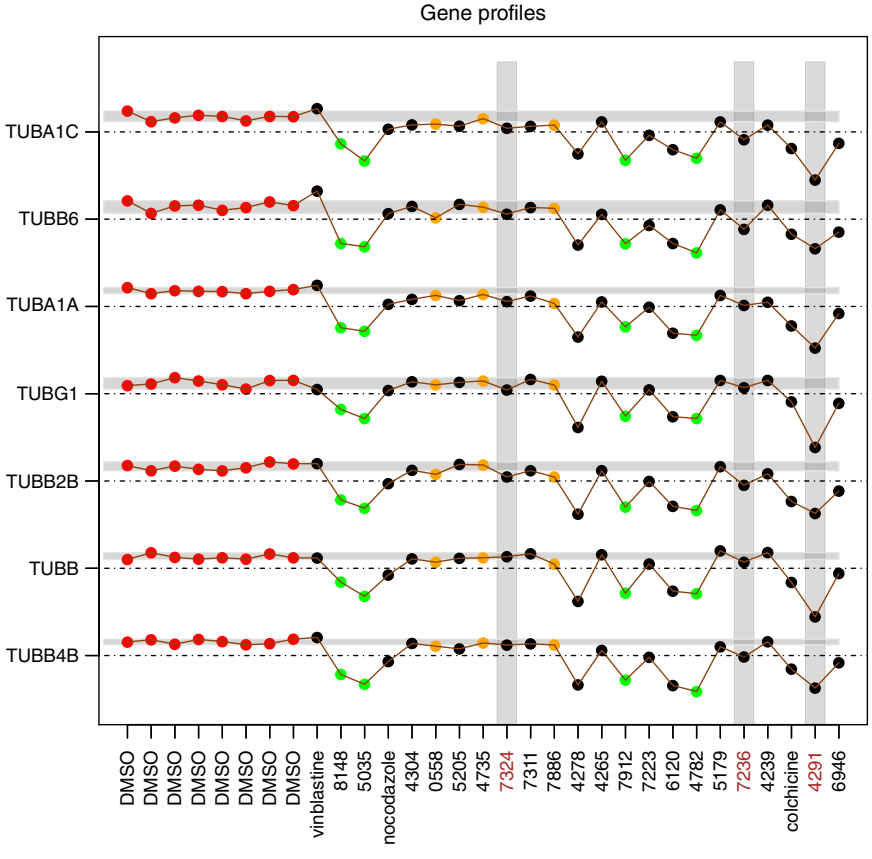


Figure 21.11 Gene expression profiles of all quality filtered informative tubulin genes for a new transcriptional experiment including the newly synthesized compounds, reference compounds, and some positives (colored green) and negatives (colored orange) from earlier profiling experiment. DMSO samples are indicated in red, and their variation is indicated with gray bars. The three compounds, indicated with gray vertical bars, showing different levels of tubulin downregulation are subsequently tested in MNT.

On the basis of this observation, a new set of 13 compounds with a substitution on R_1, either small or electron-with-drawing, was synthesized and transcriptionally profiled. It was hypothesized that these compounds have a high chance of downregulating tubulins and therefore being genotoxic. The gene expression profiling was performed in similar conditions (HEK293 cell lines at 10 μM for 8 h), and some positive and negative controls from the earlier profiling experiment together with the reference compounds were included as well.

The experiment confirmed the earlier findings (Figure 21.11). A fair amount of compounds from the set of newly synthesized ones downregulate tubulin at similar levels as the reference compounds or even stronger. Hence we expected that they would show again signs of genotoxicity in higher models.

The three compounds having some gradation in tubulin downregulation (7324, 7236, and 4291, indicated with gray bars in Figure 21.11) were tested in the MNT. Compound 7324 shows no tubulin downregulation, while 4291 downregulates tubulin the strongest. All three compounds show micronucleus formation, even compound 7324. However, the concentration at which they reach 50% relative population doubling (RPD) differs a lot between these three compounds. The RPD is 2.71, 4.17, 16.0 μM for 4291, 7236, and 7324, respectively. Since the gene expression profiling is performed at 10 μM, the exposure levels of 7324 were most probably still too low to observe significant tubulin expression changes. Hence this showed that we were able to identify chemistry motifs responsible for undesired effects, which can help us in optimizing the drug candidates.

21.8 Conclusion

21.8.1 Current Situation

Even though a lot of effort is put into testing for potential toxicity issues during all phases of drug discovery and development, compounds are still frequently stopped due to the identification of liabilities [2, 30]. The high attrition rate especially during late stages results in extensive waste of resources.

At Janssen, the early phases of drug discovery focus on identifying and understanding the desired biological activities of compounds. It is in this context that many profiling experiments such as transcriptional profiling or high content imaging are carried out. On the other hand, the majority of toxicity testing exploring the undesired properties is conducted just prior to starting preclinical development.

In this chapter we have demonstrated the feasibility to generate hypotheses regarding the different polypharmacological activities of a chemical lead series by exploring early discovery data that are generated with the intention to obtain timely insights into the desired mechanisms of action rather than flag potential toxicity issues.

Investigating the lead series designed to inhibit PDE10A identified a transcriptional signature comprising of tubulin genes that are downregulated upon compound treatment for a subset of close chemical analogs.

By combining these transcriptomic findings with a high content imaging-based signature and results from an established toxicity assay (part of preclinical

genotoxicity testing battery [31]), we have demonstrated that the observed effects are indeed linked to the formation of micronuclei after compound exposure.

Furthermore, when we tested our data with publicly available data, we were able to connect our findings to the publicly available CMAP data [7]. As such, combining all different approaches and integrating the available data allowed us to propose a hypothesis regarding the underlying genotoxicity mechanism, namely, an effect of the identified compounds on tubulins.

Furthermore, we have shown that it may be possible to identify early signs of potential liabilities across biological backgrounds as we were able to reproduce the transcriptional signature in different cell lines. Our experience confirms the value of transcriptional profiling as a means to identify toxicity of unknown compounds [32].

21.8.2 Suggested Improvements

Over the past years a number of novel screening technologies (e.g. high content imaging, various omics technologies such as whole genome transcriptome profiling) have been developed. In recent years the computational power to efficiently analyze the wealth of available data using machine learning techniques (chemogenomics) has become available as well. Together with enhancements in more human translational models (3D models, cellular cocultures, organs-on-a-chip, etc.) as well as freely available public data sources (biological, mechanistic, toxicological and chemical), we now have the opportunity to incorporate data from early screening on biological activity, pharmacokinetics, and pharmacodynamics to enable toxicity flagging at early stages.

We have shown that data obtained from biological profiling technologies such as high content imaging or transcriptional profiling that are typically executed during early phases in the discovery process can generate novel biological insights beyond the findings of a qualified toxicity assay such as the micronucleus assay. Especially when the data agrees between such independent profiling approaches, the complementarity of the data can be useful in generating hypotheses regarding the underlying mechanism of toxicity.

Furthermore, we have also shown that compound-induced biological effects may be detectable across different cell lines (here: tubulin downregulation). Therefore screening experiments conducted using omics technologies such as transcriptional profiling with the aim to study a single biological activity of a compound can be utilized to obtain information on the polypharmacological properties of a chemical structure. In other words, analyzing the data in a hypothesis-generating approach bears the potential to identify activities that would otherwise only be detected later using qualified toxicity assays. By incorporating such approaches for toxicity screening early in discovery and thereby enabling a weight-of-evidence approach [33–35], we believe it will be possible to translate an identified *in vitro* hazard much more quickly into human risk assessment, with the result of advancing cleaner and/or safer chemical structures for further development.

Based on our long-term collaborations across scientific disciplines, we also believe in the need to enhance communication between molecular toxicologists, computational scientists, biology team leads, bioinformaticians, biostatisticians, and medicinal chemists to enable decisions on how to evolve structural modifications with the aim to move away from potential liabilities while optimizing the desired activity and properties of the chemical lead series [36]. However, this requires a commitment to involve all relevant disciplines much earlier during hit series selection and lead series selection as well as regular intervals prior to finalizing preclinical development [3].

A concrete challenge we observed during the exploration of the available profiling data for the PDE10A series was the fact that these data were generated in a setting that focuses on learning about the desired properties of a compound. Accordingly, we have observed that the experimental design is regularly unsuitable for drawing conclusions in relation to toxicity as the choice of concentration is often not high enough. Still, we see an opportunity to educate colleagues about these aspects and encourage them to consider jointly with a mechanistic toxicologist the experimental designs of early studies to minimize the frequency of missing compounds that could be flagged by such potential toxicity markers. Therefore we support the notion of De Abrew et al. to include more than one concentration whenever possible, as the choice of concentration has a significant impact on the transcriptional profiles, e.g. via activation of nonspecific pathways [8]. While this is regularly done for high content imaging studies, it is not always done when using transcriptional profiling due to financial and lab-related constraints. However, approaches such as the L1000 profiling could provide a compromise in enabling the cost-effective profiling of more samples while losing some granularity due to the reduced number of transcripts that are measured [37].

21.8.3 Expected Outcome

In this chapter we highlighted a proof-of-concept study that we had conducted to demonstrate possible approaches of how to utilize screening data with the aim to improve risk/safety assessment during the lead optimization phase of the drug development process. We have shown ways of combining and experimentally verifying early flags from a hypothesis-generating, exploratory omics technology (whole genome transcriptional profiling) with other screening technologies (high content imaging) as well as a qualified toxicity assay (*in vitro* MNT) and computational approaches using publically available data (CMAP analysis) [11, 12].

Looking forward, we need to focus on developing early markers for various toxicities where possible. The findings presented in this chapter demonstrate the opportunity for creating a marker that links the transcriptional downregulation of tubulin genes with the formation of aggregates, which in turn may be an indicator of incorrect microtubule formation, which may eventually lead to aneuploidy. Even though transcriptional changes can be linked to various primary as well as secondary effects triggered by the polypharmacological properties of a compound, we believe that several other early markers – based on early stage drug

profiling data such as high content imaging or transcriptional profiling – could be developed and routinely checked within data from early discovery experiments. For example, developing a marker that indicates the potential of a compound to generate reactive oxygen species (ROS) could flag chemical structures that are likely to trigger an oxidative stress response [38], some of which are known as liver toxicants. As a result, such markers could help identify compounds likely to be linked to drug-induced liver injuries (DILI), which is still one of the main causes of late-stage drug attrition [39]. In this way a shift from late failures to a fail-early approach based on identified potential risks could be envisioned. Furthermore, we will utilize data for multiple purposes, thereby saving time and resources as selected toxicity assays would be run early to assess the correctness of potential flags.

While we observed challenges in ensuring a minimal number of false negatives, our particular marker candidate was appropriate in all observed cases: whenever both microtubule aggregates were observed and downregulation of tubulin gene expression was detected, we always also identified genotoxicity under these conditions. As such, we believe that the collaborative evaluation of profiling data from early discovery experiments has value in flagging potential issues, even though the data will likely not be able to identify all undesired properties of a given compound.

Furthermore, exploring the biological underpinnings of identified markers, as we have demonstrated in this chapter, should provide us with the mechanistic insights that could provide the basis for a mitigation strategy for additional investigative toxicity studies much earlier in the drug discovery process. The gene annotation linked to the measured transcriptional profiles allowed us to derive the genotoxicity hypothesis for the described tubulin marker. Still, further research is needed to improve on the available gene annotation, as frequently the data-driven composition of a transcriptional marker is composed of genes that are not directly interpretable due to limited understanding of the protein function encoded by the respective genes.

When looking at high content imaging data, we are aware of the challenges when attempting to biologically interpret image features. However, approaches that make use of reference compounds of known mechanism of action can be used in a guilt-by-association approach. Hereby unknown compounds showing similar effects on characteristic features of reference compounds are hypothesized to have a similar mode of action.

Still, even though early markers of potential liabilities defined for early discovery profiling technologies hold the promise to flag undesired properties, they are not likely to replace qualified assays any time soon. Rather, they will enable us to investigate possible issues early and allow us to monitor more specifically the impact of structural modifications during the preclinical medicinal chemistry optimization process. Furthermore, we also expect that likely not one single technology and probably also for some time not even a combination of early profiling technologies will allow us to flag all toxicity issues. Adding to that are also aspects such as the limited complexity of a cell lines versus a whole organ or even a whole organisms that are likely to limit how broadly these approaches can be utilized.

21.8.4 Future Opportunities

While we are already able to explore compound-induced effects on the transcriptome of cell lines or their effects on the cellular phenotype as measured by high content imaging, technological advances as well as scientific advances will continue and provide us with new opportunities to capture more and other aspects triggered by compound exposure. We have already referred to the L1000 technology [37], which is a key component of the ongoing Library of Integrated Network-based Cellular Signatures (LINCS) project of the National Institutes of Health [40]. This project aims to study biological networks and how genetic or environmental stressors can affect them resulting in diseases. For this purpose hundreds of thousands of L1000 profiles are being generated covering a range of cellular backgrounds, different perturbagens, and various concentrations of these stressors as well as genetic modifications disrupting various parts of the networks.

Advances in the field of single cell analytics (both single cell transcriptomics as well as single cell high content image analysis) are also improving the granularity with which we obtain our measurements. At the same time, advances in computational sciences and machine learning techniques begin to provide us with the tools to analyze the large amounts of available data. For instance, by applying techniques such as deep learning, we will obtain the means to identify the relevant features within the different data types. We believe this will provide us with the predictive power to focus on those compounds that have the best overall polypharmacological profile to move forward.

A concrete application that we are already undertaking at Janssen is the enrichment of screening libraries with compounds that are likely to show the desired activity but are also preselected for not having a priori defined undesired properties. This way we aim to move from biologically simple high-throughput assays to more translatable assays that are typically not only lower throughput but also more time consuming and more costly. We are convinced that it will eventually result in a more effective drug discovery process as we increase the likelihood of identifying compounds that have the desired properties needed for patients.

List of Abbreviations

AOPs	adverse outcome pathways
CMAP	connectivity map
DMSO	dimethyl sulfoxide
HEK293	human embryonic kidney 293 cells
HepG2	human hepatocarcinoma cells
LINCS	Library of Integrated Network-based Cellular Signatures
LNCaP	human prostate cancer cells
MNT	micronucleus test
PDE10A	cAMP and cAMP-inhibited cGMP 3′,5′-cyclic phosphodiesterase 10A
qHTS	quantitative high-throughput screening

QSTAR	quantitative structure–transcription assay relationships
ROS	reactive oxygen species
SK-N-BE	human neuroblastoma cells
TK6	human lymphoblastoid cells
U2OS	osteosarcoma cells

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22

The Integrated Optimization of Safety and DMPK Properties Enabling Preclinical Development: A Case History with $S1P_1$ Agonists

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22.1 Introduction to the $S1P_1$ Agonist Lead Optimization Program

22.1.1 Objectives and Challenges

The latter part of the twentieth and first decade of the twenty-first centuries saw a high level of research activity into sphingosine-1-phosphate (S1P) receptor biology with numerous pharmaceutical companies looking to exploit this understanding in the development of new drugs [1–3]. S1P is the endogenous ligand and potent modulator of the activity of a family of five S1P G-protein-coupled receptors, $S1P_{1–5}$. These receptors are known to regulate a range of biological processes including cell survival, adhesion, migration, and endocytosis, leading to physiological responses such as endothelial barrier enhancement, modulation of vascular tone, heart rate, and lymphocyte trafficking [4]. The latter two responses were of particular interest to the drug discovery program described in this case history.

Lymphocytes continuously circulate throughout the body, acting as surveillance for invading pathogens and return home to secondary lymphoid organs. To leave these secondary organs, the lymphocytes sense the S1P gradient that exists between lymph and blood. The $S1P_1$ receptor is present on the surface of lymphocytes, and agonism of this receptor results in receptor internalization and removes the ability of the lymphocyte to sense the gradient causing sequestration of these cells in the secondary lymphoid tissue. Interfering with lymphocyte trafficking via $S1P_1$, agonism represented an attractive mechanism to target autoimmune diseases such as relapsing–remitting multiple sclerosis, inflammatory bowel disease (IBD), lupus, and psoriasis [5]. However agonism of $S1P_3$ was considered undesirable due to relationships observed in animal studies with broncho- and vasoconstriction and modulation of heart rate [6–8].

At the time of this lead optimization program, the nonselective S1P receptor agonist FTY-720, fingolimod, now marketed as Gilenya® (Compound **1a**, Figure 22.1), had shown clinical efficacy as a new oral drug in relapsing–remitting

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

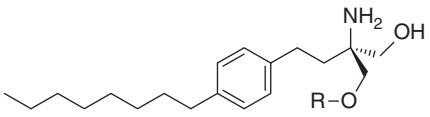


Figure 22.1 Structure of fingolimod (FTY-720) and FTY-720 phosphate.

R = H **1a** (FTY720, fingolimod)
R = $PO(OH)_2$ **1b** (FTY720-P)

multiple sclerosis and in 2010 was the first $S1P_1$ receptor agonist to have gained FDA approval. Fingolimod is a prodrug that undergoes enantiospecific phosphorylation to **1b** (Figure 22.1) *in vivo* to exert its activity. It is also a lipophilic drug able to penetrate the brain, and while this may contribute to efficacy in the CNS [9], central penetration was considered undesirable for treatment of peripheral indications such as psoriasis and IBD. The pharmacokinetics (PK) of fingolimod are characterized by a prolonged oral absorption phase and long elimination half-life (100–200 h), driven by a large volume of distribution [10, 11], which in turn drives a sustained pharmacodynamic (PD) effect of lymphocyte reduction in humans and also rodents [12]. Transient bradycardia was noted in patients and in rodent studies after the first dose with the effect in rodents attributed to agonism of the $S1P_3$ receptor. Other adverse events (including macular edema, modest hypertension, and some pulmonary effects) were observed in early clinical studies and thought to be mediated via the nonselective action of FTY-720 phosphate on $S1P_{3-5}$ receptors [9].

Due to the positive efficacy data with fingolimod in the clinic, there was urgency to discover molecules with an improved profile.

22.1.2 Overview of the Strategy and Screening Cascade

The aim of the lead optimization campaign was to discover non-prodrug, potent, and selective $S1P_1$ agonists in "drug-like" space [13–15] and with an improved profile over fingolimod. The promising anti-inflammatory activity was suitable for central and peripheral disease indications, and medicinal chemistry starting points were considered tractable. The desired product profile included a direct, non-prodrug molecule to theoretically drive a greater consistency in target site exposure due to the absence of the *in vivo* phosphorylation step required with fingolimod. Potency, coupled with a PK profile capable of delivering once daily administration, was essential to drive a low human dose to reduce attrition risk in safety and clinical studies through a low body burden. A shorter PK half-life than fingolimod was also considered desirable to drive a more controlled lymphocyte reduction profile and mitigate any potential risk associated with compromised immunity. Receptor selectivity for $S1P_1$, particularly against $S1P_3$, was essential to minimize the potential for bradycardia in the clinic.

The screening strategy initially employed by the program is shown in Figure 22.2. It is depicted "upside down" compared with traditional illustrations to emphasize the focus on the patient and the desired clinical profile, which in turn dictated the preclinical requirements and molecule profile. All animal studies were ethically reviewed and carried out in accordance with Animals

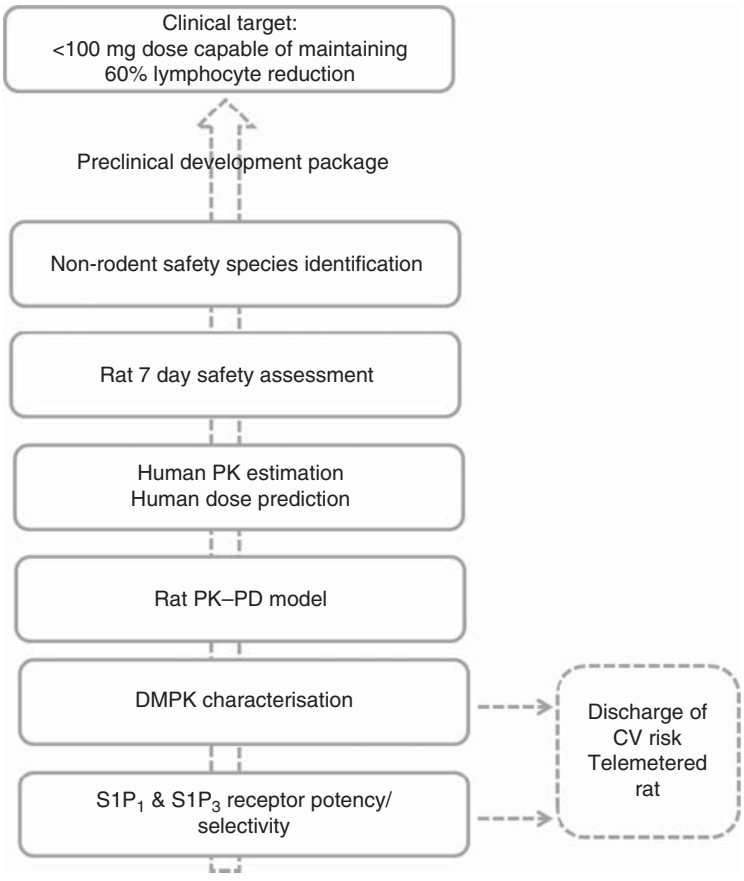


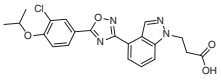
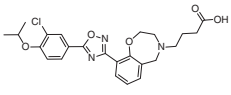
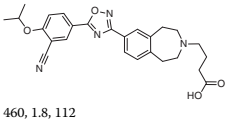
Figure 22.2 Core elements of the screening cascade for the design and selection of $S1P_1$ agonists.

(Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare, and Treatment of Animals.

22.2 Early Attention to Preclinical Safety

Intensive medicinal chemistry and the implementation of the screening strategy resulted in the successful delivery of numerous molecules suitable for evaluation in preliminary rodent safety studies. Examples of compounds progressed into such studies were compounds **2–4** [16, 17], with the key parameters summarized in Table 22.1. These compounds were in physicochemical drug-like space and had a high potency and receptor selectivity, a preclinical PK profile suitable for oral administration, no drug–drug interaction (DDI) liability, and a low human dose prediction. These molecules were progressed to rodent safety studies to assess the general toxicology profile and the potential for further development and provide an early estimation of the therapeutic index. Several molecules were progressed

Table 22.1 The overall profile of exemplar molecules entering early safety studies.

Compound	**2**	**3**	**4**
Structure			
MW, Log$D_{7.4}$, PSA	427, 2.3, 103	472, 2.4, 98	460, 1.8, 112
$S1P_1/S1P_3$ EC_{50} (μM)	0.020/>31 (>1500-fold)	0.040/>31 (800-fold)	0.008/8 (1000-fold)
Human hepatocyte CL_i ($\mu l\,min^{-1}\,10^6\,cells^{-1}$)	<7.1	11	<7.1
CYP450 inhibition (IC_{50} μM)	2C9 : 7 Others >25	>27	>40
PK rat[a]			
CL_b ($ml\,min^{-1}\,kg^{-1}$)	2.0	7.0	7.0
V_{ss} ($l\,kg^{-1}$)	1.0	1.2	1.5
$T_{½}$ (h)	7.5	3.0	2.6
F_{po} (%)	96	83	62
PK dog[b]			
CL_b ($ml\,min^{-1}\,kg^{-1}$)	4.0	26	3.0
V_{ss} ($l\,kg^{-1}$)	1.0	1.0	1.6
$T_{½}$ (h)	3.9	0.4	8.0
F_{po} (%)	94	57	69
Estimated human oral dose	≤50 mg twice daily	≤100 mg once daily	<50 mg once daily

CL_i: intrinsic clearance; PK: pharmacokinetics; CYP450: cytochrome P450 enzymes CYP1A2, 2C9, 2C19, 2D6, 3A4; CL_b: blood clearance; V_{ss}: volume of distribution at steady state; F_{po}: oral bioavailability.

a) Rat PK: IV 1 mg kg^{-1}, oral 3 mg kg^{-1}.

b) Dog PK IV 1 mg kg^{-1}, oral 2 mg kg^{-1}.

c) *Source*: From Skidmore et al. 2014 [17]. Reproduced with permission of American Chemical Society.

in parallel as this chemical template was previously uncharacterized. Compounds were administered orally, once daily, to rats at three different dose levels for 7 days. Dose levels were selected to provide multiples of the estimated human systemic exposure to ensure an appropriate therapeutic index was investigated.

22.2.1 Use of Toxicogenomics in Early Rodent Safety Studies

In addition to collecting tissue pathology and histopathology endpoints from these safety studies, toxicogenomic data were also generated. Such data provide an opportunity to identify pathways and processes affected by the test article that may be predictive of adverse findings following longer-term drug exposure (see Chapter 21 for an additional case study on the use of toxicogenomics). Data may suggest mechanisms of toxicity and "off-target" activity and are considered supplemental to clinical pathology and histopathology measurements. Transcript changes in the liver, for example, offer a means of predicting hepatotoxicity or safety events associated with dysregulation of hepatic function. Panels of genes have been identified through comparison with known hepatotoxicants that, if affected, are representative of modes of hepatotoxicity (hepatotaq). These panels include genes that code for increased expression of drug-metabolizing enzymes including the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), and the aryl hydrocarbon receptor (AhR), which are involved in the regulation of cytochrome P450 enzymes 3A, 2C, and 1A, respectively [18].

22.3 Aryl Hydrocarbon Receptor Activation Observed in Rat

The toxicokinetic data collected on the first and last days of the studies indicated that the exposure of the compounds increased in line with dose and no change in exposure was observed on repeat administration. However, analysis of the toxicogenomic data revealed an unexpected finding. Compounds **2**–**4** all caused marked increases in CYP1A1, CYP1A2, NAD(P)H quinone oxidoreductase (NQO1), and epoxide hydrolase (Ephx) genes, a gene panel indicating AhR activation (Figure 22.3). A positive response for this gene panel occurs when the upregulation of each gene exceeds its own threshold value, which was threefold for CYP1A2, twofold for CYP1A1 and NQO1, and 1.5-fold for Ephx.

The level of CYP1A1 mRNA increase in particular was very high, up to 10 000-fold the control value, and at the time of this finding was the highest in the data set at GlaxoSmithKline. Other genes in the panel (CYP1A2, NQO1, and Ephx) were all also upregulated, confirming an interaction with the AhR. It should be noted that the level of gene upregulation was comparable with that of the prototypical CYP1A inducers β-naphthoflavone (BNF) and 3-methylcholanthrene (3MC) exemplifying the magnitude of this finding. Similar observations with these structurally related compounds suggested a common mechanism. When focusing on CYP1A1 mRNA, it was clear that the magnitude of increase was related to systemic exposure (and dose), but an interesting observation at this

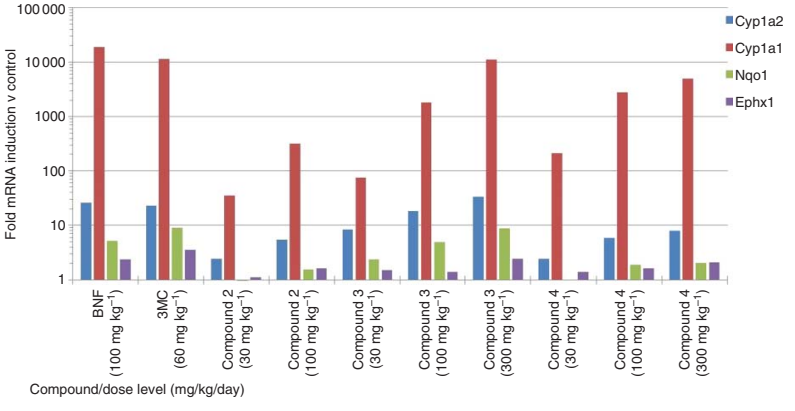


Figure 22.3 Upregulation of genes associated with the AhR gene panel for **2, 3**, and **4** following 7-day oral administration to rats. The response to prototypical inducers β-naphthoflavone (BNF) and 3-methylcholanthrene (3MC) after 4-day administration is included for reference. Cyp: cytochrome P450. NQO1: NAD(P)H quinone oxidoreductase. Ephx1: epoxide hydrolase. *Source*: From Taylor et al. 2015 [16]. Reprinted with permission American Chemical Society.

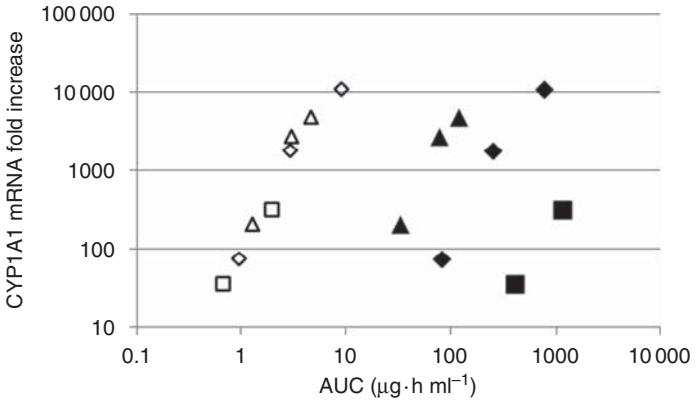


Figure 22.4 The relationship between systemic exposure (AUC) and hepatic CYP1A1 mRNA induction for compounds 2 (squares), 3 (diamonds), and 4 (triangles) following 7-day oral administration to the rat. Daily doses were 30 and 100 mg kg^{-1} for 2 and 30 and 100 and 300 mg kg^{-1} for 3 and 4, respectively. The figure illustrates the alignment of a relationship when the AUC is adjusted to represent the unbound exposure (open shapes) compared with the total exposure (closed shapes). *Source*: From Taylor et al. 2015 [16]. Reprinted with permission American Chemical Society.

early stage was that a strong relationship was observed across all compounds when plotted against unbound rather than total systemic exposure as in Figure 22.4. This observation was explored and used in the design of future safety studies.

22.4 CYP1A (Auto) Induction Observed in Non-rodent Species

Further progression of **2** required the definition of a non-rodent species for use in safety assessment evaluation. Repeated oral administration to dogs resulted in a lack of tolerability and body weight loss, and so an alternate species was sought. A repeat-dose oral study (30 mg kg^{-1}) using cynomolgus monkeys was conducted over 7 days with the objective of evaluating if appropriate systemic exposure could be achieved in this species to allow further safety evaluation. Analysis of the toxicokinetic data revealed substantial reduction in systemic exposure over the study duration. Figure 22.5 illustrates the reduction in both C_{max} and AUC on repeat administration. Similar to the findings in rat, gene expression analysis from liver samples confirmed a substantial increase of CYP1A1 and CYP1A2 mRNA up to ninefold the control value (Figure 22.6). The reduction in exposure coupled with the upregulation of CYP1A1 and 1A2 mRNA was suggestive of auto-induction, a phenomenon where the molecule induces its own metabolism. This was confirmed through experiments designed to evaluate the intrinsic clearance rate of **2** in the induced liver versus control liver using microsomes prepared from the livers of animals on the study. In control microsomes **2** was metabolically stable with no measurable disappearance of

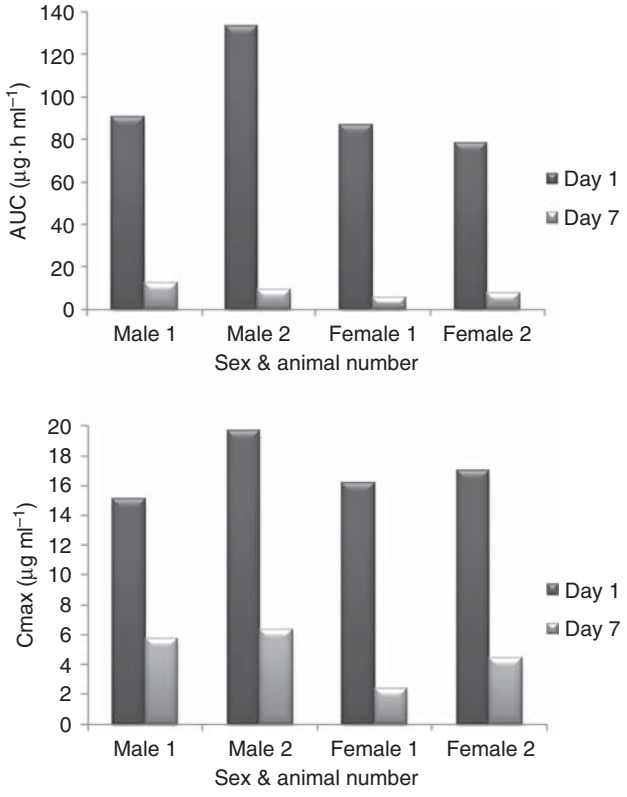


Figure 22.5 Compound **2** is an auto-inducer in cynomolgus monkeys. Toxicokinetic data (exposure, as area under the curve and Cmax) for **2** on days 1 and 7 following oral dosing at 30 mg kg^{-1} day^{-1} to monkeys. *Source*: From Taylor et al. 2015 [16]. Reprinted with permission American Chemical Society.

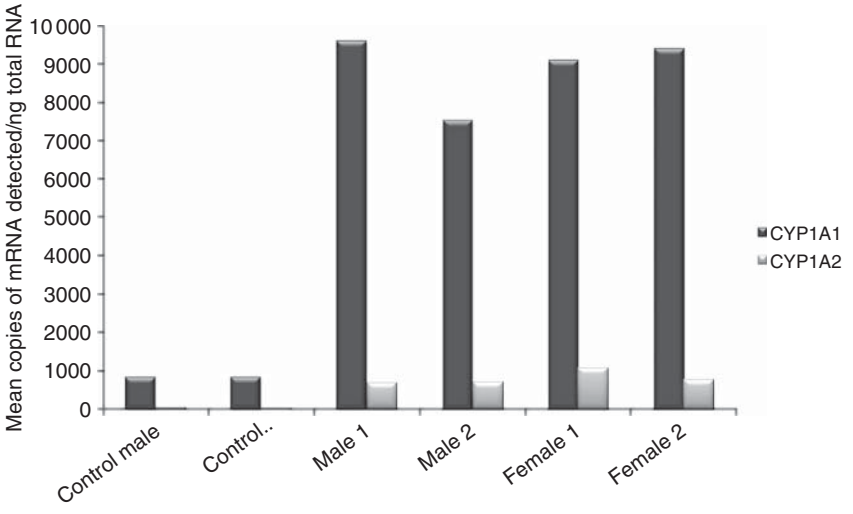


Figure 22.6 Upregulation of hepatic CYP1A1 and CYP1A2 mRNA in cynomolgus monkeys by **2** following 7-day oral dosing at 30 mg kg^{-1}. *Source*: From Taylor et al. 2015 [16]. Reprinted with permission American Chemical Society.

compound during the incubation period, whereas a high intrinsic clearance was observed in microsomes prepared from treated animals (11 ml min^{-1} g^{-1} liver). The auto-induction effect ultimately prevented **2** from progressing to further safety studies in cynomolgus monkey as adequate exposure to provide a therapeutic index suitable for development could not be achieved on chronic administration.

The extent of CYP1A1mRNA upregulation in the rat (up to 10 000-fold the control value), a finding at this level unprecedented at GSK, and a similar finding in the cynomolgus monkey prompted a review into the implications of progressing compounds that were agonists of the AhR into further development.

22.5 Introduction to the Biology and Function of the Aryl Hydrocarbon Receptor

In order to fully appreciate the strategy and approach taken in this case study, a brief introduction to the biology and function of the AhR is provided, and the impact of this information in the context of this drug development program as interpreted within GlaxoSmithKline at the time the $S1P_1$ program was active is described. More detailed information on AhR biology, function, and impact can be obtained from several review articles [19–25].

22.5.1 CYP1A Induction via the AhR

The AhR is a ligand-dependent transcription factor responsible for the regulation of gene expression in a wide range of tissues and species [19]. AhR is activated by a diverse range of endogenous and exogenous substrates and mediates numerous biological and toxicological responses [26]. Following ligand binding to the cytosolic AhR, the multi-protein complex translocates to the nucleus where the AhR ligand complex dimerizes with a related protein (ARNT). This high affinity complex then binds to specific DNA recognition sites stimulating gene transcription including those of CYP1A and other AhR responsive genes including those described earlier.

The consequences of AhR activation have been extensively characterized using high affinity ligands found as environmental contaminants. These include the potent and high affinity halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (dioxin) and the lower affinity polycyclic aromatic hydrocarbons (PAHs) such as 3MC and benzo(a)pyrene. PAHs are also present as components of exhaust fumes, cigarette smoke, and charbroiled food. Natural ligands also exist. Structure–activity relationships (SAR) from these compound classes suggest the binding pocket favors planar hydrophobic ligands though more diverse structures are also reported. Exposure to dioxin causes an array of species and tissue-specific biological and toxic events including tumor promotion, teratogenicity, modulation of cell growth, differentiation, proliferation, wasting and immune, and hepatotoxicity and dermal toxicities. The latter toxicities generally take several weeks to manifest

and can only be observed with intact animal systems, suggesting a continuous and modified gene expression profile in responsive cells [19].

22.5.2 *CYP1A Enzyme Family*

The upregulation of CYP1A enzymes is an AhR-dependent response that has been consistently observed across species and is considered one of the most sensitive AhR activation endpoints [19, 27].

The CYP1A enzyme subfamily comprises CYP1A1 and 1A2, both having high amino acid sequence conservation across rats, mice, dogs, monkeys, and human species although variation is seen in the constitutive and inducible nature of these enzymes across species and tissues. This profile impacts the location and magnitude of responses to AhR agonists, the opportunities for measurement of upregulation, and also the consequences of the findings across species in the context of drug development.

CYP1A1 basal expression is negligible yet variable. Only very low levels are expressed in the liver with CYP1A1, predominantly an extrahepatic enzyme inducible in virtually all body tissues most notably in the small intestine and lung. Higher levels are found in smokers and those having ingested chargrilled meats though other dietary factors are also involved.

In contrast CYP1A2 is predominantly a hepatic enzyme being absent or only weakly expressed in extrahepatic tissues. In humans it represents approximately 13% of total hepatic cytochrome P450 content [28] and is involved in the metabolism of up to 20% of marketed drugs plus many environmental aromatic amines [22]. The function of this enzyme and also the consequences for any change in the level of expression or activity should therefore be considered as part of a drug development program. It is inducible in tissues such as the lung and intestine with large (>60-fold) interindividual variability in CYP1A2 expression reported [29] attributable to genetic, epigenetic, and environmental factors such as smoking.

Induction of CYP1A1/1A2 via AhR-mediated pathways is generally considered to be a feedback mechanism in the maintenance of cellular homeostasis with many enzyme substrates also ligands for the AhR as is the case for the PAHs 3MC and benzo(a)pyrene. As cellular exposure to these enzyme substrates increases, enzyme induction follows to enhance the capability to detoxify the substance. As the substrate is removed, the extent of induction declines.

In vitro studies have shown that substrates of CYP1A enzymes such as benzo(a)pyrene form reactive intermediates as part of their metabolism, which are the ultimate carcinogen capable of DNA adduct formation. *In vitro* enzymology studies indicate a role for CYP1A as a perpetrator in driving these toxicities. Paradoxically, an overall protective role of CYP1A induction from oral chemical-induced carcinogenesis is observed *in vivo* [24]. As explained by Uno et al. [30], it is the balance of these processes that ultimately determines the effect. The interplay is clearly complex.

In addition to the environmental contaminants mentioned above, examples of drugs that are inducers of CYP1A2 most notably include the widely prescribed antiulcer drug omeprazole. Omeprazole, an inducer of hepatic CYP1A2 in

humans, but not rodents, is an example of a species-specific inducer. It has been used safely for more than 25 years. Minimizing exposure to cigarette smoke and dietary sources of PAHs was at one time recommended for patients on long-term omeprazole therapy, but a connection between omeprazole use and cancer incidence has yet to be described. More detail on this sequence of events is described in an article by Ma and Lu [24].

22.6 Considerations of AhR Binding and CYP1A Induction on Compound Progression

The consequences of continuing to develop molecules that are AhR agonists in the context of this drug discovery program were considered. Judgments in drug discovery are frequently problematic. The impact of termination decisions are rarely known, but an overconservative approach prevents therapeutically useful molecules from reaching patients. However, factors such as the patient population, indication, and competitive landscape should always be considered as progressing a molecule with a known risk resulting in later attrition is costly. In the case of this particular program, the molecules did not represent a first-in-class mechanism, and a "clean" profile at this early stage was preferred so as not to introduce additional risks over fingolimod.

The discussions associated with AhR activation and CYP1A induction were broadly categorized as follows: (i) increased risk of carcinogenicity in certain individuals and specific tissues, (ii) functional consequences of increased CYP1A enzyme activity, and (iii) impact of strong AhR agonism given the other emerging biological roles of the AhR.

The evidence for the role of CYP1A as a causal agent and/or detoxifier in PAH-induced carcinogenicity in mice was complex although suggestive of an overall protective effect *in vivo*. The absence of a connection between the CYP1A2 inducer omeprazole and carcinogenicity was also acknowledged though the mechanism of omeprazole induction is not fully understood and may not involve AhR binding [31]. However, the extent of CYP1A1 mRNA upregulation by the $S1P_1$ compounds was concerning, and, if translatable in human, the risk of potentiating carcinogenicity in directly exposed tissues such as the lung in smokers was considered to remain, as discussed by Nebert et al. [29] The possibility of mitigating these risks via preclinical experiments was also considered low with a clear result unlikely. The worst-case scenario was considered to be the emergence of a finding in humans in late-stage trials or postmarketing. Furthermore, the large interindividual variability in CYP1A2 expression across the population may put some individuals at greater risk, with the risk being largely unpredictable.

Induction and inhibition of cytochrome P450 enzymes have been shown to be responsible for numerous DDI in the clinic. Such interactions can limit the clinical and commercial viability of drugs, and therefore the potential is best addressed early in lead optimization [32]. P450 induction by a drug may increase the clearance of itself (auto-induction) or of co-administered drugs.

Auto-induction has the potential to result in a reduction or loss of efficacy of therapeutic agents and/or the generation of an altered metabolite profile. Focus is normally placed on CYP3A due to its importance in the metabolism of many therapeutic drugs [20] but with CYP1A2 contributing to the metabolism of around 20% of therapeutic drugs [22] there are several known DDI as a consequence of altered CYP1A metabolism [23]. Changes to the levels of this enzyme therefore require consideration in clinical practice.

Several examples of the potential for CYP1A induction to perpetrate a DDI with co-administered drugs in the clinic have been reported. Exposure to PAHs in cigarette smoke has been shown to induce CYP1A2 and increase the clearance and therefore reduce plasma concentrations of drugs including caffeine, theophylline, melatonin, clozapine, lidocaine, verapamil, erlotinib, and fluvoxamine [22, 33, 34]. Caffeine itself has also been shown to induce CYP1A2 in certain population groups [35]. A carbamazepine interaction has been reported with schizophrenic patients taking clozapine [36]. These interactions cited above for CYP1A2, while important, are usually managed in the clinic through dosage adjustment, therapeutic monitoring, and control of co-medications. In the case of smoking, this is particularly important as CYP1A2 levels can be altered abruptly via changes in smoking habits. The risk of the $S1P_1$ compounds perpetrating a DDI was acknowledged but ultimately considered a manageable issue in a clinical context. Moreover the expected clinical dose was low, reducing the likelihood of clinically significant DDI [37]. It was decided that, if necessary, a definitive DDI study could form part of the early clinical development program and, even if positive, while undesirable, this was considered manageable.

Evidence from the cynomolgus monkey study indicated the potential for these $S1P_1$ agonists to be CYP1A auto-inducers in the clinic. The potential impact of auto-induction on clinical efficacy, previously described, could ultimately result in termination. This finding would not emerge until the multiple ascending dose part of a phase I study after significant investment in preclinical development. Therefore, selecting only compounds without this property in discovery was considered essential.

In addition to enzyme regulation, numerous other physiological functions mediated by the AhR were emerging during the lifecycle of this $S1P_1$ agonist program. These included a role for the receptor in development, regulating cell differentiation and cycling, hormonal and nutritional homeostasis, coordination of cell stress responses (including inflammation and apoptosis), immune responses, aging, and cancer promotion [38]. While other literature at the time was suggesting the therapeutic potential of AhR agonism [39], the consequences of a strong AhR agonist on the emerging biological functions were not yet well defined. As attrition in late-stage drug development is unwanted and certainly costly, the uncharacterized effect of this agonist profile on longer-term administration in chronic toxicity studies and the clinic was also considered a high risk.

We contextualized these considerations for our drug discovery program relative to the desired product profile. Decisions were taken to continue progression of **3** toward 28-day safety studies but in parallel modify the screening strategy with the objective of identifying compounds with an improved profile devoid of any potential induction risk.

This decision was pragmatic as the properties of a molecule can only be modified during lead optimization. However, the decision may also be considered cautious. This was acknowledged by the team but was felt justified particularly as the compound was not a first-in-class mechanism, and in a highly competitive field, a successful molecule would likely require a "clean" profile.

22.7 Reacting to Data: Strategy Modification in Lead Optimization

The observation of AhR gene panel activation in rats and substantial CYP1A enzyme induction in cynomolgus monkey represented a body of evidence indicating this series of $S1P_1$ agonists were also agonists of the AhR in these species. However it is the effect in human systems that are of ultimate importance. The strategy to address this is now described along with the modifications to the screening cascade to bring forward compounds without the induction liability. In order to achieve this, an understanding of the structural features driving AhR agonism was required to influence the medicinal chemistry approach.

22.7.1 Evaluating CYP1A Induction in Human Systems

A human AhR binding assay was not available within GlaxoSmithKline at the time. We therefore turned to human hepatocytes as a well-characterized experimental system for studying enzyme induction *in vitro*. However, the low level of hepatic CYP1A1 expression in a human liver (recall that CYP1A1 is largely an extrahepatic enzyme in humans) meant the primary endpoint would be upregulation of CYP1A2. Due to the extensive CYP1A1 mRNA induction observed in rat liver, it was recognized that the true induction potential of these compounds may be underrepresented in human hepatocytes. Methods to study upregulation in extrahepatic tissues, where CYP1A1 is highly inducible, are less straightforward and poorly characterized due to, as an example, the complexities of obtaining metabolically competent cells from lung tissue. On balance a decision was taken to incorporate the available human hepatocyte assay into the $S1P_1$ program immediately and assume any observed CYP1A2 induction was also an indicator of CYP1A1 induction potential in other tissues. A strategic decision was also taken to introduce an AhR binding assay for future utility with this and other programs.

22.7.2 Evaluating Induction in Human Hepatocytes

Briefly, this experiment involved incubating test compounds over a concentration range with thawed cryopreserved hepatocytes for 48 h. Induction potential was assessed using dual methods of CYP1A2 mRNA levels and also catalytic enzyme activity. Catalytic enzyme activity was measured by using the rate of CYP1A-mediated deethylation of the fluorescent probe substrate 7-ethoxyresorufin where induction was represented by an increase in deethylation versus a control.

Table 22.2 Comparison of the CYP1A1, CYP1A2 (rat), or CYP1A2 (human) induction observed across various *in vivo* and *in vitro* assay formats.

	[a]Rat *in vitro*		[b]Rat *in vivo*		[c]Human *in vitro*	
Compound	**CYP1A1 mRNA**	**CYP1A2 mRNA**	**CYP1A1 mRNA**	**CYP1A2 mRNA**	**CYP1A2 mRNA**	**Catalytic activity (EROD)**
2	<1	<1	35	2	45	7.7
3	5	3	75	8	0.6	1.1
4	3	2	210	2	–	–
5	<1	<1	3	1	0.2	1.1
6	43	19	2	2	–	–
7	32	8	1	1	–	–

All data expressed as fold change versus control to enable comparison.

a) Rat *in vitro* assays conducted at 10 μM.

b) Compounds **2–5**: 7-day dosing 30 mg kg^{-1}. Compounds **6–7**: 4-day dosing 20 mg kg^{-1}.

c) Human mRNA assay at 10 μM, EROD assay at 5 μM. EROD: 7-ethoxyresorufin *O*-deethylase activity.

Source: From Taylor et al. 2015 [16]. Reprinted with permission of American Chemical Society.

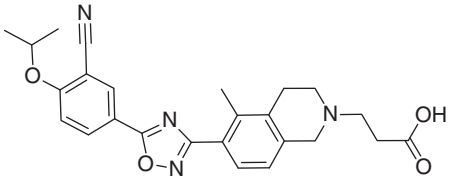
Compounds **2** and **3** were profiled in human hepatocytes. Although both compounds were considered AhR activators in rat, only **2** showed CYP1A2 upregulation in human hepatocytes, resulting in a 45-fold increase in CYP1A2 mRNA and an associated increase (eightfold) in catalytic enzyme activity (Table 22.2). While these data highlighted a potential species difference for **3** in hepatocytes, the inducing potential in other tissues was not fully discharged.

The development of **2** was terminated for reasons associated with the AhR induction potential, the primary reasons being the auto-induction in cynomolgus monkey previously described and the magnitude of exposure reduction preventing achievement of a sufficient therapeutic index. Alternative non-rodent species were also deemed inappropriate following preliminary safety studies. The development of **3** and **4** was also eventually terminated due to a variety of developability issues but not because of the AhR agonism finding alone.

Further medicinal chemistry efforts resulted in the rapid identification of **5** [40]. Based on its improved developability properties including preclinical PK and predicted human dose (Table 22.3), the compound was advanced rapidly into the human hepatocyte induction assay in addition to a rat 7-day safety assessment study. Despite the structural similarity to the predecessor compounds, the human hepatocytes assay showed no induction potential of CYP1A2 (Table 22.2), and toxicogenomic data from the rat safety study showed that over the same range of unbound AUC, no activation of the AhR gene panel occurred (Figure 22.7). These data positioned **5** as the most promising molecule for further development.

After much investment in a target, it is a common strategy to ensure that multiple candidate quality molecules are available. The search for additional molecules suitable for preclinical development continued in order to mitigate risk against a potential termination of **5** at a later stage. For example, the zwitterionic compounds presented so far (**2–5**) were restricted in distribution,

Table 22.3 The overall profile of **5** having optimal potency, receptor selectivity, PK, human dose, developability, and CYP1A properties suitable for further preclinical development.

Structure	 **5**	
MW, Log$D_{7.4}$, PSA	446, 1.7, 112	
$S1P_1/S1P_3$ EC_{50} (μM)	0.032/>40	
Human hepatocytes CLi (μl min^{-1} 10^6 $cells^{-1}$)	<7.1	
CYP IC_{50} (μM)	Five major isoforms tested >30 μM	
PK	Rat[a)]	Dog[b)]
CL_b (ml min^{-1} kg^{-1})	5	10
V_{ss} (l kg^{-1})	1.1	2.2
$T_{1/2}$ (h)	3.0	4.8
F_{po} (%)	98	53
CYP1A1/1A2 mRNA increase in rat *in vivo*	<10-fold up to 100 mg kg^{-1}	
CYP1A2 upregulation in human *in vitro*	None detected	
Bradycardia in rat	No effect up to 100 mg kg^{-1}	
Estimated human oral dose (mg, daily)	6 mg	

a) Rat PK: IV 1 mg kg^{-1}, Oral 3 mg kg^{-1}.
b) Dog PK IV 1 mg kg^{-1}, Oral 2 mg kg^{-1}.
Source: Adapted from Demont 2011 [40] and Taylor 2012 [41].

thus limiting the PD effect to the periphery. Emerging data with the CNS penetrant FTY720 suggested efficacy may also be driven via a component of S1P receptor signaling in the CNS [42, 43] prompting us to additionally explore structures containing amine features using the basicity to drive penetration of the blood–brain barrier. It should be noted that diol groups were introduced to the amines (Figure 22.8) to reduce pK_a and overall lipophilicity and elimination half-life, not with the intention of designing prodrugs to be phosphorylated in a manner similar to FTY720.

22.7.3 Screening for Induction Using Rat Hepatocytes

In order to identify a backup molecule with negligible risk of induction and given the species differences observed previously with **3** (refer to Table 22.2), the desired candidate profile was modified to exclude all signs of induction across all species and test systems. The hepatocyte induction assay was in place to cover

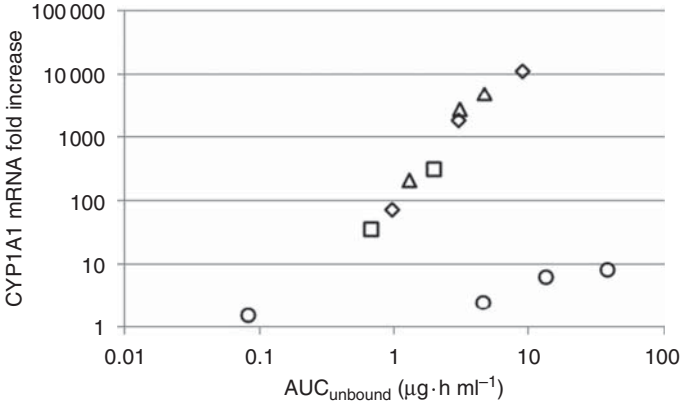


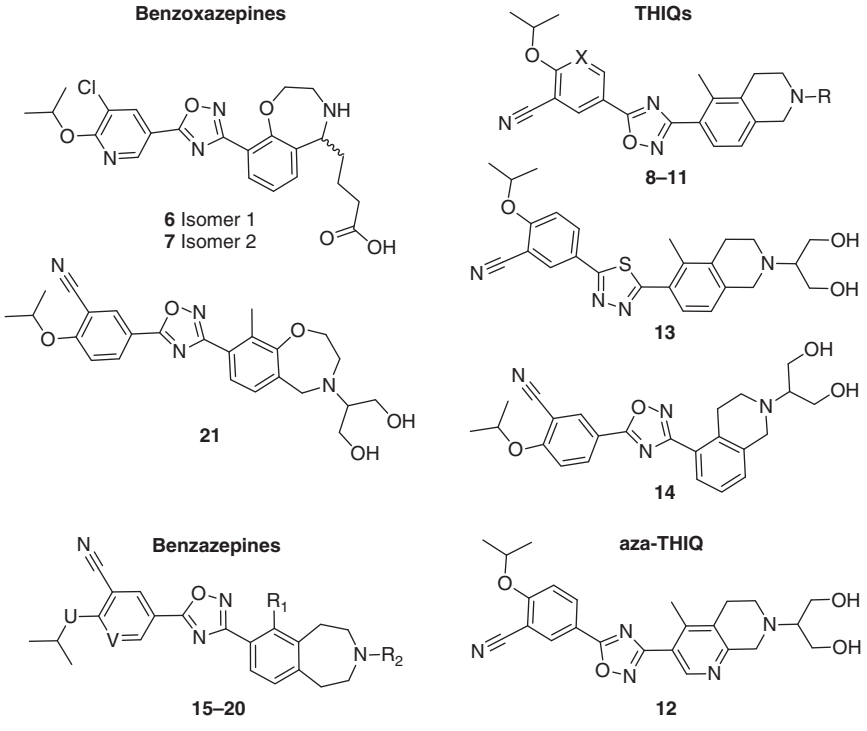
Figure 22.7 The relationship between unbound systemic exposure (AUC) and hepatic CYP1A1 mRNA for the inducers **2** (squares), **3** (diamonds), **4** (triangles) and the non-inducer **5** (circles) following 7-day administration to the rat. Daily oral doses were 1, 30, 100, and 300 mg kg^{-1} for **5** and as previously stated for **2–4**. *Source*: From Taylor et al. 2015 [16]. Reprinted with permission American Chemical Society.

human though operating 7-day rat studies using three dose levels during lead optimization for iterative screening and design purposes was unfeasible. An *in vitro* rat hepatocyte induction assay, analogous to the human assay previously described, was therefore implemented as a tool intended for the iterative screening and design of compounds in lead optimization.

The effectiveness of the rat hepatocytes assay was first tested by evaluating several compounds previously studied *in vivo* and comparing the results. The data in Table 22.2 shows that unfortunately the extent of induction observed in the rat *in vivo* was not reflected in the hepatocyte *in vitro* assay. The discrepancies were not consistently explained by comparison of systemic versus *in vitro* concentration, and despite numerous assay refinements and attempting alternative data analysis methods, an adequate explanation was not found.

22.7.4 Development of a Rat *In Vivo* Induction Protocol

As the rat hepatocyte assay was unable to reproduce the *in vivo* data, a repeat-dose *in vivo* protocol in the rat, fit for use in a drug discovery screening environment, was introduced. Considerations in the protocol design included the duration and magnitude of dosing. Previous characterization of induction in the rat [44] had demonstrated that hepatic CYP1A induction by BNF occurred after three daily administrations. We therefore designed a protocol using four daily administrations to $n = 3$ rats to ensure the maximal effect was captured. A nominal oral dose level of 30 mg kg^{-1} was selected to be representative of a dose used in future safety studies. Based on the experiences with previous compounds, this was considered sufficiently high enough to observe an induction effect yet balanced against the feasibility of compound provision in drug discovery (~150 mg for this study). In reality, due to the insufficient availability of some compounds, dose levels ranged from 15 to 30 mg kg^{-1}. Blood samples



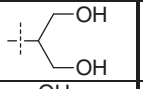
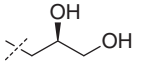
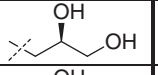
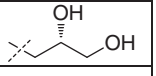
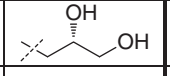
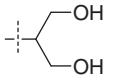
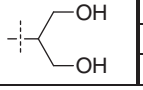
	X	R		U	V	R_1	R_2
8	CH		**15**	O	CH	CH_3	
9	CH		**16**	O	CH	CH_3	
10	CH		**17**	O	CH	CH_3	
11	N		**18**	NH	N	CH_3	
			19	O	CH	H	
			20	NH	N	H	

Figure 22.8 Structures of compounds **6–21** progressed to the 4-day rat *in vivo* induction assay. THIQ: Tetrahydroisoquinoline.

were collected after the first and fourth doses to determine the PK profile and systemic exposure. Twenty-four hours after the final dose, the animals were culled, the livers harvested, and sections prepared for mRNA/hepatotaq analysis.

22.8 Iterative Experimentation Identifies Molecules for Progression

The short 4-day induction protocol in rat was successfully implemented and used to profile several compounds across multiple subseries, as illustrated in

Figure 22.8. Due to the resource-intensive nature of this assay, compounds were first triaged using several criteria, and only those with suitable physicochemical properties, *in vitro* pharmacology, PK, efficacy, and human dose prediction were profiled. Those satisfying all these criteria and identified as non-inducers in both the rat 4-day *in vivo* study and the human hepatocyte *in vitro* assay were then considered as candidates for preclinical development.

As previously described CYP1A1 is more highly inducible than CYP1A2, and this is reflected in the magnitude of induction observed. Given the numbers of compounds profiled, we applied a criterion where a non-inducer was classified when CYP1A1 mRNA increased <20-fold and CYP1A2 mRNA increased <5-fold. A summary of the data from the 4-day study is provided in Figure 22.9, illustrating the extent of induction using CYP1A1 and 1A2 mRNA upregulation.

Systemic exposure (determined using C_{max} and AUC) was generally consistent across the 4-day dosing period, providing no evidence of auto-induction in the rat (Figure 22.10), which was in keeping with the observations drawn from the original compounds (**2–4**).

The remit of discovering non-inducer compounds resulted in substantial effort to interrogate SAR to drive iterative compound design. A literature survey

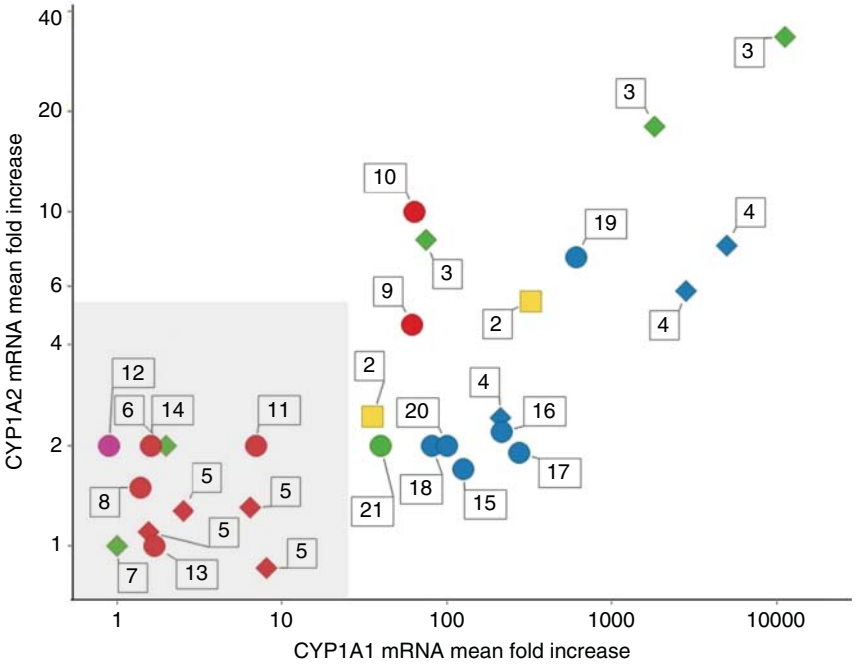


Figure 22.9 Summary of the upregulation of CYP1A1 and CYP1A2 mRNA following 4–7-daily oral administrations of various $S1P_1$ agonists to the rat. The shaded area represents the boundaries of compounds considered non-inducers at a given dose. The compounds are colored by template: THIQ (red), aza-THIQ (pink), indazoles (yellow), benzazepines (blue), and benzoxazepines (green), and shaped by class: acid (square), amine (circle), and zwitterions (diamond). *Source*: From Taylor et al. 2015 [16]. Reproduced with permission of American Chemical Society.

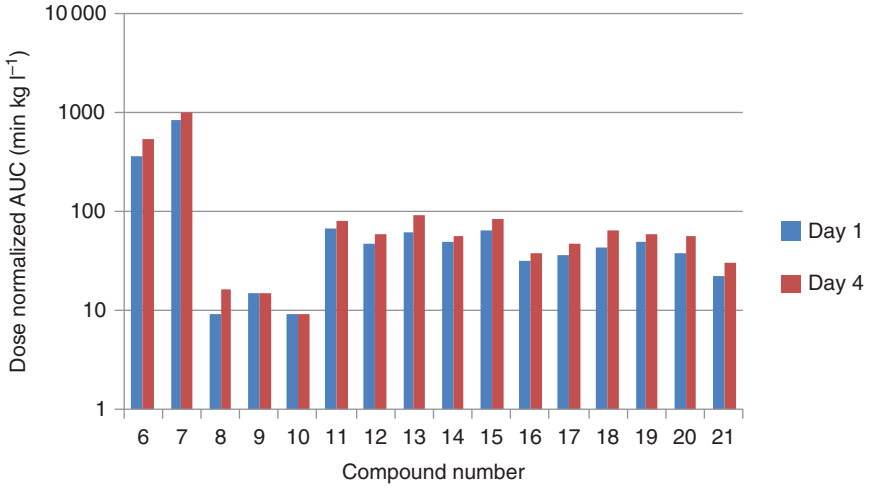
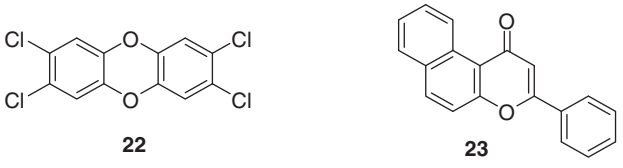


Figure 22.10 Consistent exposure (as shown by dose-normalized AUC) was observed with all compounds throughout the 4-day rat induction study, indicating the absence of auto-induction in the rat.



22 **23**

Figure 22.11 Examples of reported AhR agonists of a planar hydrophobic nature. TCDD, **22,** and BNF, **23**.

indicated that the majority of classical AhR ligands were planar with a high degree of hydrophobicity. Examples, shown in Figure 22.11, include the PAHs including **22** (TCDD) and **23** (BNF) though detailed SAR analysis of the PAHs showed that absolute planarity was not a requirement for binding [45].

Crystallography data for **3** and **5** highlighted that one of the aromatic rings in **5** (non-inducer) was out of plane when compared with **3** (inducer). The dihedral angles between the oxadiazole ring and the phenyl closest to the basic nitrogen were 18° and 5°, respectively, as illustrated in Figure 22.12. A working hypothesis followed by the team was that this "twist" in **5** was sufficient to introduce a lack of planarity and avoid AhR binding. This was also reinforced by other compounds (such as non-inducers **6** and **7**) having a carboxylic acid chain, which was also projected out of main aromatic moiety plane. Unfortunately this hypothesis did not hold uniformly as subsequent compounds (such as **9**) showed a high level of induction despite having an identical core structure to **5**. The relationship to physicochemical properties such as pK_a was also investigated, and while trends were apparent, for example, greater induction occurred with the more basic benzazepines compared with the less basic tetrahydroisoquinolines (THIQs); compounds such as the benzoxazepine **21** were not compliant to the rule.

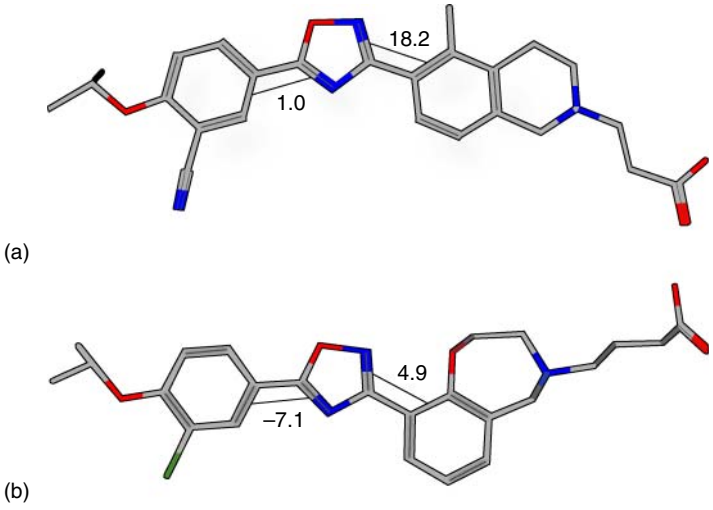


Figure 22.12 Crystallographic data for **5** (a) and **3** (b) illustrating the dihedral angles. *Source*: From Taylor et al. 2015 [16]. Reproduced with permission of American Chemical Society.

A comprehensive analysis of SAR was also conducted across many compound pairs within and across series, as reported in the literature [16]. It became apparent that the SAR was complex; for example, **9** and **10** were considered inducers, whereas the structurally similar **8** was a non-inducer, in contrast with more radical-shaped changes as in **14** that retained the profile of a non-inducer. The SAR was also extremely subtle; for example, **17** and **21** differ from **8** by only a single carbon or oxygen, respectively, and showed induction. In summary, only conclusions at the level of the "template" were defined with the benzazepines and benzoxazepines generally showing greater induction than the THIQs.

This example illustrates the type of assessments and analysis attempted to determine medicinal chemistry SAR with iterative cycles of hypothesis generation and testing. Firm relationships are always preferred, but in lead optimization, ambiguity often exists, requiring the scientist to exercise judgment. In this case links between compound structure and AhR gene panel upregulation were complex, subtle, multifactorial, and appeared to extend beyond properties such as planarity, lipophilicity, and basicity [16].

22.9 Delivery of Human AhR Agonist Assay

Unfortunately, due to the time required for development and validation, this assay did not deliver in time to be impactful for this $S1P_1$ agonist program. However, a selection of compounds were retrospectively profiled. This assay, developed using intestinal human colon adenocarcinoma cells (LS180), utilized a β-lactamase reporter gene downstream of the CYP1A1 promoter. 3MC was used as a positive control with novel compounds considered potential activators of AhR if the maximum response was >40% of the control.

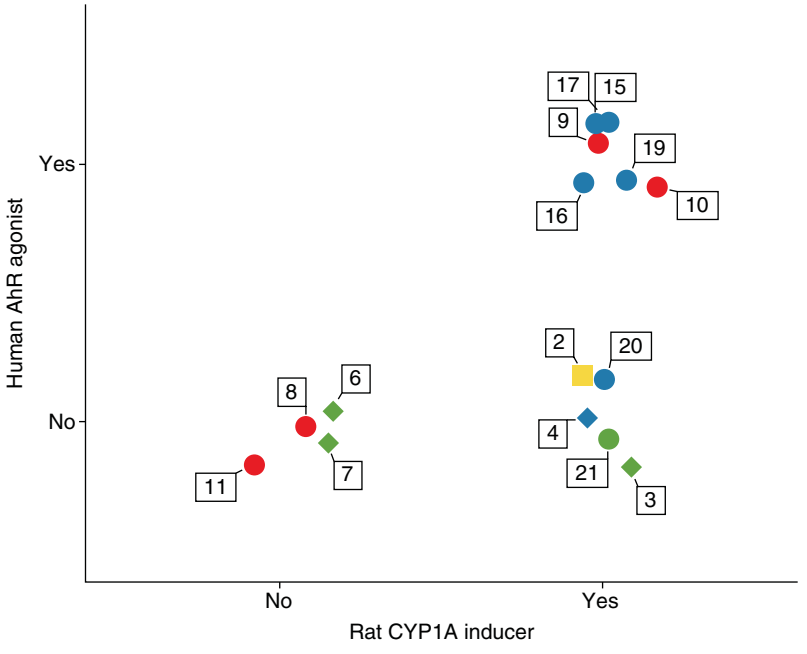


Figure 22.13 Data comparison for 15 $S1P_1$ agonists comparing agonist activity in the human AhR reporter gene assay and the rat *in vivo*. The compounds are colored by template and symbols separated for clarity: THIQ (red), indazoles (yellow), benzazepines (blue), and benzoxazepines (green), and shaped by class: acid (square), amine (circle), and zwitterions (diamond).

The potential for human AhR agonism was compared with the CYP1A inducing potential from the rat *in vivo* study. In the rat-only compounds having both <20-fold CYP1A1 and <5-fold, CYP1A2 were considered non-inducers. This limited data set shown in Figure 22.13 indicates some agreement between the assays though the rat appears to be a more sensitive indicator of induction potential using these criteria. Pleasingly, all compounds that were classified as non-inducers in the rat were also non-inducers in the human assay. These data justified our conservative strategy to design out induction across all species and assay formats.

22.10 Minimizing Cardiovascular Safety Risk Through S1P Receptor Selectivity

The potential for an improved clinical safety profile over fingolimod was an essential aspect of the program. To ensure the series of compounds under optimization did not carry a similar risk of bradycardia, telemetered rat studies were conducted early in lead optimization. This safety pharmacology model requires the surgical implantation of a telemetry device capable of remote monitoring of hemodynamic parameters. We confirmed that a series of compounds based on a promising THIQ template had the potential for >1000-fold human $S1P_1$ versus human $S1P_3$ receptor selectivity based on *in vitro* assay systems of receptor

binding [40]. This was in sharp contrast to fingolimod (see Table 22.4), which displayed equal potency at $S1P_1$ and $S1P_3$. Confirmation that the lack of potency at $S1P_3$ would drive an appropriate hemodynamic profile *in vivo* was tested with our lead compounds using the telemetered rat model. The profile for **5** is shown in Figure 22.14 from a single ascending dose experiment. In keeping with the

Table 22.4 S1P Receptor selectivity comparing fingolimod with **5,** a novel compound from lead optimization.

Compound	5	1b (FTY720 phosphate)
$S1P_1$ EC_{50} (nM)	32	4
$S1P_3$ EC_{50} (nM)	>40 000	5
Selectivity	>1250-fold	No selectivity

Source: Adapted from Demont 2011 [40].

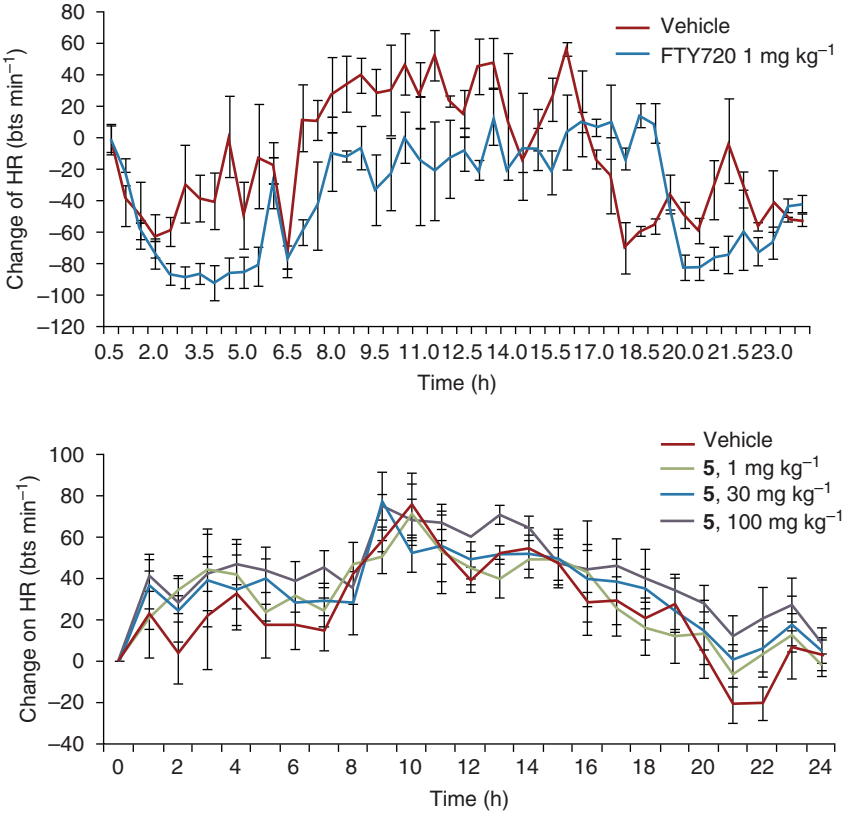


Figure 22.14 The absence of bradycardia in telemetered rats with **5** at 1, 30, or 100 mg kg^{-1} compared with **1a** (FTY720). *Source*: From Demont et al. 2011 [40]. Reproduced with permission of American Chemical Society.

in vitro receptor selectivity profile and in contrast with **1b** (FTY720 phosphate), no change in heart rate was observed [40].

22.11 Positioning Dose as the Focus of Lead Optimization

Compound failure in late-stage development is costly to the pharmaceutical industry. Analysis has been performed looking at the reasons for later stage clinical attrition with safety and efficacy remaining key factors [46, 47].

With respect to safety events, cardiovascular safety and hepatotoxicity remain a leading cause of attrition. In the case of the cardiovascular findings, the mechanistic link between QTc prolongation and blockage of the hERG channel has enabled effective screens to be established in discovery to ensure compounds with a risk are deselected early [48]. Other safety events, such as hepatotoxicity, can be idiosyncratic [49] sometimes only emerging in large-scale phase III trials or postmarketing with serious consequences for the patient and loss of return on investment for the developer. One key factor that has emerged as being linked to such events is the dose. The higher the dose, the higher the body burden, and research indicates correlations between higher doses and an increased risk of adverse findings such as hepatotoxicity [50] and DDI [37].

With respect to efficacy, understanding the relationships between concentration and response or pharmacokinetics–pharmacodynamics (PKPD) is fundamental to the prediction of drug behavior in the clinic and so should be placed at the center of drug design and selection. The development and deployment of PKPD models early in lead optimization allows for an estimation of the likely human exposures and dose. These are essential parameters in the overall integrated assessment of safety risks when contextualized with other indicators of hepatotoxicity and enzyme inhibition for example [32, 50].

For the $S1P_1$ program the PD response of interest was lymphocyte reduction, and the PKPD evaluation was positioned centrally in the lead optimization strategy [41]. A preclinical PKPD model of lymphocyte reduction in the rat was used to derive *in vivo* compound potency, which was subsequently used in conjunction with predicted human PK in a human PKPD model to estimate dose. The estimated human dose was a key factor in compound selection with the program aim to target a daily dose of ideally <100 mg. Through incorporation of PKPD in the screening cascade, several of the molecules identified had predicted doses within the desired range (Tables 22.1 and 22.4), providing confidence of minimizing other adverse events such as hepatotoxicity.

22.12 Delivery of Multiple Candidates for Development

While numerous compounds were ultimately identified as satisfying the product profile, exemplified in Table 22.3, **5** was considered the most promising, having physicochemical properties within drug-like space, >1000-fold selectivity for

$S1P_1$ over $S1P_3$, and no bradycardia observed in the telemetered rat. It displayed a favorable PK profile in two preclinical species and when coupled with nanomolar potency at the target had a low predicted human dose (<10 mg once daily), which was deemed appropriate for minimizing body burden and thus the risk of off-target findings. No evidence of CYP1A induction was observed in the rat *in vivo* nor in the human *in vitro* hepatocyte assay.

22.13 Conclusions

This case study highlights a typical lead optimization campaign where several molecule properties required investigation and iterative approaches were needed to deliver the desired target product profile. Substantial investment was made against this target with emphasis placed on early safety assessment of molecules. Toxicological consequences were identified and associated with related receptors and required optimization. Bradycardia was a known potential issue at the outset, and evaluation in telemetered rat studies was conducted early in the program cycle with lead molecules to confirm that receptor selectivity, in this case $S1P_1$ over $S1P_3$, mitigated the risk in these preclinical experiments.

A low dose reduces the overall body burden and, in turn, the risk of adverse findings such as DDI and hepatotoxicity. Human PK prediction, coupled with a translatable PKPD model, was used to provide an early estimate of the likely efficacious dose with only compounds having the potential for low dose (<100 mg) considered for further development.

An unusual finding of substantial CYP1A induction, consistent with AhR activation, was observed during early rodent safety studies. The SAR within the template was subtle with the exemplified non-inducer candidate molecule, **5**, having remarkable structural similarity to the molecules where the finding was initially observed. This highlights the detailed, iterative, and tenacious nature required in a lead optimization campaign to deliver the optimal molecule profile. This example also reveals how small structural changes can lead to a marked change and the need for continuous profiling across assays of importance. After careful consideration of the potential impact of AhR activation during late-stage development and in the clinic, the first compounds with this finding continued to progress, while the lead optimization strategy was revised to ensure that subsequent candidate molecules were devoid of this effect. This approach could be considered cautious, and others may decide on alternative courses of action. Our decision was taken with regard to minimizing late-stage attrition but also in light of the emerging positive clinical data with fingolimod and working with the assumption that another molecule entering the market with this mechanism would require a clean off-target profile. It should not be concluded from this article that AhR agonism will always be a significant cause for concern. The literature reports a complex picture with its function as a drug-metabolizing enzyme regulator. Moreover, it is clear that the functions of this receptor are far more broad reaching and the AhR may yet emerge as a therapeutic drug target [39].

For this set of compounds, *in vitro* assays were unsuccessful in predicting the extent of CYP1A induction *in vivo* in rat, so a short-term 4-day *in vivo* protocol

coupled with an aggressive pre-triage approach was used to discover compounds without induction potential. Modification of screening strategies resulted in the successful identification of several small molecule $S1P_1$ agonists that were progressed to preclinical development.

The issues arising in this case study extend across numerous areas of expertise and line functions within a drug discovery organization. For example, the assessment of bradycardia required early engagement with safety assessment and the development of combined strategies with biology from a scientific, operational, and strategic perspective. The design and operation of the 4-day *in vivo* rat protocol required expertise across DMPK and safety assessment plus medicinal chemistry scale-up and collective thinking around the SAR evaluation of induction. *In vitro* hepatocyte induction assays required DMPK resource as did the PKPD modeling and simulation. This case study exemplifies the importance of effective collaboration to rapidly identify and deliver promising new drugs for patients.

Acknowledgments

This work could not have been conducted without the expertise and dedication of the $S1P_1$ program team plus many colleagues working in the Immuno-Inflammation Therapy Area and other business partners within GlaxoSmithKline. The author is particularly grateful to Emmanuel Demont, Jason Witherington, and Mike Kelly who were key contributors to the strategy and leadership of this program. In addition to Emmanuel, the author gratefully acknowledges Dr Peter Eddershaw for critical review of this chapter.

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23

From TRAIL to ONC201: Case Study on the Safety Benefit of Developing Targeted Agents Against Cancer-selective Pathways

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23.1 Introduction: Toxicity, a Reason Behind Failed Clinical Trials

Historically, most chemotherapeutics were cytotoxic agents that targeted pathways that were operative in both normal and cancer cells. They mainly exploited the high proliferative rates of cancer cells, targeting DNA synthetic pathways that are critical for proliferation. However, given that normal cells also proliferate, especially epithelial cells of the intestine, the lymphoid system, and bone marrow, toxicities have been a major problem. The modern paradigm has been to develop therapeutic agents that target pathways where significant differences have been observed between cancer and normal cells [1].

23.2 Addressing Safety at the Onset: Targeting a Cancer-specific Pathway

23.2.1 Choosing to Activate the TRAIL Pathway as a Cancer Therapeutic Strategy

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a 21 kDa protein [2], similar to other members of the tumor necrosis family of proteins, can induce apoptosis [3] (Figure 23.1). It is expressed as a transmembrane protein, but its extracellular domain is proteolytically cleaved [4] (for one, by cathepsin E [5]) into a soluble/secreted form. Both membrane-bound and surface TRAIL can induce programmed cell death [3, 4]. TRAIL is recognized by a number of cell surface receptors, namely; death receptor 4 (DR4/TRAIL-R1), death receptor 5 (DR5), decoy receptor 1 (DcR1 or TRAIL-R3) [6], and decoy receptor 2 (DcR2

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

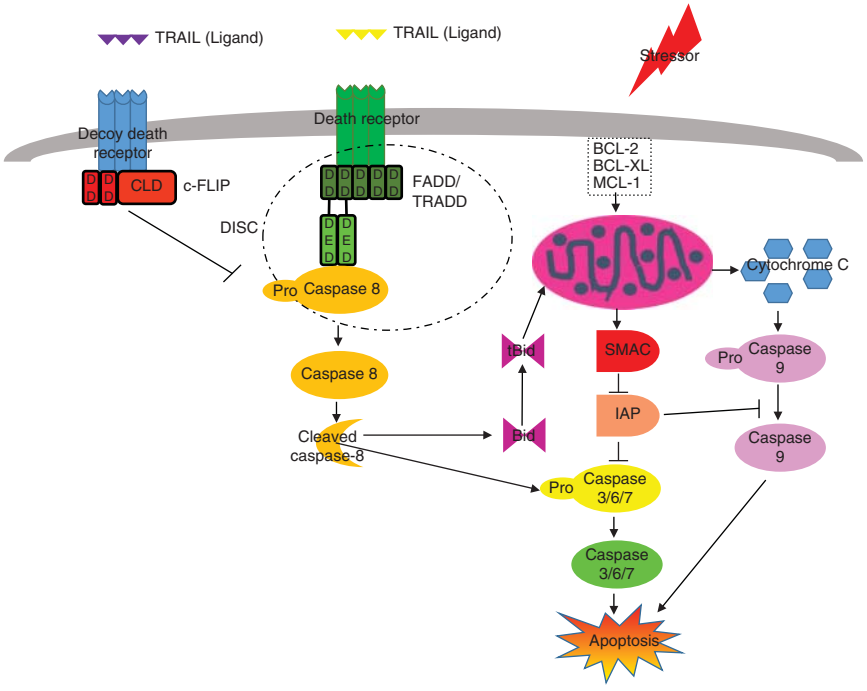

Figure 23.1 *Schematic diagram of the TRAIL signaling pathway leading to cell death*. TRAIL receptor trimerization occurs upon pathway engagement leading to caspase activation. The diagram depicts the potential for signal amplification with cross talk between the extrinsic and intrinsic pathways of cell death, and negative regulation of cell death by anti-apoptotic molecules.

or TRAIL-R4) [7, 8]. In addition, a soluble protein, osteoprotegerin, can interact with TRAIL [9]. The binding of TRAIL to its receptors facilitates receptor trimerization, resulting in a conformational change that exposes the intracellular death domains of the receptor [10]. Via homotypic interactions between death domains, the adaptor protein Fas-associated death domain (FADD) protein is recruited to the receptor [11]. Similarly, homotypic interactions between respective death effector domains in FADD and in procaspase-8 or procaspase-10 promote mobilization of these initiator caspases to the receptor. The complex formed by the receptor trimer, FADD, and procaspase-8 or procaspase-10 is referred to as the death-inducing signaling complex (DISC). The localization of procaspase-8 and procaspase-10 to the DISC facilitate caspase dimerization, subsequent caspase activation [12], and autocatalytic cleavage into both large (18 or 20 kDa, respectively) and small (10 kDa) fragments [13, 14]. The resulting large and small fragments associate into a tetramer, forming an active protease [15]. The consequences of caspase-8 and caspase-10 activation are cell type dependent. In type I cells (such as lymphocytes and thymocytes) [16], caspase-8 activation results in cleavage of the effector caspases, caspase-3 and caspase-7. On the other hand, in type II cells (such as hepatocytes), the extent of caspase-8 activation is not sufficient to induce cell death [17]. In these cells, caspase-8

cleaves the pro-apoptotic protein Bid, and truncated Bid localizes to the mitochondria and apoptosis ensues [18]. The downstream events in apoptotic cell death are common in all cells, and so the interest in the TRAIL pathway has been its selectivity in inducing apoptosis of transformed malignant cells.

23.2.2 Mechanisms Behind Cancer Selectivity

The therapeutic potential of TRAIL is largely based on exciting observations that unlike other members of the tumor necrosis family [19], TRAIL has a favorable therapeutic index [20–22]. Normal cells have redundant resistance mechanisms to TRAIL that protect them from TRAIL's pro-death effects [23]. Fibroblasts have been found to have lower levels of caspase-8. Normal cells lack the requisite ubiquitination of caspase-8 [24], a modification that is important for caspase-8 activity. Cells endogenously express inhibitors of apoptosis, such as FLICE-inhibitory protein (c-FLIP) and inhibitor of apoptosis proteins (IAP). c-FLIP, similar to caspase-8, has death effector domains that facilitate c-FLIP's interaction with FADD at the DISC. Unlike caspase-8, however, c-FLIP does not have protease activity [25]. Moreover, it blocks procaspase-8 activation at the DISC [26], at least in part, by inhibiting recruitment of procaspase-8 to the DISC [27]. Primary keratinocytes have higher levels of c-FLIP than transformed keratinocytes [28]. TRAIL resistance of natural killer and CD8(+) T cells is also attributed to high expression of c-FLIP [29]. The IAP X-linked inhibitor of apoptosis protein (XIAP) is another endogenous protein that imparts protection against TRAIL. XIAP blocks apoptosis by binding and inhibiting caspase-3 and caspase-7 [30]. Differences in the effects of TRAIL between cancer cells and normal cells motivated efforts to exploit the pathway in cancer therapeutic development.

23.2.3 Strategies of TRAIL Pathway Therapeutic Engagement

23.2.3.1 Using TRAIL

The discovery of TRAIL's cancer-selective pro-death effects spurred a flurry of research on the clinical use of TRAIL. The cytotoxic activity of TRAIL is enhanced when TRAIL molecules form oligomers [4]. LZ-TRAIL is a TRAIL variant where TRAIL has been fused to a leucine zipper motif, facilitating oligomerization [31]. Nevertheless, it was shown that untagged TRAIL, by itself, is biologically active *in vivo* as a single agent or in combination with chemotherapy [20]. This led to its approval for testing in clinical trials and in that context was known as Apo2L.0 or AMG-951/dulanermin. Unfortunately, although it was clinically safe, this untagged TRAIL was not deemed efficacious [32]. This could be in part due to an unfavorable pharmacokinetic (PK) profile, with an extended distribution half-life of only 3–5 min and an elimination half-life of 20 min [33]. By contrast, LZ-TRAIL has better PK characteristics, with an extended distribution half-life of 1.3 h and an elimination half-life of 4.8 h [34]. Another modification that improved the PK properties of TRAIL included covalently linking TRAIL to polyethylene glycol (PEG) [35, 36]. However, alternative formulations such as tagged TRAIL also had greater toxicity against normal cells including hepatocytes.

Efforts to target delivery of TRAIL to tumors have been put forth to increase the local circulation of TRAIL and reduce dilution of the agent in circulation. TRAIL has been either encapsulated in nanoparticles [37] and released from the particle or attached to the surface of the nanoparticles [38]. Cell-based delivery of TRAIL, specifically, by stem cells has also been explored. Given that mesenchymal stem cells (MSCs) migrate toward gliomas [39] (at least in part in response to factors that the gliomas secrete [40]), MSCs have been genetically engineered to produce TRAIL [41].

23.2.3.2 Using Antibodies Against TRAIL Receptors

Dulanermin, the clinically used form of TRAIL, has relatively weak agonist activity against DR4 and DR5. Moreover, it binds to the non-apoptotic receptors DcR1 and DcR2, thus potentially reducing dulanermin's efficacy. Alternatively, agonist antibodies to the TRAIL receptors DR4 [42, 43] and DR5 [44–47] have been developed. The first antibodies that were developed required crosslinking; hence, their *in vivo* efficacy was dependent on the complement component C1q and Fc receptors of immune effector cells [48]. Unfortunately, the activity of these proteins on immune cells can be affected by immunosuppressive therapies or Fc polymorphisms [48, 49]. Thus, antibodies that are able to induce apoptosis without prior crosslinking have been identified [50]. A very critical advantage in using agonist antibodies to the death receptors is their significantly longer half-lives – which can be several days [43] to weeks [51, 52]. Thus, they do not need to be administered repeatedly or continuously. Unfortunately, over the past two decades, no significant therapeutic benefit has been observed in clinical trials with these TRAIL receptor antibodies, and none have been approved by the FDA as cancer therapeutics [21].

23.2.3.3 Using Small Molecules to Activate the TRAIL Pathway

The lack of significant clinical success in using TRAIL or TRAIL receptor antibodies warrants alternative strategies to leverage the cancer-specific pro-apoptotic effect of the TRAIL pathway. Our laboratory developed a cell-based bioluminescence reporter screen to identify small molecules that can induce cells to produce TRAIL [53]. This involved expressing the luciferase gene downstream of the first 504 base pairs of the TRAIL gene promoter. In light of the high percentage of cancer cells that do not have wild-type p53, it was critical to exclude the p53-responsive element of the *TRAIL* promoter. To ensure that the induced TRAIL does not kill the cells carrying the construct, HCT116 $Bax^{-/-}$ cells were used for screening. The absence of Bax in these cells makes them resistant to TRAIL-induced apoptosis [54]. Small molecules from the NCI Diversity set II were screened at 1 μM. Twenty-nine compounds were able to induce reporter activity by >1.4-fold. Results of the screen were validated by performing reporter assays with different doses of small molecules (with the highest dose tested being 1 μM) for different durations (12, 24, 36, and 48 h). Ten of the 29 compounds increased reporter activity by greater than twofold under at least two experimental conditions tested [53].

23.3 Maximizing Efficacy and Minimizing Toxicity at the Bench

23.3.1 Decision making: Choice of Which Compound to Move Forward with (Comparison of the Different TICs) – Balancing Efficacy with Safety

Four of ten compounds (**T**RAIL-inducing compounds (TICs)) – TIC4, TIC8, TIC9, and TIC10 (Figure 23.2), that were identified in the screen have been confirmed to induce TRAIL transcription in a p53-independent manner. Two of the compounds, TIC9 and TIC10 (later referred to as ONC201), result in increased surface TRAIL expression. Not only do TIC9 and ONC201/TIC10 upregulate TRAIL, but they also induce expression of TRAIL receptor DR5. TIC9 and ONC201/TIC10 significantly induced apoptosis of HCT116 $p53^{-/-}$ cells after 72 h of treatment. Unfortunately, TIC9 also induced apoptosis of normal human fibroblasts. By contrast, ONC201/TIC10 had cancer-specific cytotoxicity even when used at 40 μM (Figure 23.3). Furthermore, no genotoxicity or alteration in cell morphology was observed in normal cells treated with ONC201/TIC10.

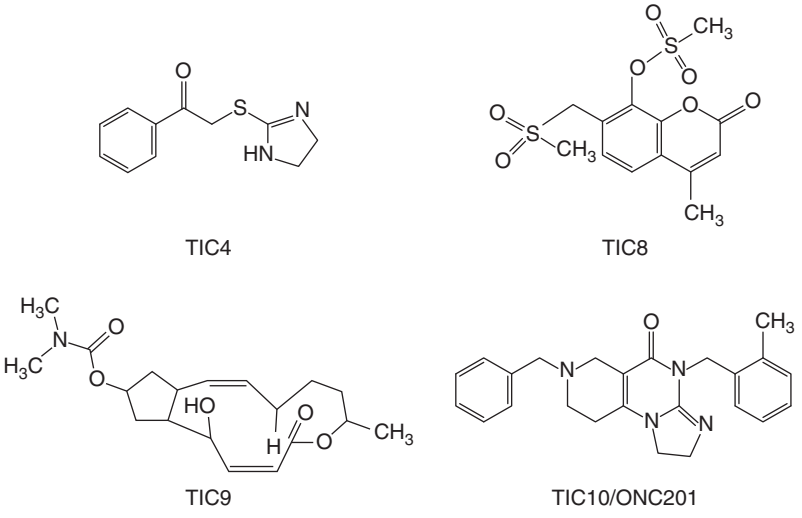

Figure 23.2 Chemical structures of some initial TRAIL-inducing compounds identified in phenotypic screen.

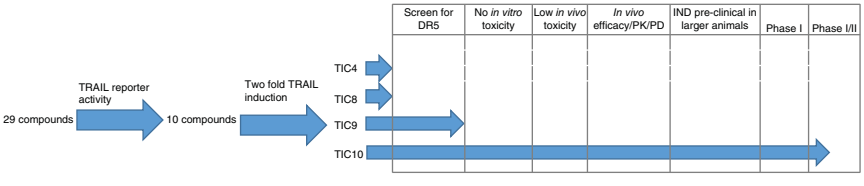

Figure 23.3 Early experience with TRAIL-inducing compounds and path toward further development of ONC201.

Given ONC201/TIC10's favorable therapeutic window, it was chosen for further development [53].

23.3.2 Expanded *In Vitro* Evaluation of Efficacy and Safety

ONC201 has been shown to be effective against multiple cancer cell types (both solid and hematologic malignancies) *in vitro* [55–59], including against cancer stem cells [57, 60]. Although there are cell types that are less susceptible to ONC201/TIC10-induced apoptosis, ONC201/TIC10 remains cytotoxic at least in part via ONC201's ability to downregulate cell proliferation and induce cell cycle arrest. Studies have confirmed that ONC201 selectively kills malignant cells, including malignant stem cells and not normal cells (or stem cells from normal bone marrow) [57, 61]. ONC201/TIC10 downregulates phosphorylation of Akt, ERK, and Foxo3A [55, 56, 59] and induces expression of TRAIL in a broad spectrum of cancer cell types [55, 58, 59]. In addition to stimulation of the TRAIL, a focus on the early signaling events revealed activation of an integrated stress response culminating in upregulation of TRAIL death receptor DR5 on the surface of tumor cells [56]. ONC201 appears to trigger a PERK-, PKR-, and HRI-dependent integrated stress response that signals through eIF2-α the upregulation of ATF4 and CHOP to activate DR5. The activation of the TRAIL and its receptor DR5 on tumor cells provides a potent mechanism for antitumor efficacy that is engaged by ONC201.

Thus, there is a mechanistic understanding of the pathway activated by ONC201 leading to TRAIL production and cancer-selective cell death. The rational design of the screening strategy based on understanding of the biology of cancer progression and the mechanisms of cell death, in addition to the therapeutic index from engagement of the TRAIL pathway, led to preclinical discovery and development of ONC201/TIC10.

Ongoing work is further exploring the role of a subclass of dopamine receptors as candidate binding targets of ONC201. Prolactin that is secreted by the pituitary gland has been detected in patients treated with ONC201 in the first-in-human clinical trial. The identification of dopamine receptors as ONC201 binding targets could be significant as GPCRs have not been previously exploited in cancer therapy. Ongoing work is further determining the contribution of dopamine receptors to the anticancer efficacy of ONC201. It is clear that drugs that emerge from phenotypic screens often have complicated mechanisms of action by contrast to small molecules that are developed to have high affinity to a pocket of an enzyme such as a kinase or that may block a protein–protein interaction. Nonetheless, ONC201 is a potent antitumor agent with already promising activity observed in the clinic.

23.3.3 Exploring Therapeutic Potential by Performing *Ex Vivo* Studies

The availability of cells from patient tumors facilitated preclinical testing of ONC201/TIC10. Cells from freshly resected tissues from a colon cancer and a glioblastoma patient were susceptible to ONC201/TIC10 [55]. Patient-derived tumor cells were also implanted in the brains of mice and demonstrated

antitumor effects of ONC201/TIC10. Furthermore, 8 primary mantle cell lymphoma samples and 18 primary acute myeloid leukemia samples were found to be sensitive to ONC201/TIC10 [57]. Peripheral blood mononuclear cells from five patients with Sezary syndrome (a form of cutaneous T-cell lymphoma) were susceptible to ONC201-induced apoptosis [62].

23.4 Leveraging Preclinical Animal Studies to Predict Clinical Performance

23.4.1 Identifying Vulnerable Tumor Types

Given TIC9 and TIC10's promising biological activity *in vitro*, their *in vivo* activities were assessed. Aside from comparing them against vehicle, TIC9 and TIC10 were studied with TIC4. Subcutaneous xenografts of HCT116 $p53^{-/-}$ cells in athymic nude mice were assessed for TRAIL mRNA and protein expression after a single intraperitoneal 25 mg kg^{-1} dose of DMSO vehicle, TIC4, TIC9, or TIC10. Confirming results of *in vitro* studies, only TIC9 and TIC10 induced TRAIL upregulation. Nevertheless, apoptosis was induced by all three TICs, including TIC4. This suggests that TIC4 has an *in vivo* pro-apoptotic effect that is TRAIL independent. Nevertheless, because of the lack of effect of ONC201 on normal cells *in vitro*, succeeding *in vivo* experiments were performed using ONC201/TIC10.

The antitumor efficacy of ONC201 has been shown to be comparable with TRAIL when used to treat HCT116 cells. To fully exploit ONC201's therapeutic potential, *in vivo* efficacy studies were performed on xenografts of different tumor types – colorectal, breast, non-small cell lung cancer, and glioblastoma [55]. ONC201 significantly inhibited tumor growth in these experiments. In addition, ONC201 was able to prolong the survival of a mouse model for high-incidence spontaneous lymphoma and leukemia of early B cells, the Eμ-myc transgenic mouse [63]. This immune-competent model extended the preclinical efficacy of ONC201/TIC10 to a hematopoietic tumor type.

23.4.2 Assessing Impact on Normal Tissue

23.4.2.1 Classical Indicators of Toxicity

ONC201/TIC10 effectively inhibited tumor growth for two weeks when administered as a single dose of 25 mg kg^{-1} orally. Administering 4× of this dose (i.e. 100 mg kg^{-1}) intraperitoneally or administering 25 mg kg^{-1} dose every week for 4 weeks did not significantly affect mouse body weight or liver histology. To further investigate ONC201's safety, immunocompetent mice were given 25 mg kg^{-1} ONC201 weekly for 4 weeks, and serum chemistry was analyzed. The results indicated that ONC201 does not significantly alter serum chemistry in non-tumor-bearing mice.

23.4.2.2 Pharmacodynamic Analyses

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate labeling (TUNEL), staining, and immunohistochemical analyses of cleaved

caspase-8 (CC8) of tumor xenograft tissues confirmed that ONC201/TIC10 induces apoptosis *in vivo*. CC8 is a biomarker of TRAIL pathway engagement and confirms activation of the extrinsic apoptotic pathway *in vivo*. Quantitative RT-PCR and immunohistochemical analyses for TRAIL showed that ONC201 induced TRAIL expression. TRAIL mRNA expression peaked as early as 48 h after the intravenous injection of 25 mg kg^{-1} ONC201/TIC10. In addition to TRAIL protein expression in tumorigenic tissue, TRAIL was also observed in the stromal fibroblasts that were adjacent to the tumor cells. This indicated that ONC201/TIC10 could induce TRAIL expression of normal cells and mediate a bystander effect in cancer cell killing. To substantiate this observed TRAIL production of normal cells, non-tumor-bearing mice were injected with ONC201/TIC10. IHC analyses of normal tissue showed that TRAIL was upregulated in the brain, kidney, and spleen. Histological assessment did not indicate toxicity, however. These *in vivo* results reflect the resistance of normal cells to TRAIL. The detection of TRAIL in the brain suggested that ONC201/TIC10 can cross the blood–brain barrier. This pharmacodynamic result revealed ONC201/TIC10's promise in treating the more recalcitrant brain tumors. This activity of ONC201/TIC10 was demonstrated using GBM xenograft studies.

23.4.3 Conducting Toxicology Studies in Accordance with Good Laboratory Practices (GLP)

To confirm ONC201/TIC10's safety, good laboratory practices (GLP)-compliant toxicology studies were performed [64]. Male and female Sprague-Dawley rats were given a single dose of 0, 12.5, 125, or 225 mg kg^{-1} ONC201 by oral gavage [61]. The lowest dose administered to rats (excluding 0 mg kg^{-1}) corresponded to 25 mg kg^{-1} in mice (a dose that has been shown to be efficacious in mice) [55]. In addition to assessing blood chemistry, tumor histology, and body weight, the following parameters/tests were monitored: food consumption, blood pressure, urinalysis, and neurobehavioral assessment [61] (via functional observational battery [65]). Rats were observed up to 18 days after ONC201 dose administration. No laboratory or clinical signs of toxicity were observed in the rats that received up to 125 mg kg^{-1} ONC201. At the highest dose tested (225 mg kg^{-1}), transient decrease in activity and abnormal gait were observed. A decrease in body weight gain and food consumption was observed in male but not in female rats.

Parallel GLP-compliant safety studies on a second animal species – beagle dogs – were performed. A single oral dose of 0, 4.2, 42, or 120 mg kg^{-1} was administered. Again, the lowest dose tested (excluding 0 mg kg^{-1}) corresponded to 25 mg kg^{-1} in mice. At this dose, no clinical signs of toxicity were observed. Administering the higher dose of 42 mg kg^{-1} caused a transient decrease in activity, emesis, vomitus, salivation, and/or soft, loose, or mucous stool and changes in fecal excretion. A decrease in food consumption was also observed at the highest dose tested (120 mg kg^{-1}) in female dogs. Based on results from the rat and beagle dog studies, the no-observed-adverse-effect level (NOAEL) following oral administration of ONC201 is that which corresponds to approximately a dose of 1.25 g in humans using standard allometric scaling. The results

from these GLP-compliant toxicity studies not only confirmed the safety of ONC201 but also provided the starting dose for a first-in-human clinical trial. This dose is 1/10th of the NOAEL [66], i.e. 125 mg.

23.5 Applying Lessons from *In Vitro* and *In Vivo* Studies to Clinical Trials

Given the broad-spectrum efficacy of ONC201 *in vitro* and *in vivo*, ONC201 entered clinical trials in 2014 and is currently being tested in Phase I/II clinical trials against both solid tumors and hematological malignancies [67]. In the first-in-human Phase I trial, patients were recruited and assigned into single-patient dose escalation cohorts. Because of the excellent safety profile of ONC201, an accelerated dose escalation design was adopted [68, 69]. The doses tested were 125, 250, 375, 500, and 625 mg. PK analyses showed that absorption was saturated at 375 mg, indicating that giving more than 625 mg ONC201 in a single dose is not needed. No adverse effects >grade 1 were observed. Thus, the recommended Phase II dose was set to 625 mg [70]. Levels of TRAIL and caspase-cleaved fragment of cytokeratin 18 were elevated in the sera of patients. This confirms ONC201's TRAIL-inducing activity. Furthermore, the detection of caspase-cleaved fragment of cytokeratin 18 indicates increased tumor apoptosis [71, 72] resulting from ONC201 treatment.

23.6 Summary

The process by which a drug progresses to clinical development is a rigorous one, designed to effectively and safely produce efficacious compounds and ones that are more effective than those on the market while demonstrating low toxicity and a desirable PK/PD profile. The development of TIC10/ONC201 to date was a methodical one – designed to produce a cancer-specific targeting compound. By using *in vitro* screening for TRAIL-activating compounds, an initial group of compounds was available for further exploration. Importantly, ONC201 upregulates both the death receptor and ligand to enhance potential efficacy. ONC201 had no toxicity in normal fibroblasts and was selected for *in vivo* based studies. As the most promising compound, based on TRAIL induction activity, efficacy, and low toxicity, ONC201 was pursued further. As with all drug development, selection of a lead compound was just the beginning. Through a large series of carefully constructed *in vivo* studies, additional valuable information was collected on the oral bioavailability of the drug, impact on tumor growth, biomarkers, and lack of toxicity toward non-tumor tissues. Potential toxicities of ONC201 were assessed at maximum tolerated dose thresholds. Preclinical studies established pharmacodynamics and PK parameters that would be further examined in patients. These detailed preclinical observations of ONC201 and additional IND-enabling studies facilitated the implementation of an accelerated clinical trial design for Phase I/II clinical trials. Clinical trial results to date yielded no >grade 1

drug-related toxicities. These promising findings would have been unlikely with compounds that were not tested rigorously in preclinical *in vitro* and *in vivo* models for safety and toxicity. Although the preclinical route was a lengthy process, we have demonstrated the benefit of using appropriate, detailed experiments that seriously consider toxicity, bioavailability, and PK/PD of efficacious compounds to bring safe drugs to the clinic. The process of clinical development continues with demonstration of single-agent safety and efficacy and with pursuit of combination therapy studies in various tumor types. While much is already known about the mechanism of action of the novel anticancer agent ONC201, with regard to TRAIL pathway activation, more needs to be learned about its direct binding targets and how they lead to anticancer efficacy. Drugs that emerge from phenotypic screens, while clearly rationally designed, often have complicated mechanisms of action. Further understanding of the molecular mechanisms of ONC201 should lead to more comprehensive biomarker analysis in the clinic that may improve patient selection and patient benefit.

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Part V

Intellectural Property

24

Patent Law Relevant to Early Drug Development

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24.1 Introduction

In order to understand patent law and its effect on drug development, it is important to have a basic understanding of the US patent system as well as the drug development process. This chapter provides an abbreviated explanation of the US patent system as well as an explanation of some of the basic concepts that underline that system. This section will also include a brief overview of the drug development process and cover some aspects of patent law that may affect this process. The purpose of this chapter is to provide the foundation for the material that follows and a language for explaining the substance of the topics that will be discussed in subsequent chapters.

24.2 Overview of Patent Protection

24.2.1 What Are Patents?

Patents are the most common way for a company to protect its technology. A patent is the grant of a property right to an inventor, issued by the US Patent and Trademark Office (USPTO). The right that a patent holder is granted is to exclude others from making, using, offering for sale, or selling the invention in the United States or from importing the invention into the United States. It is helpful to think of a patent right as being similar to a property line around a house. The patent establishes the boundaries of which the patent holder is entitled to. The terms of the boundaries are called claims, which are discussed in more detail soon. A patent does not, however, entitle the patent holder to practice or make the invention, only to exclude others from practicing or making the invention. There may be other patents or regulatory hurdles that could block the patent owner from making or using the product.

All patent applications are examined by the USPTO examiners. The examiners review the patent to decide whether or not the invention is eligible for patent

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

protection based on criteria that is discussed later on in this section. Examiners will also conduct a search of all patent and non-patent literature, such as journal publications and online references, to see if the claimed invention falls within the scope of what is already known to the public. After the patent application is filed, the contents of that application are kept confidential for 18 months. After the end of the 18-month period, the contents of the patent application are made public and published online.

24.2.2 What Is the Purpose of a Patent?

A patent is a proprietary right granted by the federal government to an inventor. Article I, Section 8, Clause 8, of the US Constitution grants Congress the power "to promote the progress of science and useful arts, by securing for limited times to authors and inventors the exclusive right to their respective writings and discoveries."[1] In analyzing how patents promote scientific progress, the courts have emphasized two mechanisms: (i) the prospect of obtaining a patent monopoly provides an incentive to invest in research to make new innovations and (ii) the patent system promotes disclosure of new inventions and thereby enlarges the public storehouse of knowledge.

The incentive to invent theory holds that, without the ability to obtain patents, scientists will not be encouraged to invent due to the costs of developing the invention. If there is no protection offered for invention, competitors could easily imitate the invention without sharing the costs of development. This is especially true in the pharmaceutical industry. Large pharmaceutical companies spend billions of dollars a year on thousands of different drug treatments; however, only a handful are selected for commercialization. If these companies were not able to protect their research, the drugs that are eventually commercialized would easily be copied and the pharmaceutical companies would not be able to profit off of their research.

The incentive to disclose argument rests on the idea that in the absence of patent protection, inventors would keep their inventions a secret in order to prevent competitors from copying them. By keeping the inventions a secret, the public is unable to gain the full benefit of new knowledge and leads to wasteful duplicative research. The patent system facilitates disclosure of research and technology by creating rights only in inventions that are disclosed to the public.

24.2.3 Types of Patents

Under US patent law, an inventor has three different types of patents available. The most common type of patent is the utility patent, as most inventions fall under this category of patent. A utility patent may be granted to anyone who invents or discovers a novel machine, process, composition of matter, article of manufacture, or any useful improvement thereof. Under the European Patent Convention (EPC), this is the only type of patent available.

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1 U.S. Constitution, Article 1, Section 8.

The less common types of patents are design patents and plant patents. Design patents may be granted to anyone who creates a new, original, and ornamental design for an article of manufacture. Design patents have been issued for products such as the iPhone and the original Coca-Cola bottle. These patents are commonly used for short-lived technologies, such as computers, which may be replaced by newer models frequently. A plant patent may be granted to anyone who invents or discovers and asexually reproduces any distinct and new variety of plant.

24.2.4 Patent Term and Scope

Because a patent right is essentially creating a monopoly, the rights of the patent holder can only exist for a fixed number of years. Utility patents are now valid for 20 years from the earliest filing date, while design and plant patents are valid for 15 years from the issue date. However, the term of a patent may be extended in certain circumstances to provide for additional protection beyond the 20-year period. This extension usually occurs to compensate for delays occurring during the filing of the patent application, or the patent prosecution, or delays caused by Food and Drug Administration (FDA) approval. For example, if a patent application does not receive a response from the USPTO within 18 months of filing a non-provisional application or utility application, the patent holder, if the patent is issued, will be entitled to an extension of its patent term equivalent to each day that the USPTO fails to file a response after the 18-month deadline. A non-provisional application is an application that establishes an invention's filing date (unless it claims the benefit of an earlier file application) and starts the official examination process with the USPTO to determine if the invention is patentable. A provisional application, in contrast, is an application that acts as a placeholder for the non-provisional application. An applicant has 12 months from the date of filing the provisional application to file the non-provisional, or utility, application.

Patent rights are limited to the country in which they are filed in. Patents are territorial by nature, and, as a result, patent rights to an invention are only protected in those countries where a patent has been issued. This is due, in part, to the different standards of patentability from country to country. As a result of this, a competitor can avoid an infringement lawsuit by simply stepping outside of the country for one element of the process. When filing for patent protection, it is important to consider which countries the product may be sold in when deciding what countries to file for patent protection in.

24.3 Requirements for Patent Protection

In order to obtain patent protection, there are several requirements that first must be satisfied for an invention to be patentable. In the United States, a patented invention is required to fall within a category of eligible subject matter, be novel and nonobvious, be useful, and enable one skilled in the art to practice the invention.[2] In the EU, a patented invention also is required to fall within a category of

2 35 U.S.C. 101, 102, 103, 112.

eligible subject matter, be novel, involve an "inventive step," and be susceptible to industrial application.[3]

24.3.1 Eligible Subject Matter

The requirement for patentable subject matter in the United States is provided in 35 USC §101, stating that, "[w]hoever invents or discovers any new and useful *process*, *machine*, *manufacture*, or *composition of matter*, or any new and useful improvement thereof, may obtain a patent therefore, subject to the conditions and requirements of this title."[4] Section 101 therefore requires that an invention must be a process, machine, manufacture, composition of matter, or improvement thereof in order to be eligible for patent protection. While this list of categories is very broad, the courts have provided three exceptions of what can be patentable, including laws of nature, physical phenomena, and abstract ideas. Within recent years, there have been several cases relating to the patentability of pharmaceuticals and other biologically related inventions. As an example, the Supreme Court in *Mayo Collaborative Services v. Prometheus Laboratories,*[5] *Inc.* held that a method of adjusting a drug dosage after observing a patient's reaction to a drug administration was a patent-ineligible subject matter under §101. Likewise, in *Association for Molecular Pathology v. Myriad Genetics, Inc.,*[6] the Supreme Court held that isolated human genes were patent ineligible under §101.

In contrast to the United States, the European patent law does not provide a definition for what qualifies as an "invention."[7] It does, however, provide a list of what does not qualify as an "invention," including discoveries, scientific theories, mathematical methods, aesthetic creations, schemes, rules and methods for performing mental acts, playing games or doing business, programs for computers, and presentations of information. The excluded category, "discoveries," is comparable with "products of nature" under US patent law.

24.3.2 Utility

The second requirement for an invention to receive patent protection is that the invention has utility. Under §101, utility requires that the invention be useful or that the invention provides some identifiable benefit. As long as the invention can be shown to have a purpose, the invention can generally satisfy this requirement. Typically, anything developed within the pharmaceutical market has some utility. However, it is important to note that in order to obtain a patent, a chemical must have actual utility, not only laboratory utility. This means that intermediates that are created in a laboratory that are not a part of the final chemical or drug are not able to be patented. Intermediates cannot be patented because an exclusive hold of intermediates could severely halt scientific research.

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3 EPC Articles 52(2), 54(1), 56, 57.
4 35 U.S.C. 101.
5 *Mayo Collaborative Services v. Prometheus Labs. Inc.*, 132 S. Ct. 1289 (2012).
6 *Association for Molecular Pathology v. Myriad Genetics, Inc.*, 133 S. Ct. 2107.
7 EPC Art. 52(2).

European patent law, on the other hand, does not require the same showing of usefulness. Instead, it requires that the invention have industrial applicability, such that it can be "made or used in any kind of industry, including agriculture."[8] This requirement has been broadly interpreted and generally serves to only exclude inventions that are purely aesthetic or that cannot work because they operate in contradiction to the laws of nature.

24.3.3 Novelty

The next requirement for an invention to receive patent protection is that it be novel under 35 USC §102.[9] In other words, the invention must be different from what already exists in the public domain, that is to say, something that is already published or otherwise made available to the public. If an invention is already known and available to the public, the idea cannot be patented because it does little to promote innovation and advance ideas. In order to determine whether an invention is novel, an examiner will review all of the materials, or "prior art," surrounding a given invention. The general rule is that any piece of prior art that discloses the claimed invention that is dated before the filing date of the patent application could potentially prevent an applicant from obtaining patent protection. The world of prior art includes all printed publications in any country and any public disclosure, such as a presentation or a public offer for sale.

European patent law has a similar novelty requirement under Article 54 EPC.[10] Article 54 proves that "an invention can be patented only if it is new." Similar to the requirement in the United States, an invention is considered to be new if it does not form part of the existing "state of the art." The "state of the art" under the EPC is considered to be absolute. Even the disclosure to a single person who is under no obligation to maintain secrecy is sufficient for a disclosure to be considered part of the state of the art.

24.3.4 Nonobviousness

The patent application then must prove to be a nonobvious invention, as required by 35 USC §103.[11] Obviousness is a very difficult concept to judge, as something that is obvious to one individual may not be obvious to another. The USPTO and US courts judge obviousness based on the standard of a person "of ordinary skill in the art," meaning, a person who is considered to have the normal skills and knowledge in a particular technical field. A person of ordinary skill in the art mainly serves as a reference for determining, or at least evaluating, whether an invention is nonobvious or not (in US patent law) or involves an inventive step or not (in EU patent law).

The standard of obviousness changed in 2007 when the Supreme Court dealt with the case *KSR International Co. v. Teleflex Inc.*[12] In *KSR v. Teleflex*, Teleflex

8 EPC Art. 57.
9 35 U.S.C. §102(a-b).
10 EPC 54(1).
11 35 U.S.C. 103.
12 *KSR International Co. v. Teleflex Inc.* 550 U.S. 398 (2007).

sued KSR for infringement on its patent for an adjustable gas pedal system that composed of an adjustable accelerator pedal and an electronic throttle control. KSR argued that the invention was obvious and, therefore, the Teleflex could not enforce its patent. The district court ruled that anyone with knowledge or experience in the industry would have considered it obvious that the two components of the pedal and the throttle control be combined. Teleflex appealed to the Court of Appeals for the Federal Circuit, which reversed the district court, finding that the court had not applied a full "teaching–suggestion–motivation test," which required the court to identify the specific teaching, suggestion, or motivation that would have led a knowledgeable person in the art to combine the two previously existing components. The Supreme Court overruled the Court of Appeals, holding that the "teaching–suggestion–motivation" test was not to be applied as a mandatory rule but that broader considerations must be made in order to determine whether a person skilled in the art would find the combination obvious.

This case is a great example of the difficulty in determining whether an invention is obvious or not. Here, two courts applying similar standards came out with different results. Currently, the "teaching–suggestion–motivation" test still shows up in questions of obviousness; however, the standard is now much broader, and it is much easier for an alleged infringer to invalidate a patent based on obviousness.

European patent law has a similar obviousness requirement, which is called "inventiveness."[13] The purpose of the inventiveness step is to avoid granting patents for inventions that are simply modifications of existing inventions. Therefore, the inventiveness step helps determine whether a particular invention is sufficiently inventive, or nonobvious enough, to be patented.

24.3.5 Enablement

The final requirement for receiving patent protection in the United States is that the invention must be fully described in the patent application. The purpose of a published patent is to teach those skilled in the art of the innovation as to enable them to advance upon the idea, thereby advancing the scientific community. In order to reach this goal, the invention described in a patent must be sufficiently clear such that a person of ordinary skill in the art could make and practice the invention. The patent application is also required to describe the best mode of practicing the invention. European patent law does not have a similar requirement to enablement.

24.4 Patent Infringement

Patent infringement is the act of making, using, selling, or offering to sell a patented invention or importing into the United States a product covered by a claim of a patent without the permission of the patent owner. A patent owner can sue an alleged infringer in federal court for patent infringement.

13 Article 52(1) EPC and Article 56 EPC.

Under 35 USC §271,[14] there are several categories for patent infringement. Direct infringement, under 35 USC §271(a), occurs when "whoever without authority makes, uses, offers to sell, or sells any patented invention within the United States or imports into the United States any patented invention during the term of the patent therefore."[15] Direct infringement does not require a showing of intent to infringe the patent or even knowledge of the patent's existence. The only requirement is that the patent is actually infringed. Infringement can be avoided simply by stepping outside of the scope of the patent for at least one element of the claim.

A patent can also be infringed if a third party induces the infringement of another, under 35 USC §271(b).[16] The statute states that "whoever actively induces infringement of a patent shall be liable as an infringer." In order for a patent holder to succeed in a case involving induced infringement, also known as indirect infringement or contributory infringement, he or she must prove that direct infringement has occurred as well as intent to induce the infringement. Induced infringement, therefore, requires that the induced infringer "knowingly aided and abetted another's direct infringement of the patent."[17] Mere knowledge of the possibility of infringement does not amount to inducement.

In addition to direct and indirect infringement, a US patent can only be infringed if a third party contributes to the infringement of another, under 35 USC §271(c).[18] 271(c) further provides that "whoever offers to sell or sells within the United States or imports into the United states a component of a patented machine, manufacture, combination, or composition, or a material or apparatus for use in practicing a patented process, constituting a material part of the invention, knowing the same to be especially made or especially adapted for use in an infringement of such patent, and not a staple article or commodity of commerce suitable for substantial non-infringing use, shall be liable as a contributory infringer."

Contributory infringement under §271(c) provides for an action of infringement based on the sale of a component of a patented product in the United States, even though the component itself does not infringe the claim of the patent. In order to establish contributory infringement, the patent owner must show direct infringement that the defendant had knowledge of the patent and that the defendant knew that the product for which the components were made was both patented and infringing and that the defendant's components had no substantial non-infringing uses.[19] This type of infringement is limited to actions taken within the United States.

The first step in determining whether a patent is infringed is to determine the meaning, or scope, of the alleged infringed claims. This process is called claim construction, in which a court will consider intrinsic and extrinsic evidence to determine the scope of a claim. Intrinsic evidence includes the language of the

14 35 U.S.C. §271.
15 35 U.S.C. §271(a).
16 35 USC §271(b).
17 *Warner-Lambert Co. v. Apotex Corp.*, 394 F.3d 1348 (Fed. Cir. 2003).
18 35 USC §271(c).
19 *Fujitsu Ltd. v. NETGEAR Inc.*, 620 F.3d 1321, 1326 (Fed. Cir. 2010).

claim, the specification of the patent, and the prosecution history of the patent. If the claim language is clear on its face, the court may turn to the specification and prosecution history to determine if there is a deviation from the clear language of the claim. If the claim language is not clear on its face, the court may refer to the specification and prosecution history to resolve ambiguity. Arguments made by the applicant during prosecution of the patent may be used to interpret the meaning or scope of a claim. If the meaning or scope of a claim remains unclear after considering the intrinsic evidence, a court may consider extrinsic evidence, which refers to expert testimony, dictionaries, and technical treatises and articles that may provide guidance as to how the claim should be interpreted.

After the court has construed the claim language, thus determining the scope of the claimed invention, a court will examine whether the accused product infringes the claim. An accused product can infringe on a patent either literally or equivalently. Under literal infringement, every limitation of a patent claim must be found in the accused product. Failure to meet a single claim limitation is sufficient to negate a claim of literal infringement.

If a claim is not literally infringed, the claim may still be infringed under the doctrine of equivalents. The doctrine of equivalents allows for a product or process to be infringed if the claimed invention claims an equivalent for each literally absent claim limitation. According to the Supreme Court in *Warner-Jenkinson Co. v. Hilton Davis Chemical Co.,*[20] in order to prove infringement of a claim under this doctrine, the patent holder must prove that any difference between the claim element and the accused product is "insubstantial" to one of ordinary skill in the art. Insubstantial differences can be proved by showing that an accused product performs substantially the same function, in the same way, to achieve the same result as the claim element under analysis.[21]

In the event that an accused product is found to infringe a valid third-party patent, two options may be available to the owner of the infringing product. First, the infringing product may be amended or altered in such a way that it would avoid infringing the third-party patent. This process is called "inventing around" and requires identifying alternative ways to functionally achieve the objectives of the desired product. However, this may not always be the best method. In situations where there are no viable ways to invent around, the patent owner may instead choose to obtain a license from the third-party patent owner. Such a license would allow the owner of the infringing product to continue making, using, and selling the product so long as the owner pays a licensing fee to the third-party patent owner.

24.5 Overview of Drug Development

The process for obtaining regulatory approval in the United States is governed by the US Federal Food, Drug, and Cosmetic Act (FDCA), a set of laws passed

20 *Warner Jenkinson Co. v. Hilton Davis Chemical Co.*, 520 U.S. 17 (1997).
21 Graver Tank & Mfg. Co. v. LindeAir Prods. Co., 339 U.S. 605, 608(1950).

by the Congress in 1938 that gives authority to the US FDA to oversee the safety of food, drugs, and cosmetics. The requirements under the FDCA became more extensive when an amendment to the Act, known as the "Drug Efficacy Amendment," was introduced in 1962 and required drug manufacturers to demonstrate efficacy in addition to safety of their drug before approval. The Amendment also required drug advertising to disclose accurate information about adverse reactions and efficacy of the drug. By requiring more extensive testing to obtain regulatory approval, the "Drug Efficacy Amendment" sought to eliminate or at least reduce the likelihood that tragedy originating from drug's usage would ensue.

The regulatory process involving new drugs is generally divided into four phases: discovery phase, preclinical phase, clinical phase, and new drug application (NDA) phase. The discovery phase begins when scientists start to look for a lead compound that could eventually become a drug candidate. This usually involves either the creation of a new molecule or the selection of an existing molecule and optimizing its structure. The alteration of a molecule may result in a molecule with different properties, which can affect its efficacy and safety. Thousands of different variations of a molecule may be tested, but only a handful may have promising characteristics. These molecules are generally the subject of a patent application.

Once the drug candidates are found, they must undergo extensive preclinical studies before they can begin clinical trials. The preclinical phase involves basic research experimentation, involving animal and human models, to obtain preliminary efficacy, toxicity, and pharmacokinetic information. Such studies are used to assist companies in deciding whether a drug candidate has scientific merit for further development as an investigational new drug (IND).

After the most promising drug candidates are selected from the preclinical phase, they may begin the clinical phase of development. Before any clinical trials can begin, the company conducting the clinical trial must file an IND application with the FDA. The IND application must include the results of preclinical studies, the drug candidate's chemical structure, the drug candidate's mechanism of action in the body, and a listing of any side effects and manufacturing information associated with the drug candidate. The application must also provide a detailed clinical trial plan outlining how, when, and where the clinical trials will take place. When the FDA decides that participants in the clinical trials will not be subject to unreasonable risks, the FDA may approve the drug candidate to begin the trials.

For a given program, only 1–5 molecules show promise to continue with clinical trials of the thousands of molecules identified in the discovery phase. This process can take between three and six years to complete, and its duration has profound implications for patent strategy considerations, as discussed in more details in Chapter 25. In particular, since the regulatory process takes several years to complete, it can significantly decrease the amount of patent term remaining on a drug. Accordingly, companies seek mechanisms to recoup some of that time lost as a result of clinical trials. Below are two mechanisms available to companies to extend their exclusivity on the market.

24.6 Extending the Life of a Product

Companies can extend their exclusivity in the market through the use of patent or non-patent exclusivities. Patent exclusivities can be granted by the USPTO at any time during the life of a drug and are dependent on the patent term. Non-patent exclusivities, on the other hand, are granted by the government once the drug product has been approved and are irrespective of the patent term.

24.6.1 Hatch–Waxman Act

The Hatch–Waxman Act, also known as the Drug Price Competition and Patent Term Restoration Act, was enacted in 1984 to establish an approval pathway for generic drugs. The Act was created to establish two objectives: (i) to make sure the public would have access to lower cost generic drugs by alleviating some hurdles faced by generic manufacturers in reaching the market and (ii) to make sure that brand-name manufacturers were properly incentivized to continue to develop new drugs.

The Hatch–Waxman Act was a response to struggle by manufacturers of both brand-name and generic drugs to develop their respective products. While manufacturers of brand-name drugs were struggling to recoup the costs of drug development in a shortened exclusivity period on the market, manufacturers of generic drugs were struggling to even reach the market. This problem was created from three issues: (i) a limited period of market exclusivity for brand-name manufacturers following FDA approval of their product, (ii) an inability to initiate generic drug development until expiration of the relevant brand-name patents, and (iii) a long and costly process to obtain generic drug approval.

Prior to the enactment of the Hatch–Waxman Act, manufacturers of brand-name drugs struggled with how the FDA review time eroded away their patent-protected exclusivity period. With little time remaining in their patent-protected period, manufacturers were subject to generic competition after limited exclusivity on the market. Brand-name drug manufacturers argued that such limited market exclusivity would hinder a company's ability to recover drug development costs and would, in turn, discourage innovation. Manufacturers of generic drugs, on the other hand, struggled with reaching the market. Generic manufacturers faced challenges that delay generic market entry beyond the expiration of the patents that protected the brand-name drugs. This delay occurred because the generic manufacturers were unable to conduct clinical trials using patented drugs without risking liability for patent infringement. Generic manufacturers could not develop and test their drugs before the patent term of the brand-name drug expired; therefore, market entry was delayed due to the lengthy process of development and clinical trials. As a result, few generic drugs were available on the market. Prior to the Hatch–Waxman Act, 19% of all prescriptions were for generic drugs, and over 150 brand-name products lacked generic counterparts, despite the lack of patent protection.

Manufacturers of generic drugs also struggled to reach the market because there was no abbreviated pathway for seeking generic approval. Generic manufacturers, instead, had to prepare and submit their own NDA and were subject

to premarket approval requirements before approval for market sale. As generic manufacturers were subject to the same costly and time-consuming approval process as the brand-name manufacturers, generic manufacturers were incurring significant expense and delays in reaching the market, allowing brand-name drugs to remain unchallenged and delayed access to lower cost drugs.

To encourage innovation of both brand-name and generic drugs, the Hatch–Waxman Act was enacted by Congress in order to strike a balance between the promotion of innovation and the improvement of access to affordable generic medicines. Several provisions are included in the Hatch–Waxman Act in order to alleviate the concerns of both brand-name and generic manufacturers. The following sections of this chapter address the various changes made by the Hatch–Waxman Act and their effect on drug exclusivity in the market place.

24.6.2 Patent Exclusivities

As mentioned previously, one common method of extending the exclusivity period of a product on the market is through the use of patent exclusivities. The initial 20-year period granted for a patent is the most basic form of patent exclusivity. Additional patent exclusivities allow the USPTO to extend the 20-year term to compensate for time lost during the patent and regulatory processes. Two different types of extensions of patent term are available: extensions for delays due to USPTO approval and extensions for delays due to regulatory approval.

24.6.2.1 Delays Due to USPTO Approval

Any patent filed within the USPTO is likely to face delays by the examiner. While this may not necessarily be the fault of the examiner, it may be due to the overwhelming amount of application files and the inability to hire competent examiners to compensate for this workload. In accordance with the Hatch–Waxman Act, the USPTO allows for one-day extensions of patent term for each day the USPTO fails to meet certain deadlines. Delays that result from actions taken by the patent holder, such as failing to respond to an office action within the required time or time taken to prolong the appeal process, are not adjusted for under this mechanism. Since patent holders do not receive exclusive rights during the time the application is being prosecuted, the main purpose is to guarantee the patent holder a term of at least 17 years.

As enumerated in 35 USC §154,[22] the USPTO follows a 14–4–4-4 rule in determining the length of time at which an examiner is expected to respond to an applicant. The examiner has 14 months from the date the applicant files the application to issue the first office action, four months from the response of the first office action to respond, four months from the applicant's notice of appeal to respond, and four months from the payment of the issue fee for the patent to actually issue. Any time taken by the examiner is included in this day-for-day extension of patent term. These delays are known as "A delays." The USPTO also

22 35 USC §154.

provides for a one-day extension for each day that the patent application is pending beyond three years from the filing date, known as "B delays." To the extent that the "A delay" and the "B delay" periods overlap, §154 provides that "the period of any adjustment granted under this subsection shall not exceed the actual number of days the issuance of the patent was delayed."

24.6.2.2 Delays Due to Regulatory Approval

Another mechanism to extend the patent term is to adjust for delays occurring during the regulatory process. The Hatch–Waxman Act provides for patent term extensions under 35 USC §156[23] for patents claiming "a product, a method of using a product, or a method of manufacturing a product" subject to regulatory delays caused by the FDA premarket approval process. Examples of products that qualify for this patent term extension include human drugs, antibiotics, biologics, animal drugs and veterinary biologics, medical devices, food additives, and color additives.

Under §156, a drug developer can recapture patent term for all delays resulting from regulatory approval and half the time lost due to preclinical studies. The extension cannot exceed five years, and the total extension may not extend the remaining patent life beyond 14 years from the date of FDA approval. One patent may be extended per product, and the patent that is extended must be valid and not expired.

24.6.3 Non-patent Exclusivities

The exclusivity of a product can also be extended through non-patent exclusivities. This type of exclusivity gives qualified FDA-approved drugs additional, competition-free time by preventing generic competitors from entering the market so long as the exclusivity period is valid.[24] However, these types of exclusivities do not prevent the FDA from approving a generic drug if the generic does not infringe on the protected change.

24.6.3.1 New Chemical Entity

New chemical entity exclusivities are offered for new chemical entities on the market where the drug does not contain an "active moiety" that has been approved by the FDA.[25] An "active moiety" is defined as a "molecule or ion, excluding those appended portions of the molecule that cause the drug to be an ester, salt including a salt with hydrogen or coordination bonds, or other non-covalent derivative, such as a complex, chelate, or clathrate, of the molecule, responsible for the physiological or pharmacological action of the drug substance.[26]"

This type of exclusivity offers a company five years of market exclusivity, which prohibits the FDA from reviewing any abbreviated new drug application

23 35 USC §156.

24 https://www.fda.gov/Drugs/DevelopmentApprovalProcess/SmallBusinessAssistance/ucm069962.htm.

25 21 C.F.R. 314.108(b).

26 21 C.F.R. 314.108(b).

(ANDA) for a generic product until the five-year period expires. The five-year period begins once the drug is first approved by the FDA. Since drug approval is not based on patent term, NCA exclusivity may or may not run concurrently with patent term. Therefore, regardless of when the patent term expires, the main goal of the NCE exclusivity is to provide the company developing the drug with a minimum of five years of market exclusivity.

Although the NCE exclusivity provides for five years of market exclusivity, the actual market exclusivity provided may be greater even in the absence of patents. Under the NCE, not only is the FDA prohibited from reviewing an ANDA during the five-year period, but applicants are also prohibited from submitting an ANDA during this period. Once an ANDA is submitted, it takes approximately 19.2 months for the FDA to approve the generic drug for commercial marketing.[27] Therefore, the actual period of market exclusivity is approximately six and a half years.

24.6.3.2 New Clinical Study Exclusivity

While NCE exclusivity is available to only entirely new drugs, another exclusivity, known as the new clinical study exclusivity or supplemental exclusivity, is provided under the Hatch–Waxman Act for previously approved drugs. This exclusivity allows companies that sponsor additional clinical trials on a previously approved drug that leads to changes in the marketed product to receive up to three years of exclusivity. Examples of changes that may qualify for this exclusivity include changes to dosage strength, formulations, route of administration, indications, or patient population.

To obtain the NCS exclusivity, companies are required to submit a supplemental application to a previously filed NDA that is directed to the change. This form of exclusivity only applies to the specific change that is provided in the supplemental application. It does not prevent a competitor from using an ANDA to sell the product as it was previously approved. While the FDA may not approve an ANDA for the same change during the three-year period, it may receive and grant tentative approval that becomes effective once the three-year exclusivity period ends.

In contrast with the NCE exclusivity, the NCS exclusivity begins once the supplemental application for the new change is approved, while the NCE exclusivity begins once the drug is first approved. Thus, companies typically apply for the NCS exclusivity as their product approaches the end of its patent term or other exclusivity period. This strategy is particularly useful when a drug changes in route of administration from being available only by prescription to being available over the counter. If the brand-name drug becomes available over the counter while the generic drugs are only available by prescription, consumers are more likely to buy over-the-counter brand-name drugs rather than obtaining a prescription from physicians.

One example of this exclusivity is seen with the drug colchicine. Colchicine[28] is a treatment available for gout, a medical condition usually characterized by

27 Food & Drug Administration 2007.

28 Yael Waknine, "FDA Approves Colchicine with Drug Interaction and Dose Warnings." July 2009.

recurrent attacks of acute inflammatory arthritis. In the United States, colchicine has been available as a generic prescription in tablet form since the nineteenth century but was never officially approved by the FDA because existing drugs on the market were not subject to the 1938 FDCA, which required safety review and approval of only new drugs. In 2007, URL Pharma conducted a randomized control trial testing its new dosage regime with colchicine in patients with gout.[29] The trial showed that a reduced dosage regimen was effective in yielding good symptom management and had fewer side effects. The FDA approved the new version of colchicine, known as Colcrys, in 2009 and gave URL Pharma a three-year market exclusivity period. As a result, URL Pharma raised the price of the drug from $0.09 to $4.85 per pill.[30] Older and unapproved versions of colchicine were subsequently removed from the market in October 2010.[31] This example demonstrates how a company can obtain an additional three-year period of exclusivity on the market by simply changing one characteristic of the drug, such as its dosage requirements.

24.6.3.3 Generic Drugs

For generic manufacturers, the Hatch–Waxman Act provides incentives to ensure that generic counterparts to brand-name drugs are available to consumers immediately upon patent expiration. This is accomplished by providing generic drug manufacturers with a safe harbor to use patented drugs for testing purposes and by providing an abbreviated approval pathway for generic drugs to obtain FDA approval.

The safe harbor provision stems from the general rule that a third party may be liable of patent infringement under 35 USC §271(a)[32] for making, using, or selling patent-prosecuted technology prior to the expiration of the patent. In *Roche v. Bolar*,[33] the Federal Circuit reaffirmed the safe harbor provision by holding that a generic drug manufacturer is liable for patent infringement for using a patented drug substance to support an NDA prior to the expiration of the patent. In *Roche v. Bolar*, a brand-name drug manufacturer sued a generic drug manufacturer for using a patented drug substance to support an application with the FDA for six months prior to the expiration of the patent. In response, the generic manufacturer argued that its use of the patented drug substance fell within the experimental use exception and was protected from infringement. The Federal Circuit dismissed this argument and found the generic manufacturer liable for patent infringement. Due to the risk of facing infringement charges, generic manufacturers were unable to initiate the testing of their products until all the relevant patent expired.

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29 FDA Orange Book; search for colchicine.

30 Kurt R. Karst (2009–2010-2021). "California Court Denies Preliminary Injunction in Lanham Act Case Concerning Unapproved Colchicine Drugs"; Harris Meyer (2009–2012–2029). "The High Price of FDA Approval". Kaiser Health News and the Philadelphia Inquirer; Colcrys vs Unapproved Colchicine Statement from URL Pharma.

31 Questions and Answers for Patients and Healthcare Providers Regarding Single-ingredient Oral Colchicine Products, FDA.gov.

32 35 U.S.C. §271(a).

33 *Roche Prods., Inc. v. Bolar Pharm. Co*., Inc., 733 F.2d 858, 863 (Fed. Cir. 1984).

In order to prevent infringement suits against generic drug manufacturers, Congress instated an exemption to the rule for generic drugs. This new exemption, codified in 35 USC §271(e)(1), is designed to ensure that generic drugs are available to consumers immediately upon patent expiration rather than only allowing generic drugs to undergo testing upon patent expiration. Section 271(e)(1) states:

> "It shall not be an act of infringement to make, use, offer to sell, or sell within the United States or import into the United States a patented invention…solely for uses reasonably related to the development and submission of information under a federal law which regulates the manufacture, use, or sale of drugs or veterinary biological products."

Another exclusivity provided for generic drugs is available under the Hatch–Waxman Act. The Hatch–Waxman Act encourages innovation of generic drugs by eliminating the need for generic manufacturers to conduct separate clinical trials. Generic drug manufacturers are only required to conduct studies showing that their drug is bioequivalent to the brand-name drug on the market. Upon showing this equivalency, generic drugs are able to submit an ANDA. The ANDA contains data that the generic drug is bioequivalent to the brand-name drug. Moreover, the Hatch–Waxman Act allows generic manufacturers to begin their studies and submit for FDA approval prior to the expiration of the patents without risk of infringement. This allows generic drugs to enter the market as soon as possible following the expiration of any relevant patent or exclusivity period.

The Hatch–Waxman Act also rewards generic manufacturers who challenge patents covering brand-name drugs with 180 days of market exclusivity. This challenge, known as a Paragraph IV challenge, arises when the manufacturer of the generic drug asserts that the generic drug does not infringe the brand-name drug's patents or that the brand-name drug's patents are invalid. A brand-name company can contest this challenge, which typically results in litigation to evaluate the generic manufacturer's claims and determine whether the market exclusivity period could continue. If successful, the generic manufacturer would be rewarded with 180 days of market exclusivity that it could share with the brand-name drug this period and the generic manufacturer could establish a dominant presence in the generic market.

The Hatch–Waxman Act has largely been considered to be a success with regard to stimulating innovation among generic drug manufactures. In general, generic manufacturers have faced fewer hurdles to reaching the market. The consequences of the Hatch–Waxman Act in terms of generic competition and other generic-based implications for originators are detailed in Chapter 26.

24.6.3.4 Orphan Drug Exclusivity

The orphan drug exclusivity is part of the Orphan Drug Act of 1982, which rewards companies for developing products to treat rare diseases or conditions with seven years of exclusivity, as well as providing them with tax credits and research grants for each orphan drug developed.[34] Orphan drug exclusivity can

34 21 C.F.R. 316.31.

be traced back to the Kefauver–Harris Amendments, which not only improved drug safety but also, consequently, increased the costs associated with bringing new drugs to the market. In response, companies focused on developing treatments that promised to bring greater profits. Orphan diseases, which affect fewer than 200 000 people in the United States, were largely ignored due to poor profit outlook.

To encourage the development of treatments to target these rare diseases, Congress created incentives for those developing drugs that target such disorders. There are three primary incentives: (i) federal funding of grants and contracts to perform clinical trials of orphan products, (ii) a tax credit of 50% of clinical testing costs, and (iii) an exclusive right to market the orphan drug for its approved use of seven years from the date of approval. This exclusivity period does not prevent the FDA from approving either another drug for the same disease or condition or the same drug for another disease or condition.[35] This exclusivity confers upon the applicant a narrow form of exclusivity that is limited to a specific drug and specific disease.

The Orphan Drug Act has been successful overall in encouraging the development of products aimed at treating medical conditions in limited populations. Despite this success, critics have questioned whether the Orphan Drug Act is necessary to encourage companies to develop orphan drugs, since orphan drugs can be quite profitable regardless. The profitability arises from the fact that, even though the orphan drug may target a relatively small number of patients, they are often sold at extremely high prices. In one study conducted in 1995 by Pharmacoeconomics [1], it was determined that each of the 11 top-selling orphan drugs earned more than $200 million within five years of being marketed. Another study, conducted by the Journal of Rare Diseases in 2008 [2], found that orphan drugs faced less generic competition overall than non-orphan drugs. These results may suggest that the potential profitability of orphan drugs encourages companies to pursue developing at least some orphan drugs without relying on government incentives.

24.6.3.5 Pediatric

Pediatric exclusivity is included as part of the FDA Modernization Act of 1997. It encourages clinical testing of drugs in the pediatric population. Before 1997, few drugs were developed or studied specifically for the pediatric population. This is a result, in part, of children being a smaller market. As a result, children were receiving treatments that had not been previously tested in the pediatric population. Some drugs that children were receiving were often ineffective and potentially dangerous, as children have important physiological differences from adults.

To encourage drug development and research for children, the FDA Modernization Act created a six-month period of exclusivity for applicants who complete pediatric studies in response to a "written request" from the FDA to evaluate the effectiveness and safety of a drug in children. This exclusivity

35 Genentech, Inc. v. Bowen, 676 F. Supp. 301 (D.D.C. 1987); Sigma-Tau Pharms. v. Schwetz, 288 F.3d 141 (4th Cir. 2002).

period is not contingent upon approval of the drug in children and does not require that the underlying pediatric study be successful. Rather, the sponsor only needs to show the FDA that the pediatric study was conducted. Further, this type of exclusivity does not attach to any specific drug but rather to all of the applicant's dosages, formulations, and indications for drugs with existing marketing exclusivity or patent life that contains the same active ingredient.[36] As a result, a sponsor who qualifies for pediatric exclusivity could potentially also have its patent, NCE exclusivity, clinical investigation exclusivity, or orphan drug exclusivity extended by six months.

Following the enactment of the pediatric exclusivity, drug companies began conducting trials of their drugs in pediatric patients. In 2007, more than 300 pediatric studies were conducted that addressed safety, efficacy, and pharmacokinetics [3]; during the same time, labeling changes for pediatric use were approved by the FDA on more than 115 products [4].

Although the exclusivity incentive acted to increase testing of drugs in children, some critics have questioned the health benefits received by children. In particular, some studies have questioned the overall quality of pediatric studies since they do not need to be subject to peer review [5]. Another study found that drugs most frequently represented in pediatric exclusivity studies were drugs that were both popular and profitable among adults but drugs that were frequently used by children were underrepresented [6]. For example, Pfizer applied for and received a pediatric extension on the active ingredient in Viagra®, which is sildenafil. Not only is sildenafil used to treat erectile dysfunction, but it is also used to treat pulmonary arterial hypertension (PAH), a disorder that affects only about 500–600 children in the United States. For treating PAH, sildenafil is marketed under the name Revatio®. By applying for and receiving pediatric exclusivity for sildenafil, the sildenafil patent was extended from March 2012 to September 2012, allowing Pfizer to receive over $1 billion in extra revenue due to the exclusivity. Moreover, as shown by the Pfizer example, the six-month pediatric exclusivity is very lucrative for drug companies. While the median cost of conducting a trial in the pediatric population is about $12 million, the median net economic benefit to the manufacturer is around $134 million, a ratio of just over 10 to 1 [7]. Overall, these findings suggest that companies may be pursuing pediatric exclusivity as a way to receive the benefits of the incentive rather than to conduct meaningful pediatric trials.

24.7 Summary

This chapter provided a basic overview of intellectual property, particularly focusing on patents, some of the fundamental principles behind them, and how the law surrounding patents has been shaped over the years by changes. Understanding the basic principles of patent law is necessary for understanding some of the more complex topics surrounding drug development.

This chapter also examined some non-patent exclusivities and their impact on drug development. The market exclusivity provisions described above have had

36 https://www.fda.gov/cder/Pediatric/faqs.htm.

an important impact on drug development in the United States. Exclusivities provided by the Hatch–Waxman Act, for example, have substantially increased the presence of generic drugs on the market, which, in turn, has reduced the cost of drugs. Other exclusivities, including orphan drug exclusivities and pediatric exclusivities, likewise, have stimulated development of drugs in areas and patient populations that are underrepresented. While the exclusivities have been successful in stimulating drug development, there are also questions about whether they encourage the development of truly novel drugs or merely reward the use of existing drugs. To address the medical needs of the public, patent and non-patent incentives need to work together to ensure that novel medicines continue to be developed.

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25

Patent Protection Strategy

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Development of a drug product is a costly and time-intensive process, taking years between the identification of a useful substance and commercialization of an approved drug product. This is in part a result of the rigorous regulatory approvals required in various countries, entailing numerous lab, animal, and clinical studies. Ensuring that a sufficient market will exist for the product and determining how best to market the drug to both consumers and the medical industry also entail significant costs. The research, development, and marketing costs related to new drug development are essentially impossible to justify if a competitor is free to simply copy the drug with only a minimal investment. Therefore, it is essential to obtain protection for the drug product and delay the entry of others into the marketplace for as long as possible.

This chapter will focus generally on intellectual property issues to consider when developing new drugs and will discuss high-level strategies to increase the value of patents as a business asset. It is not intended as a detailed guide for prosecution or enforcement of patents in any specific country; a lawyer with expertise in specific jurisdictions should be consulted for the preparation and filing of any patent applications.

25.1 Benefits of Patent Protection

Patents are legal instruments that protect inventions, conferring to the patent owner exclusive rights to practice the invention for a specified period of time (usually 20 years from the filing date of the initial patent application, which may be extended to offset regulatory issues and government delay in issuing the patent, as outlined in Chapter 24). In return, the inventor publicly discloses the invention and how to practice it. This trade-off is intended to promote ingenuity and reward new technological developments while simultaneously increasing the public's collective knowledge and enabling others to further improve upon the new technology. Patents may cover a wide variety of subject matter, including new and useful products, compositions, devices, treatments, systems, methods of making products, and methods of using products. While in some cases it

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

may be preferable to maintain an invention as a trade secret that avoids public disclosure of the invention and can protect information indefinitely as long as adequate steps are taken to maintain confidentiality, regulatory approval processes for new drugs and the potential for others to reverse engineer drug compounds and formulations generally favor patent protection.

A patent essentially confers a legally sanctioned monopoly onto its owner within the geographic area under control of the government that issues the patent. In theory, the barrier to competition provided by a patent allows the patent owner to recoup its investment in development of the invention and obtain a reasonable profit. A patent may be used offensively to block attempts by competitors to make, use, or sell the invention or to gain financial compensation for infringement. Patents can also have defensive benefits, such as preventing others from later obtaining their own patents covering the same invention or providing a threat of countersuit if threatened with another party's patent.

It is important to keep in mind, however, that securing a patent does not necessarily entitle the patent owner to practice the patent. Broad early patents in a particular field may bar exploitation of a later, more specific innovation, and subsequent patents may bar the practice of certain improvements that may have been described only at a high level in earlier patents. Therefore, it is worthwhile to investigate the risk associated with any new technology, even when it is patented.

25.2 Requirements for Patentability

25.2.1 Subject Matter Eligible for Patent Protection

The requirements for patents vary somewhat from country to country, but at their core patents are intended to protect inventions derived from human ingenuity. Patents are not intended to give an individual exclusive rights to take advantage of a discovery, law of nature, or natural phenomenon.

For instance, in the United States patent-eligible subject matter is identified by statute as "any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof."[1] Even if subject matter falls within one of these four general categories, however, it will not be entitled to patent protection if it falls within one of several recognized "judicial exceptions" to the statutory subject matter: (i) laws of nature (identified physical phenomena and mathematical relationships describing the same, such as the gravitational constant or Einstein's theory of relativity), (ii) natural phenomena (such as minerals, plants, or chemicals found in nature), and (iii) abstract ideas (such as mathematical algorithms or mental processes).[2] These are considered to be building blocks of innovation that all are free to benefit from, while patents are intended to protect inventions that utilize or apply them.

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1 35 U.S.C. § 101.

2 *See Alice Corp. Pty. Ltd. v. CLS Bank Int'l*, 134 S. Ct. 2347, 2354, 110 USPQ2d 1976, 1980 (2014); *Association for Molecular Pathology v. Myriad Genetics, Inc.*, 133 S. Ct. 2107, 2116, 106 USPQ2d 1972, 1979 (2013); *Bilski v. Kappos*, 561 U.S.593, 601, 130 S. Ct. 3218, 3225, 95 USPQ2d 1001, 1005-06 (2010); *Diamond v. Chakrabarty*, 447 U.S. 303, 309, 100 S. Ct. 2204, 2208, 206 USPQ 193, 197 (1980).

Recently, US courts focusing on patentable subject matter particularly relevant to the pharmaceutical industry have analyzed whether a discovery or product of nature has been sufficiently altered or transformed to warrant patent protection. For instance, the US Supreme Court has held that genetic sequences occurring in nature are not patentable (even if isolated from surrounding genetic material). Nevertheless, cDNA based on those sequences, new uses or applications of those sequences, and alterations of those sequences may be eligible for patent protection depending on specific circumstances because they do not exist in nature and are the result of human manipulation.[3] The Supreme Court has also held that recognition of a correlation between levels of certain metabolites and efficacy of a drug is an unpatentable "natural law"[4] that prevents researchers from patenting diagnostic methods that merely utilize well-known techniques or technology to detect or characterize metabolite levels or another naturally occurring situation. Thus, if the crucial aspect of a product or method is the discovery of a natural phenomenon, it currently will generally not be considered patentable by US courts unless more than conventional techniques are applied to that phenomenon.[5] As a result, it can be particularly difficult to patent diagnostic methods that use new versions of known technology adapted to identify a newly discovered relationship or phenomenon. These court decisions have been highly controversial, and the US Court of Appeals for the Federal Circuit (the appeals court below the Supreme Court that hears all direct patent appeals from district courts) has noted that the broad language of these Supreme Court decisions could have unintended consequences and result in the denial or invalidity of patents for some meritorious inventions.[6] As time goes on, courts will continue to define the limits of patentable subject matter in the fields of pharmaceuticals and biotechnology, and those planning patent portfolios must adapt accordingly.

25.2.2 Further Requirements for Patentability: Defining the Invention

Even if the subject matter of a patent application meets the general eligibility requirements outlined above, it must also meet additional requirements set forth in the applicable patent statute. While the specific requirements vary from country to country, these requirements generally include novelty[7] (i.e. the invention is not patented or otherwise known or available to the public), nonobviousness[8] or inventive step[9] (i.e. persons skilled in the particular art would not have easily arrived at the invention through a combination of publicly available information and routine skill), adequate written description[10] (i.e. the invention is fully, clearly, and concisely described in the patent application), definiteness or clarity of claim scope (i.e. those skilled in the art can understand what the patent covers),

3 *Association for Molecular Pathology v. Myriad Genetics, Inc.*, 133 S.Ct. 2107 (2013).
4 *Mayo Collaborative Services v. Prometheus Laboratories, Inc.*, 132 S. Ct. 1289 (2012).
5 *See Ariosa Diagnostics, Inc. v. Sequenom, Inc.*, 788 F.3d 1371 (Fed. Cir. 2015).
6 *See Sequenom*, 788 F.3d at 1380.
7 In the United States, *see* 35 U.S.C. § 102; For the European Patent Office, see EPC Art. 54 and 55.
8 In the United States, *see* 35 U.S.C. § 103.
9 For the European Patent Office, *see* EPC Art. 56.
10 In the United States, *see* 35 U.S.C. § 112.

and enablement[11] (i.e. the patent application provides sufficient information for a person having ordinary skill in the art to make or use the invention).

The requirements of novelty and nonobviousness/inventive step vary from country to country and are judged in view of "prior art"– information known or available to the relevant public prior to the filing date of the patent application. As an example, many countries around the world require absolute novelty, so that any publication of an invention destroys the right to patent the invention from a subsequently filed application. The European Patent Office (EPO), for instance, considers all information made available to the public anywhere in the world in any way (including written or oral description or actual use) before the filing date of a patent application, and it will cite such a disclosure against the application unless it falls within a very narrow exception.[12] This is true whether or not the disclosure was made by the applicant or others.[13] In the United States, by contrast, a patent will be denied if the claimed invention was patented, described in a printed publication, in public use, on sale, or otherwise available to the public before the filing date, but specific exceptions may apply if the disclosure was made by the inventor named on the application (or another who obtained the subject matter from the inventor) one year or less prior to the filing date.[14] Thus, the United States is much more forgiving than the EPO is regarding a patent applicant's own disclosure. Yet both rules are stricter than the first-to-invent patent system in place in the United States prior to 2013. Under that previous first-to-invent system, the first person to conceive of an invention was generally entitled to a patent as long as a patent application was filed within one year of the first public disclosure by the inventor or another.

The factors for determining nonobviousness or inventive step are even more varied than the novelty requirements. Generally, however, the analysis determines whether publicly available information would have made the invention obvious to a person having ordinary skill in the field to which the invention pertains. Courts decide what factors are taken into account when determining if the alleged invention requires a "nonobvious" solution or "inventive" step above the routine techniques that those of ordinary skill in the field would ordinarily employ. A patent applicant need not, and in most cases cannot, account for all of the differences in patentability rules for all countries in which it may be interested in filing, so should focus on defining the invention in a way that appears inventive regardless of the analysis employed. In addition, because any prior disclosure that is close to describing the invention may have an impact on patentability, an applicant should file an application as soon as practicable in order to minimize the chance that prior art will prevent the applicant from obtaining a patent.

The exact nature of the requirements for the written disclosure in a patent application likewise varies from country to country. Nevertheless, it can generally be said that a patent application must clearly describe the invention and

11 In the United States, *see* 35 U.S.C. § 112; for the European Patent Office see EPC Art. 83.
12 EPC Art. 54, 55.
13 EPC Art. 55.
14 35 U.S.C. § 102.

allow those of ordinary skill in the relevant field to practice the invention. The patent disclosure must also end in a series of claims that clearly define the scope of the invention to be protected. It is these claims that will be scrutinized when a patent examiner applies the prior art to discern whether the invention is novel and nonobvious.

25.3 The Significance of a Patent Portfolio

Multiple patent applications may be filed in order to protect several different aspects of the same general invention or multiple related inventions. Groups of related patents or commonly owned patents are often referred to as a "portfolio," and a strong patent portfolio can be of great importance to any pharmaceutical manufacturer by protecting investments in research and development, increasing brand awareness by providing exclusive early commercialization, and boosting profits by preventing others from entering the market until expiration of the patent rights. Patents can cover a wide variety of medicinal features, including the drug compound itself, compositions containing the drug, and methods related to manufacture or use of the drug. Not only does this protect the invention from multiple angles and increase the chances of at least some patent claims being infringed, it also gives the patent owner the option of asserting certain patents or claims without putting the entire portfolio at risk.

Patents can also be used as an asset to secure funding for further drug development, to encourage investment in the owner's company, to generate license or sale revenue, or as collateral for obtaining financing. Patents also may provide leverage in negotiating rights to the intellectual property of others, such as through cross-licensing arrangements.

Nevertheless, patent rights can often be expensive to obtain, especially if one does not limit protection to a small number of key geographic regions. For this reason, determining how and where to protect your inventions from the outset is highly important.

25.3.1 Protection of Commercial Products and Exclusive Rights

During the process of gathering and testing compounds that may have useful pharmaceutical properties, researchers will discover structural aspects, physical forms, formulations, assay techniques, chemical intermediates, methods of synthesizing, and/or methods of use that carry identifiable benefits. These aspects can be used to generate intellectual property covering multiple individual aspects of an invention. The more ways in which a drug, its manufacture, and its use are protected, the less likely it is that a competitor will be able to design around the patent portfolio.

During early stages of drug development, it is often not known what forms of the drug are most likely to be commercialized. In addition, it may be unclear whether the most valuable aspect of a new drug is its chemical structure, the methods in which it is administered, or the way in which it is created. As a result, it is usually best to describe the drug in as many different ways as possible early in

development in order to preserve the right to claim that aspect of the drug later in the patent process.

The company and the inventors developing a drug product should establish as early as possible who will have ownership of intellectual property. In most cases, companies should make sure from the outset that all employee contracts have a clause obligating the employee to assign all patent rights to the company, even where local law addresses ownership of such rights. In the case of independent contractors who may assist in the identification and development of drug products, ground rules regarding ownership also should be established before any possibly inventive work is underway. Once a potentially valuable new drug is identified or created, it may be much more difficult to negotiate with the inventors to obtain an assignment of patent rights.

Another important consideration in planning protection for a future commercial product is the geographic scope of protection. The value of a patent in a specific country depends at least in part on what activities take place in that country and the enforceability of patent rights in that jurisdiction. For instance, if a patent will only cover a method of treatment, it may have no real value in countries where the drug is made but rarely used. The patent may be even less valuable if a competitor can simply shift manufacture of the drug to a different country, particularly if the other country does not generally permit the recovery of significant economic damages and limits remedies to injunctive relief. Identifying key markets where the invention will be used and where patents may be effectively enforced is important because it is not feasible to file patents in every country around the world; the cost of doing so is prohibitive.

Another factor to consider in building a strong patent portfolio is the timing of patent applications. The prior public disclosure of an invention by another can be fatal to a patent, and even an applicant's own disclosure may create an immediate impact on the ability to obtain a patent in many countries. As a result, it is ordinarily best to file an application as soon as reasonably possible in order to minimize the possibility of the invention's disclosure prior to the filing of the application. As the invention is improved or refined, it may be possible to cover later versions with additional patent applications.

25.3.2 Monetization of Patents

There are a number of ways in which patents may create value for a company. For example, the patent owner may license patents to others, extending specified rights under the patent in exchange for royalty payments or other valuable consideration. In many cases, however, preventing others from using the technology is more valuable than the potential for license revenue. In other cases competitors may be unwilling to voluntarily license patents, and the filing of an infringement lawsuit is necessary in order to seek monetary damages for infringement and/or an injunction against the competitor's further use of the technology. Lawsuits can provide excellent leverage when licensing patents, but it must be kept in mind that it may not be easy for the patent owner to voluntarily withdraw from a lawsuit with the patent intact should the competitor refuse to concede to demands.

Due to tight governmental regulation of drug formulations, patents in the pharmaceutical industry often provide more protection against competitors than in other fields. In the US pharmaceutical industry, for instance, the Drug Price Competition and Patent Term Restoration Act, commonly referred to as the Hatch-Waxman Act, provides additional periods of exclusivity for certain drugs, extended patent term in certain cases to offset time spent in clinical trials, the ability of patent owners to sue generic drug manufacturers based on an application for regulatory approval, and a 30-month stay of regulatory approval for any generic drugs subject to patent litigation. These provisions make it much easier to exclude or delay competitors from reaching the market than in other industries, and the value of exclusivity in the market is ordinarily significantly higher than the value of a royalty stream. As a result, the pharmaceutical industry has historically lagged behind other technology sectors in patent monetization. However, for companies that do not ultimately end up marketing drugs covered by their patents or do not have the capability to service all available markets, licensing can lead to significant added revenue. In some cases, it may even make sense to liquidate a particular portfolio of patents by selling them to another party interested in enforcing the patent or further developing, manufacturing, or marketing patented products or methods.

Patents can also play a significant role in attracting investors. A patent, and the consequent right to exclude others from practicing the invention covered by the patent, is a valuable asset and can be extremely important in securing investment capital. In some cases, protection of key intellectual property will be seen as greatly increasing the value of the company due to a substantial increase in projected profit. In other cases, the total number of patents or patent families may be used as a metric by potential investors with little regard to the scope of protection afforded by the patents.

25.4 Planning a Patent Portfolio

Care should be taken to establish a patent strategy from the beginning of research and development. Patent applications should be filed before any public disclosure of the invention to ensure that rights are not impaired. Thought should be given to the geographic scope of protection and to how aggressively the invention will be pursued through one or more patent applications. The cost of patent applications can vary widely depending on the detail in which the invention is described, the number of individual aspects of the invention covered by patent claims, and the nature of the technology. A patent attorney or agent should be consulted in order to develop a strategy best suited to a company's budget and goals.

Additional considerations relevant to the nature of specific drug inventions are discussed below.

25.4.1 First- Versus Second-Generation Drugs

When a new pharmacological substance is first developed or a pharmaceutical use of a molecule is identified, the first generation of patents will ordinarily

be directed to the molecule's properties, manufacture, or uses as broadly as possible. For instance, a first-generation patent will ordinarily focus on structural or chemical aspects of the novel molecule or a treatment of a condition involving the molecule. The patent owner hopes that this will preclude competitors from making any similar drugs during the lifetime of the original patents.

Once an active pharmaceutical ingredient (API) is known, a second generation of patents is ordinarily filed to cover certain formulations or more-specific methods of use or manufacture. Because these second-generation patents should be inventive over the first generation, they need not claim priority to the first generation (which would tie them to the same expiration date as the first-generation patents). Consequently, these second-generation and subsequent patents can help to extend the time that a commercial drug or family of drugs is protected. Second-generation patents often focus on specific improvements in forms, formulations, or methods incorporating the active substance described in the first-generation patents and are intended to cover products and methods with superior efficacy or stability, optimized dosages, more efficient treatment methods, combinations with other drugs, or other improvements. While these second-generation patents are narrower than the first, they can often be helpful in extending protection to the most sought-after and up-to-date commercial embodiments.

25.4.2 Active Pharmaceutical Ingredient

An API is a molecule, substance, or mixture of substances that becomes an active ingredient in a drug product. An API is an important focus of first-generation patents because it is generally impossible for a generic firm to benefit from regulatory approval of an API without using the API itself. It is extraordinarily expensive and time-consuming to screen for unknown substances with therapeutic benefits and conduct the necessary testing to establish that they are safe and effective for use in human beings. As a result, it is usually large, well-funded companies that hold patents broadly directed to a specific therapeutic molecule or class of molecules.

A patent applicant should contemplate potentially useful variations of the API to avoid unduly narrowing the scope of patent protection. For instance, if it is determined that the therapeutic properties of a substance are attributable to one or more functional groups, the applicant should attempt to patent a genus of compounds rather than the specific species under investigation in order to prevent competitors from easily designing around the patent. By claiming a broad class of substances instead of a specific molecule, substance, or mixture, the scope of protection afforded by a patent can be significantly broadened. On the other hand, broader patent claims are more susceptible to invalidity challenges and more likely to be found to be unsupported or not enabled by a relatively narrow disclosure that establishes the effectiveness of only a small subset of the claimed ingredient.

It is optimal to file several claims of varying scope in order to best protect against a wide variety of competitors and increase the likelihood that at least one claim will be held both valid and infringed. Narrower claims directed to

individual species of a therapeutic substance are generally more effective for deterring generic competitors because they can protect the substances that will be submitted for regulatory approval while being relatively likely to result in a patent and withstand validity challenges. Broader claims encompassing a genus of therapeutic substances are better suited for deterring competition from other companies developing innovative drugs in the same field because they offer an umbrella of protection more likely to encompass particular drugs with similar structure or mode of action. Diverse sets of claims may be filed across multiple patent applications to increase the chances of quickly obtaining an issued patent. Claims to specific species may even be the subject of separate patent applications claiming a later priority date than the patent covering the genus, but it should be expected that the later application will be rejected in view of the earlier-described genus, forcing the applicant to prove that the species would not have been obvious in view of the broader genus.[15]

It may also be beneficial to separately patent specific forms (e.g. salt or crystalline forms), enantiomers, and/or polymorphs, especially where specific advantages are attributable to those variations. Specific forms may even be presented in second-generation patents or later if the form or its benefits would not have been obvious in view of the originally disclosed form.

25.4.3 Formulations

Formulations containing an API may be patented even if the API is previously known or the subject of a different patent, as long as the formulation itself would not have been obvious to a person having ordinary skill in the art at the time of the invention of the formulation. Basic formulations are ordinarily also disclosed in first-generation patents. Packaging, administration form, and dosage may also be important factors in the effectiveness or need for a specific formulation, and those aspects can be incorporated into claims to a formulation. In addition to providing a basis for narrowly claiming specific embodiments, disclosing particular formulations in a patent application can have the added benefit of preventing others from later patenting those specific formulations.

If the formulation is arguably nothing more than a mixture of known ingredients added for their known properties to achieve a predictable result, an attempt to patent the formulation will usually fail. For instance, if a new patent application attempts to cover a preexisting API combined with a known excipient, known stabilizer, and/or known tonicity agent added for their known benefits, the application will likely be denied. If, on the other hand, the application focuses on specific combinations of known pharmaceutical ingredients in a way that achieves a unique or unexpected result that would not be discovered upon routine experimentation, then a strong case for patentability can be made.

Combinations of APIs may also be patented. However, if each API is already known, some sort of synergistic effect or unanticipated result must generally be shown in order to patent the combination. Patent protection may also be possible where the combination was expected to be problematic, such as where two

15 *See, e.g. Atofina v. Great Lakes Chem. Corp.*, 441 F.3d 991, 999 (Fed. Cir. 2006).

classes of drugs were believed to interact in a potentially harmful or antagonistic manner, or where special steps were necessary to make the two APIs compatible with each other. If each API performs nothing more than its known function, patent protection will likely be denied.

Characteristics such as stability, rate of uptake, effectiveness, and lack of side effects should be considered when patenting drug formulations. It may be possible to focus on characteristics of a new formulation in addition to, or in place of, the combination of ingredients making up the formulation. These characteristics could also be used to support patentability of a formulation by demonstrating that the formulation has improved properties not present in other formulations containing the same API. However, recognition of latent or inherent properties of a well-known or obvious formulation should not result in patent protection.

25.4.4 Dosages, Administration Forms, and Treatment Methods

Even if an API or formulation incorporating an API is well known, it may be possible to obtain patent protection on specific dosages or dosage regimens, forms of administration (including carriers), other medical uses, or methods of treatment that incorporate the API. This may include new indications for use or treatment of new diseases or conditions. Even when initially patenting an API, it can be advantageous to look to the future and contemplate the delivery mechanism for the drug in order to both protect anticipated uses of the API and deter others from obtaining their own patents on such uses.

A particular dosage regimen, form of administration, or method of treatment involving a known API should be patented if it confers a unique or surprising advantage or improved result, such as increased efficacy, reduced treatment time, or elimination or reduction of side effects. If there are specific nonstandard steps that need to be taken in order for the dosage, administration form, or treatment method to exhibit a certain improvement, the likelihood of patentability increases. If the dosage, administration form, or method of treatment is merely optimized to provide a better result of the same type already known, differing from the prior art only in magnitude, obtaining a patent is less likely. For instance, unique challenges may be encountered in adapting a specific API into a sustained-release, aerosol, or solid form, making those forms patentable even though a liquid form has already been described and is well known. Patenting different forms may be difficult, however, when other compounds have been made in those forms using techniques that are also effective for the newer API, especially when known compounds are chemically similar to the new API.

Narrow claims to particular dosages or administration forms that are submitted for regulatory approval can be very effective in deterring competition from generic manufacturers unwilling to conduct independent clinical trials but may be less effective in protecting against manufacturers that independently test dosages and delivery forms and obtain separate regulatory approval. It should be kept in mind that it may be possible to claim the dosage, administration form, or treatment in terms of its effectiveness or characteristics of the results achieved rather than simply claiming the treatment steps or composition characteristics.

When patenting dosage regimens and methods of treatment, an applicant should avoid defining the regimen or treatment in a way that requires two or more different parties to perform different steps of the method. Not only does such a focus on multiple parties complicate the infringement analysis, it may make patent claims unenforceable altogether. For instance, under US law there is no infringement of a patented method where the steps of the method are not performed by a single party or under the direction of a single party.[16] As a result, a patent that requires a pharmaceutical manufacturer to provide a formulation, a doctor to prescribe a specific dosage of the formulation, and a patient to ingest the formulation may be essentially worthless. However, this problem may be possible to overcome by individually patenting specific portions of the method and focusing on the steps taken by a single actor.

25.4.5 Methods of Manufacture

It is possible to secure a patent covering the steps involved in making a drug composition, for instance, the process of synthesizing the API or the method of combining ingredients under particular conditions to create a stable drug formulation. This could be as simple as claiming the steps of combining multiple ingredients to create a specific formulation or as complicated as claiming a combination of precursor molecules and reaction conditions to yield a new molecule or composition. Methods for creating a particular form of an API can also be patented. Obtaining a patent relating to the method of manufacture can provide a drug manufacturer with the ability to sue a competitor in the country in which the drug is made, regardless of whether the drug is ever actually sold there. On the other hand, complications can arise with respect to enforcing these patents. For instance, it may be difficult or even impossible to assert such a patent where certain steps are performed by one party and others are performed by a different, completely independent party. In addition, situations where different parts of the process take place in different countries can complicate infringement analysis and enforcement. Verifying that the patented method is actually performed by the infringer may also prove difficult where the same end product can be produced by multiple alternative processes.

25.4.6 Anticipating Further Development and Variation

When filing a patent application, it is ordinarily advisable not to focus too narrowly on anticipated commercial embodiments or specific species. Specific elements of the invention should not be held out as "critical" unless they are truly necessary for any commercially viable drug, because such representations can open the door for competitors to argue that they do not infringe due to the absence of the allegedly "critical" feature.

If possible, when the invention relates to a specific molecule or substance, a patent applicant should consider whether certain functional groups, structural aspects, or other characteristics are important to the drug's function and whether

16 *Akamai Techs., Inc. v. Limelight Networks, Inc.*, 797 F.3d 1020 (Fed. Cir. 2015)(*en banc*).

it is possible to identify a broader category of compounds that includes a variety of different species having similar properties. As discussed above, it may be possible to broadly protect a genus of compounds by focusing the patent claims on shared structural characteristics or pharmaceutical properties rather than on the identity of a particular species. It may also be possible to focus patent claims on isolates or purified forms that are the focus of future research and development. However, claiming variants of a drug without proof of effectiveness may not be considered patentable, depending on the facts of a specific case. Nevertheless, even if they do not result in patent protection, such disclosed variations of the invention may provide defensive benefits by impeding the patenting of disclosed improvements by others.

25.4.7 Designing Around Prior Art

By examining features of prior art drugs, a patent applicant can determine how best to claim a new drug in a way that does not cover the prior art. This may involve focusing on the structure of the API, the combination of separate elements in a formulation, or unique performance characteristics not exhibited by the prior art. Being aware of close prior art at the beginning of the patenting process can assist in writing the application in a way that explains why the invention is superior.

Prior to filing a patent application, the applicant may wish to search prior art to understand what related drugs and technologies have already been publicly disclosed and potentially stand in the way of patentability. The results of such a search may alert the applicant that grant of a patent is unlikely in view of the prior art. In such a case, the applicant may avoid pouring substantial resources into the patent process in a futile attempt to protect the drug. On the other hand, the search may reveal that the invention has not been described in patents or other printed publications, in which case the applicant may decide to aggressively pursue patent protection. Search results can also be valuable in that they allow the patent attorney writing the patent application to identify the closest known patents and describe the invention in a way that best distinguishes the prior art.

25.5 Timing of Patent Applications

Since public disclosure of an invention can negate patentability, it is important for researchers to understand that publishing journal articles, giving presentations, and even showing a series of PowerPoint® slides in an informal setting can potentially preclude the inventors from obtaining patent coverage in one or more countries. As a result, researchers should be advised to contact patent counsel as soon as possible. Patent applications generally should be filed before any arguably public release of information regarding how the new drug product is made, even if investigation and development of the drug is ongoing.

25.5.1 Provisional and Nonprovisional Patent Applications

A "provisional patent application" is a type of application that is filed to obtain an early filing date, but does not mature into a patent without additional steps taken to convert the provisional application into a standard (nonprovisional) patent application within one year of the provisional filing date. Although there are fewer formal requirements for provisional applications than for nonprovisional patent applications, a patent will not be entitled to the benefit of the provisional application's filing date if the eventual patent claims are not adequately supported by the provisional specification. As a result, a provisional application should be drafted carefully and, whenever possible, treated as though it were a nonprovisional application.

If an invention is ready to be commercialized and the researchers foresee little change in the near future, it may make sense to simply file a nonprovisional patent application rather than a provisional application. One advantage of filing a nonprovisional application relatively early is that the process of examination will begin earlier, likely leading to earlier issuance of a patent and availability of exclusive rights thereunder. In situations where an invention is easily implemented and infringers are likely to appear almost immediately, filing a nonprovisional application as soon as possible is particularly advantageous.

On the other hand, when development is ongoing and the concept behind the drug product, method of manufacture, method of treatment, etc., is still evolving, it is ordinarily best to file a provisional application, which will allow for an additional year before the invention must be fully described in a nonprovisional patent application. Another sometimes overlooked advantage of provisional patent applications is that the term of the resultant patent is measured from the date of filing of the nonprovisional application, not the earlier provisional, meaning that while issuance of a patent from the application will be later, the expiration date of the resulting patent may be up to a year later than it would have been if the initial filing had been nonprovisional. This can be especially important in situations where an invention is most valuable later in its lifecycle. Due to long development times and regulatory approval processes, the later expiration date provided by a provisional application can be particularly advantageous in the pharmaceutical industry. Because of the minimal formal requirements, provisional applications are also often filed where public disclosure is imminent.

25.5.2 Patent Cooperation Treaty Applications and International Filings

Once the first provisional or nonprovisional patent application is filed, the applicant has a one-year window in which to file corresponding nonprovisional applications in foreign countries. Instead of filing in multiple countries, however, one may file an international application under the Patent Cooperation Treaty (PCT). An applicant also has the option to make a PCT application its initial filing. A PCT application is not fully examined as is an application filed with the patent office

of an individual country but undergoes preliminary examination and gives the applicant the benefit of an international filing date. In addition, the PCT application allows for a 30-month period from the earliest priority date to select member countries in which to file. In other words, whereas an applicant in a given country must normally file related applications in other countries within one year of the original filing, a PCT application provides the applicant with additional time in which to decide where to file national stage applications for countries that are party to the PCT. This additional time can be particularly desirable when the feasibility and market value of the drug compound are being investigated or if future developments will determine which geographic markets are most valuable. Although clear benefits to filing a PCT application exist, significant filing fees are involved that will generally increase the cost of filing a patent internationally.

Note that certain countries are not party to the PCT and as a result require the filing of an application within one year of the earliest filing, regardless of whether or not a PCT application is filed. Other countries that are party to different agreements may require a PCT application to enter the national stage in accordance with those agreements rather than directly. For instance, a PCT application may enter some countries only under a regional treaty and validating the resulting patent in that country, while an applicant that foregoes the PCT process may file an application directly. Thus, determining target countries in advance can be very helpful in formulating a filing strategy.

Similar to the PCT, the EPO allows the filing of a single patent application that can be later extended to member countries. This can be particularly advantageous when an applicant wishes to protect an invention in several European countries. The EPO will examine the application and allow an issued patent to be validated in one or more member countries, avoiding the need for parallel prosecution in multiple nations. However, if an applicant will be focusing on only select markets, it may ultimately be more cost-effective or strategically desirable to file applications directly with specific countries.

25.5.3 Factoring in Continued Development of Drug Formulations

As noted above, it is normally best to file a patent application early in order to minimize the possibility of public disclosures that could prevent patentability. However, as the drug development process continues, unforeseen improvements will ordinarily be made. As a result, it may be advisable to continue to file additional applications to cover these improvements. The term of patent protection for a particular drug may be effectively lengthened by filing a relatively broad application early in the development process and then filing applications to narrower improvements at later dates without claiming priority to the earlier application. A balance must be struck between maximizing disclosure in the early patents and allowing room for related improvements, because the earlier patent application(s) can constitute prior art that may render the later improvements unpatentable for both the applicant and third parties.

25.5.4 Accounting for Publication or Presentation of Research

Publication of research is often critical to inventors for securing funding and recognition but can be detrimental to remaining ahead of competitors and

building a patent portfolio. If research must be published or otherwise publicly disclosed, a patent application should be filed beforehand. Care should be taken to include sufficient detail in the patent application to allow those of ordinary skill in the art to practice the invention, or else the applicant may not gain any benefit from the early filing date of the application.

Inventors and patent applicants should also keep in mind that formal publication of research, such as in a journal, is not the only way in which patent rights may be impaired. Any time research is presented to a segment of the interested public, especially without an expectation of privacy, there is a chance that such a disclosure will prevent patenting of the invention. Accordingly, important details of the invention must not be revealed at symposia, on research blogs, or even in casual conversations with colleagues from other companies or academic institutions.

25.6 Prosecution of Patent Applications

25.6.1 Defining Inventions in View of the Prior Art

Once a patent application is filed with the patent office of a particular country, an examiner at that office will search for relevant prior art (information known to the public prior to the filing date of the patent application). This ordinarily involves searching for similar inventions described in earlier patents or applications and sometimes in trade publications or other available resources (such as documents available on the Internet). After analyzing the claims of the application in view of this prior art, the examiner will issue a rejection if they believe that the prior art describes or suggests the claimed invention from the viewpoint of a person having ordinary skill in that art. The examiner may also reject the claims for a variety of other substantive and formal reasons, including failure of the application to adequately describe the claimed invention, indefiniteness or vagueness of the claimed subject matter, or failure to claim an invention of the type allowed by statute. If the examiner believes that the claimed subject matter is inventive in view of the known prior art and the application meets all other requirements, he or she will allow the application to issue as a patent.

When a rejection is issued, the patent applicant has an opportunity to amend the claims of the application so that the prior art is no longer within the scope of the claims. The applicant may be able to convince the examiner through argument alone that the prior art does not read on the patent claims, and in such cases amendments will not be necessary. Once the applicant responds to the examiner, the examiner will once again analyze the claims in view of the prior art and either allow the application to issue as a patent or issue another rejection. This process can continue as necessary until either allowance or abandonment of the application, although country-specific rules may limit the number of times that an applicant may respond before prosecution is terminated.

25.6.2 The Meaning of Patent Claims: Proactive Claim Construction

Whether a patent claim reads on the prior art depends on what the words of the claim are interpreted to mean. The process of determining the meaning and scope of claim terms is referred to as "claim construction." In the United States,

examiners are required to give claims their broadest reasonable construction, while in litigation a court determines how one of ordinary skill would understand the terms (which often results in a narrower construction). One way in which an applicant can force a patent examiner to interpret a claim term more narrowly is to define that term explicitly in the patent specification. Arguments made during prosecution also may serve to define the scope of the claims and can be binding on the applicant. Therefore, providing clear definitions of key claim terms in the specification or during prosecution can be useful in ensuring that the claims will be interpreted in accordance with the applicant's intent.

One drawback of explicitly defining a claim term, however, is that the applicant takes a firm position on a claim's scope and meaning without full knowledge of competitors' future products. There can be great advantage in waiting until litigation to formally characterize the claim's meaning, because the patent owner may avoid making statement that could have unanticipated effects in certain contexts and take into account the alleged infringer's position.

25.6.3 The Importance of the Patent Specification

The written description of the invention must fulfill certain requirements, including describing the invention with sufficient specificity and in a manner that allows those of skill in the relevant art to practice it. While the specification must adequately describe the invention, the invention should not appear unduly narrow. The specification concludes with a series of claims that set the boundaries of patent coverage. However, the remainder of the specification may be used as a guide to interpret these claims and therefore should not unnecessarily characterize optional aspects as "critical" or as clearly defining "the invention."

The specification also may provide support for amendments or arguments during prosecution or may show that the inventor was in possession of a specific invention at the time of filing. While including various embodiments in the specification can be helpful if it becomes necessary to retreat to narrower claims, as mentioned above these disclosures later become prior art and can sometimes prevent the applicant from later obtaining claims in a separate application disclosing specific subsequently developed improvements. Thus an applicant must balance the benefits of disclosure with the potential for further development, because if an embodiment is not well explained, it may not be sufficiently enabled to be patented in the original application but as prior art could render later developments obvious.

The specification may limit the scope of claims if it defines the invention narrowly or demonstrates that certain subject matter was not originally considered to be part of the invention. In some countries, claimed subject matter must be limited to combinations of elements explicitly disclosed in the specification, while in other countries support for claims and claim amendments may be drawn from various different portions of the specification. An applicant should therefore make sure to explain the most important embodiments in detail rather than relying on mixing and matching of aspects from different disclosed embodiments.

25.6.4 Amendments and Arguments

In responding to rejections during examination, it may be necessary to argue that the scope of the claimed subject matter does not extend to prior art that would otherwise invalidate the claims. These arguments will likely bind the patent owner, estopping them from later asserting that the patent claims are broader than represented during prosecution. For this reason, arguments should carefully distinguish the claims from the prior art without making broad or sweeping statements that restrict the claims more than necessary. Amendments to the claims can further reinforce the notion of estoppel and should be made with care.

25.6.5 Anticipating Challenges to Infringement and Validity

There is no requirement to extensively search for prior art before filing a patent application. However, given the expense of developing, commercializing, and patenting a drug or treatment, it is often advisable to conduct an investigation into patentability prior to significant financial investment. In some countries all known material prior art must be submitted to the patent office, and so this additional searching may increase the likelihood that the patent examiner will find a reason to reject the patent application. However, this risk should not be significant in the long term, because if a patent is eventually issued and asserted against a competitor, it must be expected that the competitor will go to great lengths to invalidate the patent. Initial prior art searching can help to avoid wasting resources in pursuit of unpatentable subject matter and to focus on novel aspects of the invention.

Taking time early in the patent process to think about how a third party would respond to an infringement accusation can be of great assistance in refining and strengthening the patent claims. For instance, if a patent claim lists all of the components in a drug formulation, there may be nonessential additives that could be omitted by a competitor to provide a basis for a non-infringement argument. If so, that element should be omitted from the broadest claims if the resulting claim will still be patentable over the prior art.

Using certain words to describe a particular compound in a patent application may provide a competitor with arguments that a similar compound is not covered by that term. If the invention relates to a specific chemical structure, a competitor may use a slightly different or modified compound and still obtain the same benefit. Therefore, it may be best to focus on a class of chemicals or functional groups rather than a specific molecule. Effectively patenting a drug or treatment requires balancing interests to construct claims broad enough to be infringed by products or processes close but not identical to those in the patent to prevent easy design around, yet narrow enough to avoid all of the prior art that will inevitably be raised to challenge the patent once an infringement accusation is made. By presenting claims of varying scope, a patent owner may increase the chance of having at least one claim that survives challenges to validity but covers sufficient subject matter to discourage competition.

25.7 Extending Patent Coverage Through Additional Applications

In most countries, there is some opportunity to file related applications claiming the benefit of the filing date of an original application. In some countries, this must be done early in the process, with strict deadlines in which to take action. In other countries, a related application only need be filed before allowance or issuance of a patent. In the United States, there is almost no limit on the number of related applications as long as they are filed during the pendency of at least one application entitled to the benefit of the desired filing date. It is important to understand the local rules from the outset in order to develop an appropriate strategy to secure sufficient patent coverage from all desired perspectives.

In view of the ability to modify claims during enforcement proceedings, and a relatively low level of litigiousness, large families of related applications are less common in most countries than in the United States. The discussion that follows will focus mainly on US patent practice while also making note of general patterns that apply to other countries.

25.7.1 Continuation Applications

Continuation applications are related applications that have very few restrictions. In the United States, a continuation application may be filed at any time during the pendency of the parent application (i.e. prior to issuance or abandonment of the application), even after allowance of the application and payment of the issue fee. There is also no firm limit to the number of continuations that may be filed from a given parent.[17] In cases where some claims are rejected while others are allowed, it is appropriate to cancel the rejected claims and continue their prosecution in a continuation application. It is also possible to file a continuation application in order to present evidence supporting patentability. In addition, continuation applications are commonly filed in order to attempt to patent broader, narrower, or otherwise different claims as an invention is further developed or refined. A continuation application may even be filed in the absence of any of the above reasons, provided that such refiling is not unduly successive or repetitive.

25.7.2 Continuation-in-Part Applications

In the United States, an application may claim the benefit of the filing date of an earlier application but add new matter. This is referred to as a continuation-in-part (CIP) application. CIP applications were intended to allow a patent applicant to cover continued development of an invention. Nevertheless,

17 U.S. courts have, however, upheld rejections of claims for "prosecution laches," holding that an applicant may forfeit his right to a patent application where there are "multiple examples of repetitive filings that demonstrate a pattern of unjustified delayed prosecution." *Symbol Tech. Inc. v. Lemelson Med., Educ., & Research Found.*, 422 F.3d 1378, 1385, 76 USPQ2d 1354, 1360 (Fed. Cir. 2005). The doctrine of prosecution laches is used sparingly.

a CIP application will be entitled to the benefit of an earlier filing date only if the claims are fully supported in the earlier application. If the claimed subject matter includes elements that were not described until the CIP application was filed, the application will not be entitled to the earlier filing date and the parent application may actually be considered prior art if published prior to the filing date of the CIP. Moreover, by claiming priority to an earlier application, the term of any resulting patent will be reduced because the term is measured from the earliest filing date in the chain, not the date of issuance. As a result, a CIP application is often of limited usefulness and may provide a patent applicant with a false sense of security regarding the priority date. In most instances it is advisable to file a new application and ensure that the new matter in the application is by itself patentable over the original application. However, in cases where the applicant wishes to maintain continuity and preserve an early priority date in the event that the claims ultimately are fully supported by the earlier application, a CIP application may be beneficial.

25.7.3 Divisional Applications

Many countries allow "divisional applications" to be filed after an initial application in order to protect additional related inventions. Although they use the same terminology, the nature and requirements of these applications can vary from country to country. For example, under European Patent Convention rules applicable from April 1, 2010, to March 31, 2014, divisional applications could be filed from a pending application only within 24 months from the first rejection from the Examining Division.[18] As of April 1, 2014, the EPO removed this limitation so that divisional applications may be filed at any time during the pendency of an earlier related application.[19] However, the EPO assesses an additional fee for each subsequent generation of divisional application in order to discourage long chains of related applications that may cause uncertainty about the rights of third parties for prolonged periods of time.[20]

Strictly speaking, in the United States, a "divisional application" is an application claiming an independent or distinct invention but having the same disclosure as the parent application. In many cases, a US divisional application is filed after the patent office issues a "restriction requirement" in the parent case, indicating that there are two or more distinct inventions claimed and requiring the applicant to elect only one. The other claims subject to restriction must be canceled and can be pursued in divisional applications. These divisional applications can be filed at any time prior to issuance of the parent patent, which could be years after the restriction requirement. There are, however, practical advantages to early divisional filings because the term of the patent runs from the filing date of the first nonprovisional application to which priority is claimed.

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18 Rule 36(1) EPC, April 2010.

19 Rule 36(1) EPC, April 2014; Decision of the Administrative Council of 16 October 2013 amending Rules 36, 38 and 135 of the Implementing Regulations to the European Patent Convention (CA/D 15/13).

20 Notice from the European Patent Office dated 8 January 2014 concerning European divisional applications – amendment of Rules 36, 38, and 135 EPC and Article 2(1).

If an applicant waits until allowance of one invention to pursue another, the issuance of second and subsequent filings could be delayed by years, resulting in a loss of a significant portion at the beginning of the patent term.

In Australia, where a deadline for acceptance is set based on the date of the first examination report, divisional applications must be filed within three months of acceptance, which coincides with the deadline for third parties to request an opposition proceeding. As a result, an applicant will not be able to file a divisional application during an opposition proceeding where the request is made at the deadline, and the applicant did not earlier elect to file a divisional. As a result, it may be advisable to file a precautionary divisional application prior to that deadline.

In the pharmaceutical field, applications typically describe a family of new compounds, methods for making them, and sometimes one or more therapeutic uses. These types of claims are often considered by the US Patent and Trademark Office to be separate inventions and may be subject to restriction. Knowing that these different aspects will likely be restricted as different inventions, it often makes sense to plan to file multiple applications to cover these different aspects at the outset, increasing the likelihood that at least one application will issue as soon as possible and be enforceable while the others are pending.

25.8 Modifications to Issued Patents

Even when there are no related applications pending, there may still be a chance to broaden or narrow claims – or even add additional claims. In many countries, specific post-issuance proceedings allow modification of patents. In some, patent scope may even be modified during litigation proceedings.

25.8.1 Correcting Issued Patents Through Reissue

A US patent owner may correct minor mistakes, such as clerical or typographical errors, through a certificate of correction.[21] Major defects that affect the owner's ability to effectively assert the patent may be corrected by filing an application for a reissue patent. Specifically, US patent laws provide for the filing of a reissue application where a "patent is, through error, deemed wholly or partly inoperative or invalid, by reason of a defective specification or drawing, or by reason of the patentee claiming more or less than he had a right to claim in the patent."[22] Applicable errors include mistakes in the patent specification or drawings, failure to appropriately claim the invention, failure to perfect a claim of priority to an earlier application, or failure to properly name inventors. Similar proceedings are available in some countries to amend granted patent claims, but often the scope of the claims may not be enlarged. Additionally, some countries allow patent owners to amend claims during other proceedings, such as litigation or patent office oppositions, with certain limitations based on the types and scope of amendments.

21 35 U.S.C. § 255.
22 35 U.S.C. § 251.

A US reissue application may be filed at any time within the original patent's term in order to correct a defect in the specification or drawings or to narrow the patent's claims. However, if the patent owner wishes to broaden the scope of protection by removing limitations from particular claims or filing new claims that cover subject matter not covered by the existing patent's claims, the reissue application must be filed within two years of the issuance of the original patent. Thus, it may be beneficial to, as a matter of course, consider the potential benefit of a reissue application shortly before the two-year anniversary of the issuance of any patent.

Multiple reissue applications may be filed from a single patent as continuations of the first reissue, and multiple reissue patents may issue and all be in force at the same time.[23] For instance, during the course of a first reissue application, the patent owner may become aware of additional errors that it wishes to correct. If these errors relate to the scope of claims, the patent owner may decide to file a continuation reissue application to present new claims. As long as the first reissue to broaden the scope of the claims was filed within two years of the issuance of the original patent, subsequent reissue applications may be broadening even if filed outside that two-year window.

Despite the limitations discussed below, reissue applications can be a powerful tool to expand the scope of coverage or narrow claims to overcome prior art. Prior to initiating a lawsuit, it is advisable to consider whether a reissue should be filed in order to add new claims, particularly during the two-year window to file a broadening reissue. A patent owner should be cautious when filing a reissue application, however, because it allows a patent examiner to examine all the issued claims again in addition to the new or amended claims.

25.8.2 Limitations on Broadening Claims

US reissue applications may not completely reverse strategic decisions made during the filing or prosecution of the patent. For instance, US patent law has developed a "recapture rule" that prevents a patent owner from regaining through reissue any subject matter that was surrendered in an effort to obtain allowance of the original claims.[24] Courts consider such an amendment to be the result of a deliberate decision to surrender specific subject matter, and even though this decision may be regretted in light of subsequent market developments, it is not considered an error under the patent statute. In contrast, a failure to fully appreciate the breadth of an invention from the beginning is considered to be a correctible error.

Another instance in which courts have held that there is no error of the type that may be corrected by reissue is where the patent applicant failed to file a divisional application following a restriction requirement during prosecution of the original patent.

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23 35 U.S.C. § 251(b).

24 *See Pannu v. Storz Instruments, Inc.*, 258 F.3d 1366, 1371 (Fed. Cir. 2001)(*quoting In re Clement*, 131 F.3d 1464, 1468 (Fed.Cir. 1977); *Hester Industries, Inc. v. Stein, Inc.*, 142 F.3d 1472, 1480 (Fed. Cir. 1988).

25.8.3 Limitations on Damages

Another limitation of US reissue patents relates to the ability to enforce new or amended reissue claims against competitors that were practicing the invention prior to issuance of the reissue patent. US law affords competitors so-called "intervening rights" in situations where claims are significantly changed.

There are two types of intervening rights. The first type of intervening rights, referred to as "absolute intervening rights," is conferred by statute and ensures that

> a reissued patent shall not abridge or affect the right of any person or that person's successors in business who, prior to the grant of a reissue, made, purchased, offered to sell, or used within the United States, or imported into the United States, anything patented by the reissued patent, to continue the use of, to offer to sell, or to sell to others to be used, offered for sale, or sold, the specific thing so made, purchased, offered for sale, used, or imported unless the making, using, offering for sale, or selling of such thing infringes a valid claim of the reissued patent which was in the original patent.[25]

In other words, the patent owner cannot seek damages for acts prior to the issuance of the reissue patent or for sale or use of inventory produced prior to reissue unless a claim present in the original application and infringed by such product survived and is present in the reissue patent. This is true regardless of whether the claims were broadened or narrowed, under the theory that even if the claims were only narrowed, the alleged infringer was entitled to rely on invalidity of the original claims.

The second type of intervening rights is referred to as "equitable intervening rights." Under this type of intervening rights, a court may provide for the continued manufacture, use, or sale of products for which substantial preparation was made prior to the grant of the reissue patent to the extent that the court deems equitable for the protection of the investments made or business commenced before the grant of the reissue patent.[26]

Intervening rights may have a substantial impact on a patentee's ability to collect damages for both pre- and post-reissue acts and should be taken into account when deciding whether to seek reissue or assert a reissued patent. Due to the recapture rule and intervening rights, there can be a substantial business advantage in maintaining one or more pending applications through continuation practice just in case broader claims are desired at a later date.

25.9 Conclusion

Many variables must be taken into account when determining a patent protection strategy for new drug compounds and formulations. Due to unique factors in

25 35 U.S.C. § 251.
26 35 U.S.C. § 251.

each situation and differing laws in various countries, it is difficult to generalize a single "best" patent strategy. At the preclinical stage, it may be especially difficult to project the value of the new drug compound, formulation, or related methods. As a result, it is beneficial to keep options open and protect multiple aspects of the new product to the extent that the budget allows.

Some factors to consider early in development include the nature of the drug and its delivery methods, known alternatives already in existence, the size and location of the population in need of the drug or similar treatments, cost and other barriers to manufacturing and commercializing the drug, opportunities for further innovation and development, and potential for licensing or otherwise monetizing the invention. By using these factors to estimate the likely nature of the market for the new drug in various geographic areas, a company may begin to formulate and employ a unique patent strategy early on to maximize its ability to meaningfully protect its investment in the development of the new drug.

26

Intellectual Property: The Patent Landscape Viewed from Generic and Originator Perspectives

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26.1 Introduction

The generic pharmaceutical industry plays a very important part in the healthcare systems of most developed nations. This generic industry provides an important counterpoint to the originators by ensuring strong competition in the market. This means that notwithstanding the various legal protections afforded to innovative companies, which include the patent system and the regulatory approval system, generic competition can still have a substantial influence on the pricing of drugs. It is important to recognize that both sides of the industry, innovators and generics, are essential to the efficient functioning of the industry. Indeed, without innovators there would be no new products entering the market, and this potentially leads to needs and diseases, which remain unchallenged. At the same time, without the presence of generics in the market, many of those new products might potentially remain at prices that place them out of reach of certain markets.

The generic industry therefore serves the valuable purpose of providing an alternative source of supply of certain established drugs once the patent and regulatory protection has ceased to have effect. Many national healthcare bodies rely extensively on generic products to minimize costs in their healthcare systems. At the same time, it is essential to the future development of new drugs to have a healthy innovator industry.

26.2 Market Exclusivities That Protect Branded Drugs

There are two separate, unrelated, strands of legal protection for protecting research that are afforded to innovator drug companies and that separately provide exclusivity in the market for the originators drug product. These legal protections are the patent system and the regulatory approval system. Provisions relating to other intellectual property (IP) rights such as design rights, know-how, and trademarks also play an important role but are beyond the remit of this chapter. Although this chapter is concerned principally with the patent system

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

and the exclusivity that it affords, a brief discussion of the regulatory system is also provided for context. Although the patent system and the regulatory approval system are separate systems, the interplay and relative timings of events in the two systems frequently affects the patent strategy deployed in any particular generic drug launch situation.

The patent system protects the innovator by preventing the marketing of a drug product that falls within the scope of a granted and enforceable patent by creating a legally enforceable monopoly. It must be remembered that the mere existence of a granted patent does not necessarily mean that the claims of the granted patent are valid and enforceable against a third party who is producing a product, or operating a process, falling within the scope of the granted claims. It is also important to appreciate that the granted patent claims themselves, irrespective of whether or not they are valid and enforceable, may not necessarily cover the activities of the third party. A careful analysis of the claim language is therefore essential to determine whether or not the third-party activity falls within the scope of the claims. If it is determined that the activities do fall within the scope of the claims of a relevant patent that has been granted, then a thorough review of the prior art landscape is essential. This is necessary to determine whether or not the claims can be validly enforced against the third party in an infringement action.

The regulatory system provides a period of exclusivity for proprietary clinical data generated by an innovator company in support of their own application for a marketing authorization (MA) to sell an approved drug product. This exclusivity period is often described as the regulatory data exclusivity period. In the United Kingdom and Europe, this period lasts 10 years from the date of the MA for the drug and comprises 8 years of data exclusivity followed by 2 years of market exclusivity. The 8-year data exclusivity period is calculated from the date of the first MA in Europe during which a generic company cannot file an application for approval based on the innovator's data. After 8 years, the generic can then use the innovator's data in an application for regulatory approval but cannot market their product until the expiry of the 10-year period from the first MA. In certain circumstances, a further one-year period of data exclusivity may be obtained when the innovator has identified a further significant indication for the drug. In the United States, the period of regulatory exclusivity is 5 years for a new drug that is a chemical entity, 3 years for new indications for existing pharmaceutical drugs, and 12 years for biologic products. The United States also provides 5 years of additional market exclusivity for antibacterial products that qualify as a qualified infectious disease product (QIDP) under the GAIN Act.

The patent system is therefore intended to provide a period of exclusivity for the innovator company by creating a legal monopoly during which the innovator company has exclusive rights to make and sell an approved drug product. The regulatory framework also provides a separate period of exclusivity, but this confers different rights on the innovator. This exclusivity period serves to prevent the use of originator company's clinical data by the generic company. The clinical data may or may not be in the public domain, but either way has been generated by the innovator in the course of its mandatory clinical evaluation of the drug at great expense. Clinical evaluation is necessary to obtain regulatory approval to market

the drug and is very time consuming and costly to conduct. The unrestricted use of such data by a generic company for the purposes of seeking approval of a generic version of the innovator's drug would provide an unfair advantage to the generic company. At the same time, from a public policy perspective, this need to protect the fair interests of the innovator must be balanced against reasonable access to medicine that might be afforded by a generic entrant on the market and the need to avoid unnecessary repetitions of animal and human clinical trials.

The regulatory exclusivity period may be the same or different from the exclusivity period afforded by the patent system. On expiry of the patent, a generic company is able to market a generic version of a drug without risk of being sued for patent infringement. However, if the regulatory exclusivity period is still running (i.e. if it extends beyond the date of patent expiry) then the generic manufacturer will not be able to rely on clinical data generated by the innovator when seeking regulatory approval for its own generic product. Instead, the generic manufacturer will have to generate their own data, and this can be a substantial barrier to market entry.

In most generic entry situations, the generic manufacturer is looking to base approval of their own drug formulation on the innovator's data by asserting that the generic version is bioequivalent to the innovator's version. For practical purposes, this means that generic entry is normally delayed so that it is effected after expiry of the regulatory data exclusivity period. On the other hand, if the regulatory data exclusivity period has expired before the date of patent expiry, then, depending on the strength of the patents covering the innovator drug product, the generic manufacturer may decide either to wait until the patent expires, or to launch at risk before patent expiry, or to challenge the validity of the patent before patent expiry with the aim of having the patent revoked. This comment is based on the assumption that the generic product is equivalent to the innovator product and would infringe a granted valid patent if launched before the date of patent expiry. Another option open to a generic manufacturer who is confident that their product does not infringe an existing patent is to launch an action for a declaration of non-infringement.

The two exclusivity periods allow the innovator to recoup the extensive investment involved in bringing a new drug to the market for the treatment of a particular disease. The research and development involved in this process can take several years and consume many millions or even tens of millions of pounds in investment.

Drug development is a high risk process with a high attrition rate. The failure rate for potential drug compounds is significant even with today's advanced methods for identifying suitable candidate drugs involving targeted approaches. Frequently, of the large number of compounds synthesized, only a handful show the desired activity. At the same time, potentially active compounds must be screened to ensure that they do not give rise to unintended side effects in addition to having the desired activity. A battery of tests and assays conducted in the laboratory is therefore required to determine and exclude an extensive range of harmful side effects.

The surviving active compounds that represent potential drug candidates must then be screened further to find compounds that have a suitable degree

of solubility and the right level of lipophilicity. This is so that they can be formulated into convenient dosage forms and be bioavailable and hence effective after administration to a patient. The metabolic degradation products are analyzed for any potential side effects. Once all of these issues have been addressed, it is then necessary to conduct small-scale and subsequently wider clinical trials to establish the safety and efficacy of the potential drug in the patient population. Approval of a drug for medical use will only be granted by the regulatory authorities such as the European Medicines Agency and the Food and Drug Authority.

The patent system and also the legal regulatory framework together serve to provide the innovator company with a period of exclusivity that is intended to compensate the innovator for the extensive investment and risk required to get a drug to the approval stage and subsequently on to the market.

A good example of a situation in which conflict arises between the interests of the state and patients in obtaining cheap medicines, and with the interests of the innovator in receiving a fair return for their extensive research and investment in developing a new drug, is illustrated by the Actavis pregabalin case.[1]

In that particular case, generic drug manufacturer Actavis marketed a generic version of pregabalin for the treatment of epilepsy and generalized anxiety disorder (GAD). However, the innovator company Warner–Lambert that had invented pregabalin had its own product (Lyrica™), which was marketed and approved for the treatment of epilepsy, GAD, and neuropathic pain. Although there was no patent protection for the compound itself, a second medical use patent was still in force for the treatment of pain. In this particular instance, the generic producer Actavis sought to try to avoid a patent dispute by entering the market only for epilepsy and GAD because of the existing patent protection for the use of pregabalin for treatment of pain. The generic version was marketed under the name Lecaent™.

This seemingly straightforward situation unraveled into complex litigation precisely because of the conflicting needs of the innovator and the end users (the health authorities and ultimately the patients). Even though Actavis notified pharmacists that the product was not indicated for the treatment of neuropathic pain and even though the summary of product characteristics (SmPC) and patient information leaflet (PIL) were limited to non-patented uses, Actavis still wound up on the wrong side of a patent infringement suit.

This happened because at the time of the dispute in the United Kingdom, 83% of prescriptions were written generically by doctors without specifying a particular brand of the particular drug. Furthermore, investigations during the course of that trial also revealed that 95% of prescriptions written by doctors do not mention the particular indication, which is being treated. This means that the pharmacist dispensing the drug to the patient has no way of knowing what indication is being treated or indeed whether a particular brand of the drug should

1 HGF updates – www.hgf.com/updates/news/2015/02/swiss-claims-skinny-labels-and-subjective-intention; Warner Lambert v Actavis [2015] EWHC 72 Pat; Warner Lambert v Actavis [2015] EWHC 223 Pat.

be dispensed. Additionally, if a drug is prescribed using an INN rather than the trade name, the National Health Service (NHS) requires pharmacies to provide the cheaper generic version of the drug. Given the significant price difference between the brand label version of pregabalin (Lyrica) and the generic version, pharmacies had a strong motivation to dispense the generic version in order to save money for the NHS who was ultimately footing the bill.

At the time of writing, this dispute is still ongoing and serves to show the effects that valid patent protection can have in maintaining exclusivity in a particular market. It also illustrates that careful analysis of the patent landscape, and the legal framework in the particular jurisdiction, is essential prior to launching a generic version of a drug. Different generic companies have different approaches and attitudes to risk when launching a new generic product into a market, and this chapter examines the various approaches adopted by both generics and innovators in order to achieve their commercial aims.

26.3 The Patent Cliff

The patent cliff is particularly relevant to the modern pharmaceutical industry since it represents the largest single challenge facing the industry. It can be defined as the significant reduction in revenue for a drug product approaching or immediately following the expiry of key patents covering the drug. The date of patent expiry is well known and eagerly anticipated years in advance by generic drug manufacturers. It is generally acknowledged that once a drug has gone off-patent, a substantial proportion of total drug sales will be lost to generic manufacturers with the originator retaining only 10% or 20% of the market. From the originator side, this is an event that must be planned for years in advance to protect revenues and maintain sales of the branded drug in the face of generic competition.

The opportunity afforded by patent expiry for generics has been estimated at $65 billion by the research and consulting firm GlobalData. The company analyzed the competitive positions of 30 leading drug companies using 25 financial metrics and identified those companies likely to be hit hardest in the period up to 2019; these include Eli Lilly and AstraZeneca™.[2]

In 2016 alone, several blockbuster drugs are facing patent expiry and hence generic competition. These include Crestor™, AstraZeneca's statin product (rosuvastatin calcium), which was approved in 2007 and is used in the treatment of high cholesterol and heart disease. This drug was one of the most prescribed drugs prior to the onset of generic competition following expiry of the rosuvastatin compound patent. AstraZeneca's revenues were challenged further due to the recent expiry of Seroquel XR™, which is used in the treatment of depression and bipolar disorder.

Another notable patent expiry in 2016 is Merck's Zetia™, which is also used in the treatment of high cholesterol particularly in patients whose cholesterol levels

2 Global data: patent cliff means pharmaceutical companies will lose $65 billion by 2019. PharmaPro News (December 10, 2014).

could not be controlled with a conventional statin. Each of these patent expiries taken individually potentially represents a multibillion pound market that has suddenly become open to generic competition. In the case of AstraZeneca, Crestor and Seroquel XR accounted for annual revenues of over $7 billion, so the loss of these two drugs presents a major challenge to the business and puts pressure on the product pipeline.

The date of patent expiry therefore represents a massive opportunity for generic manufacturers. Consequently there is significant competition among generic companies to be the first to launch a generic product. In the United States, the Hatch–Waxman Act of 1984[3] provides certain incentives for generic manufacturers to launch generic products. For example, the first generic company that files an abbreviated new drug application (ANDA) with a paragraph IV certification and successfully launches and defends a paragraph IV action (Patent Term Restoration Act of 1984, Section 505(j)(5)(B)(iv)) against the innovator is granted a 180-day exclusivity period to be the sole generic supplier on the US market. In the event that more than one company files an ANDA on the same day, the 180-day exclusivity is granted to all of the companies. This 180-day period of "semi-exclusivity" is hugely valuable to a generic entrant and represents an enormous incentive to be the first generic on the market. Consequently, the originator is frequently met by multiple generic challenges at the point of patent expiry.

The generic companies are able to plan their generic drug launch strategies years in advance of the actual date of patent expiry since this term is known from the outset. Significant effort is put into analyzing the marketplace, the strengths and weaknesses of the patent portfolio, and the regulatory dossier (since these issues are important in deciding the timing of the generic launch). In some instances drug launches are delayed until after patent expiry, whereas in other cases launches may occur prior to patent expiry. The United States has an unusual approval and litigation landscape that arose following the Hatch–Waxman Act that defined procedures for generic manufacturers to challenge innovator patents and also provided an incentive for challenging those patents. Filing of ANDAs is frequently referred to as paragraph IV filings in reference to the legislation (see footnote 3).

During the lead-up to patent expiry, innovator companies generally deploy a strategy that involves emphasizing the merits of the brand drug product in an attempt to persuade doctors to continue to use the innovator product. In certain jurisdictions, such as the United States, direct advertising to patients can form part of the approach used to mitigate the effects of a looming patent cliff.

In other instances, an innovator may attempt to promote a next-generation product that it has developed in-house in order to lessen the impact of the patent cliff. Examples of this are AstraZeneca's substitution of the *S*-enantiomer of omeprazole for the earlier racemic omeprazole and Pfizer's extensive marketing of Lipitor™ (atorvastatin) in the run-up to patent expiry.

The expiry of patent coverage on Pfizer's Lipitor, a cholesterol-lowering drug, had a devastating effect on Pfizer's revenue from this drug and consequently on

3 Drug Price Competition and Patent Term Restoration Act 1984 (Public Law 98-417), known informally as Hatch Waxman Act.

its share price. Pfizer suffered a 19% drop in share price in the financial quarter in which Lipitor went off-patent.[4]

Not only do generic versions of new drugs represent a threat to the revenue stream for innovator companies but also alternative new treatments from competitor innovator companies may sometimes replace existing treatments. An example of this is Eli Lilly's Cialis™ treatment for erectile dysfunction, which competed alongside Pfizer's Viagra™ for market share. Another challenge to the innovator comes from more effective or safer treatments that can also be substituted for existing patented treatments.

However, the situation for the innovator companies is not as bad as first envisaged because the drug landscape is evolving. As more emphasis is now being placed on biologics, the appearance of generic biological drugs, known as biosimilars, has changed the financial balance in the market. Historically, patent expiries were based on small molecules that were relatively easy to synthesize and formulate. Generic companies could therefore rapidly develop a generic version of a small molecule drug and enter the market. The issue was generally one of cost, and the ability to produce a generic product economically was a key indicator of commercial opportunity and success. The approval process is relatively straightforward, provided that bioequivalence can be demonstrated for the generic product and the approval process does not require a complete reevaluation of the product. In contrast, the situation is more difficult for a complex active such as a biologic. The generic may struggle with manufacturing an equivalent biological molecule or have problems demonstrating equivalence. This presents a much larger barrier to entry than the technical challenges when seeking to provide a generic version of a small molecule.

Over recent years several biological drugs have been approved, and some of these biologics are now reaching the point of patent expiry. Generic manufacturers face far more difficult challenges in bringing by similar versions of such drugs to the market precisely because of the need to demonstrate equivalence with the original drug. Whereas with a small molecule, a generic version of a drug may be substituted at will for the original branded drug, it is not always the case that a biosimilar can be substituted for the brand biotech drug. Furthermore, under current legislation because a biosimilar drug is not truly identical to the original biological drug in the way that a small molecule generic will be, it may not be marketed under the same drug name. Consequently, the innovator has a further advantage in the market in terms of product recognition relative to the new biosimilar drug.

26.4 Paragraph IV Issues

The paragraph IV system is unique to the United States although a number of other countries have implemented similar provisions: for example, Canada and South Korea have introduced patent linkage procedures in which the originator lists the patents covering the approved products. In the United States it is a

4 Pfizer races to reinvent itself. The New York Times (May 1, 2012).

requirement of the approval process by the US Food and Drug Administration (FDA) that the innovator company must list any patents involving composition of matter claims (i.e. compound claims), formulation claims, and method of use claims (i.e. claims directed toward the use of a particular active ingredient in the treatment of a specified indication). These patents must be listed in the FDA's published list of Approved Drug Products with Therapeutic Equivalence Evaluations – the so-called *Orange Book* – which is nicknamed on account of the color of its cover.

An applicant bringing a new generic drug to the market is then able to file an ANDA but must certify relative to all patents listed in the Orange Book for that drug that either (i) the FDA should approve the generic drug after expiry of the last patent or (ii) the generic drug either does not infringe any of the patents listed in the Orange Book or the patents listed in the Orange Book that are relevant to the product are not enforceable against the generic manufacturer.

Innovators expend a substantial amount of energy determining which patents should be listed in the Orange Book and which patents should not be listed. This area is the subject of a substantial amount of litigation in its own right.

In the majority of cases, the ANDA is filed with a paragraph IV certification. The innovator company is notified of the application and then has 45 days to file an action for patent infringement against the generic manufacturer. If the innovator does not commence litigation for patent infringement, the FDA will proceed to approve the ANDA. More usually, the innovator vigorously defends the action and commences litigation; this is commonly known as paragraph IV litigation. The legislation requires that the FDA cannot then approve the ANDA until after the earlier of either the expiry of a 30-month period or the successful defense by the generic manufacturer of its claim for patent invalidity or non-infringement.

If the generic manufacturer is ultimately successful in the invalidity or non-infringement action, it is then granted a 180-day period of market exclusivity. This means that the generic company is the only company (or group of companies if more than one company files an ANDA on the same day) that can market a generic version of the drug during this period. This is a huge incentive for a generic company to launch a paragraph IV action since it gives the generic the first mover advantage. In addition, during this exclusivity period, the innovator is the only other product on the market, and the generic therefore only needs to price its generic version sufficiently below the innovator price in order to generate sales. This pricing level during the 180-day exclusivity period is invariably at a level that is much higher than the ultimate generic price level once the market is open to all generic players. Thus, the generic company can reap additional profits that it might not otherwise have had during this initial exclusivity period. In the case of a multibillion dollar drug product, the resulting additional profits can be substantial and more than compensate for the risks and costs associated with paragraph IV litigation.

Equally importantly, it can establish a brand presence during this exclusivity period and ultimately gain momentum for the product relative to later generic versions. Once the market is open to other generic entrants following expiry of the 180-day period, the price is likely to collapse significantly. Profits can still be made by the various generics at the new lower price level, but the real rewards are obtained following a successful paragraph IV challenge during the exclusivity period.

26.5 Injunctions

One of the most significant concerns that a generic company has when contemplating the launch of a generic drug is that of being prevented from launch or having a recently launched drug withdrawn from the market. This will arise because of a preliminary injunction being granted by the courts to the originator. A preliminary injunction is normally granted pending full trial of patent infringement. However, in some cases the matter does not ultimately progress to full trial, and the preliminary injunction becomes final.

An injunction may be granted to an originator on application by the originator in cases in which the launch of a competing generic version would present a risk of substantial damage to the originator's market. This damage usually takes the form of price erosion of the originator's drug; in other words, the originator can no longer sell the drug at its original price and must sell at a lower price following generic launch. The risk is that the previous higher price cannot then be subsequently regained with consequent loss to the originator in the event that either a generic drug is allowed to be launched or is allowed to remain on the market if it has already been launched at risk.

The originator would normally seek an injunction as soon as they become aware that a generic company is planning a launch. This awareness is frequently triggered by the issue of an MA for a generic version of the drug to the generic company.

It is important to remember that patent infringement is handled on a national basis under the provisions of national law. This means that infringement of a granted European patent in one of the territories in which it has been validated is handled by the national courts of that particular jurisdiction. There are differences in procedure between the different jurisdictions throughout Europe although many of the principles underlying the decisions of the various national courts concerned in the granting of injunctions are common to the different jurisdictions. A discussion of the variations in national law throughout the European Union (EU) concerning patent infringement is beyond the scope of this work. The discussion below outlines some of the factors that would be considered by a court such as that in the United Kingdom when assessing whether or not to grant an injunction. Many of these principles are reflected in the practices adopted by other courts in Europe such as the Dutch or German courts.

It should also be remembered that once the proposed Unified Patent Court (UPC) comes into existence, it will be possible to conduct patent infringement and patent revocation proceedings centrally before that court. The decisions of the UPC, once instituted, will have an effect across all of the signatory countries. This means that it will not be necessary for an originator to engage in separate national patent infringement proceedings in each territory in which patent infringement might be occurring or be necessary for a generic to seek revocation in each territory in which a generic might wish to launch a drug.

Shortly before the time of writing, it had been anticipated that the UPC would formally come into existence early in 2017 and consequently that a centralized system would sit alongside, and run in parallel to, the ability to litigate patents on a national basis. This was expected to provide a convenient forum for litigating patents. At the same time, it was expected to lead to possible changes in strategy

in the way originators prosecute and assert their key drug patents. Similarly, it was expected that this might lead to changes in the strategy that generic companies adopt in relation to certain drug launch in response to originator's actions.

There have been extensive debates in the patent community concerning different strategies once the UPC begins its work. Shortly after Brexit, it was considered that those discussions had effectively become moot since the implementation of this centralized system was expected to be delayed by at least a few years. The recent referendum in the United Kingdom that led to a decision for the United Kingdom to leave the EU initially had a substantial impact on the implementation of the UPC system. The United Kingdom was one of the three mandatory signatories required for implementation of the UPC, and the proposed departure of the United Kingdom from the EU immediately following Brexit placed significant uncertainty as to the likely timing and structure of the UPC. This was because it was thought that the United Kingdom would no longer be party to the discussions (unless a political agreement was reached with the remaining member states to allow the United Kingdom to remain part of the system).

Interestingly, by early 2017, the political landscape had shifted once again. Somewhat unexpectedly, it was then anticipated that the United Kingdom would ratify the UPC in or around April 2017, notwithstanding the vote to leave the EU. This raises a number of very interesting political and legal questions beyond the scope of this article regarding the delegation of legal decision making from the United Kingdom to the EU authorities in this limited sphere of IP. Equally interesting is the fact that the third mandatory signatory required in order for the UPC to be implemented, Germany, is likely to ratify the UPC after the United Kingdom has already done so. However, some German commentators in the IP field that this author has spoken to have privately expressed the opinion that Germany may choose not to ratify. History will decide whether or not this is the case. For now, the expectation is that the UPC will come into force within the next one or two years. This will have a substantial impact on both patent filing strategies and patent litigation strategies as discussed below.

The advent of the new UPC is likely to alter significantly the patent litigation landscape in Europe when it does come into existence because it will immediately allow third-party challengers the ability to invalidate a patent with something approaching a Pan-European effect. Revocation in those European countries that have signed up to the UPC will then be achieved in a single action. Similarly, it will be possible for patent proprietors to pursue actions for patent infringement and for injunctions covering the UPC signatory via a single action. Of course, it will still be necessary to conduct litigation in relation to territories outside of the UPC on a national basis. Patent proprietors will therefore start to develop strategies to take account of the need to "opt in" or "opt out" key patents in their patent portfolios. It is expected that differences in the way individual originator companies handle the patent filing strategies and the choices of which patents they opt in or opt out of the system may present generic companies with additional opportunities to challenge granted patents. It is also expected that the validity of individual patents may be open to challenge on the legal basis of whether they have been properly opted in or opted out in much the same way as the validity of supplementary protection certificate (SPC) filings is challenged. SPCs provide

a mechanism to extend the lifetime of a particular patent covering an approved drug in Europe by up to 5 years, and their importance is discussed later in this chapter.

In a related point to the anticipated impact of the UPC on the patent litigation landscape, it is also noted that the situation surrounding SPCs has yet to be clarified and that there is no new regulation that has been drafted sitting alongside the UPC. However, it is the case that the new UPC system will be applicable to SPCs. This raises two very important issues. The first issue concerns the matter of how to treat SPCs based on existing and future classical European patents. The second issue concerns SPCs based on new unitary patents.

In the first case, SPCs referencing newly granted classical European patent as the basic patent will, under the terms of the UPC agreement, all be subject to the UPC's exclusive jurisdiction unless they are opted out. This opt-out will be available for a limited period of 7 years in the same way as newly granted and existing European patents may be opted out during the first 7 years of the UPC system.

The second issue concerns the extent to which the territory of the SPC based on a unitary patent must match the territories in which MA has been obtained for a new drug.

When seeking regulatory approval, it is possible to obtain either a single MA covering all of the EU via a procedure known as the centralized procedure, or separate MAs can be obtained on a national basis. Presently, there is no link between the regulatory pathway for obtaining an EU-wide MA via the centralized procedure and the legal mechanism for obtaining Pan-European (in signatory territories) patent coverage via the unitary patent. A unitary SPC does not therefore exist and is not currently contemplated.

If a unitary SPC were to be created, either it would be necessary for it to be granted on the basis of any EU MA irrespective of how it was obtained by the originator, or it would have to be based on a centralized MA or separate national MAs in all of the signatory territories. If the former approach were to be adopted, this would represent a relaxation of the legal requirements, and if the latter were adopted, this would represent a more burdensome requirement.

We can expect to see a great deal of political lobbying from both sides of the industry, and this author does not anticipate a quick resolution of the issue. It is expected that the current SPC system will coexist alongside the UPC during at least part of the 7-year opt-out period while the Brexit negotiations are completed and political attention can then be turned to a unitary SPC regulation. No doubt, there will be litigation surrounding the interplay between these two systems until the two are harmonized.

For all of the above reasons, the advent of the UPC is expected to have a substantial impact on the way in which patent prosecution is conducted in Europe since the strategies adopted during prosecution may have an impact on the ultimate jurisdiction for subsequent litigation.

Returning now to the current system in which injunctions are handled nationally by the courts in the territory in question, an originator will usually apply to a court for an injunction as soon as they become aware of an existing or potential threat. Injunctions can be sought urgently on an *ex parte* basis in the event

of an imminent drug launch. In such circumstances, only the applicant for the injunction (the originator) is present in the court, and the generic has no opportunity to put their side of the argument before the court. The originator is under a duty to state both the positive aspects and also the weaknesses of their own case. In other cases, both parties may attend the injunction hearing and make representations to the court as to the relative merits of their arguments.

The court has to balance a number of competing interests when assessing whether or not to grant an injunction. The first test that the court applies is intended to establish whether or not there is a serious issue to be tried. This means that there has to be a *prima facie* case that there is a patent in force and that it is reasonably clear, without undue investigation by the court or the need for expert testimony, that patent infringement is occurring. The parties are required to put forward brief arguments as to why it is very clear on the basis of the available facts that infringement is or is not occurring.

The court also needs to consider the balance of convenience relative to the two parties. This means considering whether damages, if awarded to the originator instead of granting an injunction, would be sufficient to compensate the originator in the event that the generic company was allowed to place the product on the market (or continue marketing an already launched product) and then ultimately be found to be liable for patent infringement. The court has to consider whether the originator's market for the drug would be irreparably damaged if no injunction is granted. On the other hand, the court has to assess whether or not the generic would be irreparably damaged by the grant of an injunction against it instead of the generic simply being awarded a cross-undertaking in damages to compensate it for loss of profit during the injunction period when launch was prevented.

The court has to consider, for example, whether the generic may have lost an advantage relative to other generics in being the first to the market. The advantage of being the first generic is both financial and reputational and may not always be quantifiable. The award of a cross-undertaking in damages may not, in some circumstances, be able to compensate the generic for loss of the opportunity to launch the drug if an injunction is granted. Similarly, in the event that an injunction is issued preventing further sales of an already launched generic drug, a cross-undertaking may not be sufficient to provide adequate compensation.

As well as attempting to establish the quantum of the damages in the two competing scenarios to the extent that it is possible to do so, the court would also need to look at the ability of each party to be able to pay damages in the event that there was a finding against them. One or other parties may have to pay security for costs into the court if the financial position of the party is not particularly strong.

Finally, the court will also look at the relative merits of the parties and will also consider the conduct of the parties. This means that if a particular party has acted diligently but nevertheless ends up in litigation proceedings, they will not necessarily be treated adversely; equally, if the court considers that the party was reckless or had disregard for the law and had not taken adequate steps to avoid patent infringement by invalidating relevant patent rights, then the court is far more likely to grant an injunction.

A number of recent decisions in the UK courts emphasize the importance of clearing the way and the effect that not doing so will have on a court's willingness to issue an injunction. In the Merck versus Teva case,[5] Teva was prevented by injunction from marketing a generic version of efavirenz because they had taken no action to clear the way. In fact, the company had obtained an MA but refused to indicate to Merck when launch was planned. Furthermore, Teva had previously launched other generic drugs before patent expiry without taking the trouble to overcome relevant patents either by revocation or by negotiated agreement. In that particular case, a significant factor in the decision to grant an injunction against Teva was the fact that they had made no effort to overcome or circumvent relevant patent rights. A similar issue arose in Warner–Lambert versus Sandoz[6] and the court granted an interim injunction against Sandoz to prevent them from marketing generic pregabalin because they had given no real notice to Warner–Lambert of a proposed generic launch.

In the event that all of the above issues are finely balanced, the court will then generally base any decision on maintenance of the status quo. This means either granting an injunction to prevent launch of a generic version of a drug from being launched that has not already been launched or allowing the continued marketing of a generic drug that has already been launched by the generic company.

26.6 The Generic Company's Goals

The aim of the generic company can be stated quite simply as the desire to produce a generic version of an approved product at sufficiently low cost to take market share from the originator.

Even though the generic company does not have to stand the costs of research and development into discovering a new chemical entity or proving its safety and efficacy, there are still considerable costs associated with being second into the market with a drug. The generic company has to develop its own novel formulations unless it chooses to produce its own version of the approved formulation. In such a case, there is no argument that the formulation will infringe any relevant valid patent. The generic company then has either to wait until expiry of the patents covering the formulation or to seek to challenge the validity of the patent through invalidity proceedings. In addition to the above, the generic company also has to create its own packaging and branding and then seek approval for its generic product. The approval is usually on the basis of the innovator's data used during the original approval process and necessarily takes place after expiry of the data exclusivity period. There are also circumstances in which the validity of the data exclusivity period itself may be challenged by litigation, but this is outside the scope of this particular chapter.

In the run-up to patent expiry for an approved drug, the generic company would normally review the patent landscape and assess the strengths and weaknesses of the patent portfolio. The decision to wait for patent expiry or to design

5 Mark v Teva [2013] EWHC 1958 (Pat).

6 Warner Lambert v Sandoz [2013] EWHC 3153 (Pat).

around or challenge relevant patents that are currently in force depends on the strength of the patent protection and also on the company's attitude to risk. In addition, there may be commercial factors such as the generic competitor landscape that point in favor of one particular approach.

Generic companies will also evaluate the overall commercial landscape and the patent landscape across a number of different drugs in order to identify "low hanging fruit" opportunities where patent coverage may be weak or nonexistent. In those cases there may or may not still be regulatory data protection which is in force.

The generic company's appetite for risk and also its experience of litigation plays an important part in the ultimate strategy that is deployed. A number of generic manufacturers have very sophisticated launch strategies and have substantial experience of patent litigation in Europe and the United States. Generally, those companies are well placed to understand the patent landscape and the risks and opportunities associated with challenging unexpired patent rights through the various national courts, national patent offices, and centralized patent systems such as the European Patent Office (EPO) or the Eurasian Patent Office.

26.7 Strategies Adopted by Innovators

Innovator companies quite naturally want to protect their investment in research and development in bringing a new drug to the market. Once the identity of a new active compound is known and its manufacturing route and formulation is in the public domain, it becomes very easy for a generic copy of the drug to be produced without an excessive amount of research. The patent system therefore aims to allow the innovator the period of market exclusivity during which this investment can be recouped. The problem arises when trying to balance, on the one hand, the need for a fair return to the originator in order to promote innovation and, on the other hand, the need to provide access to medicines at reasonable cost for a wide variety of patients. The latter aim is achieved by having a healthy, competitive generic drug market that sits alongside the innovators. Establishing this balance is a political minefield and the subject of extensive and continuing debate between the various stakeholders.

In the period leading up to and subsequent to the drug launch and prior to expiry of the patent term, the innovator company needs to maintain its exclusivity on the market and maximize its return. The patent filing and prosecution strategy of patents that cover various aspects of the new drug plays an essential part in maintaining the exclusivity. Ultimately, the majority of the value of the drug to the innovator is protected by the main composition of matter patent covering the drug itself. Various "follow-on" or "secondary" patents may be obtained for developments around the drug compound, and the value of these depends greatly on the strength of the composition of matter patent itself and also on the relative expiry dates of the composition of matter patent and any subsequent secondary patents. If a strong secondary patent can be obtained, then the franchise for the drug product may be extended for a number of years beyond the basic

patent expiry date. In financial terms this can be worth £100s millions or even £s billions to the originator. Subject matter that is capable of protection by one or more secondary patents includes intermediates, processes, formulations, medical uses, isomeric forms of the drug, salt forms, polymorphs, and solvates. These are discussed in turn in more detail below.

In an ideal scenario, the innovator will have obtained, or be in the process of obtaining, protection for various developments around the drug in addition to composition of matter protection for the active drug itself. It is an inevitable fact of the development process that different types of innovation occur at different stages of the discovery process, with the earliest innovation being the identification of the drug compound itself with later developments relating to product presentation and use. As more information becomes known about the compound and how it will be used, then further opportunities present themselves as discussed below. These different opportunities then effectively provide the innovator with a continuing timeline for filing new patent applications.

Developments are occurring on a continuous basis from the moment of lead optimization and drug candidate nomination right through to the stage of clinical trials and the approval process and beyond. Indeed, development of the drug product is a continuous process that occurs in response to clinical and market needs and is maintained subsequent to the launch of the approved drug on the market. The opportunity therefore arises for the innovator to be able to file patents at various stages of the drug development process along a relatively extended timeline in order to capture innovations as and when they arise.

The degree to which these further opportunities are pursued and exploited varies from one innovator company to another, depending on their internal corporate strategy. Some innovators are fairly pragmatic about the ultimate value of such "follow-on" patents and selective in choosing subsequent developments for patent coverage, whereas other innovators adopted a far more aggressive and speculative stance in attempting to cover as many incremental developments as possible. Of course, in the latter case not all of these incremental developments are capable of being granted or if granted are highly likely to be invalidated by the courts subsequently.

The patent system enables a variety of innovations around the drug product to be protected. Novel intermediates prepared *en route* to the active may be covered by patent claims directed toward the compounds *per se*, and patent claims may also be obtained for a process either for making a new compound or indeed for making known compounds. The existence of patent claims for novel intermediates provides strong patent protection that is capable of blocking a competitor from following the manufacturing process through to the active drug. The intermediate compounds must be new compounds in order to be capable of patent protection.

It is also possible to obtain patent protection for particular dosage forms of the active ingredient. For example, the presentation of the drug in a dosage form such as a tablet, capsule, powder, etc. may provide some clinical benefit, which means that the particular formulation can then be patented. The patent claims would effectively cover the active drug product in combination with various pharmaceutical excipients when presented in a particular dosage form.

In other circumstances, the nature of the drug itself may lead to technical challenges when formulating the drug for administration to a patient. This type of difficulty arises when a drug is either more or less soluble than would be ideal for formulating, leading to problems with bioavailability absorption when administered, and therefore requires the use of particular excipients to manage that problem. Other problems arise when a drug is relatively stable or is likely to be unstable once in contact with the gastric media. Dosage forms can therefore be designed for immediate release on ingestion, for delayed release such that they are not released until they reach the lower part of the GI tract, or for sustained release in order to provide a continuous steady dose of the active drug. The types of dosage forms that are suitable vary according to the particular indication being treated and the nature of the drug being formulated. However, all of these different factors provide an opportunity for an originator to protect innovations around the dosage form of the drug.

Isomeric forms, different salt forms, solvates, and polymorphs all represent patentable subject matter that is potentially also available to the originator. Thus, different salt forms, solvate, or polymorphs that may have improved solubility or bioavailability or other clinical or formulation benefits are patentable in principle. One well-known example of this concerns omeprazole, the proton pump inhibitor, which was originally marketed in racemic form before its *S*-enantiomer was subsequently developed and launched as a single isomeric form of the same chemical entity. A whole series of patents was filed around the single isomer covering the material itself and formulations of the material. The later filing date for these patents meant that the expiry dates of the *S*-omeprazole patents therefore extended well beyond the original expiry date for the racemic omeprazole.

The patent system also allows the protection of the use of a new or known compound in medicine. Patent claims covering this type of subject matter are normally known as "medical use" claims. In the case of a new drug compound that was previously unknown, it is possible to obtain claims for use of the drug in medicine in general terms as well as claims toward the use of the drug in treating a particular disease. In the case of a compound that is already known, it is also possible to obtain patent coverage for the use of that known compound in the treatment of a new disease and indeed for further new diseases that are subsequently discovered. Patent claims to this type of subject matter are known as second medical use claims.

One of the most well-known examples in recent years concerns the failed angina drug sildenafil (Viagra), which was subsequently approved for use in treating male erectile dysfunction. Since the compound itself had been known for a considerable time at the point at which it failed to be approved for cardiac use, the patent term on the compound itself was largely spent. However, the discovery that this compound had a second hugely valuable medical use meant that patent protection was essential for protecting the exclusivity of sildenafil. In that particular example, key patents were of the second medical use type directed toward the use of sildenafil in treating male erectile dysfunction. Of course, the patent exclusivity sat alongside the regulatory data exclusivity with the two periods running in parallel. In that case, the data exclusivity was also an

important component of protecting that particular drug given various problems with the patent position that subsequently came to light.[7]

In Europe the language of medical use claims is usually in the form of "use of drug X in the treatment of disease Y," whereas in the United States, this type of claim is normally presented in the form "method of treating disease Y by administering drug X." Different jurisdictions around the world have different provisions as to how these types of claims may be presented, with some following the US format and others following the European format. A number of jurisdictions, including Argentina, Uruguay, Venezuela, Colombia, India, Pakistan, and Indonesia do not allow any medical use claims. The availability of medical use claims in particular territories is relevant to an originator's overall strategy for protecting a new drug.

A related type of patent claim that can also be obtained in some countries concerns dosing regimens. For example, if a particular drug X is used to treat a disease Y by administration of the drug in a certain dose three times a day, it is possible to obtain patent protection for a suitable formulation that can be administered only once a day for treating the same disease. The justification for such a patent might be better patient compliance or better clinical efficacy or reduced side effects. Again, this provides an originator with an opportunity to protect further innovations around the drug and its therapeutic use.

A synthetic process must contain some novel and inventive technical features to enable process claims to be obtained for a new or existing process. Process developments may include a change in the route itself, using different starting materials and reagents, or maybe a development of an existing route using different conditions and/or solvents or catalysts. In either case, provided that the new route or the improved version of the existing route provides some technical benefit, then the process may be the subject of a patent. This type of protection is particularly important if there is only a single route to a particular end product.

More usually, several different routes exist to an end product and patentable improvements reside in optimizing the process conditions of a particular route to increase the yield of the final drug product. This reduces the material costs and allows the drug to be produced at lower cost. In other cases, one particular route may offer a better yield or fewer steps than competing routes and thus represent a large cost advantage relative to those of other routes. This is particularly so in the case of a structurally complex drug that may have more than one stereocenter (i.e. more than one chiral atom). Pfizer's sertraline is an example of such a drug as this compound has two chiral centers, and consequently four possible isomers can be produced in the chemical synthesis. In such a case, the production of the "wrong" isomers represents waste product. It therefore becomes necessary to design a diastereoselective synthesis to the "correct" isomer or engage in lengthy and wasteful separation processes to obtain the desired single isomer.

The existence of drugs in enantiomeric or diastereomeric form presents further opportunities for an originator to file additional patent applications around the drug under development. Such patent applications might be directed toward particular isomers themselves as active ingredients, processes for obtaining

7 Lilly Icos v Pfizer [2002] EWCA Cir 1.

improved relative ratios of isomers or obtaining isomers in single isomeric form, or methods of separating isomers. The practice of filing secondary patents directed toward these types of improvements is widely used in the industry.

Figure 26.1 indicates the different types of patent claims that are potentially available to an innovator when embarking on a drug discovery program.

Secondary patents are generally harder to obtain and usually require good evidence of the alleged improvement in order to be granted. In addition, such patents are harder to enforce and are not as strong as the original composition of matter patents because by the time they are filed, there is usually a substantial amount of information available about the drug product and its therapy. Thus the prior art landscape for a patent directed toward development such as these is frequently much more crowded than the prior art landscape surrounding the original drug.

Secondary patents are inevitably narrower in scope than the earlier filed composition of matter patent for the drug. At the same time, provided the commercial product is adequately covered, they can provide a useful extension to the monopoly available to the originator. The interplay between the scope of

Figure 26.1 Patentable subject matter in a new drug portfolio.

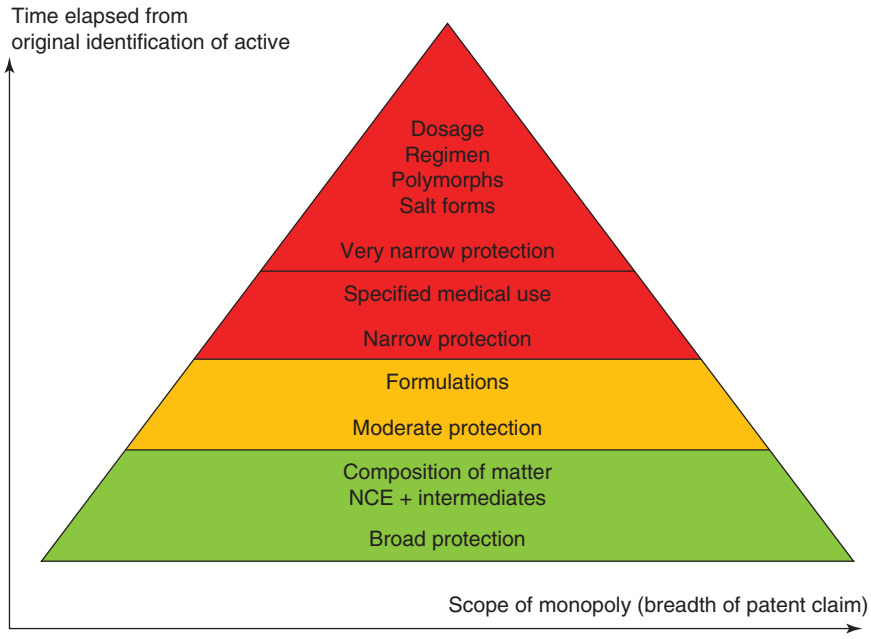

Figure 26.2 Relationship between time of filing and scope of claim for a new drug patent portfolio.

the various types of patent and the filing date can be represented as shown in Figure 26.2.

The strategy of extending the patent monopoly around a particular drug has been called "evergreening." In fact, there is a spectrum of approaches adopted by different originators to protecting innovative developments during the preclinical and approval stages of research. It is entirely legitimate for the originators to protect reasonable developments that are the subject of extensive and costly research. At the same time, there are examples of abuse of the patent system in which minor and perhaps unmeritorious development becomes the subject of patent applications and possibly even of granted patents. It is those examples that are perhaps the intended target of the label "evergreening." In certain instances, overzealous use of the patent system to block competitors may result in a referral to the competition authorities in Europe and/or the antitrust authorities in the United States because of the ultimate effect it may also have on patients.

Interpretation of the meaning of this term therefore depends heavily on an individual's standpoint within the industry. This can be seen from the different perspectives of the generic and the originator. On the generic side, the industry body Medicines for Europe (formerly the European Generic Medicines Association) considers evergreening as unacceptable because it represents the stockpiling of patent protection by obtaining separate 20-year patents on multiple attributes of the same product. In contrast, GlaxoSmithKline's public policy position considers the term "evergreening" to be "an inherently pejorative term. It is used by some to convey the false impression that research-based pharmaceutical companies

abuse the patent system by obtaining patents on what are characterised as minor improvements to existing medicines."[8] A more neutral position appears to be adopted by Wikipedia, which defines it as "a variety of legal and business strategies by which technology producers with patents or products that are about to expire return rent from them by taking out new patents."[9]

In keeping with the sentiment at the beginning of this section, innovative companies will therefore employ strategies of filing secondary patents at various stages throughout the development process. In certain cases, developments that are conceived at a relatively early stage in the timeline may be kept back until later in order to push the eventual patent expiry date for any patent that might be granted on the development further into the future. This strategy has to be balanced against the risk of an increasing body of prior art and competing research by outside entities on the drug and on the therapeutic area. This may have adverse consequences for the future patentability of that particular development.

For example, external organizations such as universities and clinical research organizations may be conducting independent research and therefore not be under any duty of confidentiality. It is also possible that generic companies may be actively conducting research into the subject matter in anticipation of a launch date a few years in the future, and the more proactive generic companies may well seek their own patent protection for such developments. The timing of the filing of any secondary patent applications in relation to a particular development will also depend on the nature of any earlier patent applications that have already been filed in relation to the drug. For example, the prior art status of earlier filed pending patent applications concerning innovation around the drug that have not yet been published is different from the prior art status of earlier published patent applications for drug developments that might be pending but as yet unpublished.

The timing of the filing of any patent applications for subsequent secondary patents may therefore be dictated by the timing of publication of earlier applications. The innovator will therefore balance considerations around the existing prior art landscape and possible future publications (both internal and external) and the consequent need for an early filing date for the development against the desire to obtain a later patent expiry date by delaying the filing of a secondary patent application. Substantial effort will be devoted to optimizing this strategy in the light of the particular commercial circumstances for the drug development program and also in the light of the prior art landscape.

The fundamental fact remains that any patent, irrespective of whether it is a patent for a new drug compound or a secondary patent based on some additional information about the method of producing the drug or use of the drug, still needs to satisfy the basic criteria for patent protection. In that sense, an original composition of matter patent has exactly the same status *vis-a-vis* validity and enforcement as a secondary patent and is judged against exactly the same legal standards.

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8 Glaxo – https://www.gsk.com/media/2949/evergreening-policy.pdf.

9 Wikipedia – https://en.wikipedia.org/wiki/evergreening.

Patents for approved drugs, together with agrochemicals, are in the unique position of having their lifetime extended in various jurisdictions. In Europe, it is possible to extend a pharmaceutical patent by up to 5½ years using an SPC that allows an extension of up to 5 years and a pediatric extension that allows a further extension of 6 months. The corresponding provisions in the United States allow an extension of up to 5 years (this is separate for the patent extension that can be obtained for any patent in the United States to compensate for prosecution delays in the USPTO). In either case, the extension is available in the statutes as a public policy decision in order to compensate originators for the substantial delays normally encountered in obtaining approval to market a pharmaceutical product. In Europe, for example, the public policy decision was taken to allow a maximum of 15-year patent life and that is the basis of the maximum term for an SPC being 5 years. The term is calculated by considering the period between the filing date of the patent and the date of approval of the drug, subtracting 5 years from that time period and then adding the resultant time period to the end of the normal 20-year patent life. In the case of drugs that are approved early on in their lifetime, the additional term is consequently either nonexistent or very small, but in the case of the drug that is approved later in its patent lifetime, then the SPC term added to the patent can be valuable. Indeed, an SPC is in principle available for any patent that covers an approved drug for which approval was granted more than 10 years after the filing date of the patent. This type of patent extension can be used to extend patents covering the drug itself, a combination of drugs, processes for making the drug, and uses of the drug in particular indications.

Consequently, originator companies devote considerable resources to obtaining patent term extensions where available and to developing suitable secondary patents that may also be capable of supporting a patent term extension. There is a general restriction in the law governing the provision of patent term extensions in Europe (Article 3(c) Regulation No. 469/2009) that stipulates that only a single SPC may be granted to an originator in respect to an approved product even if there are several possible patents belonging to the originator that could in principle be used as the basis for the SPC. It is therefore important to evaluate the strengths and weaknesses of each of the basic patents that could be used to support an SPC in order to determine which is most likely to be enforceable. It is not simply an automatic decision of choosing the latest expiring patent to extend because that patent may be weaker, and therefore more open to challenge, than an earlier expiring stronger patent.

Patents that have been subjected to patent term extensions can also be challenged and invalidated in exactly the same way as any other patent on the basis of prior art or because of some lack of internal validity. There are various reasons why a patent may lack internal validity such as lack of sufficiency (the invention not being properly described) because the invention does not provide any technical benefit or because subject matter that extends beyond the application originally filed has found its way into the application during prosecution to grant. Extended patents may be challenged in exactly the same way on these grounds in addition to legal challenges that are also possible regarding the legality of the filing of the SPC or other patent term extension. These legal challenges are invariably based on an interpretation of the law (Article 3(c) Regulation No. 469/2009) and

concern issues such as whether or not the drug product falls within the definition of a medicinal product according to the relevant statute and whether the MA was a valid MA.

Differences in the ease with which a secondary patent can be obtained and also the ease with which they can be enforced are simply a function of the prior art landscape and the supporting data contained within the patent. Supporting data is invariably needed to demonstrate a particular technical benefit and therefore justify the technical reason for the patent. Similarly, there are many instances of situations where the law is perhaps unclear on a particular aspect and consequently where applications for patent term extensions have been sought. In some of those cases, patent term extensions that have been granted are subsequently found to be invalid because they are based on an incorrect interpretation of the law. This means that there are instances in which any granted patent, whether it relates to an initial composition of matter or a secondary patent to a process or some other development, may be open to revocation. It also means that there are instances in which a granted patent that has been extended to compensate for delays in regulatory approval may also be open to challenge.

The grant of a patent or indeed an extension to a patent is not a guarantee by the patent office of the validity of the patent. This point is relevant to the discussion of generic company strategy because not all patents that are granted are valid and enforceable. This means that there are opportunities for alert, proactive generic companies to launch a generic drug product before the normal expiry of the patent term or extended patent term. Part of the evaluation of the commercial and legal landscape in advance of the launch involves a thorough assessment by the generic company of the strength of the patent and regulatory exclusivities afforded to the drug. At the same time, the originator will have been conducting exactly the same analysis to make an assessment as to when their exclusivity period is likely to expire, notwithstanding the nominal patent expiry date. This information will also be used to inform the originator's ongoing patent origination and prosecution strategy as well as its strategy in preparation for future litigation.

26.8 Strategies Adopted by Generic Companies

Once the identity of a new active compound is known and its manufacturing route and formulation in the public domain, it becomes relatively easy for a generic copy of the drug to be produced without an excessive amount of research. The patent system therefore aims to allow the innovator the period of market exclusivity during which this investment can be recouped. However, generic companies will be conducting extensive research on currently approved drugs that are scheduled for patent expiry.

In this respect, the generic companies have many years advanced warning of the anticipated date of patent expiry and regulatory data exclusivity expiry. The generic companies are aiming to bring their version of the generic drug to market as quickly and cheaply as is possible in order to maximize their returns on

their own investment in producing a generic product. The generic price is usually substantially lower than the originator price that is charged during the exclusivity period. In addition, further price erosion may occur as further generic companies launch their own competing generic products and gradually saturate the market. In certain cases, generic companies have a strategy of pursuing niche areas where there is likely to be less generic competition. However, for the major blockbuster drugs that are coming up for patent expiry, there is generally substantial generic competition. Each generic company wishes to be the first to launch, a generic product.

Generic companies may have expended considerable effort in investigating aspects of the approved drug and the commercial manufacturing process. Many will also have investigated different synthetic routes to the drug or variations of the existing route that might avoid the risk of patent infringement. Similarly, an analysis of the commercial drug formulation will have been performed, and the generic company may choose to launch either an identical formulation or a formulation that it may assert is bioequivalent but which is based on either slightly different proportions of the drug and excipient components or that employs some different excipients. Assuming that patent protection is still in force for secondary patents to processes and or drug formulations, then the decision as to whether or not to follow the approved commercial process and to use the approved commercial formulation will depend on the patent position.

Some generic companies will choose to launch at risk; however, given the attitude of the courts, especially in the United Kingdom, regarding the need to "clear the way" and ensure that there are no relevant patent rights, this approach is usually less common than a structured approach of patent monitoring and challenging selected patents or waiting for patent expiry. The likelihood of the grant of an injunction against the generic is a significant consideration when assessing the launch position during the pendency of a relevant patent.

The originator may become aware of a rival generic product following an application by a generic company for an MA for a generic version of the product. This will normally occur after expiry of the regulatory data exclusivity period but before expiry of the patent exclusivity period. The generic company will rely for its MA on the data used by the originator in gaining approval for the original version of the drug. This means that the generic version of the drug is likely to be either identical to or very similar to the original version of the drug if the generic is able to use the originator's regulatory data for approval. This means that there is a high likelihood of patent infringement of any patent that is still in force at that time. Of course, the generic may simply be obtaining this MA in readiness for launch after expiry of the patent exclusivity period. Equally well, the generic may be planning to launch before the expiry date of the patent exclusivity period. This may be a launch "at risk" or a launch following an attack on the validity of the patent. In either case, the originator is effectively on notice of a risk to its franchise for the drug.

Once an MA has been issued to the generic, the originator will usually contact the generic company for clarification regarding its marketing intentions. The generic company is under no obligation to provide this information. At this stage, the originator will be in the process of making extensive preparations for litigation

should it be necessary to apply for a preliminary injunction at short notice. In the event that no response is received from the generic company, in certain cases it is possible for the originator to seek a preliminary injunction to prevent launch. Each case is judged on the particular facts and merits of the case. The behavior of the generic will influence the balance of convenience that is considered by the court. It is therefore important for the generic company to do as much work as possible by removing (invalidating) relevant patents prior to launch of a generic drug. This will allow the generic to demonstrate to the court that they have acted in a reasonable manner and will reduce the risk of an injunction being granted against them. Equally, if the generic company has built up a body of evidence by thorough prior art searching and/or experimental evidence showing that a particular patent is clearly invalid, then this evidence can be persuasive in preventing a court in granting an injunction.

Since an injunction may be granted to the originator if it can be alleged that there is likely to be substantial damage to the market, the generic needs to be able to demonstrate to the satisfaction of the court during a preliminary hearing that either there is a clear case of patent invalidity or clearly show that patent infringement is not occurring. This means the generic company must have a complete and accurate view of the patent landscape before any launch is anticipated. Ideally, such work should be done well in advance of any MA being granted for the generic since the grant of the MA is likely to be a trigger for an action by the originator.

The simplest and safest course of action for any generic company is to wait for expiry of the patent exclusivity period. However, generic companies frequently wish to launch earlier than that or at least be in a position to launch as soon as patent expiry occurs. In certain cases, a generic company may choose to challenge the validity of the patents. This is normally done through the national courts of the jurisdiction in question since, frequently, the date of approval of the originator drug is many years after patent grant. This means that the time period for filing any post-grant patent opposition to invalidate a recently granted patent is likely to have long expired. In those cases where opposition is still possible, this can be a powerful and relatively economical way of revoking a relevant patent *ab initio*.

The normal situation, however, is that patents of interest are usually being scrutinized and challenged many years after the window of filing oppositions or other post-grant challenges in national patent offices has expired. This means that it is left to the national courts to deal with both issues relating to patent infringement and also challenges to patent validity that are mounted by generic companies. It is possible to challenge the validity of a patent in a number of jurisdictions in the national patent offices during the pendency of the granted patent, but this route is never used because of the complexity of the issues at stake and the high value of the subject matter being litigated. Even if such an action were to be commenced in a national patent office, it is highly likely that the patent office would take the decision to refer it to a national court.

The generic company planning a launch before patent expiry therefore either has to ensure that they are not liable for patent infringement or be confident that the patent can be invalidated in revocation proceedings. The generic

product may be designed so that it does not infringe the relevant patent in the case of a secondary patent such as a formulation, salt form, or process patent. Alternatively, it may well be the case that the product falls exactly within the scope of the relevant patent in which case the generic company then has the burden of seeking revocation of the patent.

A good strategy for a well-prepared generic company to adopt in such circumstances is to prepare a strong invalidity attack against an originator and approach the originator before approval of the generic version of the drug to see if a negotiated position can be agreed. If not, and assuming that the generic company is confident of its position concerning the validity of the relevant patent, the generic can then make effective preparations for launch of the drug once marketing approval has been obtained but without progressing as far as effecting an actual launch of the drug. The generic company can then launch a challenge to the validity of the relevant patent in the knowledge that it will immediately be countersued for patent infringement. At this point, in order to avoid any risk of an injunction, the generic company can then demonstrate that while it is ready to launch a generic product, it will instead give a firm undertaking that it will not launch the product in return for a cross-undertaking in damages. If the generic company is then ultimately successful in revoking the patent, it can claim damages for loss of sales in respect to the period in which it would have been able to launch a drug but for the existence of the undertaking not to do so. The court will assess the size of the market and the distribution network and reach of the generic and make an assessment as to what proportion of the total sales might otherwise have been due to the generic. The anticipated sale price, which would obviously be less than the originator's price during the exclusivity period, would then be estimated. Damages can then be calculated from this data and would then be awarded to the generic. The generic would effectively have obtained revenue without ever having to launch and sell any product although it is essential that they would have been in a position to do so.

An example of this approach arose in the GSK versus Glenmark case[10] in which Glenmark sought revocation of GSK's Malarone™ patent because it was seeking to launch a generic version before patent expiry. In that particular case, proceedings were initiated before patent expiry (but did not actually conclude until after patent expiry) for patent revocation. There was a counterclaim for patent infringement by GSK, and Glenmark agreed not to launch the drug on the market in return for a cross-undertaking in damages in the event that the patent was ultimately found invalid. At trial, and subsequently at the appeal hearing that followed, the patent was considered to be invalid, and Glenmark were awarded an undisclosed sum in damages to compensate for not having launched their generic Malarone.

This strategy may be quite effective for a generic company because, in the event of success, the generic has effectively been compensated for loss of sales without ever having to have distributed any product. If the particular drug target has been carefully chosen and there is unlikely to be a significant amount of further generic competition, pending resolution of the dispute, this approach can

10 GSK – Glaxo Smith Kline v Glenmark [2013] EWHC 148 Pat.

be beneficial to the generic company taking the initiative to challenge the patent. One disadvantage of being the first generic company to secure revocation of the patent is that it effectively opens up the entire field for any other generic competitors. Thus, in the case of a drug that is likely to be the target for a number of generic companies, this approach may or may not be appropriate. The ultimate decision on whether or not such a strategy is suitable in any particular case depends entirely on a commercial assessment of the market. However, if the first generic company is well prepared for launch in advance of the litigation, they may still benefit from a first mover advantage.

The latter case in which the generic product is likely to infringe a relevant patent is fairly typical of the normal situation that arises because generic products aim to be as similar as possible to the originator product. This is partly to ensure market penetration and partly to be certain of obtaining regulatory approval on the grounds of the generic drug being bioequivalent to the originator drug. The need for bioequivalence is important to the generic drug manufacturer because it avoids the need for the generic to conduct extensive studies for the purposes of obtaining regulatory approval. Instead, the generic can simply base their approval on the clinical data provided by the originator during the regulatory approval process, provided that the data exclusivity period has expired. Consequently, the issue of whether or not patent infringement is occurring is generally quite finely balanced because the generic version will either be identical or be closely similar to the originator product.

As part of the preparations for generic launch, the generic drug company will conduct extensive prior art searching and obtain opinions from an outside counsel as to the validity of the patent. Depending on the outcome of those investigations, the generic company may then choose to launch a preemptive challenge to the validity of the patent in the national courts. This action for patent revocation is likely to be met by a counterclaim from the originator for patent infringement. One benefit of this approach is that the commencement of litigation may lead to a settlement between the originator and the generic that retains the patent in force and at the same time allows the generic involved access to the market in return for an undisclosed license. In many cases this may be a free license provided that the generic company terminates its challenge to the validity of the patent. Any agreements concluded on this basis must be carefully examined since, in certain circumstances, these types of agreements can be considered to be anti-competitive and may be challenged by the competition and antitrust authorities.

A comprehensive evaluation of the prior art landscape will involve extensive patent monitoring to understand the originator's IP position. This will involve regular searching of the patent literature to establish which of the originator's patents, if any, are relevant to the generic's own product. During the evaluation of a suitable commercial formulation by the generic company, the generic company will perform an assessment as to whether or not the proposed formulation is likely to infringe any of the originator's patents. The analysis will also involve an evaluation of third-party patent rights to ensure that there are no other relevant third-party patents that might give rise to unwanted litigation.

A detailed knowledge of the originator's IP position may also reveal opportunities for the generic to obtain their own patent coverage for specific developments

around the drug. This may include, for example, new formulations of the drug, new processes for producing the drug, and new salt forms and polymorphs. The generic may choose to file their own patent applications for these developments, effectively preventing the originator and other generic competitors from marketing products covered by these patents. Such patents may even have value in cross-licensing situations and allow the generic company to negotiate with the originator for better access to the drug compound.

A generic company will not only search for prior art in the patent literature but will also search through the scientific literature. It is frequently the case that useful prior art documents that have not been identified by a patent office come to light during litigation between parties to invalidate a patent. This is not a reflection of the quality of the patent examination process. The national patent office in most countries generally do a thorough job of searching and examining patent applications and have extensive libraries and databases. However, it is impossible for the patent offices to identify every single potentially relevant piece of literature that might lead to patent revocation. The results of the patent examination and the ultimate grant of a patent are based on thorough searches of the patent literature and some limited scientific literature by the patent office.

This is a pragmatic approach, which is intended to ensure that, as far as possible, the patents that are granted are likely to be valid (or at least there is no reason to believe that they are invalid). As a matter of public policy, it is considered that a motivated third party seeking to invalidate a patent, such as a generic company, will perform the necessary searching to determine if there is any literature that is more relevant than that considered by the patent office during the prosecution of the patent to grant. The third-party challenger can then bring this prior art to the attention of the national courts during any challenge to the validity of the patent. It is generally accepted that the system could not really operate in any other way since it would be impossible for national patent offices to devote the necessary resources to be in a position to state beyond doubt that every patent granted was valid. In the prelaunch period, the generic has the opportunity to conduct wide-ranging searches and also to perform experimental tests about the subject matter of the patent as part of its preparations for any challenge to the validity of the patent.

In an alternative strategy, if the generic company is confident that their formulation does not infringe any relevant patent rights, there is the option to seek a declaration of non-infringement from a court. In the first instance, the generic would contact the originator with details of their product and the proposed launch and ask for confirmation from the originator that this will not constitute patent infringement. If the originator does not provide the requested confirmation, the generic may then apply to the court for a formal decision on the point.

A related type proceedings concerns action for declaration of invalidity. A generic company can apply to the court for a declaration of invalidity if it considers that an originator's patent lacks validity. The basis for such an application would usually be relevant prior art that is prejudicial to the maintenance of the patent.

Generic companies are frequently finalizing their launch strategies either toward the end of the normal patent term or during the period of a patent term extension. Part of the analysis of the strength of the patent will also involve an analysis as to the strength and validity of any patent term extension that has been granted. There are numerous examples in the recent case law before the national courts, and also numerous referrals to the Court of Justice of the European Union (CJEU), on the issue of the validity of SPCs granted for pharmaceutical products. Many of the requests for revocation of the patents have been upheld, but this fact simply reflects the huge value that a potential SPC can provide to the originator, and consequently the fact that SPC extensions will be sought wherever possible even if the chances of success are marginal.

26.9 Conclusion

The patent system provides both opportunities and challenges to the originator and to the generic.

It is important to recognize that relevant third-party patents can be overcome by negotiation or litigation. Many granted patents for approved drugs may not stand up to scrutiny in the face of a motivated challenger expending substantial resources investigating the prior art and the technical landscape.

Originator companies need to strike a fair balance between protecting developments of their innovations with secondary patents and at the same time avoiding potential allegations of abusing the patent system by pursuing this strategy too extensively. It is reasonable and proportionate for significant developments around a new drug to be protected, and the patent system is intended for this purpose. It is legitimate for originators to seek to protect developments arising from preclinical evaluation, and the issue is simply one of degree. Returning to fundamentals, if there is a genuine technical effect arising during the development phase, for example, perhaps due to an improved formulation or salt form, which will manifest itself in a measurable patient benefit, then it is right that this should be capable of protection within the framework of the IP system. This should allow an originator to obtain a reasonable return on their substantial investment both in the discovery phase and in the preclinical phase.

Equally, the generic companies have an obligation when bringing a generic product market to investigate thoroughly the patent landscape. If any relevant patent rights are found, then the onus is on the generic company to address the issues, whether it is by negotiation, design around, or a challenge to the validity of the patent rights. The generic company should expect to find that the patent landscape around a development candidate or an approved drug will include secondary patents based on technical improvements. The difficulty arises in sorting those that are meritorious of patent protection from those that are not.

The industry as a whole is changing as both originators and generics have expanded their areas of interest into the other's territory. Increasingly, the lines dividing originator and generic are becoming blurred, and there are many originator firms that have substantial business interests in the generic market

also. Similarly, there are a number of generic firms that are now originating their own drug candidates. In addition, many of the generics now pursue sophisticated patent origination strategies. Some of these are intended to protect their own innovations, and some of them are intended as either blocking or negotiating tools. The field of patent law will therefore continue to develop and evolve as increasingly complex innovations are brought to the market.

27

Patent Considerations in Collaborative Drug Development

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27.1 Introduction

There are several types of intellectual property (IP), which may be at issue in collaborative research, including patents, trade secrets, trademarks, and copyrights. This chapter will focus on patents and trade secrets. While trademark and copyright concerns may arise, such concerns would largely be the same as the ones occurring in any course of research and publishing and which the readers are more than likely sufficiently aware, e.g. the copyright protection of journal articles and treatises, which must be respected. As will be seen below, while there are definite pitfalls to be avoided with IP during collaborative drug development, often there is not a "right answer." Rather, the parties should be aware of the various concerns and risks and then make decisions based on business priorities, having weighed those risks and taken the best steps possible to mitigate any negative consequences from problems that arise.

There are several issues that can arise in the course of a collaborative research relationship and that can create problems resulting in costly litigation, jeopardizing the IP rights, and/or damaging the relationship and goodwill between the parties to the collaboration. Some of these issues are considerations of patent infringement. This may arise because one party does not have a clear understanding of the permitted scope of using the proprietary technology of the other party. There may also be considerations of protection of propriety information. This issue sometimes creates a problem when there is a collaboration between a university research laboratory and a corporate research facility. The university research laboratory typically wants to disclose new discoveries quickly to bring more recognition to the research group, thereby attracting more funding. Companies, on the other hand, typically want to maintain the confidentiality of a new discovery, at least until such time that discovery is considered to be developed enough to be patentable and possibly commercially viable. As a result, there are often nondisclosure agreements in place in a collaborative research effort, which may prevent a party from disclosing the invention. A third area in which problems often arise with collaborative research regards the ownership of new inventions. Problems can arise when research agreements do not clearly define the ownership

Early Drug Development: Bringing a Preclinical Candidate to the Clinic, First Edition.
Edited by Fabrizio Giordanetto.

rights to newly developed inventions under the research agreement. In addition, as will be seen below, the wording of agreements must be carefully considered to ensure that the agreement legally provides for what was intended by the drafter. This chapter will consider these issues, as well as some steps that may be taken to mitigate the risk of these problems.

IP is important in collaborative research for a number of reasons. First, it is important to have proper protection for any collaboratively developed research. As noted above, patent protection is considered to be a vital component of pharmaceutical drug development. The IP is an essential asset in both attracting and conducting collaborative research in the pharma arena. It is important to ensure that proper steps are taken to protect that asset, whether in the form of patents obtained for newly developed technology or the protection of trade secreted information, which may be shared in the course of the collaboration. The simple reality is that a pharmaceutical company will have limited (if any) interest in entering into a collaboration if adequate protection in the form of a patent cannot be obtained for any new technologies developed as the result of the collaboration.

From the opposite perspective, prior to entering into any new research area, it is important to know what patent exclusivity already exists in that area. No pharma company wants to invest in research that is not at least arguably free from infringing another entity's patents.

27.2 What is Intellectual Property?

27.2.1 Patents

A patent system in the United States is provided for in Article I, Section 8, Clause 8 of the US Constitution, which states, "To promote the Progress of Science and useful Arts, by securing for limited Times to Authors and Inventors the exclusive Right to their respective Writings and Discoveries." Title 35 of the US laws codifies the statutes, which govern patents and defines "patent eligible" subject matter, the requirements for a patent application, how patents may be enforced, defenses against patent infringement, etc. Patents are often mislabeled as being a "monopoly." This is somewhat of a misnomer. A patent (whether in the United States or another country) provides to the patent owner an exclusive right to prevent others from making or using the claimed invention, for a limited period of time, in the country in which the patent has been granted. Generally, in most countries, the term of a patent is 20 years from the filing date of the patent application. In exchange for this limited exclusive right, the inventors must disclose to the public, in the form of the patent specification, how to make and use the invention. From the disclosure, third parties may develop improvements on the invention, thus promoting technology advancement. The limited exclusivity given by the patent is used as an incentive for inventors to disclose their invention to the public, rather than maintaining the technology as a trade secret, thus allowing others to understand and improve on the technology.

Patent protection is considered to be a vital part of the pharmaceutical industry. While some market exclusivity is given by the US Food and Drug Administration (FDA) to the filer of a new drug application (NDA), the protection given by a patent is generally regarded as being of a greater value because it is longer in length (20 years from the date of filing of the patent application) and may be broader in protection, going beyond the specific active compounds of the NDA. An entire body of law has developed, which is directed solely to role of patents in the pharmaceutical industry, as detailed in Chapter 24. Patents, in particular, are important in collaborative reach for drug development from two perspectives, i.e. protecting the new development versus possibly infringing a third party's patent in the course of the new drug development.

Some form of patent protection for inventions exists in virtually every country. Many countries are part of the World Intellectual Property Organization (WIPO), which provides global standards and services relating to IP. However, while there is a patent system in most countries and most countries have harmonized key provisions in their patent laws, for example, giving a patent a 20-year term measured from the filling date of the patent application, there are some important differences particularly in the fields of pharmaceuticals and medicine. For example, in India, a compulsory license may be given to a third party to practice an invention if the patent owner is not "working" the invention within three years after patent grant. Under the provisions for compulsory licensing, the Indian Patent Office granted compulsory license to the Indian generic manufacturer Natco Pharma for Bayer's patented anticancer drug Nexavar in March 2012. The compulsory license was affirmed by the Intellectual Property Appellate Board (IPAB) in March 2013 [1]. Various countries (e.g. Brazil and Thailand) have also imposed compulsory licenses for AIDS drugs.[1]

Another fundamental difference related to pharmaceutical patents regards the ability to patent a method of treating a human or animal having a disease or condition. In the United States, it is permissible to patent a method of treating a human or animal. However, in many (if not most) other countries, method of treatments may not be patented. A novel pharmaceutical compound or formulation may be patented in these countries, but a method of treatment *per se* may not be patented.

Issues such as compulsory licenses and the differences in the scope of protection afforded by various countries may be important considerations in entering into a collaborative research project. For example, one party may have the mind-set of obtaining a patent to the invention in virtually every country to potentially maximize the value of the patent portfolio. The other party may not see any value in spending the money on obtaining broad global protection, particularly in what may be considered, at best, developing markets. As will be discussed below, the parties to the agreement must have a concordance in where patent protection will be obtained for any developed technology.

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1 Bridges, published by the International Centre for Trade and Sustainable Development. Vol. 11, No. 3 (2007).

27.2.2 Trade Secrets

Some technology is not amenable to being protected by a patent. This is often the case with manufacturing/production technology. It is often very difficult to prove the manufacturing process used to make a product for the purposes of establishing patent infringement. Alternatively, the process may be so specialized and difficult to reverse engineer that more/longer protection may be found in maintaining the technology as a trade secret. A trade secret can protect a technology in perpetuity, unless independently developed by a third party, or adequate protection is not given to the trade secret, allowing a third-party access to the technology. The value of trade secrets was demonstrated in a case surrounding the hormone replacement therapy Premarin. In 2005, Wyeth sued Natural Biologics, Inc. for trade secret misappropriation under the Minnesota Uniform Trade Secrets Act.[2] Wyeth had a method for producing bulk natural conjugated estrogens for use in Premarin. The method had been in use by Wyeth since 1942 and was protected by a trade secret. In 1994, Natural Biologics began communicating with a former chemist of Wyeth and within a year "developed" a process for making a conjugated estrogen, which was the same as Premarin. The court held that Wyeth had taken reasonable efforts to maintain the secrecy of the process, evidenced, in part, by the fact that the process had remained a secret for over 50 years, and Natural Biologics only acquired the process through misappropriation. The court found that the misappropriation of the trade secret constituted irreparable harm, which warranted a permanent injunction against Natural Biologics.

27.2.3 Trademark and Copyright

Trademark and copyright issues also play a role in the pharmaceutical industry. This chapter will only briefly discuss the role of trademarks and copyrights in the pharmaceutical industry, as these are usually not at issue in collaborative drug development. "A trademark is generally a word, phrase, symbol, or design, or a combination thereof, that identifies and distinguishes the source of the goods of one party from those of others. A service mark is the same as a trademark, except that it identifies and distinguishes the source of a service rather than goods."[3] Trademarks play a large role within pharmaceuticals, most importantly by creating drug identity for a new drug. The International Trademark Association released a short overview of the importance of trademarks within pharmaceuticals, which may be found at http://www.inta.org/Advocacy/Documents/INTAPharmaceuticalTrademarksPublicHealth2007.pdf.

Copyrights protect artistic and literary works and also play a role in the pharmaceutical industry, although not as important a role as trademarks. Copyrights most often arise in advertising media associated with a drug product. One important area where the question of copyrights has played a role with pharmaceuticals is with regard to drug labels. The FDA requires a generic drug to copy the label of the brand drug for prescribing information. However, the drug label is an original

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2 395 F.3d 897 (8th Cir. 2005).

3 From the USPTO "Protecting Your Trademark, Enhancing Your Rights Through Federal Registration" https://www.uspto.gov/sites/default/files/documents/BasicFacts.pdf.

written work of the innovator company. Can a generic drug company copy the prescribing label of the brand drug without creating a copyright infringement issue? This issue was addressed by the US courts in 2000, and it was held that a generic drug company cannot be held liable for copyright infringement by copying the reference label as required by the FDA and the Hatch–Waxman Act for an ANDA submission.[4]

27.3 Before the Research Begins

Before any research begins in a collaborative relationship, a clearly written research agreement is needed. Having a well thought-out research agreement in place is important to protect the interests of the parties, both collectively and individually, and mitigate the costs to the parties in the event of a disagreement. There are several key issues that the research agreement should address, which are addressed in detail below. However, before drafting and signing a joint development or joint research agreement, some earlier steps need to be considered.

Having a written collaborative agreement in place prior to beginning the research is also important to be able to maximize the patent protection of the invention. Not having a written research agreement in place before the research commences can jeopardize the patentability of the invention. In this regard, 35 U.S.C. §102(c) states:

> ***35 U.S.C. 102(c) Conditions for patentability; novelty.***
> (c) COMMON OWNERSHIP UNDER JOINT RESEARCH AGREEMENTS.—Subject matter disclosed, and a claimed invention shall be deemed to have been owned by the same person or subject to an obligation of assignment to the same person in applying the provisions of subsection (b)(2)(C) if—
>
> 1) the subject matter disclosed was developed and the claimed invention was made by, or on behalf of, 1 or more parties to a joint research agreement that was in effect on or before the effective filing date of the claimed invention;
> 2) the claimed invention was made as a result of activities undertaken within the scope of the joint research agreement; and
> 3) the application for patent for the claimed invention discloses or is amended to disclose the names of the parties to the joint research agreement. (emphasis added)

The Manual of Patent Examining Procedure (MPEP) goes on to explain in §2156 that, "In order to invoke a joint research agreement to disqualify a disclosure as prior art, the applicant (or the applicant's representative of record) must provide a statement that the disclosure of the subject matter on which the rejection is based

4 *SmithKline Beecham Consumer Healthcare, L.P. v. Watson Pharmaceuticals*, Inc., 211 F.3d 21 (2nd Cir. 2000).

and the claimed invention were made by or on behalf of parties to a joint research agreement under AIA 35 U.S.C. 102(c). The statement must also assert that the agreement was in effect on or before the effective filing date of the claimed invention, and that the claimed invention was made as a result of activities undertaken within the scope of the joint research agreement."

Thus, if party A and party B are in discussions for a possible joint development project, but party A starts working on the project prior to the joint research agreement being in place, without the scope of the research being defined, the early work done by party A may be prior art against the ultimately developed invention. The type of problem that can arise was seen with *Oddzon Products, Inc. v. Just Toys, Inc.*,[5] in which the Court of Appeals for the Federal Circuit confirmed that secret prior art can be used as "prior art" for determining whether an invention may be obvious and therefore unpatentable. In this case, a confidential design for a toy football with fins having approximately the following shape was disclosed to an inventor (see Figure 27.1).

The inventor was "inspired" by this idea and proceeded to develop the following modified design that was subsequently patented (see Figure 27.2).

The court held that this secret disclosure was prior art to the party receiving the information with respect to the patented design. In explaining how such prior art is considered in an obviousness analysis, the court explained, "This result is not illogical. It means that an invention, A′, that is obvious in view of subject matter A, derived from another, is also unpatentable. The obvious invention, A′, may not be unpatentable to the inventor of A, and it may not be unpatentable to a third party who did not receive the disclosure of A, but it is unpatentable to the party who did receive the disclosure."

The result of this is that "secret" information may be prior art under 35 U.S.C. § 102(f) and 35 U.S.C. §103 to someone who actually knows about an invention. Similarly, in *E.I. du Pont de Nemours & Co. v. Phillips Petroleum Co.*,[6] the court held that certain secret laboratory work of Phillips Petroleum (certain polymers secretly made in its laboratory) was prior art against a subsequent invention by du Pont for purposes of novelty and for purposes of determining obviousness. Thus, if the research is commenced by one party to the joint development agreement prior to the agreement being in place, that preagreement work may be prior

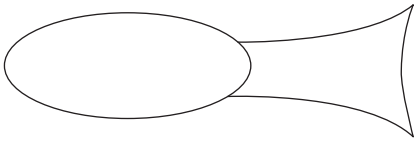

Figure 27.1 Prior art: Previous toy design.

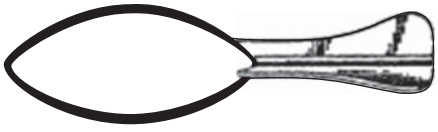

Figure 27.2 Modified toy design of invention.

5 *Oddzon Products, Inc. v. Just Toys, Inc.*, 122 F.3d 1396 (Fed. Cir. 1997).
6 *E.I. du Pont de Nemours & Co. v. Phillips Petroleum Co.,* 7 U.S.P.Q.2d 1129 (Fed. Cir. 1988).

art against the later work done under the joint research agreement, even if the predevelopment work is not disclosed to any third party.

27.3.1 Nondisclosure Agreements (NDAs)

Somewhat related to the issue of trade secrets are nondisclosure agreements (NDAs). It would be impossible to enter into a collaborative relationship without the parties first sharing at least some of their respective knowledge and expertise for each side to determine whether the collaborative relationship would be desirable and beneficial. However, how do the parties have such discussions without having a research agreement in place? Before any discussions start, the parties should both sign a nondisclosure agreement. Various sample nondisclosure agreements can be found online. The nondisclosure agreement, of course, protects any confidential information that is shared between the parties. However, provisions of the nondisclosure agreement may go beyond the sharing of what may be regarded as trade secret information. One or both parties to the agreement may not want it made public that they are discussing the possible collaboration. The area of the research may be a new drug field for one of the two parties, and that party may want their interest in the field to remain confidential from their competitors for as long as possible. A short mutual nondisclosure agreement will also allow the parties to come to the table to discuss a possible collaboration without a concern that the subject and nature of these initial discussions may become shared with an outside party.

27.3.2 Is a Freedom to Operate Search Needed?

When new drugs are being developed, in addition to protecting any new innovations, the parties must be aware of possible patents owned by third parties. The patents of a third party are often an issue when the new drug product is a new formulation or a new use for an otherwise known drug. In such situations, the innovator of the original drug may still have patent protection on the active pharmaceutical ingredient (API).

Before follow-on drug development is commenced, it is always important to start with a freedom to operate (FTO) analysis. Thus, one of the first questions that should be asked in discussing a potential collaborative relationship is whether an FTO opinion has been obtained. Related to the FTO, potential third-party players who are also working in the field should be identified and evaluated with a risk assessment. For example, the level of risk associated with a third-party National Institutes of Health (NIH) researcher working in the same area as the field of the drug development does not carry the same risk as a competitor company. However, even if an identified third-party player is open to licensing a potential blocking patent, taking such a license will incur additional costs. The collaborative agreement will need to address who will assume such costs.

An initial FTO is typically done in-house by someone at one or both of the companies involved in the collaborative follow-on drug development. However, once patents of concern are identified, it is important to have a patent attorney legally evaluate any possible problems of infringement.

27.3.3 Scope of the Collaboration

As much as possible, the research agreement should define the scope of the collaboration. What are the parties planning on jointly investigating? Who is responsible for what aspect of the research? Are there bounds or limits on what may be permissibly done under the collaborative research? It is important to consider and as much as possible address these questions in the research agreement to (i) prevent disagreements later, (ii) have a better understanding if one party has gone beyond the permitted bounds of the research agreement, and (iii) maximize the protection of the IP.

With any collaborative research, each party contributes a unique skill set and body of knowledge, with the goal being that the respective contributions will synergize to lead to new developments. Before entering into the collaboration, there needs to be mutual understanding as to what skills, expertise, and knowledge each party will contribute. With this, each party contributes proprietary knowledge, which may be in the form of trade secrets or know-how. To protect the contributions of the parties, the research agreement should define the project under investigation.

Defining the project upfront in the research agreement is important because research is inherently organic in nature and the objective may somewhat evolve over time. For example, Company A and Company B enter into a research agreement to develop new compounds with an efficacy in treating disease X. During the course of the collaboration, Company B notes a jointly developed compound, which shows promising activity to disease Y. Problems can arise with regard to the newly observed activity. Can one of the parties independently pursue the activity against disease Y? What if only Company B was responsible for the observation regarding disease Y, but this observation could have only been made using the propriety knowledge contributed by Company A? If the research agreement clearly defines that the scope of the research is for investigating compounds in the context of disease X and that proprietary knowledge contributed by one party may not be used by the other party outside the defined scope of the research, arguably Company B cannot pursue an investigation of disease Y without the permission of Company A. It is important to note that research agreements may also be modified as needed. As such, an addendum or revision may be made to the agreement if the parties jointly agree to also investigate disease Y.

Having the scope of the research defined is also important in protecting any patentable inventions that come out of the collaboration. Under US patent laws, certain prior work of the parties to a joint research agreement cannot be used as a basis for denying patentability of the invention developed under the research agreement.[7] However, the requirements to take advantage of this provision of the US patent laws include that "the claimed invention was made as a result of activities undertaken within the scope of the joint research agreement."[8] Thus, under the scenario above, if the investigation of disease Y is pursued by only Company B or if the research agreement is not modified to include the investigation of disease

7 35 U.S.C. §102(c).
8 35 U.S.C. §102(c)(2).

Y (as soon as the initial observation is made), it would be more difficult to obtain a patent protecting the use of the compound to treat disease Y, because the prior work of the parties may be used as prior art in determining whether the treatment of disease Y is patentable.

27.3.4 Trade Secrets

As noted above, in the course of a collaborative relationship, each party contributes their specialized knowledge and skill set. This knowledge often contains propriety and trade secret information. It is important that the agreement set forth requirements for protecting such information. One mistake that is often made in such agreements is that the agreements simply state something along the lines of "due and appropriate care must be taken by the parties to protect any proprietary information received from the other party." However, what is "due and appropriate care"? What one party deems as "due and appropriate care" the other party may find woefully inadequate. This disparity of perspective often arises when the parties to the research agreement are diverse entities, e.g. a large company and a university or a small start-up and a large company.

Any collaborative agreement should define what steps should be taken to protect proprietary trade secrets that are shared between the collaborators. These steps should include things such as:

1) *Physical security of the information* – Courts will always look at what steps were taken to protect the trade secrets in determining whether or not information warrants trade secret protection. One of the first things that will be considered in the event of a breach of a trade secret and release of proprietary information is whether physicals steps were in place to protect the information. Thus, the collaborative agreement should set forth what physical security must be provided for any propriety information. The collaborative agreement should address, for example, the kind of general access given to laboratory space to outsiders (e.g. whether guests must be escorted at all times); whether the material needs to be kept in some kind of secure locking containment when not being used; whether electronic copies must be kept on a secure server; whether copies (electronic or paper) of documents may be made; whether the confidential information must be clearly marked as being "confidential"; and whether transmission of information between the parties must be done in a secure encrypted manner. This list highlights some of the considerations for physical security and is certainly not exhaustive. However, a consideration of this list also shows how there may be significant divergence between the parties in how the physical security issues may be handled. Standard procedures at pharmaceutical companies require that all visitors sign in at a reception desk and be escorted throughout the building with clear visual "visitor" identification being worn. In addition, facilities are typically locked between areas, requiring a swipe card or key code. Information cannot be copied onto portable devices, such as a laptop or USB drive, and must be kept on secure servers. Universities and some small companies rarely have such physical security measures in place. In most

universities people, including guests, wander freely through the buildings and may be questioned only if they enter a particular laboratory space where people are present. With universities, information is often copied to portable devices to allow people to work on projects offsite. Thus, there can be a significant discrepancy between the parties as to what is considered adequate protection of information in terms of the physical security provided.

2) *Who has access to the information?* – The agreement should clearly set forth who may have access to the proprietary information. The safest course of action is to limit access to proprietary information on a "need to know" basis. Logically, the more people who have access to information, the more difficult it becomes to maintain control over the information. In addition, if people without a need to know are allowed access to the information, it becomes more difficult to prove that the information was in fact a trade secret in the event of inappropriate release of the information. However, even with defining the access to information on a "need to know" basis, there may be a dispute as to who "needs to know" the information and how far that permission for access extends. For example, what happens if one of the parties works with a contract research organization (CRO) for a portion of the research? Can the propriety information be shared with a CRO?
3) *What must be done with the proprietary information at the conclusion of the research project?* – Typically, the collaborative agreements call for the return of any propriety information with the destruction of any copies that may have been made.
4) *CROs* – The current paradigm of pharmaceutical research today very often involves CROs. For purposes of this chapter, the CRO may be the partner in the research or may be involved as a third party conducting some aspect of the work for one of the two parties to the research agreement. Some additional considerations come into play with CROs and confidential information. The research agreement should include provisions requiring that prior approval of the other party must be obtained before a party may engage with a CRO to perform some aspect of the research project. If one party to a research agreement desires to have some work carried out by a CRO, the CRO should be vetted for the handling of confidential information. In addition, there is a concern that the CRO may also do work for a competitor of one of the parties, in which case that party may not want confidential information to be sent to the CRO.

27.3.5 Procedures for Making Public Announcements and Publications

One important issue that often arises during a collaborative research project, particularly in the fields of pharmaceuticals and medicine, involves the dissemination to the public of information regarding developments made during project development. However, this critical issue is oftentimes not addressed in the research agreement. The parties to the agreement may have divergent interests when it comes to making announcements regarding the technology. For example, there is a clichéd paradigm in universities of "publish or perish." Thus, the view in most academic settings is that any reasonable development should be published as

soon as possible to build the *cv* of the principal investigator and give the research laboratory more credibility, which hopefully translates into more funding coming in to support the research. With small to midsize companies, the pressure to issue press releases regarding product development stems from the need to satisfy the shareholders and/or to attract investment funding. Thus, many smaller companies may be tempted to put the best spin possible on development and issue a press release with that information.

Large pharmaceutical companies, on the other hand, do not have the same pressures for attracting funding, whether through research grants or investors. Large pharmaceutical companies are able to wait until the technology is more fully developed and a patent application has been filed before issuing any press release. The general practice in large pharma is to maintain all information as secret until the needed patent application(s) has been appropriately filed.

However, problems can arise from the early release of information regarding the development of a new pharmaceutical. In *Genzyme Therapeutics Prods. LP v. Biomarin Pharm. Inc.*,[9] patents owned by Genzyme were invalidated, in part, based on a press release by the inventor, who was at Duke University, regarding the approval of the drug for Orphan Drug Designation by the FDA and the intent to treat patients with the drug in a clinical trial. The press release predated the filing of the patent applications directed to the biopharmaceutical drug and was therefore held to be prior art against the invention.

Any collaborative research agreement should have clear provisions regarding the public dissemination of any information about the project, whether through, e.g. the publication of a journal article, an oral presentation at a meeting, or a press release. It is advised that all public release of any information regarding the project be withheld until an appropriate patent(s) has been filed. Even after the patent application has been filed, the public release of information should be mutually approved by both parties to the agreement to avoid information, which should be maintained as confidential or withheld until a later time, being inadvertently released. It is also suggested that the provisions regarding the public release of information have an appropriate and practical notice provision. This scenario is most often seen when one of the two parties to the research agreement is a university laboratory. The notice provision regarding public disclosure allows for adequate time for the parties to evaluate the substance of the disclosure and to prepare and file a patent application prior the disclosure if necessary. This author has seen too many instances of one party to an agreement telling the other party that some kind public disclosure is imminent, e.g. notifying the other party that one of the researchers is scheduled to speak at a research conference a week hence, and he or she plans to speak on the developments under the project. The parties then scramble to cobble together a patent application to protect the invention being disclosed. However, in such rushed circumstances, the disclosure in the patent application often ends up being not as broad as desired or does not adequately protect the full scope of the invention. Thus, it is recommended that the collaborative research agreement requires notice of a minimum of 60 days prior to any public disclosure. It is also recommended that the party planning

9 *Genzyme Therapeutics Prods. LP v. Biomarin Pharm. Inc.*, 119 U.S.P.Q.2d 1022 (Fed. Cir. 2016).

the disclosure be required to provide a full copy of the intended disclosure to the other party to the agreement for review.

27.3.6 Ownership

A key provision to any collaborative research agreement addresses ownership of the technology developed pursuant to the agreement. The importance of addressing the ownership of the technology is based, in part, on US patent laws, under which each inventor is presumed to be a 100% owner of the invention, unless there is an agreement otherwise. 35 U.S.C. §262 states, "In the absence of any agreement to the contrary, each of the joint owners of a patent may make, use, offer to sell, or sell the patented invention within the United States, or import the patented invention into the United States, without the consent of and without accounting to the other owners." This means that each inventor can separately develop, market, license, etc. the invention, including licensing or selling the invention to the competitor of the other party to the research agreement. The danger of not setting forth the ownership of any developed technology can be seen from the court case of *Ethicon v. US Surgical*.[10]

With the *Ethicon* case, Dr. Yoon was a medical doctor and inventor who invented devices for endoscopic surgery. In 1980, Dr. Yoon asked Mr. Choi, who was an electronics technician, to help him with some projects. Mr. Choi was not paid nor was there any contractual obligation for Mr. Choi to assign his patent rights to Dr. Yoon. Similarly, there was no written research or collaborative agreement between Mr. Choi and Dr. Yoon. In 1982, the collaboration ended, and Dr. Yoon filed a US patent application on a "safety trocar" (surgical instrument). With the patent application, Dr. Yoon named himself as the sole inventor. In 1985, the patent issued and Dr. Yoon granted Ethicon an exclusive license to practice the patent and manufacture and sell safety trocars falling under the patent.

In 1989, Ethicon, as the exclusive licensee of Dr. Yoon's patent, sued US Surgical for infringement of the patent. Through the course of discovery, US Surgical learned of Dr. Yoon's relationship with Mr. Choi in developing the invention. US Surgical then met with Mr. Choi and separately obtained their own license from Mr. Choi to use safety trocars, to which he contributed the development. US Surgical then went to the court and filed a motion to have Mr. Choi added as an inventor of the patent. The court agreed that Mr. Choi should have been named as an inventor and therefore added Mr. Choi to Dr. Yoon's patent as a coinventor of the safety trocar. As noted above, under US laws each inventor is presumed to be a 100% owner of the invention, unless there is an agreement otherwise. There was no research agreement or contractual obligation between Dr. Yoon and Mr. Choi. As such, Mr. Choi, as a coinventor, was free to license the patent as he so chose, including licensing the patent to US Surgical, the direct competitor of Ethicon (the licensee through Dr. Yoon). Based on the license they obtained from Mr. Choi, US Surgical was able to have the infringement suit dismissed on the basis that Mr. Choi, as a joint owner of the patent, had granted US Surgical

10 *Ethicon v. U.S. Surgical,* 135 F.3d 1456 (Fed. Cir. 1998).

a license to use the invention. As a licensee to the patent, US Surgical could not be infringing the patent.

The case of *Board of Trustees of the Leland Stanford Junior University v. Roche Molecular Systems Inc.*[11] demonstrated that not only is important to have the ownership of any developments included in the research agreement, the wording of such provisions can also be critically important. At issue in the *Stanford v. Roche* case was the critically important and lucrative technology involved with quantifying HIV using PCR to evaluate therapeutic efficacy of HIV drugs. There were three patents involved in the case all having the title *Polymerase Chain Reaction Assays for Monitoring Antiviral Therapy and Making Therapeutic Decisions in the Treatment of Acquired Immunodeficiency Syndrome*. The technology was for the most part developed by researchers at Stanford University and Cetus (which became part of Chiron). In 1988, Dr. Holodniy joined Stanford as a research fellow. As part of his employee agreement as a research fellow, Dr. Holodniy signed an agreement titled *Copyright and Patent Agreement* (CPA), obligating him to assign his rights to any inventions to Stanford. When Dr. Holodniy joined the Stanford laboratory, he had no prior experience with PCR techniques. As a result, in February 1989, Dr. Holodniy began regular visits to Cetus over several months to learn PCR techniques and to develop a PCR-based assay for detecting HIV. As a part of his visits to Cetus, Dr. Holodniy was required to sign a *Visitor's Confidentiality Agreement* (VCA) with Cetus. The VCA stated that Dr. Holodniy "will assign and do[es] hereby assign to CETUS, my right, title, and interest in each of the ideas, inventions, and improvements" that he may devise "as a consequence of" his work at Cetus. Eventually, Dr. Holodniy's research with Cetus resulted in an assay that used PCR to measure quantitatively the amount of plasma HIV RNA in samples from infected humans. The new PCR assay was the basis for the patents at issue in the dispute with Roche.

In December 1991, Roche purchased the PCR assets of Cetus. The purchase included the agreements that were executed between Stanford and Cetus, including the VCA signed by Dr. Holodniy. Roche then began manufacturing HIV detection kits employing the patented RNA assays. Stanford asserted ownership of the technology as the assignee of the patents covering the assay and offered Roche an exclusive license to the patents. Ultimately, Stanford sued Roche in the Northern District of California, alleging that Roche's HIV detection kits infringe its patents. Roche answered and counterclaimed, in part, that Roche, in fact, possessed ownership, license, and/or shop rights to the patents through Roche's acquisition of Cetus's PCR assets. At issue were the "competing" agreements signed by Dr. Holodniy, with the first agreement being signed as part of his employment with Stanford and the second agreement signed while visiting at Cetus. In resolving who owns the patents (Stanford or Roche), the court looked at the language of the two agreements signed by Dr. Holodniy.

The agreement signed with Stanford stated, "I **agree to assign or confirm in writing** to Stanford and/or Sponsors that right, title and interest in … such inventions as required by Contracts or Grants." (emphasis added). The VCA

11 *Board of Trustees of the Leland Stanford Junior University v. Roche Molecular Systems Inc.*, 583 F.3d 832, 842 (Fed. Cir. 2009).

agreement with Cetus, on the other hand, stated that Dr. Holodniy "**will assign and do[es] hereby assign** to CETUS, my right, title, and interest in each of the ideas, inventions and improvements." (emphasis added). The differences in the language of the two agreements may not seem significant. However, the court found that the two agreements had fundamentally different interpretations. The court held the contract language in the Stanford agreement that Dr. Holodniy "'agree[s] to assign' reflects a mere promise to assign rights in the future, not an immediate transfer of expectant interests." Also weighing into the court's decision was Stanford's *Administrative Guide to Inventions, Patents, and Licensing*. The Guides stated: "Unlike industry and many other universities, Stanford's invention rights policy allows all rights to remain with the inventor if possible." The court found that the guide expressed a clear intent on the part of Stanford that ownership rights to inventors should remain with the inventors if possible. In contrast to the Stanford agreement, the language in the Cetus VCA of "I will assign and do hereby assign to CETUS, my right, title, and interest in each of the ideas, inventions, and improvements" was seen by the court as an immediate assignment of future inventions to Cetus. Thus, when Dr. Holodniy signed the VCA with Cetus, he was, in fact, transferring to Cetus the rights to any invention developed as part of his work at Cetus. As a result Cetus had right and title to the PCR assay in question, and through the acquisition of the PCR assets from Cetus, Roche acquired the rights to the patents.

These two cases serve to show how important it is to have ownership of any developed technology that clearly worked out and set forth in the collaborative research agreement before the collaborative research begins. The pitfall to not having the ownership issues clearly defined is that a competitor to one of the parties to the research agreement may obtain rights to the technology. There is no "right" way to handle ownership issues. Oftentimes the ownership of patents that were developed through a collaborative research agreement may be constrained by the overarching governing policies of one of the parties to the research agreement. For example, some universities have a policy of not outright assigning or selling patents. However, they may be willing to grant an exclusive license. On the other hand, a pharmaceutical company will not likely be interested in the technology unless they have complete control over the patent, with the right to sue for infringement. This requires that they receive ownership or an exclusive license of the patent. While there is no single "right" way to handle the ownership of any invention that results from a collaborative agreement, how the ownership will be handled must be determined prior to the research beginning. As seen with the *Stanford* case, parties who are amiably cooperating on a research project may ultimately have a falling out. This author has typically seen that the collaborative relationships dissolve when the invention proves to have significant value and one party wants to be able to capitalize on that value in a way that may be at odds with the other party. If the research agreement clearly sets forth who owns the technology, these issues may be more easily resolved.

A related issue to ownership regards inventorship and employee agreements. As seen with the *Ethicon* case, in the absence of a contractual obligation otherwise restricting the actions of an inventor, an inventor may sell or license a patent as they so choose. There are two steps that should be taken to avoid the *Ethicon*

scenario. First, clear employee agreements should be in place and be required to be signed. The employee agreements should clearly state that any inventions developed during the course of employment are the property of the employer. Before signing any collaborative research agreement, a party should confirm that the other party has employee agreements in place and that they are consistently signed. The language of the employee agreement should also be reviewed to ensure that the language does not follow the wording used by the Stanford agreement and that the agreement is not a promise to assign in the future. It is also recommended that when any invention is developed, the parties should formally review the inventorship with all of the people who were involved with the project. After determining who the named inventors should be, the remaining people on the project ideally should also review and sign off on the inventorship determination report. Having the noninventors sign the inventorship determination helps to prevent a disgruntled employee from leaving the company and later asserting that they should also be named as an inventor and possibly have rights to the invention. As soon as a patent application has been drafted, it is also advisable that the inventors be required to sign a formal assignment document, which assigns all rights to the invention globally to the named assignee(s).

A related issue to ownership, which needs to be addressed in the joint development agreement, is the licensing of the technology developed under the agreement. Whether the parties agree to jointly own any patents arising out of the research or one party owns the patent, any restrictions on the ability to license the technology should be set forth in the agreement. For example, if there are particular third parties to whom a party to the agreement does not want a license granted or particular fields covered by the patents where a party does not want any third-party access through licensing, the agreement should so state.

27.3.7 Dealing with Problems

Unfortunately, regardless of how well written a research agreement may be, disagreements over the interpretation of the provisions of the agreement or an outright breach of the agreement by one party may still occur. In addition, companies failing, merging, and/or acquiring another company and/or assets are commonplace occurrences in the business world. Consider the situation with Stanford and Roche. Stanford had an amicable relationship with Cetus, and Cetus seemed to have no issue with Stanford filing the patent applications to the PCR assay with Stanford being the owner. However, Roche, who acquired the PCR assets of Cetus, had a much different perspective and had no interest in licensing the technology from Stanford and instead asserted ownership by asserting the Cetus VCA signed by a Stanford researcher.

As a result, one important section to be included in any research agreement should address the resolution of any disputes among the parties. If there are clear dispute resolution provisions, any disputes can hopefully be resolved more quickly and at a lower cost to the parties overall. As with many considerations for a research agreement, there is no absolutely "right answer," and what may be workable for one set of parties may be untenable for another. However, there are certain variables regarding dispute resolution that should be considered and addressed in the research agreement.

For example, dispute resolution provisions should define whether disputes may be resolved via litigation or arbitration or mediation. Most agreements tend to favor using binding arbitration (possibly preceded by mediation). With mediation, an independent mediator works with both parties to try to reach a mutually satisfactory resolution to the dispute. A mediator does not "render a judgment" for or against either party. Rather, the mediator essentially listens to both sides, weighs the evidence presented by both parties, and tries to guide the parties to reach a mutually agreeable resolution on their own. With arbitration, an independent arbitrator hears testimony, considers evidence from both sides, and renders a decision in favor of one party. Thus, an arbitrator is acting as an adjudicator, although the evidence presented is much less than that presented in a trial and an arbitration moves much faster than a litigation. Another advantage to using arbitration is the technical expertise of the arbitrator. Civil court judges in the United States rarely, if ever, have any technical background. Juries similarly have no technical background. Pharmaceutical inventions by nature involve very complex science. Thus, one drawback seen with resolving disputes in civil courts is the lack of technical expertise of the adjudicating forum. This drawback can be avoided by using an arbitrator. Arbitrators are selected and approved by both parties to the dispute. Importantly, arbitrators are typically selected for their technical expertise as well as their legal expertise. Thus, an arbitrator in a dispute involving a pharmaceutical joint development or collaborative research agreement may be an experienced patent attorney with a technical educational background in pharmaceutical chemistry or biotechnology. With arbitration, the research agreement should also set forth the source of the rules that will govern the arbitration, i.e. the International Chamber of Commerce or the American Arbitration Association.

By agreeing to binding arbitration in a research agreement, the parties are agreeing that the decision of the arbitrator is "binding" and final. Thus, the party who loses in the arbitration cannot then file a complaint in a district court on the same issue. As noted above, arbitration is often favored in research agreements because it is much cheaper and faster than going through a civil litigation. It is also favored because of the technical nature of subject of the agreement.

Another consideration for dispute resolution involves venue. If a dispute is resolved in court, where will the case be heard? There is generally a perception that courts may be more favorable to parties based within their jurisdiction, particularly if the party is a large company bringing significant revenue into the state. Alternatively, certain jurisdictions are known to be very "pro-patent." If the collaborative agreement is between international entities, it is recommended that the venue to hear any dispute be in a country neutral to both parties. Having said that, before agreeing to a particular venue, the parties should be aware of how the governing law of that country (or state if the dispute is resolved in a US state court) will affect the their respective rights.

27.3.8 Costs

One of the most important issues a research agreement needs to address is costs. This may seem like a basic parameter to most people; however, when many parties

think of provisions in a research agreement regarding costs, they think of funding the research. For example, a university or small company finds incentive to enter into a collaborative agreement with a large pharmaceutical company in the monetary support for research provided by the large pharmaceutical company. However, the relevant costs associated with collaborative drug development go beyond funding the research.

27.3.8.1 Prosecution Costs

One significant cost associated with pharmaceutical technology development is the cost of protecting any invention that comes out of the research. The estimated cost for obtaining a patent in Australia, Canada, the European Union (in which the patent was validated in Germany, France, Great Britain, Italy, Netherlands, and Sweden), Japan, Mexico, and the United States is approximately $220 000.[12] This estimate was created on the assumption that the invention is an average invention in terms of complexity and that there are 20 claims. Pharmaceutical patents are typically at the high end of complexity with more than 20 claims and often longer than average examination times. In addition, this figure does not include the costs associated with a possible FTO search and patentability search, which may also be done. Nor does this estimate the costs associated with the drafting of the patent application. Including these additional expenses, the costs for an obtaining patent even on a limited global scale would be significantly higher.

This cost estimate is based on filing in only six major patent offices, with validation in six European countries. The Patent Cooperation Treaty (PCT) currently has 151 contracting states. There are numerous other countries that are not members of PCT in which separate patent applications would need to be filed, including Taiwan, Venezuela, Argentina, and Jordan. Thus, if one party to a research agreement has in mind that they want to obtain "global patent" protection, there needs to be a resolution between the parties as to what is meant by "global patent" protection. Some questions that should be considered may include the following. What are the key markets? Where are the key manufacturing sites? Where are clinical trials likely to take place? Where are the infringing drugs likely going to be made? Answering some of these questions may help identify the key countries where patents are needed.

However, along with mutually determining where patent applications will be filed, the parties also need to set forth in the joint development agreement who will be responsible for the costs associated with the IP. If there is a great disparity in patent strategy (e.g. in which countries the patent(s) will be filed), the party desiring the greater protection may need to bear a greater amount of the cost for obtaining the patents. On an issue somewhat related to costs, the research agreement also needs to set forth who will control the prosecution of any patent application. Will decisions be made jointly or will one party have control? With many pharmaceutical collaborative research agreements, one party often has a predominate interest in commercializing any technology that comes out of the research. For example, the research agreement may be between a pharmaceutical

12 Estimate obtained using Global IP Estimator from https://www.quantifyip.com/.

company and a university, where the interest of the university is funding from the pharmaceutical company and a royalty-free license to continue to use the technology for further research. The pharmaceutical company, on the other hand, has the goal of a commercial product. In such a scenario, it is often the case that the pharmaceutical company both pays for and controls the IP that comes out of the research.

In addition, the parties may want to consider what options are available for the other party if the party who has the controlling interest in the IP decides not to file a patent application. Can the other party file an application at their own expense? If they do file an application at their own expense, who owns the patent that issues? The author of this chapter is aware of a situation where two parties entered into a research agreement, stating that party A would be the owner of any IP resulting from the joint research. When party A chose not to file a patent application on the technology, party B to the agreement filed a patent application at their own expense and bore the costs for the prosecution of the application. After the patent issued, party A asserted ownership of the patent, much to the dissatisfaction of party B, who paid for the patent. However, under the joint research agreement, the rights to the patent lay in party A, regardless of the fact that party B had assumed the cost for obtaining the patent, which they were not under any obligation to do under the agreement.

As with many other aspects of the research agreement, who pays for and controls the IP are business decisions to be made by the parties to the research agreement; however, these decisions should be made prior to signing the research agreement and should be included in the final agreement.

27.3.8.2 Enforcement Costs

The section earlier discusses the costs associated with obtaining a patent, which may be very high if global protection is sought. However, overshadowing the cost of obtaining the patent is the cost associated with enforcing the patent, particularly in the United States. This issue is discussed in greater detail below; however, a significant cost that may arise (potentially the largest cost associated with a pharmaceutical invention) is the cost associated with enforcing the patent against possible infringement.

In 2013, the American Intellectual Property Law Association estimated that the cost of an average patent lawsuit, where $1 million to $25 million is at risk, was $1.6 million through the end of discovery and $2.8 million through final disposition [2]. The costs associated with pharmaceutical patent litigations are typically on the high end of the range. Joint development agreements should address how responsibility for the costs associated with enforcement of a patent will be determined. One oversight of many joint development agreements is how costs will be handled with post-grant proceedings at the US Patent and Trademark Office (USPTO). When post-grant costs are discussed, most people immediately jump to litigations and the costs associated with litigations. However, there are also options available to third parties for attacking the validity of a patent before the USPTO.

The majority of patent litigations also have corresponding invalidity post-grant proceedings filed by the accused infringer. Though a fraction of the cost of a

pharmaceutical litigation, a post-grant proceeding is still a significant cost (on the order of $300 000–$1 000 000 depending on the complexity of the patent), which should be included in the collaborative agreement.

27.3.9 Indemnifications

When one party is joining with another party in a collaborative drug development project and there is a sharing of knowledge and expertise, the parties often want an indemnification clause built into the agreement. As noted above, it is important to have an FTO analysis before entering into the joint development project. However, very few parties want to spend the time and expense of conducting their own FTO search on the other party's technology. Instead they will rely on the FTO search and analysis performed by the other party with regard to the other party's technology. However, with such reliance there will likely be a request for an indemnification clause. An indemnification clause generally states that after due diligence and to the best of their knowledge, use of the technology of the party providing the indemnification will not infringe any third party's patent. In addition, if the second party to the agreement is found liable for infringing a third party's patent for use of the technology of the first party to the agreement, the first party will compensate the second party for any damage they incur as a result of the infringement.

As an example, Company A has expertise in the formulation of APIs, which are difficult to obtain in a liquid dosage form. Company B has a new API (Drug X), which ideally would be in a liquid dosage form because it is intended for pediatric patients, for whom swallowing a solid dosage is often very difficult. Company B approaches Company A to jointly develop Drug X in a stable liquid form. As part of the joint development of the liquid formulation, Company B may require an indemnification from Company A that using the process and formulation of Company A for obtaining a liquid dosage form will not infringe any third party's patents. Similarly, Company A may require an indemnification from Company B that working with the API will not infringe any third party's patents, i.e. while the specific API may be novel, a third party may have a patent that broadly claims a genus of compound encompassing the API. Because Company A and Company B are in the best positions to know what third-party patents may be of concern with regard to the formulation and the API, respectively; they will rely on each other's due diligence and FTO, with an associated indemnification.

27.3.10 Sharing Technology

One particular area where issues of IP arise and should be considered is with regard to biological materials obtained from an entity such as the American Type Culture Collection (ATCC). In the course of pharmaceutical drug development, biological materials from the ATCC may be used for a variety of purposes. For example, with traditional small molecules relevant cell lines may be used in preclinical drug development to screen potential compounds of interest for efficacy and/or toxicity. As part of ordering material from the ATCC, the investigator is required to sign a material transfer agreement (MTA). The MTA is four pages

of legal language, which, unfortunately, many investigators sign without reading or, if read initially, the provisions and obligations are quickly forgotten. However, there are provisions of the MTA of ATCC that have important ramifications for collaborative research. First, there is a general "commercial use" clause of the agreement, which states:

> Any Commercial Use of the Biological Material is strictly prohibited without ATCC's prior written consent. Purchaser acknowledges and agrees that Purchaser's use of certain Biological Material may require a license from a person or entity not party to this MTA, or be subject to restrictions that may be imposed by a person or entity not party to this MTA ("Third Party Terms"). To the extent of ATCC's knowledge of the existence of any such applicable rights or restrictions, ATCC will take reasonable steps to identify the same, either in ATCC's catalogue of ATCC Material and/or through ATCC's customer service representatives, and to the extent they are in the possession of ATCC, ATCC shall make information regarding such Third Party Terms reasonably available for review by Purchaser upon request. Purchaser expressly acknowledges that if there is a conflict between this MTA and the Third Party Terms, the Third Party Terms shall govern. Use of the Biological Materials may be subject to the intellectual property rights of a person or entity not party to this MTA, the existence of which rights may or may not be identified in the ATCC catalogue or website, and ATCC makes no representation or warranty regarding the existence or the validity of such rights. *Purchaser shall have the sole responsibility for obtaining any intellectual property licenses necessitated by its possession and use of the Biological Materials.* https://www.atcc.org/en/Documents/Product_Use_Policy/Material_Transfer_Agreement.aspx

Thus, at the outset, when purchasing materials from the ATCC, the investigator should be aware that (i) commercial use is not permitted and (ii) a separate license may be needed from the original depositor. The question of commercial use may come into question in collaborative research when, for example, an investigator at a university is conducting a research that is funded by a for-profit commercial entity, such as a pharmaceutical company. "Commercial Use" is, in part, broadly defined under the MTA as being "the use of the Biological Material: (i) to provide a service to a person or entity not party to this MTA for financial gain." Arguably, if a university investigator is using biological material obtained from the ATCC in the course of research funded by a pharmaceutical company (who is likely not party to the MTA), such a use would be in violation of the MTA and license granted by the MTA. The ATCC MTA also specifically addresses collaborative research projects with the following clause.

> Non-commercial Use Collaborative Research Project. Except as provided above, Purchaser may only transfer Modifications and Unmodified Derivatives, subject to any Contributor rights and CAR restrictions, to Transferee(s) in Purchaser's Investigator's collaborative research project, so long as such Transferees agree to be bound by the terms and conditions of

this MTA as if a Purchaser hereunder and to not further transfer such materials. For purposes of clarity, collaborative research project(s) shall not include any Commercial Use. No subsequent transfer of such materials is permitted without ATCC's written permission. Upon completion of any collaborative research project, the Purchaser shall require Transferee to either destroy such materials or return them to Purchaser's Investigator. Collaborative research projects include, but are not limited to Industry Sponsored Academic Research but permitted use hereunder extends only to basic and discovery research related to, directly under, or in direct collaboration with Purchaser's Investigator's research project. If parties working on a collaborative research project need to obtain Progeny, they should obtain ATCC Material directly, as ATCC Material and Progeny may not be transferred.

If such materials are transferred, Purchaser agrees to provide written notice to ATCC of any such transfer within a reasonable period after such transfer following the instructions available at: www.atcc.org/transfer so that ATCC may maintain a chain of custody of such material. Purchaser assumes all risk and responsibility in connection with the transfer of such materials. Except as specifically provided in this section, Purchaser shall not distribute, sell, lend or otherwise make available or transfer to a person other than the Purchaser's Investigator or an entity not party to this MTA, the Biological Material, as defined above, for any reason, without ATCC's prior written agreement. Purchaser assumes all risk and responsibility in connection with the receipt, handling, storage, disposal, transfer, and Purchaser's and its Transferees' use of, the Biological Materials including without limitation taking all appropriate safety and handling precautions to minimize health or environmental risk.

Thus, if in the course of a collaborative research project an investigator wishes to share biological materials obtained from the ATCC, there are several provisions attached to such a transfer of material including:

1) The transferred material may not be used for a commercial use,
2) The transferee may not further share the material,
3) The transferee must destroy or return the material when the collaborative research product is done,
4) The original purchaser must notify the ATCC of the transfer of the material,
5) The purchaser assumes all risk associated with the transfer.

A scenario may easily be envisioned in which an investigator at a university purchases biological material from the ATCC (e.g. a cell line). The investigator is involved in a collaborative research project with another university investigator, with whom the biological material is shared, without telling the ATCC. Several years later, the collaborative project has concluded, and the second investigator has decided to form a start-up company based on his research. He takes with him to the new facility the cell line originally obtained from the first investigator. The cell line is now being used in a commercial use, after the conclusion of the

collaborative project. The first investigator has likely violated several provisions of the MTA, including failure to notify the ATCC of the transfer and failure to obtain the return or destruction of the biological material after the conclusion of the research project and the commercial use of the material. While the first investigator was not the entity using the biological material for a commercial use, the MTA also include the provision of 5 above, wherein the purchaser assumes all risk and responsibilities associated with the transfer.

27.4 After the Research Ends and the Patent Issues

27.4.1 Litigations

Oftentimes parties enter into a joint research agreement with disparate end goals in mind. This is to be expected because what is viewed as a benefit to the collaboration is likely different for each party to the agreement. However, despite these disparate end goals, it is generally understood by all parties that patent protection is a critical asset to come out of the joint research, particularly in the field of pharmaceutical drug development. The research project will likely be viewed as valueless by at least one party to the agreement unless any inventions are protected by patents. However, while all of the parties to such joint development agreements will acknowledge the importance of the patent protection and agree to cooperate with regard to such patent protection, the future ramifications of such agreements are often either overlooked or not considered at great length.

27.4.1.1 Required Participation in a Litigation

In the United States, a lawsuit for patent infringement may only be brought by the owner of the patent. In this regard, 35 U.S.C. §281 states, "A patentee shall have remedy by civil action for infringement of his patent." The requirement that the patent may only be enforced by the patent owner has significant ramifications on joint development agreements, depending on how the ownership of any resulting patents is structured in the agreement. For example, a joint research agreement between a university and a pharmaceutical company, in which a significant amount of the initial research is conducted at the university, may be structured to provide that the university will retain ownership of the patent, with the pharmaceutical company receiving an exclusive, royalty-free, field-of-use license for a particular disease or set of diseases. It must be noted that an "exclusive field-of-use license" is not the same as a full exclusive license of the patent. A full exclusive license of the patent has no restrictions on the licensee and for legal purposes in the context of infringement litigations is regarded as being equivalent to an assignment or transfer of ownership of the patent. Thus, with a full exclusive license, the licensee has standing to enforce the patent. However, an exclusive field-of-use license is the situation in which the patent is licensed only to a single party for a particular use or aspect of the invention, but the patent may be licensed to other entities for other uses. For example, if

the partner pharmaceutical company (Company A) has an exclusive field-of-use license for using the invention in the area of neurological diseases, the patent owner may not license the patent to another party for neurological diseases; however, the patent owner may license the patent to other entities (e.g. Company B or Company C) for the field of nonneurologically related cancers.

What happens if the second pharmaceutical company, Company B (or some other party), starts using the technology in the field of neurological diseases and is therefore infringing the patent in the area where the partner pharmaceutical company has the exclusive field of use? As noted above, 35 U.S.C. §281 states that the "**patentee** shall have remedy by civil action for infringement of his patent." However, the Company A is not the "patentee"; they are only an exclusive *field-of-use* licensee (i.e. a nonexclusive licensee when considering the totality of the patent).

In most situations such as this, where the ownership of the resulting patent is not shared, joint research agreements include cooperation clauses. In the scenario presented above, any joint research agreement should include provisions requiring that the university agree to be a party to an infringement lawsuit brought by Company A. Of course, such provisions also state that if Company A elects to bring an infringement lawsuit, Company A is responsible for the costs associated with the lawsuit. This may seem like a reasonable and equitable solution – the university agrees to be named as a party because the infringement suit cannot be brought without them; however, since Company A is paying for everything, the university is amenable with being named to the suit. However, there are ramifications to this that go beyond the overt expenses with the litigation assumed by Company A. As a party to the litigation, university personnel are more readily accessible to be deposed. Patent infringement litigations are very involved proceedings, typically with weekly teleconferences to discuss the strategy and progress. This level of involvement may be disruptive to the normal course of business of the university. In addition, as the patent owner, the university has their own interest in the strategy taken in the litigation because the value of the patent to the university goes beyond the revenue from the license from Company A. The university has an interest in maintaining the validity of the patent's claims and in the way in which the claims are interpreted, so as to not jeopardize the revenue stream from the other licenses. As a result, the university will likely want to engage their own counsel to act as an advisor to the litigation and monitor the litigation to protect the interest of the university. Company A, however, will not assume the cost of counsel separately engaged by the university. Thus, the university will, inevitably, have costs associated with the litigation that they must bear. Finally, there is the intangible cost to the university of having their name associated with litigation. The university may not want a reputation of being litigious. In addition, the accused infringing party may be an entity with whom the university has a relationship in other fields of research. Being a party to the litigation may have an impact on that relationship.

Thus, while one party may enter into a collaborative research agreement with short-term goals in mind (such as funding), there may be long-term ramifications that extend far beyond the life time of the collaboration.

27.4.1.2 Who Will Enforce the Patents?

A second issue that may arise in collaborative drug development, which is somewhat related to the issue of participation in the litigation, is the question of how enforcement of any patents will be decided. With drug development, the ultimate goal is typically to have an approved drug that, in the United States, is listed in the Orange Book. However, the party who commercializes the drug may not be the patent owner or may only be a joint patent owner. The agreement should set forth who has the right to enforce the patent and what is the required cooperation from the other party. Even if the drug company is the patent owner, the patent may list inventors from the other party (e.g. a university) to the agreement. Even though the drug company, as the patent owner, can enforce the patent on their own, the cooperation of the other party will still be required because the inventors, including the inventors from the other party, will more than likely be deposed by the accused infringer. Thus, built into the enforcement provisions must be a cooperation clause, wherein there will be cooperation between the parties to the research agreement to enforce the patents, even if only one of the parties actually owns the patents.

27.4.2 Patent Term Extension

An important consideration for pharmaceutical patents is possible patent term extension, under 35 U.S.C. §156, which provides for an extended term for patent claims directed to approved drug products/medical devices and approved methods of using and/or making the drug products/medical devices. An application for patent term extension may not be filed until the drug product has been approved. It is typically some years after a patent has granted that clinical trials are completed, and final drug approval has been obtained, making the patent eligible for patent term extension. However, there is a very tight window of 60 days after drug approval for filing an application for patent term extension. The USPTO is clear that this date may not be extended for any reason. An additional requirement of patent term extension is that the application for the extension is filed by the patent owner, who may not be the holder of the new drug application. Thus, an application for patent term extension requires communication and cooperation by the patent owner (assuming the patent owner is not the NDA holder). This cooperation is typically required after the joint research project has ended because of the delay between the patent filing and the time for filing a patent term extension application. As a result, the collaborative research agreement needs to include provisions for prospective cooperation even after the research has been concluded. Such provisions are important because there may be a change in personnel with one of the two parties, which would make establishing the need to cooperate more difficult, at a point when time is an important consideration. Alternatively, as a potentially bigger problem, the relationship between the previously joined parties to the cooperative drug development may have soured, and the parties may not be willing to provide any cooperation beyond what was set forth in the joint research agreement.

27.5 Termination of the Relationship (Death and Divorce)

All collaborative relationships ultimately end, whether through a planned termination at some identified point in the future, the failure of one party to survive (*death*) or the premature termination of the relationship by one party (*divorce*). Any well-thought-out collaborative research agreement should contemplate both the expected termination of the agreement and unexpected termination of the agreement.

As discussed earlier, a joint research agreement should set forth the scope of the project. What is the goal? What are the milestones and what are the target dates for the milestones? However, once the goal of the project is achieved, it is likely that the relationship will end. The agreement should address the winding down of the agreement. One important aspect of the winding down is the return of any confidential information or proprietary material. A planned termination of the joint research agreement is usually fairly straightforward because the parties usually have the end game in mind when they enter into the relationship. Of greater concern is the unplanned and/or premature termination of the relationship.

As much as possible, the agreement should set forth reasons for early termination by one party "for cause." A basis for the early termination by one side may be a failure to meet the milestones set forth in the agreement, for example, the project is simply not working. Alternatively, one party may not be adequately providing resources as required by the agreement. With both of these examples, one party may be regarded as having breached the agreement. Generally, when one side perceives a breach in the joint development agreement by the other side, the party perceiving the breach is required to give notice of the breach to the other party with some reasonable period provided for the other side to cure the breach.

The agreement should also address what will happen in the event of a merger and/or acquisition (M&A) by or of one of the parties. If Company A is acquired by (or even acquires) a third-party company, does that give Company B the right to terminate the agreement? Generally, M&A are a basis for possible termination of the agreement by the other party. If Company A is acquired by a competitor of Company B or Company A acquires a competitor of Company B, Company B will want the ability to terminate the relationship with Company A. However, an M&A should not mean an automatic termination of the agreement. Rather, the parties should have the ability to exit the relationship upon an M&A. One question for consideration, however, is whether there should be any penalty if the party terminating the agreement upon an M&A is the party involved in the M&A. For example, Company A is in a joint development agreement with Company B. Company B provides specialized technology for producing biopharma products. Company A then acquires or merges with Company C, who also has technology for producing biopharma products. The technology Company B contributed to the joint development project is now redundant with the in-house knowledge of Company A, and Company A wants to terminate the relationship with Company B. Company B has thus been damaged by the M&A of Company A, and Company

C and Company A/C should have a penalty for early termination under these circumstances. Also of concern in this situation is the proprietary knowledge of Company B, to which competitor Company C may have access, unless proper provisions are in place in the agreement to protect the proprietary information of Company B.

A further consideration for early termination of an agreement, whether through breach, M&A, bankruptcy etc. is what happens to the IP rights, particularly if the agreement calls for the patent ownership to reside in the party who breached the agreement, was involved in the M&A or failed. The agreement may include a "buyout" option, whereby the one party has a right to buy the IP rights of the party.

In addition, the continued use of background technology after termination should be considered. As discussed above, with joint research agreements, both sides are typically bringing specialized knowledge to the table. Termination of the agreement should address whether the parties may license a continued use of the background technology obtained from the other party.

27.6 Conclusion

As seen from other chapters, IP is a vital and integral aspect of drug development. Collaborative drug development projects have unique and varied IP considerations. These considerations arise as soon as the parties contemplate entering into a joint research and development project. These considerations involve the interaction between the collaborating parties and include the protection of shared information, ownership, and protection of any jointly developed technology, enforcement of any patents obtained for the jointly developed technology, the resolution of disputes between the parties to the agreement, etc. There are also considerations regarding third parties to the agreement, including issues such as possible infringement of a third party's patent, interaction with third parties in the course of developing the technology, and possible infringement of patents obtained by a third party. Only by keeping such IP concerns in mind before entering into and while drafting a collaborative drug development agreement can the value of the IP (and therefore the drug product) be maximized, while the risks are mitigated.

List of Abbreviations

ANDA	abbreviated new drug application
API	active pharmaceutical ingredient
ATCC	American Type Culture Collection
CPA	Copyright and Patent Agreement
CRO	contract research organization
FDA	Food and Drug Administration
FTO	freedom to operate

IP	intellectual property
IPAB	Intellectual Property Appellate Board
M&A	merger and/or acquisition
MPEP	Manual of Patent Examining Procedure
MTA	material transfer agreement
NDA	new drug application or nondisclosure agreement
NIH	National Institutes of Health
PCT	Patent Cooperation Treaty
USPTO	US Patent and Trademark Office
VCA	Visitor's Confidentiality Agreement
WIPO	World Intellectual Property Organization

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