ADVANCES IN DRUG RESEARCH

Volume 26

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DRUG RESEARCH

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PREFACE:
ADVANCING DRUG RESEARCH

Drug research advances at a rate that never fails to amaze scientists who, like ourselves, are mature enough to discern a deep perspective and still fresh enough to experience awe and wonderment. As for drug researchers, they advance in their enterprise by a combination of expertise and creativity. These two statements may seem contradictory. However, they represent two sides of the same coin—drug research as a human endeavour in which scientists take both the part of actor and audience. In particular, writers of scientific reviews play such a dual role, on the one hand, observing and collecting, on the other, constructing and asserting,

> . . . because science consists not simply of a collection of true facts about the world, but is the body of assertions and theories about the world made by people who are called scientists. It consists, in large part, of what scientists say about the world whatever the true state of the world might be (Lewontin, 1993).

And when it comes to constructing and asserting, the present volume is rather unique. As will become rapidly apparent to the many faithful readers of *Advances in Drug Research*, it differs from all previous volumes in that none of the chapters focuses on a specific class of drugs. In other words, the present volume possesses the singular characteristic of containing only general chapters presenting conceptual and/or methodological advances. Such a feature would find justification, were any required, in the pregnant words of the philosopher Mary Midgley:

> We already have far more facts than we can handle. What we need most is to improve our ways of sorting and relating them—to work on the concepts, to philosophize (Midgley, 1991).

The book opens with a chapter by Kier and Testa whose subject is drug research as a whole. This is covered in an unconventional and even provocative way by the adoption of a stance few drug researchers will be familiar with, namely that of complexity and emergent properties. Some readers may be astonished by the thesis advocated in this chapter — that the now well-established science of synergetics and complexity allows chemical and pharmacological phenomena to be seen in a new perspective, thereby

procuring original ways to conceive, probe and interpret them. But whatever their reaction, our readers cannot remain indifferent to the new paradigm.

The second chapter carries the testimony of a scientist who has devoted most of his professional career to drug design. As a pioneer and successful drug researcher, Tute has much to tell us, and he does so with great elegance and authority while reflecting upon the present and future of drug design. Because his experience is both industrial and academic, Tute manages to blend the fundamental and applied objectives of drug design and to demonstrate their complementarity, and indeed their inseparability.

The third and fourth chapters stand at the very cutting edge of methodology, explaining and evaluating the present state of the art in transgenic animals and xenobiotic-metabolizing human cells, respectively, as pharmacological and toxicological tools. Both Bürki and Lederman in their chapter, and Crespi in his, manage to condense an impressive amount of information in a relatively limited number of pages. The clarity of their presentation, the critical and prospective tone of their message, will convince our readers of the interest and potential of these new biological tools.

The fifth and final chapter is quite a novelty for *Advances in Drug Research*, and a most welcome one. Compliance, pharmacovigilance and related issues have become important disciplines now that the role of pharmaco-socioeconomics is fully recognized as an essential aspect of drug research—in this case, research whose objective is to optimize the therapeutic use of existing drugs rather than to discover new agents. In his chapter, Urquhart places patient compliance in the broad context of an evolving health care environment. His contribution, written in an easy and almost oral style, will be an eye-opener to those numerous bench and computer workers who consider research to end, and development to begin, when a drug candidate enters clinical trials.

As Wolpert recently noted,

> we may be thought of doing "science" in our everyday life by setting up hypotheses and testing them against experiment; but this is not science since there is no need for theory—only imaginative trial and error is required to achieve the right "taste" (Wolpert, 1993).

This is a decisive statement telling us that there is much more to science than merely setting up hypotheses and testing them. Creating theories is the crucial step from which hypotheses and then tests can emerge. To various extents, all chapters in this book give preponderance to theory, and then go on to consider hypotheses and facts. The editors are therefore proud to have

assembled, and the authors to have contributed to, a volume of noteworthy scientific content. May our readers share our *satis factum*, our satisfaction.

BERNARD TESTA
URS A. MEYER

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Complexity and Emergence in Drug Research

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1 General Background

1.1 INTRODUCTION

Over the past half century, scientists have become increasingly knowledgeable about interactions between drug molecules and biological systems. This has been a result of many major advances in methods of study, both experimental and computational, and in models of relevant systems. These advances include new applications of existing paradigms, technological developments enriching the data-gathering processes, and the creation of entirely new concepts to comprehend drug-related phenomena. As a consequence, we are now better equipped to move towards the elusive objective of "rationally" designing new drugs.

Within the past quarter of a century there has also been a revolution in science in regard to the way we view **systems** in general, their properties and global behaviour, and the components into which they can be decomposed. Such a view is contrary to the Newtonian concept that the whole can be defined as some simple, linear combination of its parts. In other words, a **post-Newtonian** view has emerged having at its core the belief that systems at every level of size and organization have exclusive properties that are indivisible attributes of the whole and which emerge from non-linear and dynamic interactions among the components of the system. A system exhibiting these characteristics is said to possess complexity, i.e. it is complex in a new meaning of the word. Furthermore, systems exist in a nested hierarchy where one system becomes part of the ingredients producing the emergent properties of the next higher complex system.

When we reflect on these prominent areas of development, several questions do arise which challenge drug researchers. For example, how have

the concepts of this post-Newtonian paradigm implicitly influenced the development of the current state of thinking in drug research? Do the concepts of complexity and emergence have a place in the methods and models of modern drug research? And are there some identifiable places in the hierarchy of systems investigated by drug researchers where these concepts should now play a recognized and explicit role?

We present these questions as an introduction to this writing. In the following pages, we will first outline the current concepts operating in drug research in general and medicinal chemistry in particular. Secondly, we will elaborate on some of the new concepts associated with complexity and emergent properties. Thirdly, we will see how we can answer some of the questions posed above, linking current and new concepts. And finally, we will explore possibilities for the enrichment of drug research from these post-Newtonian concepts.

1.2 PRESENT CONCEPTS IN DRUG RESEARCH

The evolution of methods and models in technology and theory has brought drug research to its current state. But what is this current state? We begin here with a brief answer by identifying the hierarchy of systems of interest to us in this activity and continue by describing the progress associated with each system.

1.2.1 Systems of Interest

Drug researchers have staked out a portion of the spectrum of systems in nature as focal points for studies and models. The full spectrum of systems now investigated by all branches of science, and defined by both size and complexity, begins at the level of "ultimate" particles (quarks, strings, etc.) and extends right through to the Universe. A hierarchy of physical systems thus exists which we feel incompetent to define, simply noting that a hierarchy of biological systems "branches" out of the sequence of inanimate systems, as discussed below.

We can say of the full spectrum of systems that each system is complex, i.e. itself composed of subsystems, and that it is an ingredient of a larger system. Each system loses characteristics in the development of the emergent properties of its next-up neighbour, those things lost being subsumed into the characteristics of the more complex neighbour. Perhaps only some philosophers of science would embrace this complete spectrum of nature's systems in their studies and models.

Drug researchers by contrast are interested in just that segment of the spectrum of complex systems where in their view the phenomena of drug

actions take place. With the advent of theories of chemical reaction mechanisms, the electron and the proton have become the least complex systems of interest in drug research, while the most complex systems studied would be populations and societies (as relevant for example in pharmacogenetics and pharmacovigilance). The spectrum of complex systems of interest in drug research, a subset of the totality of all existing systems, would thus be:

. . . subatomic particles . . . atoms . . . molecules . . . macromolecules . . . membranes and macromolecular assemblies . . . organelles . . . cells . . . tissues . . . organs . . . systems of organs . . . organisms . . . populations and societies . . .

We shall return later to some complex systems in this segment and detail some explanation.

1.2.2 Milestones of Progress

Associated with the segment of complex systems that are identified with drug research are the conceptual and technical advances that have illuminated these systems and that have brought us to our present state of understanding and skill. We identify some of these and show in Fig. 1 their relationship to some complex systems in our realm of interest.

These advances fall into two major categories. The first are those which produce an increase in understanding of a particular system without considering higher levels of complexity. Examples include quantum mechanics, a paradigm for describing the behaviour of electrons, and topological structure coding, a paradigm for describing molecules. The second category of advances are those which form a **bridge of greater understanding** between two systems in this hierarchy. Examples would include molecular orbital theory which links atoms and molecules, and enzyme and receptor theories which link molecules and biomacromolecules.

Drug design is one discipline within drug research that calls heavily upon information far from the complex systems that we directly manipulate to elicit changes. We employ for example information at the complexity level of cells or organisms in order to establish relationships with molecular structure. An in-depth understanding of this source of information is not the gift of all drug designers, who accept pharmacological data on faith as representing the measurable biological effects of chemical compounds as a function of their molecular structure (Testa, 1984).

Each of the advances in Fig. 1 has its roots in an effort to describe the ingredients of a complex system, and is built upon the Newtonian concept of reduction to parts in an effort to understand the whole system. It is to this

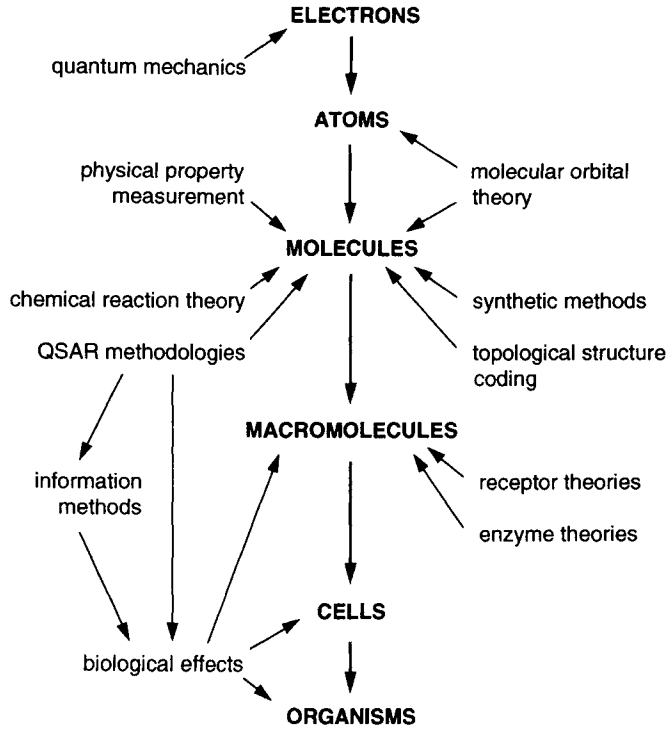

FIG. 1. The hierarchy of complex systems relevant to drug research, shown here associated with some of the theoretical and technical advances in this field.

Newtonian view of the world that we now turn our attention before introducing the post-Newtonian view.

1.3 NEWTONIAN PHILOSOPHY

1.3.1 Reductionism

The dominant philosophy in science for the last three centuries owes much to the epoch-making contributions of Isaac Newton (1643–1727). In regard to the nature of material objects, which we can generalize as systems, he viewed them as possessing properties that result from the **additive contributions** of their parts. This led to the belief that the route to the understanding of nature was through the dissection of a system to the parts followed by the study of these parts. This dissection is also performed in time, which is broken and sampled in a number of snapshots. Such a process is referred to as **analysis**, and the underlying philosophy as **reductionism**. In general then,

according to this view, the properties of the whole—and hence its behaviour—are the sum of the properties of the parts.

This philosophy has had a profound effect on the methods of inquiry in virtually every field of human endeavour. It has guided scientists to pursue the pattern of study: dissect, identify, classify, and dissect further. The logical extension of this approach is to search for the ultimate, ahistoric particle as the fountainhead of creation; the basic ingredient with which to model and understand the universe. This is the **addition/unification** sequel to reductionism, whereby parts are then reassembled into additive models.

1.3.2 Additive Models

Once a system has been dissected into parts, attempts at reassembly can be made to achieve some degree of understanding. This is done by building models made of two ingredients, namely **parts** and **linear interactions** between them. In this context, we can define a model as an effort to decouple and to discard events between ingredients that have no perceivable effect on the system under study. A model mimics a system in a simplified manner because it calls upon fewer states. A model is thus an abbreviation or a subset of the system it addresses (see also Section 3.3.1, and the enlightening review on models by Boxenbaum, 1992). In such linear additive models, small causes can only have small effects, while big causes necessarily elicit big effects.

The building of additive models begins with the portrayal of a system in an **equilibrium state**. This is true whether we are using a kinematic or a thermodynamic definition of equilibrium states. Each system is assumed to wind down to its lowest level of variable behaviour, a general statement that embraces a number of phenomena depending upon the level of complexity considered. This leads to a static model in which there is a sharp delineation between an event and its absence. There is a clear illumination of discrete events modelled to be cause and effect.

Along with the reduction–unification concepts, there have arisen ways to view nature using concepts such as thermodynamics and equilibrium. Forces such as enthalpy and entropy have been defined and invoked as integral parts of the consideration of ensembles of particles. Equilibrium states thus came to be regarded as the outcome of dynamic processes.

1.4 POST-NEWTONIAN PHILOSOPHY

1.4.1 The Need to Outgrow Reductionism

The limitations of the reductionistic philosophy became apparent near the end of the 19th century. The recognition of the diversity of life following the

contributions of Charles Darwin (1809–1882) called attention to the great difficulty in describing living systems with additive, linear models based on ingredients. At the turn of the century, Henri Poincaré (1854–1912) concluded that the accurate prediction of the trajectory of three or more interacting bodies was impossible, revealing further limitations of reductionism.

In this century, prodigious conceptual leaps were made with quantum uncertainty, Kurt Gödel's incompleteness theorem, and deterministic chaos, all of which are irrevocably incompatible with the clockwork universe of Newton and Laplace. The recognition of self-organization of states far from equilibrium as pioneered by Ilya Prigogine has dispelled any remaining belief that the reductionistic approach should offer a route to understanding a system in its globality and complexity. The concept of fractional dimensions (fractals) has brought with it a genuine revolution in our manner of viewing and describing a diversity of phenomena, and has now found many applications in the pharmaceutical sciences (Koch, 1993).

1.4.2 The Concepts of Complexity and Emergence

There has evolved over the past three decades a set of general concepts that have revolutionized the way we regard and study systems in nature. Their basic premises run counter to the Newtonian reductionistic approaches and might thus be labelled post-Newtonian concepts. The central theme of this new philosophy is the recognition that the behaviour and properties of a system are **non-linear combinations of the subsystems**. Such a system is endowed with complexity and displays specific properties that emerge from **dynamic interactions** between the subsystems. We discuss briefly complexity and emergence as the two pillars of post-Newtonian thought.

We define **emergent properties** of a system as those that are possessed by the system itself in its globality, but are neither properties of the components nor linearily derivable from them. Emergent properties arise from the interactions between the constituents of a system, when these transactions are of such a nature and magnitude that the individuality of the parts is subsumed in an unrecognizable form within the larger system. In other words, emergent properties are seen in systems whose component parts interact with such intricacy that they cannot be predicted by standard linear equations.

As an example, the ingredients of a photograph are dots arranged in some characteristic pattern. A study of individual dots may be of interest and will tell us something about them. It will, however, tell us nothing about the object having been photographed. The picture portrayed in the photograph is an emergent property of the dots. The individuality of the dots is lost; only the ensemble of dots has significance as a recognizable picture.

Closer to home, and as discussed in detail later, we can cite the properties of a molecule as being emergent and not predictable from a linear combination of atomic properties. The identity of the individual atoms in a molecule is lost in the extensive electronic interactions that occur when a molecule is formed. The physical properties of the atoms are no longer of significant value in understanding the molecule. Other steps must be taken if we are to achieve some understanding.

Emergence, in other words the existence of emergent properties, is the specific characteristic of, and the necessary condition for, systems said to display **complexity**. Emergent properties and complexity are thus two manifestations of the same phenomenon and have come to be recognized as characteristics of nature at every level. But whether emergent properties are also the sufficient condition for complexity remains to be clarified.

1.4.3 The Study of Complex Systems

The Newtonian approach to understanding nature is based first on the reduction of a system to its parts and second on the study of these parts, in the belief that the information so obtained can be reassembled additively to understand the whole. In post-Newtonian philosophy this is an incomplete procedure. Dissection of a complex system to the ingredients will give us the basic building blocks but will not reveal how they are ordered or organized in developing the emergent behaviour of the original complex system. To approach this goal one must attempt to **model the organization *per se*** in a process called **synthesis**.

As outlined above, model building is also performed in the Newtonian paradigm, but the differences with the post-Newtonian paradigm are worth restating. In the reductionistic approach, analysis into parts is by far the more important and informative step, with unification considered as an accessory and "downhill" task. Within the post-Newtonian paradigm, analysis remains an essential undertaking, but only insofar as it is the obligatory precursor to synthesis, the decisive step which justifies analysis and will bring forward understanding and knowledge. As expressed by Sheldrake (1989) in another context, one way of thinking about creative synthesis involves looking from below, from the bottom up, seeing the emergence of ever more complex forms. The other approach is to start from above, from the top down.

In post-Newtonian research, both analysis and synthesis are thus essential in order to complete a circle of research with any possibility of enhancing our understanding of a complex system. This is illustrated in Fig. 2, which shows the circle of research in the understanding of the emergent behaviour of a complex system such as an aeroplane. We can study in great depth the components of this complex system, revealing vast amounts of information

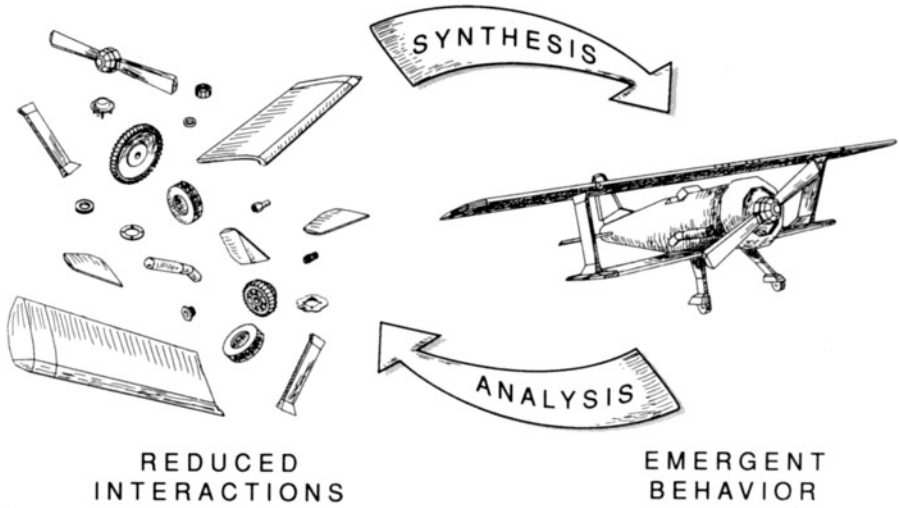

FIG. 2. Analysis and synthesis: a circle of research.

about the alloys, the plastics, even the molecular structures of these ingredients. But we will have exceeded the ability of such information to provide us with an understanding of why an airplane flies. In general we may exceed the logical depth in the series of circles of research and as a consequence we will lose information value.

This notion of exceeding logical depth and the loss of information value can be illustrated by a hierarchy of familiar systems (Fig. 3). Each circle of research is formed around a complex system on the right and an ingredient system on the left, participating in the donation of properties and behaviour to that complexity. If we explore systems too much to the left in this hierarchy, we go too far in logical depth and will lose information value needed to understand a specific complex system. As an example, in Fig. 3 we deduce that a study of the amino acid content of a system will enrich our understanding (through analysis and synthesis) of the emergent properties associated with enzyme function. However, if we were to extend our analysis to the study of the atomic structure of those amino acids, we would exceed the logical depth of the circle of research embracing the enzyme function, and the information value of these studies would be low.

A consideration of this concept is of utmost importance in designing studies leading to information for drug research. It will be one of our prime considerations in the next sections where we explore some complex systems of interest in drug research. Our intention now is indeed to compare the post-Newtonian concepts with current attitudes in the field of drug research. This will bring into focus the underlying rationale for some of the current work and will provide some bases for possible improvement.

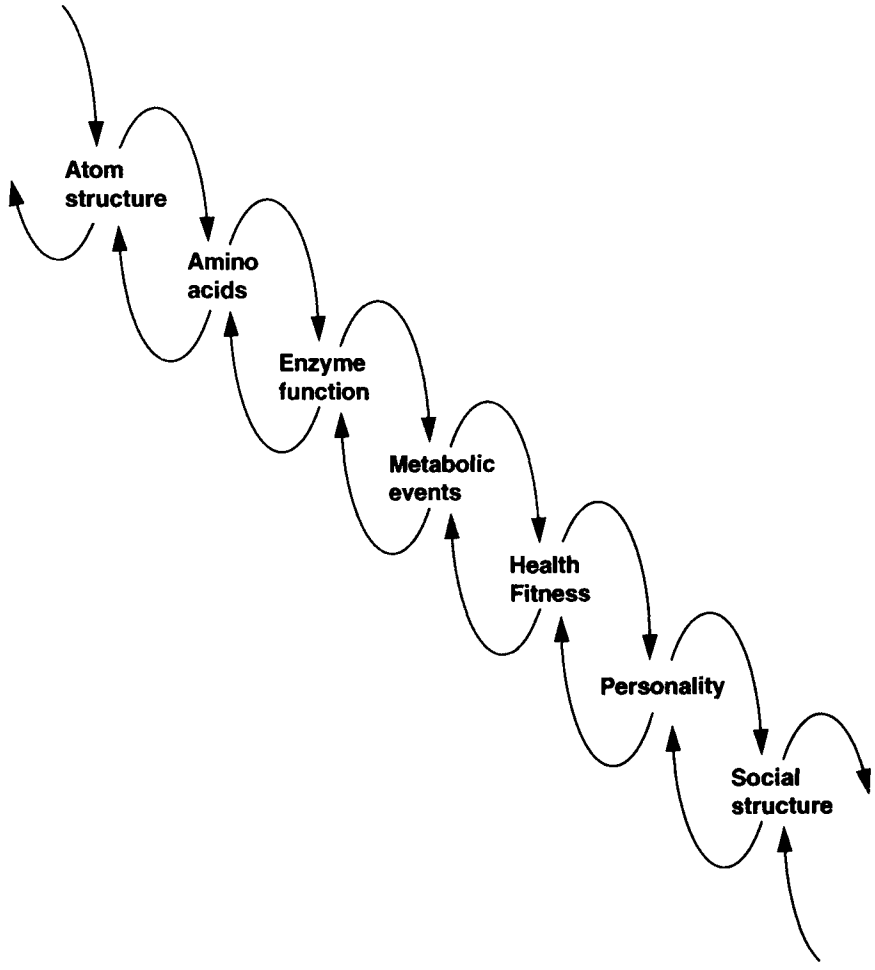

FIG. 3. Hierarchy of circles of research from the atomic level to the social level.

1.4.4 Some Properties of Complex Systems: Self-organization, the Butterfly Effect, Adaptability and Probabilistic Advantages

It must be emphasized that the complex systems at all levels in our hierarchy are **dynamical** (interactive). There is a constant motion of the whole and of the parts. Therefore relationships, interactions or transactions that take place do so as stochastic events. This is precisely what Poincaré described, who was perhaps the major founding father of post-Newtonian science. The fact that purposive encounters do occur as a part of biologically significant processes may be viewed either as a result of purely random events, or it may be characteristic of the behaviour of complex systems poised at the edge of chaos.

A degree of preferentiality can be modelled by invoking conditions where probability is focused in the direction of some distinct outcome. The suggested curvature of proteins forming a concavity that enlarges the target site of a ligand and facilitates its binding (Blum *et al.*, 1988) is one such enhancement of an outcome. The notion that ligands can slide along a formed landscape on the surface of a macromolecule (Eigen, 1990) is another example. At the molecular level the shuffling of the atoms of the heptane isomers takes place on an energy surface in which there are just nine minima (**attractors**) capable of occupancy by the 7 carbons and the 16 hydrogens.

The sources of these **probabilistic advantages** arise as emergent properties of complex systems. They are the consequences of the transactions themselves, operating to modify the probabilities one would see in the parts.

This modification of probabilities, or dissolution of improbabilities, exists at two distinct but strongly intertwined levels, that of internal relations between the parts, and that of external relations with the system's environment. Concerning the former, we may generalize from the above discussion that one emergent property common to any complex system is a capacity at restructuring the probabilities of encounters among the parts, resulting in a behaviour known as **self-organization**. In other words, a complex system is created when interacting parts encounter attractors in the space of morphogenetic rules. And once created, some complex systems are believed to be able to evolve to states of particular interest, namely self-organized criticalities. This is particularly evident with living systems (Ito and Gunji, 1994).

Complex systems being dynamical, they can indeed exist in a number of states between frozen order and destructive chaos. The most interesting region between these extremes is now known as the **edge of chaos**, but was earlier referred to simply as chaos or deterministic chaos. In these critical states, the reaction of a complex system to external causes is not predictable. There is absolutely no connection between the strength of a cause and the strength of an effect, with big causes having small or large effects, and small causes being able to elicit small or very strong effects. This latter phenomenon is known as the **butterfly effect** and is particularly evident in meteorology and biology.

Another property characteristic of the evolution of complex systems is their **adaptability to external conditions**. In other words, complex systems are not only dynamical but also adaptative. Adaptability is an emergent property of major consequence in the evolution of complex systems. As a matter of fact, and just like self-organization from which it is not dissociable, adaptability to external conditions results in a modification of probabilities, as exemplified by ecosystems (Burns, 1994). This is of utmost significance in biology, allowing biological systems to evolve to **purposive outcomes**. In drug research also, adaptability is of major but as yet

unrecognized significance, since as discussed below drug–receptor and drug–enzyme complexes must be viewed as complex systems.

2 Some Complex Systems of Interest in Drug Research

2.1 A LOOK AT COMPLEXITY AND EMERGENCE IN MOLECULES

Systems analysis at the molecular scale is the everyday task of chemists, who may not always be fully aware of the hierarchical connections between micro- and macrovariables so cogently discussed by Rabitz (1989). Here, we are looking at one aspect of this complexity, namely structural and molecular properties of relevance to drug research.

2.1.1 Atoms and Molecules

In Fig. 1, we have displayed the complex system called a molecule as being composed of ingredients at the atomic level of complexity. It can be argued quite convincingly that the properties of atoms are lost or subsumed as the properties of molecules emerge (Testa and Kier, 1991). As a matter of fact, the properties of molecules cannot be presaged from a simple sum of atom properties. A non-linear set of equations is necessary to characterize a molecule from its atom-level ingredients; this is the general scheme of molecular orbital theory. At the same time, it must be recognized that these equations were developed from electron properties which are surrogates for the atom.

The model of a molecule derived from the circle of research (Fig. 4) must be critically evaluated as to whether it contains the information associated with the emergent behaviour of the molecule. In other words, the question must be asked whether information from the atom level exceeds the logical depth necessary to understand the emergent properties of molecules as they relate to drug research (Kier and Hall, 1992).

2.1.2 The Existence of an Intermediate Level of Complexity between Atoms and Molecules

The analysis of molecules has traditionally focused directly upon atoms for information concerning emergent properties, although some thoughts have been given to a possible intervening level of complexity. There is occasionally tacit recognition that commonly occurring **ensembles of atoms** (moieties) appear in molecules and that these are responsible for recognizable phenomena. Some of these ensembles of atoms are referred to as **functional**

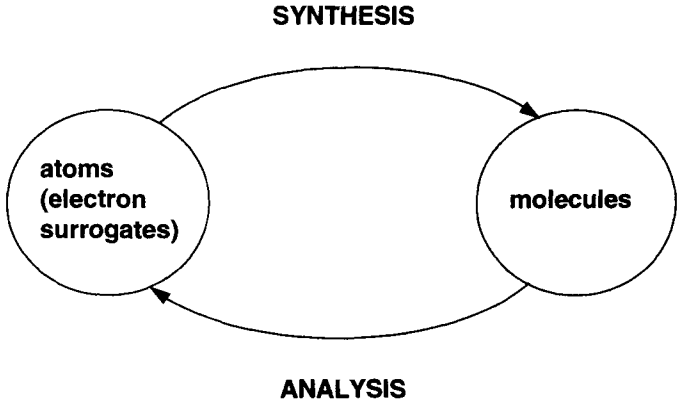

FIG. 4. The conventional model of molecules derived from a single circle of research.

groups because of their identification with distinctive roles. Such groups are often regarded as being indivisible, that is they are treated as an entity. Thus –OH, $-NH_2$, –COOH, $-SO_2NH_2$, $-CH_3$, $-CF_3$ and $-NO_2$ are seldom dissected into their constituent atoms for study or modelling. Phenomena are ascribed to these groups as emergent properties (e.g. acidity, resonance or rotor behaviour) not recognizable additively from the atoms of which they are composed.

The **pharmacophore** is another example of a species with a distinct level of complexity lying between the systems described as atoms and those described as molecules. The existence of a pharmacophore is an emergent property defined by a pharmacological receptor and this confers upon it the identity of a complex system.

2.1.3 Topology and Property Predictions

Another emergent property of moieties is certainly their topology. This attribute is often correlated or even identified with molecular properties referred to collectively as **steric effects**. A study of the atom content of a moiety or pharmacophore produces information which cannot be summed in a simple way to portray this attribute of a molecule. As an example, the three isomers of pentane have exactly the same atom content, but of course these molecules differ significantly in their topology and associated properties. A non-linear combination of the atomic constituents is necessary in order to derive a meaningful understanding of the emergent properties. The field of chemical topology has recognized this and has made contributions to

the understanding through analysis and even synthesis of models of these ensembles of atoms.

The contributions from chemical topology in the form of fragment identification, analysis and synthesis of models correlating to molecular properties have been only partially successful, perhaps because the synthesis employed may not be the best reflection of the collective interactions in the moiety. In molecular connectivity we see encouraging signs that the analysis has defined useful ensembles of atoms but we feel that the synthesis of indices encoding emergent structures must be improved (Kier and Hall, 1976). Recently, molecular topology has been rendered more rigorous with the topological analysis of electron localization functions and the recognition of a number of non-bonding, core and bonding **attractors** (Silvi and Savin, 1994).

Another area where this intermediate level of moieties has been invoked is the use of molecular fragments to predict certain physical properties of molecules (Lyman, 1982). In one example, the prediction of partition coefficients as a measure of lipophilicity is steadily evolving in several laboratories (see below). The central theme of these efforts is the dissection of a molecule into fragments followed by an evaluation of their individual contribution to the physical property. From there a simple summation of contributions (i.e. increments), mitigated by a variety of factors encoding constitutive properties, is made to model the property (Rekker, 1977; Hansch and Leo, 1979).

These studies have analysed the molecules to the proper logical depth for subsequent syntheses, but the latter have been conducted with a basically Newtonian spirit. The constitutive factors designed to encode various intramolecular interactions between fragments are limited in scope and generality, while the methods used thus far have not achieved a significant level of rigor. **Lipophilicity** is certainly a property in which the emergent behaviour of moieties is operating in a dynamic manner to produce the measurable result (van de Waterbeemd and Testa, 1987; Gaillard *et al.*, 1994). Future improvements in these methods may benefit from a better understanding and modelling of such dynamic interactions.

A new approach to the estimation of **molecular electronic charges** (an emergent property) has been built around small molecular fragments. This is referred to as a "Lego" approach (Walker and Mezey, 1993). These intermediate level ensembles are evaluated and then integrated into a constructive scheme which predicts the property for the whole molecule. By reduction of a molecule to this level of complexity, the logical depth is not exceeded and the information value is significant in the synthesis of the emergent electronic properties.

The above examples lead us to recognize that there is at least one intervening level of complexity lying between the two complex systems of atoms and molecules. These intermediate systems must be recognized,

evaluated and integrated into any study of phenomena associated with drug design and molecular properties. We can thus expand upon Fig. 4 by inserting these intermediate structures, as shown in Fig. 5.

2.1.4 The Case of Large Molecules

In the present section, we consider molecules of "usual" size, i.e. of molecular weights (MW) of the order of 10^2. In Section 2.3 we shall consider emergence in macromolecules (MW of the order of 10^4–10^6). Between these two levels, however, we believe that there exists an intermediate level of large molecules (MW of the order of 10^3) which may display emergent properties not seen in smaller molecules. What we have in mind here as an example is a property labelled "**chameleonic behaviour**", whereby a large molecule is able to display large variations in polarity and lipophilicity due to very marked conformational changes as a function of solvent.

In chemical terms, large molecules may exist in two families of conformers, one hydrophilic and the other lipophilic. In polar solvents such as water, these large molecules can expose their polar groups while masking their hydrophobic groups (hydrophobic collapse). In non-polar solvents, on the contrary, the hydrophobic groups are exposed while the polar groups are masked (hydrophilic collapse). Cyclosporin A offers an example of this behaviour (El Tayar *et al.*, 1993). This molecule has the approximate shape of a ribbon with the ends bound together to form a ring. This ribbon carries polar groups on one side and hydrophobic groups on the other. In non-polar solvents, the ring exposes peripherally its hydrophobic groups while the polar groups point inside and form intramolecular hydrogen bonds. In water, cyclosporin A has its polar groups exposed to the outside and available for intermolecular hydrogen bonds, while the hydrophobic groups form a hydrophobic core at the inside of the molecule. Another relevant example is that of morphine *O*-glucuronides (Carrupt *et al.*, 1991a).

2.2 A LOOK AT COMPLEXITY AND EMERGENCE IN AGGREGATES OF MOLECULES

Isolated molecules are but a fiction (Amann and Gans, 1989). Special techniques are required to observe a single molecule, and even in this case interactions with a probe are indispensable. When it comes to drug research, the most relevant state of molecules is the dissolved state.

2.2.1 Emergent Properties of Water

The prototype of complexity and emergent behaviour (within our realm of interest and circles of research) is **liquid water**. Short-range order and local

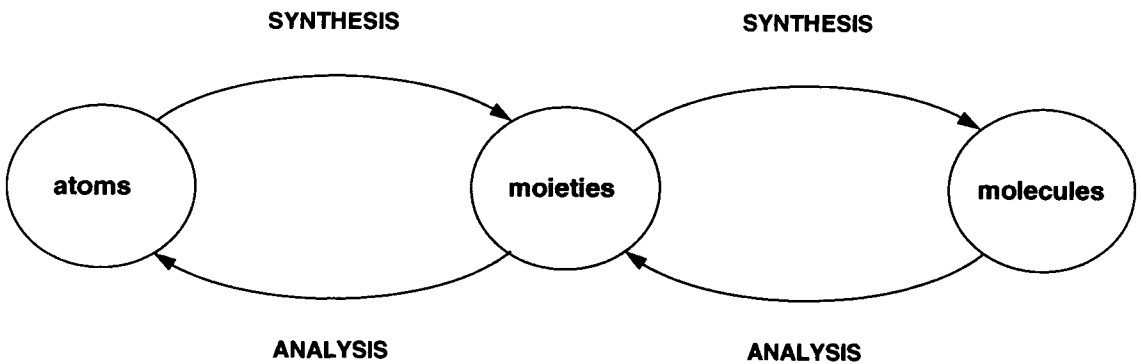

FIG. 5. An improved model of molecules derived from two circles of research.

TABLE 1

Some characteristics and attributes of the molecule of H_2O and of bulk water

H_2O	Water
Conservative state	Three states
Is discrete	Offers up a mobile proton
Forms stoichiometric hydrates with a solute molecule	Dissolves a molecule within an indefinite ensemble
Has a defined topology	Constantly changes its topology
Cannot surround and isolate anything	Surrounds and isolates solute molecules
Is in equilibrium	May receive dissipated energy or provide reaction energy
Has electronic properties	Has physical properties

structure have been studied with a variety of techniques (e.g. Kusalik and Svishchev, 1994). Molecular dynamic simulations (Stillinger and Rahman, 1974) and synthesis using cellular automata have presented a model of water as an ensemble of ever-changing quasi-macromolecules where detailed structural features are impermanent (Kier and Cheng, 1994).

As a result many different functioning structures may appear within the transient architecture of liquid water. This makes it possible for liquid water to perform in several roles. Water is thus comparable to a supramolecular assembly, and indeed it has been postulated to act as a template for macromolecular systems (e.g. nucleic acids) which have evolved and have breathed life into non-purposive molecular assemblies. In Table 1 we compare some characteristics and attributes of bulk water and the molecule of H_2O.

2.2.2 The Soluble State

The conventional approach to the understanding of chemical events is to model a system with the reagents (reactants) as isolated participants. On occasion, recognition is made of the presence of a dimer or a hydrate as a functioning member of a chemical interaction (reaction). But these are exceptions. The role of the solvent (e.g. water in a biological reaction) is usually neglected because it is poorly understood (Testa, 1984).

Upon reflection, it becomes clear that a solute is immersed in a solvent that itself possesses some architecture, a fact particularly true for water (see above). Molecular reactions or interactions do not occur *in vacuo* but must take place with the solvent as an intimate participant. Within this architecture a molecule in an aqueous or organic solution is not like a

molecule in a gaseous, liquid or solid state, but it is part of a complex system which is altogether strongly interactive, dynamic and stochastic. In water or another solvent, solute molecules are surrounded, shielded and bonded to various degrees, and they are isolated at least temporarily from their own kind. This is the soluble state of a compound, a state characterized at the molecular level as consisting of **aggregates**, defined as short-lived, probabilistic ensembles of solute molecules(s) and solvent molecule(s).

Under these conditions, aggregates display emergent properties (e.g. the state of being soluble, see above) not seen in the neat state. The soluble state also exhibits emergent properties such as diffusibility, conductivity, partitioning between solvents and interfacial activity (Testa and Kier, 1991). What is lost here are many physical properties which have no meaning in the soluble state, namely properties of the crystal, gas or bulk liquid.

The participation of the solvent in a chemical reaction or in a molecule–macromolecule interaction must now be actively considered in any model of emergent properties of macromolecules. Water, by virtue of its adaptable nature through exchange of ephemeral hydrogen bonds, may play a regulatory role in the availability of solute molecules to self-associate, to interact or to participate in chemical changes. Accordingly, we add the molecular aggregates to our hierarchy of complex systems (Fig. 6).

2.3 A LOOK AT COMPLEXITY AND EMERGENCE IN BIOMACROMOLECULES

2.3.1 Residues

Biomacromolecules (MW ca 10^4–10^6, see Section 2.2) are large molecular entities which play essential roles in living processes. Biomacromolecules which come to mind are the nucleic acids, the proteins (including lipoproteins and glycoproteins) and the glycopolymers. As discussed below, biomacromolecules exhibit emergent properties which form the substratum of living processes. We consider biomacromolecules as the smallest systems which can truly be considered as biological. In other words, the level of biomacromolecules is the lowest one for biological systems.

Synthetically and biosynthetically, a macromolecule is produced by covalently binding together a large number (approximately 10^2–10^4) of monomeric units, i.e. nucleotides, amino acids or sugars (Goodsell and Olson, 1993). In an analytical perspective, the first step in determining the structure of a macromolecule is to establish the nature and connectedness of the constituting monomers. The earlier methods of achieving this relied on cleavage reactions to separate monomers, which understandably have been designated as residues. However, such a designation is far from innocent and in fact conveys a strong reductionistic undertone since it equates a macromolecule with a long **additive** sequence (linear or branched) of

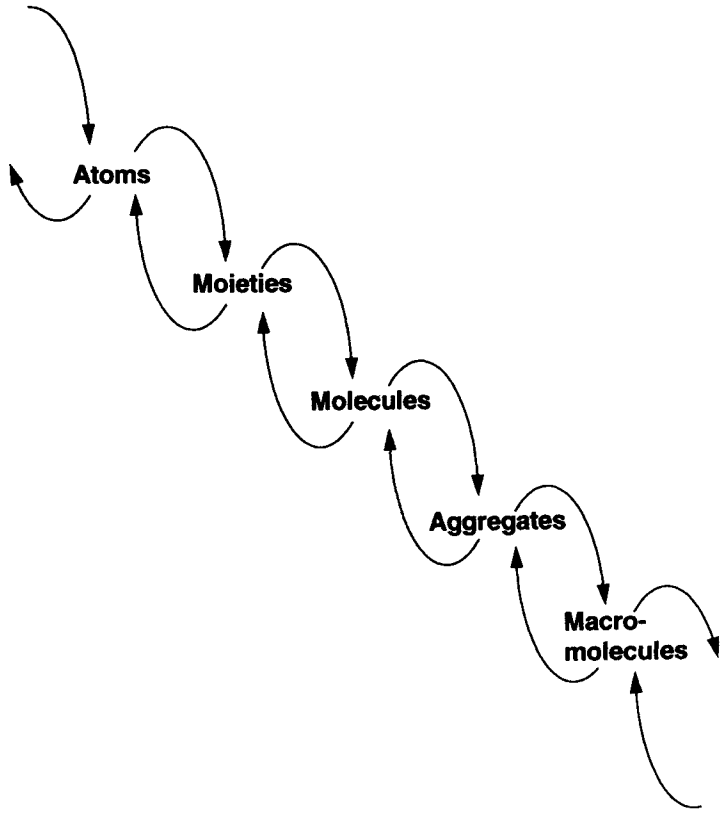

FIG. 6. Hierarchy of complex systems in the field of chemistry.

residues. Such a view is not erroneous but simply incomplete. The point we are making here is that the incompleteness of the additive view is greater than generally recognized, as best understood when considering a number of macromolecular properties.

2.3.2 Additive and Non-additive Properties of Biomacromolecules

It is a trivial statement that properties such as molecular weight and volume are additive. At the other extreme, **conformational freedom** (defined as the number of discrete allowed conformations) is not an additive property. Indeed, this number increases exponentially with the number of residues, while the conformational possibilities of the monomers decrease when they lose their individuality to become bound as residues. Thus, additivity is found to break down when conformational behaviour is considered. In

principle, the conformational hyperspace of monomers, residues and macromolecules can be computed and compared, but the reliability of the results decreases exponentially as the number of atoms increases linearly (a consequence of limitations in computing power). We conclude that macromolecules give indications of complexity in terms of an emergent conformational behaviour, whose exploration however is severely restricted.

While the number of possible conformers of a macromolecule is practically unlimited, it is a common observation that macromolecules in biological systems occupy only an extremely limited portion of the conformational hyperspace open to them. As a result, they exhibit well-defined shapes which confer upon them the emergent property of functionality (see Section 2.3.3).

The fact that only one or a very few **conformational minima** are effectively occupied by biomacromolecules must be seen as an emergent property in itself. This topic has been given particular attention in proteins and is connected with the problem of **protein folding** (Moult and Unger, 1991; Gething and Sambrook, 1992; Frauenfelder and Wolynes, 1994; Sali *et al.*, 1994). In some cases, protein folding in cells is done with the help of other proteins (e.g. chaperones, see Georgopoulos, 1992), other proteins, having been denatured experimentally, will refold *in vitro* in the absence of macromolecules and recover their native topology within an amazing short time (sometimes a matter of minutes). These processes result from complex and very poorly understood interplays of **intramolecular forces** involving long-range electrostatic interactions (e.g. ionic bonds), and shorter-range interactions such as hydrogen bonds and van der Waals forces. Hydrophobic influences also play an important role, for example with the burying of hydrophobic areas (Dill, 1990).

The well-defined shapes assumed by proteins and other biomacromolecules are far from rigid. In fact, a remarkable degree of **flexibility** often exists such that macromolecules oscillate around their conformational minima, and do so by moving residue side chains, segments of the backbone, and entire domains (Gerstein *et al.*, 1994). This flexibility is also a property essential to the correct functioning of biomacromolecules, and what we observe is an amazing fine tuning between conformational determinism and conformational freedom. In addition, it must be realized that this fine tuning is dependent on **intermolecular interactions** with the biological environment (e.g. aqueous media such as cytosol, or lipidic media such as membranes). In fact, the conformational minima of macromolecules can only be understood as **basins of attraction** rather than simple attractors in the hyperspace of intramolecular and intermolecular interactions. This is the language of the science of complexity, chaos and emergent properties. How these basins of attractions are coded in the genes and their products, for example in the amino acid sequence of proteins (the "second fundamental code of molecular biology", Thornton *et al.*, 1991), remains a

deep mystery. We review this "second code" as "order-out-of-chaos" resulting from higher level interactions between residues. For example, context has just been shown to be a major determinant of β-propensity (Minor and Kim, 1994). Such higher level interactions are by nature not linear ones, implying that they will yield partly but not completely to reductionistic approaches.

In sum, both intramolecular interactions (proximal and distal, long and short range) and intermolecular forces (with water, ions, other molecules) condition the conformational behaviour of macromolecules and are themselves influenced by it (Eisenberg, 1990). Furthermore, these intramolecular and intermolecular interactions are strongly interdependent (Testa, 1984; Testa and Kier, 1991). But there are other macromolecular properties which are of relevance, namely the various **macromolecular fields** (e.g. the polar/electrostatic, hydrophobicity and lipophilicity fields), which decrease with distance according to complex functions and can be represented in very simplified forms as distributions on the macromolecular surface of polar/electrostatic and lipophilic/hydrophobic regions (Carrupt *et al.*, 1991b; Gaillard *et al.*, 1994). And here, another level of complexity can be mentioned, since intramolecular interactions, intermolecular forces, conformational behaviour and macromolecular fields are all interdependent properties which, when considered synthetically, hint at underlying complex physical systems (Fig. 7). In fact, a more appropriate statement is to say that these various interdependent properties are nothing more than very partial and analytical descriptions of the highly complex entities known as biomacromolecules.

2.3.3 Functional Properties of Biomacromolecules

Why are proteins so big? This question has recently been asked by Goodsell and Olson (1993) in a thought-provoking minireview on the **size, shape and function** of soluble proteins. In fact, a number of complementary explanations, none of which is incompatible with any other, have been put forward (Goodsell and Olson, 1993) or can be envisaged to answer this question and throw light on the structure–function relations of biomacromolecules. We briefly review several of these explanations, which are related to ligand binding (1 and 2) or to macromolecular functions (3, 4 and 5).

(1) One explanation put forward by some authors is that a large surface area may serve as a funnel, trapping the ligands (substrates, agonists, etc.) in a two-dimensional milieu which offers a higher probability of finding the target site than a three-dimensional milieu. This is also the thesis of Eigen (1990) and Blum *et al.* (1988), mentioned in Sections 1.4.4 and 3.1.1, respectively.

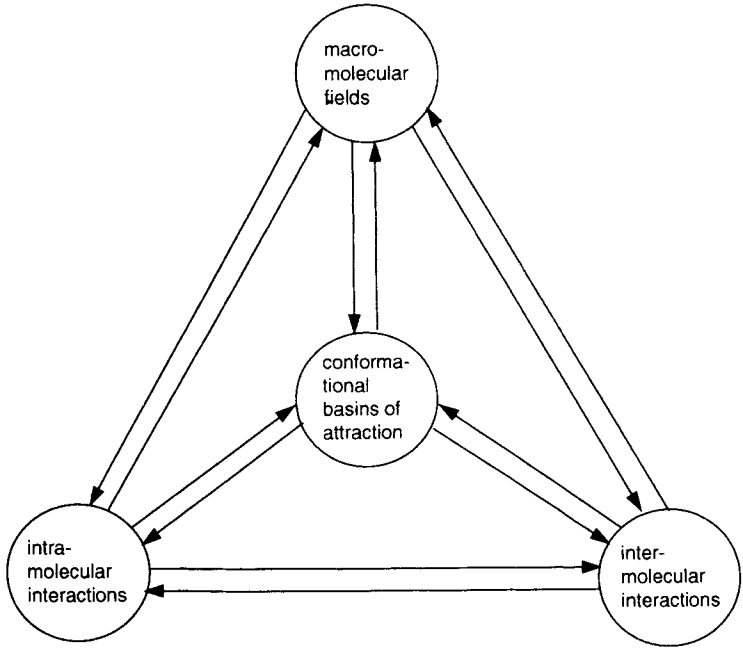

FIG. 7. Interdependent properties of biomacromolecules as partial representations of complex systems.

(2) A second reason is that a large surface arrayed with ionic and dipolar groups may generate a strong electrostatic potential that will guide charged ligands (Leckband *et al.*, 1992), as demonstrated for superoxide dismutase (Getzoff *et al.*, 1992). Similarly, the grooves of DNA are characterized by a very strong electrostatic potential which attracts cations (Pullman, 1989).
(3) A third reason is that a sufficient length of polypeptide is needed to enforce the shape of a protein, and particularly the precise stereoelectronic architecture of its active site. In enzymes, for example, this allows coupled vibrations and energized motions that contribute to catalytic mechanisms and an exquisitely fine-tuned stabilization of transition states (Kraut, 1988; Havsteen, 1989; Retey, 1990; Knowles, 1991; Tonge and Carey, 1992; Williams, 1993).
(4) Also related to function, and strongly connected with points (3) above and (5) below, is the electron transfer within a macromolecule and between macromolecules. A fine-tuned alignment (in direction, distance and, significantly, in modulated redox potential) is required for functional groups and chelated transition metal ions to form a toboggan on

which electrons can cascade down to the substrate (Tollin and Hazzard, 1991).

(5) And finally, it has been stressed that macromolecules must not associate at random to form non-functional aggregates. Rather, they must bind with very high affinity and utmost selectivity to other macromolecules to channel ligands and substrates (e.g. metabolic channelling), to receive or donate electrons (e.g. the transport chains that feed electrons to oxidoreductases such as cytochrome P450), to transmit signals to other functional macromolecules (e.g. receptors linked to G proteins), to exhibit cooperative behaviour, to be regulated, etc. (Hollenberg, 1990; Collins *et al.*, 1992; Welch and Easterby, 1994). Nothing short of a very large contact surface will ensure this extreme selectivity (Goodsell and Olson, 1993).

As a matter of fact, all five explanations above have a common denominator, namely **molecular recognition**, be it in the binding step and/or in the activation step (see Section 3.1.3). Biomacromolecules are big because only then can molecular recognition combine very high efficiency (fast turnovers) and very high selectivity. At this point, we should remember that molecular recognition is the single most essential phenomenon in biology, since it underlies such fundamental phenomena as transfer of information, information gain and self-replication, to name but a few (Sarai, 1989; Rebek, 1991).

In conclusion, biomacromolecules have evolved to be big because from their size and structural variety arise a number of emergent properties that allow them not only to function with utmost efficiency, but to do so in a manner fully controlled by the higher levels of complexity characteristic of living systems (see Section 2.4).

2.4 A LOOK AT COMPLEXITY AND EMERGENCE IN CELLS, ORGANS AND ORGANISMS

Cells are not just bags of enzymes, nor are they little men in test tubes. Trivial as these statements may seem, they summarize the unconscious or conscious belief of many scientists, that cell investigations can offer all there is to know about organisms, and that the study of biomacromolecules holds in store a comprehensive understanding of cells. These statements may also reflect the fashionable belief that the entire secret of organisms and even populations lies in genes, or the anti-science campaigns of those who preach that drug research can continue without animal experiment.

In this section, we outline a few of the many emergent properties of cells, organs and organisms.

2.4.1 *Biological Membranes*

The most significant level between macromolecules and cells is that of biological membranes, an ensemble of structures whose complexity and simplicity, fluidity and stability, as well as adaptability and resilience, will continue to puzzle biologists for many years to come (Rand and Parsegian, 1989; Devaux, 1991; Pastor *et al.*, 1991; Stoffel, 1990; Seydel *et al.*, 1994). It is here, in the study of natural membranes, that the science of complexity can find one of its most obvious objects of study in the area of biological sciences. Indeed, biological membranes are neither in the solid nor in the liquid state, but in metastable equilibrium between these two states, in a very strong analogy to the phase transitions seen in physical systems. In the vocabulary of the science of complexity, biological membranes are poised at the edge of chaos. In such a **state of criticality**, a system (any system) is no longer a linear assembly of parts with the output/change being proportional to the input, but displays creative properties with, for example, a small input producing a large change, or a large input being dampened to little or no output (see Section 1.4.4) (Ito and Gunji, 1994).

A system in a state of criticality has one essential property which explains the term "creative" used above. This property is the **processing of information**, whereby

> . . . a complex adaptive system [is characterized] by its capability of functional self-organization, based on processing of information. If one asks where does this information come from and what is its primary semantics the answer is: information generates itself in feedback loops via replication and selection, the objective being "to be or not to be". (Eigen, 1994)

2.4.2 *Organelles, Cells, Tissues, Organs and Organisms*

Complex adaptative systems such as membranes are said to self-organize, but this must not be misunderstood. Indeed, self-organized criticality at each hierarchical level of biological systems is not reached independently of other levels, but in a fully coordinated and integrated manner. The readily recognizable levels are listed in Section 1.2.1, and those to be briefly considered here appear in the title of this section.

Each of the hierarchical levels in living systems can be viewed both as a system and as a subsystem; each in fact is a multilevel hierarchical system integrated with many of its kind into a higher system (Kurganov and Lyubarev, 1992; Kurganov, 1993). Again, such a statement may seem rather obvious to the biologists and pharmacologists who grant themselves time for reflection. What is at stake here, however, is the realization, as explained at length in Section 1.4, that complex adaptative systems are not created by the linear addition of their constituting subsystems, but have evolved from

non-linear interactions between their subsystems which bring them to a state of criticality at the edge of chaos where emergent properties govern their behaviour. Eigen's **hypercycle** offers a possible model, certainly a simple one compared to biological reality, of how such complex systems may self-organize and function. Three examples are discussed here to illustrate the issue of emergent properties in biological systems.

(1) The function of many enzymes is to regulate the levels, and their variations with time, of the innumerable **endogenous compounds** (e.g. hormones and neurotransmitters) that control growth, homeostasis, differentiation, movement, apoptosis and other biological phenomena (Nebert, 1990, 1991). The activities of these enzymes are themselves highly subject to regulation, e.g. by induction, activation, increased or decreased supply of cofactors, product inhibition, uncoupling and degradation, all of which are controlled by the biological phenomena just mentioned. Feedback loops of unimaginable complexity thus operate permanently to link all biological levels, offering to the mind the intuition of a multi-dimensional regress of hypercycles within hypercycles. Our understanding of metabolic regulations may progress significantly with the advent of the new tools of molecular genetics and cellular biology, but we believe (although this is not the issue here) that a system (i.e. us) cannot know itself completely as a result of Gödel's incompleteness theorem.

(2) The most complex system we have recognized is said to be the **human brain**. It has been suggested that the healthy state of the brain (as well as that of the heart) is chaotic, and we can be confident that without the science of complexity, chaos and emergent properties, we shall not go far in understanding cerebral functions such as information acquisition, processing and storage. As a resonant tribute to the potential of the science of complexity, recent experimental work has demonstrated that spontaneously bursting networks of neurones exhibit chaotic behaviour. What is more, chaos could be experimentally controlled, thus opening far-stretched opportunities for the treatment of epilepsy (Moss, 1994; Schiff *et al.*, 1994).

(3) The **concept of time**, or rather of many times, is a central issue in complex systems and particularly in biological systems (Boxenbaum, 1986). It is difficult for us, living beings endowed with consciousness, not to use our conscious time as a universal meter and then to be misled into believing that this time of ours is relevant at all biological systems from macromolecules to human societies. Many time scales operate from subatomic time to physiological and psychological time, as cogently discussed by Alexis Carrel (1935), and there have been attempts to link them to size, e.g. body mass is correlated with heart beat which in turn is related to life expectancy (the human being standing out as an

exception) expressed in our time scale. But perhaps time is related less to mass than to complexity as an expression of information processing and creation, in which case relative time becomes an emergent property of complex systems and can no longer be viewed as marginal in the study of such systems.

It follows from all issues discussed in Section 2 that, as we progress in our scientific explorations and creations, the dimensions opened by the new science of complexity will unavoidably become part of our mental universe. This will affect not only the way scientists interpret their results, but above all the way experiments are designed and planned in our reality, both physical and virtual. How complexity and emergent properties can effect drug research is examined, sometimes provocatively, in Section 3.

3 Applications to Drug Research

3.1 A LOOK AT COMPLEXITY AND EMERGENCE IN DRUG–ENZYME AND DRUG–RECEPTOR SYSTEMS

3.1.1 Preformed or Induced Binding Sites?

Does the binding site of a receptor exist in the absence of a ligand (agonist or antagonist)? Does the active site of an enzyme exist in the absence of a substrate? Do these recognition sites have a permanent existence (i.e. are they preformed), or do they appear under the influence of the molecules they bind? The model of binding sites evoked by their ligands has been postulated for serum albumin but is far from being fully accepted by the majority of pharmacologists (Honoré, 1990).

The present sources of information are X-ray images of crystallized proteins holding substrates or inhibitors. Indeed, without such a **two-body analysis** there would be no way to identify with certainty a recognition site among an enormous number of combinations of structured features within a macromolecule (see for example the many ambiguities existing in locating ligand binding sites in 7-transmembrane receptors, Schwartz, 1994). The revelation of the possible location of a recognition site is synonymous with the actualization of its existence. Thus, and to put it simply and provocatively, do we create a recognition site by finding it?

3.1.2 Empty Sites en route to Binding

A binding site in a receptor or enzyme protein is seen as a constellation of amino acid side chains and peptide bonds in the backbone. The spatial

vicinity of any two of these binding groups is related to the dynamic character of the protein macromolecule and to the critical structural role played by water molecules (Sreenivasan and Axelsen, 1992; Timasheff, 1992). In the absence of a substrate or ligand, this constellation must be viewed as dynamic and deterministic, producing as it does a more or less fluid architecture of protein moieties and water molecules which should be understood as an attractor (or rather a basin of attraction) in morphogenetic space. In other words, the existence for a sufficient duration of a meaningful constellation of recognition groups depends upon a conjunction of critical events such as (i) a combination of interdependent conformational changes, and (ii) the interaction between water and protein sites to mask or unmask the latter (Pavlic, 1987; Quiocho *et al.*, 1989; Goodford, 1991; Rand *et al.*, 1993). A constellation of recognition groups is said to be meaningful when it allows long-distance recognition of the ligand or substrate (see Section 2.3.3).

The realization of the significance of critical circumstance (i) has been the thesis of Blum *et al.* (1988) in a recent study on the **curvature of enzyme surfaces**. This curvature is seen as having a guiding effect on the target focusing of a ligand. This ability of an enzyme or receptor to contort itself into a particular shape produces an enlarged surface area to which a ligand may be attracted. Once within the domain of influence of this target area, the ligand takes a low-energy path to the binding site. This emergent property is thus one that may be of great value in depicting the dynamic events facilitating an encounter between a ligand and a binding site.

A comparable yet broader view on the facilitation of such encounters has been presented by Behling and Jelinski (1990) following spectroscopic studies with acetylcholine binding to its receptor. These authors have integrated the **membrane** in their experimental design, arriving at a dynamic model whereby acetylcholine first binds to the membrane where it adopts its bioactive conformation, and then rapidly diffuses along the membrane to the receptor. This model is stimulating and promising because it considers a higher level of organization than a receptor viewed in isolation, and also because it reminds us of the membrane environment of many receptors and enzymes, of membrane criticality, and of the regulatory roles of still higher levels of biological organization (see Section 2.4).

The **role of water** with protein molecules, stated in (ii) above, can be illustrated by some dehydration–rehydration studies of lysozyme reviewed by Finney (1986). The interpretation of the experimental data leads to several conclusions. A certain amount of water is essential at key sites in the enzyme; the water facilitates a flexibility that is critical for the enzyme function; and finally, the water permits a proton redistribution as the polar and charged groups revert to the aqueous solution order.

Another example of (ii) above can be found in the conclusion of Kuhn *et al.* (1992), who proposed that the deep grooves in protein surfaces are formed by protein–water interactions.

3.1.3 The Binding and Activation Steps

The binding of a ligand to such a recognition site must create a viable **ligand–binding site complex system** endowed with functional capacities. To this end, the ligand must trigger additional critical events, namely (iii) the conformational and electronic adaptation in the binding site [i.e. the so-called "induced fit" (Koshland, 1968) or "zipper" models (Feeney *et al.*, 1974)], followed in the case of an agonist or a substrate by the **activation step** (e.g. production of second messenger or catalysis (Testa, 1984; Hollenberg, 1990; van Gelderen *et al.*, 1994).

Briefly stated, an effective encounter between the agonist (or substrate) and the receptor (or enzyme) necessitates the critical events (i), (ii) and (iii) outlined above. If we reflect on the enormous number of possibilities that these events may produce, we wonder if a receptor or active site is ever formed. Of course they must be but how does the "memory" of the critical events manifest itself? One possibility is that there must always be a low concentration level of ligand or substrate participating in maintaining an operating site. The presence of an even occasional ligand or substrate molecule may lead, we postulate, to an increase in the ordering of matter, which cascades into an increased ordering of the architecture of the protein. As a consequence, the relaxation process which may occur after a reversible ligand–receptor encounter is never allowed to progress too far away from the set of critical events in (i), (ii) and (iii).

In contrast, a prolonged absence of a ligand or substrate from the "battlefield" might produce, we postulate, a significant departure of the biophase from the criticality (i), (ii) and (iii) (e.g. **dormant receptors**, see Ariëns, 1991). Thus the "start-up" time of a receptor or enzyme following the arrival of a ligand or substrate may be much longer. What happens if abstenance of drug–receptor encounters lasts for too long is a matter of speculation.

The receptor or enzyme, in the light of this model, is thus a complex, dynamic system which possesses the **emergent property of function**, as a non-linear combination of the parts (the ligand and the macromolecule) operating in a time-coordinated manner. It comes into existence as a functional entity when the parts are coincident in time and juxtaposed, but does not pre-exist, we believe, in a passive equilibrium state, anticipating a ligand. Its existence must be regularly "primed" to keep the ingredients near the critical state so that it can quickly and accurately respond as a receptor or enzyme.

One example of this possible existence of a hierarchy of receptor states has been discussed by Frauenfelder (1988). He reviewed studies on the binding of substrates and ligands to myoglobin. The process follows a power law, characterizing the protein as a complex system. Nuclear magnetic resonance (NMR) analyses revealed a number of conformational substates,

sharing the same overall structure but differing in local arrangements. The transitions between substates have been called proteinquakes, an analogy with stress-relieving earthquakes. These substates perhaps play the role of intermediates in the stepwise ordering of an enzyme or receptor beyond equilibrium, discussed above. Near-critical substates may exist, awaiting the arrival of a few more molecules of ligand to carry the receptor system beyond criticality, a necessary event to produce an effect.

Some receptors are known to exist in equilibrium between two functional states. For example, the dopamine D2 receptor has high-affinity and low-affinity states, while the opiate receptor has a functional, agonist-binding state and a silent, antagonist-binding state. Endogenous factors as well as drugs may influence the equilibrium, and it would be interesting to investigate what the mechanisms involved have in common with the above scenario.

3.2 A LOOK AT THE QSAR PARADIGM

3.2.1 The Genesis and Use of SAR Models

A large amount of effort is being directed towards the development of computer-based models in the pursuit of the goal of rational drug design. The key word in this broad area of research is "model". Following the discussion in Section 1.3.2, we offer this definition: "A model is the result of efforts to isolate and remove interactions between ingredients of a complex system that are judged to have little or no influence on an observable under study." The essential point in the present context is that much information is lost when going from a real system to a manageable model.

The only realizable goal in science is to create models which, despite their essential incompleteness, are both right (i.e. self-consistent) and relevant (i.e. in correspondence with the system being described). This goal is accomplished using information gathered by perturbing a system with a probe and measuring or computing the output as observables. The source of the observables is either from direct measurement of states of a system, or from prediction of values of states based upon previously developed models. Thus, we can perturb a series of liquids energically and record their boiling points, or we can utilize an earlier established model such as molecular orbital theory to predict boiling points. From either source, we have the makings of a model of some aspect of this information.

3.2.2 Evolution and Limitations of the QSAR Paradigm

In the last three decades, three variants of a predictive drug design paradigm have come upon the scene, which are called collectively **quantitative**

structure–activity relationships (QSAR). The first variant, called the Hansch approach, uses physical properties to define a set of molecules and then attempts to find linear relationships between one or more of these properties and a set of numerical values reflecting some biological activity. Unfortunately the word "structure" is used in the label of this paradigm, while it is clear that this is not correct (Testa and Kier, 1991).

The second variant of QSAR is the use of actual structural descriptors, such as molecular orbital indices or topological codes, to define numerically the structure of a molecule and to find linear relationships with numerical biological data (Kier and Hall, 1976, 1992).

The third variant, the most recent one, was born from the recognition of the importance of the three-dimensional structure of drugs (Cohen, 1985), and has evolved to three-dimensional QSAR (3D-QSAR). **Comparative molecular field analysis** (CoMFA) is to date the most sophisticated tool in 3D-QSAR (Cramer *et al.*, 1988; Gaillard *et al.*, 1994).

The QSAR paradigm was designed to generate information to guide the synthesis of candidate molecules for a particular biological class. However, the information so obtained is limited to the variety in properties and structure within the molecular set. But more important in the present context (and poorly recognized) is the fact that there is no clear information linkage between the complexity level of the active molecules and that of the cell, organ or organism where the activity is measured. The logical depth is too great because the emergent property of the biological response has gone through too many levels of intervening complex systems to have any hope of achieving some meaningful understanding. As discussed by Levy and Boddy (1991), the **hydrid character** of pharmacokinetic parameters (the same is true of pharmacodynamic parameters) increases when going from macromolecules to organs to the whole body. Only in the case of activities measured with isolated enzymes or receptors is the logical depth limited enough to allow some sound interpretations, as already emphasized by us (Testa and Kier, 1991).

Drug designers must accept these facts not as a shortcoming but as an opportunity to achieve heuristic, Newtonian models of optimal molecular structures by additive assembly of moieties. Thus we jump across many circles of research and find parallel behaviour, which guides us to practical objectives. The danger exists in attempting to build some mechanistic interpretation into the models. Years ago, Kier (1971) cautioned against such extrapolations from molecular orbital data to receptor understanding.

3.3 A LOOK AT MOLECULAR MODELLING

With the advent of high-speed computing machines, it is now possible to process and capture vast amounts of information from theoretical models. In

the arena of drug design, various (non-synonymous) terms have surfaced to label these efforts, such as computer-assisted molecular design, computer-aided drug design, molecular modelling, molecular graphics, computational chemistry, etc. (Balbes *et al.*, 1994). The processing is both algorithmical and visual, the latter lending itself to the continuous monitoring of dynamic processes.

There are fundamentally two types of models that are developed under the plethora of rubrics aluded to earlier. We shall now spend a little time with each, pointing out their characteristics and giving a brief criticism.

3.3.1 Static Models

The static models may take the form of QSAR equations (see Section 3.2), or they may be visualized through the use of computer graphics. The graphic information generated is derived from a reductionistic analysis of a complex system. Because of the limitations in our ability to study directly molecules, proteins, receptors and others, this information invariably owes much to theoretical predictions obtained from computational chemistry approaches, e.g. molecular orbital theory and molecular mechanics. One of the earliest derivatives of this kind of information was the development of the concept of receptor mapping using molecular orbital theory (Kier, 1967, 1970, 1971). Marshall (1987) has expanded on this general approach utilizing the full power of computer graphics to visualize the results. The CoMFA approach discussed in Section 3.2 is a further extension of the static molecular model derived from theoretical models.

One significant limitation of such reductionistic models lies in their inability to reveal the dynamic nature of the systems they portray. These models describe artificial conservative states with no revelation of dynamic transactions and alterations of states. Of course the illusion of movement can be graphically fabricated by collectively changing the coordinate frame, but the transactions within the system are still unaltered. While useful information may be derived from such models, it is imperative that their genesis be well known to the drug designer.

3.3.2 Dynamic Simulation Models

A dynamic simulation uses the ingredients dissected from a complex system to attempt the construction of a model which exhibits dynamic attributes, i.e. emergent behaviour. Such a simulation is more complicated than the static models from which it is derived. Attempts are made to incorporate into these simulations the transactions and interactions that we know are there but which elude a precise definition. Comparisons are made with observables to ascertain some degree of the quality of a model.

Molecular dynamics, Monte Carlo simulations (Haile, 1992), and very recently applications of cellular automata to drug research (Kier and Cheng, 1994) have shown the way for this general approach. By creating a dynamic simulation, we complete a circle of research and introduce a greater degree of realism into the model. Emergent properties, self-organization and the interactions operating within a system may be revealed and productively modelled. The agreement with observations depends upon the judicious choice of ingredients and interaction parameters or rules. This is clearly an expanding approach to the understanding of complex systems.

3.4 THE DRUG–ORGANISM COUPLE AS A COMPLEX SYSTEM

3.4.1 Pharmacokinetic and Pharmacodynamic Events

Drug action is usually divided into three phases, namely (i) the **pharmaceutical phase** (which comprises all physical and chemical processes determining the fraction of the dose available for absorption), (ii) the **pharmacokinetic phase** (which comprises all biological processes determining the fraction of the dose available for action), and (iii) the **pharmacodynamic phase** (which comprises all biological processes involved in the drug's effects). In fact, the pharmacokinetic phase can be considered to comprise all effects exerted by a biological system upon a drug, while the pharmacodynamic phase comprises all effects exerted by a drug upon a biological system. A schematic representation of these definitions (Fig. 8) is certainly appealing to our Aristotelian and Cartesian education. But does it sufficiently account for the complexity of biological reality?

At the molecular level, the discrimination between pharmacokinetic and pharmacodynamic events vanishes since both types involve the same intermolecular interactions between a drug molecule and biological sites. These interactions are: first the long-distance forces of recognition such as ionic and ion–dipole bonds; then short-distance binding forces such as van der Waals interactions and hydrophobic bonds; and finally (but not always) the breaking and forming of covalent bonds (covalent binding to enzymes and receptors, substrate biotransformation). In other words, pharmacokinetic and pharmacodynamic events emerge at the level of functional biomacromolecules, i.e. that of the smallest biological systems (see Section 2.3; Testa, 1987).

3.4.2 A Transactional View of Drug–Organism Interactions

But the point we want to make in Section 3.4 is a different one, namely that the couple made up by a drug and an organism has some properties which

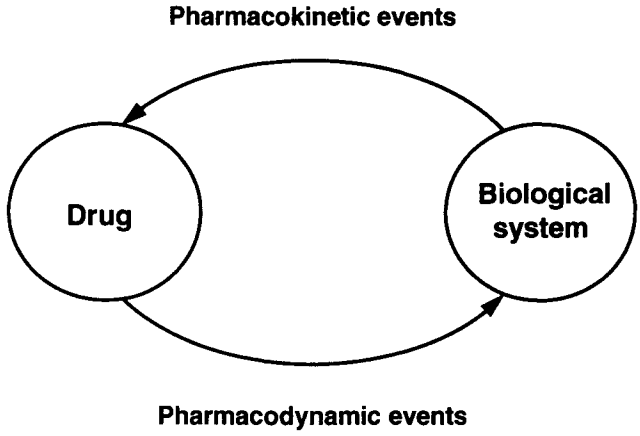

FIG. 8. Scheme representing a drug and a biological system acting upon each other through pharmacokinetic events and pharmacodynamic events (Testa, 1987).

are not the linear addition of the properties of the two partners in isolation. Indeed, when a drug is given to an organism or any biological system, the two partners enter into a type of interaction known as a **transaction**. The latter is defined as "the simultaneous and mutually interdependent interaction between multiple components" (Capra, 1983).

Consider in Fig. 9 a biological system B and a drug D in their initial states B_0 and D_0. Following the pharmaceutical phase, the biological system acts upon the drug by processes of absorption, distribution and mainly biotransformation (Fig. 9A). As a result, the drug now exists in a number of states D_i (Fig. 9B), for example by having entered various physiological compartments or having been metabolized. One or several of these D_i states are active states [active compound(s) at site of action] in which the drug acts on the biological system (Fig. 9C). The result of these actions is to change the biological system into state B_1 (Fig. 9D). In a subsequent step, interactions take place between the drug in the D_i states and the organism in the B_1 state (Fig. 9E). Several outcomes exist which are not depicted; for example, the D_i states can become void due to excretion, and B_1 can either revert to B_0, remain, or lead to another state (Testa, 1987).

In some *in vitro* biological systems such as subcellular preparations, simple pharmacokinetic (e.g. biotransformation) or pharmacodynamic events (e.g. enzyme inhibition) can be seen (Seydel *et al.*, 1994). In more elaborate biological systems such as cellular preparations and multicellular organisms, a number of well-known situations correspond to Fig. 9C–E. Thus, prolonged administration of neuropharmacological agents changes the overall sensitivity of receptors, i.e. the drug acts first on B_0, then on B_1. The types of experiments most adequately illustrating Fig. 9 are metabolic

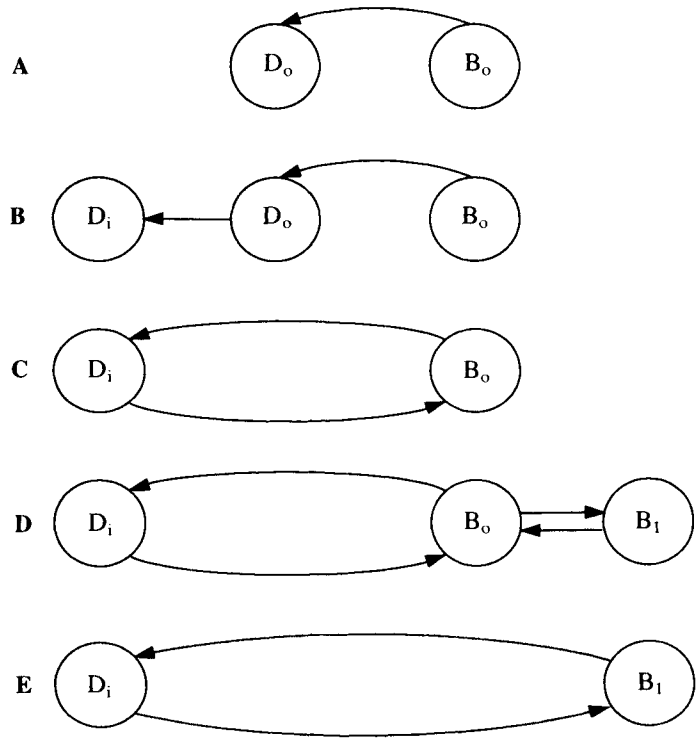

FIG. 9. Schematic translational interpretation of Fig. 8. As a result of their mutual interactions, a drug D and a biological system B exist in a number of states as explained in the text (Testa, 1987).

studies involving enzyme induction (or inhibition) where a drug is used both as an inducer (or inhibitor) and then as a substrate. Note that Fig. 9 only describes situations involving a single drug; the simultaneous or sequential involvement of two or more agents must lead to transactional situations of greater complexity.

Individually, each event (symbolized by an arrow) in Fig. 9 can be classified as pharmacokinetic (arrows pointing to the left) or pharmacodynamic (arrows pointing to the right). However, such a fragmented view neglects all-important transactions and regulations which remain hidden when the drug–organism system is analysed as a linear sequence of pharmacodynamic and pharmacokinetic events. Studies inspired by a transactional view (see above) have been quite fruitful in advancing our understanding of biological regulations. Perhaps future developments in pharmacodynamic–pharmacokinetic modelling will be able to progress far beyond the traditional dose–concentration–effect relations and integrate the transactional dimension schematized in Fig. 9.

3.5 PREDICTION OF CLINICAL EFFECTS FROM PHARMACOLOGICAL DATA

The prediction of clinical effects from pharmacological data is the Holy Grail of many pharmacologists. But because pharmacological data can be obtained from biological systems at different levels of complexity, there are a number of rungs in the ladder of extrapolation to clinical effects. And how, the reader may wonder, is this pursuit relevant to the context of our discussion on complexity and emergent properties?

3.5.1 In vivo *and* in vitro *animal data*

Much has been published on the extrapolation of *in vivo* data from animals to humans. These include pharmacokinetic data (e.g. half-lives, plasma concentrations, clearances and rates of metabolism) and pharmacodynamic data (e.g. effective and toxic doses). Two excellent reviews present many examples and insightful discussions on isometric and allometric relationships, time scales, interspecies pharmacokinetic and pharmacodynamic scaling, and physiological models (Boxenbaum and D'Souza, 1990; Chappell and Mordenti, 1991).

Such approaches are satisfactory in some cases and fail in others. Reasons for failures are seldom well understood, but various explanations—however partial—come to mind, including:

(1) **qualitative differences between species**, e.g. in the nature of enzymes and sites of action;
(2) the many other **physiological and exogenous factors** that affect drug metabolism and response (Balant *et al.*, 1990; Testa, 1995);
(3) the role of **chronopharmacological factors** (Bélanger, 1993).

To understand the causes of such failures and achieve better predictability, pharmacologists have for many years been busy studying many if not most of the biological factors mentioned above. This has been done mainly in simpler *in vitro* experimental models where these factors can be investigated largely in isolation, an apparently paradoxical situation since the research front has thus moved away from the high level of complexity of human organisms it aims at modelling. However, this paradox is understandable if we describe scientific progress with the help of a metaphor, that of a hypersurface where the shortest path between two points never appears as a direct, straight line.

In fact, what we have been and are observing is the analytical phase in the quest for clinical predictions. As stated repeatedly in previous sections, this phase is indispensable because it alone can provide information on the parts of a system. For example, our understanding of interspecies differences in receptor and enzyme specificities had to await the advent of molecular

biology and the concept of **orthologous genes**. According to Nelson *et al.* (1993), an orthologous gene in two species refers to a gene known with certainty to correspond to the ancestral gene which existed before the evolutionary divergence of the two species. Mutations occurring after the divergence have resulted in the two gene products differing not only in their primary amino acid sequence, but also in their specificities (ligand specificities for receptors, substrate and product specificities for drug-metabolizing enzymes).

3.5.2 Human Data

Closer to the target of clinical effects are data obtained in humans. Here of course the problem of interspecies differences is avoided, but other problems become foremost. One of these is genetic differences between humans, with the discovery of **genetic polymorphism** in enzymes (Meyer *et al.*, 1990) and receptors (Strange, 1994). Ethnopharmacology was born with the discovery that variations in genotypes and phenotypes between human populations are observed that cause many **interethnic differences** in drug responses (Kalow and Bertilsson, 1994).

Phenotyping patients is one approach that can be followed to predict fast or slow metabolism of some drugs in such persons. Yet even within a given phenotype, large interindividual differences are always found when comparing plasmatic half-lives, rates of elimination or other pharmacokinetic parameters. Combinations of factors (age, diet, life style, health state, etc.) are usually evoked as very partial explanations for interindividual differences in drug disposition and therapeutic response. In coming years, **genotyping** for receptor variants may become feasible, offering an additional explanatory variable.

3.5.3 Creative Extrapolation?

As illustrated above, analytical reasoning dominates current clinical pharmacology in that many efforts are directed at isolating and understanding the various factors that influence clinical effects. This is indeed the **analytical phase** outlined above, the indispensable phase of data gathering. But the objective is the prediction of clinical effects from results obtained with experimental model systems, and to this end a **synthetic phase** is necessary.

Whether starting from *in vivo* or *in vitro* data, and from animal or human data, extrapolation to clinical effects is currently performed by combinations of **mutually independent factors** (treated as independent variables), or even by simple **monofactorial equations**. Such an approach is clearly not erroneous, witness its many fair predictions, but it is a mere approximation,

the best we have at present. The point made here is that we should start devoting greater efforts to the synthetic phase. This could be done for example by trying to develop more complex models which explicitly take into account the interdependence and even instability of the factors on which extrapolations are based.

Perhaps an inclusion of exogenous factors into physiological models, if feasible, might allow improved approximations. If and when such a stage is reached, workers will be confronted with the next difficulty, which we believe to be the **interdependence of exogenous and physiological factors**. This is the ultimate challenge, the creation of a complex predictive model (here a model of clinical effects) in which all controlling factors are interdependent and influence each other to a lesser or greater degree.

In other words, a time will come when the problem of extrapolation will no longer be understood as calculating a dependent variable from independent variables, but as predicting the response of a complex system (here a patient) from that of a complex subsystem (here a pharmacological experiment). And since emergent properties characteristic of complex systems represent qualitative jumps that cannot be extrapolated, reasoning by analogy or with creative models (e.g. artificial neural networks, cellular automata) will have to be developed. Only the future will tell us whether these speculations belong to science or science fiction.

4 Concluding Remarks

The main reasons for pursuing drug research are curiosity, scientific achievements, financial opportunities, as well as medical and humanistic concerns, stated with uncertain weightings. Our focus of attention in this article has been on the scientific aspects of drug research. In the light of the current **scientific (r)evolution**, epitomized by post-Newtonian philosophy, there is much challenge and fascination in seeking to apply the concepts of complexity and emergence to drug research taken as a multidisciplinary and global endeavour. As far as we are aware, such an attempt on a broad scale is offered here for the first time.

The **short-term objective** of drug research is obviously the creation of new medicinal agents to meet current therapeutic needs. This objective will always be important and may even take primacy over any other consideration especially in cases of urgency such as the AIDS pandemia. There is, however, a **long-term objective** that runs through every effort in drug research. That is the generation of knowledge to help us understand some of the phenomena associated with the complex systems that confront drug researchers.

The achievements recorded in drug research are significant and growing at an encouraging rate, thus satisfying in a general way the above stated

objectives of drug research. In particular, the short-term objective of drug discovery has always been accompanied by the long-term objective of creating new knowledge, thus paving the road for future discoveries. We must be vigilant, however, to the possibility that we remain static and miss the growth curve made possible by entirely new ways of perceiving nature. Indeed, our world views and **paradigms** condition which questions we ask from nature, and how we conceive and design our scientific investigations. By being open to complexity and emergent properties in biological systems, we will know how to ask more profound questions, fashion dynamic syntheses, and recognize order where none was yet apparent.

Among many of the topics considered in this article, there is evidence of persistent Newtonian philosophical traditions, characterized by the struggle to model complex systems from the additive reassembly of ingredients. There is also evidence of probing too far below a complex system to identify ingredients, thus exceeding the logical depth legitimate for the generation of information useful in understanding the system under study. Another observation arising from our critique is that the **dynamic nature of everything** is usually neglected in attempts to model by synthesis the emergent properties of some complex system. Neglect of this reality leaves us with incomplete, static models whose limitations will gradually become intolerable.

The concepts of complexity and emergent properties, discussed here within the context of drug research, must become integral parts of every step in this collective effort as it is practised today and as it evolves to newer methods tomorrow. However, and this remark is so important that it was kept for the end, these new concepts are not meant to replace the traditional mechanistic approaches that have proven so successful in drug research. Rather, the post-Newtonian vision must blend with the Newtonian philosophy to enlarge and enrich our mental world. As so aptly stated by Edward O. Wilson, the father of sociobiology (quoted in Lewin, 1993):

> We needed to understand how parts of the system work before we could look at the whole. But now it's time to look at the whole once again. . . . I'm saying there is something genuinely emergent about the behavior of a complex system . . .

This is the direction to which we have tried to point.

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Drug Design: the Present and the Future

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1 Introduction

The essence of drug design is the reasoned extrapolation of our knowledge of "lead" structures or targeted receptors to suggest novel structures with defined characteristics, as potential drugs.

This chapter cannot attempt a comprehensive coverage of such a topic; rather, the intention is to provide guidelines with appropriate references to the literature for methods and examples. Design will be distinguished from discovery and development, and then consideration will be given to the methods by which knowledge can be accumulated and processed to suggest structures that may have the desired characteristics—potency, selectivity, safety and delivery being paramount among those that will be considered.

For our purposes, design begins after discovery, and may or may not be dependent on it. The use of lithium for treatment of endogenous depression came about through a serendipitous discovery, not through the application of any design principles. An improved drug may subsequently be designed, based upon a knowledge of the mechanism of action of lithium, encompassing knowledge of the physicochemical properties of lithium and the nature and distribution of a lithium receptor, and of the distribution and effects of lithium in the body. Penicillin G was discovered, and subsequently many so-called semi-synthetic antibiotics were designed following close study of the relations between structure and properties of β-lactam derivatives. Yet more structures were designed through knowledge of the biological target for the β-lactams, an enzyme crucial to survival of bacteria. Other derivatives have been designed through knowledge of the bacterial defence mechanisms, in particular β-lactamases.

The design of drugs is best accomplished through knowledge of both structure–activity (or property–activity) relationships, and the target receptor (or receptors). In the past, the designer has most frequently relied only on knowledge of structure–activity relationship (SAR). Increasingly, we are accumulating knowledge of receptors and potential receptors through the isolation, sequencing and structure determination of macromolecules. We are now able to contemplate drug design through knowledge of macromolecule structure and function only, in the absence of any ligand. Drug design can begin by imagining a complementary structure that would be likely to bind to a particular region of a macromolecule known to be involved in a normal or pathological process. Such a structure can be synthesized, and the predicted affinity confirmed. The geometry of interaction may be investigated in favourable cases through X-ray crystallography. In parallel, one would determine the consequences of interaction: is the ligand an agonist, an antagonist, or without effect? Favourable answers to these questions would lead to a cycle of structural modification, synthesis and testing until the desired objectives of potency, selectivity, safety and delivery characteristics had been met.

Design refers to the proposal of a structure in order to achieve the objectives of binding to a macromolecular target site (in the extreme case no ligand may be known), or to achieve the enhancement in potency, the selectivity, the safety and the delivery characteristics that are deemed necessary.

Development refers to the reality of preparing a drug for the market place. In development, it will be realized that predictions of potency, selectivity and particularly safety based on our current knowledge fall far short of the desired perfection. Potency is often compromised to provide selectivity. Safety will depend on our very limited knowledge of metabolic pathways, and on hopeful extrapolations from animal to human responses. Delivery will depend on the skills of formulation, to compensate for physicochemical characteristics such as insolubility that may have been unavoidable in the designer's quest for a sufficient level of potency. At the end of the day, every drug represents a compromise. But better drugs can always be designed.

Design begins either with the choice of a "lead" structure, or with the probing of a relevant macromolecular receptor site in order to suggest such a structure. We shall consider how the lead may be chosen from amongst a miscellaneous collection of active compounds: for a number of reasons, the most potent compound may not make the most suitable prototype for subsequent modification. Certain principles can be applied to suggest those modifications that are likely to be productive in terms of enhancing potency or increasing the likelihood of a safe and acceptable drug. As the structural database grows, with corresponding biological knowledge on potency and selectivity, so various statistical techniques can be applied in order both to gain mechanistic insight, and to optimize activity. Classical and contemporary methods of choosing the lead, principles to be applied in modification, and methods for optimization of activity will be discussed and exemplified.

This review will not include immunological approaches to therapy, nor will it include any discussion of gene engineering, or the potential use of anti-sense oligonucleotides.

It will be seen that drug design requires three ingredients: the empirical, the experimental and the theoretical.

2 Goals of Design

The way in which design principles are applied depends crucially on the goals set, the background knowledge and the availability of relevant screening procedures.

Design can be used to achieve an enhancement in *potency*, should a suitable receptor binding assay be available. This presupposes knowledge of the receptor, at best from an X-ray diffraction study of the receptor-

containing macromolecule co-crystallized with an inhibitor. Currently there are some 800 protein structures known at sufficient resolution, and this number is increasing at a rate of about 60 per year. If detailed knowledge of the receptor at the molecular level is not available, design for potency must rely on being able to distinguish those molecular properties, or structural features, that determine action at the receptor from those that determine pharmacokinetics. Knowledge will be required of how the organism is likely to affect the drug molecule. Features determining receptor binding may then be dissected out through a certain amount of empirical synthesis, followed by relevant screening and a quantitative SAR (QSAR) study for diagnosis and optimization of the desired activity.

Design can be used to achieve *selectivity*. In the case of a chemotherapeutic agent, such as an antibacterial or antiviral drug, such selectivity may be achieved through design based on knowledge of differences in cytology, biochemistry or metabolism between host and invading organism. The achievement of *selective toxicity* with an anti-infective agent intended for short-term, acute treatment is a design problem with many options for solution. In contrast, a pharmacodynamic agent required to treat hypertension, or a cytotoxic drug for cancer treatment, presents a different problem with fewer options. Design here may be required to achieve differences in binding to isoenzymes or to receptor subtypes, perhaps at the expense of a decrease in potency (binding) to the desired target. We shall examine the design principles that can be applied to this objective.

Design can be used to tackle the many problems involved in drug *delivery*, from solubility and formulation to distribution and metabolism. Problems relating to the acceptability of a formulation, some predictable toxic effects, persistence in the host (half-life and its relation to frequency of dosage) and persistence in the environment (stability and toxicity to fish, etc.) can be considered as design problems. Traditionally, metabolism has been studied as part of the clinical phase of a drug development programme. More frequently, it is now considered as an integral part of compound evaluation during animal studies. Also, of course, knowledge of metabolism can contribute to the design of drugs with altered pharmacokinetic profiles and particularly to the design of prodrugs.

2.1 RECEPTOR BINDING

A preamble into theory is necessary before we can discuss current work, and how this can be applied to design of more potent compounds.

2.1.1 Thermodynamics of Drug–Receptor Interactions

Drug–receptor interaction depends on optimal steric and electrostatic complementarity of their combining surfaces. Quantification of the binding

is usually made through the equilibrium dissociation constant K_i. For the most simple case of a reversible bimolecular drug (D)-receptor (R) interaction, expressed as:

$$D + R = DR \tag{1}$$

then the relations between frequently quoted equilibrium constants are:

$$K_{equilibrium} = K_{association} = [DR]/[D][R] \tag{2}$$

$$K_i = K_{dissociation} = [D][R]/[DR] \tag{3}$$

The potency of a reversibly binding inhibitor or antagonist is often measured as an IC_{50} value to displace binding of a labelled ligand to the receptor site. The relation between IC_{50} and K_i is given by the Cheng–Prusoff equation:

$$IC_{50} = K_i(1 + [L]/K_L) \tag{4}$$

where [L] is the concentration of the labelled ligand and K_L its dissociation constant. Frequently, IC_{50} is the concentration of an enzyme inhibitory drug required to reduce the reaction velocity of an enzyme to 50% of its value in the absence of inhibitor. In this case,

$$IC_{50} = K_i(1 + [S]/K_m) \tag{5}$$

where [S] is the substrate concentration and K_m is the Michaelis constant of the substrate.

Thermodynamic analysis offers an insight into the molecular events underlying drug–receptor interactions (Testa *et al.*, 1987; Raffa and Porreca, 1989). The thermodynamic quantification of binding may be obtained through application of the familiar equations (6) and (7)

$$\Delta G^0 = RT\ln K_i = 2.303RT\log K_i \tag{6}$$

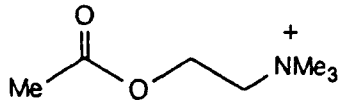
$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{7}$$

where ΔG^0 is the standard free energy change of the reaction, ΔH^0 is the enthalpy change and ΔS^0 is the entropy change; T is the absolute temperature and R is the gas constant. There are two useful "rule of thumb" methods for converting K_i to binding free energy, and IC_{50} differences to differences in binding free energy. The factor $2.303RT$ is approximately 1.4 kcal/mol at body temperature, so to convert K_i to binding free energy, for example, a K_i of 10^{-8} M is equivalent to a binding free energy of

1.4(−8) or −11.2 kcal and the difference in free energy of binding between two molecules, $IC_{50}(X)$ and $IC_{50}(Y)$ is simply

$$\Delta\Delta G = 1.4[\log IC_{50}(X) - \log IC_{50}(Y)] \quad (8)$$

A fascinating example of the value of applying equation (8) comes from the work of Höltje and Kier (1975), who considered the binding of acetylcholine (**1**) and a series of analogues in which the onium head had been replaced by *t*-butyl, isopropyl, ethyl and methyl groups, to the enzyme acetylcholinesterase. It had long been accepted that the acetylcholine–acetylcholinesterase interaction involved the positively charged, onium head group of acetylcholine interacting with some anionic receptor site such as a glutamic or aspartic acid residue in an ionic bond. Calculation of expected differences in binding energy assuming an anionic receptor site, modelled as an acetate anion, did not match with free energy differences to be expected according to equation (8). Rather, a receptor modelled as a benzene ring provided a much better fit to the data, leading to the outrageous postulate that the "anionic site" of acetylcholinesterase is something akin to a phenylalanine residue! This suggestion has recently been confirmed through X-ray analysis of the binding pocket of the enzyme (Sussman and Silman, 1992).



(1)

Knowledge of the intrinsic contribution, as binding free energy, from an ionic, polar, hydrogen bond or any other type of non-covalent "bond" can be used in drug design. Höltje and Kier used this knowledge to predict the nature of the receptor. If the receptor is already known, such knowledge can allow the design of novel drugs or improved analogues through matching complementary features and calculating the expected increments in free energy of binding.

2.1.2 Experimental Approaches

The intrinsic binding free energy (including enthalpic and entropic, or including desolvation) of a functional group can be determined experimentally. The usual approach is to find pairs of structures, X–Y–Z and X–Y, for which the difference in binding to a receptor can be traced solely to the contribution of the single functional group, Z. It is assumed that the

loss in overall rotational and translational entropy caused by bimolecular association is the same for X–Y–Z as for X–Y, an assumption known as the anchor principle (Page and Jencks, 1971; Page, 1977). With a possible correction for "freezing out" the rotor if the link Y–Z is a flexible one, then the difference in binding of the two structures represents the intrinsic binding provided by Z.

Recent developments in molecular genetics have enabled the intrinsic binding of groups present in the receptor to be measured by way of site-specific mutations. If crystallographic studies have also been made so that the geometry of the binding site interaction is known, then a comparison of equilibrium binding constants can yield intrinsic binding contributions. Fersht (1984) discussed the mutation of Tyr-34 to Phe-34 in the enzyme tyrosyl tRNA synthetase, thus removing a hydrogen bond donor site. This lowered the binding energy for substrate by only 0.55 kcal/mol. This low value for the intrinsic binding of a phenolic OH group was very surprising to a generation of medicinal chemists who had been using standard values of enthalpies of formation for different types of bond (coulombic, van der Waals, or hydrogen bond) to estimate approximate strengths for drug–receptor interaction. Such enthalpies are quite inappropriate, for what is involved is the interaction of solvated drug with solvated receptor in which movement of water molecules and perhaps also counterions must be considered. An appropriate inventory for a hydrogen bond would be represented by equation (9):

$$R{\cdot\cdot}OH_2 + D{\cdot\cdot}HOH = R{\cdot\cdot}D + HOH{\cdot\cdot}OH_2 \qquad (9)$$

in which the driving force is not so much the enthalpy difference between the left- and right-hand sides, but the entropy gain as the bound water molecules are released to bulk water. The effect of environment on energetics of the hydrogen bond has been studied by Sneddon *et al.* (1989) using a thermodynamic simulation method. For formation of the single hydrogen bond between two molecules of formamide, the calculated free energy change is −8.44 kcal/mol in carbon tetrachloride, but only −0.34 kcal/mol in water.

Overall binding is even more complicated for a salt bridge between, say, carboxylate anion in a receptor and protonated amine in a drug. Both functions would be solvated, and both could have counterions associated with them, which also would be solvated. Complexation would be accompanied by exchange of ion pairs, and an alteration in the degree to which each ion is exposed to bulk solvent, which has a considerable effect on free energy (Gilson and Honig, 1988). Fersht (1984) has pointed out that hydrogen bonds and salt bridges (because they involve exchanges) may contribute surprisingly little to binding energy, but they do of course provide specificity for the interaction. An instructive case study is provided by the

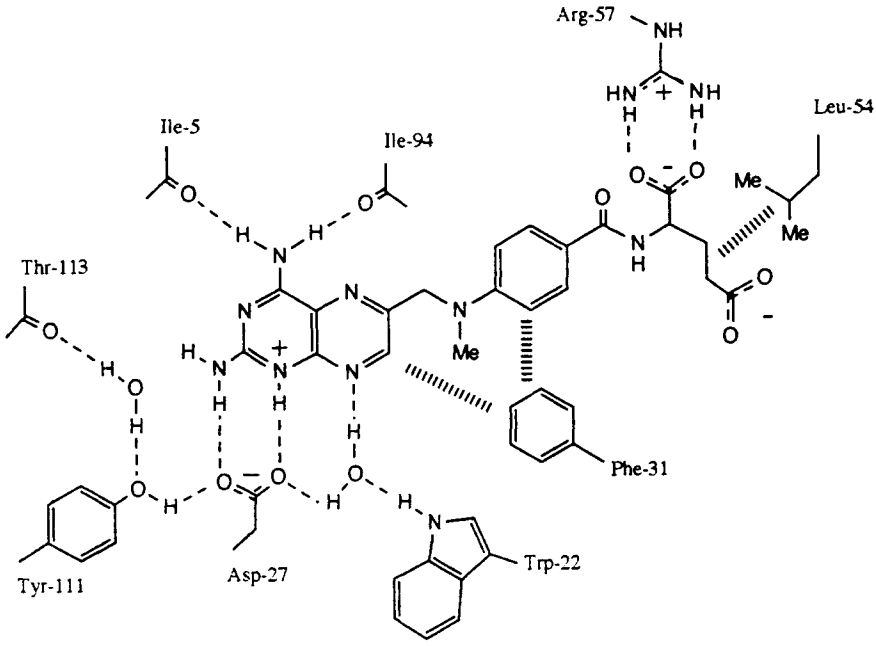


FIG. 1. Schematic drawing showing some of the interactions between the inhibitor, methotrexate, and the active site of dihydrofolate reductase from *E. coli*.

binding of the antineoplastic drug, methotrexate, to its receptor in the active site of dihydrofolate reductase. The geometry of binding is known with precision from the crystallographic investigations of Bolin *et al.* (1982) on complexes with bacterial enzymes from *Lactobacillus casei* and *Escherichia coli*. Figure 1 shows some of these binding interactions in schematic form for the *E. coli* enzyme. Note particularly the salt bridge between Asp-27, and the protonated pteridine ring of methotrexate, and the presence of two water molecules acting as bridges between drug and receptor. Mutagenesis of Asp-27 to Asn-27 or to Ser-27 removes the potential for salt bridge formation and perturbs the position of these two waters (Howell *et al.*, 1986). The dissociation constant increases correspond to binding free energy decreases of only 1.8 kcal/mol for Asn-27 and 4.4 kcal/mol for Ser-27. Theoretical methods (free energy perturbation calculations of Singh and Benkovic, 1988) show that the binding energy of the charge–charge interaction is counteracted by the cost in solvation of the charges, so that the salt bridge is not such a significant factor in stabilization of the ionic, as compared to the neutral (Asn-27, Ser-27), complexes.

The importance of non-polar residues to binding of methotrexate has also been examined, both by site-specific mutation (Taira and Benkovic, 1988)

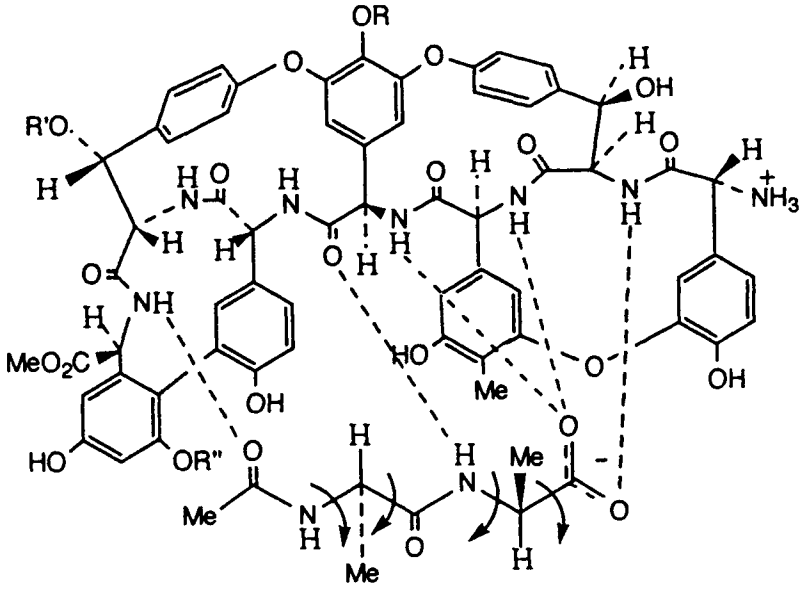


FIG. 2. Schematic drawing showing the hydrogen bonds between *N*-acetyl-D-ala-D-ala and the antibiotic, ristocetin-A.

and free energy perturbation studies (Singh and Benkovic, 1988). Replacement of Phe-31 by Tyr-31 or Val-31 costs 1.8 and 5.1 kcal/mol, respectively. A calculation on the mutation of Phe-31 to Ser-31 suggests binding would be reduced by as much as 9.7 kcal/mol, and that Leu-54 to Gly-54 would cost 4.0 kcal/mol. Clearly non-polar (dispersion and hydrophobic) interactions are as important to binding as are polar (hydrogen bond or coulombic) interactions.

The partitioning of free energy contributions in the explanation (and for design, the prediction) of binding constants is a subjective matter. Different workers choose different definitions, e.g. of "hydrophobic" binding, which may or may not include dispersion interaction, and different approaches to factorization of enthalpic and entropic components.

In a series of papers, Williams and co-workers (Williams *et al.*, 1991, 1993a,b; Searle *et al.*, 1993; Holroyd *et al.*, 1993) have presented their study of the binding between the cell wall analogue *N*-acetyl-D-ala-D-ala and the antibiotics vancomycin and ristocetin-A. Figure 2 is a schematic of the hydrogen bonds involved in the interaction with ristocetin-A, which also brings the methyl groups of the peptide into "hydrophobic" contact with aromatic rings of the antibiotic. For *N*-acetyl-D-ala-D-ala and for some simple analogues and derivatives complexing with these antibiotics, equilib-

rium data and values for changes in free energies, enthalpies and entropies were derived both using microcalorimetry (Rodriguez-Tabar *et al.*, 1986) and by spectroscopic methods (Williams *et al.*, 1991). The geometry of the complexes was deduced from nuclear magnetic resonance (NMR) studies, in combination with molecular modelling. By invoking the anchor principle, and with some simplifying assumptions, Williams has shown how drug–receptor binding energy may be estimated. Williams divides free energy of binding according to equation (10) into the components defined and exemplified as follows:

$$\Delta G = \Delta G_{T+R} + \Delta G_r + \Delta G_h + \Delta G_{vdW} + \Delta G_{conf} + \Sigma \Delta G_p \qquad (10)$$

ΔG is the binding free energy for the bimolecular association A + B in aqueous solution.

ΔG_{T+R} is the unfavourable change in overall translational and rotational free energy on binding. This is largely entropy, and is estimated as 14 kcal/mol for binding between a drug of molecular weight (MW) >100 and a macromolecular receptor (Page, 1977; Andrews *et al.*, 1984; Williams *et al.*, 1991). It can be considerably less for bimolecular association of smaller molecules (Searle and Williams, 1992).

ΔG_r is the unfavourable decrease in entropy due to freezing out internal rotations in A and B. Williams *et al.* (1993a) estimate 0.5–1.2 kcal/mol per rotor. Andrews *et al.* (1984) derive an average 0.7 kcal/mol per rotor. A typical rotor is a Csp^3–Csp^3 bond; methyl groups still enjoy freedom of rotation when bound, and an amide bond is considered rigid—neither therefore count as rotors. A formally single bond in a conjugated system (such as the central bond in 1,3-butadiene) would have some double bond character and therefore be partially restricted. Holroyd *et al.* (1993) count such a bond as half the normal rotor value.

ΔG_h is the hydrophobic effect, relevant only to binding in aqueous solution. This is entropy driven and physically associated with the release of "ordered" water from the non-polar surface which is accessible to water before, but not after, binding. According to Williams, this stabilizes A.B by about 50 cal/mol/Å^2 of surface removed. Although the relationship of surface area to hydrophobic binding is a matter of some uncertainty, results of Rotello *et al.* (1993), Sharp *et al.* (1991) and Honig *et al.* (1993) support the higher figure rather than that of 25 kcal/mol/Å^2 originally suggested by Chothia (1976). Surface area can be estimated by molecular modelling.

ΔG_{vdW} is the van der Waals forces between closely packed non-polar surfaces. If complementarity is achieved, this will be favourable

(−ve), if not achieved it may be repulsive (+ve). In Williams' work on vancomycin complexes, this term was assumed to be zero for complexes deemed to have good complementarity, but without extensive non-polar contacts. It can be estimated by molecular mechanics.

Note that a useful operational measure of "hydrophobicity" is the octanol/water log P parameter (Hansch and Leo, 1979). This is a free energy-related parameter and is frequently thought of as synonymous with hydrophobicity, but it undoubtedly incorporates both ΔG_h and ΔG_{vdW} components so is not pure hydrophobicity in the sense above. Lipophilicity is a more appropriate name. Contrary to common belief, log P is mostly enthalpic (Taylor, 1990; Da *et al.*, 1992).

ΔG_{conf} is an unfavourable conformation of A and/or B which may be involved in the complex; for A and B minimum energy conformations in solution may be distorted on binding. There are many documented examples of unfavourable conformations being involved in drug–receptor interaction (Jorgensen, 1991). The energy difference can be estimated using molecular mechanics.

ΔG_p is the intrinsic binding energy between polar groups, including enthalpy and entropy (desolvation) components. Williams *et al.* (1993) calculated amide–amide hydrogen bonds as worth -0.2 to -1.7 ± 0.5 kcal/mol.

Williams' analysis points the way, but still falls short of being a general method to calculate binding energy when the geometry of a complex is known directly from an X-ray determination, or can be surmised from molecular modelling. The approach suffers from two drawbacks.

(1) The estimates for ΔG_p, derived after accounting for hydrophobic and entropic contributions, depend critically on how these latter terms are estimated; in particular, on whether entropic advantages of new vibrational modes in the complex are credited to ΔG_p, or are accounted for by a reduction in the ΔG_{T+R} term (Searle *et al.*, 1992).

(2) The three components of binding (hydrophobic, van der Waals, and polar) are insufficient. Indeed, a face-to-face π stacking interaction between the carboxyl group and ring 1 (Fig. 2) is now concluded to make a significant contribution to the binding between *N*-acetyl-D-ala-D-ala and ristocetin-A (Holroyd *et al.*, 1993). In any general scheme, such stacking interactions and also polar–non-polar interactions such as that between the quaternary nitrogen of acetylcholine and polarizable aromatic residues (Dougherty and Stauffer, 1990; Sussman and Silman, 1992) must be recognized and treated.

2.1.3 *Empirical Approach*

Andrews *et al.* (1984) have adopted a semi-empirical approach to the calculation of binding energy. They fix the loss of translational and rotational entropy in any drug–receptor association to be 14 kcal/mol (the Williams ΔG_{T+R} term) and partition binding energy ΔG (defined as a +ve quantity) according to equation (11):

$$\Delta G = -14 + n_{DOF}E_{DOF} + n_X E_X \tag{11}$$

where n_X is the number of groups X present in the drug; E_X is the intrinsic binding of group X (equivalent to Williams' ΔG_p); n_{DOF} is a count of the number of rotatable bonds which will be frozen out on binding, at an entropic cost of E_{DOF} per bond (equivalent to Williams' ΔG_r).

Binding data for a selection of 200 drugs and enzyme inhibitors were used to provide a statistical estimation of *average* intrinsic binding energies E_X for groups X, and an *average* value for E_{DOF}. Multiple regression gave the *average* intrinsic binding energies and ranges of Table 1.

Although a somewhat arbitrary choice of groups had to be made (governed by the need to include a significant representation of each group in the dataset), it is gratifying that the values of the group *average* energies are in accord with expectations from other work. The values for hydroxyl and carbonyl groups (2.5 and 3.4 kcal/mol, respectively) accord with expectations for a hydrogen bond. Charged group interactions are stronger than neutral group interactions, and values for carbon atoms are reminiscent of lipophilic binding parameters. The entropic loss for freezing a bond rotation is as used by Williams and in close agreement with a theoretical value.

An "Andrews" estimate of binding energy, using the values given in Table 1, can be useful when the structure of the receptor is unknown (see Section 3.1.1). It can give an indication of how well (compared with an *average* binding) that structure fits its receptor. Even more useful, comparison of calculated *average* binding energies for two structures X–Y–Z and X–Y will indicate how well, and with what sort of binding, group Z contacts the receptor. It is frequently found that the observed binding of a structure to its receptor can be accounted for by participation of but a small fraction of potential binding sites, which may lead to a selective "downsizing" design strategy (see Section 3.1.2).

When the structure of the receptor is known, and particularly when a ligand has been co-crystallized in the binding site, then opportunities abound for the design of tight binding ligands to make use of accessory binding locations. Thus, the trimethoprim (**2**) derivative (**3**) was synthesized to fit the pyrimidine ring into the pteridine binding pocket of the dihydrofolate reductase (DHFR) receptor site, with a carboxymethylene

TABLE 1[a]

Intrinsic binding energies (kcal/mol)

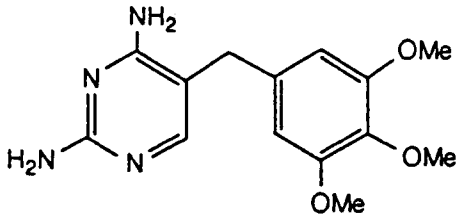
Group	Energy	Range[b]
DOF[c]	−0.7	−0.7 to −1.0
$C(sp^2)$	0.7	0.6–0.8
$C(sp^3)$	0.8	0.1–1.0
N^+	11.5	10.4–15.0
N	1.2	0.8–1.8
CO_2^-	8.2	7.3–10.3
OPO_3^{2-}	10.0	7.7–10.6
OH	2.5	2.5–4.0
C=O	3.4	3.2–4.0
O, S	1.1	0.7–2.0
Halogen	1.3	0.2–2.0

[a]Reprinted with permission from Andrews *et al.* (1984). Copyright 1984 American Chemical Society.

[b]Range of energies for six random 100-compound data sets.

[c]Degrees of internal conformational freedom.

chain of the appropriate length attached to the 3-methoxy group of trimethoprim, designed so as to reach Arg-57, the polar residue that interacts with the α-carboxy group of methotrexate (Fig. 1). This design strategy realized a 50-fold (2.4 kcal/mol) enhancement in binding (Kuyper *et al.*, 1982).



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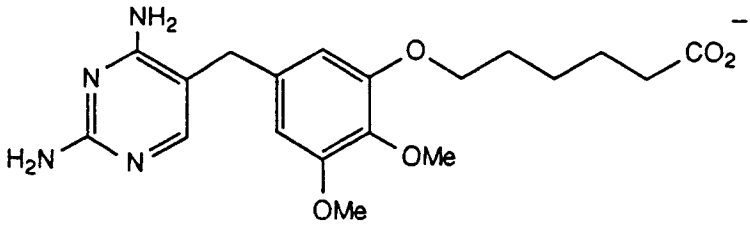


(3)

An Andrews calculation on trimethoprim (**2**) reveals a slightly better than *average* binding (observed 11.5 kcal/mol, calculated *average* 9.6 kcal/mol). Calculation of the *average* difference between **3** and **2** can be made as *four times* $C(sp^3)$ *plus* CO_2-*minus five times* E_{DOF}, which equals (3.2 + 8.2 − 3.5), or 7.9 kcal/mol. This is a poor prediction for the activity of **3**, and illustrates the fact that Andrews-type calculations will overestimate binding for drugs that do not utilize all groups for binding. (Some parts of a molecule, especially a large molecule, will act merely as spacers, or links between binding sites, and will not contribute directly to binding themselves. This will frequently be the case for long alkyl chains). An Andrews calculation may also overestimate binding for molecules that do not bind in a low-energy conformation, but need to adopt a higher-energy, unfavourable conformation in order to maximize interaction of the principal binding sites. The most useful aspect of Andrews calculations is not as a tool for absolute prediction of binding constants, but rather as a tool for the comparison of two or more structures, to gauge which is likely to be the better fit—in terms of utilizing binding groups, and/or binding in a low-energy conformation—to the receptor (see Section 3.1.1).

2.1.4 Theoretical and Molecular Modelling Approaches

As an alternative to free energy additivity schemes, but only when the receptor site geometry is known, a molecular mechanics technique can be used to optimize the geometry of interaction of a ligand—real or predicted —in a binding site. This also gives a binding energy, as the difference between total energy of the complex and the sum of energies of the separate, minimized components. The design of DHFR inhibitors from X-ray crystal structures using the sophisticated molecular mechanics program, AMBER, has been discussed by Roth (1986). It is clear from Roth's work that such a procedure can only have limited success; the pairwise potentials of molecular mechanics force fields do not include hydrophobic effects; no account is taken of entropic effects; solvation effects are usually ignored; and no account is taken in a molecular mechanics treatment of any orbital interactions of the π–π type. Moreover, no account is taken of the important difference in energy of interaction with solvent (water) and receptor.

Over the last 10 years, all these severe limitations of the traditional molecular mechanics approach have been tackled. The appropriate use of molecular mechanics, molecular dynamics, quantum mechanics and computer graphics has given in a number of cases useful qualitative data on solvation and binding, with good agreement between theory and experiment (Kollman and Merz, 1990).

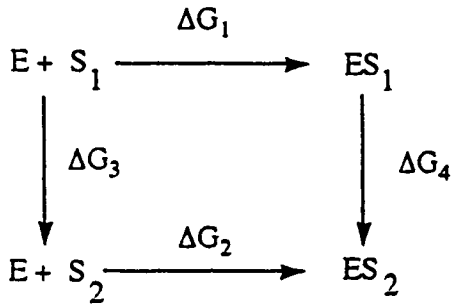


FIG. 3. Thermodynamic cycle used to analyse relative binding of ligands S_1 and S_2 to an enzyme, E. Each state is solvated by a "box" of explicit water molecules.

Relative ligand binding *in solution* of different ligands S_1 and S_2 to a receptor site or enzyme E can be estimated by the free energy perturbation method, using the principle of the thermodynamic cycle (Fig. 3), in which all processes are run *with explicit inclusion of water.*

The horizontal processes which would give ΔG_1 (binding energy of S_1) and similarly binding energy of S_2 cannot be simulated because they involve large changes in ligand position and movements of water. They can, however, be derived experimentally from K_i or K_m values. The vertical processes giving ΔG_3 and ΔG_4 can be studied by the free energy perturbation method (Singh *et al.*, 1987). This involves molecular dynamics using a sophisticated force field such as AMBER, with *ab initio* calculated charges, and the collection and statistical analysis of energy data as S_1 is transformed in small steps to S_2. Now note that the difference in binding free energy between S_1 and S_2 is given by $\Delta\Delta G_{bind}$ where

$$\Delta\Delta G_{bind} = \Delta G_2 - \Delta G_1 = \Delta G_4 - \Delta G_3$$

and ΔG_3 is in fact $\Delta\Delta G_{solv}$, the relative solvation energy of S_2 and S_1. The thermodynamic cycle perturbation method has been used to predict the relative solvation energies of molecules (Bash *et al.*, 1987) and the relative free energies of binding of ligands to enzymes. In a most exciting application, the relative binding energies of three ligands (Fig. 4, X = NH, amidate; X = O, ester; X = CH_2, phosphinate) to the active site of the zinc-dependent bacterial endopeptidase, thermolysin, have been calculated and compared with experiment (Merz and Kollman, 1989). Crystal structures of the enzyme–inhibitor complexes show that the ligands bind in a similar way in the active site, and indicate the advantage of the most powerful inhibitor (the amidate) to result from a hydrogen bond to a backbone carbonyl oxygen of the enzyme. The calculations revealed that the lower binding of the ester was due to a repulsion between the ester oxygen

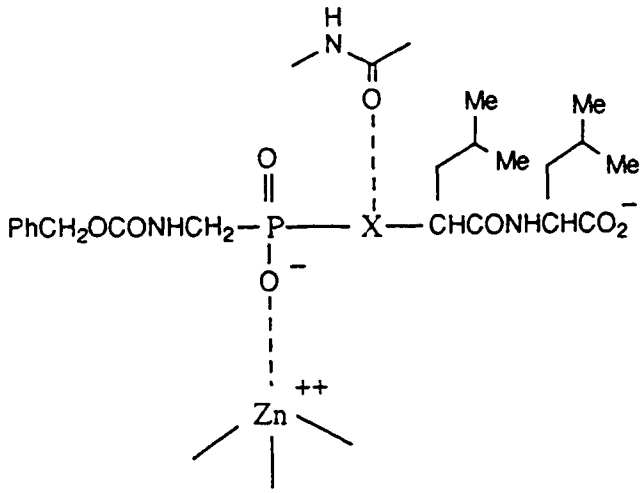


FIG. 4. Schematic drawing showing inhibitors of thermolysin and their interactions with the zinc atom and with a backbone carbonyl oxygen. Attraction occurs where X = NH, forced repulsion where X = O or CH_2.

and the same enzyme backbone oxygen, which was partially offset by an easier desolvation of the ester as compared to the amidate. The most interesting case was that of the phosphinate, found experimentally to bind just as well as the amidate, despite loss of a potential hydrogen bond donation. Comparison of amidate with phosphinate in the free energy cycle shows that although ΔG_4 is 2 kcal/mol for the NH to CH_2 perturbation, ΔG_3 is −2 kcal/mol, for the CH_2 derivative is 2 kcal/mol easier to desolvate than the amidate.

The perturbation calculations show that with proper consideration of solvation effects, theory can match experiment, and moreover can provide explanations of unexpected trends in binding data. Unfortunately, at present these calculations require hefty computer power, and a degree of expertise that exists in just a few centres of excellence. It will be a few years yet before they become routine.

Prediction of the binding energy between any two molecules in water is clearly fraught with difficulty. Many workers have therefore concentrated on calculating the *interaction propensity* properties of individual atoms within a protein or receptor site, representing this interaction propensity over the whole protein or site by a 3D contour map on a suitable computer graphic device. At its simplest level, the distribution of electrons (the charge) in the protein is calculated, and the potential electrostatic interaction with a hypothetical unit positive point charge at each of a number of points in space (grid points) is then calculated. The map represents the potential

electrostatic energy of interaction over the surface of the protein. Use of the point charge and other probes, e.g. methyl, water, to produce a variety of maps (fields) showing both electronic, steric or hydrogen bonding potential of receptor sites as an aid to ligand design will be described further in Section 3.3.

Many field-based techniques have been used in drug design. A "molecular lipophilicity potential" has been defined and can be used to calculate log P as a function of conformation, and as a field value in comparative molecular field analysis (CoMFA; Gaillard *et al.*, 1994). The HINT model and algorithms developed by Kellogg *et al.* (1991) incorporate *H*ydrophobic *INT*eraction potential. The HINT program, and its associated routines KEY, LOCK, and LOCKSMITH (Kellogg and Abraham, 1992) enable not only the potential for hydrophobic (strictly, lipophilic!), hydrophilic, and hydrogen bond interactions to be visualized by mapping, but also allow for the generation of a crude interaction energy as the two partners—receptor and drug—are docked together. Routine KEY maps "hydrophobic" and hydropathic profiles complementary to the receptor, and LOCK maps profiles complementary to a ligand. The routine LOCKSMITH combines LOCK maps, scaled according to activity of the ligands, producing a map of interaction-enhancing features extracted from the set of active structures. The idea behind HINT is to include the experimental log P octanol:water (a free energy measure of potential to interact with a solvent) as an atom descriptor, a. Pairwise interactions between atoms (i,j) in receptor and in drug are then summed, as in a molecular mechanics estimation of total energy. In HINT the interaction (b_{ij}) between any pair of atoms is given by

$$b_{ij} = s_i a_i s_j a_j R_{ij} + r_{ij} \tag{12}$$

where s is the solvent accessible surface area, a is the "hydrophobic" atom constant, and R_{ij} and r_{ij} are distance dependent functions given by

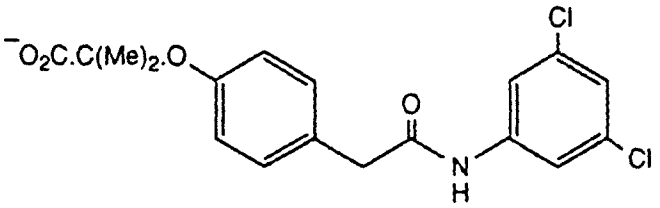
$$R_{ij} = T_{ij} e^{-r} \tag{13}$$

$$r_{ij} = A\varepsilon_{ij}\left[(r_{vdW}/r)^6 - 2(r_{vdW}/r)^{12} \right] \tag{14}$$

Thus the pairwise energy, equation (12), sums a hydrophobic/hydropathic component [equation (13)] and a Lennard-Jones type dispersion component [equation (14)]. Peculiar to HINT is the sign-flip function T_{ij} of equation (13) which examines each atom–atom interaction and corrects the sign ($T_{ij} = \pm 1$) for polar interactions, e.g. those involved between acid and base functions and in hydrogen bonds.

Wireko *et al.* (1991) used HINT on crystal coordinates of haemoglobin

complexed with a series of 10 allosteric modifiers (of oxygen uptake), including structures **4**–**6**. The order of activity was found to run parallel to the order of calculated "binding energies". In a later paper, Kellogg and Abraham (1992) showed that a KEY map made from haemoglobin correctly identifies major lipophilic, polar (hydropathic) and hydrogen bonding sites. The most effective compounds are, respectively, **4** and **6**; compound **5**, having its carbonyl group oriented in the opposite sense, has lower activity, as would be predicted from the hydropathic contours of the KEY map.



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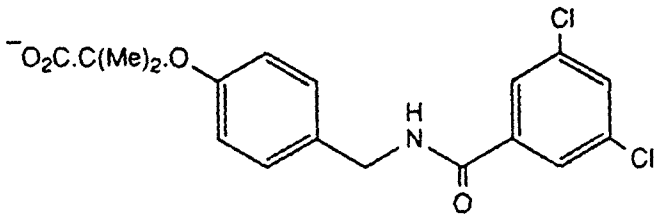

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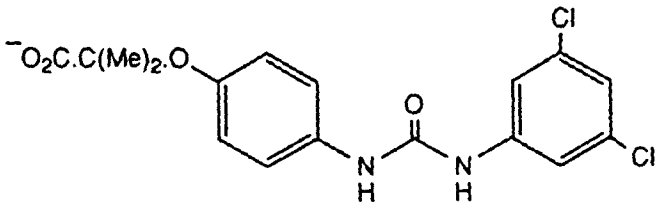


(6)

These routines should prove extremely useful in the design of high-affinity ligands for structurally characterized receptors.

2.2 RECEPTOR SELECTIVITY

2.2.1 Overview of the Problem

The non-selectivity of drug molecules is frequently a problem of greater magnitude than any perceived lack of potency. The inescapable side effects

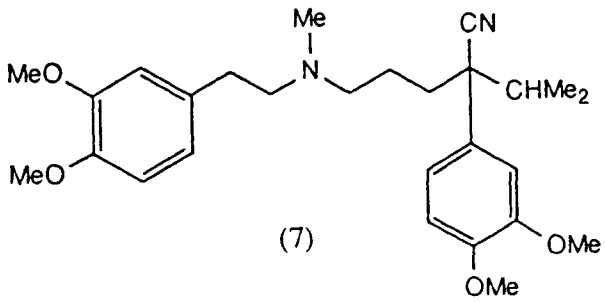
of many drugs result from an affinity to receptors other than the principal target.

In his classical text "Selective Toxicity", the late Adrien Albert (1985) has discussed the principal ways in which drug selectivity can be achieved, distinguishing the needs for selectivity in chemotherapeutic agents and pharmacodynamic drugs. Selectivity can be designed into an anti-infective drug by paying attention to differences (between host and parasite, for example) in accumulation of a toxic molecule, to differences in cytology (e.g. bacterial cell wall synthesis inhibitors), or to differences in biochemistry. According to the target, a great degree of selectivity may be designed into the drug from the beginning, by appropriate choice of chemical class or mechanism.

A case study of selectivity which is of great and continuing interest concerns trimethoprim. This antibacterial drug depends for its success on an extraordinary selectivity (30 000-fold) as an inhibitor of bacterial DHFR, rather than mammalian DHFR. This is an instance where both species, bacteria and humans, possess the target enzyme. Very high homology exists between the two enzymes, and the receptor sites differ very little in detailed structure. Some conclusions as to the origin of such specificity were drawn by Matthews *et al.* (1985) on the basis of X-ray analysis of complexes with bacterial (*E. coli*) and chicken liver enzymes, the key finding being that residues on opposite sides of the active site cleft in the chicken enzyme are about 1.5–2.0 Å further apart than are structurally equivalent residues in the *E. coli* enzyme. But, studies on the chicken liver enzyme may not be relevant to the human enzyme. Indeed, crystal studies of Oefner *et al.* (1988) on complexes of human DHFR with folate, methotrexate and trimethoprim itself do not support the explanation given by Matthews, and so we are still not sure of the basis for the species selectivity exhibited by trimethoprim.

2.2.2 *Selectivity of Pharmacodynamic Agents*

Selectivity of a pharmacodynamic agent is not easy to achieve. In fact, as LaBella (1991) has pointed out, promiscuity (affinity for a great number of receptors) is to be expected, especially with molecules containing an aromatic nucleus separated from a nitrogen atom by a 2–5 atom chain, where the nitrogen may be an amine, imine or amide and is able to donate a proton in forming a hydrogen bond. This structural theme was noted earlier by Andrews and Lloyd (1982), who pointed out that many CNS drugs are of this general type. LaBella comments on the extreme case of verapamil (**7**), a calcium channel blocker currently in use for the prophylactic treatment of angina. Apart from blockade of the calcium channel receptor at a concentration of $<10^{-7}$ M, the H_1 histamine receptor is also antagonized at 10^{-7} M; serotonin and α_1-adrenergic receptors at 10^{-6} M; H_2 histamine,



(7)

α_2-adrenergic, muscarinic, dopamine, phencyclidine, serotonin transport, dopamine transport, and γ-aminobutyric acid transport receptors at 10^{-5} M; and β_2-adrenergic and noradrenaline transport receptors at 10^{-4} M. This extreme promiscuity may result from possession of the aromatic ring–carbon chain–basic nitrogen motif twice, one at each end of the molecule. The lesson from this drug is that whilst increasing the size of a molecule may increase potency by adding more binding groups, it is unlikely to provide selectivity, and indeed more probable that an "upsizing" design strategy (see Section 3.1.2) will result in a less selective, i.e. more promiscuous, drug entity. LaBella concludes:

> It is unlikely that binding site and subsite dimensions, geometry, charge environments, hydrophobic surfaces and other features will ever be known to the extent that drug design technology will yield a compound with absolute specificity for one species of functional protein. The alternative strategy, screening compounds in multiple protein-specific assays, would appear to be a practical means of estimating the pharmacological profile of any given drug, and could serve to select and, to some extent, design drugs that approach the desired specificity.

LaBella is probably right in his assertion that absolute *specificity* will never be attained. Even a reasonable degree of selectivity is a difficult enough objective of antagonist drugs designed to block pharmacodynamic receptors. The reason for this difficulty lies in the overall similarity of many of these receptors, which are G-protein-coupled receptors (GPCRs) and have evolved from a common ancestor protein. Trumpp-Kallmeyer *et al.* (1992) have analysed the peptide sequences of 39 of these receptor proteins, to reveal a common feature of seven hydrophobic stretches corresponding to membrane-spanning α-helices. Alignment of primary sequences shows a high degree of homology, which was subsequently used as a basis for constructing 3D models, based on bacteriorhodopsin as template. Bacteriorhodopsin also consists of seven membrane-spanning helices connected by hydrophilic loops and has striking structural parallels with the mammalian GPCRs, and its structure has been determined by high-resolution electron

cryomicroscopy (Henderson *et al.*, 1990). Five cationic neurotransmitter receptor sequences (5-HT_2, dopamine D_2, muscarinic M_2, adrenergic α_2 and β_2 receptors) were studied in detail, and 3D receptor models constructed. In all cases, the binding site for agonists consists of a charged aspartic acid residue located in the same position in the middle of a transmembrane helix, helix 3, and a hydrophobic pocket containing conserved aromatic residues on helices 4, 5, 6 and 7. These similarities of course explain the promiscuity of competitive antagonists at GPCRs.

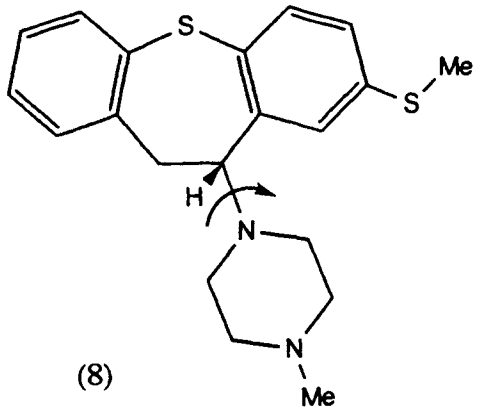
A particularly interesting feature of this work is the finding of not only hydrophobic but aromatic residues clustered at acetylcholine binding sites (first discovered in acetylcholinesterase; see Sussman *et al.*, 1991) and also at catechol and indolamine binding sites. It seems that a cluster of aromatic residues produces a hydrophobic binding site which nevertheless is able to bind ammonium ligands through a cation–π electron interaction coupled with hydrophobic desolvation. Trumpp-Kallmeyer was able to conclude that *selectivity* of recognition for any particular ligand would result from interactions—some favourable, some unfavourable—with other residues in the neighbourhood of the binding sites on the proteins concerned.

2.2.3 Potency-based Approach

What then is the drug designer to do, in order to achieve selectivity, in the face of overall similarity between receptor sites? Two knowledge-based approaches have been used, and therefore come within our definition of drug design. Both approaches rely heavily on computational techniques, and on a principle that increased selectivity will parallel increased affinity for the receptor of interest.

One approach is to use computer graphics as an aid to what is variously known as receptor mapping, or pharmacophore mapping (Humblet and Marshall, 1980; Gund *et al.*, 1987). When the 3D structure of the receptor is unknown, one can start with the assumption that molecules which have the same (high) affinity interact at the same receptor by presenting the same pharmacophore elements, or structural features, to the receptor in the same way. One can make use of computer graphics "overlays" of active ligands in order to derive a hypothesis as to which structural features contribute to binding. Having derived the hypothesis (the pharmacophore) it is necessary to synthesize and test new molecules containing all these structural features in such a way that they may be presented to the receptor in an appropriate low-energy conformation, or rigid structure. The "training set" for the exercise usually contains flexible molecules, and unless some rigid analogues are available then conformational analysis has to be undertaken to derive likely conformers for overlaying. Many methods for conformational search and energy minimization are now available (Kollman and Merz, 1990).

An example of this approach is to be found in the work of Hibert *et al.* (1988). A systematic conformational search of four serotonin (5-HT) receptor antagonists (**8–11**) was performed by "building" models of each and then by probing the energy–torsion angle relationships as torsion angles (indicated by arrows on the structures) were incremented. The SYBYL molecular modelling package was used for determination of stable conformations, and the MAXIMIN/MULTIFIT routine was used to find likely overlaps, forcing the molecular features chosen as reference to an optimized fit at the cost of some conformational energy. Reference features in this example included centroids of aromatic rings and basic nitrogen atoms, with the recognition that there are two aromatic rings as putative reference points in structures **8–10**, and two basic nitrogens in structures **8** and **11**. Following superposition, it was possible to propose geometric features—a distance between the centre of an aromatic ring and a basic nitrogen, and the



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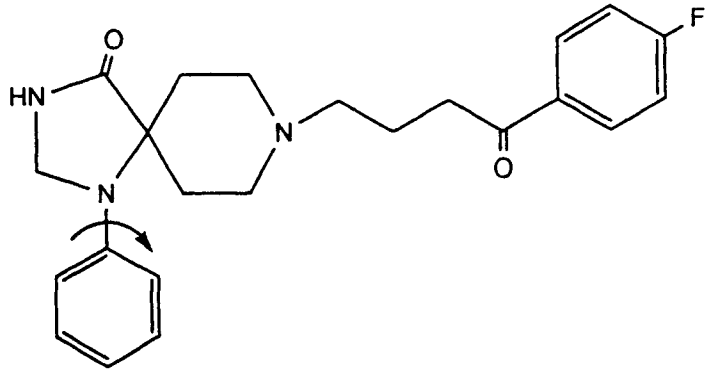
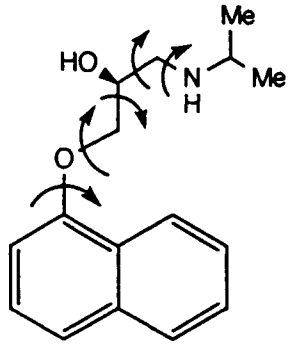


(9)

distance of the nitrogen above the plane defined by the ring—which define a possible pharmacophore for the 5-HT_{1A} receptor. A van der Waals surface was then derived, corresponding to the envelope of superimposed antagonists in their proposed active conformations. The resulting model, a volume containing reference points with properties of basicity and hydrophobicity, was sufficiently precise to allow the prediction that the *S*-enantiomer of structure **12** would fit better, and therefore be more potent, than its



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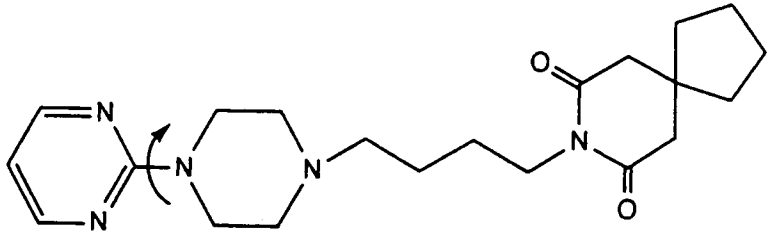

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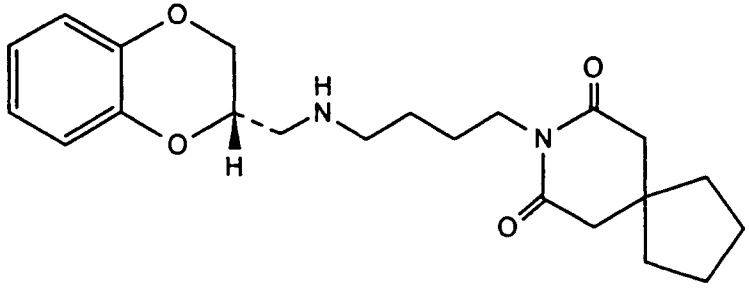


(12)

TABLE 2[a]

Affinities for central 5-HT receptor sites in rat frontal cortex (pIC_{50})

Structure and name	5-HT_{1A}	5-HT_{1B}	5-HT_2
8 (−)-methiothepin	7.02	6.74	8.20
9 spiperone	6.91	6.00	8.67
10 propranolol	6.77	6.31	5.10
11 buspirone	7.66	4.90	5.47
12 MDL 72832	9.21	6.10	6.70
(*R*)-enantiomer of **12**	7.70	5.30	6.10

[a]Reprinted with permission from Hibert *et al.* (1988). Copyright 1988 American Chemical Society.

R-enantiomer. This prediction was subsequently verified, and at the time of its disclosure compound **12** was the most active 5-HT_{1A} antagonist reported; moreover, it was found to be very selective between 5-HT receptors of great overall similarity (Table 2).

The systematic conformational search method can be prohibitive for a large number of structures and for many rotatable bonds, the compute power required being an exponential function of the number of rotatable bonds. The essence of pharmacophore generation being to generate conformers such that certain features in each molecule overlap each other, i.e. in an ensemble of all overlapping conformers these features would overlap or at least be within a short distance of each other (say, 0.3 Å), then it has been natural to apply a distance geometry algorithm. In the ensemble distance geometry method (Blaney and Dixon, 1991) all molecules are represented as a set of intramolecular distance constraints, and the set of active molecules with a set of intermolecular distance constraints between suspected pharmacophore features. Just as distance geometry has been used to solve a set of distance-bound constraints including NOE (Nuclear Overhauser Effect) distances from NMR to give 3D coordinates and hence conformations compatible with these distances (Crippen and Havel, 1988; Ghose and Crippen, 1990), so it can be used in the ensemble method to generate conformations so that the pharmacophore elements overlap between active structures. A particular advantage of the method is that it can deal well with flexible rings. Pioneering the method, Sheridan *et al.* (1986) have derived the pharmacophore for the nicotinic acetylcholine receptor from the set of agonists comprising nicotine, cytisine, ferrugine methiodide and muscarone (**13–16**, Fig. 5). The features considered to define the pharmacophore were: a cationic centre, A; an electronegative atom, B; and an atom (or ring centroid) forming a dipole with B, C. Only

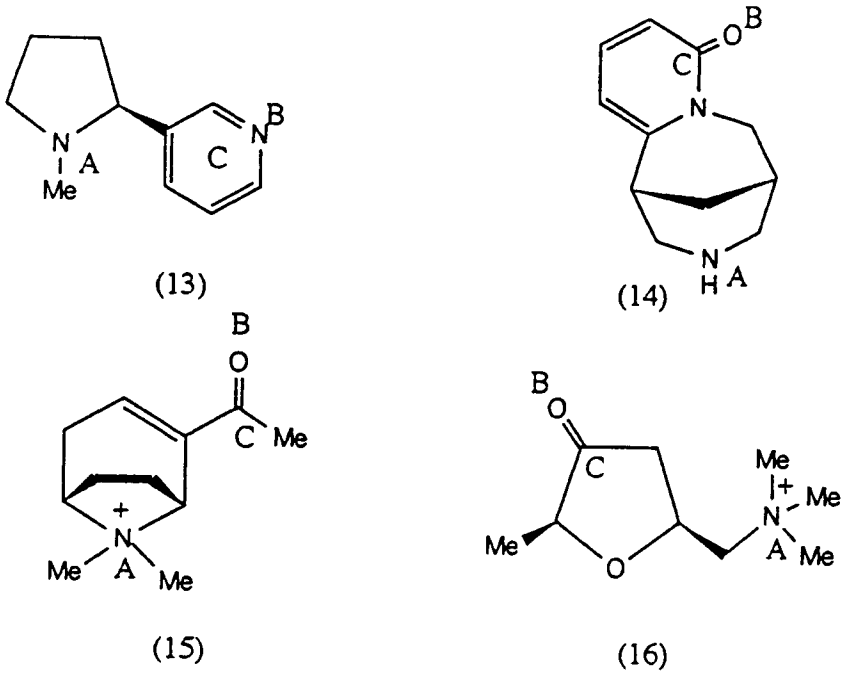


FIG. 5. Structures and features (A, B, C—see text) used to derive the nicotinic acetylcholine receptor pharmacophore.

one pharmacophore was found possible, and this was found to be consistent with a previous model.

To exemplify a second computer-based approach to designing a selective drug, we can return to our case study regarding the selectivity of trimethoprim (**2**). By studying **2** and its analogues and their activities against bacterial and mammalian enzymes, can the knowledge gained be used to design a more selective analogue? This is what Selassie *et al.* (1989, 1991) have done using the classical Hansch approach to derive QSARs against a bacterial enzyme (*E. coli*) on the one hand, and against a vertebrate enzyme (chicken liver) on the other. QSARs were constructed for the two enzymes from the same set of 68 congeners of **2**. The equations were quite different; for the vertebrate enzyme, a hydrophobic parameter was found necessary for a certain substituent position, indicating a limited hydrophobic interaction and hence a particular shape for a hydrophobic pocket. In contrast, for the bacterial enzyme hydrophobic factors seemed unimportant, and only steric factors (parameterized through the molar refractivity) were found to be significant. The X-ray studies of Matthews *et al.* (1985) provided a gratifying rationalization of the QSARs, which were used to predict the

selectivity of several analogues. By simply inserting values for the relevant physicochemical parameters into the two equations to predict potency, and subtracting one result from the other, indications are that the 3′-C_2H_5, 4′-CH_2OH, 5′-$NHCOCH_3$ analogue might be equipotent and about six-fold more selective than trimethoprim itself. We still await synthesis and, hopefully, verification of this prediction.

In the absence of any detailed knowledge of the receptors, two QSARs can, in principle, be used to maximize selectivity in the design of new congeners. This has not often been reported, probably because certain conditions must be met for QSAR studies of this nature; namely, an extensive set of congeners, known to be acting by a similar mechanism, exhibiting good variance in substituents, parameter values and activity.

2.2.4 Selective Partitioning Approach

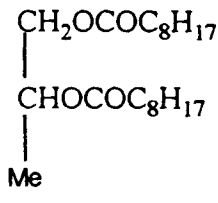
As Albert has stressed, selectivity between species (e.g. human and parasite) is normally achieved by making use of differences either in biochemistry or in accumulation. Differences in accumulation of a drug may result from differential affinity for the tissues involved (excluding any active transport mechanisms).

For pharmacodynamic agents acting within one species (human), selectivity may also be achieved by differences in accumulation, when similar receptors are located in cells or in membranes of different character. The neurotransmitter receptors, for example, are similar, but are found in a variety of highly differentiated cells whose membranes are unlikely to possess the same physical characteristics.

In a very thoughtful investigation of solvent systems to model membrane characteristics, Leahy *et al.* (1989, 1992) have argued that two receptors sited in different tissues (or membranes) could exist in environments that are very different in hydrogen bonding character: one may be surrounded by amphiprotic groups (as in a protein) or by proton donors; the other may be surrounded by proton acceptors (as in a phospholipid membrane).

Since 1964, octanol:water has been used to model membrane transport and membrane accumulation (Fujita *et al.*, 1964). Whilst generally satisfactory, and particularly good for modelling membranes consisting largely of protein, some membranes are undoubtedly better represented by an alternative solvent. So, Leahy argued for the use of propylene glycol dipelargonate (PGDP, **17**) as a proton acceptor solvent to model phospholipid (**18**) membranes.

Leahy measured partition coefficients in the PGDP:water system, and argued that selectivity may be achieved by designing molecules to interact with the receptors themselves in the same way (common pharmacophore)



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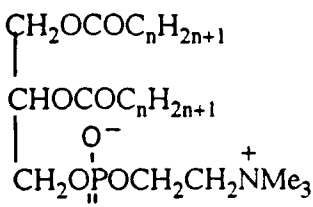


(18)

but which possess ancillary substituents able to interact differentially with the surrounding medium (tissue or membrane). A strong proton acceptor, such as *N*-methylimidazole or the carbonyl group of an oxoheterocycle, would be repelled by PGDP but attracted by octanol, leading to a large difference in log *P* between the two solvent systems. If the receptor to be selectively occupied were located in an amphiphilic, protein environment, such an ancillary group would be expected to provide a selectivity advantage over similar receptors found in a phospholipid environment.

To design for selectivity, then, one should follow the relationship between selectivity and the difference in log *P* between two relevant solvent systems, and maximize this difference by choice of appropriate substitution. Young *et al.* (1988) have made use of Δ log *P* between octanol and cyclohexane systems to model successfully accumulation in brain tissue, giving support to this new, QSAR-based approach to selectivity enhancement. Related to this approach is the older and well-established principle that for keeping drugs away from the CNS, one should design them to have lipophilicity either much less than, or much greater than, a log *P* value of 2.0 in the octanol:water system (Hansch *et al.*, 1987; see also Section 2.3.1).

2.3 RECEPTOR ACCESS

Once administered, by whatever route, a drug must usually cross one or more membranes before the required biophase and target receptor are reached. Design principles can be applied to help access the biophase, and depend on knowledge of the mechanisms involved.

The vast majority of drugs must rely on passive transport. This involves both free movement in solution (including filtration of small, and relatively polar solutes through pores in membranes) and partitioning. It is clear that the distribution, and particularly the rate of passage through semi-permeable membranes depends to some extent on molecular size, but is most critically dependent on solute charge and partition coefficient. Our current knowledge of the relationships between structure and pK_a, and between structure and partition coefficient, can be used in design to promote passive transport.

Occasionally, a drug may be transported by way of a specific membrane carrier protein. Such a carrier protein, or transporter, may assist in the passage of polar or charged molecules (inorganic ions, amino acids, some peptides, endogenous neurotransmitters, some pyrimidines *and some drugs*) across *particular* membranes. Two processes can be distinguished. Facilitated transport involves passage of a molecule down its concentration gradient and requires no energy input, an example being the transport of glucose from blood into erythrocytes. Active transport will move a molecule against a concentration gradient and requires energy, most frequently supplied by ATP hydrolysis or by respiration (Walsh, 1976). Active transport is highly developed in bacteria and has been utilized in the design of some intracellularly acting antibacterial agents.

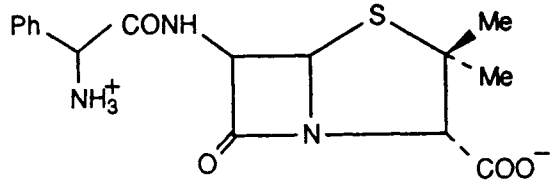
2.3.1 Passive Diffusion

The so-called "pH partition principle" was enunciated by Brodie (1964). To pass readily through a biological membrane, a molecule should be uncharged. This is to avoid either repulsion by a charge of the same sign, or attraction to a charge of the opposite sign. Either situation would prevent passage across the charged layer on a membrane surface. The pK_a of the drug should therefore be such as to allow a good proportion of the drug to be unionized at the pH of the membrane biophase. The unionized fraction will partition into the membrane at a rate dependent on its partition coefficient, and likewise will partition out of the membrane, and hence through a series of membranes, at a rate dependent on partition coefficient.

The pH partition principle is a very general one, and should not be taken to imply that an ionic species will not undergo partitioning into a lipid membrane. It is probable that many highly lipophilic cations will form ion pairs with a physiological counterion such as chloride, and the ion pair will then partition into the membrane (Murthy and Zografi, 1970).

The internally charge-compensated zwitterion is the predominant form, at physiological pH, of many amino acids and also of drug molecules such as ampicillin (**19**) and tetracycline (**20**). An effective internal charge neutralization can lead to a reasonably lipid-soluble species, lipophilicity being dependent on the distance between charges (Tsai *et al.*, 1991). Evidence from lipophilicity measurement and molecular modelling suggests that the dopamine antagonist raclopride exists in the organic phase as the internally charge-compensated, hydrogen-bonded zwitterion (**21**). Log *D* is 1.33 at pH 7.6, and would be expected to be much lower if the two charges were separated and independently solvated (Tsai *et al.*, 1993).

The drug designer may do well to provide internal charge compensation, rather than to seek a salt formulation when faced with a strongly cationic or anionic drug candidate. A good example of this ploy comes from the work



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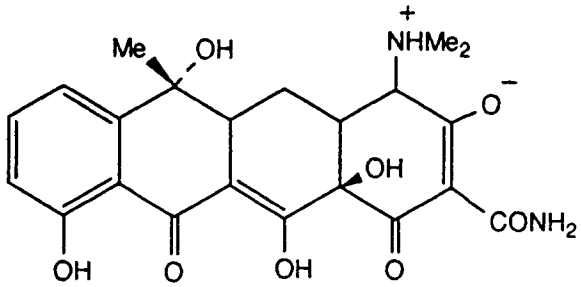

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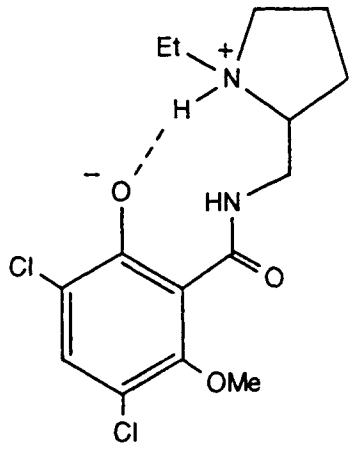

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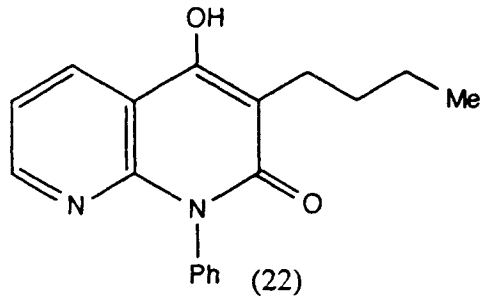
of Blythin *et al.* (1991). A series of substituted 1,8-naphthyridones, e.g. **22**, have been developed as inhibitors of the release of leukotrienes, and have potential use as anti-allergy drugs. Two problems with **22** were lack of solubility and low plasma levels after oral dosing. Both problems were addressed by the preparation of zwitterionic analogues, such as **23**, which



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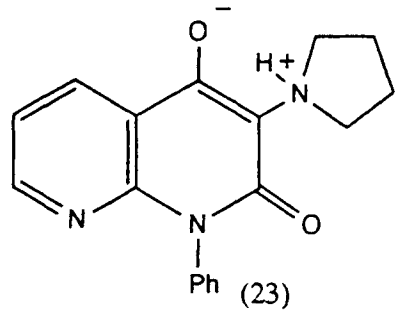


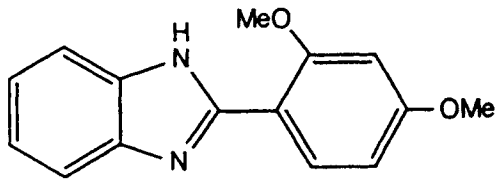
(23)

gave excellent blood levels. This compound, as Sch 37224, has entered clinical trials for the treatment of asthma.

In practice, it is necessary to discover the optimum transport characteristics, both pK_a and partition (log P) through a QSAR study involving the route of administration and biophase in question. Through such studies, more specific principles have resulted and can be used in drug design.

Through studies of hypnotics, anaesthetics and CNS depressants in particular, it has been proposed that an octanol:water log P of 2.0 ± 0.3 is the ideal lipophilic character to design into a neutral molecule for passive penetration into the CNS (Gupta, 1989). (For acidic or basic molecules, log D is often measured at "physiological" pH and taken as the relevant value. Thus, a log D of about 2.0 at pH 7.4 is the ideal for weak acids and bases, and probably also for zwitterions.)

To keep molecules out of the CNS and so ensure minimal non-specific CNS depression (sedation) then log P should be kept well away from a value of 2.0. Hansch has studied the role of lipophilicity in drug transport and also in drug metabolism (see Section 3.1.8) and has set forth the "Principle of Minimal Hydrophobicity in Drug Design" (Hansch *et al.*, 1987). Hansch contrasts log P (or log D) values for first-generation H_1 antihistamines (which penetrate the CNS and lead to sedation as an undesirable side effect) with the more recently designed analogues of lower log P, which are non-sedative. A fascinating and much quoted example of the use of log P in



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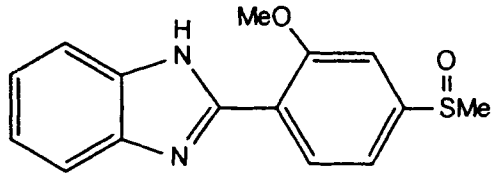

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design to exclude compounds from the CNS has been related by Hansch. The cardiotonic drug candidate AR-L 57 (**24**) caused "bright colour vision", clearly a CNS side effect, in volunteers. The analogue AR-L 115(**25**) was prepared, in which replacement of the *p*-OMe group by the more polar, bioisosteric group S(O)Me reduced the log *D* value from 2.59 to 1.17 (measured at pH 8.0). The side effect was almost eliminated, and **25**, sulmazole, was taken to clinical trials.

Designing molecules to have log *P* either much lower or much higher than 2.0 will not guarantee non-penetration of the CNS (witness morphine, log $P = 0.07$; and tetrahydrocannabinol, log $P = 3.78$). The principle is however sufficiently well established to deserve attention in drug design.

The influence of chemical and biological factors on brain uptake of drugs has been reviewed by Audus *et al.* (1992).

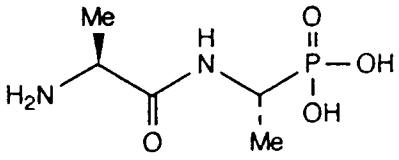
2.3.2 Active and Facilitated Transport

Nicotine (log $D = 0.45$ at pH 7.4) very rapidly enters the CNS, but this is believed to involve facilitated transport (Spector and Goldberg, 1982). Facilitated or active mechanisms are often suspected when transport seems not to follow the pH partition concepts, and can be proved by showing an energy requirement to exist, or that the carrier can become saturated, or that transport is competitively inhibited by a substance of similar structure. Special mechanisms exist for the transport of essential nutrients into the body, e.g. through the gastro-epithelial membrane, or for the selective disposal of excess electrolytes and polar metabolites from the body, e.g.

through the kidney tubule. Little is known about the transporter proteins involved, and it is by accident rather than design that some drugs may be carried towards their target by such means. The active transport of chloroquine into erythrocytes (Fitch *et al.*, 1974) is largely responsible for its success as an animalarial drug, but was certainly not a designed feature.

Molecular studies have been made on some neurotransmitter transporters, which regulate extracellular and synaptic neurotransmitter concentrations. These transporters have 12–13 transmembrane domains, and there is a high degree of homology between the noradrenaline, dopamine and GABA systems (Amara and Pacholcyzk, 1991). Whereas such studies may eventually help in the design of more selective CNS agents, i.e. to selectively block uptake of a particular neurotransmitter (Tejani-Butt, 1993), it is inconceivable that we could design new drugs to make use of these systems for access to a receptor site.

Active transport is highly developed in bacteria and in other free-living unicellular organisms. Well-refined structures of seven periplasmic proteins that serve as initial receptors for active transport in Gram-negative bacteria have been obtained (Quiocho, 1991). These have provided a detailed understanding of the atomic interactions associated with the specific recognition and binding, prior to uptake, of sulphate, phosphate, leucine, mono- and oligosaccharides. Such knowledge could conceivably be used to design new antibacterial drugs, precedent having been established with the development of alafosfalin (**26**). This drug uses the peptide transport system to gain access, and is then cleaved to aminoethylphosphonic acid which inhibits alanine racemase and uridine 5′-diphosphate-*N*-acetylmuramoylalanine synthetase, so disrupting bacterial cell wall synthesis (Grappel *et al.*, 1985).



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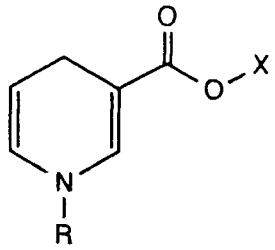
2.3.3 Chemical Delivery Systems

By the term "chemical delivery system" (CDS) we shall here refer to all derivatives of a "parent" drug which enhance access to the intended site of action by modification of physicochemical properties, and which deliver the parent by chemical or enzymic action in the biophase itself. The term "prodrug" is also often used, but this term really has a wider meaning:

"prodrugging" can be done not only for drug targeting, but also to improve the overall properties of a parent with respect to problems of formulation, such as solubility, stability or taste. The drug alafosfalin (**26**) incorporates a CDS, designed to make use of active transport. Most CDS systems are designed to convert a polar parent to a relatively lipophilic, well-transported drug, using a chemically or enzymically labile linkage, frequently an ester or amide. The linkage should be stable until the biophase is reached, and design must take into consideration the enzyme systems available in the biophase to affect subsequent release.

Bodor and Brewster (1983) first used the term CDS, in describing the use of dihydropyridine ester- (or amide)-linked prodrugs such as **27** (X-OH is the parent) which can partition readily into the CNS, there to be oxidized to pyridinium salts (**28**), which are effectively trapped in the biophase because of their extreme polarity, and which then undergo enzymic or chemical hydrolysis of the now very labile ester link to release active drug.



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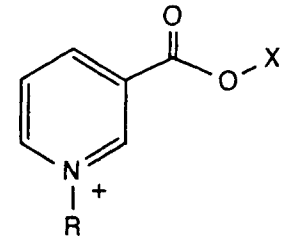

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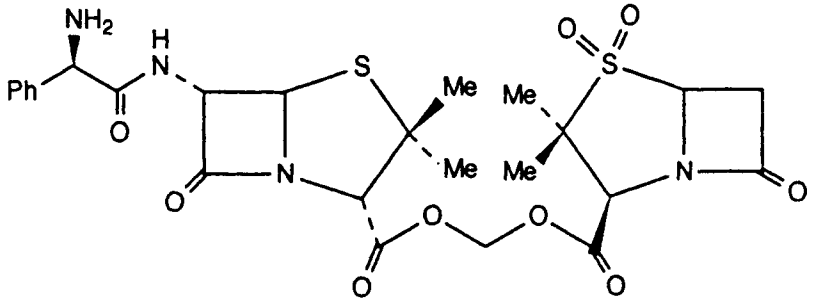
Though there are no drugs yet on the market from this particular system, the pyridine–pyridinium redox CDS has been widely exploited experimentally to deliver hormones and drugs to the brain (Bodor and Farag, 1984; Shek *et al.*, 1987). By using a variety of groups, R, in **27** from, for example, methyl through to octyl, the lipophilicity can be adjusted to provide optimum properties for passive transport.

Examples of the CDS strategy can be found in reviews by Sinkula (1975), Bodor (1984), Pardridge (1985) and Bodor and Kaminski (1987). Of particular interest is sultamicillin (**29**), an orally delivered, mutual prodrug of the β-lactamase inhibitor sulbactam (see Section 3.4.2) with the antibiotic ampicillin, introduced to the market in 1987 (Hara *et al.*, 1988). Also, an experimental strategy for delivery of the anti-HIV drug, AZT, is intriguing: the active reverse transcriptase inhibitor produced *in vivo* from AZT is the 5′-triphosphate, which is dianionic and cannot diffuse across cell membranes. Prodrug diphosphoesters (**30**, R = Me, Et, Pr^i, Bu^t) have been prepared which can traverse membranes and then may be enzymically converted directly to AZT-5′-monophosphate (Freeman *et al.*, 1993).



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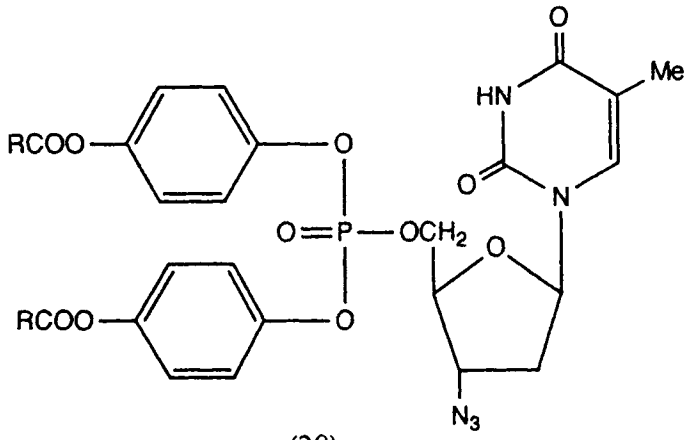

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3 Methods of Design

Design begins after discovery. By discovery we mean the identification of a structure, or structures, that will produce a desired pharmacological or biochemical response in a primary screen, or that will bind tightly to a relevant receptor site.

Many "active" molecules may well be identified. These may be natural products, or existing drugs, or come from the screening of available compounds. Such screening may be directed towards analogues of a particular structural prototype: analogues of agonists can be screened to find antagonists, analogues of enzyme substrates can be screened to find inhibitors. The screening may be quite random, or may depend on selection of available structural prototypes. Alternatively, a modern strategy is to use a high-throughput screen and a compound library, perhaps initially screening mixtures and only later identifying the active component (Houghten *et*

al., 1991). It will then be necessary to choose which molecule to use as the "lead"—that will be modified to achieve design objectives as set out in a product protocol.

3.1 OPTIMIZATION OF THE DRUG

After choosing the "lead", one may use knowledge of the receptor site to achieve potency or selectivity advantages. One may even be able to generate the lead based on knowledge of receptor structure alone (see Sections 3.2 and 3.3). Whether or not such knowledge of the receptor site is available, the methods outlined in this section can be applied in order to optimize drug properties to the point of selection for preclinical or phase 1 study.

3.1.1 Choice of a Lead

A few caveats are in order as to what defines a lead. Firstly, a lead is more than just a compound that shows a defined level of activity in a primary screen. The screen must have been validated; usually this will be by obtaining the expected responses from pharmacological standards or known drugs. Any reasons for false positives must be understood. Certain substances such as chemically reactive or unstable compounds, protein denaturants, membrane destabilizing agents or uncouplers of oxidative phosphorylation will record as "active" in a great variety of screens. These must be recognized and eliminated by suitable secondary procedures.

Secondly, and this applies particularly to structures automatically generated from knowledge of the receptor site (Section 3.3), the question must be asked, how amenable is this structure to synthesis and to the synthesis of derivatives and analogues? Is there a chemical "handle" such as carboxylic ester or amide, amino or hydroxyl group?

Thirdly, literature and particularly patent searches may be necessary to establish the likely difficulty of achieving novelty as the lead is modified towards the design objectives.

A number of structures may well satisfy the above criteria, none of which relates to the actual potency of the active compounds. It would be tempting, then, to select the most potent compound as the lead. It is much better, though, if we can assess which structure is the best match to the receptor site, whose structure is presumably unknown. The empirical approach of Andrews *et al.* (1984) to calculate *average* binding energy was discussed in Section 2.1.3. If the primary screen, or some secondary assay, can give relative binding data, this can be used to estimate binding free energy. If then the observed binding of a molecule is significantly greater than the Andrews calculated average binding energy, the deduction can be made that

most functional groups in the molecule are interacting favourably with the receptor and/or that the drug acts in a low energy conformation. The match to the receptor is good, and such a molecule represents a good lead. Conversely, and this will be the case with many large and conformationally mobile structures, observed binding may well be much less than the calculated *average* binding. Some functional groups may not be contributing to binding, and/or this structure has to assume a relatively high energy conformation in order to bind. This situation would require the next step in design to be the progressive deletion of functional groups to determine which are involved in binding (downsizing approach, see Section 3.1.2) and the synthesis of rigid analogues to determine the binding conformation (see Section 3.1.4). In a follow-up paper, Andrews (1986) discusses his method of choosing the best matched structure as the lead, and gives several examples. It is clear that when a number of similar structures are available, judicious calculations can indicate both the role of specific functional groups, and likely biologically active conformations.

3.1.2 Upsizing and Downsizing

With the exception of bioisosteric replacement and some forms of introduction of conformational constraint, all molecular modifications used to optimize the lead compound can be divided into "upsizing" or "downsizing". Historically, it is the upsizing of relatively simple agonist molecules such as acetylcholine or histamine that has given us many useful acetylcholine or histamine antagonist drugs. Upsizing starts with a relatively simple structure and adds groups or makes substitution of small groups for larger ones. Possible advantages of upsizing are:

(1) increase potency by binding to accessory site;
(2) addition of protective group prevents metabolism so improves pharmacokinetics (stability, half-life);
(3) addition of supplementary group modifies overall lipophilicity or solubility, so improves transport or facilitates formulation;
(4) improve selectivity by hindering fit to alternative receptor;
(5) reduce renal elimination;
(6) gain novelty advantage (patentability).

Conversely, the lead may already have sufficient potency (binding to a target receptor) and the perceived problems are pharmacokinetic or toxicological. We may consider downsizing, the possible advantages being:

(1) of a polypeptide, is likely to increase absorption and half-life;
(2) remove a site of metabolic vulnerability;
(3) reduce biliary clearance (see Section 3.1.8);

(4) increase solubility or decrease lipophilicity;
(5) easier synthetic target;
(6) finally, the systematic deletion of groups can determine which ones are involved in binding.

3.1.3 Bioisosteres

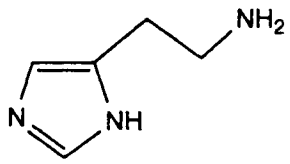
The concept of bioisosterism is at the heart of drug design. As a concept, it existed in the minds of medicinal chemists long before the term was introduced (Friedman, 1951).

At some point in drug design it is usual for the chemist to pose the question: here I have a structure, which is both potent and selective, but will not make a good drug because—and there may be one or many reasons —how can I replace a part, or all, of this structure with an alternative structure that is equivalent in maintaining the favourable features, but removes the unfavourable? Unfavourable features may involve metabolic instability, toxicity, insolubility or merely the lack of novelty. At the simplest level, a methyl substituent may be metabolically vulnerable. Replacement by chlorine—of similar size and lipophilicity—may overcome this difficulty if size and liphophilicity are the important properties for a desirable receptor interaction. "Of similar size" is covered by the term isostere. But to have similar biological properties needs the term *bio*isostere. Hence the definition by Thornber (1979): "Bioisosteres are groups or molecules which have chemical and physical similarities producing broadly similar biological properties."

Note "broadly similar" biological properties. Bioisosteric replacement must allow a number of properties to remain, and some to be altered. Thus, one may allow selective binding to a receptor to remain, but alter an agonist to an antagonist. This is the classical approach to many drugs that was pursued long before the term "bioisostere" was invented.

Bioisosteric replacement can be made from a position of knowledge, if the desirable properties of the substituent or substructure to be changed have been characterized. Such properties can include (with typical parameters): (a) size (volume, molar refractivity, surface area, Taft E_S); (b) shape (Verloop length and breadth, bond angles, interatom distances); (c) lipophilicity (log P, π, f); (d) solubility (log S); (e) ionization state (pK_a, σ); (f) tautomeric equilibrium (e.g. keto:enol ratio, imidazole N^{τ}-H: N^{π}-H ratio); (g) charge distribution (dipole moment, electrostatic potential); (h) chemical reactivity (HOMO, LUMO); (i) hydrogen bonding ability (α_2, β_2 of Abraham *et al.*, 1991).

Bioisosteric replacement of a substituent or group can be made without *a priori* knowledge of desirable properties, provided that at least two (but preferably more) groups giving the required response can be identified, and



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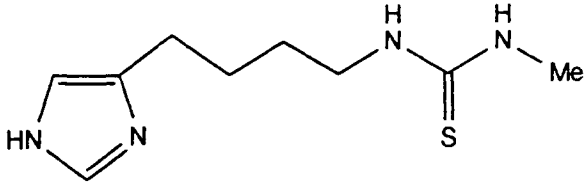

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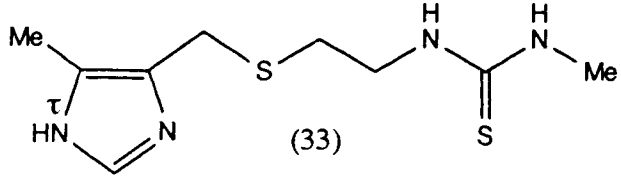

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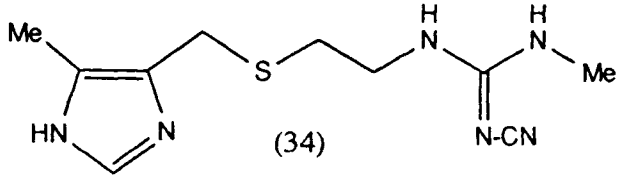
a broad range of property parameters can be assigned. With a database of alternative groups that are also parameterized for these properties, a nearest neighbour or cluster analysis can be used (Hansch *et al.*, 1973). This will identify similar groups or clusters of groups, based on trial selections of parameters that are likely to be important to the desired activity. Groups clustering with the "actives" can be chosen as potential bioisosteres.

Alternatively, one may make *ad hoc* replacements based on the now considerable literature on bioisosteres. Many examples are given in the reviews of Thornber (1979), Lipinski (1986) and Burger (1991).

A good example of the repeated application of bioisosteric replacement concerns the design of the histamine H_2 receptor antagonist, anti-ulcer drug cimetidine. Based on the classical idea that an antagonist might be developed as a derivative of the agonist histamine (**31**), the chain between the imidazole ring and amino group was increased to $-(CH_2)_4-$ and the amino group replaced, first by *N*-methylguanidine, and then by a guanidine bioisostere, thiourea, to give burimamide (**32**). Burimamide was further modified by introduction of a methyl group into the imidazole ring, and bioisosteric replacement of methylene by sulphur, giving metiamide (**33**), both changes encouraging a preponderance of the N^{τ}-H imidazole tautomer which had been identified as necessary both for potency and H_2 versus H_1 receptor selectivity (Black *et al.*, 1974). The thiourea group of metiamide was subsequently shown to be responsible for an alarming degree of toxicity in experimental animals, so further bioisosteric replacements were tried, including nitroguanidine and cyanoguanidine, the latter giving the extremely successful cimetidine (**34**).

Subsequent to the development of cimetidine, H_2 antagonism was discovered in compounds with rings other than imidazole. In particular, the drug ranitidine (**35**) was developed. It would appear that the di-



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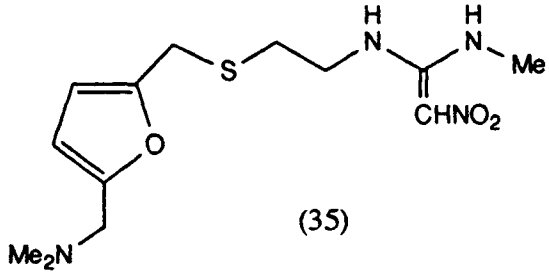


(35)

methylaminomethyl-furan grouping is a bioisostere for imidazole, but this is not so. Molecular modelling of a number of H_2 antagonists by Höltje and Batzenschlager (1990) indicates that ranitidine, with its protonated dimethylamino group, makes a strong salt link with an anionic site in the receptor, a site not reached by the imidazole ring system of cimetidine. Thus, rather than bioisosteric "equivalence" being achieved, ranitidine loses the imidazole interaction, but gains the somewhat stronger, alternative interaction with an anionic site.

The design and use of peptide bioisosteres—peptidomimetics—has been reviewed by Giannis and Kolter (1993). Peptidomimetics are required (a) to be metabolically stable, (b) to have high bioavailability and (c) to have minimal side effects, while maintaining or even improving upon the potency of the peptide. Surrogates considered range from simple amide bond isosteres, such as a *trans*-substituted ethylene linkage, and dipeptide analogues to the imitation of conformation through β-turn mimetics or even the complete global restriction of conformation, as exemplified by structures **40** and **41** discussed in Section 3.1.4.

3.1.4 Conformational Constraint

A few drug molecules, such as steroids, tetracyclines and morphine, are rigid, but for most a degree of conformational flexibility is involved, and they exist in solution as an equilibrium mixture of conformers.

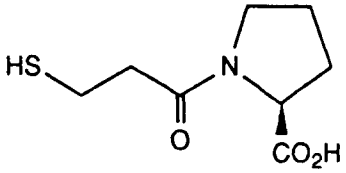
Knowledge of the receptor-bound conformation of a ligand is of great help in drug design. Constraints can then be introduced to encourage

preponderance of the receptor-bound conformation in solution. If the preferred solution conformation of the molecule is also that required by the receptor, then the free energy of binding is more favourable in terms of both enthalpy and entropy. On complex formation, conformational entropy is lost [recall Andrews' (1984) estimate of the *average* entropy loss of 0.7 kcal/mol for each Csp^3–Csp^3 bond in a molecule when fixed on a receptor—see Section 2.1.2].

It follows that for a totally rigid ligand there will be no loss of conformational entropy, and provided that receptor and ligand surfaces are entirely complementary in charge, hydrophobicity and dispersion effects, then binding should be maximized. However, it is a gross oversimplification to believe that a totally rigid molecule will make the best drug for any given application. Williams (1977) has pointed to the flexibility of endogenous transmitters such as noradrenaline and acetylcholine, and to the super-flexible peptide hormones, and has argued that the flexibility of a bioactive molecule, be it agonist or antagonist, might be a requirement in order to reach, by successive movements or "wriggling", its final binding site. A receptor need not be on an open (e.g. cell) surface, but may be relatively buried, e.g. the active site pocket of a globular protein within the cell. Whether the potential ligand binds or does not bind to such a site, within a limited time, will depend on whether it can reach it, and conformational flexibility may be a prerequisite.

The drug designer is as usual faced with making a compromise. Rigidity favours binding, but flexibility may be needed for access! The best strategy may well be to encourage the desired conformation (if known from receptor complementarity or from pharmacophore modelling) without rendering the molecule totally rigid. Conformational design to synthesize *flexible* molecules with a defined shape has been discussed by Hoffmann (1992). Conformation-determining factors can be built into a molecule, often by no more than the introduction of a methyl substituent, to encourage the main chain of atoms (or chain linking pharmacophoric groups) to lie predominantly in one direction. Such tendencies can readily be predicted using the rule-based approach advocated by Hoffmann, or by computer graphic investigation using molecular mechanics.

Conformational control has played a major part in the design of inhibitors of the angiotensin-converting enzyme (ACE). This has been reviewed recently in *Advances in Drug Research* (Lawton *et al.*, 1992). The basic requirements for an ACE inhibitor are present in the early lead compound (**36**): a zinc liganding group (–SH), a carbonyl group acting as hydrogen bond acceptor, and a carboxyl group regarded as binding to a positively charged centre in the enzyme. Introduction of a methyl group gave the first clinically useful ACE inhibitor, captopril (**37**). In captopril, the methyl group directs the conformational preference of the main chain and binding is increased 10-fold. The conformation of several inhibitors was studied by



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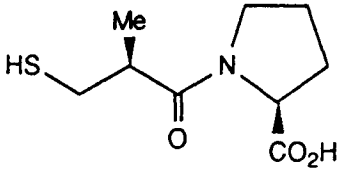

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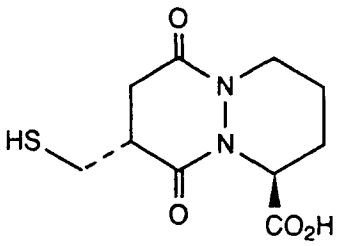

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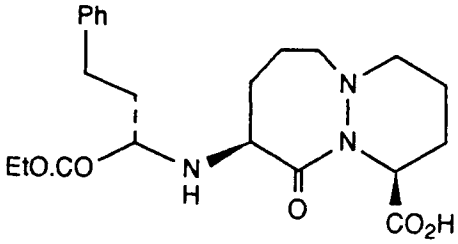


(39)

Hassall *et al.* (1984) using NMR techniques. Guided by the results, they synthesized a series of bicyclic analogues, in which the chain of atoms connecting the –SH and $-CO_2H$ groups was constrained still further: the *cis–trans* isomerization of the proline amide bond of **37** was fixed *trans* by **38**. Further design by the same group resulted in the clinical agent cilazapril (**39**) in which conformational constraint has again been achieved by ring closure, the –SH ligand has been replaced by the bioisostere $-CO_2H$, which in turn has been esterified to the prodrug group $-CO_2Et$; additional hydrophobic binding has been achieved by a phenethyl substituent. Compared with captopril, cilazapril is more potent and provides a longer-lasting effect *in vivo*.

The conformational analysis and superimposition of ACE inhibitors have now provided a model for the active (receptor) site of ACE which can be used for the design of further inhibitors (Andrews *et al.*, 1985). A low-energy conformer of captopril was expected, and now found, to be a best fit to the receptor. In modelling, it is a reasonable first assumption that a low-energy conformer will be utilized, but—and especially with large molecules utilizing several binding groups—this is not always so. Higher-energy conformers, and the effect of both solvent and the receptor itself in stabilizing any conformation of a flexible ligand must be considered. Recently, the effect of binding on ligand conformation has received much attention, and cases of profound and unexpected conformational change have been discovered by NMR and X-ray measurements on the complex

(Jorgensen, 1991). As a consequence of the fact that flexible molecules distort to form optimal interactions with receptors, much frustration is likely if one attempts to design drugs by analogy only to flexible ligands, rather than with inclusion of rigid analogues.

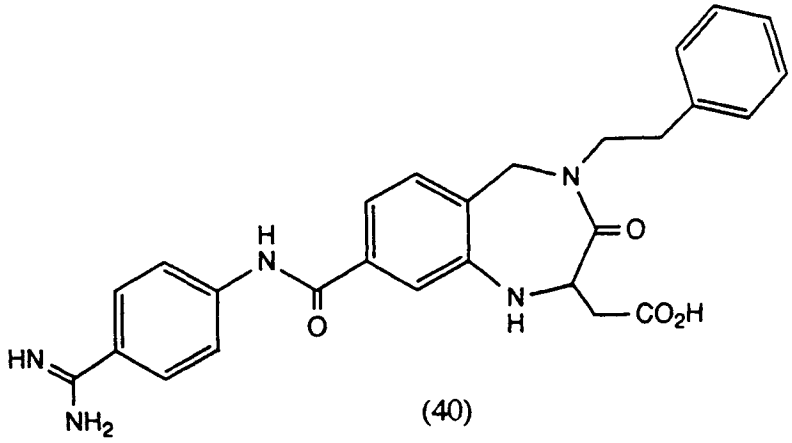
The most severe problems exist with peptide ligands, and this is particularly the case for the high-throughput screening of peptide libraries. Once an active peptide has been discovered, it will be necessary to design a suitable non-peptide analogue to avoid the problems of poor absorption and sensitivity to peptidases that confound the direct use of peptides as drugs. It will be necessary to know the conformation of the peptide lead.

Exhaustive conformation/activity studies have been made by Gurrath *et al.* (1992) as a preliminary to the design of antagonists to the cellular binding of extracellular proteins containing the Arg–Gly–Asp (RGD) sequence. This sequence forms a recognition/binding site which complexes with cell surface receptors. In order to find the binding conformation of the RGD sequence, a number of cyclic penta and hexapeptides, all containing the RGD sequence, were synthesized. Apart from cyclization, further conformational constraint was introduced by incorporating D-amino acids and proline residues, as X(X′) into c(RGDFX(X′)). Conformation was determined by extensive NMR measurements and by molecular dynamics calculations.

The RGD sequence was thus constrained into a variety of sheet or β-turn structures, which were unequivocally determined for each peptide. The biological activity of 18 cyclic peptides was then compared with that of a linear standard, GRGDS, in inhibition assays of tumour cell adhesion. An increase in activity of up to 100-fold was observed for just two cyclic pentapeptides, all others showing a decrease in activity. This identified the required conformation of the RGD backbone.

Ku *et al.* (1993) have reported the design, synthesis and biological activity of the very potent fibrinogen receptor antagonist, **40**. This compound was designed as a non-peptide equivalent (peptidomimetic—see Section 3.1.3) of the constrained RGD-containing cyclic peptide (**41**), with consideration of both compositional and conformational features. Both **40** and **41** have affinity at the nanomolar level, suggesting that the constrained conformation of **41** reflects the receptor-bound conformation of the agonist.

A type II β-turn was identified as the necessary conformation of pro-leu-glycinamide (**42**), an endogenous tripeptide which modulates dopamine receptors by enhancing the binding of agonists (Sreenivasan *et al.*, 1993). Evidence for this conformation was provided by NMR and X-ray, but finally by synthesis of constrained analogues, including **43** which incorporates a γ-lactam β-turn mimic developed by Freidinger *et al.* (1989). Compound **43** was found to be 10 000 times more potent than **42** in enhancing the binding of the agonist 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene.



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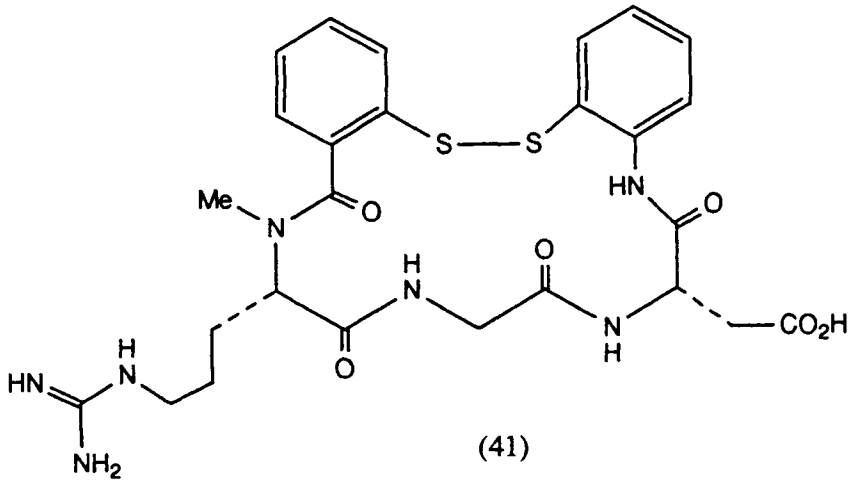
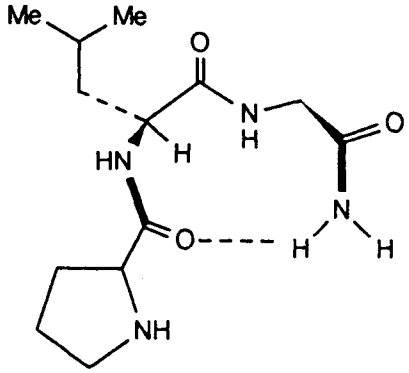


(41)

Marshall (1992) has reviewed the range of recognition motifs commonly used by peptide ligands, as determined by NMR and X-ray methods. Two extremes have emerged as most common. As in the two examples just quoted, interaction is frequently dominated by side chains of the peptide being held in β-turns. Alternatively, interaction can occur with a linear sequence, involving not only side chains but also the peptide backbone in binding, as for example in recognition of the substrate and inhibitors of HIV proteinase. Marshall concludes that if one hopes to determine the biologically relevant conformation of a flexible ligand, it is imperative to include the receptor in the study, either directly through NMR or X-ray investigation of the complex, or indirectly through study of the binding of conformationally constrained analogues.



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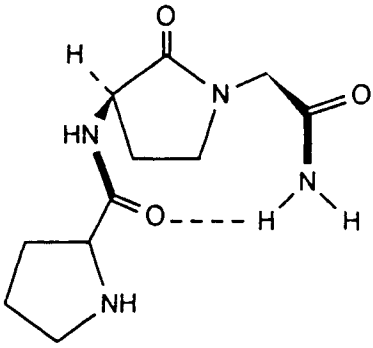

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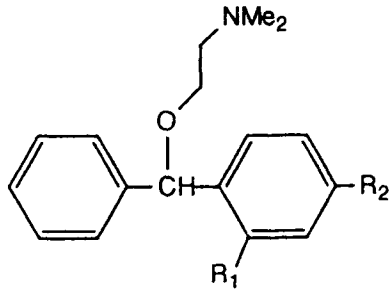
3.1.5 Chirality and Design

Enantiomers, diastereoisomers and stereoisomers in general are different chemical compounds. They may exhibit both quantitative and qualitative differences in their interaction with a target receptor. They may be transported, stored, metabolized or excreted in quite different ways (Crossley, 1992). Given this, it is amazing that racemic mixtures continue to be tested in the hope that some conclusion can be reached as to the "activity" of the separate components. There are huge pitfalls to the development of racemates, and it is rarely justified. The required activity of thalidomide resides in one isomer, and the devastating teratogenic potential resides in the other (Ariëns, 1986; Gross, 1990). The development of a single enantiomer is not exempt from stereochemical problems: one must be certain that racemization does not occur *in vivo*. This problem and the particular case of thalidomide, which racemizes in 1–2 h in the body, have been discussed by Testa *et al.* (1993).

The reluctance to perform any synthetic modification which introduces a chiral centre, though understandable because of the labour involved in performing a separation, might well be replaced by a positive desire to do so. This is, in part, because of the fact that many methods for enantiospecific, or enantioselective synthesis (Brown and Davies, 1989) and for chiral separation are now available, but principally, it is because the separation and testing of homochiral materials can lead to improvements in potency, selectivity, stability and safety. Such improvements have in the past been achieved as empirical discoveries, consequent to resolution. With current knowledge, some improvements may be *designed* by the appropriate introduction of asymmetry. In order to illustrate this, it is helpful to use a nomenclature introduced by Lehmann *et al.* (1976). This nomenclature designates which enantiomer is which from a biological activity point of view.



(44)

The more active isomer (where activity refers to binding affinity at a defined receptor) is designated the *eutomer*, the less active is the *distomer*. The ratio of activities is the *eudismic ratio*, and its logarithm, the *eudismic index*, is then proportional to the difference in binding free energy between the enantiomers. The eudismic index is a quantitative measure of chiral discrimination.

Reference has already been made to captopril (**37**). Substitution of **36** by a methyl group and separation of the *S*-enantiomer, the eutomer (**37**), yields an increase in binding of 10-fold. The corresponding *R*-enantiomer, the distomer, is 100-fold less potent, a eudismic index of 2.0 reflecting a difference in binding free energy of some 2.8 kcal/mol. Part of this difference results from conformational steering in the eutomer, part from the inability of the methyl group in the distomer to utilize a lipophilic binding site.

The introduction of asymmetry, followed by resolution, can be used to gain selectivity from a promiscuous molecule. Closely related receptors may differ only in the shape or size of their non-polar (hydrophobic) areas. Because of this, it makes sense to try to exploit differences in hydrophobic binding. Ariëns (1986) exemplifies this by referring to derivatives of diphenhydramine (**44**, $R_1 = R_2 = H$). Table 3 is compiled from the data of Rekker *et al.* (1971) and shows how a 10-fold selectivity of diphenhydramine for the histamine receptor has been translated into an increase in potency and more than 100-fold selectivity towards the histamine receptor in the (+)-methyl derivative, and to an increase in potency and more than 100-fold increase in selectivity towards the acetylcholine receptor in the (−)-*t*-butyl derivative. The data provide a good example of "Pfeiffer's Rule" (Pfeiffer, 1956) that enantiomeric potency ratios increase with potency. Many examples of this rule have been reviewed by Lehmann (1986).

The testing of pure optical isomers can give much insight into the nature of a receptor. An example concerns the anaesthetic gases. It is widely believed that anaesthetic gases exert their action by a structurally non-specific perturbation of the fluid character of nerve membranes. This view

TABLE 3[a]

Activities (pA_2) and eudismic ratios of optical isomers for diphenhydramine derivatives

R_1	R_2	Isomer	Anticholinergic activity	Antihistaminic activity
H	H		6.68	7.62
Me	H	+	6.14	8.76
Eudismic ratio			1.9	77.6
Me	H	−	5.86	6.87
H	CMe_3	+	6.03	6.36
Eudismic ratio			1.20	2.3
H	CMe_3	−	8.12	6.00

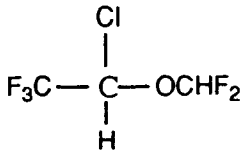
[a]Data of Rekker *et al.* (1971). Reprinted with permission from Ariëns (1986).

has been supported by numerous QSAR studies, establishing correlations between anaesthetic potency and hydrophobicity. Some QSARs have also suggested that anaesthetic receptors contain a polar site, which can accept a hydrogen bond (Hansch *et al.*, 1975; Abraham *et al.*, 1991).

In contrast, much recent evidence suggests that these supposedly "non-specific" agents might act by binding directly to a particularly sensitive protein, rather than a phospholipid membrane, as target (Franks and Lieb, 1994). For example, the enzyme luciferase is inhibited by clinically effective concentrations of anaesthetics (Franks and Lieb, 1984).

The "specific receptor" viewpoint has been strengthened by the testing of optically pure isomers of isoflurane (**45**) on molluscan nerve ion channels (Franks and Lieb, 1991). At the human ED_{50} for general anaesthesia, the *S*(+)-enantiomer is twice as effective as the *R*(−)-enantiomer both in eliciting the potassium current and in inhibiting a current induced by nicotinic acetylcholine receptor activation, in neuronal cells excised from a snail. When tested for anaesthetic activity in mice, the *S*(+)-enantiomer of isoflurane was found to be 50% more effective than the *R*(−)-enantiomer following intraperitoneal injection (Harris *et al.*, 1992). These investigations were made possible by the prior work of Meinwald *et al.* (1991) who carried out a microscale resolution of halothane (**46**), enflurane (**47**) and isoflurane (**45**) by gas chromatography on capillary columns of a chiral cyclodextrin derivative.

With molecules more complex than the gaseous anaesthetics, it is often the case that enantiomers differ not only in configuration, but also in conformation. The effect can be profound when the chiral centre is in a vinylic relationship as depicted by **48** or the aromatic equivalent **49**.



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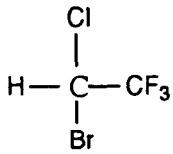

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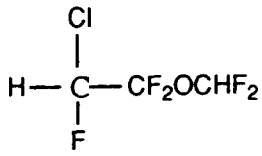

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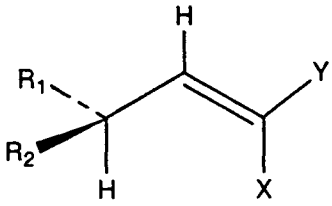

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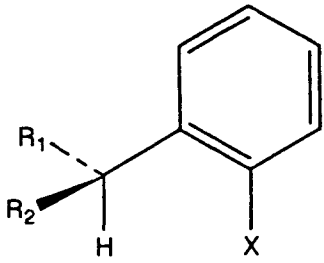

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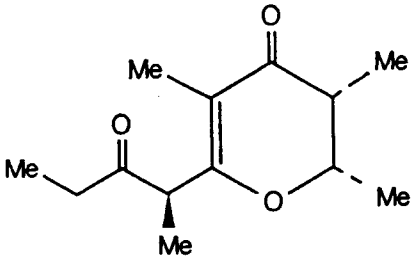

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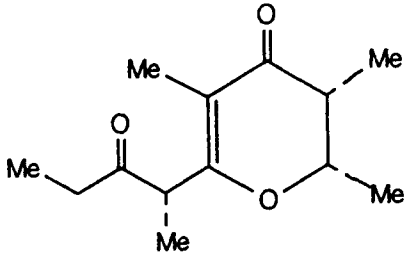
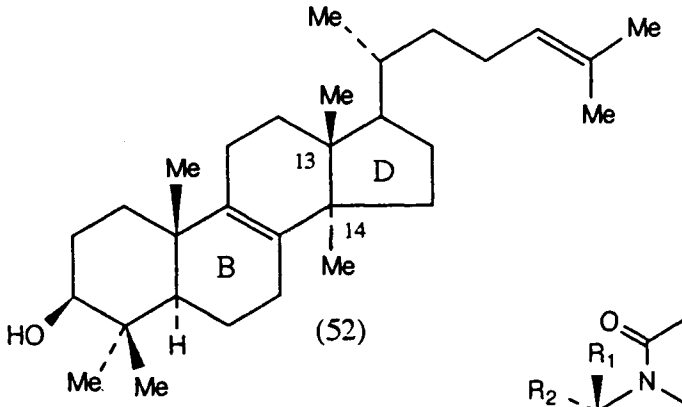


(51)

In these structures, the strongly preferred conformation is one in which the H–C–C=C–X unit lies in one plane. Interchanging R_1 and R_2 (where R_1 may be a main chain and R_2 a methyl group) will encourage the main chain to change direction by approximately 120°. Enantiomers incorporating such a system can exhibit large, even qualitative, differences in activity. Hofmann (1992) quotes the example of stegobinone (**50**), the sex hormone of the drugstore beetle. The configurational change from **50** to the enantiomer **51** may seem trivial when considered as a swap of the methyl and hydrogen groups, and this is how it is portrayed in the structural



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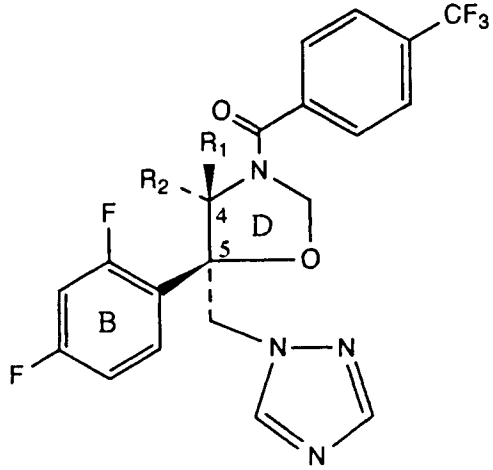


(53, R_1 = Me, R_2 = H)

(54, R_1 = H, R_2 = Me)

diagrams. This is misleading, for the allyl system determines conformational preference such that the hydrogen remains in the plane of the double bond, and it is the methyl and propionyl groups that should be swapped in position. The spatial arrangement for the propionyl side chain relative to the six-membered ring is completely different in the two epimers. The epimer **51** is an antagonist of **50**.

Introduction of a chiral centre into a ring can also lead to unexpected conformational effects, which can be exploited in drug design. There has been much exploration of azole (imidazole, triazole) derivatives as antifungal drugs over the last 20 years. Knowledge that such azoles act through inhibition of lanosterol C14-demethylase, a step crucial to fungal biosynthesis of ergosterol (the essential sterol in the fungal membrane), has prompted comparisons of the structure of inhibitors with the structure of the substrate, lanosterol (**52**).

Hata *et al.* (1991) designed diastereoisomers **53** (4*R*, 5*R* isomer, R_1 = Me, R_2 = H) and **54** (4*S*,5*R* isomer, R_1 = H, R_2 = Me). Crystallographic studies of **53** and **54** showed that optical differences caused differences in ring conformation, and that only the markedly more potent isomer, **53**, was

superimposable on lanosterol in such a way that when rings B and D of lanosterol were overlaid on rings B and D of **53** then the methyl group at C4 was superimposed on the C13 methyl group of lanosterol. Such a superimposition can now be used to suggest further derivatives in which the lanosterol structure is mimicked even more closely.

3.1.6 Design for Safety: Surrogates for Toxophores

Molecular modification can be used to eliminate the potential for toxicity from a candidate drug. This requires knowledge of the chemical mechanisms of toxic action, both direct and indirect (via metabolic activation), so that one may recognize the potential toxophore.

The qualifying word *potential* is used, because toxic action will only result from completion of a sequence of events. These events will typically involve exposure (short or long, acute or chronic, and dependent on route of administration), absorption and distribution, metabolism, and finally interaction of the drug or its metabolite with a sensitive site (often DNA, perhaps a functional protein) or within a sensitive tissue (e.g. liver, kidney, eye). Design criteria may differ between drugs intended for short-term use, such as anti-infective agents, and those which may be taken over a long period. Age, sex, and many other factors conspire to determine the outcome following exposure to a *potentially* toxic molecule (Ariëns, 1980).

The potential for direct toxicity, as manifested by acylating or alkylating groups, or by double bonds in conjunction with a carbonyl function that will thereby act as Michael acceptors, is easily recognized.

Indirect toxicity is not so apparent. It most often results from the biotransformation of what may appear to be a relatively unreactive molecule to a highly reactive intermediate, such as an epoxide or a radical species, through Phase 1 metabolism. Occasionally, a Phase 2 (conjugation) step is involved.

Fortunately, there is now a comprehensive body of knowledge on the metabolic reactions that produce reactive (toxic) intermediates, so the drug designer can be aware of what might occur, and take steps to circumvent the possibility. Nelson (1982) has reviewed the classes and structures of drugs whose toxicities have been linked to metabolic activation. Problem classes include aromatic and some heteroaromatic nitro compounds (which may be *reduced* to a reactive toxin), and aromatic amines and their N-acylated derivatives (which may be *oxidized*, before or after *hydrolysis*, to a toxic hydroxylamine or iminoquinone). These are the most common classes, but others are hydrazines and acyl-hydrazines, haloalkanes, thiols and thioureas, quinones, many alkenes and alkynes, benzenoid aromatics, fused polycyclic aromatic compounds, and electron-rich heteroaromatics such as furans, thiophenes and pyrroles.

The drug designer must consider the susceptibility to, and consequences of, the principal Phase 1 metabolic reactions: *hydrolysis*, *reduction* and *oxidation*. Susceptibility to oxidation can be calculated using semi-empirical molecular orbital theory. Ease of oxidation is reflected by the energy of the HOMO, and the probable site of oxidation can be predicted from calculation of the electrophilic superdelocalizability. Likewise, ease of reduction is related to LUMO energy, and probable site to nucleophilic superdelocalizability (Loew and Burt, 1990).

Unfortunately, the outcome of oxidation is not only dependent on the ease with which it will occur (susceptibility) but is also dependent on the overall lipophilicity of the molecule (see Section 2.3.1). The enzymes most frequently implicated in the transformation of compounds to reactive toxins are the cytochrome P450s. Because the determinants of binding (lipophilicity, steric complementarity, presence of polar functions) vary in importance across the various P450 isoenzymes, the outcome of metabolism is currently much more difficult to predict than the susceptibility.

Classical QSARs have been developed within classes of compounds, including aromatic nitro compounds. The mutagenicity (Ames test) of a set of 188 aromatic nitro compounds has been correlated with log P and energy of the LUMO, together with indicator variables to account for steric effects (Debnath *et al.*, 1991). Computational approaches to predicting toxicity have been reviewed by Dunn (1988), Richard *et al.* (1989) and Benigni *et al.* (1989). Classical QSAR through regression analysis is of limited use, for the problem is one of classification. Does the structure have toxic potential or not? Pattern recognition methods and discriminant analysis have been used to analyse databases of toxicity and derive relationships which allow one to predict the probability of any structure being toxic, according to protocols defining mutagenicity, teratogenicity, carcinogenicity, skin irritation, etc. A proviso with such methods is that the substructural features and properties of the query structure are well represented in the database from which the predicting model was derived. In drug design, structures requiring toxicological prediction often exhibit unique chemistry, so this proviso is not satisfied. The TOPKAT toxicity prediction program (Enslein *et al.*, 1986) will warn the user when this occurs, and classify structures as to their probabilities of being toxic. Such a program can be useful, but cannot replace an experimental investigation of metabolism.

Until recently, Phase 2 (conjugation) reactions have largely been ignored as a source of toxic derivatives of a drug. Conjugation normally involves the addition of a hydrophilic or acidic moiety to the drug itself, or to its Phase 1 metabolite, and will encourage rapid urinary and biliary excretion. Over the last decade, however, there have been reports of a significant number of conjugation reactions which do not facilitate elimination, but rather result in the formation of lipophilic products which can accumulate and in some cases are toxic.

In particular, drugs containing an alcoholic hydroxyl group can be esterified with an endogenous long chain fatty acid; other drugs containing a carboxyl group, or giving rise to a carboxyl group through a Phase 1 oxidation or hydrolysis, may form an ester through the hydroxyl group of cholesterol; and glutathione conjugates may be transformed to toxic thiols or may be oxidized to lipophilic sulphones. Such reactions have been reviewed by Caldwell and Parkash (1993).

Having established susceptibility to a metabolic transformation which might lead to toxicity, the designer must either remove the offending group (downsizing, see Section 3.1.2), replace the toxophore (bioisosteric replacement, see Section 3.1.3) or block the metabolic transformation. Substitution of a fluorine or trifluoromethyl group will often block metabolism at or near the substituted site. Alternatively, introduction of a methyl group could either block or provide an alternative safe route of metabolism via oxidation of the methyl group. These and other strategies have been discussed by Nelson (1982) and Armstrong (1988).

3.1.7 Design for Delivery: Absorption

The absorption of a drug by the oral or by the parenteral route usually depends on passive transport (see Section 2.3.1). The drug should be sufficiently lipophilic to partition into a membrane, but also sufficiently hydrophilic to pass across the membrane, and also sufficiently soluble. The first requirement for absorption is that the drug be in solution.

Yalkowsky and Morozowich (1980) have discussed the physicochemical factors which predispose to solubility, and the various ways to achieve absorption. These include formulation (not a design problem) and also the control of physical properties through the use of ionizable moieties (which will generally increase solubility or allow formulation as a soluble salt). The prodrug approach (see Section 2.3.3) may be used, but of course brings with it the problem of releasing the active moiety at the right time and place. An ionizable function in the drug can cause problems in transport, either through preventing passage through charged membranes, or by providing a site for chelation with a metal (absorption of tetracyclines is prevented by calcium). The first approach to consider should therefore be the adjustment of solubility and partition coefficient, by making changes in any part of the structure not directly involved in receptor binding.

For liquids, many studies have shown inverse linear relationships between solubility and partition coefficient. But most drugs are solids, and for these solubility depends also on the ease of breaking cohesive forces within the crystal, hence relationships include a function of the melting point as a correction term.

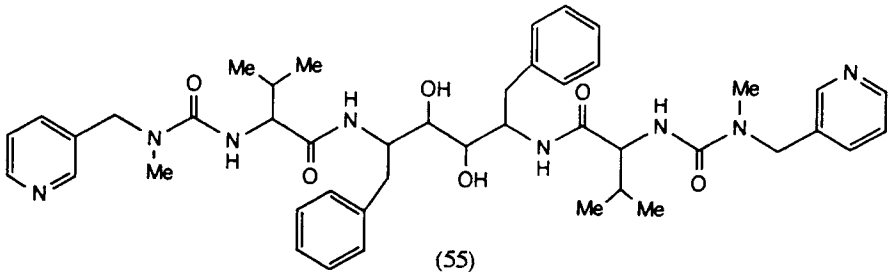
Suzuki (1991) has developed an estimating system for both the partition

coefficient and for aqueous solubility. Equation (15) correlates molar solubility, S, with log P and melting point for a wide range of non-ionic compounds. T_m is the melting point in °C; if the compound is a liquid at 25°C, then T_m is set equal to 25.

$$\log 1/S = 1.050 \log P + 0.00956(T_m - 25) - 0.515 \quad (15)$$
$$(n = 497,\ r = 0.976,\ s = 0.505)$$

This equation covers a wide range (from −1.96 to 10.49) of log $1/S$ values and chemical classes, and includes 348 liquids and 149 solids.

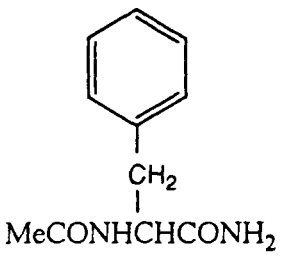
The bioavailability of a drug can depend critically on solubility. The formulation of a drug for intravenous administration demands solubility. As equation (15) indicates, solubility can be improved either by adding hydrophilic groups [a good example of this approach is provided by the work of Thompson *et al.* (1992)] or by destabilizing the crystal structure. The latter can frequently be done by removing some of the potential for intermolecular hydrogen bonding. The *N*-methylation of an amide, or substitution of a carbamate oxygen by *N*-methyl, may increase log P only marginally but by disrupting crystal structure can provide a substantial increase in solubility. An example of this ploy comes from the development of the bis-*N*-methylurea (**55**) from a bis-carbamate analogue, which gave a 100-fold increase in solubility, without detriment to either enzyme inhibition or antiviral activity, so enabling intravenous formulation for a clinical trial of this HIV protease inhibitor (Kempf *et al.*, 1991).



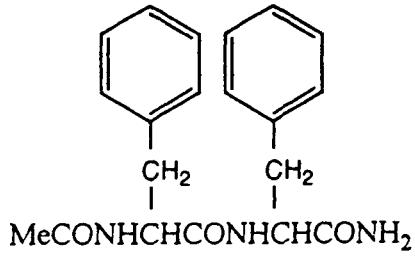
(55)

It may be better to increase solubility by disrupting crystal structure, rather than by adding polar groups. The reason for this is that for absorption into the membrane, the drug must be desolvated. A polar group may hinder both initial absorption, and subsequent transport. In a study of the intestinal absorption of peptide derivatives, Karls *et al.* (1991) found that absorption was most influenced by the number of hydrogen bonding sites, the major determinant of desolvation energy.

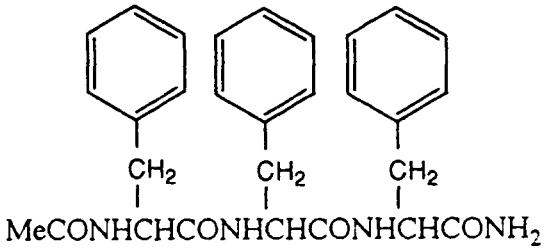
Two series of radiolabelled D-phenylalanine derivatives, **56–58** and **59–62**, were synthesized. The molecules are uncharged and enzymatically stable


CH2
MeCONHCHCONH2

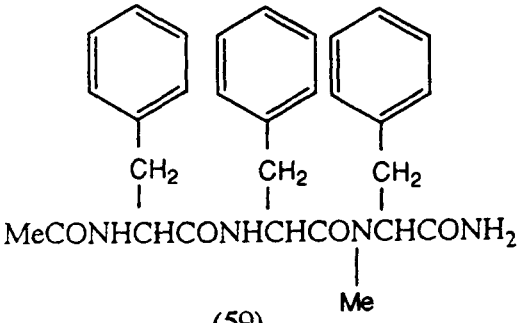
(56)


CH2
CH2
MeCONHCHCONHCHCONH2

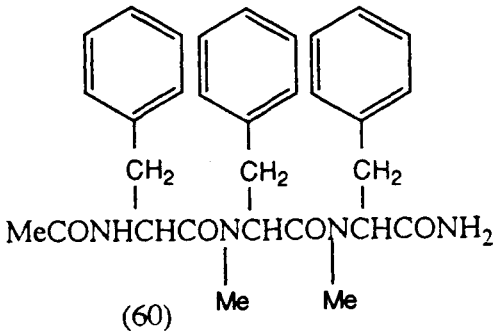
(57)


CH2
CH2
CH2
MeCONHCHCONHCHCONHCHCONH2

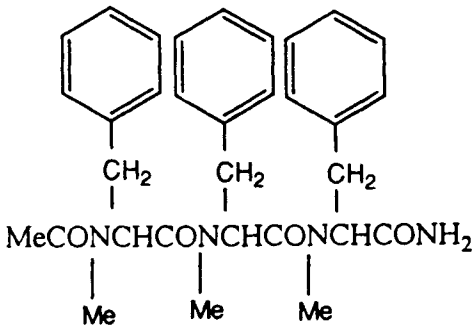
(58)


CH2
CH2
CH2
MeCONHCHCONHCHCONCHCONH2
Me

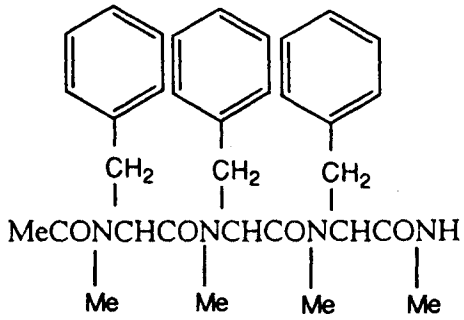
(59)


CH2
CH2
CH2
MeCONHCHCONCHCONCHCONH2
Me
Me

(60)



(61)



(62)

due to the D-configuration. Both absorption through the gut and clearance by liver and kidneys were monitored in rats. Some of the results are presented in Table 4.

In the first series, log *P* increases from 0.1 to 2.3 and one might have expected absorption to increase, as is found with many other classes of compound (Austel and Kutter, 1983). Absorption actually falls, and could be due to the increase in molecular weight or to the increasing difficulty of desolvation with increase in hydrogen bonding sites. In the second series, log *P* and molecular weight only increase marginally as N–H groups are substituted and one must conclude that it is the reduction of hydrogen bonding potential that causes the steady increase in absorption.

3.1.8 Design for Delivery: Metabolism and Excretion

In the context of designing drugs, it is not sufficient to adjust the physicochemical properties so that the drug can reach the site of action; it is

TABLE 4[a]

Absorption of model peptides in rats

Peptide structure	MW	log *P*	H-bond number[b]	% absorption[c]
56	206	0.1	5	77 ±4
57	353	1.2	7	58 ±3
58	501	2.3	9	13 ±1
59	515	2.6	8	22 ±1
60	529	2.5	7	30 ±2
61	543	2.9	6	31 ±1
62	557	3.2	5	44 ±2

[a]Reprinted with permission from Karls *et al.* (1991).
[b]Each N–H and carbonyl C=O are assigned a value of 1.
[c]Intestinal absorption following intraduodenal injection.

necessary to ensure that it remains there long enough and in sufficient concentration to exert useful activity.

Drugs are removed (cleared) from the system by three major pathways. Following the appropriate phamacokinetic investigations, total clearance may be expressed as the sum of clearance by renal excretion, by biliary excretion and by metabolism (Gaillot *et al.*, 1990). Unfortunately, and for a variety of reasons (Armstrong, 1988), experiments in animals often give a false picture of what will happen in humans. All too frequently, drug development requires "fixing" an excretion or metabolism problem that is recognized only in human studies. An alternative development candidate is then selected, or the chemist is recalled to adjust the structure in an attempt to fix the problem. The extreme difficulty of predicting excretion and, particularly, metabolic routes and rates in humans is the reason for many workers having pronounced that drug design is an impossible concept. But such a view is contrary to the spirit of this article! Taking the view that drug design is not a totally cerebral exercise, but must also involve appropriate and relevant *in vitro* and *in vivo* experimentation along the way to determine likely metabolic and other clearance mechanisms, then total drug design is a reality.

Renal clearance occurs by passive, glomerular filtration of free drug, and/or by active tubular secretion. A design strategy to forestall the first process would be to limit water solubility, increase log *P* (or log *D*) and increase protein binding. Tubular secretion is an active process with separate mechanisms for acids and bases; unfortunately it occurs with relatively little structural specificity and is independent of whether drug is free or largely protein bound. A design strategy would be to avoid ionic

species (particularly acids) and to consider bioisosteres (see Section 3.1.3) in order to minimize tubular secretion and maximize tubular reabsorption. Tubular reabsorption is a passive process which occurs with uncharged molecules.

Biliary clearance is a complex process and still not fully understood, although it is known that different active transport mechanisms are involved for anionic, cationic and neutral compounds (Klaassen and Watkins, 1984). Two very important determinants are size (molecular weight) and polarity. For all compounds with a molecular weight <300, or with very high molecular weight (proteins), there is very little biliary excretion. Low molecular weight compounds are reabsorbed when initially secreted in bile. Above a molecular weight of 300, there is a species variation for anions but little species variation for cations. Biliary excretion is usually only a serious problem in humans for anions of molecular weight >500 and for cations >200. It is rarely a problem for neutral, lipophilic compounds which can be reabsorbed.

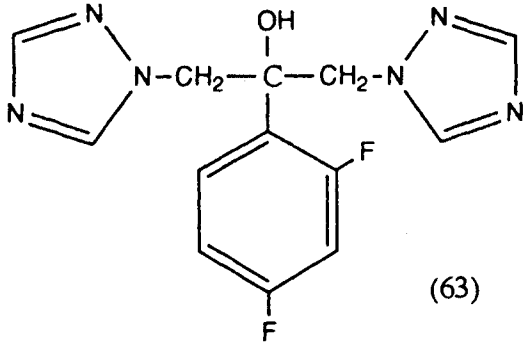
Metabolism (metabolic clearance, hepatic clearance) is a major source of divergence between *in vitro* and *in vivo* activity and a prime concern of drug design is therefore to use metabolically stable molecular fragments. Such fragments will in general not be "unmetabolizable" but will be stable in the right molecular environment. This usually means a molecule of reasonably low lipophilicity. When a "fix" has to be performed on the otherwise ideal drug which is removed too quickly by metabolic action, then a stable bioisostere might be sought (see Section 3.1.3) or else metabolism might be blocked by introduction of steric hindrance, substitution of a sensitive site or decrease of chemical reactivity.

Metabolic reactions generally occur with more lipophilic compounds; in particular oxidation which lowers lipophilicity and favours subsequent renal excretion of the metabolite, and oxidation followed by conjugation which can give higher molecular weight, ionic materials which will undergo subsequent biliary excretion. The key for drug design is to avoid the high lipophilicity which clearly enhances both liver microsomal localization of drugs, and their binding to a variety of metabolizing enzymes. Positive correlations between lipophilicity (as log P or log D) and metabolic transformations have frequently been observed, and have been reviewed by Austel and Kutter (1983) and by Seydel and Schaper (1986). A particularly revealing study of β-blocking drugs was made by Drayer (1984). Following oral dosage to humans, Drayer found that the first pass effect (metabolic clearance by the liver) was negligible for log P values <1, but thereafter increased markedly in parallel with increasing log P. Recall here the "Principle of Minimal Hydrophobicity in Drug Design" set forth by Hansch *et al.* (1987).

There is now a vast literature on metabolic reactions and their substrate specificity that can be of help in making an informed choice of metabolically

stable groups (Armstrong, 1988; Caldwell and Mitchell, 1990). Unfortunately, little is yet known about the 3D structures of the enzymes concerned. Among these, the central role of the cytochromes P450 continues to attract much research effort. Crystal structures of the free, substrate (camphor)-occupied, and inhibitor-bound bacterial cytochrome P450cam have been published (Poulos *et al.*, 1986, 1987; Poulos and Howard, 1987) and have provided the structural framework for building models of human isoenzymes (Gotoh, 1992). Kinetic studies and computer modelling are gradually revealing substrate SARs for both P450cam (Fruetel *et al.*, 1992) and for human P450s (Smith and Jones, 1992); see also the review by Koymans *et al.*, 1993a).

Until our knowledge is more complete, design for stability will continue to rely upon a semi-empirical choice of metabolically stable fragments such as halogenated aromatics, $-CF_3$, $-CF_2-$, and replacement of groups such as imidazole by a stable bioisostere such as 1,2,4-triazole as used in the design of the systemic antifungal drug fluconazole (**63**).



(63)

Fluconazole was designed following much earlier work on imidazole derivatives, which had been shown to inhibit a crucial step in biosynthesis of ergosterol, the essential sterol of the fungal membrane. Imidazoles were shown to be poorly effective *in vivo* because of rapid and extensive metabolism. Note the design features:

(1) Replacement of imidazole by triazole removes a metabolic site (triazole is more stable towards electrophilic attack).
(2) The symmetrical use of a second triazole removes an asymmetric centre, so obviating the need for resolution. Triazole and hydroxyl groups keep overall lipophilicity low. The hydroxyl is sterically hindered, hence does not undergo conjugation.
(3) Use of difluorophenyl rather than, for example, dichlorophenyl or other substituted phenyl, provides metabolic stability and keeps overall lipophilicity within bounds.

The result is a neutral molecule of moderate lipophilicity (log P of 0.5) which is well absorbed orally, has a plasma half-life of 25 h (allowing once-a-day dosing), giving high blood levels, low protein binding and recovery of unchanged drug in the urine (Humphrey *et al.*, 1985; Feczko, 1992).

3.1.9 QSAR

Successful development of a drug, from choice of the lead structure through preparation and testing of derivatives and bioisosteres to selection of the clinical candidate, requires that properties be optimized for potency, selectivity and delivery.

Optimization needs to be tempered by compromise. The most potent structure *in vitro* may be too polar, or too lipophilic, for delivery to the target *in vivo*. Compromise and drug selection may be achieved by developing an SAR for each desirable attribute.

The development of a useful QSAR from physical, chemical and biological data requires that the data are assembled, examined, correlated and re-examined many times in an effort to find relating characteristics and express these in a correlation equation. A good correlation can lead directly to an explanation of biological activity changes in a series of molecules, expressed in physicochemical or structural terms. This is especially valuable if the receptor has not been characterized by direct methods. Steps in generating a QSAR might well be:

(1) Assemble the data.
    Choose the "training set".
      Make omissions.
(2) Consider likely models.
    Choose descriptors.
      Make assumptions.
(3) Derive the QSAR.
    Choose the appropriate statistical method.
      Make predictions.
(4) Test and refine the QSAR.
    Choose alternative data sets and/or descriptors.
      Make decisions.

All the above steps of QSAR generation depend on the quality and quantity of the data, which will grow in volume and change in precision as the investigation proceeds. At the outset, choice of "training set" is of importance. Rather than simply including those compounds that are already available or readily synthesized, attention should be given to designing a test series that possesses maximum variance for all activity descriptors (indepen-

dent variables). Moreover, in order to separate the effects of, say, lipophilicity and size of a substituent on potency, the parameters chosen to represent these properties should not vary in a parallel manner (they must be orthogonal). Design of a test series presents a challenge for the chemist, who may need to prepare some unusual derivatives, but will pay handsome dividends when the time comes to interpret the results (Pleiss and Unger, 1990).

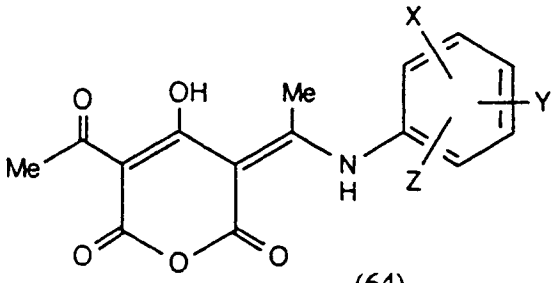
Although it is desirable to include as many data (compounds) as possible in developing a model, it is wise initially to omit compounds with unique characteristics, e.g. the one acid in a set of basic compounds, an insoluble derivative, or an unstable or readily metabolized analogue. Such compounds will be recognizable as outliers once a robust equation has been generated, but until then their inclusion might obscure a relationship.

There are two schools of thought in model building. One method is to search for correlations amongst a vast array of physicochemical and structural variables, with no preconceived notion of mechanism. This approach avoids bias and may detect an unexpected relationship, but there is a danger of finding chance relationships that can be misleading (Topliss and Edwards, 1979). The alternative method is to suggest a physical model and make a choice of appropriate descriptors to test that model.

A physical model usually predisposes to physicochemical descriptors, such as pK_a, log P or molar volume for the whole molecule, or the equivalent descriptors for substituents on a common molecular framework. But different structures can have the same or similar property values, and we are interested in designing *structures*. So at some stage we must choose structural descriptors (atom types, substructural fragments, connections, or indices from molecular orbital calculations) or at least relate structure to property in order to design the appropriate *structure*.

A physical model involves the assumption that all compounds have a common response-determining step, and commonly also assumes that metabolic changes are not occurring.

To derive the QSAR, an appropriate statistical technique must be chosen. The choice will depend on the quality and the quantity of the data. Multiple linear regression analysis has been the preferred tool, and has the huge advantage of providing a correlation equation that is readily interpretable in terms of a mechanism. But, in cases where data include inactive compounds, and when activity measurements are qualitative rather than quantitative, then a discriminant analysis or pattern recognition technique must be chosen. Such techniques have been much used for treating toxicity data; they result in relationships that can be used predictively but offer little mechanistic insight (Benigni *et al.*, 1989). For very large data sets and variables with high covariance, a principal components (PC) or partial least squares (PLS) procedure may be most suitable (Ståhle and Wold, 1988; Cramer *et al.*, 1988a). For the 3D QSAR method of CoMFA, the molecules



(64)

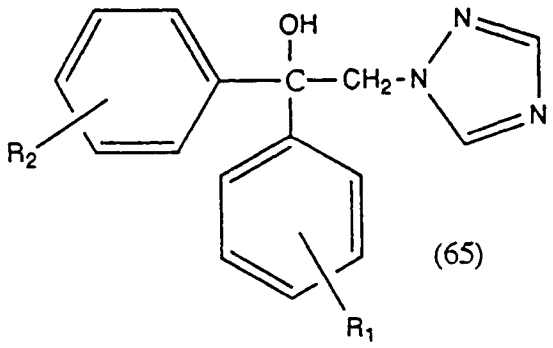
in a series are normally represented by their steric and electrostatic fields, generated by a probe technique such as GRID (Section 3.3.1). The descriptors are steric and electrostatic potentials at points on a 3D lattice within which the molecules are aligned. Other descriptors, such as molecular lipophilicity potential (Gaillard *et al.*, 1994), may also be considered, and a vast amount of data must be analysed for the selection of those potentials which account for variation in activity. The PLS method is used, together with cross-validation to maximize the likelihood that results have predictive validity (Cramer *et al.*, 1988b; Bush and Nachbar, 1993).

The ultimate test of a QSAR is of course to make predictions and verify them experimentally. More compounds will thereby be prepared, the data set will be expanded, the QSAR will be refined and its limitations found. No QSAR "goes on forever", e.g. a linear relationship between receptor binding and lipophilicity (as log *P*) will break down when the molecule either becomes too big to fit within the receptor site, or too insoluble for delivery.

Decisions can be made on the basis of QSARs for progression of a particular compound into safety evaluation and clinical trial.

Cramer *et al.* (1979) used QSAR to develop a series of anti-allergic pyranenamines (**64**). For early members of the series a graphical representation of the variation in potency with hydrophobicity (π) and electronic effects (σ) was used to guide further synthesis towards compounds of lower hydrophobicity and negligible electronic influence. A rather simple linear equation in π and σ was constructed for the first 19 derivatives, but some outliers (considerably more active than predicted) were noticed. These observations were followed up by further synthesis and a new QSAR discovered, incorporating additional descriptors for hydrogen bonding and volume effects, to fit 98 compounds. By following the trends predicted in these equations, *a potency enhancement of 4 orders of magnitude was obtained* in going from the primary set to the most potent compound synthesized!

The compound selected for clinical trial from this final set was not in fact



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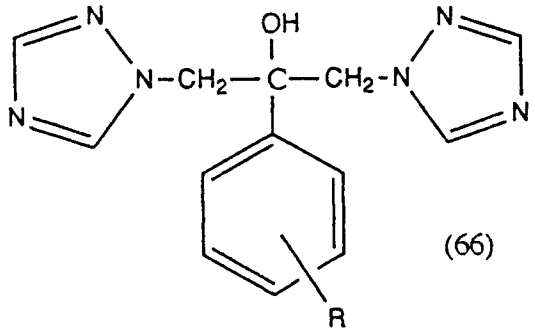


(66)

the most potent 3,5-[NHCO(CHOH)$_2$H] derivative but rather the 3-NH$_2$,4-OH compound; still potent, but a better choice for oral administration.

Klopman and Ptchelintsev (1993) have used structural descriptors to analyse the antifungal activity and the toxicity (teratogenicity) of a set of 71 mono- (**65**) and bis- (**66**) triazole alcohols. The Multi-CASE (Multiple Computer-Automated Structure Evaluation) method was used for data analysis. The program automatically generates structure descriptors, which are evaluated for their ability to discriminate between classes of activity (or toxicity), including inactive or non-toxic compounds. A therapeutic index was calculated for all members of the training set, as the ratio of teratogenic to antifungal potency measurements.

The program was able to predict, correctly, the teratogenicity and antifungal potency of some key compounds not included in the training set. *In particular, the program correctly predicted fluconazole (**63**, see Section 3.1.8), now marketed as an antifungal, to be one of the most potent and safe antifungals of the triazole class.*

Nine examples of the successful application of property-based QSARs to drug design have been discussed by Fujita (1990). In three of these examples, compounds selected on the basis of QSARs have been marketed.

3.2 CHARACTERIZATION OF THE RECEPTOR

Section 3.1 has outlined traditional methods of drug design which are largely independent of knowledge concerning the structure of the receptor itself, or the receptor macromolecule.

Over the last decade, drug design has switched dramatically towards the determination and use of knowledge concerning the receptor itself. The first attempts to make use of receptor knowledge in this way were made by Beddell *et al.* (1976, 1984) and Abraham *et al.* (1984). These two groups made use of the crystal structure of human haemoglobin. Compounds were designed and fitted to the protein, using molecular modelling techniques, in the region of a known binding site for the cofactor 2,3-diphosphoglycerate, which regulates oxygen binding by causing a conformational change. Highly active, potential antisickling agents were thus designed, based on knowledge of the structure and function of haemoglobin.

Once a receptor site is characterized structurally (and in the case of enzyme active sites, mechanistically), such information can be used for the *de novo* design of a lead compound (Section 3.3).

When mechanism is understood, mechanism-based inhibitors such as transition state analogues and suicide inhibitors (Section 3.4) may also be designed. Recent determination of the crystal structure of a complex of penicillin G with a deacylation-defective mutant β-lactamase from *E. coli* shows how such antibiotics are recognized *and how they are destroyed* (Strynadka *et al.*, 1992).

Advances in protein crystallography, NMR, biotechnology and in computational methods have opened up this approach, which has been called structure-based drug design. It depends upon the determination of macromolecular structures and their liganded complexes to atomic accuracy. Experimental data are now available from X-ray, neutron diffraction and NMR for over 800 protein structures (Thornton, 1992) and for many nucleic acids. Many of these data are available from the Brookhaven Protein Database (Bernstein *et al.*, 1977) which currently contains nearly 2000 coordinate sets for proteins and other biological macromolecules. Protein targets for structure-based drug design have been reviewed by Walkinshaw (1992) and Navia and Murcko (1992). The reviews deal with proteins (and some of their ligands) that are of functional importance in immunology, endocrinology, disease states such as cancer, disorders of blood and circulation, genetic disorders, inflammatory and respiratory disease, and infection.

3.2.1 Crystallographic Methods

Crystal structures solved by X-ray or neutron diffraction provide a static picture of the receptor macromolecule, and of uncomplexed and liganded

receptor sites. It is important to study both free and liganded forms of active site, since a considerable conformational change can sometimes occur on binding, and may be of functional significance. To study a liganded form, potential ligands should have solubilities high enough to allow for stoichiometric binding to the high protein concentrations required for preparation of the crystals. Provided suitable crystals can be grown—and this is still the main limitation—protein crystallography can provide structures for any size of molecule. Frequently, the proteins have been obtained in sufficient quantity and purity for crystallization and analysis only by using some recent advances in biotechnology, in particular cloning and mutation techniques (Erickson and Fesik, 1992).

A most compelling example of the use of crystallographic methods concerns the development of drugs to treat the human immunodeficiency virus (HIV), the causative agent of AIDS. An HIV-specific aspartyl protease, necessary for virus maturation, has been the subject of intense study over the last 5 years. Its structure was solved simultaneously by industrial (Navia *et al.*, 1989) and academic groups using synthetic (Wlodawer *et al.*, 1989) and cloned (Lapatto *et al.*, 1989) protein. The first crystal structure of a complex of the protease with a substrate-based inhibitor was solved shortly afterwards (Miller *et al.*, 1989) and revealed details of a large conformational shift in the protein on binding. The protein is a symmetrical dimer, each half contributing one of the necessary two catalytic aspartic acid residues. A so-called "flap" is formed from a contiguous sequence of residues in each half, and these two flaps move to embrace the substrate (and inhibitors) when binding takes place. This mechanism has been confirmed by examination of more than 120 different HIV protease/inhibitor complexes in at least 17 laboratories. Many compounds have been designed and synthesized, and some have entered clinical trials, on the basis of analysis of these crystal structures (Wlodawer, 1992; Greer *et al.*, 1994).

Erickson *et al.* (1990) designed a symmetric inhibitor, based on the two-fold (C_2) symmetry of the protease active site. This substrate analogue, designated A-74704, proved to be both highly potent (with a K_i of 4.5 nM) and highly selective (selectivity ratio of 10^4 over other aspartyl proteases). Figure 6 shows in diagrammatic form the multiple hydrogen bond contacts between inhibitor and enzyme, including two hydrogen bonds made through a bound water molecule. The benzyl and isopropyl side chains of the inhibitor bind respectively in hydrophobic S1 and S2 (and S1′ and S2′) subsites.

The buried water molecule noticed in the crystal structure of this and many other inhibitor complexes is tetrahedrally coordinated to both the inhibitor and the "flaps" of the enzyme. Randad *et al.* (1993) have used molecular modelling based on the crystal structures to "incorporate" the water oxygen atom as the carbonyl oxygen of novel, cyclic ureas (**67**). They reasoned that displacement of the water should be energetically favourable

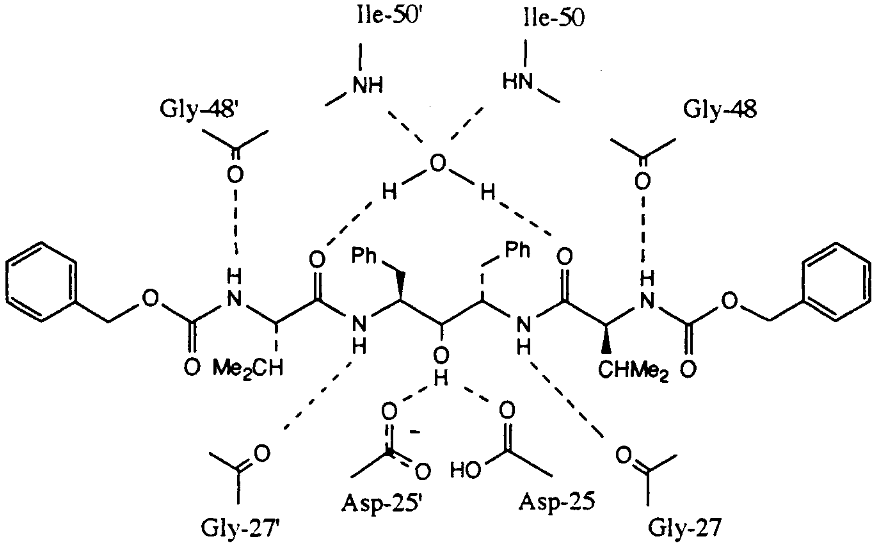
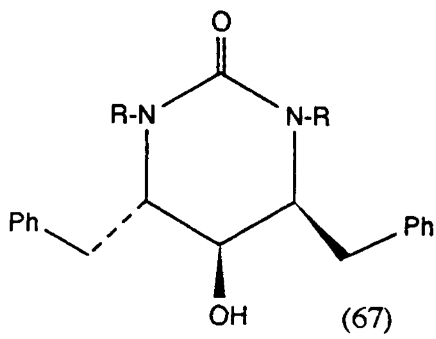


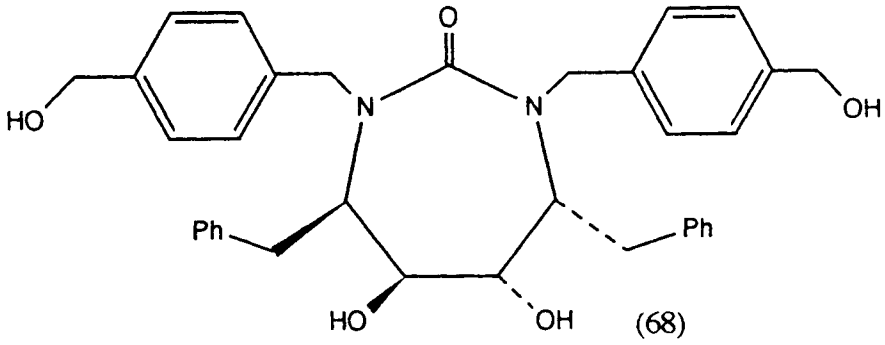
FIG. 6. A symmetrical inhibitor designed to fit the symmetrical HIV protease active site.



(67)

(Timasheff, 1992) for these structures, which can now bind directly between aspartic acids and the flaps, and moreover conversion of a flexible, linear inhibitor into a rigid, cyclic structure with its restriction in conformational freedom would provide an entropic advantage (see Section 2.1).

Lam *et al.* (1994) have also used this trick of both mimicking and displacing the water molecule. They designed a series of 7-membered ring cyclic ureas, including **68**, which is a potent enzyme (K_i = 0.27 nM) and virus inhibitor, and also has significant oral bioavailability. This compound is currently under clinical investigation. Crystal structures of 10 of these cyclic urea complexes with protease have been determined, and show that, as the



(68)

modelling predicted, the inhibitor links the catalytic aspartic acids to the flaps via a hydrogen bond network that does not include an intervening water molecule.

Abbott, Roche and Merck Laboratories now have protease inhibitors in clinical trials against AIDS, all spawned by structure-based design. Other companies with clinical trials candidates are Agouron Pharmaceuticals (targeting thymidylate synthase, for treatment of cancer and psoriasis), Biogen (targeting thrombin for anticoagulant therapy) and Sterling Winthrop (targeting the rhinovirus coat protein to treat the common cold). BioCryst is one of several new companies whose resources are directed totally to structure-based design. BioCryst has designed inhibitors of purine nucleoside phosphorylase as potential drugs for treatment of arthritis, psoriasis and cancer (Ealick *et al.*, 1991; Bugg *et al.*, 1993).

3.2.2 NMR Methods

Apart from crystallography, NMR is the only technique which can provide the atomic resolution needed for structure-based drug design. Moreover, NMR in solution may afford data more relevant to the biophase.

The investigation of protein (and receptor) structure, ligand–receptor binding, and differences between the conformation of an unbound, but solvated, and receptor-bound ligand by NMR needs an amalgamation of the skills of molecular biologists, spectroscopists and computational chemists.

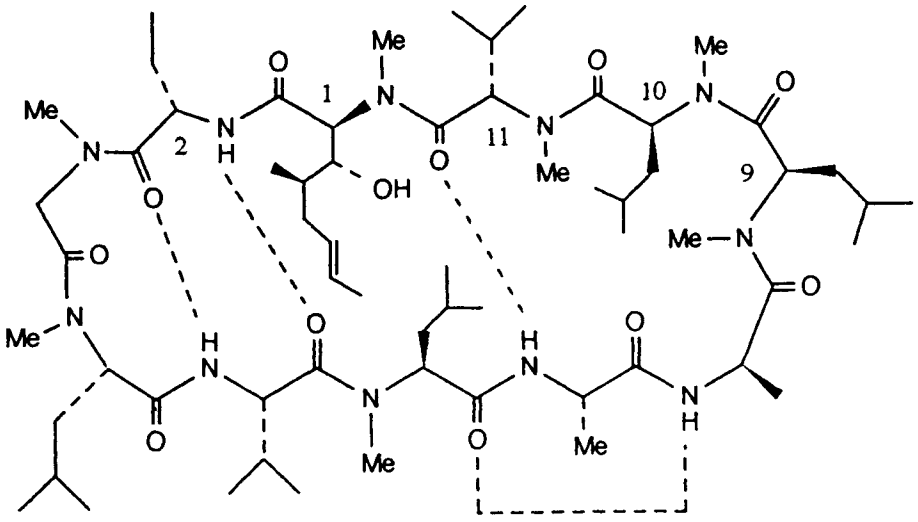
The molecular biologist may be called upon to prepare a suitable quantity of protein for the investigation, by cloning and expression. If the protein is expressed in bacteria, then by growing the bacteria in a medium containing ^{13}C-glucose or ^{15}N-ammonium chloride the expressed protein may be enriched in ^{13}C or ^{15}N. The large heteronuclear couplings observed from isotopically enriched proteins are needed to resolve peaks which overlap in a 2D spectrum.

With the introduction of high field (>400 MHz) spectrometers, and the advent of a whole array of NMR techniques, it has become possible to obtain all resonance assignments as well as a very large number of inter-proton distances, to be used as constraints in the subsequent structure determination. Peptide NMR has been reviewed by Williamson and Waltho (1992), protein NMR by Erickson and Fesik (1992), and Marshall (1992) has given an account of the 3D structure of peptide–protein complexes determined by both crystallographic and NMR techniques. Marshall has stressed the importance of having the relevant environment for determination of the receptor-bound conformation of the ligand, upon which information drug design will depend. The conformation of unbound ligand in solution, or ligand in the crystal state, frequently differs from the conformation bound to the receptor (Jorgensen, 1991). Modern NMR techniques allow the determination of this conformation.

For weakly bound ligands (exchanging rapidly) the transferred NOEs due to interaction between ligand and receptor can be measured. For strongly bound ligands, isotopic labelling of the ligand will allow recognition of signals due only to the ligand and not to the receptor. Both techniques give distance constraints that can be used to calculate the bound conformation. Heteronuclear NMR techniques can also distinguish between binding and non-binding regions of a ligand; crucial information for drug design. It is the non-binding regions of the ligand which should be modified to provide pharmacokinetic advantages, whilst binding regions should be retained in a rigid analogue or bioisostere.

The final stage in determining structure from NMR involves computing. The measurements, primarily NOE but also J-coupling and NH exchange, provide distance constraints which must be used to calculate a consistent time-averaged solution structure, or family of structures. With peptides, it is only rarely that a single, "rigid" conformation will exist or be preferentially populated. For proteins, major problems are caused by the large amount of data, and by the relative mobility of solvent-exposed residues.

The two methods used to calculate structures from distance constraints are distance geometry (programs such as DISGEO, DSPACE, DISMAN, DIANA, DGEOM) and restrained molecular dynamics (programs such as CHARMm, XPLOR, AMBER, SYBYL, GROMOS). Distance geometry uses covalent constraints (bond lengths and angles) as well as NMR-derived constraints to provide starting structures, which are then refined using molecular dynamics. A molecular dynamics simulation can take many forms, and may or may not include solvent effects. Usually, so-called simulated annealing is performed, in which the starting structure is heated, equilibrated (with application of the NMR distance constraints), then cooled and finally energy minimized. Currently there are many different protocols in use for final refinement of the structure. These arise from differences in availability of software, differences in the way electrostatic interactions and



(69)

solvation are handled, and differences in expertise of the investigator. Consequently, NMR-derived structures must continue to be treated with caution.

A most persuasive example of the value of NMR investigations concerns the determination of free and bound conformations of the immunosuppressant cyclosporin A (**69**). This drug binds to a 165-residue cytoplasmic protein, cyclophilin A, and thereby blocks a signal transduction pathway leading to T-lymphocyte activation (Schreiber, 1991; Walsh *et al.*, 1992). Both X-ray and NMR investigations (in apolar solvents) of uncomplexed cyclosporin A show the existence of one *cis* peptide bond and four intramolecular hydrogen bonds from NH to O=C (Van Duyne *et al.*, 1991). Two groups (Weber *et al.*, 1991; Fesik *et al.*, 1991) have used ^{13}C-resolved NOE measurements followed by distance geometry and molecular dynamics protocols to study the drug–protein complex. They were able to identify those parts of cyclosporin A that bind to cyclophilin A (residues 1, 2, 9, 10, 11) and to determine the bound conformation. It transpires that the unbound structure is turned inside out in the complex, with loss of all four intramolecular hydrogen bonds, isomerization of the 9,10-peptide bond from *cis* to *trans* and exposure of nearly all polar groups to the aqueous environment. The bound state has now also been studied by X-ray crystallography of the complex (Mikol *et al.*, 1993), which finds a similar structure to that deduced from NMR.

It has been suggested that the conformation of cyclosporin A observed in

the bound state is induced by the solvent, i.e. it pre-exists in aqueous solution, and is not therefore induced by binding to the receptor. Evidence that this is indeed the case has come from the measurement of partition coefficients and from molecular dynamics simulations (El Tayar *et al.*, 1993).

NMR investigations have also been made on the complex of cyclosporin A with another cyclophilin, cyclophilin B, which is highly homologous to cyclophilin A. The only difference between the complexes exists in the vicinity of residue 11, suggesting that selectivity could be achieved in a derivative of cyclophilin A by modification at just this position (Erickson and Fesik, 1992).

3.2.3 Homology Modelling

In the absence of crystallographic or NMR data, predictive techniques based on protein primary sequences can be used to elaborate crude 3D models. Such models will suggest that certain amino acid residues are involved in forming the active (receptor) site. The assignment of structural or functional roles to particular residues can be tested by site-directed mutagenesis, and the model can be further refined by consideration of SAR among ligands.

The number of known protein sequences is expanding exponentially; currently at about 26 000, it is expected to reach 100 000 by the turn of the century (Rost and Sander, 1993), contrasting sharply with the number of proteins whose 3D structure is known at the tertiary level of organization, and consequently the number of receptor sites known at atomic resolution.

The most successful approach to predicting secondary and tertiary structure of proteins from their sequences is homology modelling. Preferably, the sequence in the unknown structure is matched to a similar sequence in another protein whose 3D structure is known. The match will not be exact, for the sequences will be of different lengths, and highly homologous regions or exactly matching pairs will be separated by different numbers of amino acids. Where the unknown protein is one of a family of proteins with a common function, prediction can be made at better than 70% accuracy by use of multiple sequence alignments (Subbiah and Harrison, 1989; Rost and Sander, 1993). In order to "convert" the known structure to a 3D model of the new sequence there will in general be four steps in the modelling process.

(1) Match obvious catalytic or structural residues, hydrophobic regions, glycine and proline residues. Modify (mutate) residues accordingly.
(2) Make deletions to enable matching of other residues. The deletions must not be in regions predicted to form helix or sheet structural motifs.
(3) Make insertions of unmatched sequences of residues, which will then comprise turns and loops between helix or sheet regions. The particular

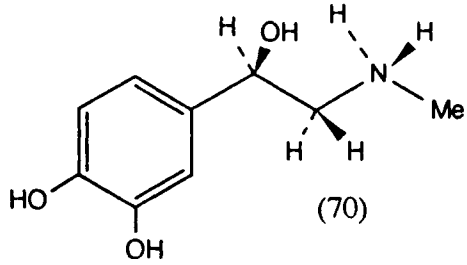
problems of modelling loops in homologous structures, and particularly in antibody molecules, have been addressed by Thornton (1990).

(4) Refine the structure by use of constrained molecular dynamics and molecular mechanics routines.

Homology modelling is not an exact technique. Especially, when the extent of sequence homology (exact matches and matches between amino acid residues of similar property, e.g. hydrophobic, polar, acidic, basic) is low, then more attention will be paid to structural rather than sequence similarities and to prediction of structure for unmatched sequences. In such cases, and always when there is no crystal structure of a member of the family to provide a template, then total reliance has to be placed on the experience of the investigator or in one of the many computer programs now available. The principal methods have been reviewed by Sternberg (1986) and Blundell *et al.* (1987a).

Crude 3D models of several GPCRs have now been constructed, using homology methods based on bacteriorhodopsin as template (Hibert *et al.*, 1991; Trumpp-Kallmeyer *et al.*, 1992; Humblet and Mirzadegan, 1992). For example, a model of the β_2-adrenergic receptor has been constructed by Maloney-Huss and Lybrand (1992), using the entire sequence from the hamster. The model is consistent with results of site-directed mutagenesis experiments, and affords an explanation of the stereoselectivity of adrenaline: the naturally occurring *R*-isomer (**70**) can be positioned in the model such that the protonated amine forms an ionic interaction with Asp-113, and the aromatic hydroxyls form hydrogen bonds with Ser-204 and Ser-207 (these serines are conserved in GPCRs that bind catecholamines, and mutation of either reduces agonist activity). The catechol ring stacks between Trp-286 and Phe-290, and the β-OH group hydrogen bonds to Ser-319.

In a similar vein, homology modelling has been extensively used to derive 3D active site models for several cytochromes of the extensive P450 family, based on the high-resolution crystal structure of the bacterial P450cam (P-450-101) as template (Lewis and Moereels, 1992; Koymans *et al.*, 1993b). These models have been used to explain substrate specificity.



(70)

Although homology-based models have been used to explain SAR, there is still a long way to go before such models can confidently be constructed to the precision required for *de novo* drug design. Principally, this is because of the current difficulty in predicting tertiary structure, and in allowing for changes in receptor conformation that sometimes accompany ligand binding. Homology modelling of HIV protease, for example, was done before X-ray crystallography had been accomplished: subsequent comparisons of the model and the crystal structure show very accurate reproduction of several functionally significant regions, but these did not include the flaps, extensive domains which fold down upon substrates and inhibitors to complete binding via a crucial water molecule (Weber, 1990).

3.2.4 Antibody-directed Design

Many known drug receptors, and many prospective drug targets, exist as molecular arrays within membrane-bound macromolecules that cannot be readily crystallized; neither can they be isolated or purified for the application of NMR methods. Moreover, even if an amino acid sequence were available, rule-based methods for the prediction of secondary structure, being derived as they are from a database of soluble proteins, cannot be applied with any confidence to the membrane-bound state.

The techniques of crystallography, NMR and homology modelling can however be applied to antibodies and to antibody fragments. Through monoclonal technology, idiotypic antibodies (Ab-1) to receptor sites can be selected and produced without the need to isolate the target. Through the use of these antibodies, various kinds of receptor can be recognized, isolated, purified, quantified and biochemically characterized (Tung, 1981). Many drugs and toxins have been coupled with monoclonal antibodies to confer target specificity in the quest for antitumour agents, but in spite of impressive activity of such compounds in experimental animals, they have not so far shown clinical utility (Upeslacis and Hinman, 1988; Hinman and Yarranton, 1993).

Antibody-directed drug design is a novel concept that has as its starting-point the selection of an antibody to the receptor macromolecule that in itself has functional (drug-like, agonist or antagonist) activity. Such an antibody can be considered to carry a mirror image (or footprint) of the target receptor both in a spatial and in an electrostatic sense. Moreover, by using Ab-1 as antigen, an anti-idiotypic antibody (Ab-2) can be raised as a positive image. This *might* incorporate a bioisostere of the original antigen (receptor). That this hope will not always be realized was shown by X-ray analysis of a complex of an antilysozyme antigen-binding fragment (F_{ab}) with its anti-idiotope F_{ab} by Bentely *et al.* (1989). As discussed by Perutz (1992), there is a topological resemblance, but no chemical resemblance,

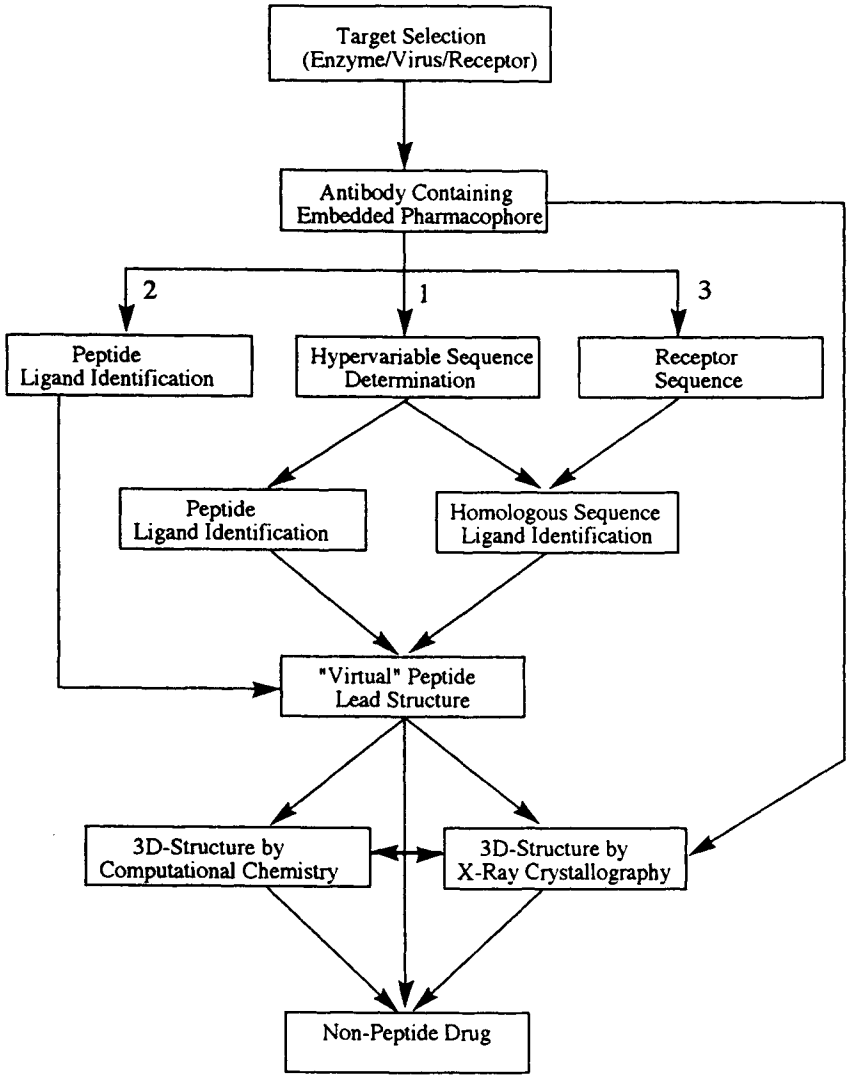


FIG. 7. Antibody-directed drug design methods. Reprinted with permission from Wolff and Maggio (1991).

between the domains of the original lysozyme, and the domain of the anti-idiotope in contact with the antilysozyme F_{ab}. Topological complementarity of lysozyme and of the anti-idiotope to the lysozyme anti-body have in this case been achieved in quite different ways.

Figure 7, adapted from a forward-looking review by Wolff and Maggio

(1991), illustrates how the information embedded in Ab-1 (or possibly in Ab-2) can be extracted and used in drug design.

In addition to the direct information obtainable from X-ray crystallography on Ab-1, Ab-2, their fragments and complexes, three other techniques may be used. Referring to Fig. 7, these are as follows.

(1) The amino acid sequence of the hypervariable loop is determined, and homology modelling then used to find a plausible secondary structure (Martin *et al.*, 1989).
(2) Peptides complementary to the surface of Ab-2 (which acts as a receptor bioisostere) are selected from a peptide library (Lam *et al.*, 1991; Houghten *et al.*, 1991) using molecular immunology detection methods. Such peptides are expected to be receptor antagonists or agonists (Scott and Smith, 1990).
(3) Key amino acids in the hypervariable loops of Ab-2 are found by homology matching with sequences in the original macromolecular antigen (Williams *et al.*, 1988).

Either directly (2) or indirectly (1 or 3) peptides will be selected as ligands having affinity for the receptor. The biologically active (receptor binding) conformation of the peptide must now be determined through X-ray, NMR or computational methods. Finally, it is desirable that a non-peptide surrogate be designed.

Structure-based design through the use of monoclonal antibodies to reflect receptor structure is in its infancy, but is a most exciting method which has the potential to provide both potent and, especially, selective drug entities. It will require the combined skills of molecular biology, protein crystallography and computational chemistry and much good fortune in obtaining suitable antibodies.

3.3 PROBING THE RECEPTOR

A 3D picture of the receptor protein, from crystallography, NMR or modelling, can be used to design a potent (high affinity) ligand.

First attempts to tailor-make ligands to macromolecules relied on physical (ball and stick or wire) models laboriously constructed from atom coordinate data. Such an approach was successfully applied by Beddell *et al.* (1976) to a binding site on human haemoglobin. By inspection of the model and application of chemical intuition, complementary ligands were imagined, built and manually docked. Though physical models are still useful, they have been largely replaced by the electronic models that can be called from a database, or built, and subsequently modified and examined at a computer graphics workstation.

3.3.1 Computer Graphics and Design

A physical model is capable only of representing atomic connections and giving a crude appreciation of the shape and accessibility of a binding site. Through computer graphics, shape and shape complementarity to potential ligands can be assessed by calculation and display either of the van der Waals surface or of a water-accessible surface. Electrostatic, hydrophobic and hydrogen bonding potential can also be calculated and added to the display. One particularly useful technique is to map electrostatic potential onto a molecular surface; another is to use colour coding to distinguish polar and hydrophobic regions.

Goodford (1985) introduced the first and still the most frequently used quantitative technique to examine the environment of a protein, in order that putative binding sites may be identified and characterized. The program GRID first assigns appropriate van der Waals radii and partial charges to all atoms. Next, small fragment "probes" are placed are regularly spaced grid points throughout any desired region (such as a known active site) to determine, through molecular mechanics principles, an interaction potential. By choosing the appropriate probe, likely interactions with charged, neutral, hydrogen bond acceptor or donor groups, and water molecules can be determined in position and strength. GRID has been extensively tested on a variety of enzyme–inhibitor complexes and solvated proteins, and has been found to reproduce accurately the positions of liganding groups and water molecules (Boobbyer *et al.*, 1989). Related programs are HSITE (Danziger and Dean, 1989) which focuses on hydrogen bonding sites, and HINT (Kellogg *et al.*, 1991) which has been discussed in Section 2.1.3 and which reproduces hydrophobic binding potential with a small probe, or hydrophobic binding between the macromolecule and a potential ligand.

The GRID program has been influential in the design of very potent inhibitors of influenza virus neuraminidase (von Itzstein *et al.*, 1993; Taylor and von Itzstein, 1994). It was proposed over 30 years ago that inhibitors of neuraminidase might be useful as antiviral drugs against influenza, through interference with the functions of neuraminidase (which are to aid transport of virus through the mucosal lining of the upper respiratory tract, and to aid release of virus from infected cells). Neuraminidase splits *N*-acetylneuraminic acid (NANA) from glycoconjugates on the cell surface, and so removes a recognition and binding site for another influenza coat protein, haemagglutinin. The mechanism of glycosidic cleavage, illustrated in Fig. 8, is of SN1 type, involving an intermediate endocyclic cation which has planar geometry about C2 (Chong *et al.*, 1992).

The 2-deoxy derivative of NANA, known as DANA (**71**), was synthesized by Meindl and Tuppy (1969) and shown to be a reasonable inhibitor of the enzyme ($K_i \sim 10^{-5}$ M) by virtue of being a transition state analogue (Section 3.4.1). Although DANA is not sufficiently potent to show antiviral activity

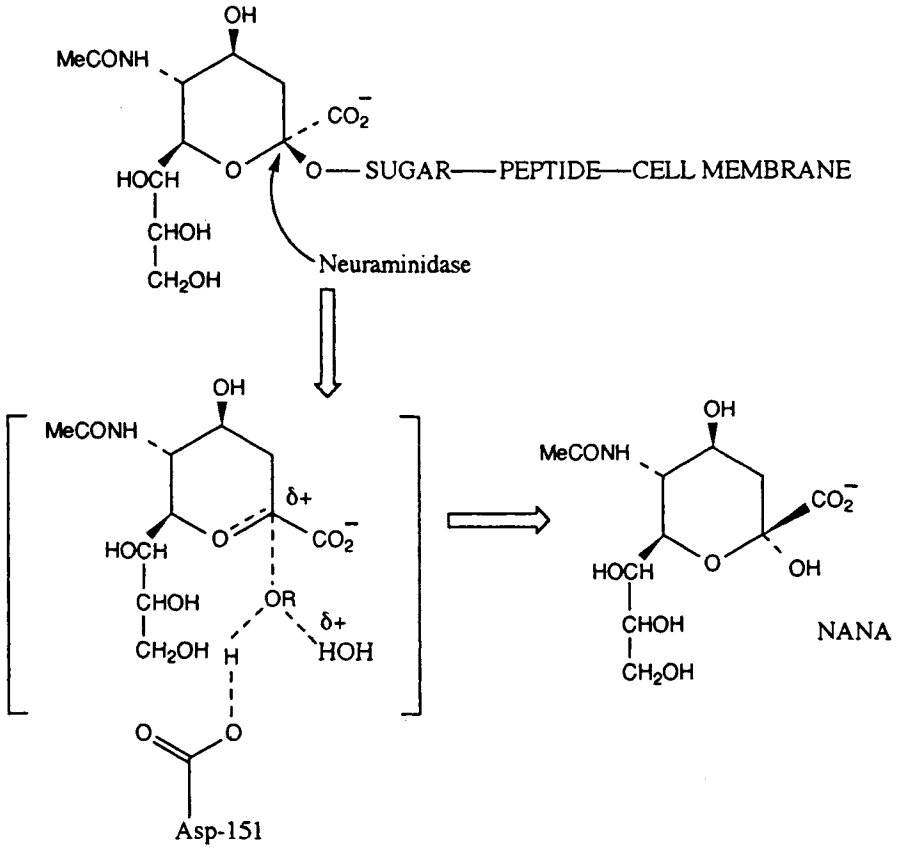
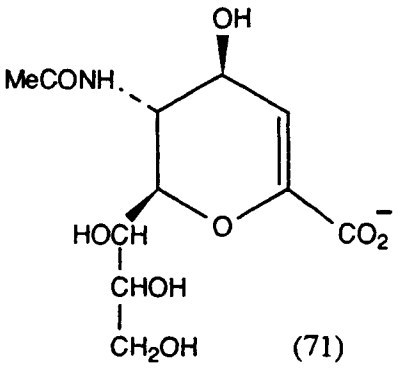


FIG. 8. Glycosidic bond cleavage by neuraminidase passes through a cationic intermediate with planar geometry about C2. Reprinted with permission from Chong *et al.* (1992).

in vivo, it did prove to be an invaluable lead compound in the search for more potent analogues. A crystallographic analysis of the binding of NANA (Varghese *et al.*, 1992) and of DANA (Bossart-Whitaker *et al.*, 1993) to influenza neuraminidase revealed an empty pocket near the 4-hydroxyl group of the sugar ring. Using GRID calculations with a variety of probes, Von Itzstein *et al.* (1993) found that a protonated primary amine probe identified a strong binding site within this pocket. It followed from this that replacement of the 4-hydroxyl by a basic (hence protonated) amino function should produce an increase in binding, and so the 4-amino and 4-guanidino (**72**) analogues were synthesized. The latter proved especially potent, with K_i of 2×10^{-10} M against the enzyme. Compound **72** also proved to be an effective inhibitor of influenza in mice and ferrets, but only by intranasal



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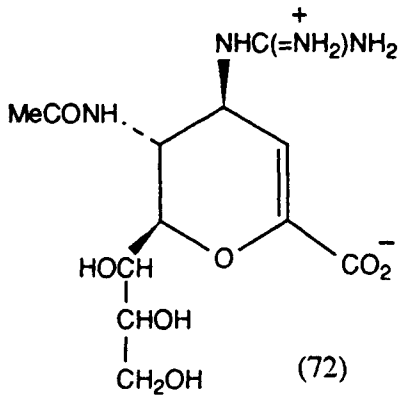


(72)

administration. It is not surprising that the compound was not effective following intraperitoneal administration; such a polar compound would undoubtedly be very rapidly eliminated from the system. Nevertheless, **72** represents a very significant advance in the eventual design of an effective antiviral drug.

3.3.2 Ligands Built by Computer

An intermediate goal of structure-based drug design is to suggest ligands which may bind strongly to the receptor sites revealed by, for example, crystallography. To this end, computational tools have been developed which can analyse the active site and then automatically screen a database of potential ligands for goodness of fit, or else "grow" a potential ligand within the site.

Programs such as GRID will suggest "hot spots" within a protein, where appropriate probe atoms would experience a strong attraction. To convert an array of attracting, probe atoms into a viable ligand raises enormous problems, e.g. what connecting fragments to use, how to control the connection geometry so that favourable interactions are maintained, and how to avoid steric clashes within the site while making the connections.

Over the last 5 years, impressive progress has been made, and many programs have been described, as summarized in Table 5.

Two main approaches have been followed. One is to search structural databases for molecules, or fragments, which satisfy minimal steric and electrostatic criteria; the other is to construct new molecules in the site, from a "seed" atom or fragment, satisfying the binding criteria as the ligand "grows". Typical of the first approach are the programs BUILDER, ALADDIN and DOCK. Using the DOCK program to search 10 000 structures from the Cambridge Crystallographic Structure Database (CSD),

TABLE 5

Programs for *de novo* ligand design based on receptor structure

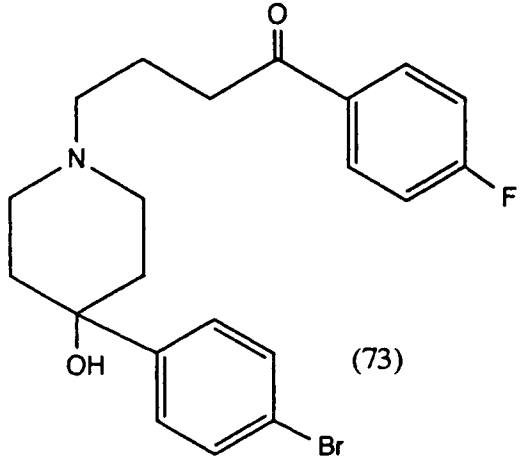
Program	Method	Requirements	Features	Validation	Citations
DOCK	Sphere-matching algorithm. Systematic search for conformational, preferences	User-supplied database or CS Database may be used	Identifies complementary steric and H-bonding features	Orientation of methotrexate to DHFR and netropsin to DNA	Kuntz *et al.* (1982), Leach and Kuntz (1992)
ALADDIN	Matching to a 3D pharmacophore	User supplied database GENIE language	Fast computer search of database	Finds known dopamine agonists	Van Drie *et al.* (1989)
CAVEAT	Connect binding fragments positioned in the active site, with cyclic connecting fragments	Database of cyclic connecting fragments, and a known ligand to the binding site	Searches for a cyclic template to mimic a known ligand	Plausible mimics to the α-amylase inhibitor, tendamistat, found	Bartlett *et al.* (1989)
BUILDER	Database search identifies complementary molecules. These are joined on an irregular lattice	GRID or HSITE or similar program and database, e.g. CSD	Final structure needs refinement by energy minimization. No prediction of binding energies	HIV protease active site filled with skeletal structures	Lewis *et al.* (1992)
SPROUT	Database search identifies a template to fill site. Appropriate hydrophobic and electrostatic functions added	Database of linear, branched and cyclic templates	Variety of techniques may be used to explore cavity space	Trypsin and HIV protease inhibitors	Gillet *et al.* (1993)

CLIX	Connection of binding fragments. Identifies structures from CSD having binding fragments in right positions	GRID to identify binding fragments. CSD	Can suggest modifications to structures in CSD to improve binding	NANA binding to haemagglutinin	Lawrence and Davis (1992)
LUDI	Connection of binding fragments. Uses library of linking fragments and small bridging groups	GRID as option to identify binding fragments. Large database of linking fragments	Determines hydrophobic interaction sites	Builds methotrexate into DHFR. Finds benzamidine binding to trypsin	Böhm (1992)
GEMINI	Builds a peptide ligand from a seed structure	Protein structure database	Applicable only to peptides	Orientation of peptides bound to proteinases	Singh *et al.* (1991)
GROW	Sequential build-up of peptide ligand from a seed amino acid	Database of conformations of aminoacids	Conformation enthalpy and solvation effects included	Finds crystal structure of peptides bound to *Rhizopus* pepsin	Moon and Howe (1991)
LEGEND	Build up structures from randomly seeded atoms and randomly selected torsions	MM2 force field	Energy minimization in the site after assigning charges	Promising DHFR inhibitors generated	Nishibata and Itai (1991, 1993)
GENSTAR	Build-up method using sp^3 carbons, followed by appropriate heteroatom substitution	Many user-specified variables to input	Simple scoring method to select candidates	Generates structures similar to known inhibitors of HIV protease, FK506 binding protein and carbonic anhydrase	Rotstein and Murcko (1993a)

TABLE 5 (cont'd)

Programs for *de novo* ligand design based on receptor structure

Program	Method	Requirements	Features	Validation	Citations
GROUPBUILD	Randomized build-up from seed fragment or inhibitor, using fragments, testing for complementarity to site	Fragment database and force field. SASA program to calculate solvent accessible surface	Entropic and solvation effects considered, refinement by energy minimization	Generates structures similar to known inhibitors of HIV protease, FK506 binding protein and carbonic anhydrase	Rotstein and Murcko (1993b)
CONCEPTS	Places a group of fragments in the site; these are linked using a molecular dynamics routine. Acceptance based on molecular mechanics energy of ligand–site complex	Molecular dynamics and AMBER force field	Use of molecular dynamics. No necessary dependence on libraries of fragments	Finds ligands similar to known inhibitors for FK506 binding protein and HIV protease	Pearlman and Murcko (1993)



(73)

DesJarlais *et al.* (1990) identified haloperidol (**73**) as a potential ligand for HIV protease. This hit was based on an unusual orientation: although the hydroxyl group lies between the active-site aspartic acid residues, the long axis of the crystal structure conformation of haloperidol does not lie along the backbone of known peptide-based inhibitors, such as that represented in Fig. 6 (Martin, 1992). Haloperidol was subsequently confirmed to be an inhibitor of protease (K_i = 0.1 mM). Although not highly potent against the enzyme, both the novel mode of binding suggested by computation (though not yet confirmed by crystal X-ray) and, particularly, the fact that haloperidol has good bioavailability makes this a good lead, justifying use of the DOCK program.

The well-characterized active sites of HIV protease and DHFR have been much used to validate some of the programs listed in Table 5. Typical of the methods that "grow" a structure from a seed atom or fragment (or a known inhibitor) is the program LEGEND, which has generated many potential ligands for DHFR (Nishibata and Itai, 1993). A flow chart of structure generation by LEGEND is represented in Fig. 9. Molecules are grown by adding atoms one by one, checking each by iterative force field calculations. Heteroatoms are introduced by changing the initially placed carbon atoms according to electrostatic potential values, so as to form hydrogen bonds or maximize electrostatic attraction between site and ligand.

Using a guanidino group as the seed, output structures similar to several known DHFR inhibitors were generated, such as **74** which may be compared to trimethoprim (**2**).

Currently, the programs available are all able to suggest a great many potential novel ligands to target sites. Unfortunately, many would represent very difficult synthetic challenges! Many would contain toxophores, or be unstable structures. A major difficulty still remains in making a selection of

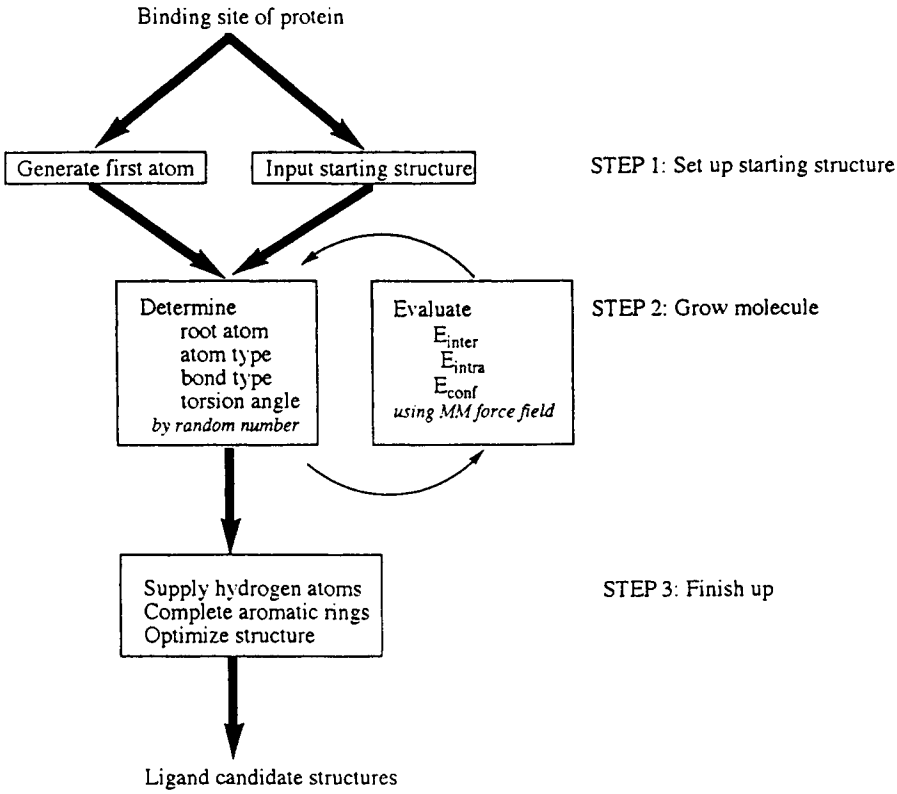
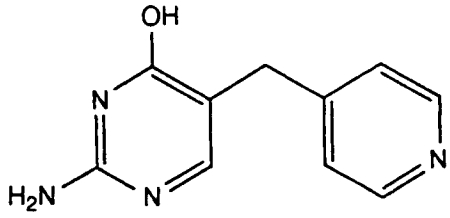


FIG. 9. Flow chart of structure generation by LEGEND. Reprinted with permission from Nishibata and Itai (1993). Copyright 1993 American Chemical Society.



(74)

the potentially most potent ligands: this requires better ways of incorporating estimates of hydrophobic binding, solvation effects and entropic effects within the selection algorithms. The program GROUPBUILD is most interesting in this regard, using as it does a calculation of solvent-accessible surface area to estimate solvation effects and hydrophobicity for both the binding site and potential ligands.

Despite the problems that remain, it is clear that with the tremendous increase in number of receptor sites that are being characterized by X-ray, the computational approach to *de novo* ligand and eventually drug design will assume great importance in the years ahead.

3.4 MECHANISM-BASED DESIGN

The design target for a drug is frequently an enzyme, whose inhibition will lead to the desired biological response. It may be that reversible inhibition by a weakly binding inhibitor is sufficient, but it is more likely that strong, or even irreversible binding through formation of a covalent link with the drug molecule will be required in order to achieve the necessary potency and selectivity.

The key to designing either tight binding or irreversible ligands is to understand the mechanism of the enzymic transformation. First, it is necessary to identify the *reacting entities* (nucleophilic and electrophilic groups, key water molecules, metal ions, coenzymes); second, one must recognize the importance of the favourable binding interactions between the *non-reacting parts* of the substrate and the enzyme. These binding interactions reach a maximum in the transition state, and may occur over a considerable distance—well separated from the reacting entities. These interactions are particularly necessary to compensate for the unfavourable entropy loss on binding which accompanies flexible substrates, such as proteins.

3.4.1 Transition State Analogues

Transition state analogues are essentially stable molecules which resemble, in geometry and in charge distribution, metastable intermediates of the enzymic reaction. The actual transition state of the reaction will be close in structure to the metastable intermediate, and will quite likely vary slightly between different substrates accepted by the same enzyme. There will not be a unique transition state for all transformations catalysed by one particular enzyme, neither of course will there be a unique transition state for different enzymes catalysing the hydrolysis of peptide links in a protein. There will nevertheless be some similarities in mechanism, and so structures containing a tetrahedral centre have been designed to inhibit a variety of proteinases, where a tetrahedral intermediate is always presumed. Differences exist in the pathway to, and breakdown of, the tetrahedral intermediate, and its stabilization, between thiol and serine proteinases, zinc proteinases, and aspartic proteinases.

Detailed knowledge of how each enzyme carries out its chemical task is

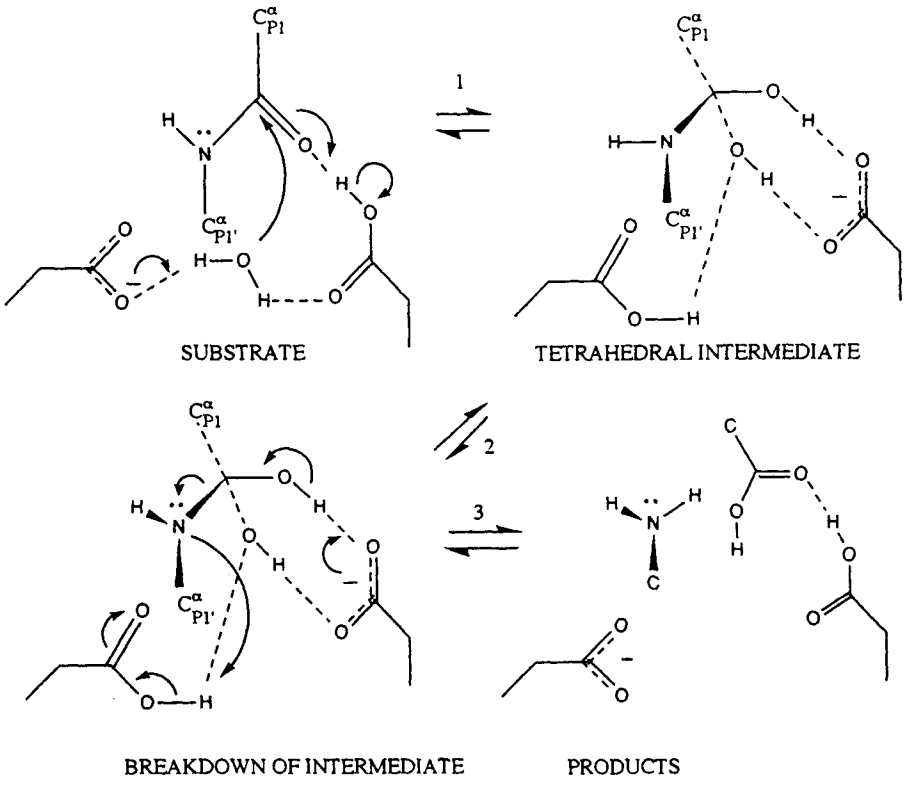


FIG. 10. Postulated mechanism of hydrolysis of a peptide substrate by an aspartic proteinase. Stabilization of the tetrahedral intermediate depends heavily on hydrogen bonding interaction with serine and threonine residues (not shown). Reprinted with permission from James *et al.* (1992). Copyright 1992 American Chemical Society.

gradually accumulating, thanks largely to crystallographic analysis of enzyme complexes with empirically designed transition state mimetics. The aspartic acid proteinases have received much attention, because of the importance of renin in blood pressure control, and of HIV protease as a target for the therapy of AIDS. From such studies (Blundell *et al.*, 1987b; Fraser *et al.*, 1992; James *et al.*, 1992) a scheme has been proposed for the catalytic pathway of peptide hydrolysis. In the formation of the tetrahedral intermediate, one aspartic acid residue acts as a general base, the other as a general acid, and the nucleophile is the central water molecule found between these two carboxyl groups (Fig. 10).

Inhibitors of HIV protease have been designed (Dreyer *et al.*, 1989; Rich *et al.*, 1990; Roberts *et al.*, 1990; Tam *et al.*, 1992; Martin, 1992) based on

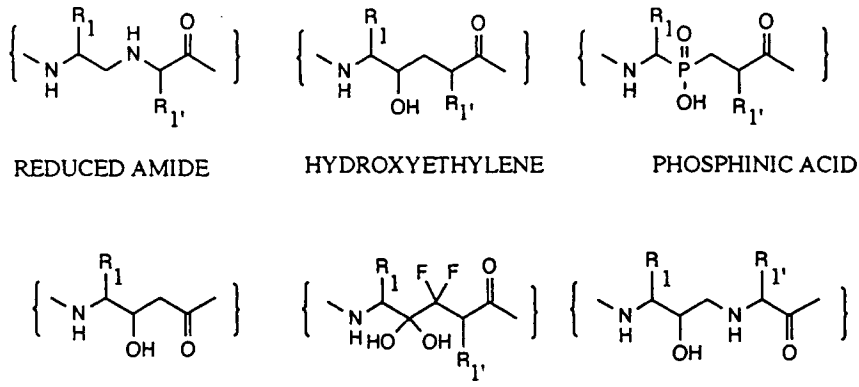
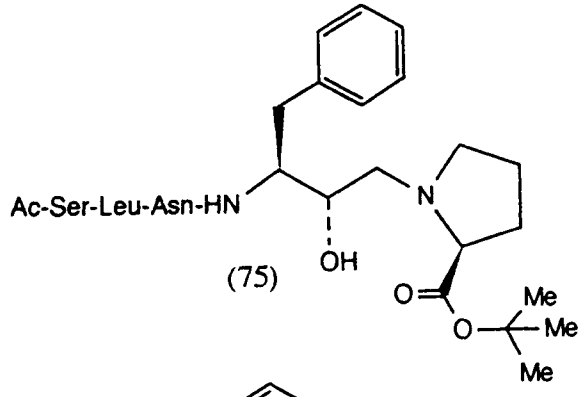


FIG. 11. Some transition state analogues incorporated into inhibitors of renin and of HIV protease.

the incorporation of transition state analogue fragments of Fig. 11, which are mimetics of the tetrahedral intermediate shown in Fig. 10. The principal structural feature in most transition state analogues designed to inhibit HIV protease is the critical hydroxyl group, shown by X-ray analysis to bind to both aspartic acid groups in a mechanistically related fashion. Generally, an (*S*)-hydroxyl enantiomer has been preferred, as revealed by the crystal structure of the compound known as JG-365 (**75**) complexed to the enzyme (Swain *et al.*, 1990). But it has been shown that the preferred configuration for tight binding depends on the whole structure, and contacts made throughout the binding cleft. The very potent hydroxyethylamine-containing inhibitor from Roche, Ro-3959 (**76**), has the (*R*) configuration at the hydroxyl group and an IC_{50} of 0.4 nM against the enzyme, compared with an $IC_{50} > 100$ nM for the (*S*)-enantiomer (Krohn *et al.*, 1991). Crystal studies show that in both **75** and **76** the hydroxyl group is located between the aspartic acids, but the adjacent methylene groups fit into the active site in a quite different manner.

The lesson for drug design is to synthesize both enantiomers of a chiral transition state analogue (see Section 3.1.5) or be guided by appropriate crystallographic and modelling studies. For an extended binding site, multiple binding modes must always be considered (Rich *et al.*, 1991).

Transition state mimics have generally been synthesized first on an empirical basis: tetrahedral analogues if the mechanism involves a tetrahedral intermediate; planar analogues if the enzyme catalyses an SN1 displacement, as exemplified by the synthesis of DANA (**71**) for the inhibition of neuraminidase. Recognition that transition state geometry and



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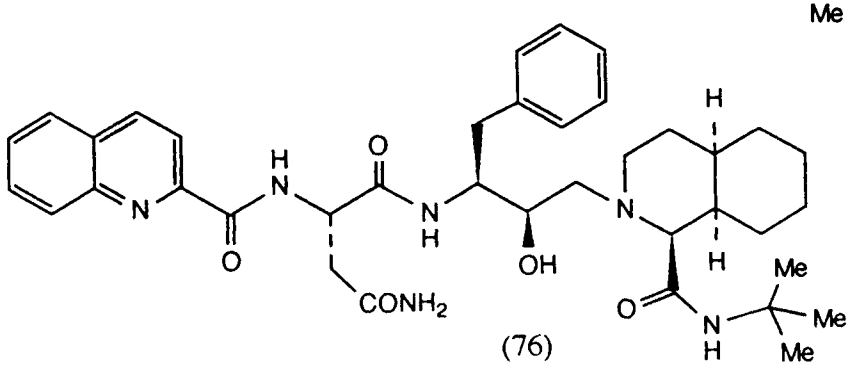


(76)

charge have been successfully simulated in the inhibitor can come from two sources. Firstly, binding affinity is generally 10^2–10^5 times greater than for a corresponding ground state substrate structure (Douglas, 1983). In certain cases, binding can be considerably more: for example, transition state analogue inhibitors of adenosine deaminase are known with K_i values up to 8 orders of magnitude lower than the K_m value for the substrate, adenosine (Kati and Wolfenden, 1989). Secondly, this tight binding can be slow in onset. This is because "substrate-like" recognition is followed by slow reorganization of enzyme and substrate conformation until the "transition state analogue combination" is achieved. Referring again to Fig. 10, substrate recognition and binding is fast, but steps 1 and 2 necessarily involve conformational changes in both enzyme and substrate in order to achieve correct alignment of orbitals for C–N bond cleavage in step 3.

As our understanding of the detailed mechanism of enzyme catalysis advances, so will the number of effectively designed transition state analogues. Rational design elements will be: (a) to predict the transition state (metastable intermediate) geometry, (b) to synthesize a prototype inhibitor and obtain a crystal of the enzyme–prototype complex for analysis, and finally (c) to use the crystal study to refine knowledge and design the ultimate drug molecule.

3.4.2 Mechanism-based inhibitors

The surest way to inhibit an enzyme is to block the active site irreversibly by chemical reaction with some active species to form a covalent bond. Thus, iodoacetate will irreversibly inactivate thiol proteases by forming the stable carboxymethyl mercaptan. Iodoacetate is of course non-selective (many other enzymes would be inactivated), toxic (many sensitive sites would be alkylated) and moreover the "drug" itself is unstable due to its very reactivity.

Active site-directed (hence selective) irreversible inhibitors have been designed by incorporating a reactive group into a substrate analogue. But such compounds may not be totally selective, and still may be both toxic and unstable.

Mechanism-based inhibitors (also known as suicide inhibitors or as k_{cat} inhibitors) are actually substrates for their target enzymes. A reactive group is only "revealed" by enzyme action: it is therefore not subject to hydrolysis until it has been revealed in the vicinity of the enzyme. The ability of the inhibitor then to inactivate the enzyme will depend upon relative rates of: (a) covalent bond formation with the enzyme, (b) diffusion of the reactive entity away from the enzyme, and (c) hydrolysis.

Suicide inhibitors have been reviewed by Rando (1974), Metcalf (1981) and Walsh (1982, 1984). Suicide inhibitors of cytochrome P450s have been reviewed by Ortiz de Montellano (1988).

Despite the obvious attractions, very few drugs have yet been realized from this approach. A notable exception is sulbactam, synthesized by the Pfizer research group as a β-lactamase inhibitor, and subsequently found useful in combination with β-lactam antibiotics for the treatment of resistant bacteria [see sultamicillin **29**, Section 2.3.3]. The mechanism of inactivation of β-lactamase by sulbactam has been investigated by Brenner and Knowles (1981) and is presented in Fig. 12. The formation of an acyl-enzyme intermediate occurs with opening of both 4- and 5-membered rings. The intermediate can undergo hydrolysis to release the enzyme (path A) but can also tautomerize to place the double bond in conjugation with the ester function, so stabilizing the system towards hydrolysis (path B). Alternatively, the desired irreversible inactivation occurs by attack of a nucleophilic group of the enzyme, followed by fragmentation (path C).

Although detailed structural as well as mechanistic knowledge of an enzyme is desirable, it is by no means necessary in order to design a suicide substrate. This has been shown by Myers and Widlanski (1993) who have designed a simple inhibitor of human prostatic acid phosphatase (PAP), an enzyme that is believed to be involved in the regulation of androgen receptor activity in prostate cells. Since the enzyme shows a preference for hydrolysis of aryl phosphates, the 4-(fluoromethyl)-phenyl phosphate (FMPP) was prepared as a substrate that would, on hydrolysis by the

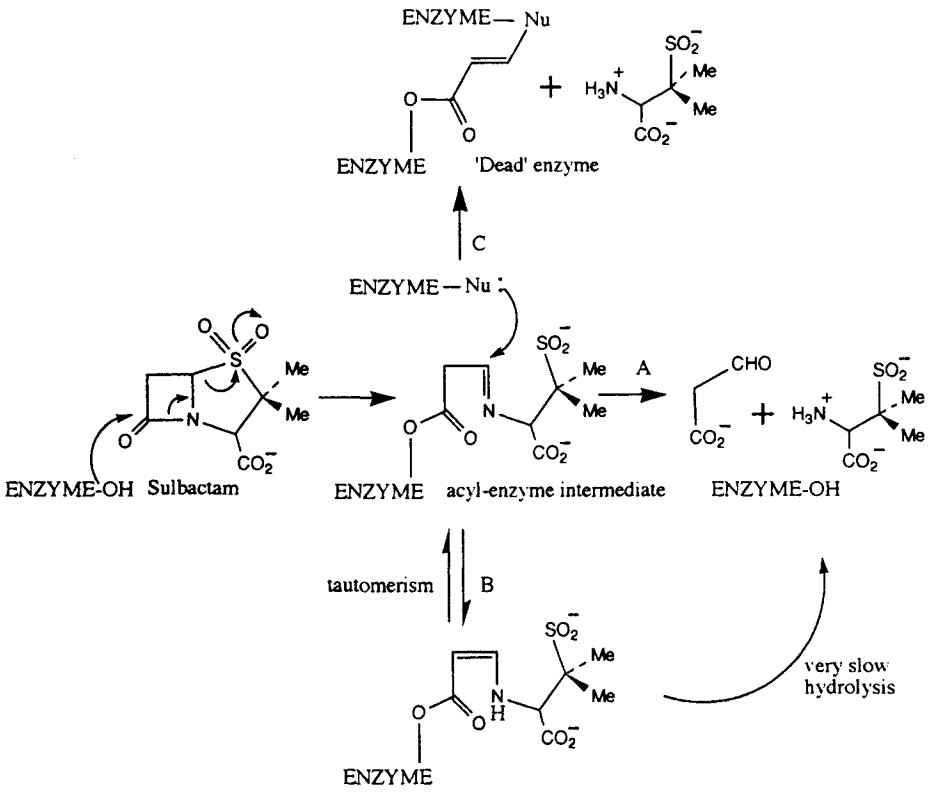


FIG. 12. Scheme to show inactivation of β-lactamase by the suicide inhibitor sulbactam.

enzyme, release the unstable intermediate 4-fluoromethylphenol. This intermediate can either diffuse away from the enzyme, or by base catalysis suffer elimination of fluoride to afford the highly reactive *p*-methylene quinone. This too can diffuse away, but may be available long enough to alkylate a nucleophilic group somewhere within the active site of the enzyme (Fig. 13).

True enough, treatment of PAP with FMPP resulted in a time-dependent inactivation of the enzyme. Competitive inhibitors of PAP protected against inactivation. The authors suggest that FMPP represents a useful basic structure which can be incorporated into the design of more specific phosphatase inhibitors: for example, the modified tyrosine **77** could be incorporated into a particular peptide to give a suicide substrate that is selective for a protein phosphatase which preferentially hydrolyses that peptide.

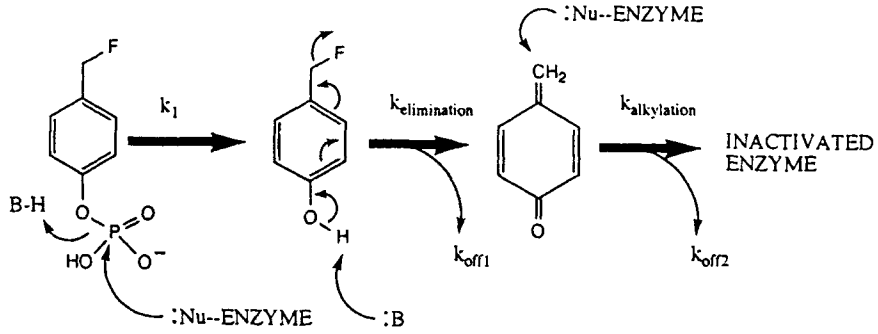
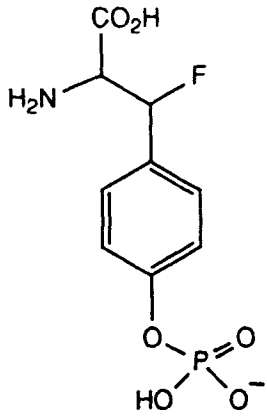


FIG. 13. Scheme to show inactivation of phosphatase by a suicide inhibitor. Reprinted with permission from Myers and Widlanski (1993). Copyright 1993 by the AAAS.



(77)

The fact that few drugs have been developed from the suicide inhibitor approach is a reflection on the likely toxicity of some reactive intermediates (such as the methylene quinone above) should they not react immediately with the target enzyme. However, the potential for design of such agents remains. With knowledge of the fine structure of the enzyme active site, it should be possible to prepare suicide substrates with greater selectivity for their target. Should this selectivity (and hence non-covalent binding affinity) for the target persist in the reactive intermediate, then it should not diffuse away before reaction, and inactivation, have occurred.

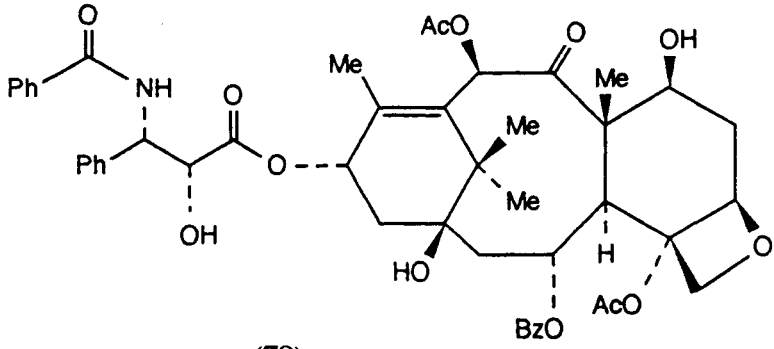
4 The Future

The changing pattern of disease, coupled with a steady increase in our understanding of physiological and biochemical mechanisms and macromolecular structure, will continue to provide new targets for drug design.

In the anti-infective area, increase in resistance to established antibiotics will require that new drugs be developed. The HIV pandemic and resulting AIDS syndrome have opened the door to a host of opportunistic infections. Exacerbated by HIV, tuberculosis is having a savage effect on the lives of millions of people, and new drugs are needed to combat resistant strains (Zhang *et al.*, 1992). The incidence of malaria is increasing (Brown, 1992) and we badly need a new generation of drugs for this disease. We still have no drug at all to treat the common cold, and only a poor and limited therapy for influenza. Macromolecular targets have already been identified for all these diseases.

In the developed countries, we are seeing a frightening increase in pulmonary disease: asthma, bronchitis and rhinitis. Certain peptides (the tachykinins) are known to be involved as mediators of the inflammatory response in these conditions: the primary structures of tachykinins are known, and some antagonists of their action have been identified. This field is open for rapid development (Lowe and Snider, 1993). A novel approach to anti-inflammatory agents targets cell adhesion, mediated by specific cell surface glycoproteins. The structure, biochemistry and enzymology of the adhesion molecules, and the inhibition of cell adhesion, is another growth area for drug design (Brackenbury, 1990; Rao *et al.*, 1991).

We sorely need new and improved anticancer drugs. Much excitement has been generated by the development of taxol (**78**), a macrocyclic natural product with a novel mechanism, the stabilization of microtubules (Suffness, 1993). The total synthesis of taxol has only just been accomplished, after nearly two decades of research. The chemistry described (Nicolau *et al.*, 1994) not only gives the solution to a formidable synthetic challenge but also opens the way to design novel taxoid derivatives and study the SAR. Hopefully, a part structure can be synthesized which will retain the antitumour activity in a compound that is more readily available and more easily administered.



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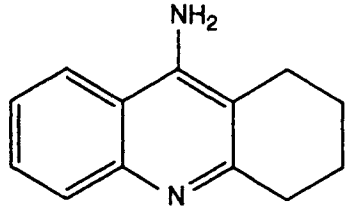

(79)

Degenerative and cognitive disorders are set to increase as we face an ageing population. Of these disorders, Alzheimer's disease (AD) has received, and will continue to receive, much attention (Dolmella *et al.*, 1994). The aetiology of AD is not understood, but there are two promising avenues of research in progress. One concerns the role and biogenesis of the glycoprotein amyloid which is deposited in AD plaques (Krafft, 1993). Another focuses on the role of acetylcholine (John *et al.*, 1993), and the acetylcholinesterase inhibitor tacrine (**79**) has just been licensed for the specific treatment of AD in the USA. The structure of acetylcholinesterase has recently been determined by X-ray crystallography (Sussman and Silman, 1992), opening the way for structure-based design of more effective tacrine analogues.

It is quite clear, from a brief survey of some disease targets, that the corresponding drug targets are likely to be known macromolecules, or the enzymes involved in their metabolism. The contemporary increase in emphasis on structure-based drug design is set to continue.

I expect protein crystallography and NMR, and NMR investigations of protein–ligand complexes, to play the major role in drug design that it has already assumed in the enormous effort that has gone into finding compounds capable of interfering with the HIV virus. Since peptides do not in themselves make good drugs, but may be used as tools to identify their many receptors, I expect the continuing use of peptide and other small molecule "libraries" to identify new leads, and further advances in the design of peptidomimetics, which will retain or mimic both the functionality and receptor-bound conformation of the peptide ligand.

Computational tools will be required to support structural investigations, with better algorithms for the prediction of tertiary structure. Current programs, both those using molecular mechanics and those based on quantum mechanical principles, will in future be required to incorporate automatically the influence of surrounding solvent. A most exciting computational area is that of *de novo* design of ligands to known macromolecular receptor sites. Better programs are needed, in particular programs that do not rely on a user-supplied database of fragments of molecular probes.

Such a program should be able to assess both solvation and entropy effects in making an estimate of the binding free energy of ligands.

Classical QSAR will continue to play its part in the optimization and selection of drug candidates. A fundamental difficulty with classical (property-based) QSAR is an over-reliance on the relevance of hydrophobicity, electrostatic and simple bulk steric effects as determinants of relative potency. We know that conformation is crucially important, but this is ignored in the classical approaches. The need for a structure-based QSAR method which also incorporates conformational flexibility might be met by development of a neural network (Livingstone and Salt, 1992; So and Richards, 1992) or machine learning program (King *et al.*, 1992).

Above all, drug design is multi-disciplinary: never more so than now. We need skilful chemists to prepare complex molecules, perhaps incorporating unusual substituents in order to explore variations in physical property. We need conventional and "molecular" biologists, pharmacologists and physical pharmacists. We need crystallographers and spectroscopists for small molecule and macromolecular investigation of structure, and we need the backup of computational chemists with access to databases of 3D structural information, and physicochemical constants. With such expertise, knowledge will continue to accumulate, and there can be no doubt that new and improved drugs will be designed.

We will find the cure.

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This chapter is dedicated to Steven and Kelly.

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Transgenic Animals as Pharmacological Tools

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LIST OF ABBREVIATIONS

ES	Embryonic stem
FIAU	1(1-2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil
G418	Geneticin
HAT	Hypoxanthine–aminopterine–thymidine
HPRT	Hypoxanthine phosphoribosyltransferase
HSV	Herpes simplex virus
PCR	Polymerase chain reaction
6-TG	6-Thioguanine
YAC	Yeast artificial chromosome

DEFINITIONS

Transgenic animal:	animal that has integrated foreign DNA into its germ-line as a consequence of experimental introduction of DNA (Palmiter and Brinster, 1986).
Targeted mutant:	mouse carrying a genetic modification in a selected gene as a consequence of homologous recombination of a transfected targeting vector with the corresponding target gene (includes "knock-out" and "knock-in" mice). Homozygous knock-out mice are also named null mutants.

1 Introduction

Currently, progress in several biological disciplines is clearly technology driven. This is in particular the case where genetically modified animal models are used to probe the complexity of a higher organism. The spectrum of methods for stably altering the genetic constitution of an animal, in particular of laboratory rodents, is being extended by methods of unprecedented precision. Whereas classical transgenic animals carry experimentally introduced genetic elements randomly inserted in their genome, today's mouse models may carry a single targeted point mutation in a selected locus. Combined models are being evaluated, where a predetermined mutation will be restricted to a particular tissue, due to the tissue-specific activation of a recombination event. These possibilities, to specifically modify the physiology of an animal by experimentally altering its genetic constitution, have opened the way to re-evaluate a number of fundamental biological questions concerning development and the function of complex systems, the most prominent being the immune system. Immunological tolerance, autoimmunity and, more recently, host defence

mechanisms have successfully been explored using a wealth of genetically modified animal models.

A number of genetically modified animals have been established as models for human genetic diseases, the most prominent examples currently being the models for cystic fibrosis (reviewed by Smithies, 1993). Also, for diseases involving abnormalities in gene expression which may not be due to a primary event of genetic nature, experimental animal models are instrumental in defining the role of individual components in the evolution of the disease, a notable example being Alzheimer's disease (for review see Aguzzi *et al.*, 1994).

Obviously, the availability of methods to generate specific animal models will also have a considerable impact on more applied disciplines, such as pharmacology. A better understanding of complex *in vivo* networks, in the normal and pathological state, will in particular result in the identification of relevant drug targets in order to prevent or correct pathological deviations from normal physiological states, and in a better understanding of drug actions. Animals with drug receptors of human structure may be better suited to test for drug availability and efficacy *in vivo* than the normal animal. Further, animals with modified metabolic pathways, or animals carrying specific reporter genes, may serve as sensitive indicators of unwanted drug-induced side effects (for reviews see Liggitt and Reddington, 1992; Liggitt *et al.*, 1992).

In designing an animal model, the biological problem has to be compared with the technical strategies available and the best-suited approach has to be chosen. In order to provide the reader with an up-to-date overview on available methods to modify the genetic constitution of an animal, the first part of this review concentrates on the technical aspects of establishing an animal model. The different methods are outlined, references for detailed descriptions are provided, and the potential application and the inherent weaknesses and limits are thoroughly discussed. The presentations cover pronuclear microinjection to generate transgenic animals, as well as the use of murine embryonic stem cells to introduce targeted mutations into the germ-line of mice. The approaches are grouped with regard to their suitability to induce so-called gain-of-function or loss-of-function modifications (see also Table 1).

In the second part, the review will focus on the three concepts (illustrated with selected relevant examples) mentioned above:

(1) animals for the identification of relevant drug targets;
(2) animals for the testing of drug efficacy;
(3) sensitive animal models to monitor for unwanted drug-induced side effects. As the use of genetically modified animal models in pharmacology is still in an exploratory phase, the potential future applications and impacts of refined animal models are discussed in Section 6.

TABLE 1

Approaches available to induce gain-of-function and loss-of-function models

Gain-of-function	Transgene with constitutive promoter	pm	Tissue-specific overexpression, dependent on regulatory sequence of transgene (Section 3.1.1)
	Transgene with inducible promoter	pm	(Section 3.1.2)
	Targeted, in frame insertion of coding sequence	gt	Gene replacement, tissue-specific expression depending on target locus (Section 3.2)
	Trans-activator system	pm	Tissue-specific activation (Section 3.1.3)
	Cre/lox-mediated activation of gene or transgene	gt/pm	Tissue specific, depending on CRE transgene expression pattern (Section 3.1.4)
	Targeted insertion of point mutations	gt	(Section 2.2.3)
Loss-of-function	Expression of antisense RNA and ribozymes	pm	Reduction in level of gene expression (Section 4.1.2)
	Expression of dominant negative mutation	pm	(Section 4.1.1)
	Toxigenics: toxin expression, HSV-1 *tk*/ganciclovir	pm	Cell ablation: constitutive, conditional (proliferating cells) (Section 4.1.3)
	Cre/lox inactivation of gene or transgene	gt/pm	Tissue-specific gene disruption (Section 4.2.2)
	Gene disruption (knock-out models), heterozygous, homozygous	gt	Gene disruption affecting all tissues (Section 4.2.1)

pm, by pronuclear microinjection; gt, by gene targeting; gt/pm, combined approaches.

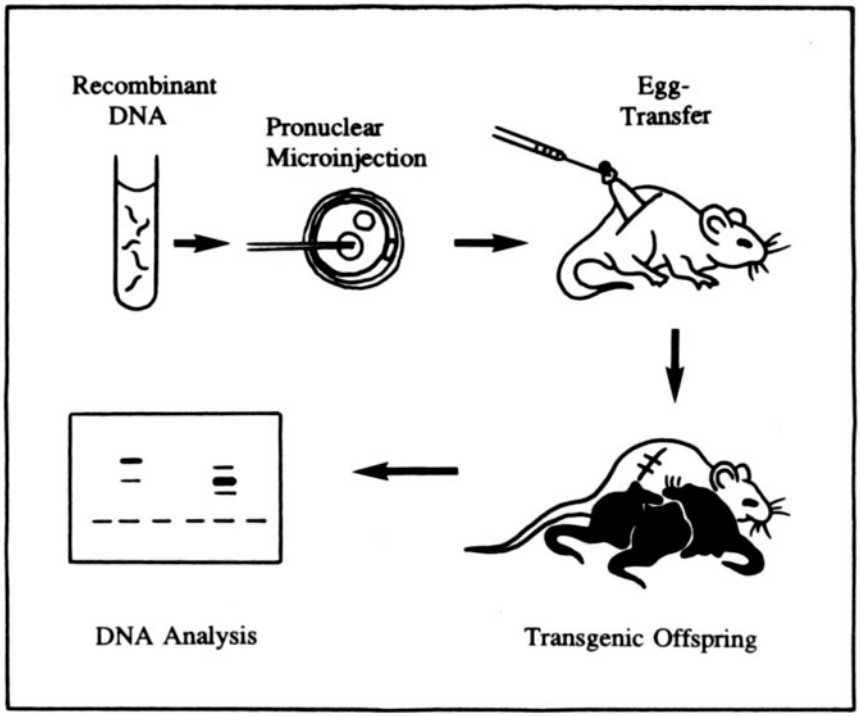

FIG. 1. Outline of the generation of transgenic mice via pronuclear microinjection. Transgenic animals carry the transgenes randomly integrated (hemizygous, tandems, dominant).

2 Methods to Generate Genetically Modified Animals

Three different methods have successfully been used to transfer genes into animals:

(1) the direct microinjection of recombinant DNA molecules into one of the pronuclei of the freshly fertilized egg (Fig. 1);
(2) the transfection of foreign DNA by retroviruses or retroviral vectors into embryos at various stages of development;
(3) the use of genetically transformed embryonic stem cells as vehicles (Fig. 2).

Although technically simple, the use of retroviruses and retroviral vectors has not found widespread application. This is mainly due to the size limitations for transfected DNA (about 10 kb), as well as to the unresolved problems of reproducibly expressing a virally transfected eukaryotic gene. However, the methods of pronuclear microinjection and the use of embryonic stem cells are being successfully applied by a growing number of research groups.

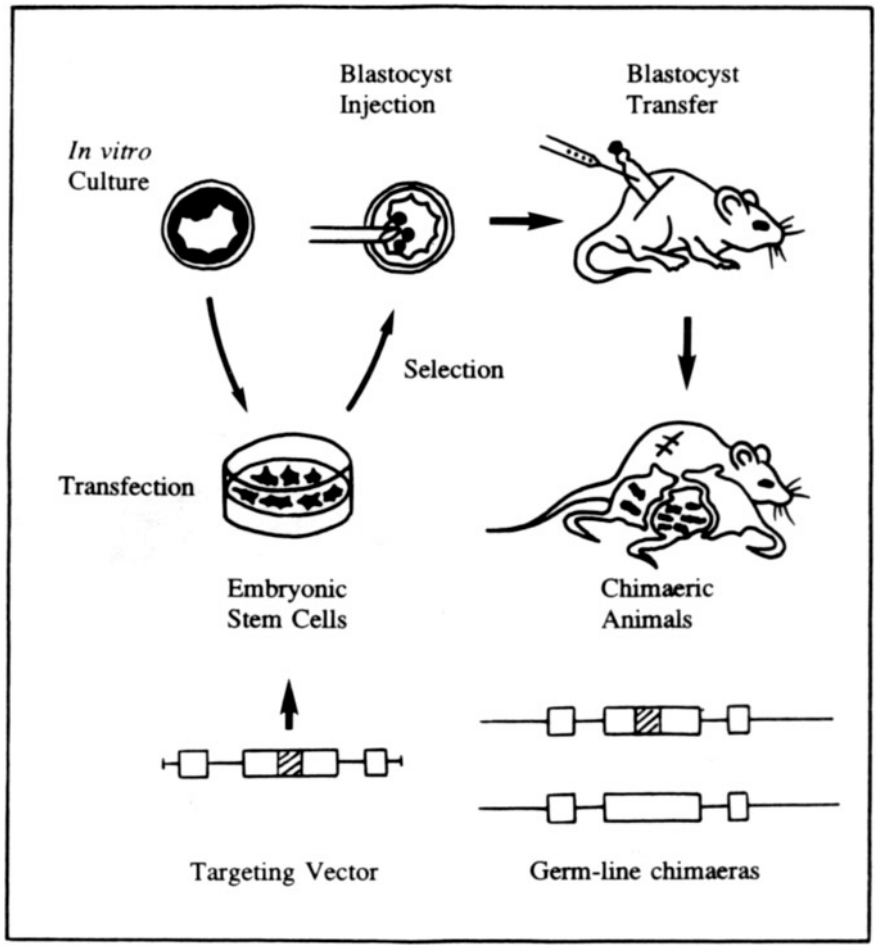

FIG. 2. Outline of the generation of mouse chimeras using ES cells. Germ-line chimeras transmit to their offspring selected, targeted mutations (homologous recombinations, heterozygous, usually recessive).

2.1 PRONUCLEAR MICROINJECTION

From experiments with cultured cells, it is evident that transformation frequencies are usually low, unless the DNA is injected directly into the nucleus (Anderson *et al.*, 1980; Capecchi, 1980). As early embryonic stages of mammals are available in limited numbers only, the microinjection method is the method of choice for transfecting foreign DNA into these target cells. Among the early developmental stages, the uncleaved zygote is the ideal target. For example, in the mouse egg, the pronuclei are large in diameter (15–20 μm), well visible, and they are present for hours during the

afternoon of day 1 of development (Gamow and Prescott, 1970). Except for a slightly retarded cell cycle, the same holds true for rat eggs.

The basic methodology of microinjection into mouse eggs was described as early as 1966, by Lin. The first report of a successful application of the microinjection approach to integrate foreign cloned DNA sequences into the developing mouse was published by Gordon *et al.* (1980), followed by several reports of successful generation of transgenic mice by the same method (see Fig. 1; reviewed by Gordon, 1983; Palmiter and Brinster, 1986; Jaenisch, 1988). The factors affecting the efficiency of the microinjection approach were subsequently optimized by Brinster *et al.* (1985). The optimal conditions entail injection of a few hundred linear DNA molecules, in a buffer solution composed of 10 mM Tris–HCl, 0.25 mM EDTA, pH 7.5, directly into one of the pronuclei of eggs derived from hybrid mouse strains. This corresponds to a concentration of about 1–2 ng DNA/μl buffer for a molecule of about 3 kb and an injection volume of about 2 pl. Under these conditions, about 10–30% of the mice that develop to term after transfer into the reproductive tracts of pseudo-pregnant foster mothers inherit one or more copies of the microinjected DNA. Due to the loss by direct mechanical damage and developmental arrest in the uterus, this corresponds to about two to five transgenic animals per 100 microinjected and transferred eggs. The method is outlined in Fig. 1. Molecular analysis reveals that the foreign DNA copies, the transgenes, are stably integrated in the genome of the transgenic carrier mouse, also called the founder. Integration takes place at an apparently random site, usually in a head-to-tail tandem array of several copies (up to several hundred). When a mixture of two different transgenes is coinjected, usually copies of both types cointegrate into the same site. The molecular mechanism of transgene insertion has not been characterized in detail, but may be based on non-homologous repair–ligation mechanisms (Bishop and Smith, 1989; Derbyshire *et al.*, 1994). In the founder animal, the integration site is present in a hemizygous form. Usually, the founder animal carries the integration locus in all its cells, indicating an insertion of the transgenes before the first cleavage division of the egg. Occasionally, a founder animal is revealed to be a mosaic of cells with and without a transgene insertion, or to carry more than one integration site per cell (Brinster *et al.*, 1985). These animals are identified by an unexpected transmission frequency of the transgenes to the offspring, which is 50% for an animal with one integration site in all its cells. Transgene insertion may disrupt a murine gene, thereby causing an insertional mutation. Such insertional mutations may be revealed as lethal mutations or as phenotypic aberrations when a transgene locus is crossed to homozygosity (reviewed by Gridley *et al.*, 1987; Meisler, 1992). As the identification of the affected endogenous gene is difficult and has been achieved in a few cases only, such mutations will not further be considered in this review.

In view of the relative unpredictability of transgene expression, in general, several transgenic lines have to be generated for each construct. Each line has to be carefully analysed for the pattern and level of the transgene expression as well as for stability after germ-line transmission. Selected lines with appropriate expression may then further be expanded for the biological studies in mind. Identification of a suitable transgenic line, including the characterization of the first generation offspring, will therefore take about 6–8 months from the time of microinjection of the transgene.

The equipment and experimental procedures used to generate transgenic animals are accurately described in laboratory handbooks by Hogan *et al.* (1986) and Wassarman and De Pamphilis (1993).

2.2 GENE TARGETING IN EMBRYONIC STEM CELLS

The development of techniques for the selection and screening of homologous recombination events in mammalian cells and the application of this technique to murine embryonic stem (ES) cells has made it possible to alter the germ-line of mice in a predetermined way. This so-called "gene-targeting" procedure, the exchange of an endogenous allele of a target gene against a mutated copy, allows the introduction of specific mutations into the gene of interest. These mutations may include the inactivation of the gene (knock-out, loss-of-function), the replacement by another gene (knock-in, gain-of-function), the deletion of a gene segment or the introduction of subtle mutations. Gene targeting followed by the introduction of the mutatant ES cells into an early embryo, as outlined in Fig. 2, therefore provides a powerful tool for generating mutant mouse strains with defined genetic modifications. So far, this methodology is restricted to mice, as germ-line competent ES cells have been established for this species only.

2.2.1 Embryonic Stem Cells

ES cell lines that keep their pluripotency after transfection and selection procedures are essential for the introduction of selected targeted mutations into the germ-line of mice. Undifferentiated ES cells are established *in vitro* from the inner cell mass of explanted blastocysts (Evans and Kaufman, 1981; Martin, 1981; Ledermann and Bürki, 1991). ES cells are maintained in a pluripotent state by coculturing with mitotically inactivated feeder cells, such as embryonic fibroblasts, and the addition of a differentiation-inhibiting activity called leukaemia inhibitory factor (LIF; Smith and Hooper, 1987; Williams *et al.*, 1988). The developmental potential of ES cells may be investigated *in vitro* by omitting LIF from the culture medium, or *in vivo* by reintroducing ES cells into early embryonic stages. After microinjection of ES cells into morulae or into the blastocoel cavity of a blastocyst, the ES

cells participate in normal development of the embryo and contribute to all three germ layers including the germ-line of the resulting chimeric animal (Evans and Kaufman, 1981). Alternatively, to form chimeras, ES cells can simply be aggregated with host morulae (Wood *et al.*, 1993a,b), thus omitting the technically intricate microinjection step. Most of the germ-line-competent ES cell lines used so far in gene targeting experiments (D3, CCE, AB1, E14TG2a) have been derived from blastocysts of sublines of the mouse strain 129/Sv. Recently, ES cell lines derived from inbred mouse strains other than 129/Sv have been successfully used: C57BL/6-derived ES cell lines (Bruce 4, Köntgen *et al.*, 1993; BL/6-III, Zou *et al.*, 1993; Kägi *et al.*, 1994) and a hybrid C57BL/6 × CBA ES cell line (TT2, Saga *et al.*, 1992). From the moment of ES cell transfection to the birth of potentially homozygous carriers of an ES cell-introduced genetic alteration, it will take approximately 12 months (i.e. second-generation offspring of the germ-line chimera).

2.2.2 *Types of Targeting Constructs*

Two types of constructs have been used to modify target genes in ES cells: replacement-type vectors and insertion-type vectors (see Fig. 3). Replacement-type vectors contain part of the genomic target gene sequence with a mutant foreign sequence, such as a positive selection marker, inserted within the coding region. The vector is linearized outside the region of homology. Such vectors recombine with the target gene by a double cross-over event, resulting in the replacement of the chromosomal DNA by the targeting construct. This type of vector is commonly used to disrupt target genes but also to replace the target gene by another gene, e.g. a reporter gene (Le Mouellic *et al.*, 1990; Mansour *et al.*, 1990), or to induce large deletions of the target locus (Mombaerts *et al.*, 1991; Zhang *et al.*, 1994).

Insertion-type vectors are linearized within the region of homology. Such vectors are inserted entirely into the target gene locus by a single cross-over event, leading to a partial duplication of the homologous DNA. The position of the linearization site will affect the structure of the recombinant allele. Therefore, for the design of an insertion-type vector it is important to consider the potential RNA species and coding possibilities of the recombinant allele (Hasty and Bradley, 1993). With this type of vector the target gene can either be inactivated or mutated through the introduction of subtle mutations.

2.2.3 *Introduction of Subtle Mutations*

Several strategies have been designed to introduce subtle mutations (e.g. point mutations) into the target locus without leaving selectable sequences

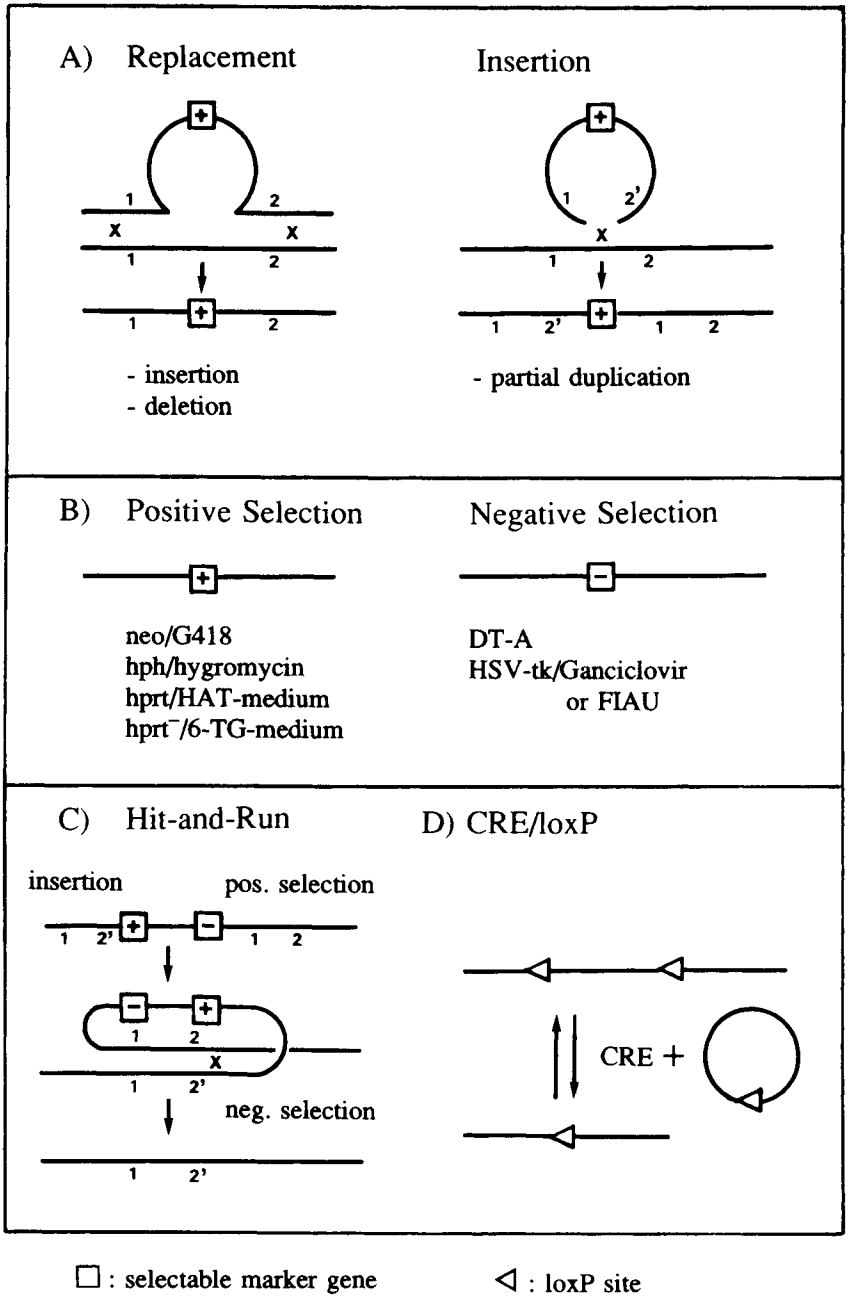

FIG. 3. Representation of (A) the two basic types of targeting vectors, (B) positive and negative selection markers, (C) the "hit-and-run" approach to introduce subtle mutations, and (D) the CRE/loxP recombinase system.

in the target gene, to analyse gene function and for the correction of specific gene defects in gene therapy (for review see Doetschman *et al.*, 1987; Thompson *et al.*, 1989; see also Fig. 3).

The first method described was the direct microinjection of the vector DNA with only minor alterations in the target gene into ES cells (Zimmer and Gruss, 1989). Because of the high transfection frequency observed with this method (20%), it was not necessary to use a selection marker. However, this method is technically very difficult to perform and no laboratory could successfully reproduce this procedure, nor have these cells been reported to be transmitted through the germ-line.

An alternative method, the "hit and run" or "in and out" strategy, requires two steps of recombination and is based on an insertion-type vector containing a subtle mutation in the target gene and a positive and a negative selection marker in the plasmid backbone (Hasty *et al.*, 1991; Valancius and Smithies, 1991). In the first step, the construct is inserted into the target gene by a single cross-over. This process is selected with the positive selection marker. In the second step, the endogenous gene is replaced with the modified DNA by intrachromosomal homologous recombination. This process may be directly selected due to the loss of the negative selection marker.

A much simpler method for introducing subtle mutations into the target gene has been developed by Reid *et al.* (1991) and Davis *et al.* (1992). The selection marker and the target construct containing minor alterations are located on separate DNA fragments and introduced simultaneously into ES cells by coelectroporation. ES clones with a homologous recombination event in the target locus together with a random insertion of the selection marker have been identified. The selection marker will be separated from the targeted allele during meiosis following transmission through the germ-line.

Still another approach to replace a selected gene sequence by a slightly mutated sequence makes use of hypoxanthine phosphoribosyltransferase (HPRT)-deficient ES cell lines. Here, in the first step, a selected gene sequence is replaced by the *hprt* gene. Transfected cells can be positively selected in hypoxanthine–aminopterine–thymidine (HAT) medium. In the second step, the *hprt* gene is replaced by a mutated gene sequence. The resulting ES cells are again HPRT-deficient and can be positively selected in 6-thioguanine (6-TG)-containing medium (see Section 2.2.5).

2.2.4 Site-specific Recombinase Systems

In a recently developed method, site-specific recombinases from bacteriophage or yeast have been used to introduce subtle mutations or deletions

into a target locus (Kilby *et al.*, 1993; Gu *et al.*, 1993; Nagy *et al.*, 1993). The 38 kDa CRE recombinase from bacteriophage P1, which has been shown to be more efficient in ES cells than the yeast recombinase FLP (Gu *et al.*, 1993), catalyses a *lox*P-dependent site-specific recombination in both prokaryotic and eukaryotic cells. In the first step, a sequence flanked by two 34 bp *lox*P sites in the same orientation can be inserted into a target locus. In the second step, it is efficiently removed from the chromosome by subsequent expression of the CRE recombinase. For the introduction of subtle mutations, for example, the selection marker flanked by two *lox*P sites is inserted into an intron of the mutated genomic targeting sequences. This construct is used to replace part of the target locus by homologous recombination. After positive selection, the CRE enzyme is transiently expressed in the targeted ES cell clones. CRE catalyses a *lox*P-dependent recombination thereby excising the potentially disruptive selectable marker gene, leaving a single *lox*P site in the intron of the mutated target gene (see Fig. 3).

2.2.5 Enrichment for Gene Targeting Events

The targeting construct is introduced into the ES cells by electroporation. This method is technically very simple and leads in most cases to single copy integrations of the exogenous DNA which is advantageous for gene targeting experiments (Smithies, 1986). However, the major disadvantage is the low transformation efficiency (10^{-3}) and therefore it is necessary to include a positive selection marker in the targeting construct. The most commonly used positive selection marker is the prokaryotic neomycin phosphotransferase (*neo*) gene driven by strong promoters such as the phosphoglycerate kinase-1 (PGK-1) promoter, the RNA polymerase II (pol II) promoter (Soriano *et al.*, 1991), the human β-actin promoter or the weaker herpes simplex thymidine kinase promoter including polyoma enhancer fragments (pMC1; Thomas and Capecchi, 1987). The expression of the *neo* gene confers resistance to geneticin (G418). The second positive selection marker commonly used in gene targeting experiments is the prokaryotic hygromycin B phosphotransferase (*hph*) gene. Its expression can be selected for with hygromycin B. Alternatively, the use of *hprt* minigenes as the selectable marker in HPRT-deficient ES cells has been described (Reid *et al.*, 1990; Selfridge *et al.*, 1992; see also Section 2.2.3).

Homologous recombination occurs approximately 1000-fold less frequently than non-homologous recombination, therefore methods have been developed to enrich for homologous recombination events. A widely applied method includes the use of a positive and a negative selection marker and does not require the expression of the target gene in ES cells (Mansour *et al.*, 1988). The targeting construct is based on a replacement-type vector

containing the positive selection marker within the region of homology and the negative selection marker at one or both ends of the homologous DNA. Genes for the negative selection that have been used in this approach include the herpes simplex virus thymidine kinase gene which converts nucleoside analogues, such as ganciclovir or 1(1-2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU), into toxic metabolites, and the diphtheria toxin A gene (Yagi *et al.*, 1990). In the first step, the positive selection is applied for cells that have integrated the targeting construct in their genome. In the second step, the negative selection is applied against cells with a random insertion of the construct, since cells carrying a targeted integration event must have lost the terminal negative selection marker. Positive–negative selection enhances the ratio of targeted to non-targeted events only by 5–10-fold due to occasional loss of the negative selection marker during random integration.

The enrichment for homologous recombination events can be increased by using a positive selection marker lacking 5′ (promoter) or 3′ (polyadenylation signal) regulatory sequences, provided the target gene is expressed in ES cells. In most cases, the marker gene will be efficiently expressed only when inserted into the target locus after homologous recombination, and not after random integration. The use of a promoterless selection marker in the targeting construct enhances the ratio of targeted to random integration by 50–100-fold, whereas a selection marker lacking a polyadenylation signal may enhance the ratio by 7–50-fold (reviewed by Bradley *et al.*, 1992).

Another strategy for enrichment of homologous recombination events was demonstrated by Lindberg *et al.* (1993). In this approach, an artificial splice acceptor site was placed in front of the coding sequence of a promoterless neomycin resistance gene and this cassette was inserted into the first intron of the target gene. As expected, the insertion resulted in the splicing of the first exon to the neomycin coding sequence, thereby competing with the normal splicing, and the generation of a fusion protein.

2.2.6 Double Knock-out of Target Genes

The two alleles of the target gene can be inactivated consecutively in ES cells through two rounds of homologous recombination. This can either be obtained by using two constructs containing different positive selection markers (Te Riele *et al.*, 1990; Mortensen *et al.*, 1991), or by increasing the G418 concentration after the first round of homologous recombination (Mortensen *et al.*, 1992), thereby selecting for cells with the selectable marker also inserted in the second allele, most probably due to somatic recombination of the two alleles. Using this approach, gene function may be studied directly in ES cells *in vitro* or in a chimeric *in vivo* situation, or in the RAG-2 blastocyst complementation assay (see Section 4.2.2).

2.2.7 *Identification of Targeted ES Cell Clones*

Two types of screening procedures are used to identify ES cell clones carrying a targeted integration of the construct DNA: polymerase chain reaction (PCR) and Southern blot analysis (Southern, 1975). Both methods rely upon the specific juxtaposition of vector components and target locus sequences after homologous recombination.

The PCR is the most sensitive method for distinguishing cells with a targeted integration from the majority of cells carrying a random integration of the target DNA (Mullis and Faloona, 1987; Frohman and Martin, 1990). Two oligonucleotide primers are used to amplify a specific fragment created by the homologous recombination event. One primer is complementary to sequences unique to the target locus and the other is unique to sequences within the targeting construct (such as the selectable marker gene). PCR amplification of the expected fragment is possible only when these primers are correctly juxtaposed by a homologous recombination event.

Southern blot analysis is used to confirm the PCR results. The genomic DNA is isolated from PCR-positive ES cell clones. With the choice of restriction digest and probes for hybridization, the wild-type allele can readily be distinguished from the targeted allele since predicted novel restriction fragments are generated by the homologous recombination event.

2.2.8 *Parameters Affecting the Frequency of Homologous Recombination*

Considering the variety of approaches chosen, published frequencies of homologous recombination (targeted insertion versus random insertion) are difficult to compare directly. However, the major parameters influencing the frequency of homologous recombination in ES cells include the target locus itself or the locus region, the length of homologous DNA used in the targeting construct, transcriptional activity of the target locus and the penetrance of the selectable marker (reviewed by Frohman and Martin, 1989; Fung-Leung and Mak, 1992). A crucial parameter seems to be complete homology between target gene sequences and targeting sequences. This is indicated by observations that the use of isogenic DNA vectors can greatly increase the frequency of homologous recombination. Sequencing has revealed that even minor polymorphic variations between the donor and recipient target DNA may reduce the efficiency of gene targeting (Van Deursen and Wieringa, 1992; Te Riele *et al.*, 1992).

The equipment and experimental procedures used to generate targeted mutant mice are described in laboratory handbooks by Joyner (1993) and Wassarman and De Pamphilis (1993).

3 Gain-of-function Models

3.1 RANDOMLY INSERTED TRANSGENES

3.1.1 Constitutive Promoters

A transgene inserted into the germ-line of animals may comprise an intact gene in its genomic configuration, including its own regulatory elements, promoter, enhancer and locus control region. Alternatively, a transgene may comprise coding sequences in the form of a cDNA, combined with homologous or heterologous regulatory sequences. In this latter case, the addition of intronic sequences may be mandatory for efficient transgene expression (Brinster *et al.*, 1988; Palmiter *et al.*, 1991; Whitelaw *et al.*, 1991). Despite a wealth of useful transgenic animal models, the predictable and tissue-specific expression of the introduced transgene remains in many cases a considerable problem. The randomly integrated transgenes are subject to a number of *cis*- and *trans*-acting factors which influence their expression: the flanking host sequences, *de novo* methylation (Jähner *et al.*, 1982; Engler *et al.*, 1991), the presence of prokaryotic vector sequences (Townes *et al.*, 1985), or the interference of different elements of recombinant transgenes (Swanson *et al.*, 1985). Dominant regulatory elements, referred to as the locus control region (LCR), have been identified for a minority of (*trans*-) genes, including the genes of the globin cluster (Grosveld *et al.*, 1987) and the *CD2* gene (Greaves *et al.*, 1989). As these LCRs may be located at considerable distance from the coding region of a gene, they are usually not included in a genomic segment cloned in a plasmid vector (about 10 kb) or a cosmid vector (about 30 kb). However, genes located on yeast artificial chromosomes (YACs; about 200–300 kb) are likely to be accompanied by the corresponding LCR. The availability of YACs and the possibility to inject these long DNA molecules into pronuclear mouse eggs in order to generate transgenic mice may solve some difficulties in reliably expressing transgenes (Gaensler *et al.*, 1993; Schedl *et al.*, 1993). YACs may however comprise unidentified genes in addition of the gene of interest. This may create unwanted effects in a YAC transgenic animal.

Animals with a gain-of-function due to the expression of a transgene under control of a constitutive promoter represent the majority of transgenic models. These animals can be used to illuminate the properties of a complex *in vivo* system by perturbing an element due to overexpression or out-of-time or out-of-place expression of a protein. In this way information on the development, the normal functions or the malfunctions of a complex system can be gained. This strategy has in particular successfully been applied to the exploration of immunological self-tolerance, oncogenes and

cancer, and mammalian development (for review see Hanahan, 1989). In addition, the generation of transgenic models to study disease states has greatly contributed to a further definition of genetic predispositions and early events in the development of cardiovascular diseases (Barrett and Mullins, 1992a), diabetes (Lipes and Eisenbarth, 1990), endocrine disorders (Stewart, 1993) and atherosclerosis (Breslow, 1993).

3.1.2 Inducible Promoters

In studying the function of genes, where the (over)-expression induces a deleterious phenotype, e.g. early in development, it is necessary to be able to control the activity of that transgene. This can be achieved by using an inducible promoter. Two such promoters have been used extensively: the mouse mammary tumour virus (MMTV) long terminal repeat, and the metallothionein promoter (for review see Palmiter and Brinster, 1986). The generally unpredictable tissue specificity and the relatively high basal level of expression have limited their use. In addition, the stimuli used, steroids or heavy metals, exhibit undesired side effects. More recent studies have identified *cis*-acting elements from several genes that respond to other physiological and environmental stimuli: altering the carbohydrate and protein content of the diet can markedly alter the activity of the PEPCK promoter (McGrane *et al.*, 1988); hypoxia induces the erythropoietin gene (Semenza *et al.*, 1991); reduced temperature induces a mitochondrial uncoupling protein in brown fat (Boyer and Kozak, 1991); and aromatic hydrocarbons or phenobarbital induce a cytochrome P450 transgene in the liver (Jones *et al.*, 1991; Ramsden *et al.*, 1993). The potential use of inducible promoters is therefore limited to a few cell types only.

3.1.3 Trans-activation Systems

To overcome the limitations with inducible promoters, specificity can be provided by a binary system where an exogenous trans-activating factor controls the expression of a so-called responder transgene. The trans-activating factor can be placed under the control of a regulatory element with a defined temporal and tissue-specific expression pattern. By crossing such an animal with an animal harbouring a transresponder gene, offspring with a predefined transgene expression pattern can be generated. The trans-activating system may be derived from yeast (GAL4/UAS control elements; Ornitz *et al.*, 1991) or from HSV-1 (VP16 trans-activator, IE promoter; Byrne and Ruddle, 1989).

Recently, this latter viral trans-activation system has been modified to a tetracycline-responsive system (Gossen and Bujard, 1992). *In vitro*, the trans-activation of a luciferase responder gene could be regulated over up to

five orders of magnitude, depending on the concentration of antibiotic in the medium. Such a system might allow the very strict control of the activity of an individual transgene. However, when transferred into a transgenic *in vivo* situation, trans-activation by tissue-specific trans-activator expression has not been successfully achieved (personal observation).

3.1.4 Recombinase-activated Genes and Transgenes

In several laboratories, a trans-activation system based on the structural restoration of a disrupted gene or transgene is being evaluated. For this approach, site-specific recombinases (see Section 2.2.4) are expressed by a transgene in order to induce the deletion of disruptive sequences flanked by recombinase-specific sites either from a targeted non-functional gene or from a non-functional transgene (Orban *et al.*, 1992). Deletion of the disruptive sequence will then activate the previously silent gene or transgene in all cells where the recombinase is expressed. The approach seems particularly promising for the tissue-specific reactivation of targeted genes in knock-out mice.

3.2 TARGETED INSERTION OF TRANSGENES

The generation of gain-of-function mutants with random integration of the transgene via the ES cell route (Robertson *et al.*, 1986; Gossler *et al.*, 1986) has not found widespread application since the generation of transgenic animals via pronuclear microinjection is much faster and less laborious. However, the ES cell route to transgenesis offers a way of overcoming the problem of unreliable transgene expression. By a targeted insertion of transgene sequences, the foreign sequences are placed under control of the regulatory elements of that locus. The transgene sequences may be inserted, leaving the target locus functionally intact or, alternatively, they may replace part of the target gene, thereby disrupting the endogenous gene. An endogenous murine gene may thus be replaced by a reporter gene, e.g. the *Escherichia coli lacZ* gene. Le Mouellic *et al.* (1990) have replaced one copy of the murine homeobox gene *hox3.1* by the *lacZ* reporter gene and Mansour *et al.* (1990) have introduced the *lacZ* gene into the murine *int-2* locus so that the endogenous promoter of the targeted gene controls the reporter gene. The expression of these genes can be followed *in situ* throughout embryogenesis of the mutant animal. Homozygous embryos will allow the visual assessment at the cellular level of gene inactivation effects in transgenic mice.

An endogenous murine gene of choice can also be replaced by human sequences (Stacey *et al.*, 1994). This concept of replacing the endogenous

murine gene by human sequences via gene targeting or via the combination of knock-out mice with mice overexpressing human transgenes will become increasingly important for the *in vivo* testing of drug efficacy (see Section 5.2.2).

4 Loss-of-function Models

4.1 RANDOMLY INSERTED TRANSGENES

The induction of a loss or reduction of specific function by transgene expression is not trivial and has found restricted applications. However, a few interesting concepts have been realized: overexpression of a receptor to reduce free ligand (Hofmann *et al.*, 1988); expression of transgenes encoding dominant negative mutations; expression of antisense RNA (or a ribozyme); or expression of a toxic polypeptide. These latter concepts are discussed in more detail.

4.1.1 Dominant Negative Mutations

Genes with dominant negative mutations encode mutant polypeptides that inhibit the normal function of the wild-type protein (Herskowitz, 1987). Such inhibitory variants may be designed by mutating, for example, sites for oligomerization, substrate binding, catalysis, membrane association or phosphorylation. Several dominant negative mutations have successfully been expressed in transgenic mice. A transgene encoding a mutated form of pro-alpha(1) collagen has induced a dominant negative trait (Stacey *et al.*, 1988). Expression of as little as 10% mutant collagen in transgenic foetuses resulted in a lethal phenotype due to a reduced type I collagen content, a phenotype corresponding to the inherited human disease perinatal lethal osteogenesis imperfecta. This observation suggests that the presence of non-functional subunits may severely inhibit the correct assembly of multimeric structures.

By expressing human sickle cell haemoglobin (HbS) in transgenic mice, irreversibly sickled cells have been observed in the blood of animals with high levels of HbS (Greaves *et al.*, 1990). By breeding similar mice to β-thalassaemic mice, animals with decreased haematocrits, elevated reticulocyte counts, lower haemoglobin concentrations and splenomegaly, which are all indications of the anaemia associated with human sickle cell disease, were observed (Ryan *et al.*, 1990). In HbS transgenic mice, the mutant haemoglobin molecules, when present at high levels (80% of total haemoglobin) and when erythrocytes are deoxygenated, are polymerizing

and causing a deformation of the erythrocytes to a characteristic sickle shape.

Examples of mutant monomeres acting as dominant negative mutants were described by Lavigueur *et al.* (1989). A mutant *p53* tumour suppressor gene was expressed in transgenic mice. Such mice reproducibly succumb to a variety of tumours. Dycaico *et al.* (1988) succeeded in reproducing in transgenic mice a neonatal hepatitis by expressing the human allele of α1-antitrypsin carrying the dominant mutation responsible for the disease in humans.

4.1.2 Expression of Antisense RNA

Although antisense RNA (minus strand RNA complementary to messenger RNA) has been shown to effectively repress the expression of specific genes of *in vitro* cultured cells at the translational level, with transgenic mice only a few successful attempts have been reported. In one case, the myelination of the central nervous system of transgenic mice was significantly reduced, resulting in a shiverer phenotype, by expressing an antisense basic myelin protein mini-gene in myelin-producing cells (Katsuki *et al.*, 1988). In a recent case, a transgenic mouse with impaired type II glucocorticoid receptor function was generated by partially eliminating gene expression with type II glucocorticoid receptor antisense RNA (Pepin *et al.*, 1992a).

Theoretically, the expression of catalytic RNA targeted to specific messages by antisense sequences flanking the catalytic domain should also lead to a decrease of the message level. Such ribozymes may reduce RNA levels more efficiently than antisense RNA because they act enzymatically and even low levels may efficiently degrade target molecules (Cotten, 1990). This principle has successfully been realized in transgenic mice by expressing an insulin-promoted glucokinase ribozyme. In pancreatic islet cells of these animals reduced levels of glucokinase (GK) mRNA, and reduced GK activity (at least 3-fold reduction) was observed (Efrat *et al.*, 1993).

4.1.3 Toxigenics

A still different approach to induce a loss-of-function in transgenic animals makes use of the cell toxicity of the diphtheria toxin or ricin subunit A. By fusing the structural gene for the toxin subunit to a cell type-specific promoter, it should be possible to induce autonomous death of all cells in which that specific promoter is active. This targeted cell ablation, also named toxigenics (Beddington, 1988), has been applied to generate transgenic mice exhibiting microphthalmia (reduced lens mass), pancreatic rudiments or dwarfism, depending on the tissue specificity of the promoters

used: gamma-crystallin promoter, elastase I promoter and growth hormone promoter, respectively (Breitman *et al.*, 1987; Palmiter *et al.*, 1987; Behringer *et al.*, 1988). An incomplete penetrance of toxicity and a highly variable expression of the toxin gene constructs make interpretations difficult. However, certain pathological conditions where particular cell types are reduced or absent may well be mimicked by genetic cell ablation in transgenic animals.

The conditional ablation of specific cells has been achieved by treating transgenic animals expressing HSV-1-TK with the antiherpetic drug ganciclovir. In lymphoid tissue of mice expressing HSV-1-*tk* under control of an immunoglobulin promoter, administration of ganciclovir led to a massive, but reversible, destruction of B- and T-cell lineages (Heyman *et al.*, 1989). This method allows for induction of ablation at precise moments of development. Ablation is however restricted to proliferating cells expressing HSV-*tk* (Evans, 1989).

4.2 TARGETED INSERTION OF DISRUPTIVE SEQUENCES

4.2.1 Constitutive Knock-outs

The generation of mutant mice carrying targeted gene disruptions via homologous recombination between exogenous DNA and its endogenous homologue has now found widespread application as probe for gene function. The number of these "designer mutant mice", also called knock-out mice or null mutants, that are generated in the fields of immunology, cancer genetics, neurobiology and developmental biology is steadily increasing (for reviews see Rossant, 1991; Erickson, 1993; Aguzzi *et al.*, 1994; Grant and Silva, 1994). The knock-out technique has been especially attractive for immunologists since the disruption of genes involved in immune function is unlikely to be lethal and the effect of such a knock-out on the immune response can be readily studied. Several genes involved in immune cell function and immune cell activation/regulation have been inactivated by gene targeting. The classes of proteins encoded by these genes include surface receptors and ligands, recombination activation factors and cytokines. The effects of individual knock-outs range from minor to more broad defects in immune cell development (reviewed by Yeung *et al.*, 1993; Huang, 1993).

Gene targeting technology has also been used in attempts to generate mouse models for human diseases caused by a single gene defect. The first demonstration that ES cells could be used to generate animals with genetic alterations associated with a human inherited disease was the generation of HPRT-deficient mice (Kuehn *et al.*, 1987; Hooper *et al.*, 1987), a potential model for Lesh–Nyhan syndrome. This syndrome is caused by a deficiency

of the enzyme HPRT involved in the purine salvage pathway and is characterized by behavioural alterations including self-injurious behaviour and mental retardation. Although, contrary to expectation, HPRT-deficient mice do not develop behavioural abnormalities, this mouse model could be improved by the administration of an adenine phosphoribosyltransferase (APRT) inhibitor, since this enzyme is more important in the rodent purine salvage pathway than in humans (Wu and Melton, 1993).

A second example for the generation of a mouse model for a single gene defect in humans is the production of cystic fibrosis (CF) mutations in mice (Clarke *et al.*, 1992; Snouwaert *et al.*, 1992; Dorin *et al.*, 1992). CF is caused by a defective CF transmembrane conductance regulator (*cftr*) gene which encodes a chloride channel. Depending on the targeting construct used, the animals homozygous for the *cftr* disruption display a range of classical symptoms of CF patients including failure to thrive, intestinal mucus obstructions and some pancreatic abnormalities. However, these animals do not reflect the whole human condition since they also show additional pathology that has not been reported in humans and, on the other hand, lack pathology seen in humans (for review see Smithies, 1993). Nevertheless, these mice will be important models to develop novel therapeutic approaches, including gene therapy approaches.

The third example involves the generation of a mouse model for Gaucher's disease, a lysosomal storage disorder in humans. The disease is caused by an autosomally inherited deficiency of the enzyme glucocerebrosidase (β-D-glucosyl-*N*-acylsphingosine glucohydrolase). Homozygous deficient mice show a more severe phenotype than observed in the human condition and die within 24 h of birth. Therefore, mutant mice carrying less deleterious mutations will be more adequate models for the frequently observed milder presentations of Gaucher's disease (Tybulewicz *et al.*, 1992).

One general finding with knock-out mice is that an observed mutant phenotype is often less severe than expected, and/or detectable only in a subset of the tissues which normally express the target gene. This has been observed in a variety of knock-out mice (e.g. *wnt-1* -/-,*en-2* -/-, c-*abl* -/-, c-*src* -/-,*Il-2* -/-; for reviews see Gridley, 1991; Rajewsky, 1992). The explanation for this observation is the existence of functional redundancy between genes (Gridley, 1991; Doherty, 1993; Strohman, 1994). For example, *wnt-1*, *en-2*, c-*abl* and c-*src* are all members of multigene families and the expression of related genes in overlapping domains may account for the lack of a mutant phenotype in regions where the mutated gene is normally strongly expressed. A possibility to investigate the redundancy problem is the generation of double (or, if necessary, multiple) knock-out animals. This has been demonstrated for two members of the *myo*D family of myogenic transcriptional regulators: *Myo*D and *myf*-5. Single knock-out mice for these genes develop fairly normal amounts of muscle (Rudnicki *et al.*, 1992; Braun *et al.*,

1992) whereas in double-knock-out animals no muscle is formed (Rudnicki *et al.*, 1993). In contrast, *Il-2* and *Il-4* double-knock-out animals have not exhibited any cumulative phenotype, indicating functional independence of IL-2 and IL-4 (Sadlack *et al.*, 1994).

Another important finding with knock-out mice is the fact that the genetic background of the induced mutation might affect the phenotype of the homozygous mutant mice. Ramirez-Solis *et al.* (1993) reported incomplete penetrance and variable expressivity of a *hox*b-4 (*hox*-2.6) mutation in a 129SvEv/C57BL/6J hybrid background compared to a complete penetrance of the induced mutation in an inbred 129SvEv background. Harvey *et al.* (1993b) demonstrated the alteration of the spectrum of tumours developed in *p53*-deficient mice with different genetic backgrounds. These effects might be due to polymorphic modifier genes in different mouse strains.

4.2.2 Tissue-specific Knock-outs

Mice with constitutive knock-outs lack a particular gene product in all tissues and during ontogeny. This may be problematic from two points. If a gene product is missing throughout the earliest stages of ontogeny, compensatory mechanisms may be built up which may not easily by mobilized when the gene function is lost in the adult stage only. Second, a missing gene function may be lethal early in development. In this case, it will be impossible to study the effect of absence of the gene in later stages or in the adult organism. These problems can partially be overcome by strategies to create animals with gene deficiencies in part of their tissues only.

Cells with a double knock-out of a particular gene may be studied in a chimeric situation. In this respect, an elegant method, named RAG-2-deficient blastocyst complementation, to study the role of a target gene product in T- and B-lymphocyte development, has been described by Chen *et al.* (1993). Injection of normal ES cells into RAG-2-deficient blastocysts will lead to the development of a normal population of exclusively ES-derived lymphocytes in the chimeric animal, since RAG-2-deficient mice lack mature B- and T-lymphocytes. Using ES cells with homozygous mutations, RAG-2-deficient blastocyst complementation could provide a physiological assay to directly determine the potential role of almost any gene in the development and/or function of lymphocytes in the chimeric animal.

Even more elegantly, site-specific recombinases may be used to structurally disrupt a gene which had previously been provided with recombinase-specific sites, for example targeted to intronic sites (see also Section 2.2.4). Deletion of flanked exons, and consequently gene inactivation, will however

be restricted to tissues where a transgene encoding the recombinase is active (Orban *et al.*, 1992). As transgene expression usually is not fully penetrant, this approach may be problematic for cells and organs with a considerable regeneration capacity, such as the bone marrow or the liver, because the wild-type cells most likely will displace the transgenic cells.

5 **Pharmacological Models**

5.1 IDENTIFICATION OF RELEVANT DRUG TARGETS

Many highly successful therapeutic drugs have been selected and developed by entirely empirical approaches. For many of these drugs the molecular drug target, the drug receptor, and the exact mode of action have not been elucidated. However, understanding the specific targets of action may be critical in differentiating therapeutic efficacy from undesired drug-related toxicity, but also for the identification of agents with greater specificity.

This shall be illustrated by summarizing the characterization of the immunosuppressive effects of cyclosporin (CsA, Sandimmune), FK506 and rapamycin (for reviews see Morris, 1991; Schreiber and Crabtree, 1991; Bierer *et al.*, 1993; Kunz and Hall, 1993). These agents bind endogenous intracellular receptors, termed immunophilins, and for CsA and FK506 the resulting complex interacts with the protein phosphatase calcineurin to exert the immunosuppressive effect, most notably on T-cells. The agents block T-cell activation by interfering with the transcription of a number of cytokine genes. All of these immunosuppressive agents interfere with the interleukin (IL-2) pathway, CsA and FK506 by a different mode of action to rapamycin (Bierer *et al.*, 1990; Dumont *et al.*, 1990). IL-2 is considered so central to T-cell activation that much effort has been concentrated on elucidating the IL-2 pathway. Recent findings with IL-2-deficient knock-out mice, however, put the crucial importance of IL-2 in the establishment of an *in vivo* T-cell responses into question (Kündig *et al.*, 1993). In these IL-2-deficient mice, primary and secondary cytotoxic T-cell responses against vaccinia and lymphocytic choriomeningitis virus were within normal ranges. In contrast, *in vitro* secondary antiviral T-cell response were absent unless IL-2 was added, confirming the crucial role of IL-2 *in vitro*. However, the normal *in vivo* immune responses in IL-2-deficient mice questions the importance of IL-2 as defined by *in vitro* studies. Contrary to expectation, the development of T- and B-cells lineages is also not noticeably disturbed in IL-2/IL-4 double deficient mice (Sadlack *et al.*, 1994). These recent findings may indicate that immunosuppressive agents exert their effect by actions on processes independent of the IL-2 pathway. The understanding of the *in vivo* mechanisms responsible for T-cell activation ultimately may lead

to the definition of alternative drug targets and the identification of immunosuppressive drugs of greater specificity and reduced toxic side effects (Bierer *et al.*, 1993; Schreier *et al.*, 1993).

In summary, genetically modified animals are instrumental in understanding complex *in vivo* interactions, in the normal and the pathological forms. This knowledge is indispensable for the identification of drug targets for the intervention with not yet understood processes as well as with diseases with undefined onset and slow progression, as for instance autoimmune diseases or Alzheimer's disease.

5.2 MODELS FOR DRUG TESTING

Among the many transgenic disease models, relatively few have been used to evaluate drug-mediated therapeutic interventions. These are listed in Table 2. Most of the models exhibit viral or human drug receptors and are described in the following sections.

5.2.1 Viral Drug Receptors

Viral diseases remain elusive targets for pharmacological intervention. Although many aspects of viral replication and propagation can be studied *in vitro*, testing for the efficacy of candidate antiviral drugs is greatly facilitated by the availability of relevant animal models (Field and Brown, 1989). Several transgenic animals have been established in order to study aspects of human viral diseases. Three different concepts have been realized:

(1) expression of a viral receptor to make the transgenic mice susceptible for human virus infection;
(2) expression of the complete proviral genome in order to mimic the pathologies observed in patients;
(3) expression of isolated viral genes in order to dissect the viral genome with regard to pathology.

For instance, the human poliovirus receptor has been overexpressed in a wide range of tissues in transgenic mice (Ren *et al.*, 1990; Koike *et al.*, 1991). Inoculation of these mice with poliovirus resulted in viral replication in the central nervous system and in the development of a disease analogous to paralytic poliomyelitis.

To study HIV-related pathogenesis, transgenic mice harbouring the complete HIV proviral genome have been generated (Leonard *et al.*, 1988). These mice developed a fatal disease reminiscent of human AIDS, and infectious virus could be isolated from skin, spleen and lymph nodes. HIV is

TABLE 2

Transgenic models for drug-mediated therapeutic interventions

Transgene (species)	Model	Drug(s)	Reference
mren-2 (rat)	Hypertension	Captopril	Ohkubo *et al.* (1990), Barrett and Mullins (1992b)
mren-2 (rat)	Hypertension	DuP753	Bader *et al.* (1992)
hAOGEN, hrenin infusion (rat)	Hypertension	Ro42-5892	Ganten *et al.* (1992)
hMDR1 (mouse)	Multiple drug resistance	Quinidine	Mickisch *et al.* (1991)
Type II glucocorticoid receptor antisense (mouse)	Depression (endocrine changes)	Desipramine	Pepin *et al.* (1992b)
HIV-1-tat/HIV-1-LTRhαAT reporter gene (mouse)	AIDS	Antiviral drugs	Mehtali *et al.* (1992)
HIV-1-*rev*/HIV-1-RREp24 (mouse)	AIDS	Antiviral drugs	Unpublished
Il-2-lacZ reporter gene (mouse)	Immune suppression	Cyclosporin A	Brombacher *et al.* (1994)
cftr –/– (mouse)	Cystic fibrosis	Somatic gene therapy	Smithies (1993), Rosenfeld *et al.* (1992)

a complex retrovirus, being expressed as a single transcriptional unit, but regulated by a multitude of *cis*-acting control elements as well as viral and cellular *trans*-acting proteins, most notably the viral Tat and Rev proteins (for review see Kräusslich, 1992). Expression of the viral trans-activator Tat in transgenic mice induces skin lesions resembling Kaposi's sarcoma (Vogel *et al.*, 1988).

Regarding the crucial role in controlling viral gene expression, Tat and Rev are promising targets for drug-induced blockage of virus propagation. Binary transactivator systems for both regulatory proteins are being used *in*

vitro for screening purposes. A Tat-controlled system has been introduced in a double transgenic mouse. In this model an LTR-CAT (long terminal repeat-choline acetyl transferase) reporter gene is activated by Tat expressed in the eyes of animals (Khillan *et al.*, 1988). Alternatively, a binary transgenic mouse system expressing the HIV-*tat* gene exclusively in lymphocytes has been described by Mehtali *et al.* (1992). In this system a reporter gene encoding a secreted variant of human α1-antitrypsin (α1-AT) readily permits the quantitative determination of plasma levels of human α1-AT in double transgenic animals. Such mouse models might serve to test the *in vivo* efficacy of LTR- or Tat-specific inhibitors. A Rev-controlled transgene expression has recently been observed in lymphocytes of double transgenic mice (unpublished observations). Also these mice will serve for the *in vivo* testing of potential Rev inhibitors. Such mice will allow extensive pharmacological studies using a viral drug target but avoiding the handling of virally contaminated animals.

5.2.2 *Humanized Drug Receptors*

Potential obstacles to the extrapolation of pharmacological data from animals, in particular laboratory rodents, to humans are not only, for example, differences in biochemical pathways, differences in organ systems and physiological processes, but also structural differences at the molecular level. These structural differences may, for instance, drastically influence the interaction of a potential antagonist with receptors of rodent versus human origin. To overcome structural differences transgenic techniques can be used to introduce drug targets of human structure into rodents, either on wild-type or on a knock-out background. In a more sophisticated design, by using gene targeting approaches, murine gene(s) might be directly replaced by human sequences (Stacey *et al.*, 1994). Although this concept has not yet been widely applied, one example illustrates the potential of animal models with humanized drug targets. Human renin and angiotensin genes were expressed in transgenic rats (Ganten *et al.*, 1992). The human enzymes did not cross-react with the rat renin–angiotensin system and vice versa. Whereas a murine renin transgene in rats caused a fulminant hypertension (Mullins *et al.*, 1990), the human renin transgene did not, despite qualitatively similar renin expression patterns. The unique species specificity of the renin substrate reaction is advantageous for developing and testing human specific renin inhibitors; e.g. infusion of human renin in human angiotensin transgenic rats led to an increase of angiotensin II and an elevation of blood pressure. This could be antagonized by the human-specific renin enzyme inhibitor Ro42-5892 (Hoffmann-La Roche). Rat renin also elevated blood pressure and rat angiotensin II, however, this effect was not antagonized by the human renin inhibitor. Another example are mice transgenic for the

human multidrug resistance gene *MDR*1, encoding the cell surface multidrug transporter P-glycoprotein. Mice have been created that express P-glycoprotein in their bone marrow, which become resistant to the leukopenia induced in non-transgenic animals by chemotherapeutic drugs (Galski *et al.*, 1989; Mickisch *et al.*, 1991). Bone marrow resistance in MDR1 mice is reversed significantly by simultaneous administration of known multidrug transporter inhibitors such as quinidine. These mice should prove valuable as a rapid test system to determine the efficacy of anticancer agents, especially those that reverse multidrug resistance (see also Table 2).

The coexistence of a rodent and a human enzyme–substrate or receptor–ligand system may in many cases lead to interferences due to cross-reactions. Therefore, to test for drug efficacy, the murine elements will have to be removed by gene disruption. In this case, it has to be demonstrated that the human molecules can functionally replace the murine analogues. Rodents with humanized drug receptors may speed up the *in vivo* testing of therapeutic agents and particularly replace testing with primates.

5.3 MODELS TO PROBE FOR DRUG-INDUCED SIDE EFFECTS

The drug-induced side effects to be discussed in this section are toxicity, oncogenicity and genotoxicity. Any of these effects usually prevents further development of a drug, therefore rapid *in vitro* test systems have been developed to screen for drugs with severe side effects. A second test series is usually carried out using rodents. Subtle side effects may necessitate repetition of the experiments using larger numbers of animals in order to statistically establish the findings. Transgenic animals sensitized due to a genetic predisposition might reduce the duration of the test phases as well as the number of animals used. Based on this concept, several transgenic animals have been generated and evaluated for use in drug testing.

Mice carrying one disrupted allele of the porphobilinogen deaminase gene, encoding the third enzyme in the haeme biosynthesis pathway, are currently being tested for their capacity to metabolize potentially toxic drugs (Lindberg *et al.*, 1993). At the same time, these mice are being evaluated for their validity as a model for human acute intermittent porphyria.

Several transgenic mice harbouring oncogenes, so-called oncomice, have been proposed as sensitive indicator organisms for oncogenic compounds. Breuer *et al.* (1989) described a transgenic mouse line overexpressing the *pim*-1 oncogene in lymphocytes. These mice are predisposed to develop T-cell lymphomas, but only to the extent that about 10% of the mice develop a lymphoma within 240 days. The mice were tested for their susceptibility to *N*-ethyl-*N*-nitrosourea (ENU), a chemical carcinogen. After a single low dose of ENU nearly all transgenic mice developed T-cell

lymphomas within 200 days. These mice are currently being evaluated as sensitive test animals to assess the oncogenic potential of different chemical compounds. Transgenic mice expressing oncogenes in skin or mammary tissue have been tested for their sensitivity to chemical induction of skin or mammary gland tumours (Spalding *et al.*, 1993). Alternatively, mice with an induced disruption of a tumour suppressor gene, e.g. *p53*, may serve as sensitive indicator animals (Harvey *et al.*, 1993a). For introduction in a toxicity programme, indicator strains to test for the oncogenic potential of chemical agents ideally should exhibit a low spontaneous frequency of tumour development and a transgene-expressing set of cells sensitized towards different classes of carcinogenic agents. Such validated strains might drastically reduce the number of animals necessary to establish drug-induced oncogenic side effects.

With regard to testing for genotoxic effects, a number of transgenic animals harbouring reporter constructs flanked by bacteriophage lambda sequences have been established. The bacteriophage sequences allow the rapid recovery and recloning of indicator transgenes, usually bacterial *lacZ* sequences, from mutagen-treated organs (Gossen *et al.*, 1989; Provost *et al.*, 1993). These systems allow the fast recovery of *in vivo* induced gene mutations and the direct correlation of mutational mechanisms with, for example, cancer or ageing (for review see Gossen and Vijg, 1993). Again, testing will be possible with significantly reduced numbers of animals.

In addition to the above models, transgenic animals offer the possibility for investigating the potential toxicity of protein or peptide drugs. In this respect, transgenic animals are suitable to study the effects of chronic exposure and of ectopically expressed protein. Examples where inappropriately expressed proteins have resulted in significant toxicity include mice transgenic for granulocyte–macrophage colony stimulating factors (Lang *et al.*, 1987), transforming growth factors (Sandgren *et al.*, 1990; Jhappan *et al.*, 1990; Matsui *et al.*, 1990) and IL-4 (Lewis *et al.*, 1991).

6 Outlook

For the next decade, molecular biology will remain the driving force for the ongoing revolution in biology, medicine and pharmacology (Pyeritz, 1992). Molecular biology has provided fine analytical tools to inventorize the molecular make-up of cells and organisms. In particular, genetic engineering allows refined molecular mechanisms by introducing genetic modifications into cells and animals. Therefore, currently, our insight into complex biological systems is growing exponentially. Moreover, methods to modify the genetic make-up of higher organisms are being refined with breathtaking speed. The shortcomings of the currently available methods may soon be overcome. Targeted insertion of transgenes will allow precise control of

tissue specificity of expression. Inducible trans-activator or recombinase systems may allow the spatial and temporal control of transgene expression or rearrangement, respectively (see Sections 3.1.4 and 4.2.2). The application of YAC-based methods may allow the insertion of complete gene clusters and eventually the replacement of large portions of the genome of the target cell. All this will lead to the establishment of ever more refined animal models to study complex *in vivo* systems, to understand genetic components of complex diseases and to study pathogenesis. The insight will lead to the definition of relevant drug targets, thus enabling a rational and efficient development of diagnostic, preventive or therapeutic intervention, be it drug or gene mediated. Empiric approaches to drug development will increasingly be replaced by selectively improved methods. As outlined in the present review, transgenic systems as pharmacological tools are of practical use with regard to several aspects:

(1) Genetically modified animals serve as disease models for human diseases.
(2) Relevant drug targets can be identified by stepwise modifying of candidate genes.
(3) Relevant drug receptors are replaced *in vivo* by human structures.

TABLE 3

The various types of transgenic animals relevant to a rational drug-finding and drug-development process

Identification of relevant drug targets, selection of drug receptor	Transgenic animals to probe complex *in vivo* systems, disease models
⇩	
In vitro screenings for potential drugs	
⇩	
Tests for *in vivo* efficacy	Transgenic animals with viral or humanized drug receptor
⇩	
Drug testing for toxic, oncogenic and genotoxic potential	Metabolic mutants, oncomice, transgenic mice with indicator genes for genotoxic effects

(4) Introduced mutations affecting drug metabolism allow the study of drug metabolism, biotransformation and eventual undesired toxic side effects.
(5) Models with genetically induced sensitivity to oncogenic transformation serve as sensitive indicators of carcinogenic agents.
(6) Transgenic animals carrying easily recoverable indicator genes serve as *in vivo* systems to rapidly assess the genotoxic potential of a substance.

In Table 3 the different models are listed with regard to their potential contribution to a rational drug discovery and development process.

In our opinion, the most profound impact will emanate from the insight and understanding of some of the highly complex *in vivo* systems. Despite the difficulty of applying linear genetic thinking to complex interactions (Strohman, 1994), refined transgenic animals will help to elucidate many of the not yet understood biological processes. A better understanding of the intricate genetic and epigenetic control mechanisms governing normal development as well as tissue homeostasis in the adult will eventually lead to the design of refined interventions to support the systems in regaining control after onset of disease processes.

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Xenobiotic-metabolizing Human Cells as Tools for Pharmacological and Toxicological Research

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1 Introduction

Metabolism often plays a central role in the efficacy of a drug and/or the toxicity associated with exposure to a drug or other xenobiotics. Metabolism may convert a prodrug to the pharmacologically active form, and may be a primary determinant of peak plasma concentration and half-life. Alternatively, competition for metabolism may be the mechanism for a drug–drug interaction.

The primary purpose of enzymes involved in xenobiotic metabolism is the conversion of a foreign compound into derivatives which are more water soluble and hence more easily eliminated from the body. Xenobiotic metabolism is typically divided into two phases. Phase I metabolism involves functionalization, or the introduction of a functional group for subsequent conjugation by Phase II enzymes. The cytochrome P450 class of mixed function oxygenases are the principal enzymes involved in Phase I metabolism (Gonzalez, 1988; Guengerich, 1988). However, other enzymes, such as flavin monooxygenases (Ziegler, 1988; Cashman *et al.*, 1992), may also play a role. Phase II metabolism usually involves conjugation with a highly water soluble moiety such as sulfate, glutathione or glucuronic acid which further increases the water solubility of the xenobiotic.

The cytochrome P450 system is the principal enzyme system for the metabolism of lipophilic xenobiotics. It is a heme-containing, membrane-bound, multi-enzyme system which is present in many tissues *in vivo* but is present at the highest level in liver. A coenzyme, cytochrome P450 NADPH oxidoreductase (OR), is essential for P450 catalytic function and cytochrome b_5 may stimulate catalytic activities of some enzymes. In human liver, it is estimated that there are 15–20 different xenobiotic-metabolizing cytochrome P450 forms. A standard nomenclature, based on relatedness of the amino acid sequences, has been developed (Nelson *et al.*, 1993). The most recent

nomenclature for the different human cytochrome P450 forms will be used in this chapter. Certain P450 forms are known to be polymorphic in humans and some are regulated in response to exposure to environmental agents. Competition for metabolism by some P450 forms is the mechanism of some clinically significant drug–drug interactions.

Identification of the human enzymes involved in the metabolism of a series of investigational compounds (e.g. candidates for drug development) can help direct the choice, among the alternatives, of the best investigational compound to take into development, can help focus *in vivo* studies to specific areas of concern and can help elucidate the mechanisms for interindividual variability in response or toxicity. A major challenge is to understand the role human cytochrome P450 forms play separately and together in overall xenobiotic metabolism. Because of the central role of cytochrome P450 enzymes in drug metabolism, and the unique difficulties in obtaining versatile mammalian cell systems to study xenobiotic metabolism, this chapter will primarily focus on cytochrome P450 expression in cell culture.

A variety of different approaches have been developed to study form specificity in cytochrome P450-mediated metabolism, including use of human tissue fractions, whole cells, tissue slices and cloned/expressed enzymes. These different approaches can be divided into two classes: (1) primary (or fresh) tissue-based and (2) long-term cell culture-based (established cell lines or cDNA expression). Because of the central role of cytochrome P450s in drug metabolism and also in carcinogenesis, considerable effort has been devoted by many laboratories to the development of model systems to study human P450 metabolism (for reviews see Gonzalez, 1988; Guengerich, 1988; Gonzalez *et al.*, 1991a; Langenbach *et al.*, 1992). In recent years great progress has been made in the development of cell culture systems which contain xenobiotic-metabolizing capacities. This progress has been made possible through the use of recombinant DNA techniques. Complementary DNA (cDNA) encoding nearly every major human P450 form has been isolated and sequenced. These cDNAs have been heterologously expressed in a variety of systems including bacteria (Barnes *et al.*, 1991), yeast (Oeda *et al.*, 1985), mammalian cells (Doehmer *et al.*, 1988), insect cells (Tamura *et al.*, 1992) and intact organisms (Komori *et al.*, 1993). There currently appears to be no one "ideal" heterologous expression system. The usefulness and appropriateness of the expression system to be used is dependent on the intended application(s). The methods for cytochrome P450 expression have been discussed extensively in Waterman and Johnson (1991).

Most of the cDNA expression systems offer a standardized system suitable for routine use in the laboratory. The cDNA expression systems are stable, homogeneous and, importantly, make human enzymes as available as non-human material. These systems can provide, at relatively low cost,

valuable information about potential routes of human metabolism. Applications to the prediction of human metabolism dictate a requirement that the cDNA-expressed enzyme faithfully reflect the catalytic properties of the native enzyme.

In general, a relatively limited array of enzymes has been expressed in any one particular cDNA expression system. Therefore production of complete metabolite profiles with cDNA-expressed enzymes is still problematic. Also, there is incomplete knowledge as to the average contribution of one P450 enzyme to overall P450 content and metabolism. However, given the rate of progress in developing these systems, the generation of complete metabolite profiles from Phase I enzymes should become possible in the near future.

Established cell lines may natively contain the xenobiotic-metabolizing enzymes or these enzymes can be introduced via recombinant DNA or other methods. There are several reports of levels of xenobiotic-metabolizing enzymes in various cell lines (Weibel *et al.*, 1977; Diamond *et al.*, 1980; Tong *et al.*, 1983; McGregor *et al.*, 1991; Bayad *et al.*, 1991; Kulka *et al.*, 1993). Established cell lines have been found, in general, to have relatively low levels of a limited array of xenobiotic-metabolizing enzymes. Human tissue samples have higher levels of enzymes but are highly variable; levels of specific enzymes can vary due to exposure to inducing agents, polymorphisms or methods of handling the tissue. Use of human tissue still has the unique advantage that it is currently the only "complete" system and also provides important information regarding properties of the native enzymes which is invaluable in "validating" the catalytic properties of cDNA-expressed enzymes. The use of human tissue-based approaches to the analysis of human xenobiotic metabolism has been reviewed recently (Wrighton and Stevens, 1992) and will not be discussed in extensive detail here.

A focus of this chapter will be on human cell expression; human cells expressing human xenobiotic-metabolizing enzymes have the potential to be the most accurate *in vitro* model of prediction of toxic effects in humans. Human cell expression of human cytochrome P450 enzymes has been the focus of my laboratory and several other laboratories. Where appropriate and relevant, data from other, non-human cell systems and applications beyond xenobiotic (drug) metabolism will be discussed. Expression of individual enzymes should be more than a means to produce reagents containing single, active cytochrome P450 enzymes. It is desirable to utilize the enzyme in the context of the whole cell and thus more fully understand the role of the products of xenobiotic metabolism in the homeostasis of the host cell. In addition, some systems offer the capacity to introduce multiple enzymes which provides a means to more fully understand the dynamics of sequential or parallel multienzyme pathways.

2 *In vitro* systems

2.1 GENERAL CONSIDERATIONS

In vitro systems with varying levels of complexity have been used to study xenobiotic metabolism. These include purified proteins, subcellular fractions/cell lysates, intact whole cells, tissue slices and whole organ culture. Advantages and disadvantages of the approaches are summarized below.

2.1.1 Purified Proteins

Within the cytochrome P450 system, purification and reconstitution represented a critical step forward in the field (Lu and West, 1980). Purification led to a better appreciation of the large number of enzymes in the cytochrome P450 family, allowed assignment of specific catalytic activities to specific enzymes and allowed the production of specific antibodies for the quantitation and selective inhibition of specific P450 forms by Western blot and immunoinhibition, respectively. Purification from primary tissues is labour intensive, results in low yields of "pure" protein and there are often lingering doubts regarding whether the P450 preparation is truly pure and does not contain a comigrating enzyme. Reconstitution can be tricky and the lipid composition can affect catalytic activity of the P450 enzyme (e.g. Halvorson *et al.*, 1990). Purification from primary tissues has become almost unnecessary because expression of the appropriate cDNA in the appropriate P450 null background provides the equivalent of single P450 enzyme system.

2.1.2 Tissue Lysate/Tissue Fractions

Tissue lysate (or homogenates), post-mitochondrial supernatants and microsomes offer several practical advantages for the study of xenobiotic metabolism. The principal advantages are that the human tissues provide a "complete" system containing all the enzymes in ratios found *in vivo*, and tissue fractions are stable in relatively long-term storage. Within the different types of tissue fractions, microsomes provide an enrichment of the membrane-bound enzymes, and post-mitochondrial supernatants provide a means to study both membrane-bound and soluble enzymes. Tissue fractions are easily prepared from a variety of tissues including human liver and can be cryopreserved for several years. This allows detailed characterization of the tissue prior to use with xenobiotics of unknown routes of metabolism

and provides a level of convenience needed for a system which is to be used routinely. Characterization of a human liver microsome bank includes quantitation of the relative levels of the different human P450 forms by Western blot or with enzyme-specific substrates.

The homogenization of a primary tissue produces a preparation which contains the "averaged" properties of the individual cells which originally comprised the tissue. Moreover, the cofactor requirements and buffer compositions used with tissue fractions can vary substantially from physiological, or intracellular, conditions. Latency is introduced for some Phase II enzymes including UDP-glucuronosyl transferases. There are clearly different "optimum conditions" for different enzymes, yet *in vivo*, all enzymes must function under the same conditions. One issue which remains to be addressed is the magnitude of any bias which may be introduced when comparing the relative role of different enzymes under individual "optimum" conditions to the relative role under physiological conditions.

Cell or tissue lysates represent the crudest tissue fraction. Use of lysates is practical when the level of enzyme is relatively high. However, lysates have a tendency to form aggregates, which limits the maximum protein concentration which can be used and also limits the time for which the reaction to be studied is linear. Typically, protein concentrations of less than 3 mg/ml must be used and incubation time is limited to about 30 min. This can be an important limitation for substrates which are metabolized very slowly. In addition, cell fractionation is not limited to primary tissues. Fractions can be prepared from cDNA-expressing cell lines. This can provide a level of convenience to the researcher relative to the demands of maintaining multiple cell lines for extended periods of time.

Identification of the P450 form(s) responsible for the metabolism of a xenobiotic can be achieved by correlation of the rates of metabolism of the new entity with the rates of metabolism for marker substrates for specific enzymes in different liver specimens with the levels of the different P450 forms (for review see Wrighton *et al.*, 1993b). A typical approach involves the selection of multiple human tissue preparations with "high", "medium" and "low" levels of the enzymes of interest. The rate of metabolism of the new entity is assessed in these samples and correlation analyses are performed versus the different marker activities. The enzyme with the highest correlation with the metabolism of the new entity is likely to be the principal enzyme involved in its metabolism. Alternatively, or in addition to correlation analyses, the roles of specific enzymes can be analysed by selective immunoinhibition or chemical inhibition of different P450 forms. The use of chemical inhibitors has been reviewed recently (Halpert *et al.*, 1994).

However, correlation and inhibition studies in human liver microsome samples are inherently less sensitive than studies with individual enzymes.

With the former approach, one is measuring the reduction in the rate of a metabolism. With the latter approach, one is measuring the presence of metabolites above a zero background. As a practical matter, enzymes which contribute less than 20% to overall metabolism can be difficult to identify by correlation analysis or inhibition studies.

2.1.3 Whole Cells

Whole cells (for example, primary hepatocytes or genetically engineered cells) provide the simplest intact system for the study of xenobiotic metabolism (Ratanasavanh *et al.*, 1986; Daujat *et al.*, 1991: Berry *et al.*, 1992). Intact cells provide physiological concentrations of cofactors and ionic strength.

Primary human hepatocytes in particular have not yet proven to be compatible with cryopreservation (Utesch *et al.*, 1992), therefore studies must be performed when the tissue becomes available. Characterization of the enzyme composition of the tissue derived from an individual must be conducted in parallel with characterization of the "unknown" xenobiotic. This can lead to the devotion of considerable experimental effort to studies which, in the end, do not meet quality control criteria. Despite these limitations, human hepatocytes are uniquely suited for studies of cytochrome P450 regulation and also provide the only current system which maintains a balanced and physiological ratio of cofactors and individual Phase I and Phase II enzymes.

Whole cells have substantial advantages in applications to toxicology. However, many primary cells have a limited life span, and do not replicate in culture. This limits toxicology applications to short-term studies. Genetically engineered cells overcome this limitation. They represent a stable dividing population which permits longer-term studies.

2.1.4 Tissue Slices

Tissue slices and whole organ culture maintain many spatial aspects of the intact tissue and, like whole cells, maintain the linkage between Phase I and Phase II enzymes (Sipes *et al.*, 1987). Precision-cut tissue slices are more easily and reproducibly prepared than primary hepatocytes. However, viability limitations often restrict studies to a few hours' duration. Histological examination of the material after exposure to a xenobiotic is possible with tissue slices.

2.2 PRACTICAL CONSIDERATIONS

A number of practical considerations affect the choice of experimental approaches to address a specific question. In general, these practical considerations tend to have a greater impact on applications to toxicology (where specific endpoints may not be measurable in all cell types) than studies of xenobiotic metabolism (where the principal requirement is enzyme activity). However, applications to toxicology should be considered when making a choice of an experimental approach as specific toxicological issues may develop from the metabolism studies or in other aspects of the safety assessment processes.

Practical considerations include the following.

(1) The level of expression present in the *in vitro* system and how that relates to expression levels found *in vivo*. Enzyme concentration can affect the useful range which can be examined for many parameters, including concentration range, incubation time and suitable analytical methods. The slower the rate of metabolism, the higher the level of enzyme expression necessary to achieve detectable levels of metabolites. Very high (relative to *in vivo*) expression may lead to the generation of metabolites not expected to be found *in vivo* by forcing metabolism down a non-physiological pathway. If metabolism is to be detected via the loss of the parent compound, higher levels of P450 expression are needed.

A related consideration is whether the anticipated mechanisms of toxicity are based on binding to DNA or protein. Higher levels of P450 expression are generally needed to observe toxicity based on protein binding than to observe toxicity based on DNA binding.

The level of enzyme needed can influence the choice of preparation used for the study. Microsomal preparations from cell cultures allow the use of higher concentrations of active enzyme per unit volume than use of whole cells or cell lysates. The use of whole, viable cells allows the use of longer incubation times but at a lower enzyme concentration per unit volume. In addition, adequate oxygen transfer and nutrient concentrations are needed to maintain culture viability. These requirements impose limitations on cell concentration. In addition, microsomes cannot be efficiently prepared from all cultured cell types. We have found that standard microsome preparation procedures as used for human or rodent liver were unsuitable for isolating active enzymes from human lymphoblasts, and this appears to be a general property of cultured cell lines. Specific catalytic activities in microsomes were lower than for whole cell lysates. This loss of activity appears to happen in other mammalian cell systems which has led to the common use of whole cell lysates.With human lymphoblasts, shortening the length of

the centrifugation for sedimentation of the microsomes has resulted in preparations which retained high specific activity (Penman *et al.*, 1993). The general applicability of this approach to systems beyond human lymphoblasts remains to be determined.

(2) It is more desirable to measure toxic effects in the host cells expressing the P450 enzyme. This approach to toxicity studies is inherently more sensitive than alternative approaches such as coculture of P450-expressing cells and target cells or the use of extracellular metabolizing systems which require mass transfer of reactive metabolites from the metabolizing cell to the target cell. Such mass transfer is often inefficient and thus much higher P450 expression levels are needed to observe toxicity.

(3) The nature of the toxicological endpoint to be measured. Some endpoints are more readily measured in certain cell types or cell lines. For example, the human hepatoma cell line HepG2 forms colonies with very low efficiency. Therefore some genetic toxicology assays, such as the induction of gene locus mutations, cannot be easily measured in this cell line.

(4) The host cell line should be sensitive to the class of toxins. Some cell types and cell lines are resistant to certain classes to toxins. For example, mouse CYP1A2 was expressed in wild-type CHO cells and the cells were found to be insensitive to heterocyclic amine-induced genetic damage. Heterocyclic amines are known to be activated by CYP1A2. Significant induction of genetic damage was only observed when the cytochrome P450 was introduced into a DNA repair-deficient CHO cell (Thompson *et al.*, 1991).

(5) Potential complications due to the presence of cellular "baggage" enzymes in the host cell. Native P450 enzymes are present which may compete for, or interfere with, metabolism by the cDNA-expressed enzyme. Other native xenobiotic-metabolizing enzymes (transferases, hydrolases, etc.) might be present or absent. An example of the importance of other enzymes is provided by Wolfel *et al.* (1991). Rat CYP1A2 was expressed in V79 cells; however, aromatic amines, which are known to be activated by CYP1A2, were not mutagenic to CYP1A2-expressing V79 cells. The reason for the lack of mutagenicity was that the V79 cells used were substantially devoid of *N*-acetyl transferase activity. This conjugation step converts the CYP1A2-generated, *N*-hydroxyl metabolite to a more potent electrophile. When a V79 derivative cell line which had retained *N*-acetyl transferase activity was used for CYP1A2 expression, aromatic amine mutagenicity was observed. Human cells, relative to non-human cells, offer the advantage of containing only human enzymes. Therefore metabolism by endogenous, "baggage" enzymes represents potential human "real life" metabolism.

2.3 DESIRABLE SYSTEM CONFIGURATION

As discussed in the previous section, the most desirable system configuration is dependent on the experimental question to be addressed. However, the following general guidelines are appropriate.

(1) Human cell hosts are better than non-human cell hosts. Non-human cells have not been demonstrated to offer practical advantages regarding the maximum level of P450 expression which can be achieved. In addition, human cells contain human "baggage" enzymes which produce potential human metabolites. This avoids committing time and resources to identification of non-human metabolites. In addition, cell lines with low levels, or absent, endogenous cytochrome P450 are preferred.
(2) Use the system with the highest available expression levels. This will at the very least simplify analytical procedures and shorten incubation times. Levels of cytochrome P450 expression which can be quantitated spectrophotometrically allow more accurate quantitation of expression levels for comparisons among different P450 forms (on a nmol enzyme basis).
(3) Use the system with the greatest number of enzymes available. This will allow the development of as complete a picture of hepatic metabolism as possible.
(4) Use a system which allows the preparation of subcellular fractions. Microsome preparation provides a 5- to 10-fold enrichment in cytochrome P450 expression levels and also permits longer incubation times. This enrichment and concomitant increase in metabolizing capacity per unit volume may be important in detecting metabolism of slow turnover substrates.
(5) It is often desirable that the system has the capacity to introduce additional enzymes. This allows introduction of additional enzymes for the reconstruction of enzyme pathways or examination of the potential for protection from an observed toxicity by a Phase II enzyme.
(6) Measure toxic effects in the target cell expressing the cytochrome P450 enzyme. This approach is inherently more sensitive than use of extracellular metabolism systems or coculture approaches. Therefore one is more likely to observe an effect at physiological exposure concentrations.
(7) Use whole cells for metabolism studies. This avoids the use of non-physiological buffer and cofactor compositions.

2.4 POTENTIAL APPLICATIONS

In applying *in vitro* systems to questions of pharmacology or toxicology, it is essential to remain within and draw conclusions supported by the ex-

perimental capabilities of the system. *In vitro* systems are useful for understanding the mechanism of an *observed* toxicity (i.e. is a P450 generating an electrophile and, if so, which P450 is responsible?) and are useful for determining which human P450s produce which metabolites. But it is not currently appropriate to conclude, in the absence of *in vivo* data, that because a P450 is observed to generate an electrophile from a xenobiotic *in vitro*, and that P450 is expressed in liver, that the xenobiotic will be hepatotoxic. For example, other enzymes normally present *in vivo* but absent *in vitro* may serve to protect the host cell. Data from other systems are needed to complement the data from the cDNA-expressed enzymes and place these results into context.

Many applications of *in vitro* systems for new drug discovery, drug development and safety assessment are possible. The choice of the specific applications depends to a certain extent on the desired properties of the new drug, for example, increased metabolic stability, increased metabolic lability, avoiding polymorphic metabolism or drug–drug interactions which affect the safety and/or efficacy of related drugs, identification of metabolites (or, more importantly, pharmacologically active metabolites) and finally studies of the mechanisms of clinically observed toxicities.

The use of *in vitro* systems for studies of metabolic stability, metabolic lability, avoiding polymorphic metabolism or drug–drug interactions represents a "metabolic triage" as part of the decision-making process for determining which of several drug candidates, with comparable efficacy, should proceed to development. Studies of metabolic stability/lability are best performed with systems with complete complements of metabolizing enzymes, because *a priori* knowledge of the specific enzymes involved in biotransformation is often lacking. Therefore human tissue preparations or mixtures of cDNA-expressed enzymes are best used for this application.

Generation of potential human metabolites for structural identification prior to administration of the drug candidate to humans can be performed with either cDNA-expressed enzymes or tissue fractions. This allows identification of potential human metabolites and development of appropriate analytical methods prior to clinical trials. Generation and identification of pharmacologically active human metabolites early in the development process can be beneficial for obtaining appropriate patent protection.

Identification of the cytochrome P450 form(s) principally responsible for the metabolism of a drug is useful for predicting potential drug–drug interactions or excessive interindividual variability due to polymorphism in enzyme expression (First *et al.*, 1991; O'Reilly *et al.*, 1992; for review see Murray, 1992). Competition for or induction of metabolizing enzyme are causes of drug–drug interactions. Knowledge of the cytochrome P450 forms responsible for metabolism will allow focusing on clinical drug–drug interaction studies to other drugs known to be metabolized by or known to induce the same enzyme. There is growing concern among regulatory

agencies regarding drug–drug interactions (Peck *et al.*, 1993). Often, early in drug development, the structure of metabolites and appropriate analytical methods are unknown. Useful preliminary information regarding affinity for different enzymes can be developed very early in drug discovery/development through the use of inhibition studies with model substrates and cDNA-expressed enzymes.

In vitro systems are useful for hypothesis formulation for subsequent *in vivo* studies. *In vitro* systems can identify likely candidate enzymes and guide development of experimental approaches to examine the importance of the candidate enzymes in the aetiology of human disease. Fundamental questions, such as whether humans produce the potentially toxic metabolite and if this metabolite is toxic to a human cell can be answered, *in vitro*, without the need to expose humans to the xenobiotic. *In vitro* systems can be used to determine which enzymes are responsible for the conversion of a protoxin to its toxic intermediate, and which enzymes may efficiently deactivate the toxic intermediate or represent competing pathways of metabolism to a non-toxic species. With this knowledge, predictions can be made as to potential environmental factors, P450 inducers or inhibitors, or hereditary factors (polymorphisms) which may influence the balance between the pathways for specific individuals. Relatively good correlations of alkylating agent carcinogenicity between rodents and humans have been reported (Dedrick and Morrison, 1992). For compounds of unknown human carcinogenicity, *in vitro* systems should prove useful in establishing the relevance of a toxic effect observed in animals to potential toxicity in humans.

3 General Approaches to cDNA Expression

There are two general approaches to cDNA expression, transient expression and stable expression. Transient expression systems are typically based on a viral vector; the host cells are infected with cDNA-bearing virus and the cDNA-derived protein is produced. At some point a maximum level of cDNA-derived protein expression is obtained and the protein is harvested for use in incubations. Viral vectors often have cytopathic effects on the host cells which usually precludes analysis of xenobiotic-induced toxicity to the host cell. Stable expression systems can be based on integrating or episomal vectors. With both stable expression approaches, homogeneous, clonally derived populations of cells stably expressing the cDNA are identified and characterized. The properties, advantages and disadvantages of the two approaches are discussed below.

3.1 TRANSIENT EXPRESSION SYSTEMS

The first transient expression system for cytochrome P450 expression was the COS cell system which couples monkey cells with an SV40-based expression vector (Zuber *et al.*, 1986). The cDNA of interest is introduced into a plasmid expression vector under control of a heterologous promoter. The vector is introduced into COS cells via electroporation or another method allowing DNA uptake. The plasmid vector replicates within the cells and cDNA-derived protein accumulates within the cell.

The principal advantage of COS cell transient cDNA expression systems is that they provide a rapid means for producing catalytically active protein. The introduction of the cDNA into the vector is simple, based on rapid bacteria-based molecular biology. The vector DNA is then used to directly transfect the host cells. COS cell expression suffers from limitations in expression level (which tends to be low) and overall yield, since a limited number of cells can be transfected and only about 10% of the cells take up the transfected vector molecule. The practical aspects of COS cell expression are discussed in Clarke and Waterman (1991).

Other viral-based transient expression systems include vaccinia virus (vv)/HepG2 cells (Gonzalez *et al.*, 1991b) and baculovirus/insect cells (BEV; Gonzalez *et al.*, 1991c). Both of these systems offer substantially higher expression levels compared to COS cell expression but both of these systems are considerably less rapid. For both vv and BEV the cDNA is delivered to the host cell via an infectious virus. The production/purification of the cDNA-bearing virus is time consuming. The cDNA to be expressed is first introduced into a plasmid and the plasmid is transfected into host cells infected with virus. The plasmid is incorporated into the virus via recombination. cDNA-bearing virus must be identified and purified. Viral stocks are produced and titred. These stocks are used to infect host cells for cDNA-derived protein production.

In the case of BEV expression there are additional technical difficulties. The level of cDNA expression is much higher than the cellular capacity to provide either haeme or the NADPH cytochrome P450 oxidoreductase (OR) coenzyme necessary for catalytic activity. Therefore haeme, typically in the form of haemin, must be added to the culture media during P450 production. Such supplementation increases the fraction of cDNA-derived protein which contains haeme; however, a substantial fraction usually still remains as catalytically inactive apoprotein. The OR limitation has been addressed in two ways. The cDNA-expressed P450 protein can be reconstituted with purified OR (Buters *et al.*, 1994) or virus containing OR cDNA can be coinfected/coexpressed with virus containing the P450 cDNA (Tamura *et al.*, 1992).

All transient expression systems are limited in their applications to

toxicology. In both vv and BEV the expression period is brief (a few days) and is followed by destruction of the host cell by the virus, therefore xenobiotic-induced toxicity to the host cell cannot be measured. Due to differences in infection ratio among individual cells, expression on the cellular level is non-homogeneous, further complicating toxicological studies. In these transient expression systems toxicological investigations are usually limited to macromolecular binding studies.

3.2 STABLE EXPRESSION SYSTEMS

Stable expression systems offer homogeneous expression at the cellular level with the capability to integrate toxicity measurements into studies of xenobiotic metabolism. A variety of host cells with different tissues of origin or from different mammalian species have been used. A judicious choice of host cell allows the measurement of many toxicological endpoints. Compared to transient expression systems, it takes considerably more effort to develop cell lines stably expressing cytochrome P450 cDNAs. Additional effort is usually required to develop cell lines with sufficiently high expression levels to have a versatile system for the production of metabolites and for toxicology studies. In general, the levels of expression obtained in stable cell lines are somewhat lower than can be achieved in the better transient expression systems.

It is now usually easy to achieve expression levels of a few pmol P450/mg microsomal protein, a level substantially lower than that typically found in human liver. This level of expression allows studies with very sensitive enzyme assays, and toxicology studies involving DNA-binding electrophiles. It is considerably more difficult to achieve stable expression levels over 10 pmol/mg microsomal protein. This higher level is necessary for spectrophotometric quantitation of P450 levels and also allows applications to the study of metabolism and toxicity of many more potential substrates. Some stable expression systems offer robust expression levels; expression of 160 pmol P450/mg microsomal protein has been reported (Penman *et al.*, 1993).

Two types of vectors have been used for stable expression, integrating and episomal vectors. Within the category of integrating vectors, simple DNA transfection and retroviral infection have been used successfully. The integrating vectors provide a system where expression of the cDNA is maintained even in the absence of selection for the vector. The integration site in the genome and the number of integration events per cell can influence expression level and the stability of expression, therefore it is usually necessary to screen multiple clonal populations in order to find the one which is "best". The episomal system which has been used successfully is based on the OriP sequences derived from Epstein–Barr virus (Sugden *et*

al., 1985) and uses human B-lymphoblastoid cells as a host cell line. With the episomal system, the vector is not integrated into the cellular genome and selection for the vector is necessary for long-term stability. Vectors with independent methods for selection and different copy numbers are available which allow the introduction of multiple cDNAs expressed at different levels (Crespi *et al.*, 1990a, 1991c). The episomal system offers the advantage that expression levels and stability of expression from the vector are not influenced by integration site as they are with integrating vectors. Therefore, very little variability is usually observed among clonal isolates from bulk transfected cells, and the need to screen many clones for expression level and stability of expression is usually avoided.

3.3 PROPERTIES OF THE HOST CELL

As stated earlier, properties of the host cell are a relevant consideration, because the properties of the native cell line influence which applications are possible with the system. Important properties include the following.

(1) The background cytochrome P450 complement of the cell line and the conditions under which these enzymes are expressed. CYP1A1 is sometimes expressed in cultured mammalian cells upon treatment with appropriate inducers (Diamond *et al.*, 1980; Crespi *et al.*, 1985).
(2) Other enzymatic activities which are present in the cells, e.g. transferases, hydrolases, etc. which may also serve to metabolize the xenobiotic. In addition, the level of endogenous OR should be adequate for P450 catalytic activity. OR appears to be expressed in virtually all mammalian cell lines, however the levels can vary substantially (Sawada *et al.*, 1991). Alternatively, OR coexpression can be performed.
(3) The nature of the toxicological endpoints to be measured. For example, macromolecular binding or enzyme leakage can be measured in virtually any cell type; the induction of chromosome level events (aberrations, sister chromatid exchange and micronuclei) can be measured in any replicating cell line; while the measurement of gene locus mutations or malignant transformation is possible in a much more restricted array of cell lines.
(4) The growth requirements and properties of the host cell. Cells requiring foetal bovine serum will be more expensive to use than cells for which a cheaper serum source is adequate. Cells which grow in suspension are much easier to scale up to large cultures than cells which are anchorage dependent.
(5) Other properties may also be desirable, for example endogenous expression of a receptor, transporter protein or other process which may be the target for action of the xenobiotic.

4 Challenges

4.1 SYSTEM DEVELOPMENT

There are two challenges to developing a comprehensive panel of cells expressing human P450s. The first is the multiplicity of human cytochromes P450 expressed *in vivo*. There are 15–20 distinct, xenobiotic-metabolizing cytochrome P450 enzymes expressed in human liver, and each enzyme has to be regarded as a separate project. The second challenge is achieving expression at levels which are adequate for the intended studies.

An appropriate goal is to achieve expression levels for a particular cDNA-derived protein which are comparable to the average level in human liver. However, true parity is not always essential. P450-mediated protoxin activation is usually detectable at lower P450 expression levels, and the unlimited supply of cDNA-derived material allows the utilization of high protein concentrations for metabolite generation.

Based on our experience in human lymphoblasts, one needs at least 1 pmol P450/mg microsomal protein in order to observe toxic effects in a stably transfected cell line. In fact, protoxin activation is one of the most sensitive indicators of successful cDNA expression in mammalian cells, often detecting an effect at levels below detection by enzyme assay or Western blot. However, at these low expression levels the system has very limited applications to studies of xenobiotic metabolism. In order to have a system which is useful for drug metabolism, one needs at least 10-fold higher expression levels, or about 10 pmol P450/mg microsomal protein. At this level, primary metabolites of high or moderate turnover substrates are easily detected, particularly if radiolabelled material is used. This is about the minimum level necessary for spectrophotometric determination of P450 content. Few stable expression systems have achieved expression levels which permit spectrophotometric determination of P450 content. A system of greater utility for studying drug metabolism should contain about 50 pmol P450/mg microsomal protein. At this expression level secondary metabolites are readily detected, low turnover substrates can be studied and metabolism can often be measured by loss of the parent compound.

The expression levels (as P450 content) discussed above assume adequate, but not necessarily saturating, cytochrome P450 reductase levels, and reasonable linearity of the system (i.e. linear metabolism in microsomes or lysates for at least 30 min). If a system is linear for shorter time periods, higher expression levels are needed; if the system is linear for longer periods of time, lower expression levels are adequate.

Complicating the goal of achieving versatile expression levels is the variability in expression efficiency for the different P450 cDNAs. We have observed a nearly 100-fold range in expression levels for different P450s using vectors which are otherwise identical. Differences in protein expres-

sion level can be due to differences in transcription rate, mRNA stability, translation rate or protein stability, all of which can vary among different P450 forms. An initial transfection/expression study for cDNA expression can yield a cell line which can be versatile for most intended studies, or alternatively the low expression level can result in a cell line which is completely useless. In addition, some cDNAs appear to be quite refractory to expression in some systems, and some cDNAs appear to be expressed at adequate levels but the catalytic activity is much lower than should be expected.

4.2 DATA INTERPRETATION

The major advantage of an *in vitro* system is that it represents a simplified system which allows the experimenter to address questions which cannot be tested *in vivo*. These systems can allow analysis of activation or metabolism at the single enzyme level. They can test proposed pathways of metabolism or activation. Such studies are not practical with *in vivo* systems. The major disadvantage is that *in vitro* systems are a simplified system and the results can be easily over-interpreted. *In vitro* systems cannot model the pharmacokinetics or toxicokinetics of xenobiotic exposure *in vivo*. In addition, there may be other, unappreciated enzymes or factors which influence metabolism/toxicity *in vivo* which are not present in the *in vitro* system.

In vitro systems containing human xenobiotic-metabolizing enzymes can provide qualitative data, such as the human metabolites which may be produced *in vivo* and which enzymes are capable of producing these metabolites. When comparing quantitative aspects of metabolism among different cytochrome P450 forms in a cDNA expression system, the data can be interpreted in two contexts:

(1) metabolism per unit P450 enzyme, i.e. nmol product per nmol of that P450 form in the incubation;
(2) contribution of metabolism by a particular P450 to overall metabolism (or hepatic metabolism).

The first approach provides a simple interpretation of the capacity of a particular P450 form to metabolize a xenobiotic relative to other P450 enzymes. Comparison is straightforward, requiring knowledge of the expression level in the cDNA expression system and verification of linearity of the system. It may also be appropriate to adjust data for differences in OR levels if these levels vary for the different cDNA-expressed enzymes. Unless the enzyme specificity is absolute or nearly absolute, this approach cannot establish the "principal" involved in metabolism because all enzymes are not equally abundant *in vivo*.

The second approach establishes which enzyme is principally responsible

for metabolism by providing a correction for the abundance of the P450 being analysed relative to the other P450 enzymes expressed *in vivo*, but requires an analytical framework for extrapolation of the relative metabolic rates in the cDNA expression system to the relative metabolic rates *in vivo*. Methods for relation of data from cDNA-expressed enzymes to human liver microsomes are discussed below and applied in Section 7 of this chapter.

It is also desirable to extend the analysis from relative rates of metabolism to absolute rates which determine important *in vivo* parameters, such as clearance. Recently, a framework for extrapolation of *in vitro* clearance data to *in vivo* clearance has been proposed for drugs cleared principally by the cytochrome P450 system using the rat as a model (Houston, 1994). In this approach, data reported for *in vitro* clearance using rat liver microsomes or rat hepatocytes were transformed by a scaling factor which is based on the amount of microsomal protein (or hepatocytes) in the *in vitro* incubation relative to the comparable amount of material *in vivo*. A good *in vitro* to *in vivo* correlation was obtained, although the correlation was poorer both for *in vitro* data obtained with microsomes relative to hepatocytes and for rapidly cleared drugs relative to slowly cleared drugs. The best correlation was obtained for slow *in vivo* clearance drugs and with hepatocyte data. This report suggests that an analogous framework for *in vitro* to *in vivo* extrapolation can be developed for humans using data from primary tissue preparations. Therefore, it seems reasonable to conclude at this time that, if means can be developed for extrapolation of data obtained from individual, cDNA-expressed enzymes to the balance of enzymes present *in vivo* (human liver microsomes for example), it should be possible, using the same methods for *in vitro* to *in vivo* extrapolation with human tissue preparations, to further extrapolate data from cDNA-expressed enzymes to the *in vivo* situation.

The better correlation with *in vitro* data obtained from hepatocytes (relative to microsomes) deserves further investigation. This difference may be due to a difference in catalytic activity *in vivo* relative to "optimal" conditions *in vitro*. It is possible that use of non-physiological buffer conditions will overstate or understate the contribution of a particular P450 to overall metabolism. For example, the role of cytochrome b_5/OR in catalytic activity is influenced by ionic strength (Schenkman *et al.*, 1994; Voznesensky and Schenkman, 1994) which is a variable in microsmal incubations but not in the intracellular space of a hepatocyte. This suggests that performing incubations of cDNA-expressed enzymes under conditions which mimic intact cells may substantially improve correlations.

The relative importance of different enzymes serves to define the "principal" enzyme(s) as that which is most important. The principal enzyme is that whose **activity** is primarily responsible for the metabolism of the xenobiotic. The significance of the "principal" enzyme is that modulations in the level of this enzyme are most likely to have an effect on the

biological response (plasma concentration, clearance, toxicity, etc.). The properties which determine which enzyme (of potentially many with overlapping substrate specificity) is the principal enzyme are the enzyme's affinity (apparent K_m), capacity (apparent V_{max}) and relative abundance. V_{max} and K_m both must be considered since humans are rarely exposed to concentrations of xenobiotics which are saturating to cytochrome P450 enzymes (i.e. consideration of V_{max} alone is inadequate). The V_{max} divided by the K_m defines the intrinsic clearance of the enzyme. Intrinsic clearance multiplied by the enzyme abundance defines the **contribution** of one enzyme to overall metabolism. The contribution of the individual enzyme divided by the sum of all contributions establishes the relative contribution. The enzyme with the largest relative contribution to metabolism is the principal enzyme. Contribution for an enzyme at any substrate concentration(s) can be described by the equation:

$$\text{contribution} = [(V_{max} \times S)/(S + K_m)] \times \text{enzyme abundance}$$

When the substrate concentration is less than 1/2 the lowest K_m, the relationship can be adequately described as:

$$\text{contribution} = [(V_{max})/(K_m)] \times \text{enzyme abundance}$$

The need to consider all the enzymes which metabolize a xenobiotic underscores the importance of having a comprehensive set of enzymes expressed in the heterologous system. For the purposes of this discussion, only cytochrome P450 substrates will be considered. For the cytochrome P450 system, the individual enzymes are better characterized (at this time) than with other, Phase II enzymes.

In theory, successful measurement of the three components of the lower equation, for all of the enzymes involved in metabolism, allows calculation of the contributions of the enzymes to overall metabolism. At this time, measurement of V_{max} can be problematic with cDNA-expressed enzymes and the *in vivo* abundances of all human enzymes have not been characterized.

The apparent K_m is readily measured for cDNA-expressed enzymes. To date, most K_m values for cDNA-expressed enzymes have shown relatively good agreement with K_m values for the same enzyme in human tissue preparations. In principal, apparent V_{max} (per unit enzyme) can be measured in any system which permits spectrophotometric determination of P450 content (alternatively quantitative Western blot can be used to quantitate P450 levels). However, in practice a host of other factors including levels of OR, levels of cytochrome b_5, ionic strength, organic solvents and membrane composition can affect apparent V_{max} of the enzyme. Careful experimentation can "optimize" the incubation conditions in order to maximize metabolite production from an enzyme during an incubation. However, it is unclear exactly how these "optimized" conditions

FIG. 1. The testosterone 6β-hydroxylase activity (expressed as turnover number) for cDNA-expressed CYP3A4 using yeast, bacteria and mammalian cell systems. Yeast-based expression with coexpressed human cytochrome P450 reductase and human cytochrome b_5 or native, yeast cytochrome P450 reductase and cytochrome b_5 (Peyronneau *et al.*, 1992). *E. coli*-GSH: *E. coli*-based expression where the enzyme was purified and reconstituted with and without the addition of glutathione (Gillam *et al.*, 1993). Human lymphoblast-based expression with endogeneous cytochrome P450 reductase (Crespi *et al.*, 1991a) and with coexpression of cytochrome P450 reductase cDNA (C. Crespi, unpublished observation). Vaccinia virus (vv)/HepG2 cell expression data from Buters *et al.* (1994).

relate to the activity of the enzyme in its native environment. The data in Fig. 1 illustrate the variability in enzyme turnover numbers which have been reported for CYP3A4 expressed in different systems. In general, mammalian cell expression yields higher turnover numbers than microbial expression. However, the enormous range (>1000-fold overall and >20-fold for "optimized") indicates that integration of data based solely on P450 contents is currently problematic.

In addition, the abundance of the different cytochrome P450 enzymes in human liver has not been fully defined. Recently, important progress has been made in the definition of total and relative abundance of different cytochrome P450 enzymes in human liver. Shimada *et al.* (1994) performed a quantitative Western blot analysis of 60 liver samples (30 Caucasians and 30 Japanese) for the levels of CYP1A2, CYP2A6, CYP2B6, CYP2C (individual forms not analysed), CYP2D6, CYP2E1 and CYP3A (individual forms again not analysed, although CYP3A4 should comprise the vast majority of CYP3A proteins). In this analysis, 72% of the total cytochrome P450 content was accounted for in the Western blot analyses. The variability among the individual samples was substantial, with the standard deviation typically 50–100% of the mean. The abundances of the individual

TABLE 1

Composition of human liver microsomes as reported by Shimada *et al.* (1994)

Parameter	Value (mean ± SD)
Total P450	344 pmol/mg
Cytochrome P4501A2	12.7 ± 6.2% of total P450
Cytochrome P4502A6	4.0 ± 3.2% of total P450
Cytochrome P4502B6	0.2 ± 0.3% of total P450
Cytochrome P4502C	18.2 ± 6.7% of total P450
Cytochrome P4502D6	1.5 ± 1.3% of total P450
Cytochrome P4502E1	6.6 ± 2.9% of total P450
Cytochrome P4503A	28.8 ± 10.4% of total P450
Amount accounted for by analyses	72 ± 15.3% of total P450

cytochrome P450 forms, as reported by Shimada *et al.* (1994), are given in Table 1.

The principal limitation of these data is the lack of definition of the individual forms for the CYP2C subfamily. Analysis of this subfamily has remained problematic due to high cross-reactivities of all of the distinct forms with most antibody preparations. In addition, Western blot analysis does not distinguish between active and inactive forms of the protein. Furthermore, distinct enzymes may have different affinities for coenzymes necessary for catalytic activity, which will serve to unlink abundance of the protein and its catalytic activity. Therefore the assumptions must be made that the ratios of active to inactive protein are similar for all forms and that all forms have similar affinities for coenzymes. These assumptions may not be justified. However, even with these limitations, the study of Shimada *et al.* (1994) contributes greatly to our understanding of relative enzyme abundance in human liver. In addition, the relative abundance data, coupled with the absolute P450 content (per unit protein) and the turnover numbers for enzyme-specific substrates (per unit protein), can provide an estimate of the turnover number for individual enzymes in the human liver membrane environment. This provides an important benchmark for evaluation of turnover number data from cDNA-expressed enzymes.

Since a "principal" P450 is defined by its catalytic activity, and activity per unit enzyme is variable for the same enzyme/substrate when the enzyme is expressed in different heterologous systems, it seems more appropriate to establish relative contributions based on enzyme **activities** in the incubations of cDNA-expressed enzymes and human tissue preparations.

The use of relative catalytic activity of the subject enzymes in different systems integrates any differences in V_{max}, active enzyme abundance and efficiency of coupling to coenzymes, between the systems, into a single term.

A critical parameter for the development and application of this approach is the use of enzyme-specific assays for the assessment of the catalytic activities of individual enzymes in human liver microsomes. Cytochrome P450 form-specific assays have been developed for *in vitro* measurement of most human cytochrome P450 forms. Many of these specific assays will be discussed in Section 6.

With this approach, the ratios of specific (or marker) catalytic activities in human liver microsomes to cDNA-expressed enzymes provide relative activity factors (RAFs). The RAF provides a means to relate the activity of the cDNA-expressed enzyme to the activity of the enzyme in its "native" environment. RAF is simply a ratio of enzyme activity or the two systems as used by the investigator. The RAF is calculated as:

$$\text{RAF} = \frac{\text{mean } V_{max} \text{ for specific substrate for human liver microsomes}}{V_{max} \text{ for specific substrate for cDNA expressed}}$$

For the calculation of a RAF, one takes the ratio of catalytic activities with a saturating concentration of substrate. While the use of *in vitro* intrinsic clearance for the two systems would also be appropriate, given that the K_m values are similar for cDNA-expressed and native enzymes, and RAF is a ratio, K_m does not affect RAF.

By using specific catalytic activities, the differences in V_{max} due to any factor are automatically compensated for in the calculation. Because this method establishes *relative* contribution, there is no *a priori* need to use the same units for both components of the RAF calculation as long as the same units are used within the cDNA-expressed enzyme data set and the human tissue data set. Moreover, knowledge of the P450 content in either the cDNA expression system or in human liver microsomes is not necessary in order to make an interpretation.

RAFs are applied to rates of metabolism at non-saturating conditions, or the *in vitro* intrinsic clearance (V_{max}/K_m) as measured for each of the cDNA-expressed enzymes which are found to metabolize the xenobiotic. The RAF-modified rate of metabolism for the individual P450 enzymes then provides the basis for determining which is the principal P450. The choice of "mean rate for specific substrate for human liver microsomes" is based on the large amount of data available for xenobiotic metabolism with human liver microsomes. Human liver microsomes are much more available than good-quality human hepatocytes. Therefore, even though data from hepatocytes correlated better with *in vivo* clearance, the limited data set for this system restricts its usefulness. In addition, given the large variability among tissues from different donors, the data from a few tissue samples are likely to be an inadequate sample. The mean rate of metabolism should be derived from a relatively large number of individuals. The mean rate can be determined by pooling microsomal samples from different individuals with

equal contributions on a unit protein basis, or taking the mean value from the individual samples from a panel of human liver microsomes.

The relative contribution of the individual P450 forms which have been found to be active towards a xenobiotic can be expressed as:

$$\text{relative contribution}_i = (\text{metabolism by form}_i) \times (\text{RAF}_i)$$

Metabolism in the above equation is defined as either the rate of metabolism at non-saturating substrate concentrations, toxicological parameters such as the initial slope of a dose–response curve in a toxicity assay or the slope of a mutation dose–response curve, or the intrinsic clearance for the enzyme. The P450 form with the highest relative contribution is predicted to be the principal P450.

There are several important caveats to this approach.

(1) This approach makes the assumption that any effects on V_{max} are independent of substrate, i.e. the rank order of rates of metabolism is the same for a particular cDNA-expressed enzyme and the same enzyme present in human tissue preparations, and any factor which affects V_{max} for one substrate also does so equally for other substrates. The validity of this assumption has not been rigorously tested but for most enzymes an appropriate set of test compounds is available.
(2) This approach determines *relative* contribution, not *absolute* contribution. Therefore it does not predict parameters such as *in vivo* clearance.

The prediction of the principal P450 may be all that is possible for toxicological endpoints because of the multifactoral nature of toxic effects such as carcinogenesis. However, it may be possible to develop enzyme-specific absolute scaling factors (ASFs) based on the ratio of *in vivo* intrinsic clearance for an enzyme-specific drug and *in vitro* intrinsic clearance for the same drug under the specific conditions of incubation. Intrinsic clearances are most appropriate for calculation of ASF values because the enzymes usually will not be saturated *in vivo*, and absolute drug concentration at the site of metabolism cannot be measured (i.e. where was the measurement made relative to the K_m), therefore K_m and V_{max} should be considered in both components of the ratio. Also, since the activity of the cDNA-expressed enzyme is being directly related to the activity of the enzyme *in vivo*, any effects on *in vitro* enzyme activity due to deviations from physiological conditions, ionic strength, cofactor concentration, coenzyme concentration, etc.) should be automatically compensated for in the analysis.

Finally, since an RAF approach can be used to interrelate the roles of individual enzymes, SAF values may only be needed for a subset of the enzymes shown to be active. These enzymes may be able to serve as reference points, with RAF-based calculations establishing roles of the other enzymes.

Two examples of application of this relative contribution approach are included in Section 7 of this chapter.

5 Criteria for Validation

Proper validation of cDNA-expressed enzymes is clearly essential for the appropriate use and acceptance of data from these systems. In addition to the discussion of this point contained herein, other discussions have been published by Remmel and Burchell (1993) and Goldberg *et al.* (1993).

Basic characterization of the expressed enzyme must be performed in order to assure that the catalytic properties of the cDNA-expressed enzyme faithfully reflect the properties of the enzyme as present in human tissue. While there is no *a priori* reason to expect different catalytic properties for a full-length, cDNA-expressed enzyme, unexpected results have been obtained upon occasion. There is currently a limited, but acceptable, data set of kinetic parameters determined in human tissue preparations with which to perform these comparisons. Since cDNA expression systems usually contain single enzymes, one needs kinetic data from either a purified human P450 preparation (whose properties can themselves be influenced by reconstitution conditions) or an enzyme-specific substrate coupled to human liver microsomes. Data from human tissue preparations using substrates which are not enzyme specific are of limited utility for validation studies unless the contribution of individual forms can be determined by appropriate kinetic plots. With specific substrates and human liver microsomes, turnover numbers are often problematic because the level of active enzyme is unknown or cannot be determined. V_{max} values or turnover numbers usually are only available for purified, reconstituted enzymes or cDNA-expressed enzymes. However, turnover numbers may be estimated using the data in Shimada *et al.* (1994). Turnover numbers obtained from purified reconstituted enzymes should be viewed with caution. While these enzymes are optimally reconstituted with lipid and OR, turnover numbers are usually only slightly higher than whole liver microsomes where the specific P450 represents only a fraction of the total P450. In general, one should expect turnover numbers from cDNA-expressed enzymes 2- to 3-fold higher than observed for purified/reconstituted enzymes.

5.1 METABOLISM APPLICATIONS

Characterization of the catalytic properties of the enzymes should focus on the same parameters which will be considered *in vivo*. The most important parameter is usually intrinsic clearance by the enzyme (V_{max}/K_m). Therefore the apparent K_m and V_{max} need to be verified for the expressed enzyme.

Verification of kinetic parameters is of critical importance if the primary sequence of the enzyme has been modified in order to achieve expression (as is often the case for bacterial systems). Certain P450 forms have been relatively easy to express (CYP1A2 for example) while others (CYP3A4) have been difficult to express in catalytically active forms. In addition, some enzymes have a relatively large number of specific substrates while others have a much more limited number.

Determination of the apparent K_m is simple for cDNA-expressed enzymes. Naturally, kinetic plots should indicate the presence of a single K_m. Experimental variables will likely introduce a 2- to 3-fold variation in K_m values between systems. Variations of more than 3-fold should be investigated further.

Measurement of V_{max} requires quantitation of P450 content by spectrophotometry (preferred) or by quantitative Western blot. Quantitative comparison of V_{max} data for cDNA-expressed enzymes to purified reconstituted enzymes provides a basis for determining the performance of the enzyme in the system. The data in Fig. 1 provide an illustration of the differences in turnover number which can be obtained in different systems. Data are graphed for CYP3A4-catalysed testosterone 6β-hydroxylase activity for yeast, *Escherichia coli*, human lymphoblast (with and without OR coexpression) and vv/HepG2 cell-expressed enzyme. The level of activity in yeast microsomes with native (yeast) OR and cytochrome b_5 was extremely low. The turnover could be substantially increased through coexpression of mammalian cytochrome b_5 and OR (Peyronneau *et al.*, 1992). *E. coli*-expressed CYP3A4 (with the amino terminus modified) was only active upon purification of the protein and reconstitution with OR and cytochrome b_5. The catalytic activity was very low. Surprisingly, the addition of glutathione to the incubation stimulated the enzyme (this effect did not occur in human liver microsomes; Gillam *et al.*, 1993). In contrast to the microbial systems, mammalian cell-expressed CYP3A4 has substantially higher turnover numbers (Crespi *et al.*, 1991a; Buters *et al.*, 1994). Therefore, while the expression levels in mammalian cell systems are lower than in microbial systems, part of this difference is compensated for by the higher intrinsic activity of the mammalian cell-expressed enzyme.

This analysis can be extended to estimated turnover numbers for CYP3A in human liver microsomes. Yamazaki *et al.* (1993) reported data for a panel of 18 human liver microsome samples. In this panel, the mean P450 content and testosterone 6β-hydroxylase activity were 307 pmol/mg and 1070 pmol/(mg min), respectively. By using the percentage of total P450 which is CYP3A in Table 1, 28.8%, a turnover number for CYP3A4 in human liver microsomes was estimated—12 per minute. The turnover numbers for CYP3A4 expressed in mammalian systems tend to be at or above this value while turnover numbers for CYP3A4 expressed in microbial systems tend to be below this value.

Another consideration is the rank order of rates of metabolism for different substrates for the same enzyme. At present there are insufficient data to determine if there are differences in rank orders of rates of metabolism for the cDNA-expressed enzymes from different systems relative to human liver enzymes. If rank orders of rates of metabolism for cDNA-expressed enzymes are indeed different from human liver microsomes, it will be nearly impossible to determine the "principal" P450 using the RAF approach.

5.2 TOXICOLOGY APPLICATIONS

Validation in the context of toxicology assays is more problematic. The traditional approach to validation of an *in vitro* toxicology assay is to develop a standard protocol and criteria for a positive response and then correlate the response in the *in vitro* assay to those observed *in vivo* in animals. The problem is that the human enzyme/human cell systems were developed because the animal systems do not adequately model human metabolism. Therefore animal data are largely inappropriate for validation of human enzyme-based systems. There are few data for human toxicity which are appropriate for making comparisons for P450-expressing cultured cells. It appears unlikely that such data will become available in the near future. Therefore at this time only a "component" approach to validation is possible. With this approach, the catalytic properties (apparent K_m, turnover number) of the enzyme are verified relative to data for the enzyme as it is present in human liver microsomes. This is the same validation discussed above for metabolism studies. The endpoint of the toxicology assay should also be validated. For example, if mutagenesis is studied, the mutants scored should be verified as being true mutants and it should be verified that they were induced by the treatment. In this way the properties of the two key components, the cDNA-expressed enzyme and the toxicological endpoint, are validated. More selective validations should become possible as the roles of specific human enzymes in clinical toxicities become better understood.

The use of cDNA-expressed cytochrome P450 enzymes permits the incorporation of additional control conditions. Cells bearing the vector only (without cDNA) can serve as a control to verify that activation is mediated by the P450 enzyme. In addition, the use of specific enzyme inhibitors can also verify that the P450 is mediating the observed toxicity, i.e. the addition of an enzyme inhibitor mitigates the effect. In this way, each cell line can serve as its own control. Finally, direct measurement of metabolites in the incubation medium for the toxicology assays provides additional support for a P450-mediated toxic effect.

6 State of the Art

In this section the properties and significance of individual cytochrome P450 forms and the state of the art for cDNA expression of each form are discussed. The focus of this discussion is on human cell expression of the human P450 forms. However, for many of these forms data for only one or two human cell expression systems are available. Where appropriate and where data are available, some expression data from other mammalian cell and non-mammalian cell systems are discussed.

One difficulty in framing this discussion is a lack of commonality in units for the expression systems. For example, the same substrate may not have been examined in all systems or activity may be expressed per mg total cell lysate protein, per mg cytosol-free cell membrane protein, per mg microsomal protein or per million cells. In this section, activity levels will be compared in the units originally reported. The following values, as determined in the human lymphoblast system, may be used to compare among the alternative methods of enzyme preparation: cytosol-free membranes provide about a 2-fold enrichment in activity, microsomes provide 5-fold enrichment in activity and there are about 7 million cells per mg total protein. These ratios may differ somewhat for other mammalian cell systems but they are unlikely to be off by more than 2-fold.

Prior to reviewing the significance and state of the art for expression of the individual human P450 forms, a brief summary of the two most commonly used human cell expression systems, vv/HepG2 cells and human lymphoblasts, is provided. In addition to these two systems, human P450 enzymes have been stably expressed in other mammalian systems including V79 cells, HepG2 cells, mouse fibroblasts, and other cell types. These data will be discussed within the context of the specific P450 forms expressed.

Vaccinia virus has been used successfully for the expression of a large number of mammalian cytochrome P450s. Vaccinia virus has a large genome which precludes direct insertion of the cDNA using standard recombinant DNA techniques, therefore the cDNA to be expressed is first introduced to a plasmid called an insertion vector. The vector contains the necessary element for propagation in bacteria, a promoter for the expression of the cDNA, flanking elements for the vv thymidine kinase (TK) gene and the bacterial β-galactosidase gene for colormetric identification of recombinant virus. The insertion plasmid and wild-type virus DNA are transfected into CV-1 cells for integration of the insertion vector into the vv genome. Identification of recombinant virus is made based on colormetric identification of product of the β-galactosidase gene and inactivation of the vv TK gene. Recombinant viruses are then plaque purified. Final verification of the recombinant virus is based on assay of the cDNA-encoded protein. Vaccinia virus can be used to infect a wide host range. Cytochrome P450 expression is

most commonly performed in HepG2 cells because this cell line is relatively rich in OR and good catalytic activities can be obtained without need for reconstitution with additional OR. The technical aspects of vv expression can be found in Gonzalez *et al.* (1991b).

Our laboratory has focused on development of human cells which express human P450 (Crespi *et al.*, 1990a,b,c, 1991a,b,c, 1993; Davies *et al.*, 1989; Penman *et al.*, 1993, 1994). Human B lymphoblastoid cells have been used because of the relative ease of culture and scale-up of this anchorage-independent cell type, and the availability of a flexible extrachromosomal vector system which works well in this cell line (Sugden *et al.*, 1985). Two means of selection for the vector have been used, resistance to hygromycin B and resistance to l-histidinol. Vector copy numbers vary depending on the means of selection (5 per cell for hygromycin B and 40 per cell for l-histidinol). The level of cDNA expression is determined by the strength of the promoter and the vector copy number. cDNAs encoding human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 have been transfected into the lymphoblastoid cells using the extrachromosomal vector system. Most cell lines have used the herpes simplex virus TK gene promoter, which is a strong promoter in this system, and a vector conferring resistance to l-histidinol.

The particular cell line used in our laboratory, AHH-1 TK+/− cells (Crespi and Thilly, 1984), is a versatile indicator cell line for a variety of *in vitro* toxicological end points (Crespi *et al.*, 1993; Crofton-Sleigh *et al.*, 1993). Therefore, P450-mediated metabolism can be readily related to toxic or genotoxic effects at the cellular level. This cell line has endogenous CYP1A1 which is detectable upon treatment with appropriate inducers. A unique strength of the system is the ability to express multiple cDNAs in the same cell. Up to five different cDNAs have been expressed simultaneously (Crespi *et al.*, 1991c).

6.1 CYP1A1

6.1.1 Properties

CYP1A1 appears to be expressed at very low levels (if at all) in human liver. Wrighton and Stevens (1992) report that only one of 50 human liver samples contained protein immunoreactive to an antibody raised against rat CYP1A1. Murray *et al.* (1993) used CYP1A1-specific and CYP1A2-specific antipeptide antibodies and found no evidence for CYP1A1 expression in human liver microsomes. However, McKinnon. (1991) reported detection of CYP1A1 mRNA in 11 of 23 human liver samples studied.

There is good evidence that CYP1A1 is inducible in extrahepatic tissues

by cigarette smoking and polycyclic aromatic hydrocarbons (PAH) exposure (Pasanen *et al.*, 1988). This protein has been detected by Western blot in human lung microsomes (Wheeler *et al.*, 1990) and has been partially purified from human lung tissue (Shimada *et al.*, 1992). CYP1A1 marker activities are 7-ethoxyresorufin *O*-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH), but in human liver, CYP1A2 is the primary contributor to EROD activity and multiple forms contribute to AHH activity. In human lung, levels of EROD are quite low, about 1 pmol product/(mg min), which is consistent with the low overall P450 content in human lung (~10 pmol/mg; Prough *et al.*, 1977). There is substantial overlap in immunogenicity and substrate specificity between 1A1 and 1A2 which has complicated form-specific analyses. Due to the low abundance of CYP1A1 in human liver and its low abundance in extrahepatic tissues (even in smokers), it seems unlikely that CYP1A1 will be the principal enzyme for the clearance of a xenobiotic. However, it is possible that CYP1A1 will be found to have a principal role in the metabolic activation of protoxins in specific target tissues.

Human CYP1A1 cDNA was first isolated by screening a cDNA library produced from TCDD-treated human breast carcinoma cells (Jaiswal *et al.*, 1985) and has been isolated from other sources (Quattrochi *et al.*, 1985; Kawajiri *et al.*, 1986).

6.1.2 cDNA Expression

Human CYP1A1 has been expressed in several of the heterologous expression systems including yeast, human lymphoblasts, V79 cells and human fibroblasts. The EROD activity in V79 cells expressing CYP1A1 is 50 pmol/(mg total protein × min) (Schmalix *et al.*, 1993). In human lymphoblasts, EROD activity of 155 pmol/(mg microsomal protein × min) was obtained (Penman *et al.*, 1994). EROD activity in human fibroblasts expressing CYP1A1 cDNA is 1.2 pmol/(10^6 cells × min) or approximately 8.4 pmol/(mg lysate protein × min) assuming 7×10^6 cells per mg lysate protein (States *et al.*, 1993). Human CYP1A1 has been expressed in COS-1 cells (McManus *et al.*, 1990). Unfortunately, EROD activities were not reported and expression levels cannot be quantitatively compared to other systems. The human lymphoblast system is the only mammalian system which has a sufficiently high expression level to permit spectrophotometric detection of P450 content. Human CYP1A1 expression in the vv/HepG2 system has not been reported. Monkey CYP1A1 has been expressed in Chinese hamster cells (Sawada *et al.*, 1993). Rodent CYP1A1 has been expressed and characterized in a variety of mammalian cell backgrounds including V79 cells (Dogra *et al.*, 1990), with vv vector using mouse and human cells as host (Battula *et al.*, 1987; Tsyrlov *et al.*, 1993), mouse

hepatoma cells (Puga *et al.*, 1990) and repair-deficient CHO cells (Trinidad *et al.*, 1991).

CYP1A1 has also been expressed in yeast (Eugster *et al.*, 1990; Sengstag *et al.*, 1994) and EROD activities of 223 pmol/(mg min) have been reported. The turnover numbers for EROD in yeast-expressed CYP1A1 (1.4/min) were substantially lower than observed for human lymphoblast-expressed CYP1A1 (7.6/min). Apparent K_ms were quite similar (92 nM and 87 nM in yeast and human lymphoblasts, respectively). Modified CYP1A1 has also been expressed in *E. coli* (Guo *et al.*, 1994). The expression level of CYP1A1 in *E. coli* (per mg membrane protein) was comparable to that obtained with human lymphoblasts (about 30 pmol/mg in both systems). The turnover number for EROD for *E. coli*-expressed CYP1A1 was quite low in isolated membranes, but could be increased to 8/min if the protein was purified and reconstituted. The apparent K_m for *E. coli*-expressed CYP1A1 EROD activity, 580 nM, was about 6-fold higher than that for the yeast- or human lymphoblast-expressed enzymes. It is not clear whether this difference is due to the base substitutions necessary to obtain expression in *E. coli*, or some other cause.

cDNA-expressed human CYP1A1 has been used to study the metabolic activation of PAH (McManus *et al.*, 1990; Schmalix *et al.*, 1993; Penman *et al.*, 1994), heterocyclic amine mutagens (McManus *et al.*, 1990; Probst *et al.*, 1992), aflatoxin B_1 (AFB), and the tobacco smoke nitrosamine, NNK (Penman *et al.*, 1994). As with rodent CYP1A1 and CYP1A2, human CYP1A1 is generally more active than human CYP1A2 towards PAH with CYP1A2 is generally more active than CYP1A1 towards heterocyclic and aromatic amines.

6.2 CYP1A2

6.2.1 Properties

CYP1A2 is the major CYP1A subfamily protein in human liver, because of the lack of CYP1A1 in human liver. Thus, many substrates for both CYP1A1 and CYP1A2 are in fact "CYP1A2 specific" in human liver microsomes. The purification of CYP1A2 protein from human liver has been reported (Distlerath *et al.*, 1985). CYP1A2 catalyses EROD, phenacetin *O*-deethylation, acetanilide 4-hydroxylation and caffeine 3-demethylation (Butler *et al.*, 1989). EROD is the most commonly used "CYP1A2-specific" assay. Caffeine 3-demethylation has been used as an *in vivo* probe of CYP1A2 levels (Visitisen *et al.*, 1991). CYP1A2 is inhibited by several quinolone antibiotics (Fuhr *et al.*, 1993). The theophylline analogue furafylline is a specific, mechanism-based inhibitor of CYP1A2 in

human liver (Sesardic *et al.*, 1990; Kunze and Trager, 1993; Clarke *et al.*, 1994).

CYP1A2 appears to play a major role in the metabolic activation of a variety of aromatic amine and heterocyclic amine promutagens/procarcinogens (Butler *et al.*, 1989; Aoyama *et al.*, 1989a). CYP1A2 levels appear to be modulated by environmental factors. CYP1A2 protein levels and catalytic activity are positively correlated with the amount of cigarette smoking (Sesardic *et al.*, 1988; Kalow and Tang, 1991). Omeprazole has been found to induce CYP1A2 levels in hepatocytes and variably *in vivo* in the liver of some individuals (Diaz *et al.*, 1990). CYP1A2 cDNA was first cloned by Jaiswal *et al.* (1986).

6.2.2 cDNA Expression

Human CYP1A2 has been expressed in several heterologous systems including bacteria, yeast, human lymphoblasts, V79 cells, vv/HepG2 cells and mouse hepatoma cells. EROD activity provides a basis for comparison among the various expression systems and with enzyme levels in human liver microsomes. The mean EROD activity in microsomes from h1A2v2 human lymphoblastoid cells (Penman *et al.*, 1994), cytosol-free membranes prepared from V79 cells expressing human CYP1A2 cDNA (Wolfel *et al.*, 1992) and the mean of several panels of human liver microsomes is given in Fig. 2. Human lymphoblasts have provided the highest-level mammalian cell expression of CYP1A2. Human CYP1A2 has also been expressed in COS-1 cells (McManus *et al.*, 1990); the expressed enzyme was found to activate

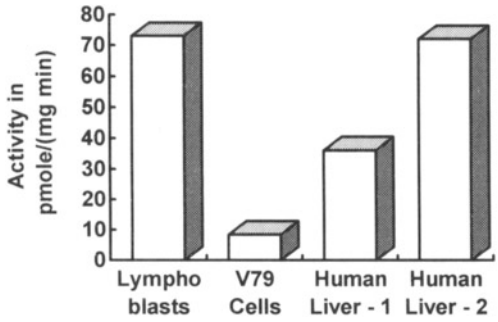

FIG. 2. 7-Ethoxyresorufin *O*-deethylase activity for cDNA-expressed CYP1A2 in human lymphoblasts (Penman *et al.*, 1994) and V79 cells (Wolfel *et al.*, 1992). Comparison to the mean levels for two human liver microsome panels (Human Liver-1: Murray *et al.*, 1993; Human Liver-2: Yamazaki *et al.*, 1993).

heterocyclic amines. However, due to the lack of data for a comparable substrate, the levels of expression are not easily compared to those achieved in other systems. Similarly, human CYP1A2 has been expressed in mouse hepatoma cells (Puga *et al.*, 1990). Acetanilide 4-hydroxylase activities were comparable to those observed in mouse liver microsomes. Rodent CYP1A2 has been expressed in several systems including several different mammalian cell lines (Battula, 1989; Wofel *et al.*, 1991).

Human CYP1A2 has been expressed in several microbial systems including bacteria and yeast (Sandhu *et al.*, 1994; Sengstag *et al.*, 1994; Guo *et al.*, 1994). As with human CYP1A1, the turnover numbers for *E. coli*-expressed human CYP1A2 are higher upon purification and reconstitution.

6.3 CYP2A6

6.3.1 Properties

CYP2A6 is the principal human coumarin 7-hydroxylase (Yamano *et al.*, 1990). In contrast, the rat CYP2A orthologues do not show substantial activity for coumarin 7-hydroxylation. Coumarin 7-hydroxylase is a sensitive, specific probe for the measurement of CYP2A6-derived catalytic activity. We (Crespi *et al.*, 1990b) and others (Pearce *et al.*, 1992) have found that the catalytic activity of CYP2A6 is markedly inhibited in phosphate buffers. Therefore, if phosphate buffers are used routinely, any contribution by CYP2A6 to overall metabolism may be underestimated. The use of different buffer compositions may be the cause of the large range of *mean* activity levels in panels of human liver microsomes (Fig. 3). All reports with high activity specified Tris buffers in the methods section. To date, CYP2A6 has not been found to have a prominent role in the metabolism of drugs, however CYP2A6 was recently reported to metabolize the non-steroidal aromatase inhibitor fadrozole (Walter and Hundal, 1994). CYP2A6 has been found to metabolically activate some nitrosamines (Crespi *et al.*, 1990b, 1991b) and CYP2A6-mediated metabolism has been found to contribute significantly to overall human hepatic metabolism of nitrosamines (Yamazaki *et al.*, 1992). CYP2A6 has also been found to metabolically activate AFB; however, this enzyme appears to be a low affinity form and is presumably a minor contributor to total human hepatic metabolism (Crespi *et al.*, 1991a). CYP2A6 has also been found to oxidize butadiene (Duescher and Elfarra, 1994) and to play a minor role in the activation of the anticancer agents cyclophosphamide and ifosphamide (Chang *et al.*, 1993). CYP2A6 and CYP2E1 often have overlapping substrate specificities, e.g. for nitrosamines and butadiene, and are sensitive

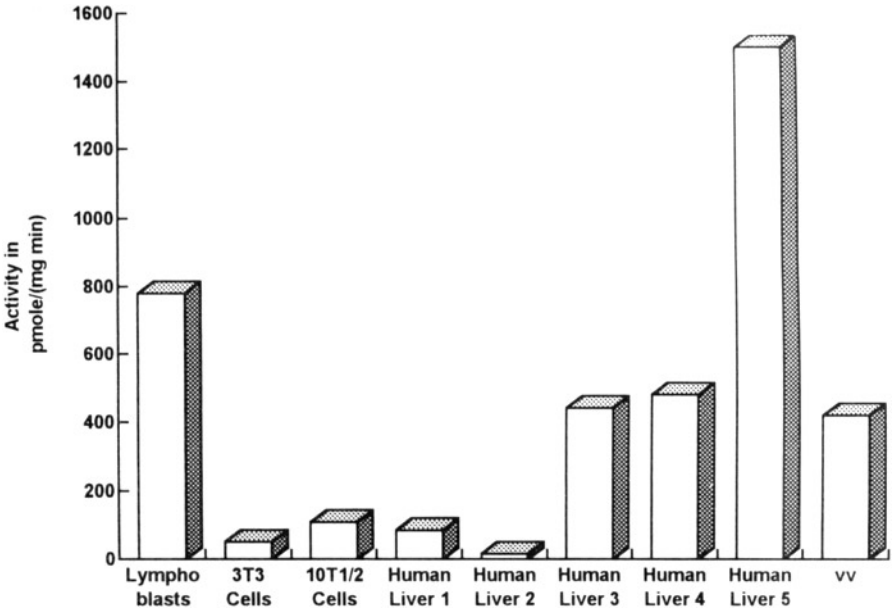

FIG. 3. Coumarin 7-hydroxylase activity for cDNA-expressed CYP2A6 in human lymphoblasts (Crespi *et al.*, 1990b, when microsomes are prepared according to Penman *et al.*, 1993), vv/HepG2 cells (Waxman *et al.*, 1991), 3T3 mouse cells (Salonpaa *et al.*, 1993) and 10T1/2 mouse cells (Tiano *et al.*, 1993). Comparison with the mean levels for five panels of human liver microsomes. Liver 1: Murray *et al.* (1993); Liver 2: Yamazaki *et al.* (1993); Liver 3: Wrighton *et al.* (1993b); Liver 4: Yun *et al.* (1992); Liver 5: Pearce *et al.* (1992).

to similar inhibitors (Halpert *et al.*, 1994). Therefore establishing the relative roles of CYP2A6 and CYP2E1 in human tissue preparations can be problematic.

The CYP2A6 enzyme was purified and reconstituted by Yun *et al.* (1992). They report that levels of catalytic activity vary more than 100 fold among 20 different human liver samples. They also report that CYP2A6 is a minor P450 comprising about 1% of total human hepatic P450. Shimada *et al.* (1994) report that CYP2A6 comprises 4% of total liver P450. If one uses the reported mean P450 content and coumarin 7-hydroxylase activity for a human liver panel [440 pmol/(mg min) and 317 pmol/mg respectively; Wrighton *et al.*, 1993b] and assumes that 4% of the total P450 is CYP2A6, then the turnover number for enzyme is 35/min. Such a turnover number is 17 fold higher than the purified reconstituted CYP2A6 (Yun *et al.*, 1992) but comparable to the turnover number for cDNA-expressed CYP2A6 (Fig. 4).

The complete CYP2A6 cDNA was isolated by Yamano *et al.* (1989b) and

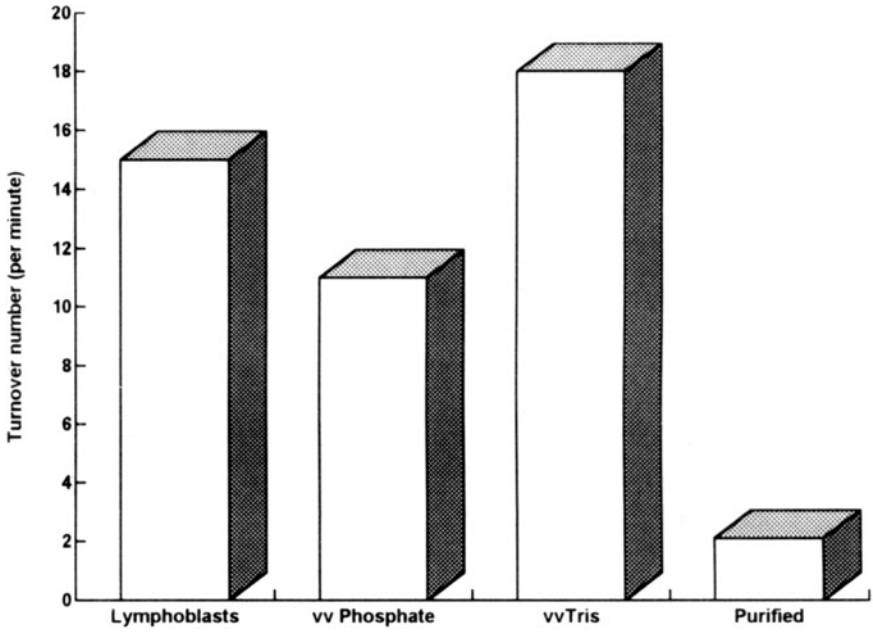

FIG 4. Turnover number for cDNA-expressed CYP2A6 and purified/reconstituted human liver CYP2A6. Data from human lymphoblasts ("lymphoblast", Crespi *et al.*, 1991a), vaccinia virus using phosphate buffers ("vv Phosphate", Yamano *et al.*, 1990), vv using Tris buffers ("vv Tris", C. Crespi and F. Gonzalez, unpublished observation) and purified/reconstituted human liver CYP2A6 ("purified", Yun *et al.*, 1992).

Crespi *et al.* (1990b). A partial cDNA isolate was previously reported by Phillips *et al.* (1985) as one of the first human P450 cDNA sequences reported.

6.3.2 cDNA Expression

Human CYP2A6 has been expressed in several heterologous systems including vv/HepG2 cells, human lymphoblasts, C3H 10T1/2 mouse cells and 3T3 mouse cells. Interest in this enzyme has been primarily due to its potential role in the activation of nitrosamine procarcinogens. CYP2A6, as measured by coumarin 7-hydroxylase activity, has been expressed at the highest levels in human lymphoblasts and vv/HepG2 cells (Fig. 3). Somewhat lower expression levels were reported in the retroviral expression systems (Tiano *et al.*, 1993; Salonpaa *et al.*, 1993). CYP2A6 has been found

to metabolically activate *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, AFB, and NNK to species which are cytotoxic and mutagenic to human B-lymphoblastoid cells (Crespi *et al.*, 1990b, 1991b). The observation of CYP2A6-catalysed activation of aflatoxin B1 to DNA-binding intermediates was also reported using 3T3 cells (Salonpaa *et al.*, 1993) and vv/HepG2 cells (Aoyama *et al.*, 1990). The observation of the activation of NNK by CYP2A6 to a gene mutagen has been extended to measurement of malignant transformation induced by NNK in CYP2A6-expressing C3H 10T1/2 cells (Tiano *et al.*, 1993).

6.4 CYP2B6

6.4.1 Properties

Characterization of CYP2B6 protein levels in human liver has been somewhat problematic. Many antibody preparations prepared against rodent CYP2B forms appear to cross-react poorly with the human form. In addition, a CYP2B6-specific or CYP2B6-selective substrate probe has not yet been identified. Therefore, the tools for analysis of human CYP2B6 expression are not fully available.

Yamano *et al.* (1990) analysed CYP2B6 mRNA levels and protein levels using a cDNA probe and an anti-rat CYP2B1 antibody developed in rabbits, respectively. Thirteen human liver samples were studied and CYP2B6 mRNA and protein were present at high levels in only two liver samples.

Yamazaki *et al.* (1993) analysed CYP2B6 protein by immunoblotting using an anti-monkey CYP2B antibody (Ohmuri *et al.*, 1993). They reported immunoreactive protein in eight of 18 human liver samples. It is interesting to note that these same liver samples also showed elevated levels of CYP3A protein, in fact, CYP2B and CYP3A expression were relatively well correlated ($r = 0.75$). This may indicate common mechanisms of regulation and also may complicate correlation analyses of CYP2B or CYP3A protein levels with catalytic activities for a human liver panel. Shimada *et al.* (1994) report that CYP2B6 comprises only 0.2% of total human cytochrome P450 in a panel of human liver microsomes. Most of the specimens in this study had negligible expression of CYP2B6. Chang *et al.* (1993) used a different antibody preparation (anti-rat CYP2B1 developed in rabbits) and detected immunoreactive protein in two of three human liver samples [using 30% of the protein loading of Yamano *et al.* (1989a)]. Band intensity on the Western blots was greater than the intensity of cDNA-expressed CYP2B6 from human lymphoblasts (discussed below). Therefore with this antibody reagent, in a limited number of human liver samples, CYP2B6 protein is present. In this same report, CYP2B6 was identified as one of several human hepatic P450s which is capable of activating cyclophosphamide and

ifosphamide. CYP2B6 appears to be more prominent in the activation of cyclophosphamide than in the activation of ifosphamide. Orphenadrine, a selective inhibitor of rodent CYP2B enzymes (Reidy *et al.*, 1989), also appears to be a selective inhibitor of human CYP2B6, although at lower affinity. A cDNA has been isolated encoding the CYP2B6 protein (Yamano *et al.*, 1989a).

6.4.2 cDNA Expression

Yamano *et al.* (1989a) expressed CYP2B6 cDNA using the vv system. They reported that the cDNA-expressed enzyme was capable of 7-ethoxycoumarin *O*-deethylase activity. Recently, CYP2B6 was found to metabolize styrene (Nakajima *et al.*, 1994). Using the same expression system, Waxman *et al.* (1991) reported that CYP2B6 was not a steroid hydroxylase, which is a property of rodent CYP2B subfamily proteins. CYP2B6 was found to be one of five human P450 forms capable of metabolizing AFB to a DNA-binding intermediate (Aoyama *et al.*, 1990).

CYP2B6 cDNA has been expressed in human B lymphoblastoid cells using the episomal vector system. Microsomal P450 contents were detectable spectrophotometrically at 55 pmol P450/mg protein. In addition to 7-ethoxycoumarin *O*-deethylase activity,7-ethoxy-4-trifluoromethylcoumarin *O*-deethylase (DeLuca *et al.*, 1988; Buters *et al.*, 1993) activity was readily detected in microsomes from CYP2B6-expressing cells [mean level 190 pmol/(mg min)]. This substrate is CYP2B6 specific in human liver microsomes. However, kinetic differences between different enzymes may allow establishment of the contribution by CYP2B6. CYP2B6 cDNA-expressing human lymphoblastoid cells have been used to study the activation of cyclophosphamide and ifosphamide to metabolites which are cytostatic to the P450-expressing cell line (Chang *et al.*, 1993).

6.5 CYP2C PROPERTIES AND cDNA EXPRESSION

Unlike the CYP1A, CYP2A and CYP2B subfamilies in humans where only a single enzyme is present in human liver, the CYP2C subfamily contains multiple members including CYP2C8, CYP2C9/10, CYP2C18 and CYP2C19. One member of the CYP2C subfamily, CYP2C19, is polymorphic in humans; the prototypical substrate for this polymorphism is the 4′-hydroxylation of *S*-mephenytoin (Kalow, 1986; Wrighton *et al.*, 1993a; Goldstein *et al.*, 1994). The substrate specificity and antibody cross-reactivity of these enzymes often overlaps, which can complicate P450 form-specific analyses using approaches other than cDNA expression. The application of cDNA expression approaches has been instrumental in the elucidation of the roles of the different CYP2C subfamily members in the

specific metabolic pathways. Therefore, for this subfamily of enzymes, the properties and cDNA expression have been combined into a single subsection.

CYP2C9 appears to be the most mass-abundant protein in the CYP2C subfamily in humans. CYP2C9 and CYP2C10 are very similar proteins with only two amino acid differences in the deduced protein sequences. However, the 3′ non-coding sequences of the respective cDNAs are very different, which has led to the different designations of the proteins. cDNA expression studies have indicated that CYP2C9 and to a lesser degree CYP2C8 are capable of tolbutamide hydroxylation (Relling *et al.*, 1990). CYP2C9 is capable of the 7-hydroxylation of (*S*)-warfarin. Based on studies in human liver microsomes and cDNA-expressed P450 using vv/HepG2 cells, CYP2C9 appears to be the principal P450 responsible for modulating the anticoagulant properties of the biologically more potent warfarin isomer, (*S*)-warfarin, *in vivo*. Competition for CYP2C9-mediated metabolism plays a role in drug interactions involving warfarin (Rettie *et al.*, 1992). There is a relatively common allelic variant of CYP2C9 (Arg144Cys) which exhibits reduced capacity metabolism of (*S*)-warfarin while having normal ability to metabolize tolbutamide (Rettie *et al.*, 1994). CYP2C9 is also responsible for the hydroxylation of the non-steroidal anti-inflammatory drug diclofenac (Leeman *et al.*, 1993). Sulfaphenazole (Back *et al.*, 1988) and sulfinpyrazone (Miners *et al.*, 1988) are selective CYP2C9 inhibitors.

CYP2C8 has recently been established at the principal enzyme responsible for the 6α-hydroxylation of taxol, the principal route of metabolism for this anticancer drug (Rahman *et al.*, 1994). For this study, a combined approach of correlation analysis with levels of immunoquantitated CYP2C8 protein in human liver microsomes, inhibition analyses in human liver microsomes, and vv-expressed proteins established that CYP2C8 was the principal enzyme for taxol 6α-hydroxylation and that this enzyme catalysed the reaction at high turnover (30/min).

CYP2C18 has been examined as a candidate for the (*S*)-mephenytoin 4′-hydroxylase polymorphism. Romkes *et al.* (1991) demonstrated that cDNA-expressed CYP2C18 4′-hydroxylated (*S*)-mephenytoin at a rate above background. However, CYP2C19 has recently been established as the protein responsible for the (*S*)-mephenytoin 4′-hydroxylase polymorphism (Wrighton *et al.*, 1993; Goldstein *et al.*, 1994). Population studies have demonstrated that 3–5% of Caucasians and about 20% of Asians are poor metabolizers of (*S*)-mephenytoin (Kalow, 1986). The molecular basis for this polymorphism has recently been established (de Morais *et al.*, 1994).

While many CYP2C proteins have been expressed using vv/HepG2 cells (Rettie *et al.*, 1992), yeast (Romkes *et al.*, 1991; Kaminsky *et al.*, 1993; Lopez-Garcia *et al.*, 1993) and *E. coli* (Sandhu *et al.*, 1993), human CYP2C proteins have not been reported to have been stably expressed in any mammalian cell systems.

6.6 CYP2D6

6.6.1 Properties

CYP2D6 was the first polymorphic P450 associated with drug metabolism in humans. Some 5–10% of Caucasians are deficient in this enzyme (Mahgoub *et al.*, 1977; Eichelbaum *et al.*, 1979). The poor metabolizer phenotype behaves as an autosomal recessive trait. CYP2D6 has a principal role in the oxidation of many drugs containing basic amine functionalities, including beta-blocking drugs (bufuralol, propranalol, metoprolol, timolol), anti-arrythmics (sparteine, encainide, flecainide, mexiletine), tricyclic antidepressants (imipramine, desipramine, nortriptyline) and many other drugs (dextromethorphan, perhexiline, codeine) (Eichelbaum and Gross, 1990).

A cDNA containing the entire CYP2D6 coding sequence has been isolated and the molecular nature of the poor metabolizer genotype has been characterized (Skoda *et al.*, 1988; Gonzalez *et al.*, 1988b; Hanioka *et al.*, 1990; Kagimoto *et al.*, 1990; Gough *et al.*, 1990).

The CYP2D6 gene has also been isolated and sequenced (Kimura *et al.*, 1989). Comparison of the sequences of the original CYP2D6 cDNA reveals one base difference (adenine versus guanine at position 1120 of the cDNA) which results in a methionine at position 374 in the cDNA isolate and valine at the same position in the genomic isolate. Ellis *et al.* (1994) report different ratios of metoprolol metabolites (α-hydroxyl versus *O*-demethyl) for methionine and valine CYP2D6 enzymes with the ratio for the valine protein being closer to that observed with human liver microsomes. The effect of this base substitution on catalytic activity for other substrates has not been reported.

6.6.2 cDNA Expression

CYP2D6 cDNA has been expressed in a variety of heterologous expression systems including human lymphoblasts, vv and yeast (Crespi *et al.*, 1991b; Tyndale *et al.*, 1991; Ellis *et al.*, 1992; Penman *et al.*, 1993). These systems have been used to probe the substrate specificity of the CYP2D6 protein and also established that CYP2D6 is capable of activating procarcinogens. Procarcinogen activation by CYP2D6 provides a mechanistic hypothesis for the association of the CYP2D6 extensive metabolizer phenotype with increased risk for smoking-induced lung cancer (Ayesh *et al.*, 1984; Caporaso *et al.*, 1989). The human lymphoblast and vv expression work used the methionine variant of CYP2D6.

The highest levels of expression of CYP2D6 protein have been observed in the human lymphoblast system. A spectral P450 content of 160 pmol/mg microsomal protein has been reported (h2D6v2 cells; Penman *et al.*, 1993).

The mean (+)-bufuralol 1′-hydroxylase activity in microsomes from h2D6v2 human lymphoblastoid cells [935 pmol/(mg min)] is about five times that observed in human liver microsomes [200 pmol/(mg min); Dayer *et al.*, 1987; Wrighton *et al.*, 1993b; Shimada *et al.*, 1994]. Apparent K_m for bufuralol 1′-hydroxylation and debrisoquine 4-hydroxylation were comparable for the cDNA-expressed enzyme and in human liver microsomes (Penman *et al.*, 1993). The turnover numbers for cDNA-expressed CYP2D6 in human lymphoblasts were comparable to (bufuralol) or slightly lower than (debrisoquine) those observed for the purified reconstituted CYP2D6 protein (Dislerath *et al.*, 1985; Gut *et al.*, 1986). It is interesting to note that CYP2D6 cDNA expression is enhanced by addition of dimethylsulfoxide to the media in human lymphoblasts (Crespi *et al.*, 1991b; Penman *et al.*, 1993). Curiously, CYP2D6 protein is much less active per unit enzyme when expressed in the vv system (Tyndale *et al.*, 1991). The mechanism for the relatively low activity is, at present, unknown. However, it is unlikely to be due to coupling with OR since the difference is observed also when cumene hydroperoxide is used instead of NADPH.

6.7 CYP2E1

6.7.1 Properties

The CYP2E1 protein plays a central role in toxicology and a relatively minor role in drug metabolism. CYP2E1 is important in the metabolism of many low molecular weight procarcinogens (Guengerich *et al.*, 1991). Levels of CYP2E1 are elevated in individuals consuming ethanol or isoniazid (Wrighton *et al.*, 1986). Only two drugs, chlorzoxazone (Peter *et al.*, 1990) and enflurane (Kharasch *et al.*, 1994), have been demonstrated to be metabolized by CYP2E1. The metabolism of the skeletal muscle relaxant chlorzoxazone by 6-hydroxylation is a selective probe for CYP2E1 (Peter *et al.*, 1990).

6.7.2 cDNA Expression

Human CYP2E1 cDNA has been expressed in several heterologous systems including human lymphoblasts, vv/HepG2 cells and stable expression in HepG2 cells using retrovirus. In the human lymphoblast system, CYP2E1 cDNA was initially expressed relatively inefficiently (Crespi *et al.*, 1990b). Nevertheless, CYP2E1-expressing cells were extraordinarily sensitive to the mutagenicity of nitrosamines. Recent modifications to the expression vector through the use of multiple expression units and coexpression of OR have led to the development of a human lymphoblastoid cell line with 10-fold

higher activity (C. L. Crespi, in preparation). Cytochrome P450 contents were measured at 40 pmol/mg microsomal protein. The mean *p*-nitrophenol hydroxylase activity in microsomes from h2E1/OR cells (cultured in the presence of dexamethasone to induce cytochrome P450 reductase activity) was 780 pmol/(mg min), nearly twice that observed in human liver microsomes [490 pmol/(mg min); Murray *et al.*, 1993] and 12 times that observed for HepG2 cells expressing CYP2E1 cDNA (Dai *et al.*, 1993). Similar high catalytic activity has been reported for the vv-expressed CYP2E1 (Patten *et al.*, 1992). It should be noted that both CYP2A6 and CYP2E1 catalyse *p*-nitrophenol hydroxylase; therefore human liver microsome *p*-nitrophenol hydroxylase values may overestimate CYP2E1 content due to a contribution by CYP2A6. Human lymphoblast-expressed CYP2E1 was also found to hydroxylate chlorzoxazone with a turnover number of 22/min. A modified CYP2E1 has also been expressed in *E. coli*. The specific content in *E. coli* membranes was 5-fold higher than that of human lymphoblasts but the turnover number for chlorzoxazone in *E. coli* membranes (supplemented with cytochrome b_5) was 20-fold lower. Purification and reconstitution of the enzyme elevated the turnover number by about 5-fold (Gillam *et al.*, 1994). Curiously, purification and reconstitution of *E. coli*-expressed CYP2E1 lowered the apparent K_m for chlorzoxazone 6-hydroxylation about 2.5-fold. The lower value is in agreement with the apparent K_m as measured in human liver microsomes or human lymphoblast-expressed CYP2E1 (Peter *et al.*, 1990). The mechanism for this shift in apparent K_m is unknown. The level of CYP2E1 catalytic activity in h2E1/OR cells was about 10-fold higher than that reported for CYP2E1 stably expressed in HepG2 cells (Dai *et al.*, 1993). CYP2E1 has also been expressed in NIH 3T3 cells (Nouso *et al.*, 1992). The only catalytic activity data reported were for 7-ethoxycoumarin, a non-specific substrate. This substrate has not been examined in the other cDNA expression systems.

Vaccinia virus-expressed CYP2E1 has been used to study the metabolism of nitrosamines (Patten *et al.*, 1992; Smith *et al.*, 1992). The level of expression in human lymphoblast and vv/HepG2 cells appears to be comparable.

6.8 CYP3A

6.8.1 Properties

CYP3A enzymes, principally CYP3A4 and CYP3A5, play a major role in both drug metabolism and toxicology. CYP3A proteins are the most abundant cytochrome P450s in human liver, comprising about 30% of total P450 (Shimada *et al.*, 1994). The principal CYP3A protein in adults in

CYP3A4. CYP3A5 is found in the liver of about 20% of individuals (Aoyama *et al.*, 1989b). CYP3A is also highly expressed in the intestinal epithelium (Watkins *et al.*, 1987). The levels of CYP3A4 appear to be elevated in individuals receiving glucocorticoids, macrolide antibiotics and phenobarbital (Watkins *et al.*, 1987). Certain drugs, such as erythromycin and ketoconazole, are potent inhibitors of CYP3A catalytic activity *in vitro* and *in vivo* (First *et al.*, 1991).

CYP3A metabolizes a variety of endogenous compounds, such as steroids, and a wide range of structurally diverse drugs and other xenobiotics (for review see Wrighton and Stevens, 1992). Marker activities for this enzyme in human liver microsomes include the 6β-hydroxylation of testosterone, erythromycin *N*-demethylation, nifedipine oxidation and midazolam hydroxylation. CYP3A4 catalytic activity is activated, *in vitro*, by the binding of some ligands/substrates, and recent studies indicate that the enzyme may simultaneously bind two substrates (Shou *et al.*, 1994).

6.8.2 cDNA Expression

Initial CYP3A4 expression experiments were performed in COS-1 cell (Gonzalez *et al.*, 1988a). These experiments demonstrated that CYP3A4 was the nefidipine oxidase enzyme. However, the expression levels were quite low which functionally precluded studies with low-turnover substrates.

CYP3A4 has been expressed in human lymphoblasts (Crespi *et al.*, 1991a). The expression level in this report was quite low. Recent modifications to the promoter for cDNA expression and coexpression of OR have led to a 40-fold increase in catalytic activity. The mean testosterone 6β-hydroxylase activity in microsomes from h3A4/OR cells (cultured in the presence of dexamethasone to induce cytochrome P450 reductase activity) [1000 pmol/(mg min)] is comparable to the mean values observed in human liver microsomes [1070 pmol/(mg min); Yamazaki *et al.*, 1993]. The turnover number for testosterone and CYP3A4 in the human lymphoblasts was 15/min for endogenous OR levels and increased to 44/min with OR coexpression.

CYP3A4 has been efficiently expressed using the vv system and using the baculovirus system (Buters *et al.*, 1994). With the baculovirus system, very high levels of expression were obtained and the protein was readily purified and reconstituted. Substrate turnover numbers for vv-expressed and baculovirus-expressed CYP3A4 were quite good. The turnover numbers for testosterone were 25–30/min with either system. In contrast, the turnover numbers for yeast-expressed or *E. coli*-expressed CYP3A4 were an order of magnitude lower (Brian *et al.*, 1990; Peyronneau *et al.*, 1992; Gillam *et al.*, 1993).

6.9 OTHER ENZYMES

Phase II enzymes, UDP-glucuronosyl transferases (UGT), sulfotransferases, methyltransferase, hydrolases, *N*-acetyltransferases (NAT) and glutathione transferases, often play a central role in the clearance of drugs and the detoxification of xenobiotics and in some cases the activation of promutagens/procarcinogens. These enzymes are either membrane bound (e.g. UGTs) or soluble (e.g. sulphotransferases and NATs). Considerable progress has been made in recent years in cloning and characterizing cDNAs encoding these proteins (Blum *et al.*, 1991; Coughtrie, 1992), and stable cDNA expression in mammalian cells and a variety of other systems has been achieved. As with the cytochrome P450 system, multiple forms of the enzymes exist with unique, but often overlapping substrate specificity. Certain of these enzymes (e.g. NAT2) are known to be polymorphic in human populations. In the future we can look forward to the integration of Phase I and Phase II metabolism with cDNA expression systems.

7 Examples of Applications

The cDNA expression systems can be used to address questions such as: can human enzymes metabolize a xenobiotic? What are the metabolites? Can human enzymes activate a protoxin? In order to adequately support a negative conclusion it is obvious that the range of cytochrome P450 enzymes examined needs to be as comprehensive as possible.

One application of this system to drug development is the investigation of which cytochrome P450 form(s) is primarily responsible for the metabolism of a drug or drug candidate. Such studies can establish whether a new drug is metabolized by an enzyme known to be polymorphic in humans (e.g. CYP2D6) or is primarily metabolized by an enzyme known to be often involved in drug–drug interactions (e.g. CYP3A4 and to a lesser extent CYP2C9). Early information regarding cytochrome P450 form-specific metabolism can help to plan clinical investigations. For example, will it be important to phenotype or genotype individuals with respect to CYP2D6? Or, what are the likely drugs which may cause drug–drug interactions (e.g. ketoconazole or rifampin for CYP3A4 and sulfaphenazole for CYP2C9)?

Given the multiplicity of enzymes which have the potential to metabolize a xenobiotic, it is desirable to include as many enzymes as possible in the initial screen. Production of metabolites should be analysed in a multiphase approach, with an initial analysis under conditions which maximize metabolite production and then follow-up studies with those enzymes which are found to be active. The follow-up studies can help place the relative roles of the different enzymes into perspective by determining relative affinities

and/or capacities for the different enzymes and also relating the results from cDNA-expressed enzymes to the balance of metabolism in human liver.

For metabolite analyses, either whole cells or cell fractions can be used. Both preparations have advantages and disadvantages. For example, with whole cells, the native architecture of the enzyme is maintained, the plasma membrane as a potential barrier to metabolism is maintained, "latency" of UGT is avoided, long-term (several days) incubations are possible and the intracellular environment maintains physiological ionic strength and cofactor concentrations. However, whole cells require complex media which may complicate analysis of metabolites, the oxygen requirements of the cells limit the concentration of active enzyme which can be used per unit culture, and toxicities of the compound being studied may introduce non-linearities in the system. In contrast, with microsomal preparations from cDNA-expressing cells, the use of higher enzyme concentrations per unit volume in a simpler buffer system is possible, however latency of UGTs may be present and the system is active for only a few hours.

We have routinely used a 2-day incubation with whole cells and a 2–3 h incubation with microsomes for initial screening work. In addition, it is often informative to test several substrate concentrations, separated by 10-fold for example, in order to provide an initial assessment of the relative affinities of the enzymes found to be active. Those enzymes found to be active can then be subjected to more detailed analyses including time course analyses, determination of apparent K_m and V_{max}, or inhibition analyses of model substrates by the compound under investigation. The relative affinities of the different enzymes is often an important parameter. For example, CYP3A4 may be a low-affinity enzyme for some substrates and hence drug–drug interactions will be less significant. Knowledge of the P450 contents and catalytic activities of the cell lines allows correction for the differences in content among the cell lines and, as discussed earlier, comparison to human liver microsomes. In the aggregate, these studies should provide a more and more complete picture of the relative affinities and capacities of the active enzymes. Some published reports using panels of cDNA-expressed enzymes include Czerwinski *et al.* (1991), Waxman *et al.* (1991), Rodrigues *et al.* (1994), Clarke *et al.* (1994) and Duescher and Elfarra (1994).

A second application is testing interaction with specific enzymes by inhibition analyses. Again CYP2D6 and CYP3A4 are enzymes of particular interest for this approach with drugs. cDNA-expressed enzymes offer an advantage over liver microsomes because of their consistency among preparations and the lack of potentially competing enzymes. The lack of competing enzymes also allows greater flexibility in the performance of inhibition analyses because technically simpler enzyme assays, which may not be absolutely enzyme specific in human liver preparations, can be used

successfully with cDNA-expressed enzymes where only a single enzyme is present. Inhibition analyses are particularly useful for providing information regarding the affinity of the compound for different enzymes with investigational compounds for which analytical procedures are not yet available.

There are several examples in the literature of the integration of studies with cDNA-expressed human P450 enzymes and human liver microsomes which have led to improved understanding of human enzyme-mediated activation of protoxins. Some of these examples are discussed below. Most of these reports have taken a multifaceted approach combining studies in human liver microsomes with cDNA-expressed enzymes. The general metabolic properties of these toxic xenobiotics (e.g. multiplicity of enzymes, differences in affinity and capacity, methods to compare to human liver data, etc.) apply to drugs and drug candidates as well.

7.1 TAMOXIFEN GENOTOXICITY

Styles *et al.* (1994) have examined the genotoxicity of tamoxifen using a panel of human B lymphoblastoid cells expressing individual and multiple cytochrome P450s. Tamoxifen has shown utility in treating women with breast cancer and is currently undergoing trials to assess its effectiveness as a prophylactic in women free of breast disease. Tamoxifen is a carcinogen in the female rat (Hirsimaki *et al.*, 1993), has been shown to produce modified DNA bases by ^{32}P post-labelling and can induce unscheduled DNA synthesis in rat hepatocytes (White *et al.*, 1993). Several different models of the route of metabolism to form a DNA binding intermediate have been proposed (Potter *et al.*, 1994; Pathak and Bodell, 1994). In the aggregate, these data indicate that tamoxifen is a genotoxic rat carcinogen. Tamoxifen has been demonstrated to induce micronuclei in MCL-5 cells which express five human P450 cDNAs (White *et al.*, 1993; Styles *et al.*, 1994).

The study by Styles *et al.* (1994) examined whether the genotoxicity of tamoxifen was P450 dependent and which particular P450(s) were capable of metabolic activation. Human lymphoblastoid cells expressing human CYP1A1, CYP1A2, CYP2D6, CYP2E1 and CYP3A4 cDNAs, as well as control cells with vector only, were used as target cells for tamoxifen-induced formation of micronuclei. Cells expressing CYP2E1, CYP3A4 and CYP2D6 were found to be capable of activating tamoxifen. Significantly higher tamoxifen concentrations were needed to observe genotoxicity in CYP2D6-expressing cells relative to the other cell lines.

A structural analogue of tamoxifen, toremifene, was also examined. This chemical was found to be less clastogenic than tamoxifen, however positive responses were observed for cells expressing CYP2E1 or CYP3A4 cDNAs and in MCL-5 cells. Studies with synthetic tamoxifen epoxide showed micronuclei induction in all cell lines examined.

This study clearly demonstrates that human enzymes are capable of activating tamoxifen to genotoxic species. The observation of human enzyme-mediated, human cell genotoxicity suggests a prudent level of concern regarding treatment of humans who are presently free of disease with tamoxifen. It is important to note that this study does not prove that tamoxifen is a genotoxic carcinogen in humans. For instance, Phase II enzymes may serve to substantially protect cells *in vivo* from tamoxifen's genotoxic effects and phase II enzymes were not examined by Styles *et al.* (1994). The observation of activation by CYP2E1 and CYP3A4 suggests that coadministration of specific inhibitors (disulfiram and ketoconazole for example) may redirect metabolism to pathways which do not result in genotoxic metabolites. Also, given that the levels of both CYP2E1 and CYP3A4 are modulated by environmental factors, this implies that individuals may vary substantially with respect to susceptibility to tamoxifen's genotoxic effects.

7.2 TOBACCO SMOKE NITROSAMINE ACTIVATION

The tobacco smoke-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) has received considerable study in a variety of experimental systems. This chemical is carcinogenic in rats and mice inducing lung tumours. NNK is an asymmetric nitrosamine which, when metabolized, generates methylating or pyroxylbutylating agents. NNK metabolism and carcinogenicity have been previously reviewed (Hecht and Hoffman, 1988).

NNK is metabolized by multiple cytochrome P450 enzymes in both rodents and humans. We have examined the mutagenicity of NNK in a panel of human B lymphoblastoid cell lines expressing human CYP1A2, CYP2A6, CYP2E1 and CYP2D6 cDNAs as well as control cells (Crespi *et al.*, 1991b). We observed that all P450s examined metabolically activated NNK while control cells were not mutated by NNK exposure. A more recent study extends the range to cytochrome P450 enzymes capable of activating NNK to include CYP1A1 (Penman *et al.*, 1994). The role of CYP1A2, CYP2A6, CYP2B6 and CYP2E1 in the metabolism of NNK has been verified by direct measurement of metabolism by vv/HepG2 cell-expressed cDNAs (Smith *et al.*, 1992). The apparent K_ms for the different cytochrome P450 forms were similar and relatively high. In this study, CYP2C8, CYP2C9, CYP3A4, CYP3A5 and CYP2D6 were found to be inactive. The apparent discordance between the CYP2D6 mutagenicity results and the metabolism results were examined in a subsequent study (Penman *et al.*, 1993). In this study, microsomes prepared from human lymphoblasts expressing CYP2D6 cDNA were found to metabolize NNK and this metabolism (and the mutagenicity of NNK) was blocked by the

addition on quinidine, a potent CYP2D6 inhibitor. The apparent discordance between the human lymphoblast and the vv-expressed CYP2D6 is apparently due to lower-than-expected catalytic activity of CYP2D6 when expressed in vv relative to human lymphoblasts.

In cases where multiple cytochrome P450 forms are capable of metabolizing a xenobiotic, metabolism studies in human tissue fractions are often useful in determining which P450 forms are principally responsible for the metabolism. In the study of Smith *et al.* (1992), specific, inhibitory antibodies against human cytochrome P450 forms were used to investigate the contribution of specific P450 forms to overall NNK metabolism. The antibody inhibition analyses revealed that two P450 forms, CYP1A2 and CYP2E1, equally accounted for nearly all of human hepatic NNK metabolism.

Given the relatively complete data set for NNK, this chemical provides a test of the RAF approach for determining the principal P450 responsible for hepatic metabolism from the mutagenicity data in Crespi *et al.* (1991b), and the catalytic activities in human lymphoblast microsomes and human liver microsomes. For this analysis, the initial slope of the mutagenicity dose–response curve was used as a basis for comparison. At low mutagen concentrations, this parameter is dependent on V_{max}/K_m, as is the intrinsic clearance.

The data are presented in Table 2. The slopes of the dose–response curves varied about 7.5-fold, with cells expressing CYP1A2 and cells expressing CYP2A6 being the most sensitive, and cells expressing CYP2E1 being the least sensitive. The mean catalytic activity for an enzyme-specific substrate in human liver microsomes was divided by the catalytic activity for the same substrate in human lymphoblast microsomes (more recently developed human lymphoblastoid lines have higher catalytic activity) to yield a RAF. The RAFs range from 0.23 for CYP2D6 which was expressed in human lymphoblasts at a level substantially above that in human liver, to 20 for CYP2E1, which was (at that time) expressed at levels substantially below those of human liver. Multiplication of the slope of the mutagenicity curve by the RAF provides the predicted relative contribution for each P450 form. Based on this analysis, CYP1A2 and CYP2E1 are indicated as the two principal P450s for NNK metabolism with approximately equal contributions. Any contributions by CYP2A6 and CYP2D6 are expected to be minor. The results from the human lymphoblast data are in good agreement with the results from the inhibition studies with human liver microsomes, which also found CYP1A2 and CYP2E1 to be principal P450s with approximately equal contributions (Smith *et al.*, 1992).

The above analysis predicts relative (not absolute) contributions of the different enzymes to overall hepatic metabolism. Naturally, the relative contributions of the different enzymes will vary from tissue to tissue depending on the enzyme composition in the tissue. For example, if the

TABLE 2

Application of relative activity factors to NNK mutagenicity data; prediction of the principal P450 for activation

	P450 form			
	CYP1A2	CYP2A6	CYP2D6	CYP2E1
Lymphoblast line	1A2/Hol (h1A2)	2A3/Hol (h2A3)	2D6/Hol (h2D6)	h2E1/Hol (h2E1)
Mutagenicity slope[a]	0.63	0.63	0.14	0.084
Substrate	EROD	Coumarin 7-hydroxylase	(+)-Bufuralol 1′-hydroxylase	*p*-Nitrophenol hydroxylase
Lymphoblast activity	22 pmol/ (mg min)	780 pmol/ (mg min)	470 pmol/ (mg min)	25 pmol/ (mg min)
Human liver activity[b]	66 pmol/ (mg min)	603 pmol/ (mg min)	108 pmol/ (mg min)	490 pmol/ (mg min)
RAF	3.0	0.77	0.23	20
Contribution	1.9 (46%)	0.5 (12%)	0.03 (1%)	1.7 (41%)

[a]Expressed as induced mutant fraction (per million) per μg/ml NNK exposure concentration, from Crespi *et al.* (1991a).

[b]CYP1A2: weighted average of the human liver data in Fig. 2; CYP2A6: weighted average of the human liver data in Fig. 3; CYP2D6: weighted average of the human liver data in Dayer *et al.* (1987) and Wrighton *et al.* (1993b); CYP2E1: human liver data from Murray *et al.* (1993).

target tissue is lung, there is no evidence for CYP1A2 expression in human lung (Wheeler *et al.*, 1990) while there is evidence for CYP2E1 expression (Wheeler *et al.*, 1992). Therefore, in the lung, the relative contributions of CYP1A2 and CYP2E1 will be different. Also, we have found that human CYP1A1 can activate NNK, and CYP1A1 expression is elevated in the lungs of smokers (Wheeler *et al.*, 1990).

7.3 OXAZAPHOSPHORINE ACTIVATION

The cancer chemotherapeutic agents cyclophosphamide (CP) and ifosphamide (IF) are metabolized to alkylating species by cytochrome P450 enzymes. Chang *et al.* (1993) used an integrated approach utilizing human liver microsomes in conjunction with P450 form-selective chemical and immunochemical inhibitors, metabolic studies with cDNA-expressed enzymes and cytotoxicity studies with cells expressing specific cytochrome P450 enzymes to determine the role of specific human cytochrome P450 enzymes in the activation of CP and IF.

Kinetic analyses of CP and IF metabolism in human liver microsomes indicated the presence of high-affinity and low-affinity enzymes capable of activating these chemicals. Studies with cDNA-expressed human P450 enzymes revealed that five enzymes, CYP2A6, CYP2B6, CYP2C8, CYP2C9 and CYP3A4, were capable of metabolizing CP and IF. The CYP2C enzymes were more active at low substrate concentrations and the other enzymes were more active at high substrate concentrations.

The CYP2B-selective chemical inhibitor orphenadrine and an inhibitory anti-CYP2B IgG, the CYP3A-selective chemical inhibitor troleandomycin (TAO) and an inhibitory anti-CYP3A IgG, and an inhibitory anti-CYP2A IgG were used to elucidate the relative contributions of the three high-affinity enzymes to overall CP and IF metabolism. TAO and anti-CYP3A IgG were found to inhibit IF metabolism and not CP metabolism. In an independent report, CP and IF were found to inhibit CYP3A4-catalysed testosterone 6β-hydroxylase activity in human liver microsomes (Murray *et al.*, 1994). Anti-CYP2A IgG was slightly inhibitory to both CP and IF metabolism in one liver specimen with the highest CYP2A levels, measured by enzyme assay or Western blot. Orphenadrine and anti-CYP2B IgG were inhibitory to CP and not IF metabolism in human liver samples which had immunodetectable CYP2B protein.

In the aggregate, these studies indicate that for CP, CYP2B6 is more important than CYP2A6 and CYP3A while for IF, CYP3A4 is more important than CYP2B6 or CYP2A6. The relative contribution of the high-affinity CYP2C enzymes remains to be examined.

7.4 AFLATOXIN B_1 ACTIVATION

AFB is a fungal toxin for which there is widespread human exposure through contamination of improperly stored grains. On dietary exposure, AFB is a potent carcinogen in rats and less so in mice. There is considerable evidence for AFB carcinogenicity in humans. The toxicology and significance of AFB have been reviewed (Wogan, 1973).

The role of specific human cytochrome P450 enzymes in the metabolic activation of AFB has been examined by a number of laboratories using several methods. Shimada and Guengerich (1989) correlated AFB activation to a bacterial mutagen to the levels of CYP3A protein and catalytic activity in a panel of human liver microsomes. This activation could be inhibited by anti-CYP3A IgG. High AFB concentration (three to four orders of magnitude above human dietary exposure levels) were needed in order to detect a response in the bacterial mutagenicity assay.

Forrester *et al.* (1990) confirmed these results using essentially the same methods. However, based on inhibition by anti-CYP1A and anti-CYP2A antisera they suggested an additional role for CYP1A2 and CYP2A6 in the

activation of AFB. The studies again used a relatively insensitive bacterial mutation assay which required micromolar concentrations of AFB in order to detect a response.

Aoyama *et al.* (1990) examined AFB activation using a panel of cDNA-expressed cytochrome P450s using vv/HepG2 cells. Five forms, CYP1A2, CYP2A6, CYP2B6, CYP3A3 and CYP3A4, were found to be capable of activating AFB to a species which bound to cellular DNA. Given the multiplicity of enzymes capable of activating AFB, an important question is which P450 forms are most active at low AFB concentrations.

Using a panel of human B lymphoblastoid cell lines expressing CYP1A2, CYP2A6 and CYP3A4 cDNAs and specifically developed to contain approximately equal cytochrome P450 contents (verified by quantitative Western blot), Crespi *et al.* (1991a) demonstrated that cells expressing CYP1A2 were 5-fold more sensitive to AFB mutagenicity than cells expressing CYP3A4, which were in turn 14-fold more sensitive to AFB than cells expressing CYP2A6. The rank order for mutagenicity was supported by parallel studies of AFB binding to cellular DNA. These studies suggested that CYP1A2 enzyme has a higher affinity for AFB activation than CYP3A4 enzyme. However, these studies compared AFB activation on a unit P450 enzyme basis. The relative importance of CYP1A2 and CYP3A4 to AFB activation in human liver is also dependent on the relative abundance of the two enzymes. Shimada *et al.* (1994) report that CYP3A is 2.3-fold more abundant than CYP1A2 in human liver microsomes; the difference in enzyme abundance is not sufficient to compensate for the difference in affinity.

The RAF approach can also be applied to the human lymphoblast mutagenicity data using the same approach as was used for NNK earlier in this section. The RAF approach reaches the conclusion that CYP1A2 is more important than CYP3A4. Table 3 gives the data for this analysis.

The relative roles of CYP1A2 and CYP3A4 in the activation of AFB for cDNA-expressed enzymes and human liver microsomes were thoroughly examined by Gallagher *et al.* (1994). In this study, the apparent K_m for formation of AFB-8,9-oxide (the electrophilic species) was determined for cDNA-expressed CYP1A2 and CYP3A4 (expressed in the human lymphoblast system). The relative contribution of CYP1A2 and CYP3A4 for human liver microsomal production of AFB-8,9-oxide was examined through the use of the CYP1A2 selective inhibitor furafylline and the CYP3A selective inhibitor TAO. These studies revealed that at low AFB concentrations, activation proceeds primarily through CYP1A2. CYP3A4 is a low-affinity form for AFB activation.

The studies with AFB serve to emphasize the importance of using sensitive toxicology test systems. The human cell system is sensitive to ng/ml concentrations of AFB, whereas bacteria mutagenicity test systems require μg/ml AFB concentrations for a detectable response. The lower concentra-

TABLE 1

Application of relative activity factors to aflatoxin b_1 mutagenicity data; prediction of the principal P450 for activation

	P450 form		
	CYP1A2	CYP2A6	CYP3A4
Lymphoblast line	1A2/Hyg	2A3/Hyg	3A4/Hol
Mutagenicity slope[a]	0.305	0.0045	0.070
Substrate	EROD	Coumarin 7-hydroxylase	Testosterone 6β-hydroxylase
Lymphoblast activity	2.8 pmol/(mg min)	15 pmol/(mg min)	25 pmol/(mg min)
Human liver activity[b]	66 pmol/(mg min)	603 pmol/(mg/min)	1070 pmol/(mg min)
RAF	23.6	40.2	42.8
Contribution	7.2 (69%)	0.19 (2%)	2.8 (27%)

[a]Expressed as induced mutant fraction (per million) per ng/ml AFB exposure concentration, from Crespi *et al.* (1991a).

[b]CYP1A2: weighted average of the human liver data in Figure 2; CYP2A6: weighted average of the human liver data in Fig. 3; CYP3A4: human liver data from Yamazaki *et al.* (1983).

tion is more representative of human exposure levels. With a less sensitive test system it is more likely that differences in enzyme capacity are measured and not differences in affinity and capacity. This is more likely to be especially important for CYP3A enzymes, which show "activation" of catalytic activity *in vitro* by some substrates (i.e. the need to use high substrate concentrations results in the measurement of CYP3A4-mediated effects in an artificial, activated state and thus overstates contribution by CYP3A).

8 Prospects for the Future

The field of xenobiotic metabolism is rich with a myriad of different, often competing, pathways of metabolism. Efforts at cDNA expression have only begun to develop comprehensive systems for the analysis of all Phase I and all Phase II enzymes. A comprehensive set of human cytochrome P450 and Phase II enzymes is not available in the context of a single host cell type, but given the rate of progress, we can look forward to nearly complete systems in the near future.

In addition, we can look forward to refinements in the means to extrapolate from cDNA-expressed enzymes to the balance of enzyme present in human liver *in vivo*. The examples developed in this chapter were all based on toxicological endpoints using data sets which had previously been published. While this approach shows considerable promise, clearly more extensive validation of the approach, using drugs and drug candidates, is in order. Cyclophosphamide and ifosphamide are two good candidate drugs because of the multiplicity of enzymes which metabolize these compounds.

Finally, coexpression of many enzymes within a single cell will be important technique for the analysis of the balance of Phase I and Phase II enzymes on overall metabolism and/or toxicity of xenobiotics. The feasibility of this approach has been demonstrated with the development of MCL-5 cells which coexpress five cDNAs (Crespi *et al.*, 1991c). However, an even greater multiplicity of enzyme coexpression is still needed and higher expression levels are necessary for routine use of such a system for metabolite analysis.

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Correlates of Variable Patient Compliance in Drug Trials: Relevance in the New Health Care Environment

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This chapter is dedicated to the memory of Ellen Weber, late Professor of Clinical Pharmacology at the University of Heidelberg, and one of the pioneers in research on patient compliance with therapeutic drug regimens. During most of her career, this field was of interest only to the few who, like her, needed no prompting to maintain an integrative view of therapeutics. Those who dismissed her work are now getting their lessons in integrative therapeutics from economists.

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1 Introduction

It is now well documented that a large minority of patients misuse prescription drugs in ambulatory care, mostly by underdosing in various time patterns. Despite its proven or likely medical and economic consequences, this problem has only recently begun to attract serious interest among health professionals—a timely development, because the recent changes in thinking about health care have put the focus on the outcomes of medical interventions. In ambulatory care, prescription drugs are the main intervention, and their effectiveness can be nullified or otherwise subverted by poor compliance with prescribed regimens. Thus, patient compliance is not only a crucial link between prescribing and outcomes, but also an important parameter in the overall quality of outcomes-oriented care.

Knowledge of the consequences of variable compliance should come from drug trials, where careful scrutiny and good control over the quality of data can assure accurate results. Such information is substantially lacking: it was of little interest before focus shifted to outcomes, complicated by poor methods of measurement and analysis. The new focus on outcomes, plus new methods and analyses, makes this a timely topic.

To set the stage, it is useful to put this topic in the larger context of the dramatically changing health care environment and its implications for drug research, development and clinical evaluation.

2 Overview of Changes in the Health Care Environment

Profound changes are occurring in the organization and financing of health care. National differences exist, but the forces for change are similar

throughout the industrial world. One force has been rapid growth in the cost of health care, but cost is not the sole issue: technological progress has brought us rather quickly from having few choices in low-cost care, to having many choices in high-cost care. The systems of care in the developed countries were organized for sparse-choice/low-cost care, not for the converse—another force for change.

Pharmaceuticals, in all aspects, from drug discovery to clinical use, are also under intense scrutiny, along with practically every other aspect of health care.

2.1 PRODUCERS OF CHEMICALS OR INFORMATION? DISEASE MANAGERS?

Many research-based pharmaceutical firms are presently reanalysing the bases of their business, questioning even the notion that their product can continue to be a packaged pharmaceutical. Some have already concluded that the packaged pharmaceutical can only be an intermediate, with their final product being "outcomes of disease management". This rethinking foreshadows immense transformations, as firms complete their change from producers of chemicals to producers of information—the currency in the land of disease management.

2.2 PROOF OF EFFICACY—PROOF OF VALUE

The conventional proof of efficacy is still the key to product registration and market entry, but in the new, information-intensive environment it brings no competitive advantage. Those responsible for the purchase of pharmaceuticals have begun to demand proof of *value*, which is to be found in information on the medical and economic outcomes of treatment, and the best means to those ends. Proof of efficacy can usually suffice as proof of value if it applies to the first curative medicine for a hitherto untreatable disease, but hardly so when the choice is among multiple, like-indicated, differently priced agents.

2.3 WHO DECIDES?

Another change is the diminishing role of the individual physician in pharmaceutical purchasing. Increasingly, decisions are being taken by a "formulary committee"—experts from pharmacy, medicine and other fields. With this transition, the traditionally information-sparse ("infopenic"), 5–7-minute detailing of individual physicians is fading from the scene, replaced by information-intensive ("infotensive") presentations to a com-

mittee of experts. This change is logical, because modern pharmaceuticals are complex, high-technology products that can only be caricatured in a brief detailing.

2.4 THE AGE OF INFOPENIA DRAWS TO A CLOSE

Change from the infopenic to the infotensive marketplace means that pharmaceutical research and design must meet the informational needs of this new marketplace, which seeks the very kinds of quantitative information once shunned because of the complications it brought to detailing, regulatory review and statistical analysis. Indeed, an odd pair of bedfellows, marketeers and biostatisticians, dominated drug development in the infopenic age, sharing a common but differently motivated lust for oversimplified clinical drug evaluation, apt for detailing.

2.5 ESTABLISHING THE VALUE OF VALUE

A useful metaphor, attributable to Dr Robert Temple of the US Food & Drug Administration, is that of a "pharmaceutical user's manual". In these terms, one might consider a well-written user's manual for version 5.0 of a big, widely used computer program, e.g. for word processing or spreadsheets; then, however, reflect back to how that manual looked when version 1 was being beta-tested—sparse, often confusing, and lacking many of the topics that, in later versions, were crucial to the product's wide use. In successive versions, the manual became more information intense: the expanding table of contents expressed the expanding intellectual scope of the field. Without the manual to guide informed use, copies of the disks of version 5.0 are of limited value, or worse if misuse begets costly error. To compare the value of competitive programs, one compares products item by item in the current table of contents. Then prices are compared, and the best values judged: performance relative to price. Users with special needs will interpret values differently, for they will give extra weight to certain headings in the table of contents.

Let us now apply this framework to pharmaceuticals.

Carl Peck, formerly Director of the Center for Drug Evaluation and Research at the US Food & Drug Administration, stimulated me to suggest a specific term—"pharmaco-informatics"—to express the idea of a subdiscipline of pharmacology that focuses on the format and structure of the corpus of information that differentiates a medicine from a chemical. In the terms of the previous paragraph, it concerns the structure of the table of contents of the "pharmaceutical user's manual". Transition to the infotensive age will

be rapid if most formulary committees give serious attention to these matters, but slow if they cannot look beyond prices.

2.6 WHY?

Consider the older, so-called "well-understood" drugs. In general, the older the drug, the lower the quality and quantity of information supporting its use. The "user's manuals" for most of these drugs are very thin—a reminder that years of extensive clinical usage can pass with only minor pharmaco-informatic yield. Even though some have been studied in large trials, these are usually fixed-dose, compliance-blind studies, which add little to the information on how best to use the drug, the patterns of misuse and their consequences. Some agents have gone through years of use at doses far in excess of optimal (Temple, 1989). Drug delivery systems have been the basis for a second cycle of development of some of these agents, improving their selectivity of action, expanding their pharmaco-informatic status, and giving them new value. Nifedipine is a conspicuous example (see Urquhart, 1992).

Low-priced, infopenic drugs are akin to simple software that one can buy at prices approaching the cost of a blank floppy disk. No one in the software marketplace confuses "program" with "disk", but "medicine" and "chemical" are often confused in the pharmaceuticals marketplace. This reflects the failure of pharmaceutical scientists and marketeers alike to communicate the value of the corpus of information that transforms a formulated chemical into a valuable medicine. We must do better.

Reference prices pegged to infopenic drugs are one result of this failure. I consider them one of the death throes of the infopenic age; they are completely contrary to the infotensive age, akin to tying software prices to the cost of a disk.

Let us turn to a specific set of pharmaco-informatic issues, which has to do with the development of an important new entry into the table of contents of the pharmaceutical user's manual.

3 Patterns of Misuse and their Consequences

A key value for the new pharmaceutical environment is a pharmaceutical's ability to deliver good medical and economic outcomes in the highest proportion of patients. Yet any drug's ability to effect a good outcome can be nullified by improper use, and certain patterns of misuse of some agents can create special hazards. Some pharmaceuticals are better able than others to maintain action in the face of common dosing errors. With a third or more of ambulatory patients making recurring substantial errors in dosing, a pharmaceutical's ability to "forgive" common errors in dosing is a valuable

therapeutic attribute. It is only beginning to receive attention, though it is rich in pharmaco-informatic content.

Among pharmaceutical topics, patient compliance with prescribed drug regimens was long an unwelcome orphan, for several reasons. One is that the definition of "patient compliance" was cast in purely behavioural terms, without reference to pharmacologic and therapeutic consequences of dosing that deviates from the recommended regimen—let alone any sense of how it might influence outcomes of treatment. Another reason has been that the methods of estimating compliance were, until recently, biased and unreliable. Without reliable measurements, progress in science and medicine is stymied, and there is a general reluctance to give serious consideration to matters that cannot be quantified. Probably the foremost reason for neglecting patient compliance, however, has been the long-standing fragmentation of responsibility for the outcomes of health care.

Let us consider each issue in turn: the definition and measurement of patient compliance, and the value of information about patient compliance and its correlates in a changing health care environment.

4 Defining Patient Compliance

The definition of "patient compliance" is "the extent to which the patient's actual time history of dosing corresponds to the prescribed dosing regimen", i.e. the comparison of two time series. Compliance thus subsumes both the quantity of drug taken and the timing of doses, i.e. drug exposure. Just as improper quantity, improper timing can nullify drug action or create hazard.

4.1 APPLICATIONS OF DEFINING COMPLIANCE AS DRUG EXPOSURE

Defining "patient compliance" in terms of the time history of dosing, i.e. drug exposure, has several important applications.

4.1.1 Dose–Response Relations

The first of these is the quantitative analysis of dose-dependent drug actions, for example, to infer dose–response relations from clinical correlates of variable compliance (Efron and Feldman, 1991; Urquhart, 1991a).

4.1.2 Pharmacometric Interpretability

The patient's recorded time history of dosing can be used as input to pharmacokinetic (PK) models, to project the time history of drug concentra-

tion in plasma, as Michael Weintraub's group was first to do (Rubio *et al.*, 1992). The time history of drug concentration in plasma, in turn, is the natural input to pharmacodynamic (PD) models, to project the time courses of drug actions. Implementation of this pharmacometric approach promises to eliminate much of the guesswork in prescribing—the very guesswork that results in consistent undermedication with drugs that have narrow therapeutic indices, e.g. oral anticoagulants, even in the peerless Dutch system (van den Besselaar *et al.*, 1988; van der Meer *et al.*, 1993; Urquhart *et al.*, 1994). Confident distinction between non-compliance and non-response is the key to confident prescribing, better medical management and better outcomes of treatment: better *value*.

4.1.3 Role in the Management of Medication

A third feature of "patient compliance" defined in objective, pharmacometric terms is its key role in a management approach to improving compliance of ambulatory patients—important for the one-third or so of patients in chronic pharmacotherapy who are poor or partial compliers. The management approach to such patients takes a leaf from the book of organizational management. Its essentials are: agreement between prescriber and patient about the desirability of treatment; the details of executing the drug regimen; the monitoring of the patient's dosing; and special instructions on interactions with other drugs or foods. Patients also know that they will review their dosing record periodically with the physician or pharmacist. Thus, patients carry a burden of accountability, knowing that their actual execution of prescribed drug regimens is recorded. Patient and physician or pharmacist review the dosing record periodically, seeking to fit the regimen into the patient's daily life, to maintain a regimen that can produce the desired outcomes.

4.1.3.1 Basics of the Management Approach. The key step is the comparison of what was actually done vs what was supposed to have been done. Its cornerstones are accountability, objectivity and freedom from self-reporting bias. Clearly, pharmaceuticals with greater margins for error in dose timing will be easier to use and thus especially valuable for the large minority of partially compliant patients. Pharmaceuticals that require punctual dosing for good outcomes will be troublesome for the majority of patients who are not naturally punctual in routine tasks.

4.1.3.2 Pertinent Experience in Other Fields. Management in this manner has a long record of utility in other areas of human endeavour. It is not a panacea, but the no-management alternative has a rich history of inefficiency and failure. Letting everyone follow their instincts, undirected, has

certain virtues in discovery-oriented research, but the subject here is routine therapeutics, not research. The management approach is just now in its first stages of being applied.

4.1.3.3 Failure Modes in Management. If the patient has not accepted the principle of drug treatment, then there is a need for renewed explanation and education to lay the groundwork for informed consent regarding a treatment plan. Those who fail to accept the principle of drug treatment usually take little or no medicine, and when they do take an occasional dose, it is often immediately prior to a scheduled visit to the doctor. Pre-visit dosing masks the clinical eye to otherwise poor compliance, but is readily identifiable with proper measurements (Feinstein, 1990).

Note in Proof. The management approach discussed in Section 3.1.3 had its first trial, with positive results, by Bakker *et al.*, as reported at the 1994 European Clinical Pharmacy meetings. The abstract is published in Supplement 6 of *Pharm. World Sci.*, p. G28, 1994.

4.1.3.4 Who Needs Medication Management? The well-informed patient who grants well-informed consent does not necessarily execute the dosing regimen satisfactorily. Many anecdotes testify to the prevalence of poor compliance among doctors, pharmacists and nurses. Readers can review their own experiences in trying to comply with prescribed drug regimens whose rationale they fully understood and accepted. One simple explanation is that advanced knowledge of therapeutics does not compete very effectively for priority in a busy schedule. Thus, claims that "patient education" will solve most compliance problems crash on the rocks of erratic compliance by the therapeutically well educated.

Note in Proof. Dr Carl Peck, previously Director of the Center for Drug Evaluation and Research at the US Food & Drug Administration, recently discontinued his personal use of beta-blockers for what had been generally effective prophylaxis of migraine. His reason for discontinuation, as related to the author, was recurring multi-day lapses in dosing, observed with electronic monitoring (MEMS®, APREX). As discussed elsewhere, such lapses are hazardous because of rebound hypersensitivity to endogenous catecholamines, which was an important factor in Dr Peck's decision. His understanding of clinical pharmacology is peerless, and, having self-prescribed the beta-blocker, his is the ultimate in doctor–patient relationship and communication. Dr Peck approved publication of this story, to help teach two principles: (a) education about disease and drugs is essential for the patient's making informed decision about treatment; but (b) education has little effect on the quality of the patient's execution of an agreed-upon treatment plan. This case report illustrates that abstract knowledge com-

petes poorly for priority in a busy schedule, and that punctual execution of drug regimens comes naturally only to some. Others need either a management approach, an especially long-acting, forgiving drug (or a use of a drug delivery system to confer long duration of action on a shorter-acting drug), or other means to ensure satisfactory drug exposure.

4.1.3.5 Summary. A reliably recorded time history of dosing is the cornerstone upon which patient and caregiver can cooperatively manage the medication programme, with the objective of maintaining a time history of dosing that corresponds well enough to the ideal regimen.

5 Methodologic Advances

Advances in measurement have freed the estimation of patient compliance from its long-standing dependence on methods easily manipulated by patients, whose reluctance to acknowledge poor compliance contributes to self-reporting bias, documented in many ways. The years 1986–1987 saw the introduction of chemical marker and electronic monitoring methods, which provide different but complementary estimates of the time history of dosing by ambulatory patients. These advances have been extensively reviewed (Feinstein, 1990; Pullar and Feely, 1990; Urquhart, 1990; Cramer and Spilker, 1991; Bond and Hussar, 1991; Vander Stichele, 1991; Kruse, 1992). The gist of both methods is as follows.

5.1 LOW-DOSE CHEMICAL MARKERS

Small quantities of slow-turnover, measurable agent are added to each dosage form, such that its ingestion introduces a defined quantity of marker into the body, but at doses of marker too low for it to have pharmacologic effect. Measurement of marker concentration in plasma (or quantity in urine) is a reflection of aggregate dosing during a period of a few days to a week. The method proves that marker-containing dosage forms were ingested, but can indicate only a range of time during which dosing occurred. Low-dose phenobarbital appears to be the best-validated marker (Feely *et al.*, 1987), used in the widest variety of conditions (Pullar and Feely, 1990).

Slow turnover is a key attribute. The slower the turnover, the wider the range of time during which a single measurement can indicate aggregate dosing. Also, measured plasma levels of slow-turnover agents generally avoid bias due to what Feinstein (1990) termed "white-coat compliance"—correct dosing during the day or two prior to a scheduled visit but partial or poor compliance at other times. White-coat compliance also results in the

plasma concentrations of most drugs giving an overestimate of compliance, for, unless the drug's turnover is exceptionally slow, its concentration in plasma reflects dosing only during the preceding 24–36 h—just when white-coat compliance is in full sway.

5.2 ELECTRONIC MONITORING

Microcircuitry is integrated into pharmaceutical packaging such that a time/date-stamped record is stored each time the package is used in the manner needed to remove a dose. The technique is called "medication event monitoring", for it automatically records date and time of presumptively taken doses, though it does not prove that the drug actually entered the body. In this sense, electronic monitoring is complementary to low-dose markers, which prove ingestion but cannot show timing. Electronic monitoring has been applied to eyedrop dispensing (Glover, 1976; Kass *et al.*, 1984), bulk containers for solid dosage forms (Cramer *et al.*, 1989; Averbuch *et al.*, 1990; Kruse and Weber, 1990), unit-dose "blister" packages (Eisen *et al.*, 1987; Cheung *et al.*, 1988; Potter, 1991), and nebulizers for inhalational drugs (Rand *et al.*, 1992). Figure 1 shows electronically monitored bulk containers for solid dosage forms; the circuitry is sufficiently compact not to distort the overall size, shape or weight of the package.

There is broad consensus that electronic monitoring is the best available method (Feinstein, 1990; Pullar and Feely, 1990; Bond and Hussar, 1991; Cramer and Spilker, 1991; Vander Stichele, 1991; Kruse, 1992). It does not prove dose ingestion, but the likelihood appears to be low of a patient's taking the trouble to execute the monitored manoeuvres with the package on schedule, without actually taking the dose. Several simple rules must be followed, e.g. the patient must use monitored packages as the sole sources of drug, and the dose must be ingested as soon as it is removed from the package.

6 Compliance Measurement in Clinical Trials

There is a large discrepancy between results with the pre- and post-1986–1987 methods for measuring compliance in clinical trials. Trialists persevere in estimating compliance from counts of returned, unused dosage forms, despite the fact that this technique has been shown to overestimate compliance by three methods in multiple settings (Cramer *et al.*, 1989; Mäenpää *et al.*, 1987; Pullar *et al.*, 1989; Rudd *et al.*, 1989; Guerrero *et al.*, 1993). Indeed, Pullar and his colleagues in Leeds compared chemical marker data with returned tablet counts in several hundred patients in various trial settings, and concluded that returned tablet counts "grossly

FIG. 1. Electronic medication event monitor in the process of communicating stored data from monitor to computer. The transfer occurs without wire connection between the inverted monitor and the communications interface device, which transfers data to MS-DOS-based computers via serial port. A second medication event monitor, with a somewhat larger container size, stands nearby. (Copyright, 1994, APREX Corporation; reproduced with permission.)

overestimate" compliance (Pullar *et al.*, 1989). Reluctance to return untaken doses is almost as prevalent as poor/partial compliance, so many patients opt to either hoard or discard untaken doses.

When patient compliance in drug trials is not ignored altogether, it is usually assessed by returned tablet count and found to be satisfactory in over 90% of patients—a finding that, if it were true, would make compliance a minor issue in trials. That, in turn, would justify the usual procedure of using the averaged responses of all patients as the best estimates of the drug's effects. However, the compliance estimates based on returned tablet counts simply do not jibe with the findings with low-dose markers or electronic monitoring. These new methods show substantial

underdosing by 30–40% of patients in drug trials (Pullar and Feely, 1990; Urquhart, 1990; Bond and Hussar, 1991; Cramer and Spilker, 1991; Vander Stichele, 1991; Kruse, 1992). This major discrepancy between old, bias-ridden methods and the new, essentially bias-free methods has many implications for the design and interpretation of drug trials. One of the main implications is for labelling policy.

6.1 IMPLICATIONS FOR LABELLING POLICY

With far greater prevalence of underdosing in clinical trials than detected by the older methods, all-patient averages underestimate the consequences of dosing per the recommended regimen—not only in respect to beneficial effects, but also in respect to adverse effects. As Lasagna and Hutt have noted (1991), it is a form of mislabelling to present in drug labelling only all-patient averages of drug effects, for they are diluted by poor compliance among a large minority. Such labelling misinforms compliant patients, who constitute at least a modest *majority* of treated patients. In effect, the label recommends a certain dose, but gives data on the consequences of taking a substantially lower dose (Lasagna and Hutt, 1991).

6.1.1 Cholestyramine Example

An example and model for enlightened labelling policy is the US labelling for cholestyramine (Anon., 1995a). This is based on the results of the Lipid Research Clinics Coronary Primary Prevention Trial (LRC CPPT) (1984), and includes estimates of drug efficacy at four different dosing levels:

(a) the all-patient average;
(b) full compliance with the dosing instructions;
(c) taking approximately half of the recommended dose;
(d) taking approximately a quarter of the recommended dose.

The label includes Table 1, which indicates that (b) was twice as large as (a), that (a) was about the same as (c), and twice as big as (d). As Table 1 shows, group (b) had just over half the patients, with the remainder about equally divided between (c) and (d).

This break-out of values is much more informative to the vast majority of patients than the all-patient average. Variable dosing, which occurred as well in the placebo group, though with a somewhat different distribution than in the active group, had no evident effect on the two main endpoints of the LRC-CPPT, cholesterol reduction and coronary risk reduction (Lipid Research Clinics Coronary Primary Prevention Trial, 1984).

TABLE 1

Compliance-related effects of cholestyramine on plasma cholesterol levels and risk of coronary events

Daily dose (4 g packets)	% Reduction in		% of patients in group
	Plasma cholesterol and	Coronary risk	
1–2	4.4	10.9	23.1
2–5	11.5	26.1	26.1
5–6	19.0	39.3	50.8
Average	8.5	19.0	100.0 (n = 1900)

Note: The concentration of cholesterol in the placebo group (n = 1893) was independent of measured compliance (Lipid Research Clinics Coronary Primary Prevention Trial, 1984).

Source: US labelling of cholestyramine (Anon., 1995a), based on data from the Lipid Research Clinics Coronary Primary Prevention Trial (1984); see also Urquhart (1991).

6.1.2 Gemfibrozil Example

Similar results were found in the Helsinki Heart Study (HHS) of gemfibrozil (Manninen *et al.*, 1988). Over one-third of patients were poorly or partially compliant, with placebo and active alike (Mäenpää *et al.*, 1987; Manninen *et al.*, 1988). The cholesterol fractions varied in relation to compliance with gemfibrozil, but not with placebo (Manninen *et al.*, 1988).

6.1.3 Primary vs Secondary Prevention Trials

It is important to note that both LRC-CPPT and HHS were primary prevention trials, carried out in patients whose only detectable abnormality was elevated lipid levels, and who were taking no chronic medications at the time of enrolment into the trial. In other trial situations, however, patients may be ill at the outset, and taking non-trial drugs that can influence the endpoints of the trial. These are described as secondary prevention trials. In that difficult situation, the measure of compliance with the trial agents, placebo or active, is also an approximate measure of compliance with non-trial agents, for one usually finds that patients take all or none of the prescribed agents at each point in the dosing schedule. Thus, if the non-trial agents are therapeutically important, one can expect that variable compliance with their recommended regimens will have potentially important

influence on the patient's well-being, including the trial endpoints. Thus can be created a strong correlation between compliance with placebo and trial outcomes, as discussed in detail by Urquhart (1991a), along with other aspects of compliance-stratified analysis of trials.

6.2 IMPLICATIONS FOR STATISTICAL POLICY

It has long been customary to analyse drug trials by the so-called "intention to treat" principle, wherein all patients randomized to receive a particular regimen are analysed as if they had correctly followed that regimen—irrespective of what actually happened. If, as the pre-1986–1987 methods indicated, poor compliance was only a problem in a small minority of patients, we could comfortably overlook non-compliance, for its effects would be very small. The reality, however, is otherwise, with the potential to create dilutional effects too big to ignore, resulting in mislabelling, as noted above. Thus, there is conflict between the ethical foundations of labelling policy and statistical policy. The resolution is to present the data both ways and let the informed user decide (Efron and Feldman, 1991; Feinstein, 1991; Hasford, 1991; Hurley, 1991; Lasagna and Hutt, 1991; Urquhart, 1991a).

6.3 SPECIAL CONSEQUENCES OF DRUG HOLIDAYS

A further complication for trials analysis is created by a particular pattern of non-compliance called the drug holiday—a multiday lapse in dosing, usually beginning with an abrupt halt in dosing, and usually ending in an abrupt resumption of full-strength dosing. Originally discovered by Michael Kass and his coworkers (1986a,b, 1987), studying compliance with topical ocular hypotensive drugs in glaucoma patients, drug holidays were analysed in relation to their pharmacodynamic, economic and risk implications (Urquhart and Chevalley, 1988).

6.3.1 Hazardous "Rebound" Effects

One consequence of these episodic interruptions in dosing is their potential to elicit "rebound" effects from drugs that are so prone. The term is used when cessation of dosing results in more than just a waning of drug action, but instead there occurs a temporary *reversal* of drug action, making matters worse, for a time, than the pretreatment baseline. A well-documented example is the occurrence of rebound hypertension and tachycardia (with

occasionally also angina pectoris) following the abrupt cessation of dosing with the most widely used type of beta-blockers—those that lack intrinsic sympathomimetic activity (Rangno and Langlois, 1982; Houston and Hodge, 1988; Gilligan *et al.*, 1991). Psaty and coworkers (1990) found an appreciably elevated relative risk of incident coronary disease apparently related to drug holidays with systemic beta-blockers in patients prescribed these agents for uncomplicated hypertension.

6.3.2 *Transient Overdose Effects*

An unexplored aspect of drug holidays is their potential to create transient overdose effects when dosing suddenly resumes after some days' lapse. Drugs most likely to show such effects are those that require careful upward titration of dosing at the outset of treatment, e.g. encainide, flecainide, the alpha-1 blockers, and the like. A key question is the rate at which a treated patient returns towards the drug-naive state when dosing suddenly halts. If the drug-naive state returns in a few days, then the resumption of full-strength dosing after a few days' lapse in dosing could be hazardous. One naturally wonders what role, if any, this phenomenon might have played in the results of the CAST trial of encainide and flecainide (Echt *et al.*, 1991). Recurring drug holidays are observed in only 15–20% of patients, so if serious risk were indeed concentrated in this small minority of recurrent holiday takers, it could have masked genuine benefits in patients who dose correctly.

6.3.3 *Drug Action Fades During Long Holidays*

Even if the drug is prone neither to rebound nor transient overdose effects, its actions will fade if the interruption in dosing is long enough, which brings us to the question of the differential ability of like-indicated drugs to maintain action in the face of interrupted dosing. This attribute is defined in answering the following question.

6.4 HOW MUCH COMPLIANCE IS ENOUGH?

In the face of so much variability in dosing, one naturally asks about how much latitude each drug may allow without either degradation of its beneficial effects or increase in its hazards. A few agents have been so characterized.

6.4.1 Example of Cholestyramine

The compliance-dependent efficacy of cholestyramine is defined in Table 1, as has already been discussed.

6.4.2 Combination Oral Steroidal Contraceptives

These widely used products provide another example. Their labelling in both the US and the UK has recently been modified to inform patients what steps they should take when they discover that they have missed one dose, two doses, or more than two doses (Guillebaud, 1993; Anon., 1995b). These recommendations have been based on a series of studies in which placebos were substituted for active drug in purposeful, controlled fashion (Morris *et al.*, 1979; Chowdry *et al.*, 1980; Wang *et al.*, 1982; Landgren and Diczfalusy, 1984; Smith *et al.*, 1986)—a useful trial design that, when ethically possible to use, can indicate consequences of missed doses free of concerns about biases that might lurk in purely observational studies. Some of these studies were performed in volunteers who had earlier chosen tubal ligation as a permanent method of contraception, thus allowing their hormonal responses to lapses in steroidal contraceptives to be observed with impunity.

6.4.3 Beta-blockers

A controlled withdrawal study design has been used to compare two beta-blockers in respect to their ability to maintain action in the face of a missed dose (Johnson and Whelton, 1994).

6.5 IMPLICATIONS FOR TRIAL DESIGN

Here, then, are two basic approaches to the question of ascertaining how much compliance is enough: (a) purposeful, controlled lapses in dosing, through the technique of substituting placebo for active; (b) building a correlation matrix between natural variations in compliance and trials outcomes. Each has its problems and limitations, but there can be no question about the desirability of having specific information showing how much compliance is enough and what to do when the bounds are exceeded.

6.5.1 Targeting the Partial Complier

There are also implications of the "how much is enough" question for the comparative evaluation of like-acting drugs. Agents that appear to be

indistinguishable when taken correctly, or when their all-patient averages are compared, may nevertheless act quite differently in partially compliant patients. The special needs of this large minority are usually ignored. An exception, however, is the innovative programme that Pfizer has devised for amlodipine in Belgium and Switzerland. Amlodipine is the longest-acting agent in its class, with a duration of action several times longer than the recommended 24-h interval between doses; thus, it would appear to be able to "forgive" occasional lapses in dosing of a day or two (Urquhart, 1994). As such, it could be considered to be the preferable agent for partial compliers, because it stands the best chance of being able to maintain action in the face of not-too-long, not-too-frequent lapses in dosing. Elsewhere, I have described a parameter called "therapeutic coverage", which allows one to quantify these considerations (Urquhart, 1991b).

6.5.1.1 Role of Once-daily Dosing. Once-daily dosing does not, *per se*, imply forgiveness (Levy, 1993). A crucial consideration in forgiveness is the relation between (a) the drug's post-dose duration of action and (b) the recommended interval between doses. The larger (a) is in relation to (b), the more forgiveness for delays or omissions in dosing. It is independent of dosing frequency. Indeed, a once-daily product with a post-dose duration of action little more than 24 h can be exceedingly unforgiving of common lapses in dosing. Such a product would be better given twice daily in half doses; the patient might skip a few more doses, but the focus should be on continuity of drug action, not the percentage of prescribed doses taken, which is a misconception carried over from tablet-counting days.

6.5.1.2 Role of Forgiveness. The most forgiving drug in its class can, all other things being equal, be expected to deliver the best outcomes in its class—precisely because common errors in dose timing or dose taking are least likely to undermine the drug's therapeutic actions and the outcomes that rationally follow from the maintenance of those actions. It is not yet routine to test for comparative forgiveness during premarket clinical development. It is a new entry in the table of contents of the pharmaceutical user's manual. The message is still "sinking in" that only one patient in about six is punctually compliant (Urquhart, 1994), and that, among the rest, lapses in dosing occur with varying frequencies, and may sometimes extend to 3–4 or more days. These are key findings for an outcomes-driven marketplace for pharmaceuticals. For a drug developer, it is far better to know that forgiveness is wanting and take steps to improve it, before investing in costly, long-term studies on comparative outcome.

6.5.1.3 Role of Disease Severity or Prognosis. It is also useful to know that the prevalence of poor compliance is seemingly irrespective of prognosis, e.g. with post-transplant immunosuppressants (Didlake *et al.*,

1988; Rovelli *et al.*, 1989) and tamoxifen for breast cancer (Waterhouse *et al.*, 1993). There is growing recognition in the field of organ transplantation that patient compliance is a leading cause of rejection (Didlake *et al.*, 1988; Rovelli *et al.*, 1989), which increases treatment cost 2-1/2-fold (Aswad *et al.*, 1993).

7 Conclusion

Underdosing, in various patterns, is a common feature of ambulatory care. Its prevalence makes it a natural topic, not only for investigation, but also for remedial action, based on understanding the clinical correlates of the common patterns of dosing. Drugs differ in the extent to which they forgive common errors in dosing, but such information is rarely provided. In an outcomes-oriented marketplace (Ellwood, 1988), the drug with the greatest degree of forgiveness for common errors in dosing will, all other things being equal, provide the best outcomes of treatment. Thus, it should be an integral part of clinical development to explore this property, with steps taken, if necessary, to redesign dosage forms or regimens to provide adequate forgiveness. This is a logical part of the table of contents of the user's manual for every pharmaceutical used in ambulatory care.

Forgiveness is one of a number of factors that have made relevant the measurement of patient compliance in drug trials. Others comprise a scientific framework for the measurements: a quantitative definition of compliance, reliable methods that are free of self-reporting bias, and a new environment in health care that focuses on outcomes of treatment. Perfection in compliance is not an end in itself; adequate compliance is the means to a satisfactory outcome. Understanding how much compliance is enough is important, as is communicating to patients what steps they should take when they find themselves having made errors in dosing. Comparing like-indicated agents in partially compliant patients can reveal otherwise hidden differences in seemingly me-too medicines. Thus, compliance is not just an abstract academic concept; its measurement and analysis are basic to sound therapeutics, offering new ways to design and differentiate pharmaceutical products for better, more cost-effective health care.

Thus the exploration of "misuse and its consequences" is rich in pharmaco-informatic content for the new pharmaceutical environment.

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