Methods and Principles in Medicinal Chemistry

Edited by
Eckhard Ottow and Hilmar Weinmann

WILEY-VCH

Nuclear Receptors as Drug Targets

Volume 39

Series Editors:
R. Mannhold,
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2008
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Nuclear Receptors as Drug Targets

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WILEY-VCH Verlag GmbH & Co. KGaA

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Cover Desciption:

Nuclear receptor research historically was built upon natural and synthetic modulators of the steroid receptors as a basis. Therefore the steroid skeleton was chosen as the core element of the cover illustration.

Today nuclear receptors represent one of the most important classes of drug targets. Modern drug finding methods and technologies like computational chemistry (molecular modelling picture in A-ring of steroid skeleton), molecular and cell biology (B-ring), medicinal chemistry for the preparation of steroidal and nonsteroidal modulators (C-ring) and structural biology (D-ring) have to be used in close collaboration to find novel and improved treatments for severe diseases in specific tissues (background of the graphics).

Library of Congress Card No.: applied for

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

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

Composition Thomson Digital, Noida, India
Printing Strauss GmbH, Mörlenbach
Bookbinding Litges & Dopf GmbH, Heppenheim

Printed in the Federal Republic of Germany

Printed on acid-free paper

ISBN: 978-3-527-31872-8

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ISBN: 978-3-527-31872-8

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ISBN: 978-3-527-31872-8

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Preface

With volumes on *G-Protein-Coupled Receptors* and *Voltage-Gated Ion Channels* we have started to edit volumes dedicated to important target classes. Here we introduce the third book following this concept. Eckhard Ottow and Hilmar Weinmann contribute a volume focusing on *Nuclear Receptors as Drug Targets*.

Nuclear receptors are a large superfamily of transcription factors involved in important physiological functions such as the control of embryonic development, organ physiology, cell differentiation and homeostasis. They play an important role in metabolism, homeostasis, growth and development, aging, and reproduction. Beyond normal physiology, nuclear receptors also play a role in many pathological processes, such as cancer, diabetes, rheumatoid arthritis, asthma and r hormone-resistance syndromes. Despite their long history these transcriptional regulators remain of great interest in modern drug discovery.

Nuclear receptors are soluble proteins that can bind to specific DNA-regulatory elements and act as cell-type- and promoter-specific regulators of transcription. In contrast to other transcription factors, the activity of nuclear receptors can be modulated by binding to the corresponding ligands – small lipophilic molecules that easily penetrate biological membranes. For a number of nuclear receptors, identified in recent years, no ligands are known. These so-called orphan receptors have attracted considerable interest since they could lead to the discovery of new endocrine-regulatory systems.

The target family of human nuclear receptors has a common evolutionary history as evidenced by their folding/sequence relationships and their common cellular function. Functions of nuclear receptors are highly complex and the pathways that are controlled by nuclear receptors are connected either mutually or with other partner proteins. Despite this complexity the nuclear receptor family has a long history of successful drug discovery. Recently, drug discovery in the field of nuclear receptors developed capabilities for profiling compounds within a setting much closer to the native physiological environment compared to previous studies. New technologies such as high-throughput methods in chemistry and structural biology, novel biochemical methods, and pathway analysis tools such as differential gene expression and proteomics will enable new discoveries finally leading to drugs with

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

improved therapeutic profiles. A better understanding of the ligand-induced activities that produce tissue-selective beneficial effects should enable the development of safer drugs with minimized side-effects. Furthermore, ligand discovery for the remaining orphan receptors might hold great promise. Target validation and better definition of therapeutic relevance for the remaining orphan nuclear receptors should be possible by using new tool compounds. Thus, despite its long history the nuclear receptor target family still bears tremendous potential, and nuclear receptor drug discovery should lead to highly effective and specific drugs for the future treatment of a broad variety of human diseases.

The present volume comprehensively treats nuclear receptors from the medicinal chemistry point of view. In an excellent introductory chapter, Eckhard Ottow and Hilmar Weinmann give a historic perspective on nuclear receptors as modern drug targets. The second section is dedicated to basic concepts and new perspectives in nuclear receptor research. Then, a comprehensive section individually treats estrogen, progesterone, androgen, glucocorticoid, as well as vitamin D receptors, followed by several chapters focusing on orphan and other nuclear receptors. Modern tools for nuclear receptor research are covered in the final part of the volume.

The Series Editors are grateful to Eckhard Ottow and Hilmar Weinmann for their enthusiasm in organizing this volume and to work with such a fine selection of authors. Last, but not least, we thank the publisher Wiley-VCH, in particular Dr. Nicola Oberbeckmann-Winter and Dr. Frank Weinreich, for their valuable contributions to this project and to the entire series.

April 2008

Raimund Mannhold, Düsseldorf
Hugo Kubinyi, Weisenheim am Sand
Gerd Folkers, Zürich

A Personal Foreword

Nuclear receptors have been drug targets for decades and this has led to an enormous body of knowledge about this target class and the medicinal chemistry of its small-molecule modulators. Nuclear receptors are interesting targets because of their great importance for many biological processes, with great potential for the treatment of severe diseases such as cancer, coronary heart disease and diabetes.

A further very important fact why nuclear receptors are attractive drug targets is that they are usually 'drugable', which means that the likelihood of identifying small-molecule agonists and antagonists, suitable for oral application, is rather high. Therefore, it is not surprising that numerous natural and synthetic nuclear receptor ligands, many of them belonging to the steroid structural class, are on the market. The huge economic impact of nuclear receptor targeting drugs is demonstrated by their estimated share of 10–15% of the $400 billion global pharmaceutical market. In 2003, 34 of the top 200 most prescribed drugs were targeting nuclear receptors.

The nuclear receptor family contains a large group of transcription factors, with 48 members identified in the human genome. Despite the fact that this figure is relatively small compared to the kinase or G-protein-coupled receptor target families, nuclear receptors form a very fascinating group which is still far from being completely understood in terms of its biological relevance or its modulation and control by natural and synthetic ligands.

Given such a strong motivation, this volume of *Methods and Principles in Medicinal Chemistry* attempts to present an overview on the various aspects of modern nuclear receptors research and its wide-ranging applications.

The book covers a broad spectrum of topics, ranging from pioneering research in the field of classical steroid hormones to very recently discovered orphan receptors and their modulators. State-of-the-art technologies are also discussed in the individual chapters that help to develop a deeper insight into the biochemical and pharmacological principles underlying the biological function of nuclear receptors.

In the introductory chapter a very brief overview on historic developments in nuclear receptor drug discovery from the pioneering experiments up to our current knowledge is given by Eckhard Ottow and Hilmar Weinmann.

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A field which is still far from being completely understood on a molecular level is the nuclear receptor–cofactor interaction. Luc Brunsveld, B. Vaz and S. Möcklinghoff give an introduction to our current knowledge in this field and explain why these interactions might be attractive targets for more selective modulators.

In the first chapter on classical steroid hormones Ross V. Weatherman brings more light to a special aspect of nuclear receptor regulatory systems by untangling the estrogen receptor web. Pharmacological use of a great variety of different chemotypes as subtype selective estrogens is summarized extensively by Gerrit H. Veenemann. The trilogy on the estrogen receptor is completed by V. Craig Jordan and Eric A. Ariazi who review estrogen receptors as therapeutic targets in breast cancer.

Klaus Schöllkopf and Norbert Schmees give an overview of the biology of the progesterone receptor and of the development of research in the field of steroidal progestins and recent trends in discovery of nonsteroidal selective progesterone receptor modulators.

Progesterone receptor antagonists and their potential clinical applications (e.g. in uterine myoma, endometriosis, breast, ovarian or uterine cancer, or as potential contraceptives) are discussed by Irving M. Spitz.

Androgens play an important role in male physiology due to their essential roles in male sexual differentiation, maintenance of muscle and bone mass, prostate growth, and spermatogenesis through the action of the androgen receptor. Michael L. Mohler, Casey E. Bohl, Ramesh Narayanan, Yali He, Dong Jin Hwang, James T. Dalton and Duane D. Miller review the various chemotypes of nonsteroidal tissue selective androgen receptor modulators and their clinical use (e.g. as prostate cancer treatments).

The following chapter by Heike Schäcke, Khusru Asadullah, Markus Berger and Hartmut Rehwinkel is dedicated to the glucocorticoid receptor as a target for classic and novel antiinflammatory therapy and novel glucocorticoid receptor ligands. The introduction of glucocorticoid therapy revolutionized antiinflammatory therapy. Fifty years after their initial clinical use, glucocorticoids are still the most important and frequently prescribed class of antiinflammatory drugs for various inflammatory disorders. Despite the many beneficial effects of classical glucocorticoids, however, their limitations and disadvantages seriously handicap their successful use as antiinflammatory agents. One goal of current research efforts therefore is to develop novel antiinflammatory strategies with more selective compounds, which are greatly needed.

Similarly, calcitriols, the vitamin D receptor agonists, are very well established for antiinflammatory treatment in daily clinical practice. Ekkehard May, Andreas Steinmeyer, Khusru Asadullah and Ulrich Zügel discuss the molecular and cellular principles of vitamin D action and drug discovery efforts for new vitamin D receptor modulators.

Peroxisome proliferator-activated receptors are ligand-activated receptors which regulate a number of genes involved in nutrient metabolism and energy homeostasis, and thus have served as drug targets for the treatment of metabolic diseases. Anne Reifel Miller and Alan M. Warshawsky review peroxisome proliferator-activated receptor γ modulation for the treatment of type 2 diabetes.

Retinoids are a class of polyisoprenoids that are derived by oxidative cleavage of β-carotenes of plant origin to yield vitamin A (retinol). They are essential for embryonic development and play important physiological functions, particularly in the brain and reproductive system, by regulating organogenesis, organ homeostasis, and cell growth, differentiation and apoptosis. The naturally occurring and synthetic retinoids are currently the subject of intense biological interest stimulated by the discovery of retinoid nuclear receptors and the realization of these compounds as nonsteroidal small-molecule hormones. Most retinoids that are currently used in dermatology and in oncology were discovered by chemical modifications on the basis of vitamin A structure and by biological evaluations in suitable pharmacological models. Vincent C. O. Njar provides the reader with a thorough overview of retinoids that are in clinical use.

Nuclear receptors also play an important role as drug targets in cardiovascular diseases. Peter Kolkhof, Lars Bärfacker, Alexander Hillisch, Helmut Haning and Stefan Schäfer review the mineralocorticoid receptor, peroxisome proliferator-activated receptorα and thyroid hormone receptors in detail based upon their therapeutic value, and additionally vitamin D receptor, retinoic acid receptors and retinoid X receptors as well as liver X receptors are covered with a special focus on their role in cardiovascular diseases.

The NR4A subfamily of receptors and their modulators is the topic of a chapter contributed by Henri Mattes. The NR4A subfamily of nuclear receptors has been implicated in Parkinson's disease, schizophrenia, manic depression, atherogenesis, Alzheimer's disease, rheumatoid arthritis, cancer and apoptosis. Therefore, there is currently great interest in the identification of selective modulators that may help to elucidate the mode of action of the NR4A subfamily.

Christoph Handschin describes the role of nuclear receptors, more specifically the pregnane X receptor and the constitutive androstane receptor, in the induction of drug metabolism and detoxification of drugs and other xenobiotics.

Nuclear receptor targeted screening libraries and chemogenomics approaches are a relatively recent field of research enabled by progress in cheminformatics as well as structural biology and combinatorial chemistry. J. Mestres summarizes the current knowledge and applications in this field of nuclear receptor research efforts.

As described in the individual chapters of this book, newly discovered receptors, recent insights into the effects of classical nuclear receptor action together with new technologies to explore their molecular mechanisms and to identify new modulators with finely tuned properties led to tremendous new interest in nuclear receptor research and fascinating novel results. The editors hope that the readers will share some of the excitement of this highly active field of research and the exciting emerging possibilities for the development of novel drug candidates.

We found work on this book both stimulating and thrilling, and we would like to acknowledge very much the great enthusiasm of all chapter authors in supporting this project and contributing their high-quality manuscripts within a tough schedule. Our acknowledgements go further to Dr. Frank Weinreich, Dr. Nicola Oberbeckmann-Winter and the whole staff of Wiley-VCH for their extremely professional support in the production of this monograph. Finally, we are

extremely thankful to the Editors of the series *Methods and Principles in Medicinal Chemistry*, Hugo Kubinyi, Raimund Mannhold and Gerd Folkers, for triggering this project and for giving us the opportunity to bring together this volume on *Nuclear Receptors as Drug Targets*.

May 2008

Eckhard Ottow
Hilmar Weinmann

1
Nuclear Receptors as Drug Targets: A Historical Perspective of Modern Drug Discovery

Eckhard Ottow and Hilmar Weinmann

1.1
Introduction

Nuclear receptors are a large superfamily of transcription factors involved in important physiological functions such as control of embryonic development, organ physiology, cell differentiation and homeostasis [1–3]. Apart from the normal physiology, nuclear receptors have been identified to play a role in many pathological processes, such as cancer, diabetes, rheumatoid arthritis, asthma or hormone-resistance syndromes [4, 5]. Therefore, despite their already long history, these transcriptional regulators are still of great interest in modern biomedical research and drug discovery.

Nuclear receptors are soluble proteins that can bind to specific DNA-regulatory elements and act as cell-type- and promoter-specific regulators of transcription [6]. In contrast to other transcription factors, the activity of nuclear receptors can be modulated by binding to the corresponding ligands – small lipophilic molecules that easily penetrate biological membranes. A number of nuclear receptors identified in recent years do not have any known ligands. These so-called orphan receptors have attracted considerable interest since they could lead to the discovery of new endocrine regulatory systems [7].

1.2
Short Historical Overview on Nuclear Receptors in Pharmacological Research and Drug Discovery

The first drugs in the nuclear receptor family were discovered prior to detailed pharmacological knowledge of the target class. Many compounds with clinical relevance were initially discovered by identification of biologically active compounds from natural extracts. Later, scientists used these biologically active molecules as tool compounds to derive the actual drug targets. Modern nuclear receptor-based

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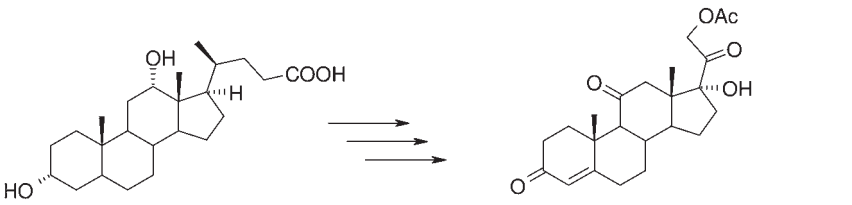
ISBN: 978-3-527-31872-8

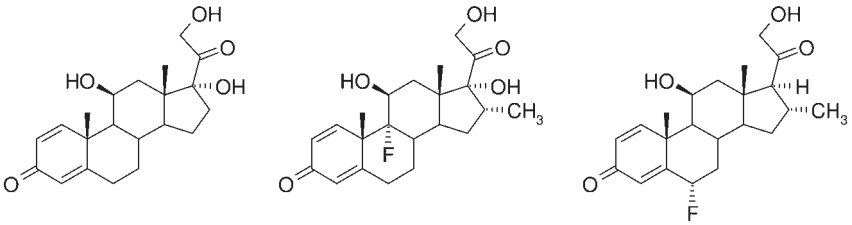
endocrinology was built in many cases on pioneering experiments with bioactive fractions from natural extracts that contained steroid or thyroid hormones [8].

1.2.1 Glucocorticoid Receptor Research

Glucocorticoid receptor (GR) drug discovery was initiated by adrenal gland extracts. These tissue extracts were used by clinicians to treat Addison's disease (glucocorticoid deficiency) [9]. From these early clinical studies it was shown that the adrenal extract was related to the maintenance of homeostatic function. It was discovered that, apart from bringing about remission from stress-related diseases, these extracts also suppressed symptoms in patients suffering from inflammatory conditions such as allergy, hay fever and asthma. At the same time cortisone was identified as an active steroidal compound by biochemical characterization of these adrenal gland extracts. In 1948 sufficient quantities of cortisone could be purified and its effects in inflammatory disease could be tested. Finally, total syntheses of cortisone were independently achieved by R. B. Woodward *et al.* [10] and an industrial research group at Merck in the early 1950s [11]. For the commercial production of cortisone a process was established by the Merck process research and development group. Given that chemical development at that time was done without the support of today's analytical tools like nuclear magnetic resonance, mass spectrometry and modern chromatographic methods the successful large-scale transformation of desoxycholic acid to cortisone acetate in more than 30 steps with 18 isolated intermediates was a tremendous achievement [12] (Scheme 1.1).



Desoxycholic acid **Cortisone acetate**



Prednisolone **Dexamethasone** **Fluocortolon**

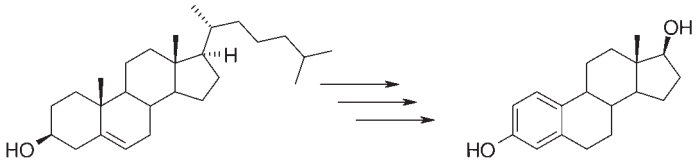
Scheme 1.1

With this early success the further evolution of the first-generation steroidal glucocorticoids was started, and the stage for later syntheses of potent synthetic steroids such as prednisolone, dexamethasone and fluocortolon was set [13].

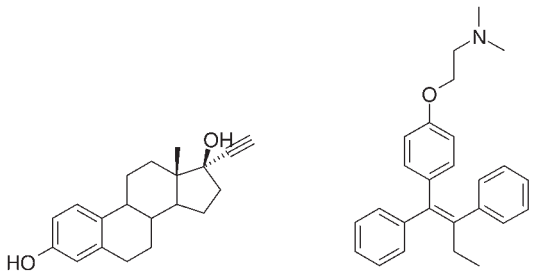
1.2.2 Estrogen Receptor Research

The first generation of drugs that targeted other steroid receptors was found by a similar history from natural sources. Estrone was isolated in 1929 as the first natural estrogenic hormone independently by the chemists E. A. Doisy and A. Butenandt from the urine of pregnant women, and its chemical structure was elucidated 1932 by A. Butenandt [14]. In 1938, H. H. Inhoffen achieved for the first time the synthesis of estradiol from cholesterol (Scheme 1.2). The first synthesis of ethinyl-estradiol, which has an improved oral bioavailability, was published in 1938 by H. H. Inhoffen and W. Hohlweg [15]. This discovery would later become one of the cornerstones of modern female fertility control and, even today, ethinyl-estradiol is an important component of many oral contraceptives.

From the very beginning, estrogen research also played an important role for other indications. It was known since 1916 that ovariectomy could decrease the incidence of mammary cancer in high-incidence strains of mice [16]. Studies of the biological effects of extracts containing estrogenic activity triggered screens for compounds with antiestrogenic effects – initially for contraception in the 1960s, but later for estrogen-responsive breast cancers. Screens for antiestrogenic nonsteroidal compounds led to the discovery of ethamoxytriphetol, clomiphene and tamoxifen. Tamoxifen finally became the gold standard for the endocrine treatment of breast cancer and the first approved cancer chemopreventative agent.



Cholesterol **Estradiol**



Ethinylestradiol **Tamoxifen**

Scheme 1.2

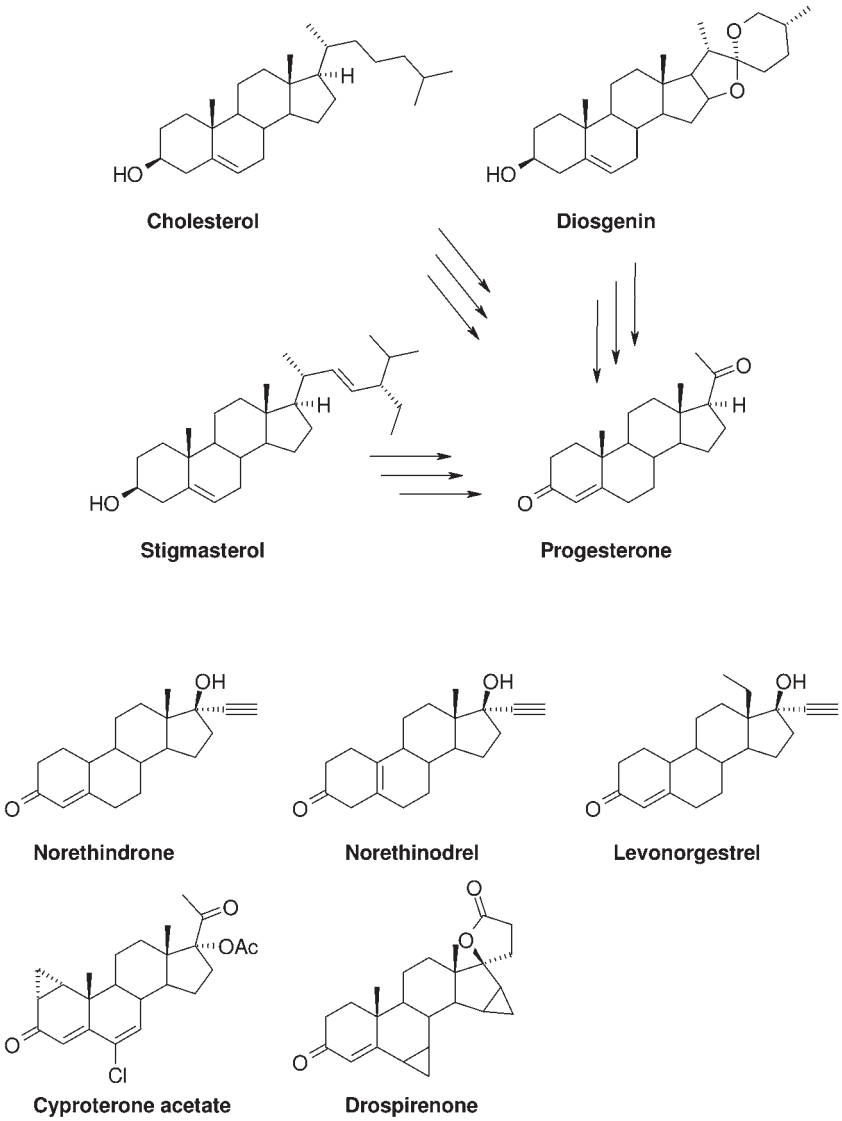
1.2.3 Progesterone Receptor Research

Progesterone was one of the first nuclear receptor hormones for which the functional role had been elucidated and used as a drug target. It plays an important role in female reproduction, controlling ovulation and maintaining pregnancy as well as in the growth and differentiation of endometrial and myometrial cells in the uterus.

The development of progesterone receptor (PR) ligands went through three important periods. In the beginning the development of steroid ligands that mimic the effect of natural progesterone was a major focus. Later on the development of steroids with improved properties and therapeutic applications was the central goal of synthetic work related to PR ligands. Nowadays, apart from further optimization of steroidal compounds, the development of nonsteroidal chemical entities with improved properties that might lead to still greater therapeutic applications is an important aspect of active drug discovery efforts [17].

As with other classic nuclear receptor hormones, identification and structure elucidation of progesterone started with the identification of a specific tissue that contained the biological activity. It was discovered at the beginning of the 20th century by surgical studies that removal of the corpus luteum led to the termination of pregnancy [18] and that extracts from the corpus luteum suppressed ovulation in rats [19]. It was demonstrated by Corner and Allen that gestational activity, including normal birth, can be restored in castrated rabbits by extracts from the corpus luteum [20]. This observation became a convenient assay to test progestational activity. Clauberg later developed a modified assay that eliminated the need for castration [21]. The isolation of purified crystalline corpus luteum hormone from sow ovaries was achieved by various groups in 1934 by applying these assays to identify the active fractions [22–25]. Ovaries from 10–15 sows were necessary to isolate enough purified hormone to induce the characteristic physiological changes in the uterus of one rabbit. Therefore, synthetic chemistry efforts were initiated to prepare the hormone in larger amounts. In these early synthetic studies readily available steroids from natural sources like cholesterol (from cow and pig fat), stigmasterol (from soy beans) and diosgenin (from Mexican yams) were used by various groups (Scheme 1.3) [26].

The observation that progesterone prevented ovulation was taken up by Haberlandt who was able to demonstrate that it could be used for fertility control [27]. In 1955, Gregory Pincus demonstrated that large doses of orally administered progesterone inhibited ovulation in women [28]. Despite its potential application as a contraceptive, the practical use of progesterone was limited by the fact that it is essentially inactive when administered orally. Therefore the discovery of a compound with contraceptive properties and better drug-like properties was the focus of synthetic efforts with progesterone as a lead structure. Inhoffen had discovered already in 1938 that 17α-ethynyl-testosterone was a weakly active progestin when administered orally [29]. More than a decade later, in 1951 C. Djerassi and a team of scientists at Synthex succeeded in synthesizing 19-nor-17α-ethynyl-testosterone or



Scheme 1.3

norethindrone [30]. Scientists at Searle reported at just about the same time the successful synthesis of the double-bond isomer of norethindrone, named norethynodrel [31]. Both compounds revealed approximately twice the activity of progesterone in animal studies when given orally. These results triggered clinical studies [32] that ended with the market introduction of Enovid (norethynodrel) in 1959 and Ortho-Novum (norethindrone) in 1962 as oral contraceptives. With the introduction of these two oral contraceptives into the marketplace, the era of

progesterone receptor ligands entered a new phase. More potent progestines, like levonorgestrel and gestodene, and progestines with a special favorable profile on other nuclear receptors, like cyproterone acetate and drospirenone, were discovered during the following decades of intense research efforts in this field [33]. Cyproterone acetate was the first progestin with a potent antiandrogenic effect and it is still in use for the treatment of prostate cancer [34]. Drospirenone is structurally related to the aldosterone antagonist spironolactone. It has not only progestagenic properties, but is also a very potent antimineralocorticoid and it also shows antiandrogenic activity [35]. This pharmacodynamic profile is quite close to the natural progesterone.

1.2.4 Other Receptor Research

As these early research efforts had resulted in modulators of the steroid receptor subgroup of the nuclear receptor family, these compounds could be used to support the purification of the receptors in the first nuclear receptor gene-cloning studies. The first human nuclear receptor that was cloned was the GR. This was achieved by using reagents made available from the purification and biochemical characterization of adrenal extracts. With purified receptor, selective antibodies were used to help isolate the corresponding cDNA [36, 37]. cDNAs with the full-length coding region of GR provided the first full-length amino acid sequence of a nuclear receptor. Around the same time three research groups independently succeeded in cloning also the estrogen receptor (ER) [38–40]. The comparison of nuclear receptor sequences from human and other species revealed conserved domains in the nuclear receptor family.

The finding that nuclear receptors could be isolated without prior knowledge of their ligand triggered research for new nuclear receptors. The number of orphan nuclear receptors rapidly exceeded the number of classical nuclear hormone receptors [41–43]. The preferred method for the identification of new nuclear receptors shifted from the laboratory to *in silico* methods due to the availability of large databases of randomly generated partial cDNA sequences known as expressed sequence tags (ESTs) and new bioinformatic query tools. Two new mammalian nuclear receptors were successfully identified through automated searches of EST databases. The pregnane X receptor (PXR) was discovered in a public mouse EST database by a high-throughput *in silico* screen for nuclear receptor-like sequences [44] and the photoreceptor cell-specific receptor (PNR) was identified in a human EST database [45]. Finally the number of known human nuclear receptors reached 48 after the PNR was isolated from EST databases. In 2001, the availability of the complete human genome sequence confirmed that there are 48 members in the human nuclear receptor family [46, 47] (Table 1.1).

The nuclear receptor superfamily can be generally divided into four major subfamilies based on their DNA-binding properties and dimerization preferences. However, this classification is rather broad and does not take into account of any evolutionary relationship between nuclear receptors. Therefore, a new phylogeny-based nomenclature approved by the Nuclear Receptor Nomenclature

Table 1.1 List of human nuclear receptors and systematic nomenclature.

Name	Abbreviation	Nomenclature	Ligand
Thyroid hormone receptor	TRα	NR1A1	thyroid hormone
	TRβ	NR1A2	thyroid hormone
Retinoic acid receptor	RARα	NR1B1	retinoic acid
	RARβ	NR1B2	retinoic acid
	RARγ	NR1B3	retinoic acid
Peroxisome proliferator-activated receptor	PPARα	NR1C1	fatty acids, leukotriene B4
	PPARβ	NR1C2	fatty acids
	PPARγ	NR1C3	fatty acids, prostaglandin J2
Reverse erbA	Rev-erbα	NR1D1	orphan
	Rev-erbβ	NR1D1	orphan
Retinoic acid receptor-related orphan receptor	RORα	NR1F1	cholesterol
	RORβ	NR1F2	retinoic acid
	RORγ	NR1F3	retinoic acid
Liver X receptor	LXRα	NR1H3	oxysterols
	LXRβ	NR1H2	oxysterols
Farnesoid X receptor	FXRα	NR1H4	bile acids
	FXRβ	NR1H5	lanosterols
Vitamin D receptor	VDR	NR1I1	vitamin D
Pregnane X receptor	PXR	NR1I2	xenobiotics
Constitutive androstane receptor	CAR	NR1I3	xenobiotics
Human nuclear factor 4	HNF4α	NR2A1	orphan
	HNF4γ	NR2A2	orphan
Retinoid X receptor	RXRα	NR2B1	retinoic acid
	RXRβ	NR2B2	retinoic acid
	RXRγ	NR2B3	retinoic acid
Testis receptor	TR2	NR2C1	orphan
	TR4	NR2C2	orphan
Tailless	TLL	NR2E2	orphan
Photoreceptor-specific nuclear receptor	PNR	NR2E3	orphan
Chicken ovalbumin upstream promoter-transcription factor	COUP-TFI	NR2F1	orphan
	COUP-TFII	NR2F2	orphan
ErbA2-related gene-2	EAR2	NR2F6	orphan
Estrogen receptor	ERα	NR3A1	estradiol
	ERβ	NR3A2	estradiol
Estrogen receptor-related receptor	ERRα	NR3B1	orphan
	ERRβ	NR3B2	orphan
	ERRγ	NR3B3	orphan
Glucocorticoid receptor	GR	NR3C1	cortisol
Mineralocorticoid receptor	MR	NR3C2	aldosterone
Progesterone receptor	PR	NR3C3	progesterone

(*Continued*)

Table 1.1 (*Continued*)

Name	Abbreviation	Nomenclature	Ligand
Androgen receptor	AR	NR3C4	testosterone
Nerve growth factor-induced factor B	NGFI-B	NR4A1	orphan
Nur-related factor 1	NURR1	NR4A2	orphan
Neuron-derived orphan receptor 1	NOR1	NR4A3	orphan
Steroidogenic factor-1	SF1	NR5A1	orphan
Liver receptor homologous protein 1	LRH1	NR5A2	orphan
Germ cell nuclear factor	GCNF	NR6A1	orphan
DSS-AHC critical region on the chromosome gene 1	DAX1	NR0B1	orphan
Short heterodimeric partner	SHP	NR0B2	orphan

Committee has been proposed for nuclear receptors in addition to the original names [48]. This nomenclature system is based upon multiple alignment procedures and phylogenetic tree reconstruction methods, which finally led to the subdivision of the nuclear receptor superfamily into seven subfamilies which are numbered from 0 to 6. The phylogenetically closest members of each subfamily are combined into groups designated by capital letters arranged in the alphabetical order and the individual genes within each group are numbered. This nomenclature system should overcome the problem of the existence of several names and abbreviations of the same gene.

With the discovery of new nuclear receptors, novel interdependencies between first-generation drugs and their targets were detected. For example, traditional pharmacological research methods had revealed earlier that thiazolidinediones (TZDs) have a clinical benefit in diabetes; however, the molecular basis for this therapeutic effect was not known. By using expression constructs derived from the isolated nuclear receptor genes, activity screens for each receptor were developed. By using such screens it was found that TZDs are potent and selective activators of peroxisome-proliferator-activated receptor (PPAR) γ [49]. After discovery of this connection, the search for a second-generation PPARγ compounds was started using an *in vitro* assay for PPARγ activation. This second-generation approach of using the receptor rather than a bioactive extract can be characterized as a 'reverse endocrinology' approach. Historically, ligands of nuclear receptors have been discovered due to their biological effects. In a modern reversal of this process, the orphan receptors can be used to identify ligands, which then can be used to study the biological role of the receptors.

In such a reverse endocrinology approach the farnesoid X receptor (FXR) could be connected to bile acid ligands. By further exploration of bile acids and other chemical probes for FXR it was discovered that FXR is linked to bile acid homeostasis, and it was postulated that FXR ligands might have beneficial effects for the treatment of cholestatic liver disease and other disorders [50].

Recently, a third-generation drug discovery effort has begun in the field of nuclear receptors research. Newer screening methods which deliver additional information

besides potency and selectivity (e.g. selective effects on gene expression) can be used to discover leads with therapeutic advantages over drugs on the market.

1.3 Recent Progress in Nuclear Receptor Drug Discovery

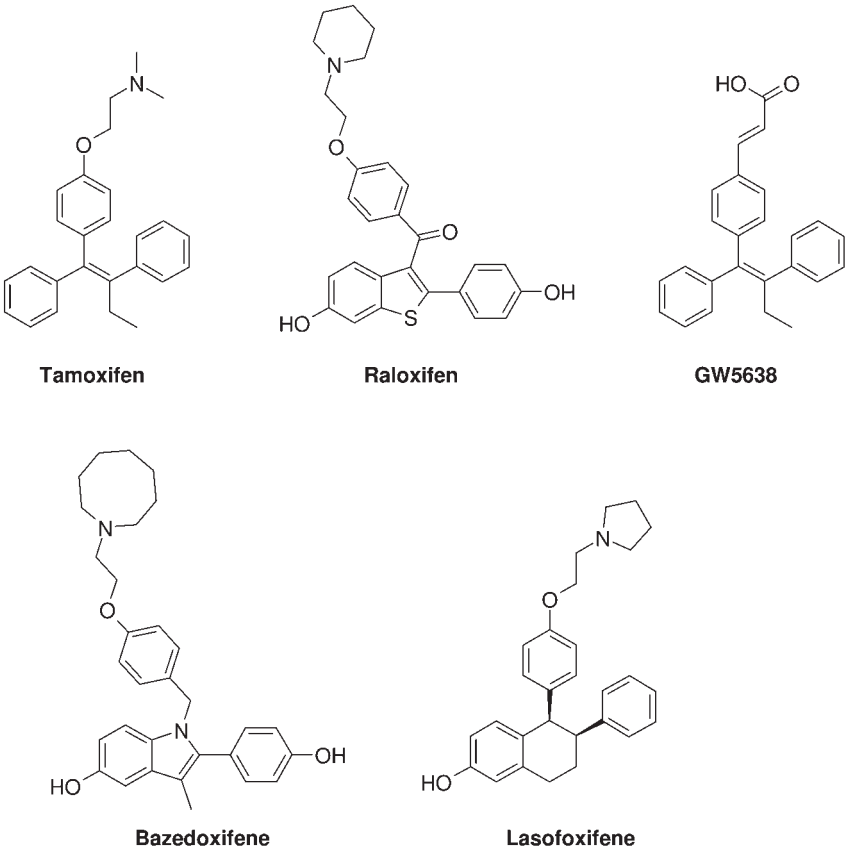
Nuclear receptors have been studied in drug discovery research for decades. Synthetic ligands for a certain receptor usually have been identified through high-throughput screening (HTS) or structure-based drug design approaches. In the second step these lead structures have to be optimized further by medicinal chemists to adjust the properties of these compounds to appropriately modulate the activities of the receptor. Ligands that display differential activities compared to the natural ligand have been referred to as selective nuclear receptor modulators. Originally this concept was demonstrated for the ER with the selective ER modulators (SERMs), 4-OH-tamoxifen and raloxifene. These SERMs retained tissue-selective agonist activity in bone tissue and on lipid profile for raloxifene, but functioned as antagonists in reproductive tissues [51, 52]. Although both molecules were originally considered to be 'antiestrogens', 4-OH-tamoxifen demonstrated a general trend toward estradiol-like activity in uterine tissue, whereas raloxifene had no such activity. These pioneering experiments related to novel ER ligands triggered studies for the identification of novel, tissue-selective synthetic modulators for several of the therapeutically relevant nuclear receptors. Overall, the current goal of nuclear receptor drug discovery efforts in many cases is to manipulate the receptor with a ligand to retain tissue-selective benefits while minimizing unwanted side activities.

1.3.1 SERMs

The stilbene derivative tamoxifen was the first synthetic nuclear receptor small-molecule modulator that demonstrated differential tissue effects. However, it has not found wide application as treatment for menopausal symptoms due to its stimulatory effects on the uterus which cause a potential risk for endometrial cancer [53]. Despite this drawback tamoxifen is still used as a treatment for ER-positive breast cancer.

Raloxifene, a second-generation SERM, was originally developed as a follow-up agent for tamoxifen in breast cancer. During development studies it was found that this modulator has significant osteoporosis protective effects without the endometrial activities of tamoxifen [54]. The molecular basis for these ER-modulating properties has been at the center of major pharmacological research activities [55]. One possible mechanism that was suggested is the differential effects of SERM bound ER to promote corepressor association versus coactivator association [56, 57]. Following a theoretical approach that small-molecule ligands can induce specific ER conformations several triphenylethylene ligands for ER were prepared and screened through an uterine Ishikawa cellular assay [58]. Compounds which decreased estrogen-mediated

Ishikawa cell stimulation were then tested in ovariectomized rats for the ability to protect against loss of bone mineral density. It was found that GW5638 had antagonist properties on the uterus and agonist activities on bone and the cardiovascular system [59]. In further experiments it was shown that the biological properties of GW5638 derive from triggering a structural conformation of ER different from the conformations imposed by other SERMs [60]. Recently, several novel SERMs (e.g. bazedoxifene, lasofoxifene) have been identified with a combination of cellular screens, primarily uterine- and breast cell-based assays [61–63] (Scheme 1.4).



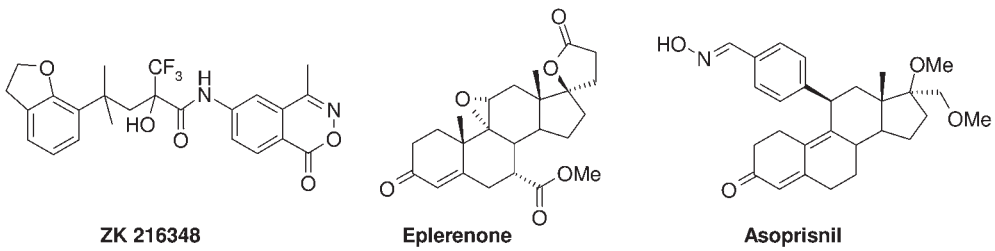
Scheme 1.4

Two other novel approaches have recently been reported in the field of ER ligand discovery. The potential of compounds as pathway-selective ligands and antiinflammatory agents was studied by the use of NFκB-driven reporter assays [64]. A second relatively recent focus for ER-directed drug discovery is related to the fact that there are two subtypes of this receptor, ERα and ERβ, which derive from two separate genes [65, 66]. Stimulated by the specific tissue distribution pattern of these two related receptors, research to find ER subtype-selective modulators for the treatment

of various diseases was triggered. Several reports have demonstrated that it is possible to identify ERβ-selective ligands [67, 68].

1.3.2 Selective GR Modulators

The classic synthetic glucocorticoids dexamethasone and prednisone are in use for the treatment of various severe diseases such as rheumatoid arthritis, inflammatory myopathies, cancers and several immunological disorders. Unfortunately, long-term treatment with these drugs often triggers serious side-effects such as fat redistribution, diabetes, vascular necrosis and osteoporosis. Therefore, major drug discovery efforts are currently ongoing to find novel chemical entities that are able to differentially modulate GR to retain the beneficial effects of glucocorticoids without the unwanted side-effects of current treatments [69]. Many of the antiinflammatory effects of GR are believed to be driven by the ability of the monomeric form of the receptor to interfere with NFκB and AP-1 function, which ultimately results in the reduction of proinflammatory cytokines such as interleukins (IL)-1, -2, -6 and -8, and tumor necrosis factor-α [70]. Various chemical ligand series which display differential GR activation have been reported. Three main methods for measurement of GR activity were used to identify these ligands: (i) direct GR binding compared to other steroid receptors, (ii) a cell-based assay measuring glucocorticoid response element-mediated gene transcription (transactivation), and (iii) cell-based assays measuring the ability of GR to regulate NFκB and AP-1-driven genes (transrepression) . Several steroid-based compounds have been shown to differentially decrease transactivation with only minimal effects toward transrepression [71, 72]. A quinoline-based series of compounds described as a nonsteroidal class of GR ligands was found to have a trend toward a preferred transactivation/transrepression profile in cellular assays. Some of these ligands also demonstrated a more promising therapeutic window for selective *in vivo* effects [73, 74]. Another nonsteroidal GR ligand, ZK 216348, showed significant dissociation of transactivation and transrepression activities [75]. Thorough pharmacological characterization *in vitro* proved that ZK 216348 is a dissociative molecule. Further *in vivo* experiments with an ear inflammatory model for efficacy and models for skin atrophy, weight gain, adrenal weight and blood glucose levels for unwanted side-effects showed an improved therapeutic profile relative to prednisone (Scheme 1.5).



Scheme 1.5

1.3.3
Other Modulator Efforts: PR, MR, AR, PPAR, FXR and LXR (see Table 1.1)

The theory of selective nuclear receptor modulation to achieve therapeutic value by an optimized activity profile different from the natural ligand has been also experimentally studied in several other receptors.

An example from the PR is the steroid ligand asoprisnil, which has been shown to produce antiuterotrophic effects with only minimal labor-inducing and breakthrough bleeding effects [76].

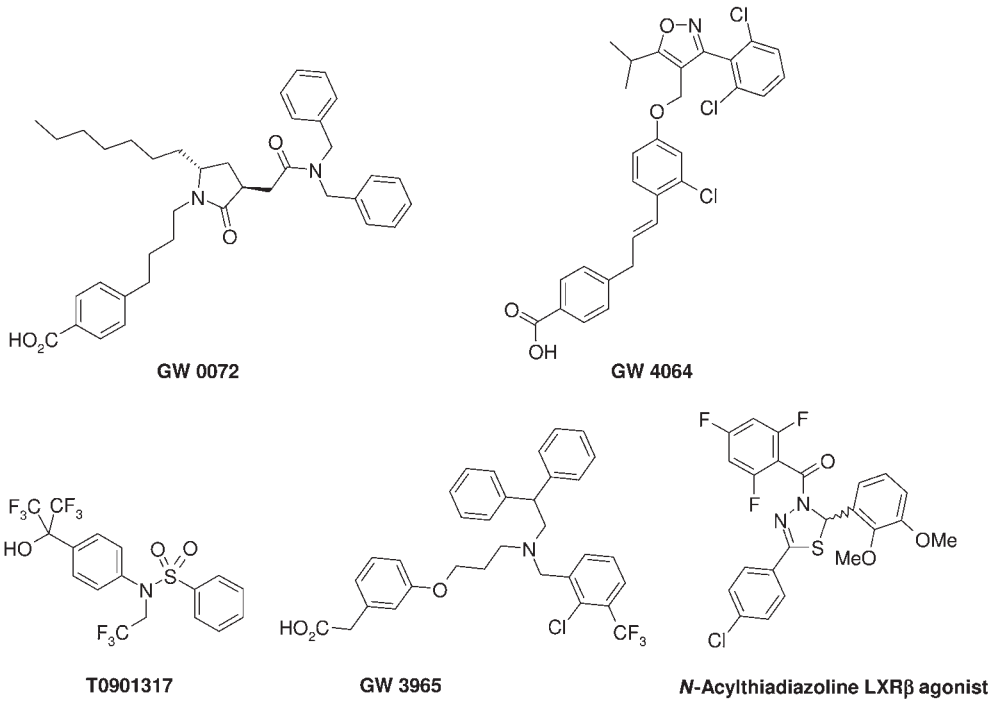
Eplerenone, a selective MR modulator, was discovered decades ago and has recently received approval as a treatment for hypertension [77]. This synthetic steroid derivative has a higher specificity for MR relative to other nuclear receptors and works as a partial antagonist of aldosterone [78].

Modulators of the AR for application in prostate cancer and possibly treating the neurological and muscular degenerative symptoms of androgen deficiency form the basis of another field of intense research efforts [79, 80]. LGD2226 is a recent example of a tissue-selective AR modulator, which seems to retain some anabolic effects on bone and muscle with reduced proliferative effects on the prostate [81].

Selective modulators for PPARγ (SPPARMs) have been successfully discovered by several groups. The first-generation TZD class of PPARγ agonists which are in use as insulin sensitizers show dose-limiting issues such as hemodilution and edema. It was found in early experiments of PPARγ activation by TZDs that these ligands activate through direct interaction with the C-terminal AF-2 helix [82]. Structural studies have also revealed PPARγ activators that bind the ligand-binding domain (LBD) using non-TZD epitopes such as the partial agonist GW0072 [83]. Ligands with specific binding and activation modes are a potential starting point for the discovery of PPARγ modulators with improved biological activities. Non-TZD-selective PPARγ modulators have been discovered which induce an altered LBD conformation compared with TZDs [84]. These compounds demonstrated qualitative differences versus traditional agonists toward gene expression in cell culture and *in vivo*, as well as toward *in vivo* physiological responses such as adipose depot size. Therefore, further research efforts to come up with novel SPPARMs may lead to compounds with improved characteristics compared to currently used therapeutics.

FXR and the LXRs are other examples of nuclear receptors for which modulators have been investigated recently [85, 86]. It was possible to identify ligands with potential novel biological activity compared to the natural ligands (e.g. the potent synthetic FXR agonist GW4064 [87]). LXRα/β are regulated *in vivo* by oxysterols and this regulation is in accordance with the role of the LXRs in cholesterol homeostasis [88]. Studies with nonsubtype-selective LXR tool compounds in animal models revealed that besides atheroprotective effects, these agonists also promote lipogenesis and triglyceride accumulation in liver. The LXR agonists T0901317 and GW3965 show differential effects on cofactor recruitment in human hepatoma cell assays. Furthermore, these two ligands show different *in vivo* effects on hepatic lipogenesis genes. As LXRα is the dominant subtype in the liver, where LXRβ is expressed at very low levels, there might be a good chance that an LXRβ-selective agonist may retain

efficacy without increasing hepatic lipogenesis. *N*-Acylthiadiazolines were reported recently as a new class of LXR agonists with selectivity for LXRβ [89] (Scheme 1.6).



Scheme 1.6

More experimental studies will be necessary to better understand the molecular basis of modulator effects both for the steroid receptor and nonsteroid receptor modulators.

In summary, these examples show the high degree of complexity required at several levels, such as high-affinity binding to the receptor, induction of conformational change or altered structural dynamics, selection of an appropriate cellular assay for measuring nuclear receptor modulation as well as selection of relevant *in vivo* models for determining any therapeutic effects. Due to structural and functional similarities within the nuclear receptors superfamily, there is a high probability that knowledge created from one receptor concerning modulation by a synthetic ligand can be applied to other members of the family [90, 91]. This increased knowledge of nuclear receptor functions will be the base for novel, safer and more effective drugs. New technologies to profile ligands are especially important for rapid progress into this direction.

1.4 Modern Methods and Technologies in Nuclear Receptor Drug Discovery

New technologies in molecular biology, structural biology, computational methods and peptide interaction methods are important tools for discovering novel drug

candidates for the modulation of specific nuclear receptors. These novel technologies enable rapid profiling of nuclear receptor ligands in a more physiologically relevant manner.

1.4.1 Cofactor Interaction Screening

Nuclear receptors do not act in an isolated way, but in complex associations with other cellular factors. Cofactor interaction screening can give valuable insights into the relationship between nuclear receptor structure and functional activity [92]. If a certain ligand changes the pattern of cofactor interaction compared to other ligands, it is likely that the different *in vitro* profile will translate into a special gene expression pattern or physiological outcome *in vivo*. Peptides representing these interactions can be prepared based on known interaction motifs or isolated through HTS of random peptide libraries. This method has been applied to characterize known SERMs and to discover ER ligands with unique properties. Affinity selection of peptides has been used to identify binding surfaces that are exposed on ERα/β when complexed with different ligands, such as with estradiol or 4-OH-tamoxifen [93]. It was discovered that the established SERMs, which are known to produce distinct biological effects, induced specific conformational changes in the receptors. Ligand screens for subtle differences between ER ligands have been performed based on nuclear receptor–peptide interactions using a high-throughput multiplexed technology, which used fluorescently encoded microspheres [94, 95]. The repertoire of novel nuclear receptor-interacting cofactors has strongly expanded in the past few years. For the rapid identification of novel interacting cofactors, genome-wide screens for binding partners have been performed in yeast and mammalian-based two-hybrid systems. More than 200 human nuclear receptor cofactors have been identified. Especially for the discovery of new specific nuclear receptor modulators these interacting cofactors are of great importance as each new cofactor has the potential to give a better insight into a particular cellular interaction and thus lays the foundation for a molecular screen for ligands that uniquely affect this interaction.

1.4.2 Microarray Technology and Gene Expression Profiling

Nuclear receptors are transcription factors and therefore monitoring ligand effects on nuclear receptor target genes is a powerful tool for drug discovery. This approach has been limited in the past due to difficulties and the related costs in measuring endogenous gene expression. It is now possible by using microarray technology to assess endogenous gene expression on a genome-wide scale and this technology has been used to define an unbiased set of nuclear receptor target genes. Microarray technology has been applied to differentiate the functions of ERα and ERβ in estrogen target organs such as bone, breast and uterus. Human U2OS osteosarcoma cells which express neither ERα nor ERβ were stably transfected with human ERα/β

to achieve overexpression of the receptors in this bone model system [96]. Two overlapping but distinct patterns of gene expression were found after treatment of the two cell lines with 17β-estradiol; 28% of the estradiol-regulated genes were ERα cell specific, whereas 11% were ERβ cell specific. These experiments enabled the functional dissection of the pathways regulated by two functionally similar receptors. Furthermore, unique sets of endogenous target genes have been identified for use in ligand-screening assays. The effects of various ER modulators on ERα and ERβ target genes have been studied in similar experiments with U2OS cells expressing either ERα or ERβ [97]. It was demonstrated by microarray analysis that raloxifene and tamoxifen regulated only 27% of the same genes in the ERα- and ERβ-containing cells. These results give clear hints that estrogens and SERMs exert tissue-specific effects by regulating unique sets of target genes. Methods for high-throughput analysis of gene expression could be an entry into screening of large numbers of compounds in a cellular environment using a cost-effective technology. Advances in glass slide preparations for monitoring transcriptional changes of several thousand genes make it possible to assess a hit from a multiwell cell treatment over a genome-wide range of genes in a cost-effective way. Slight differences between even very closely related chemical structures can be detected with such an analysis. Gene-expression profiling has been used to characterize breast cancer cells and to identify desired 'molecular fingerprints' within the experimental data [98]. In this way key 'biomarkers' can be identified that deliver information related to the phenotypic effect of a chemical modulator. The huge amounts of data generated by such an approach are challenging for bioinformatics methods. Therefore, advances in gene-expression profiling as a method for drug screening has to be complemented by progress in bioinformatics and data handling.

1.4.3 Novel Computational Methods

Nuclear receptor target genes have not only been discovered through experimental studies, but also *in silico* approaches have been developed which can accelerate nuclear receptor drug discovery. Comprehensive computational approaches can now be used to detect nuclear receptor target genes. A new computer algorithm (NUBIscan) gives the opportunity to predict nuclear receptor target sequences in regulatory regions of genes [99]. High-throughput genome-wide chromatin immunoprecipitation methods have been combined with computational methods to discover ER target genes and promoter sequences [100]. *In silico* methods for gene identification complement microarray approaches in an efficient way as they are not biased by target tissue or expression levels.

1.4.4 Structural Biology

Despite the described progress in many fields of nuclear receptor research which has also resulted in the determination of novel signalling pathways, for a

surprisingly large group of orphan receptors the ligands have remained unidentified. Structural biology contributed important novel insights into nuclear receptor-binding domains and helped to adopt some orphan receptors by crystallography [101, 102]. These studies suggest that not all of the orphan nuclear receptors use ligand binding in the classical way. Some orphan receptors lack the capacity for ligand binding, which suggests that they are regulated by alternative, ligand-independent mechanisms [103]. Nurr1 is an orphan nuclear receptor that has been studied thoroughly especially due to its essential role in developing and adult dopamine neurons. Therefore, ligands for Nurr1 could be of potential therapeutic value, but identification of Nurr1 ligands has proven difficult. Structural studies of the Nurr1 LBD can contribute to the explanation of these difficulties. Bulky and hydrophobic amino acid side-chains in the Nurr1 LBD occupy the space that form the LBD in other nuclear receptors, therefore prohibiting interaction with any ligand. The constitutive activity of Nurr1 can be explained by the position of its AF-2 helix, which is folded in an active conformation. Therefore, Nurr1 is the first example of a nuclear receptor that functions entirely independent of ligand binding.

1.5 Summary and Future Developments

Human nuclear receptors play an important role in many physiological processes such as metabolism, homeostasis, differentiation, growth and development, aging, and reproduction. This target family has a common evolutionary history as evidenced by their sequence relationship and their common cellular function [104]. Functions of nuclear receptors are highly complex and the pathways which are controlled by nuclear receptors are connected with each other as well as with numerous other partner proteins [105]. Despite this complexity, the nuclear receptor family has had a long history of successful drug discovery. Over the last decade drug discovery in the nuclear receptors field has started to develop capabilities for profiling compounds in a high-throughput manner in a setting much closer to the native physiological environment compared to previous studies [106]. New technologies like high-throughput methods in chemistry and structural biology, novel biochemical methods, and pathway analysis tools such as differential gene expression and proteomics will enable new discoveries which in the end will lead to drugs with improved therapeutic profiles. A better understanding of the ligand-induced activities that produce tissue-selective beneficial effects should enable the development of safer drugs with minimized unwanted side-effects. Furthermore, ligand discovery for the remaining orphan receptors might hold great promise. Target validation and better definition of therapeutic relevance for the remaining orphan nuclear receptors should be possible by using new tool compounds. In summary, despite its long history the nuclear receptor target family still bears tremendous potential, and nuclear receptor drug discovery should lead to highly effective and specific drugs for the future treatment of a broad variety of human diseases.

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2
Targeting the Nuclear Receptor–Cofactor Interaction

Belen Vaz, Sabine Möcklinghoff, and Luc Brunsveld

2.1
Introduction

The developmental and physiological processes regulated by nuclear receptors are the result of the interplay between the receptors, their small-molecule ligands (hormones), their response elements on the DNA and a large set of interacting proteins, the so-called cofactors [1, 2]. Up to recently, the modulation of the function of nuclear receptors concentrated mainly on ligands with agonistic, antagonistic or partial (ant)agonistic properties that bind in the binding pocket of the ligand-binding domain (LBD), also occupied by the natural ligands. These types of ligands have proven very beneficial for the treatment of various diseases and the regulation of hormone functioning, as illustrated by many of the chapters in this book. Some of these ligands have even been shown to feature tissue-specific or -selective (ant) agonistic properties, which is often of crucial importance for the separation of the beneficial effects of nuclear receptor stimulation or inhibition from unwanted side-effects, regulated by the same receptor [2]. Nevertheless, predictability and control over tissue specificity of these classical ligands is difficult. The physiological effects of some of the nuclear receptor ligands are sometimes also altered over a period of time. A typical example of this is the transition of nuclear receptor ligands used for the treatment of specific cancers from an antagonistic profile into an agonistic profile [3]. Interestingly, there are also nuclear receptors for which, up to now, no ligand, neither of natural nor of synthetic origin, has been found. These so-called orphan receptors seem not to be amenable to modulation by classical ligands.

The limitations of the current nuclear receptor ligands generate a continuous quest for new ligands with optimized profiles in both industry and academia. The requirements of the effects of these ligands on the level of the organism are usually quite clear; the translation of these requirements to the level of the conformation of the protein and structure of the ligand is, however, usually not evident. This is due to a major extent to the large plethora of protein–protein interactions between nuclear receptors and their many cofactors [1, 2]. Binding of a ligand results in a change of the

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

conformation of the LBD and with that it influences the dimerization of the nuclear receptor with other proteins [4]. There are several hundreds of cofactor proteins known, but both the molecular rules for the formation of the nuclear receptor–cofactor complexes and the physiological meaning of many of these are often still a mystery [5]. Additionally, ligand binding also influences other nuclear receptor–protein interactions (e.g. with NFκB) located at topically different positions on the nuclear receptor. Considering these issues, it is clear that the nuclear receptor–cofactor interaction is a subject of intense investigation. Both the molecular understanding of this interaction [6], its relationship with the observed physiological effects [7] and the possible inhibition of this interaction are topics at the forefront of nuclear receptor research.

The most prominent interaction of nuclear receptors with coactivators, a specific subset of the cofactors that promote gene transcription, is a so-called helix–groove interaction; the coactivator proteins feature short peptide motifs, the LXXLL motif (the nuclear receptor box), that fold into amphiphatic helices which bind to a hydrophobic groove on the nuclear receptor LBD surface. The presence or absence of this interaction is mainly regulated via the positioning of the C-terminal helix of the LBD, helix 12 (H12). Together with H3, H4 and H5, H12 forms a hydrophobic groove, surrounded by charged amino acids, which is termed the second activation function (AF-2). H12 is the most flexible helix of the LBD; the positioning of H12 accounts for the presence or absence of the hydrophobic groove on the nuclear receptor surface. Classical ligands regulate the positioning of H12 on the LBD [8]. The individual nature of these ligands determines the exact positioning and flexibility of H12, and with that the interactions with the coactivators and thus the control over gene expression. The difficulty in predictably controlling this interaction and its crucial role in nuclear receptor protein–protein interactions has initiated the search for compounds that can inhibit this interaction by directly blocking it via binding at the interaction site on the nuclear receptor.

The nuclear receptor–coactivator interaction is a rather small and confined protein–protein interaction, with clear interaction motifs, such as three hydrophobic side-chains of the amphiphatic helix of the coactivator binding in the hydrophobic groove of the nuclear receptor and a charge clamp consisting of two hydrogen bonds from the nuclear receptor to the LXXLL helix backbone. These characteristics have spurred the hope that the nuclear receptor–coactivator interaction is a drugable interaction, amenable to modulation with small molecules that directly bind to the hydrophobic groove on the nuclear receptor surface. Such ligands would be important chemical tools in the biological elucidation of this protein–protein interaction and would have the promise of yielding new types of nuclear receptor antagonist (Figure 2.1).

Two major issues have to be dealt with in the search for such types of coactivator binding inhibitors (CBIs) [9]. First of all it has to be proven whether direct inhibition of this interaction is a valid principle to antagonize nuclear receptor functioning. Apart from via the AF-2, nuclear receptors also control gene transcription via their activation function 1 (AF-1), located at the N-terminus of the protein, in a more or less ligand-independent way. This additional interaction actually results in more complex

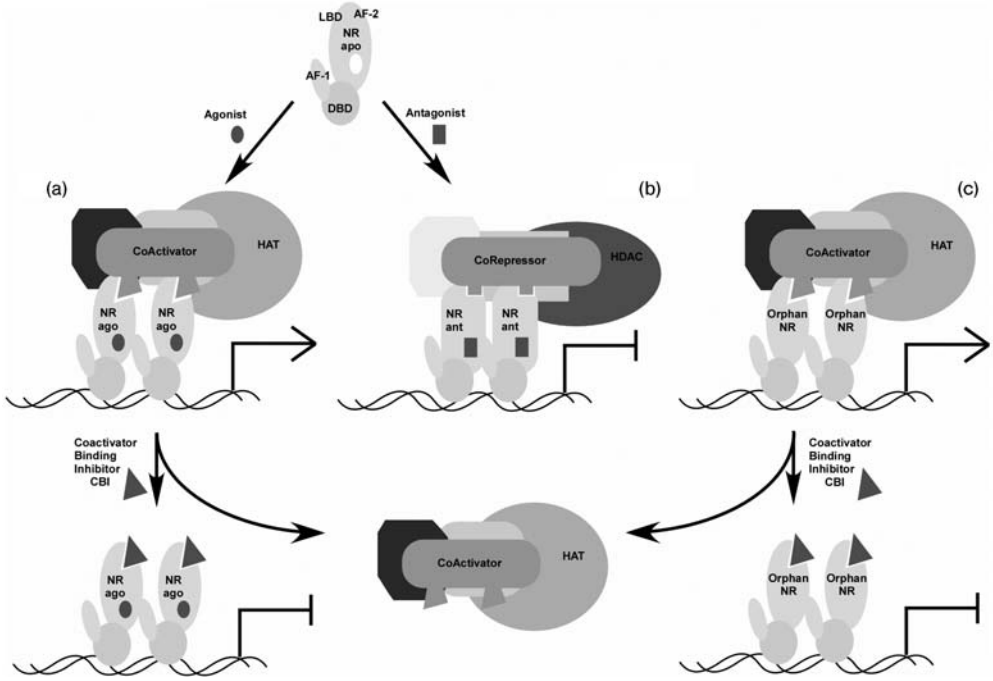


Figure 2.1 Schematic, simplified representation of the modulation of nuclear receptor functioning via classical agonists (a) resulting in the recruitment of coactivators featuring among others histone acetyltransferase (HATs) functionalities and via classical antagonists (b) resulting in the recruitment of corepressors featuring among others histone deacetylase (HDAC) functionalities. A new approach is the modulation of nuclear receptor functioning via CBIs targeting either agonist liganded nuclear receptors (a) or orphan nuclear receptors (c), resulting in the displacement of the coactivator complex.

models for the interaction between nuclear receptors and coactivators than that displayed in Figure 2.1 [10]. Depending on the type of nuclear receptor, these two activation functions have different importance. The functionality of inhibiting the nuclear receptor–coactivator interaction will also depend on the cell type and its different expression levels of the different coactivators. The second issue that requires elucidation is the question whether small-molecule ligands can be found that feature both sufficient binding affinity to the hydrophobic groove to displace coactivator proteins and that display selectivity for a specific nuclear receptor over the other nuclear receptors. The 48 human nuclear receptors and the over 300 cofactors undergo a complex combination of protein–protein interactions with overlapping specificities and affinities [6]. A successful inhibitor would require a potent and selective profile on the nuclear receptor of interest.

In this chapter we discuss both the insight that has been gained concerning the biological relevance of inhibiting the nuclear receptor–coactivator interaction and the progress made in the development of inhibitors for this interaction. The biological

relevance strongly depends on the type of nuclear receptor under study. We therefore decided to discuss this topic for specific nuclear receptors individually. Chemically different types of CBIs have been developed such as peptides and small molecules. This classification has also been used for their discussion, in order to allow a good comparison. The main focus of both topics will be on the biology and the compounds for the two estrogen receptors (ERα and ERβ), the androgen receptor (AR) and the thyroid receptor (TR), since the major part of the reported literature deals with these receptors.

2.2
Evaluation of the Nuclear Receptor–Cofactor Interaction as a Drug Target

The molecular elucidation of the nuclear receptor–cofactor interaction has greatly benefited from the availability of a number of crystal structures of these complexes, usually featuring the LBD of the nuclear receptor and a LXXLL or FXXLF peptide motif representing the coactivators (Figure 2.2) [11]. These structural studies together with biological studies [6] have resulted in understanding part of what defines the selectivity of the interaction of the nuclear receptor binding groove with the LXXLL motif. Nevertheless, it is still for the most part unacknowledged what defines the exact specificity of a certain nuclear receptor for a specific LXXLL motif and how this relates to ligand binding. Understanding the precise details of how nuclear receptors distinguish between the plethora of cofactors will help to answer the question whether selective inhibition of a nuclear receptor–cofactor interaction is feasible and whether this can be done with small molecules.

A second outstanding question is whether the direct inhibition of the nuclear receptor–coactivator interaction also leads to a functional inhibition of nuclear receptor functioning. The pharmaceutical validity of antagonizing specific nuclear receptors is unquestioned; however, classical hormone ligands often do so via different or more stringent structural mechanisms including not only the inhibition of coactivator binding. Nuclear receptors generally feature two activating functions (AF-1 and AF-2) with different functional priority in gene transcription activation. Inhibition of only the AF-2 via CBIs might not be sufficient for completely antagonizing nuclear receptor functioning. Additionally, many coactivators feature more than one LXXLL motif and bind to nuclear receptor dimers in a multivalent fashion, leading to strong binding. In order to establish the direct inhibition of the nuclear receptor–coactivator interaction as a *bona fide* pharmaceutical target, this issue requires addressing. In this section the current research that has focused on answering this question will be highlighted. Since there are significant functional differences in how the AF-2 functions among the different nuclear receptor family members, this topic is discussed using results obtained on the ER–coactivator and the AR–coactivator interactions. This selection is directed by the clear differences in AF-2 functionality, the different coactivator motifs bound by these receptors (LXXLL versus FXXLF), and by the significant amount of research interest in these two proteins in the structural biology, biochemical and cell biological fields.

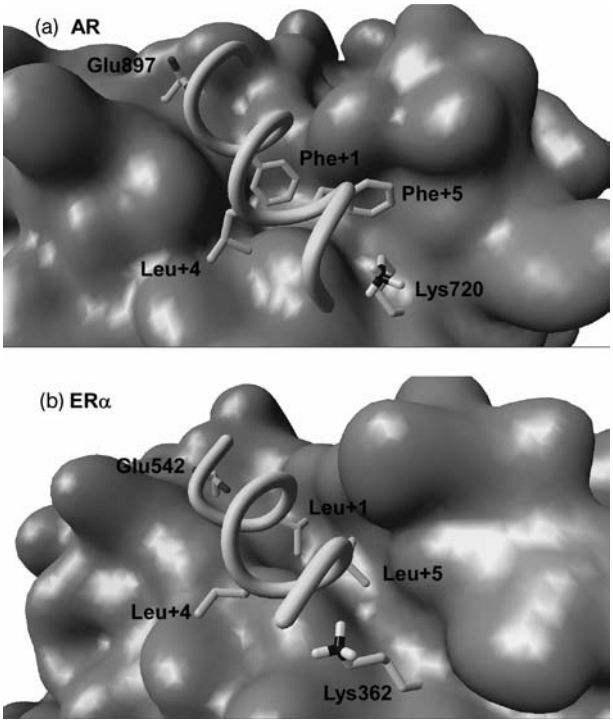


Figure 2.2 (a) Representation of the AR LBD surface bound to a FXXLF peptide motif (based on PDB ID 1T5Z) [12]. (b) Representation of the ERα LBD surface bound to a LXXLL peptide motif (based on PDB ID 3ERD) [13]. In both structures the LBD surface is displayed with the lysine and glutamic acid side-chains forming the charge clamp. The helical peptides are displayed via a tube with the side-chains of the three hydrophobic amino acids binding to the hydrophobic cleft on the nuclear receptor displayed in a stick model. For both cases the hydrophobic side-chains at positions $+1$ and $+5$ bind in a hydrophobic groove, which is deeper in the case of the AR, and the hydrophobic side-chain at $+4$ binds on a shallower hydrophobic patch.

2.2.1
Evaluation of the ER–Coactivator Interaction

The action of estrogens and analogs in regulating gene expression is mediated mainly by the two subtypes ERα and ERβ. These two isoforms share a relatively high sequence homology with the LBD featuring the lowest identity (58%), resulting in certain differences in ligand-binding specificities [14, 15]. The activity of both ERs depends on their interaction with cofactors via α-helical LXXLL motifs [13]. The inhibition of this interaction of ERs with coactivators has been investigated in the natural setting of the cell. Using both full-length ER and full-length coactivators it was shown that direct inhibition of this protein–protein interaction is possible, resulting in the inhibition of ER-mediated gene transcription [16]. With the use of phage display, specific peptides were identified *in vitro* that featured antagonistic

Table 2.1 Amino acid numbering of the LXXLL and FXXLF motifs and representative natural peptide motifs and peptide motifs isolated via phage display against ER and AR.

	−5	−4	−3	−2	−1	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10	+11	+12
LXXLL						**L**	X	X	**L**	**L**							
FXXLF						**F**	X	X	**L**	**F**							
AR18–32	T	Y	R	G	A	**F**	Q	N	**L**	**F**	Q	S	V	R	E		
SRC2–3	K	K	E	N	A	**L**	R	Y	**L**	**L**	D	K	D	D	T		
F6	E	P	L	T	L	**L**	E	R	**L**	**L**	M	D	D	K	Q	A	V
α/βV	S	S	P	G	S	R	E	W	F	K	D	M	L	S	R		
pM-622–5					L	**F**	S	N	**L**	**F**	Y	G	T	P	Y	G	A

properties regarding the ERα–coactivator and/or the ERβ–coactivator interaction [16–18]. These peptides contained the typical LXXLL motif and were shown to bind in the coactivator binding groove. Some of these peptides were shown to successfully inhibit estradiol-mediated transcriptional activity up to 50% (e.g. peptide F6, Table 2.1) when coexpressed in cells featuring ERα [16, 18]. When two of these peptides were linked via a 50-amino-acid spacer, the transcriptional activity of ER was even antagonized to below 20% [18]. These studies thus provide a cellular proof of concept that inhibition of the ER–coactivator interaction is a pharmacologically valid concept to block ER mediated gene transcription.

The inhibitory effects of the peptides were cell type dependent, highlighting the differences in importance of AF-1 and AF-2 for transcriptional activity also for ERs in different cells. Interestingly, the peptides shown to inhibit estradiol-mediated transcriptional activity were observed not to be able to block ERα transcription mediated by 4-OH-tamoxifen. Binding of 4-OH-tamoxifen in the LBD results in a conformation of the receptor that prevents LXXLL coactivators from binding, via preventing the right positioning of H12 [11, 19]. Peptides isolated against 4-OH-tamoxifen-activated ERα therefore generally did not contain the LXXLL motif (e.g. peptide α/βV, Table 2.1). Expression of these peptides in cells blocked the partial agonistic activity of 4-OH-tamoxifen liganded ERα up to 90%, while having no or only a minimal effect on estradiol-mediated transcription [16]. These and other peptides [20] generated against 4-OH-tamoxifen liganded ERα or ERβ are known to interact with a region of the LBD that is not affected by the distinct conformational effects induced by receptor agonists and antagonists [21]. This unique interaction surface may play a role in the sensitivity of ERs to coregulators in the presence of antagonists like 4-OH-tamoxifen. Thus, this is another nuclear receptor–cofactor interaction that offers the possibility for the development of pharmaceuticals antagonizing the partial agonistic activity of, for example, 4-OH-tamoxifen.

The displacement of coactivators from ERα and ERβ has also been investigated in detail *in vitro* using the full-length receptors, full-length steroid receptor coactivator (SRC) 1a and a DNA estrogen response element (ERE) [22]. It was also shown in this *in vitro* study that short LXXLL-containing peptides from p160 coactivators competed with the complete SRC1a for binding to estradiol liganded ER bound to ERE. Around

100-fold molar excess of the peptides was required to inhibit SRC1a binding by 50%, suggesting that whereas the nuclear receptor box region is a primary site of interaction between SRC1a and ER, additional contacts between the coactivator and the ligand–receptor–DNA complex, such as two LXXLL motifs of the same protein binding to the nuclear receptor dimer, and the interaction with the AF-1, contribute to the overall affinity.

2.2.2
Evaluation of the AR–Coactivator Interaction

Although the general mechanism of action of the AR is similar to other members of the nuclear receptor superfamily, there are certain significant differences in the activation of AR and its cofactor-binding characteristics. An important feature of the AR LBD is the relatively low affinity for most, but not all, LXXLL motifs and, instead, a high affinity for aromatic-rich motifs found, for example, within the N-terminus of the AR itself (FQNLF and WHTLF) [23]. The interaction of the N-terminal AR FXXLF motif with the hydrophobic AF-2 of the AR LBD is androgen controlled and an important feature for the stabilization of H12, slowing down the dissociation of the ligand from the LBD [24]. Even though the predominant role of AF-1 in AR transactivation is clear, questions still remain concerning the *in vivo* functional effects of the N/C interaction and its role in transactivation. Antagonist-binding does not initiate the N/C interaction in AR and results in recruitment of the corepressor NCoR, demonstrating dramatic differences in receptor conformation [25]. The N-terminus–AF-2 interaction has been shown to be of an important nature; however, studies have shown that especially upon binding to DNA, this interaction looses importance [26]. The importance of the N-terminus–AF-2 interaction has also been shown to depend on the type of promoter to which the AR will be recruited, leading to different functional interactions with coactivators [27]. Other proteins have also been shown to associate to the AR via its AF-2, including several coactivators (e.g. ARA70, ARA54, ARA55, etc.) [28–31]. All of these cofactors contain FXXLF motifs, and the specific androgen dependency of the interaction of these motifs with the AR LBD was confirmed by peptide interaction and mutagenesis studies [31]. It has been shown that these proteins can enhance the transcriptional activity of the AR and that these proteins can even confer transcriptional activity to antagonist-bound AR [32–34]. AR-interacting FXXLF and FXXFF peptides isolated by phage display also showed selectivity to the AR compared to other nuclear receptors, and helped to identify additional AR cofactors with FXXFF and FXXMF motifs, like gelsolin and PAK6 [35]. Other studies successfully selected several, relatively specific, AR-interacting peptides containing (F/W)XXL(F/W), FXXLY or FXXYF motifs [36, 37]. These findings raise the possibility that other, so far unrecognized, proteins interact with the AR LBD via similar motifs. Some of the identified peptides were shown to be capable of suppressing the AR N/C interaction and a selected set of these peptides was actually capable of suppressing AR transactivation [37]. It has been postulated that FXXLF-containing AR-interacting peptides can be separated into two functionally distinct groups [38] – those that functionally resemble the natural N-terminal FXXLF, but do

not have significant antagonistic properties, and those that interact with AR in the presence of any ligand and function as effective antagonists. The latter peptides were isolated using phage display against full-length AR, and showed high affinity and selectivity to the AR. This high affinity and a different functional inhibition mode could be the reasons that these peptides are able to overcome the occupancy of AF-2 with the N-terminal FXXLF motif and also effectively antagonize the AR. Some of these peptides showed almost complete inhibition of AR-mediated gene expression when coexpressed in cells, proving the possibility to effectively antagonize the AR in cells with a CBI (e.g. peptide pM-622–5, Table 2.1) [38].

Crystal structures of the AR LBD in complex with several natural and phage display-derived peptides revealed the structural basis of the FXXLF motif specificity [36]. The AR coactivator binding site is unique in that it is able to rearrange upon cofactor binding to form a longer, deeper, narrower and smoother binding groove on the surface of the AR LBD in comparison with other nuclear receptors (Figure 2.2) [36, 39]. This allows high-affinity binding of two Phe residues at positions $+1$ and $+5$ in a FXXLF motif. The backbone of a bound LXXLL peptide forms only one hydrogen bond with one of the conserved charge clamp residues, Lys720, instead of both Lys720 and Glu897 as with FXXLF peptides [12, 36]. These characteristics make the AR AF-2 domain specific for accommodation of bulky side-chains like in FXXLF motifs and less attractive for LXXLL motifs [36]. Nevertheless, it could be demonstrated that AR also binds a subset of LXXLL motifs, particularly SRC2–1 and SRC2–3, with a higher affinity than FXXLF motifs derived from the AR N-terminal repeat and ARA70 [12]. Moreover, using transfection assays, it could be proven that binding occurs via the AR LBD and leads to a relatively strong transcription activity. In fact, it is not the LXXLL motif alone that is responsible for the high-affinity binding of SRC2–3 to AR-AF-2, but also the negative charges in the four residues following the motif (Table 2.1) interact with positively charged sites on the receptor surface [12].

Mutations in the AR and coactivator overexpression play an important role in prostate cancers [40]. Mutation of V730, for example, to longer side-chains, as occurs in somatic prostate cancers, increases the binding of the SRC1–4 box LXXLL motif in the presence of typical AR agonists [39]. In prostate cancers an overexpression of p160 coactivators accounts for binding of these coactivators to the AR AF-2, overruling the AF-2 interaction with the AR N-terminus [27, 41]. Typically antiandrogens are used to inhibit the transcriptional activity of the AR in prostate cancers. However, after a certain period of time many tumors become resistant to these antiandrogens due to the high frequency of mutations in AR in advanced prostate cancer [3, 42]. The T877A mutation in the AR LBD facilitates increased binding affinities to ligands such as estradiol, progesterone and some antagonists like hydroxyflutamide (OHF) that subsequently function as agonists [43, 44]. OHF binding induces a conformational change within the T877A mutant which enables the N/C interaction of the AR and accommodates the receptor in its active conformation [45, 46]. It is, however, also reported that the AR T877A mutation results in better recruitment of other FXXLF motifs as well as an SRC3–1 LXXLL motif [47]. These data indicate that especially in specific prostate cancers the nuclear receptor–coactivator might be a valid target to be antagonized with CBIs.

2.3
Inhibitors of the Nuclear Receptor–Cofactor Interaction

2.3.1
Phage Display Peptides

Phage display is a powerful method for the creation of highly diverse peptide libraries displayed individually on the surface of small bacterial viruses called phages. This huge collection of diverse peptides can be exposed to a target protein (e.g. the LBD of a nuclear receptor) for the identification of peptide binders. By performing the phage display against different nuclear receptors or nuclear receptors bound to different ligands, peptide sequence information regarding affinity and specificity to nuclear receptor binding can be obtained. Phage display screening has been applied for the search of peptides binding the LBD of nuclear receptors at the hydrophobic groove typically binding LXXLL peptides. Several potent and nuclear receptor specific peptides have been identified (e.g. for both ERα and ERβ, and AR and TRβ).

Phage display was first applied in nuclear receptor research by McDonell *et al.* in 1999 [16, 17] and enabled the identification of peptides that could interact selectively with either 17β-estradiol- or 4-OH-tamoxifen-bound ERs. With these peptides it was demonstrated for the first time that it is possible to antagonize nuclear receptor activity by the use of compounds targeting a binding site different from the ligand-binding pocket. Together with other studies, these phage display-derived peptides also allowed the identification of the amino acid characteristics at different positions in the peptide required to induce ER selectivity in the LXXLL motif [18]. It was observed, for example, that flanking residues of the LXXLL motif determine the selectivity in terms of preference and affinity for a specific receptor. Residues located at positions −2 and −1 (Table 2.1) play a critical role in the specificity, and together with the charge clamp, determine the positioning and orientation of the motif in the hydrophobic cleft.

Phage display has also been applied for the search of peptide partners of the AR [37]. A set of peptide sequences was, for example, found that contain the (F/W)XXL(F/W) and FXXLY motifs. Known AR coregulators such as ARA 70, ARA 55, ARA54 and FHL2 as well as the N-terminus of AR also feature the FXXL(F/Y) motifs, which shows that sequence comparison of phage display peptides with known proteins is a suitable method for the identification of cofactors of nuclear receptors. The requirement for bulky hydrophobic amino acids such as Phe, Trp and Tyr at positions 1, 4 and 5 in the LXXLL motif for optimal binding to the AR was further shown by Ala mutation studies at those positions in AR coregulators [37]. Peptides binding to the AR similarly as for ER feature preferred flanking amino acids. A clear preference for a positively charge amino acid, such as Lys or Arg, at position −1 and a hydrophobic amino acid, such as Phe, Trp, Tyr or Leu, at position +6 was established.

In order to study how the AR AF-2 surface can accommodate the different bulky residues present in the FXXLF sequence and how this differentiates from binding of LXXLL motifs, Fletterick *et al.* have combined phage display with crystallography studies. A set of binding motifs was identified (FXXLF, FXXLW, WXXLW, WXXVW, FXXYF, FXXFF, LXXLL) with modulated binding affinities [36]. Using X-ray

crystallography, the structural implications of the different sequences on binding to the AR AF-2 surface were studied. In line with previous studies on AR-binding requirements [48–51], the charge clamp, constituted of residues Lys720 and Glu897, was found to define the limits of the hydrophobic cleft and both amino acids were found to contribute to the binding affinity of the motif via hydrogen-bonding contacts with the peptide backbone. The analysis of the different peptides bound to the AR established that the AR AF-2 surface adapts itself to each sequence motif. In particular, the binding of the LXXLL motif was shown to be different from the classical FXXLF motif. The flanking residues were largely disordered and the LXXLL peptide backbone forms only one hydrogen bond, with Lys720. It was shown that the AR LBD rearranges to bind the more compact LXXLL motifs via subsidiary contacts with LXXLL flanking sequences to discriminate between LXXLL motifs [12]. An independent study by McDonell *et al*. corroborated the general FXXLF preference for the AR [38]. In this study, and in contrast to previous studies on AR, the full-length AR was used for the phage display screening. Interestingly, the previously found Trp-rich motifs [36] did not show up in this study. This difference could be due to the use of the complete protein; it is described that the conformation of the coactivator-binding groove in the complete receptor could be different from the isolated LBD [38, 52, 53].

Ala scan mutagenesis of the AR FXXLF motif verified the importance of the residues at positions $+1$, $+4$ and $+5$ for the interaction with the coactivator groove [54]. In order to investigate the specific amino acid requirements at position $+4$, a systematic functional analysis has been done, demonstrating that Leu substitutions by Phe and Met are compatible with a high-affinity and specific AR LBD interaction [35]. This affinity was also maintained when these mutations were introduced in the ARA54 and ARA70 FXXLF motifs. Further studies also showed that the flanking residues at the core of the FXXLF motif determine the specific mode of the interaction with the AR LBD [55].

In summary, phage display, in combination with mutational analysis, constitutes a powerful method for developing peptide sequences that can specifically bind to a given nuclear receptor. Additionally, this methodology is useful for the identification of unknown nuclear receptor-binding proteins and for the analysis of different nuclear receptor surface conformations generated by different hormone ligands. Importantly, the nature of the protein, LBD alone or the complete protein, can have a crucial influence on the outcome of the phage display screening and on the functionality of the peptides.

2.3.2
Nonnatural Cyclic Peptides

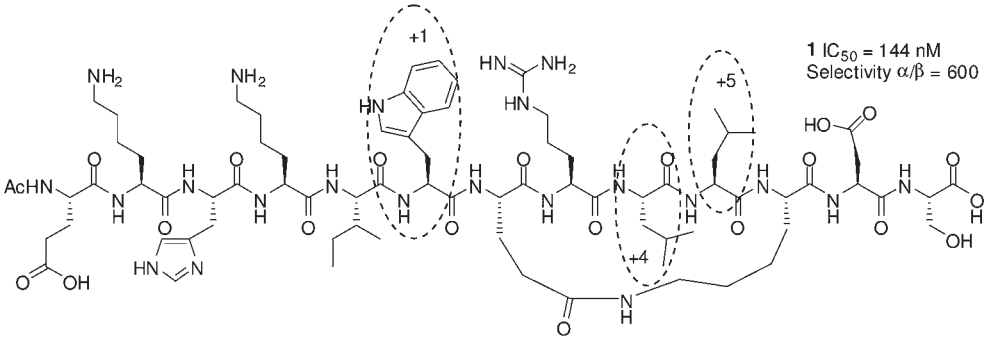
Cyclization of peptides is an established strategy to stabilize short peptides in an α-helical conformation. Concomitantly, different types of cyclic peptides have been explored as inhibitors of the nuclear receptor–coactivator interaction. Guy *et al*. have created a library of α-helical constrained peptidomimetics, based on the second nuclear receptor box found in the SRC2 coactivator, by the introduction of a macrolactam bridge [56–58]. The macrolactam bridge induces an α-helical

conformation of the peptides, thus reducing the entropic penalty upon binding as a helix to the nuclear receptor. The length, orientation and positioning of the constraining moiety were optimized with respect to an optimal induction of the α-helical conformation. A 13-amino-acid peptide was selected as starting point for the design of a library of peptidomimetics, including a set of nonnatural amino acids replacing the Leu residues at positions +1, +4 and +5. The library was screened against the interaction of SCR2 with both ERα and ERβ in the presence of estradiol and TRβ in the presence of thyroid hormone. This allowed for the study of both the binding potency and receptor selectivity. With the set of cyclic peptides, inhibitors for all receptors (ERα, ERβ and TRβ) were found, with some featuring remarkably high selectivities. A cyclic peptide was found, for example, that featured a 600-fold selectivity for hERα over hTRβ and 100-fold over hERβ (**1**, Figure 2.3). This shows that it is possible to find nuclear receptor–cofactor inhibitors that show good nuclear receptor selectively, which holds promises for the development of small-molecule CBIs. Interestingly, and in contrast to results obtained by phage display on the ERs [18, 59], potent and selective CBIs for the ERs were found that contained aromatic residues at the +1, +4 or +5 positions. These results show that phage display and peptide design and synthesis might give access to orthogonal compounds.

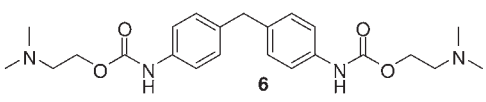
The conformation and coactivator binding of nuclear receptors depends on the molecular structure of the hormone. Therefore, the binding affinity and selectivity of cyclic peptides to ERα and ERβ were studied in the presence of different hormones [58]. These measurements allow identifying the specific subsites in the hydrophobic LXXLL-binding groove that distinguish the different conformations of the same nuclear receptor. A library, mainly with substituted phenylalanines and phenylglycines as nonnatural amino acids in positions +1, +4 and +5, was screened against the interaction of SCR2 with hERα and hERβ in the presence of three different ligands: estradiol and the two partial agonists diethylstilbestrol and genistein. Several of the cyclic peptides showed high selectivity (greater than 50-fold) for blocking the interaction of ERα with SRC2 depending on the ligand bound. These results show the possibility to generate CBIs that selectively target different conformations of the same nuclear receptor, which is of importance for the design of inhibitors for specific cell types or for nuclear receptor mutants with different conformations.

Spatola *et al.* have explored side-chain–side-chain disulfide bridges to generate helix-stabilized cyclic peptides targeting the nuclear receptor–coactivator interaction [60]. A D,L-dicysteine motif in the relative positions i, $i + 3$, resulted to be most favorable for the induction of helicity. Based on this scaffold, LXXLL-containing disulfide-bridge nonapeptides were reported with high affinity and selectivity for ERα (**2** and **3**, Figure 2.3). Introduction of nonnatural amino acids, notably the neopentyl glycine amino acid, in the cyclic peptides and optimization of binding affinity even resulted in peptide binders with a K_i of only 70 pM [61]. In a following detailed study it was shown that a high helical content of the peptide not always correlates with higher affinity to the hydrophobic ER LBD coactivator-binding groove [61, 62]. Cyclic peptides with other thiol-containing amino acids, such as homocysteine and penicillamine, were also studied. The homocysteine, featuring a longer side-chain than Cys, confers more flexibility to the constraining bridge. This

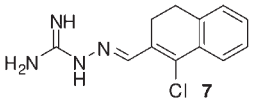
resulted in a positive effect on the binding affinity but decreased the isoform, ERα and ERβ, selectivity (**4**, Figure 2.3). On the other hand, the bulkiness introduced by the penicillamines provided additional constrain, resulting in a gain in selectivity (**5**, Figure 2.3) [61].



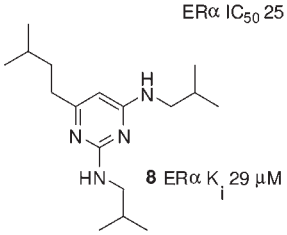
		ERα K_i [nM]	Selectivity α/β
H-Lys-D-Cys-Ile-Leu-Cys-Arg-Leu-Leu-Gln-NH_2	**2**	25	15.6
H-Arg-D-Cys-Ile-Leu-Cys-Arg-Leu-Leu-Gln-NH_2	**3**	11	7.0
H-Arg-D-Cys-Ile-Leu-Cys-Arg-Leu-Leu-Gln-NH_2	**4**	12	1.8
H-Arg-D-Cys-Ile-Leu-Cys-Arg-Leu-Leu-Gln-NH_2	**5**	78	50.9

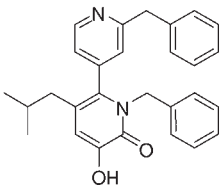


ERα IC_{50} 25 μM



ERα IC_{50} 5.5 μM





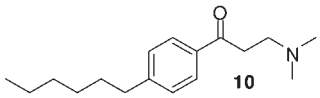


Figure 2.3 Chemical structures and binding potencies and selectivities of cyclic peptides and small-molecule CBIs for ERα, ERβ and TRβ.

Studies on peptides that are not cyclic and feature only one Cys and thus a free sulfhydryl group have revealed a strong affinity, below 60 nM in every case, to ERs. It turned out that these peptides bind to Cys residues on the LBD via disulfide bridges [61]. A similar specific targeting has been developed with small molecules (*vide infra*).

2.3.3 Small Molecules

Small molecules constitute the ultimate goal in the search for compounds that can directly block the nuclear receptor–cofactor interaction. Such compounds would not only constitute a new pharmacological and cell biological tool to modulate nuclear receptor-regulated gene transcription, but are also envisaged to provide a new entry in nuclear receptor antagonism.

There are two patents describing computational approaches for the identification of small-molecule CBIs [56, 63]. Based on crystallographic information chemical scaffolds were designed featuring pendant substituents mimicking the Leu side-chains of the LXXLL motif or Phe side-chains in the FXXLF motif. The actual binding constants of the designed compounds have, however, not yet been reported.

Shao *et al.* applied both a computational based approach and a mammalian two-hybrid screening assay to identify new small-molecule CBIs [64]. Available crystallographic information of the ERα LBD [13] was used for the *in silico* approach, and the most determining points of the interaction between the ERα LBD surface and the LXXLL motif were identified. The hydrophobic groove was described with a suitable topology, taking not only the hydrophobic interactions into account, but also the ionic interactions with the charge clamp. Using this model a virtual screening of the Available Chemicals Directory (ACD) database was performed and the selected conformers of hit compounds were docked on to the predefined binding region of the ER hydrophobic groove. Two active compounds (**6** and **7**) were identified (Figure 2.3). Compound **6**, a bulky symmetrical small molecule, effectively inhibited coactivator recruitment with an IC_{50} value of 25 μM. This structure resulted to be ERα selective, as it did not interfere with the interaction of ERβ or progesterone receptor with the coactivator SRC3. Compound **6** is, however, not membrane permeable. Compound **7** ($IC_{50} = 5.5$ μM) was identified as a CBI via the two-hybrid assay and is cell permeable. This compound inhibits endogenous ERα function in MCF-7 cells. Even though it is not clear yet whether the compound functions via direct or allosteric blocking of the interaction of ERα with coactivators, the results do show that small molecules can regulate this interaction via mechanisms different from the classical hormone functioning.

Katzenellenbogen *et al.* developed small molecules that inhibit the binding of coactivator peptides to ERα [9]. Their *de novo* design was based on a diverse set of heterocycle cores mimicking the helical scaffold of the coactivator peptides. Pyrimidines featuring side-chains mimicking Leu were found to be the most effective binders (**8**, Figure 2.3). Due to the intrinsic torsional flexibility of pyrimidine

cycles, the hydrophobic groups attached seemed to be mobile enough to adopt an optimized position and, therefore, show good affinity for the hydrophobic groove of the ER. The highest affinity obtained had a K_i of 29 μM. Further optimization of this design taking into account additional features involved in the interaction of ERs with cofactors shows great promises to achieve higher affinities for these newly designed CBIs.

The most recent contribution to the field of CBIs comes from Hamilton *et al.* and is based on their helix mimetic biaryl scaffolds [65]. By providing their scaffold with either aliphatic of benzylic substituents, CBIs were developed that showed inhibitory properties in the low micromolar regime (**9**, Figure 2.3) [66]. These are the most potent small-molecule CBIs for the ER to date, and offer ample opportunity for optimization and investigation of cross-reactivity and selectivity.

Recently, Burris *et al.* have presented another example of a small-molecule CBI [67]. Using X-ray crystallography, a second binding site for 4-OH-tamoxifen was discovered in the coactivator-binding groove of the ERβ. The crystal structure revealed a folded disposition of the compound sitting in the coactivator recognition surface of the ER, overlapping with the corresponding coactivator peptide. Although its physiological relevance needs to be proven, this is the first crystallographic characterization of a small molecule bound to the hydrophobic cleft of a nuclear receptor, which can give new insights in the effort of developing small molecules as potent inhibitors of protein–protein interactions in the context of nuclear receptors and coactivators.

Fletterick *et al.* have reported another class of small-molecule CBIs [68]. These compounds target the TRβ by covalently binding to the hydrophobic groove on the TRβ. A high-throughput library screen was performed in order to identify possible hits disrupting the TRβ–cofactor interaction. Two of the hits, both aromatic β-aminoketones (**10**, Figure 2.3), showed IC_{50} values below 10 μM. Further experimentation proved that the mode of action of this class of compounds is based on their Michael acceptor character after an internal liberation of the amino group, which leads to an α,β-unsaturated ketone. These compounds can react covalently to thiol nucleophiles displayed on the nuclear receptor surface in analogy to the previously described peptides [61]. Although the TRβ LBD surface features seven Cys residues, it turned out that the Cys located directly in the TR hydrophobic coactivator-binding groove is responsible for the irreversible inhibitor association. This selectivity supports a mechanism of action in which the inhibitor is positioned first in the cleft by hydrophobic interactions and subsequently binds to the nucleophilic Cys. The most potent compounds described in this work showed selectivities between the two isoforms of TR, preferring TRβ 12- and 50-fold over TRα, probably due to small differences in the corresponding hydrophobic grooves. Interestingly, the high potency described also enabled the inhibition of the interaction between the complete TRβ and full-length SCR2 in a cellular environment. This not only confirms the ability of the compound to cross the cell membrane and being translocated to the nucleus, but more importantly shows that small molecules, be it covalent or noncovalent, can inhibit the nuclear receptor–coactivator interaction.

2.4 Perspectives

The nuclear receptor–cofactor interaction, more specifically the nuclear receptor–coactivator interaction, has emerged as a drugable protein–protein interaction. Even though many aspects of its drugability are still unsolved, both the results obtained on the biological evaluation of its direct inhibition and the development of the first small-molecule inhibitors show the potential of targeting this interaction.

The biological validity and pharmacological effects of inhibiting the nuclear receptor–cofactor interaction depend on many parameters such as the type of nuclear receptor that is targeted, the functional state of the nuclear receptor (liganded or not), possible mutations, the cell type in which the nuclear receptor is expressed and, related to that, the available natural cofactors in the cell. In the literature plenty of proof exists that the direct inhibition of the ER–coactivator interaction, either with small molecules or with peptides, results in a characteristic antagonistic effect on the ER functioning. The ER–coactivator interaction is thus a valid pharmaceutical target. The pharmacological effects of the inhibition of the AR–coactivator interaction are much less pronounced. The partially different mechanisms via which this nuclear receptor operates makes a much less clear-cut case that the inhibition of the AR–coactivator interaction is a valid concept for antagonizing AR-mediated gene transcription. As the functioning of nuclear receptors is dependent on its environment in the cell, it is important to keep in mind that for certain combinations of nuclear receptors and cells the concept of nuclear receptor–coactivators inhibition might be valid, whereas in other cases it is not. A coherent set of rules to guide the search for CBIs does not yet exist, but general guidelines such as the important differences in inhibitors resulting from screens that utilize the complete nuclear receptor versus those that rely on the LBD only, enormously aid in the process of establishing these principles.

The first CBIs have been published recently and, considering the limited amount of optimization work performed on these compounds thus far, their moderate affinity holds great promise for significant improvement. As such, these compounds provide a very good basis for optimization, proof-of-concept and selectivity studies. Independent of the ultimate biological validity of these compounds, these recent studies show that small molecules can be found that inhibit the nuclear receptor–coactivator interaction.

The current studies on inhibiting the nuclear receptor–coactivator interaction have focused mainly on the ERs, AR and TR. With compounds developed against these nuclear receptors it becomes possible to also target other nuclear receptors (e.g. the orphan nuclear receptors). Here, CBIs might provide an entry for modulation of these nuclear receptors for which up to now no classical ligand has been found.

The most important point of attention currently is the development of new and optimized CBIs with which the concept of inhibition of the nuclear receptor–cofactor interaction can be investigated in more detail and be possibly validated for

many nuclear receptors. Therefore, the search for such compounds and their optimization, either via screening or design, will be of utmost importance. These small-molecule inhibitors will provide the tools to validate the concept of inhibiting the nuclear receptor–cofactor interaction and might result in leads to be developed in the future.

Acknowledgments

LB was supported by a Sofja Kovalevskaja Award of the Alexander von Humboldt Foundation and Bundesministerium für Bildung und Forschung. BV was supported by a post-doctoral fellowship of the Spanish Ministerio de Educación y Ciencia.

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3
Untangling the Estrogen Receptor Web: Tools to Selectively Study Estrogen-Binding Receptors

Ross V. Weatherman

3.1
Physiological Roles of Estrogen and the Challenges in Drug Discovery

Estrogens play a fundamental role in the development of the reproductive system of females, regulating the growth and function of tissues such as the breast, uterus and ovaries. The most potent estrogen, estradiol (Figure 3.1), is present in circulation from the onset of puberty to menopause, and can lead to abnormal cell growth and cancer in tissues such as the breast. Blocking and/or preventing breast cancer is the major therapeutic role of estrogen receptor (ER) antagonists with drugs such as tamoxifen and fulvestrant (Figure 3.1) as the most successful examples [1]. Osteoporosis, hot flashes and possible increases in coronary heart disease are all symptoms in menopausal women that can be treated using ER agonist-directed therapy, either through the use of estrogen replacement therapy or compounds such as raloxifene (Figure 3.1).

In both the treatment of breast cancer and menopausal symptoms, however, current estrogen-based therapies have serious side-effects in nontarget tissues that present serious challenges. Breast cancer therapies such as tamoxifen can cause hot flashes in patients and estrogen replacement therapies can increase risk of breast cancer to unacceptable levels [2]. Tamoxifen and raloxifene are selective ER modulators (SERMs), which means that they have a mixed profile in which they act as estrogens in certain tissues and antiestrogens in others. The ideal SERM profile in different tissues has not been achieved. As a result, the key to progress in the field of estrogen drug design and discovery is identifying all possible ERs and estrogen signaling pathways, and discovering their roles in specific physiological responses to estrogen-targeted drugs.

3.2
Possibility of Multiple Targets

As is the case with many other nuclear receptors, there are multiple forms of the ER in a cell. There are two major subtypes, named ERα and ERβ, which are encoded by

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

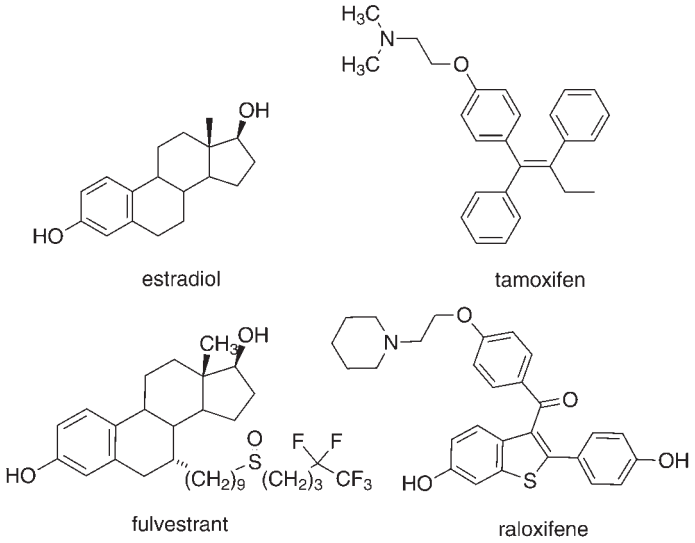


Figure 3.1 Therapeutically important ER modulators.

separate genes. As shown in Figure 3.2, there are a number of other receptors that can bind to ER ligands such as the ER-related receptors (ERRs) and a G-protein-coupled receptor (GPCR) known as GPR30. To further complicate matters, ERα and ERβ can stimulate pathways through cross-talk with other signal transduction networks either by acting in the cytoplasm or by localizing to the plasma membrane or mitochondria [3]. The goal of this chapter is to survey possible receptors and pathways that could play a role in dictating the response to ER modulators, and summarize the tools to selectively study the role of these targets and their relevance to nuclear receptor drug discovery.

3.3 ERα

3.3.1 Discovery and Characterization

The 'original' ER, ERα, was discovered in the late 1960s by Elwood Jensen and a number of other laboratories, and has been the most extensively studied ER [4]. Cloning of the human ER gene revealed the receptor to be approximately 67 kDa with an N-terminal domain, a DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) [5]. Structural information is available for the DBD and LBD, but no full-length structure has been determined, most likely due to the relatively unfolded nature of the N-terminal region [6]. A 46-kDa splicing isoform of ERα missing part of its N-terminal region has also been reported to be present in

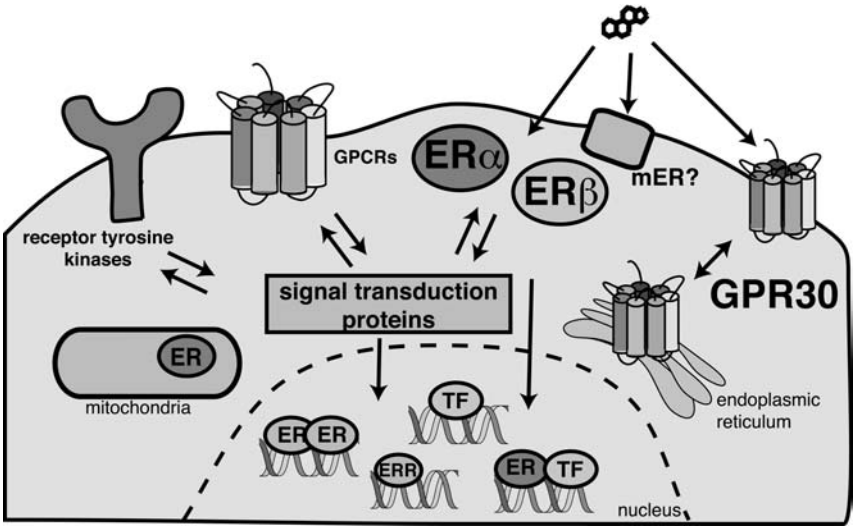


Figure 3.2 Possible targets and signaling pathways responsible for the responses to estrogen and antiestrogen drugs.

cardiovascular tissue and could play a role in estrogen-induced nitric oxide release in vascular endothelial cells [7]. The effect of ligands other than estradiol on this isoform is unknown.

The experiments performed to characterize ERα are too numerous to review here, but a classic mechanism of action has emerged from all this work for ERα. In its unbound state, ERα exists in the cytoplasm in complexes with a number of other proteins such as heat shock proteins, which dissociate after ligand binding to allow ER to translocate to the nucleus. In the nucleus, ER binds to estrogen response elements (ERE) in the promoter regions of target genes and then positively or negatively regulates transcription of the target genes. As will be discussed later, ERα also has a number of signaling mechanisms besides this classic model that may ultimately be as important in determining responses to specific drugs.

3.3.2
Expression Patterns and Response to Known Drugs

ERα is expressed widely throughout the body with significant expression in the uterus, breast and bone. Fully estrogenic compounds like estradiol and diethylstilbestrol (DES) cause ERα to act as a transcriptional activator at classic ERE-containing promoters, whereas SERMs and full antiestrogens like tamoxifen, raloxifene and fulvestrant antagonize the action of estradiol and prevent transcriptional activation [8]. Antagonists such as tamoxifen and raloxifene act by disrupting the positioning of helix 12 in the LBD to prevent coactivator binding [9]. While this explains the antiestrogen effects of these compounds, it does not explain the estrogenic effects of SERMs in many tissues.

3.3.3 Physiological Roles of ERα

Most of the pharmacology performed on ER has been performed on ERα, and there is a strong correlation between the effects of drugs on ERα at the molecular level and effects seen *in vivo*, but there are still many physiological effects that do not correlate wit ERα activity. As a result, it is important to identify the physiological processes specifically regulated by ERα. Both genetic and pharmacological approaches have been taken to address this question. A brief summary of the results from each approach follows.

The phenotypes of ERα knockout (ERKO) mice have been reviewed extensively in a number of high-quality articles [10, 11], so this chapter will only summarize results that are relevant to drug discovery. The ERKO mouse showed significant alterations in breast and uterus, suggesting ERα's central role in regulating proliferation in those tissues. The ovaries were nonfunctioning, but this is possibly due to disruption of the hypothalamic–pituitary–ovarian axis. The male ERKO mouse was also infertile, likely due to defects in testicular function. With respect to nonreproductive tissues, the ERKO mice showed decreased bone growth compared to wild-type mice, increased accumulation of adipose tissue and a marked decrease in the ability of estradiol to prevent atherosclerosis. Experiments with ERKO mice also suggest that ERα has a role in estradiol-mediated protection from injury due to ischemia (both cerebral and myocardial), but does not seem to have a unique role in the estradiol-mediated prevention of hot flashes in a common mouse hot flash model.

The other approach to selectively study the role of ERα has been the use of selective compounds. As is described in more detail in other articles and another chapter in this book, the ligand-binding pockets of ERα and ERβ are not identical. This difference has been exploited to make compounds that bind more tightly to one isoform over the other. There have been reports of both ERα-selective agonists and antagonists. The most widely used selective ERα agonist is a propylpyrazole triol (PPT) (Figure 3.3) that shows over 400-fold ERα binding selectivity and activates ERα in cell-based reporter assays [12, 13], but other compounds such as a 16α-estradiol lactone (16αLE) have also been reported [14]. The first reported selective ERα antagonist was a PPT analog known as methyl-piperidino-pyrazole with 200-fold ERα binding selectivity and 1000-fold preference for inhibiting ERα-mediated gene transcription [15]. A number of other compounds have since been reported that are generally 100-fold selective for ERα binding and over 100-fold selective for inhibiting ERα transactivation [16].

The use of selective ERα modulators has provided information similar to that obtained from ERKO transgenic mice. PPT stimulated uterine proliferation as well as ethinyl-estradiol, prevented bone mineral density loss in ovariectomized rats, lowered serum cholesterol levels and prevented hot flashes in a chemically induced hot flash rat model [17]. The use of PPT also showed that ERα is primarily responsible for the effects of estrogen on the pituitary, vasodilation, neuroprotection, cardioprotection and insulin sensitivity (reviewed in Ref. [18]). Protective effects were also

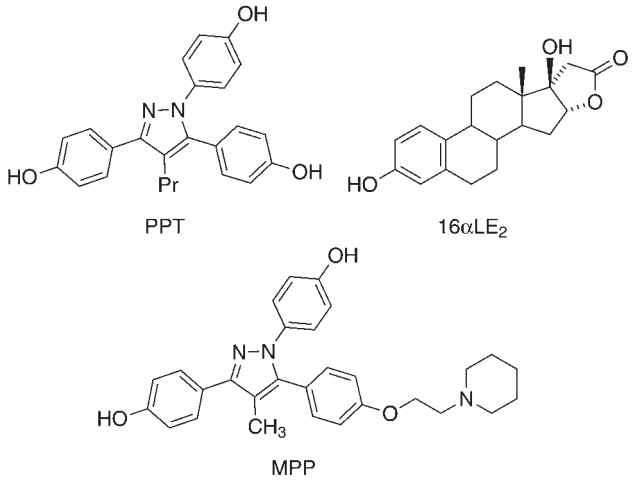


Figure 3.3 Selective ERα ligands.

seen using PPT in animal model systems for multiple sclerosis [19]. The use of the ERα-selective antagonists has focused mainly on their effects on uterine tissue and breast cancer cell lines; in both contexts, the ERα antagonists repressed proliferation [20–22].

In summary, the use of ERKO mice and ERα-selective ligands has shown that ERα has a primary role in tissues that are targets of therapeutic estrogen and antiestrogen treatment. Selective ligands and transgenic mice have also revealed new possible processes regulated by ERα. Even though it is clear that ERα has primary importance in current drug discovery efforts, the roles of the other receptors could have an important impact on the side-effect profiles of today's drugs or point to novel physiological processes regulated by estrogens.

3.4 ERβ

3.4.1 Discovery and Characterization

Nearly 10 years after the cloning of ERα, ERβ was cloned from rat prostate and found to be very similar to ERα [23]. The amino acid homology between ERα and ERβ is 97% in the DBD, but less conserved in the LBD (60%) and unconserved in the N-terminal domain (17%). Considering the similarities to ERα, it should not be surprising that ERβ operates by a similar mechanism of action to regulate gene transcription. Even so, a very important question remains – does ERβ specifically regulate any of the therapeutically important estrogen responses and can it be selectively modulated?

3.4.2
Expression Patterns and Response to Known Drugs

ERβ is also expressed widely throughout the body, but is expressed in particularly high amounts in the prostate, ovary, lung and testis, and in relatively low amounts in the uterus [24]. ERβ binds classic ER ligands as well as, if not better than, ERα [24]. Phytoestrogens such as genistein can bind to ERβ with greater affinity than they bind to ERα. Structural studies have also been performed with the ERβ LBD and different ligands, and the structures are almost identical to the structures of the ligand with ERβ.

3.4.3
Possible Physiological Roles of ERβ

Determining the possible physiological roles of ERβ and their therapeutic relevance became an area of very active research shortly after its discovery. There has been much confusion regarding different phenotypes of the ERβ knockout (βERKO) mice in different labs [18]. In the majority of the sets of βERKO mice, ERβ does not have a significant role in controlling proliferation in the mammary gland or uterus. Estradiol could still maintain bone mineral density and prevent hot flashes in the βERKO mice, suggesting that ERβ is not necessary for these tasks either. The βERKO mice do have some irregularities in heart function and response to myocardial infarction, and the females have reduced fertility that appears to be due to decrease follicle production in the ovary. The βERKO mice also have alterations in their levels of anxiety, depression and learning ability that suggest cross-talk between ERβ and serotonin signaling. Even though three independent sets of βERKO suggest that ERβ has a limited role in the tissues traditionally targeted by estrogen and antiestrogen therapy, there is another set of mice with dramatically different phenotypes [25]. It is unclear why these phenotypes are seen in this set of mice and not the other three sets.

There has also been a flurry of activity since the discovery of ERβ to make ERβ-selective ligands. The first and most widely used ERβ agonist is the diarylpropylnitrile DPN (Figure 3.4), which shows 75-fold ERβ binding selectivity [26], but other compounds include aryl diphenolic azoles like ERB-041 and the steroidal compound 8β-vinyl estradiol (8βVE2) which show better ERβ selectivity [14, 27]. To date, there have been no published reports of a truly selective ERβ antagonist. The only real selective antagonist has been a tetrahydrochrysene enantiomer that is a full antagonist for ERβ, but it is also a full agonist for ERα, so use of this compound is complicated [28].

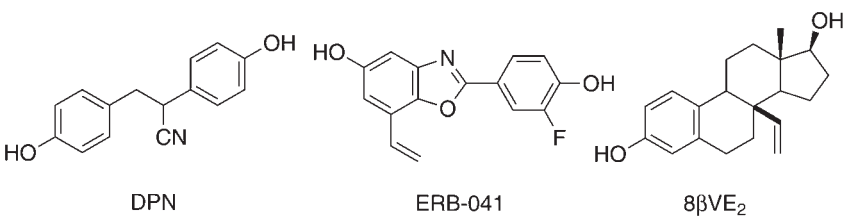


Figure 3.4 Selective ERβ ligands.

Overall, tissues sensitive to these ERβ-selective ligands have been the same responsive tissues seen in the three βERKO mouse models. No effects were seen in the breast, uterus, bone, prostate or heart [18]. The one exception to this was the steroidal 8βVE2 compound, which did show some proliferative activity in the uterus at high concentrations [14]. In the brain, DPN had antidepressant and antianxiety effects in mouse models [29, 30]. The ERβ-selective ligands also could relieve inflammation in mouse models for inflammatory bowel disease, arthritis and endometriosis [31, 32]. This suggests an exciting link between ERβ and specific inflammatory responses, although it is possible that ERα could have similar antiinflammatory effects.

In summary, the dream of ERβ-selective ligands solving all of the challenges facing ER drug discovery is unfulfilled, but remarkable progress has been made in a short time. Even though ERα seems to be the dominant ER when it comes to the pharmacology of current ER-targeted drugs, the ERβ-selective tools point to new roles for ERβ that might lead to new therapeutic opportunities.

3.5 ERR

3.5.1 Discovery and Characterization

Using probes derived from the DBD sequence of ERα, the genes for two closely related receptors were identified and named ERRs [33]. These two subtypes, named ERRα and ERRβ, were later joined by a third member, ERRγ [34]. The sequence homology between the ER and ERR family is approximately 60% in the DNA-binding region, but only 30% in the LBD. The ERR family can bind to the classic ERE, but can also bind to other response elements with significantly different sequences, suggesting that ERR signaling has some overlap with ER signaling, but also has its own functions.

3.5.2 Expression Patterns and Response to Known Drugs

The expression patterns of the different ERR subtypes are significantly different [35]. ERRα and ERRγ are found in adult brain, muscle, kidney, testis and uterus as well as in developing bone, muscle and neuronal tissue. The expression of ERRβ is much more narrow with little to no expression in most adult tissues while showing limited expression during development.

The most profound difference between the ER and ERR families is their ligand-binding profile [35]. No endogenous ligand has been identified for any of the ERRs, leaving them as orphan receptors for now. Members of the ERR family do not bind to estradiol and many of their activities appear to be ligand independent, but they can bind to synthetic ligands. All three ERR family members bind to DES at much higher

concentrations than needed to bind to ERα and ERβ [36–38], but there is debate as to whether DES acts as an antagonist/inverse agonist or whether it has no effect. The SERM 4-OH-tamoxifen binds to ERRγ in the micromolar concentration range and acts as an antagonist/inverse agonist [36, 37].

3.5.3
Possible Physiological Roles of ERRs

Selective tools have helped determine possible roles of each ERR family member. ERRα knockout mice show relatively normal anatomical features, but have much lower fat deposits than wild-type mice and are resistant to high-fat diet-induced obesity, suggesting a role for ERRα in metabolic regulation [39]. The mice also lack the ability to maintain body temperature due to defects in mitochondrial function [39]. ERRβ knockout mice die before birth, suggesting an important role for ERRβ during embryonic development [40]. The ERRγ knockout mice die within 1 week after birth due to faulty oxidative metabolism in the heart [41].

Subtype-selective compounds has also helped further understanding of the possible roles of ERR family members. A thiadiazoleacrylamide (Figure 3.5) has been reported to be a selective inverse agonist for ERRα with the ability to regulate the activity of the key metabolic coactivator peroxisome proliferator-activated receptor γ coactivator 1α, suggesting a role for ERRα in metabolism [42, 43]. A 4-OH-tamoxifen-derived inverse agonist with 25-fold selectivity for ERRγ over ERα has also been reported [44]. In addition, an ERRβ/γ-selective phenolic acylhydrazone agonist has been reported to inhibit the proliferation of prostate cancer cells, suggesting a therapeutic application for ERRγ-selective ligands [45, 46].

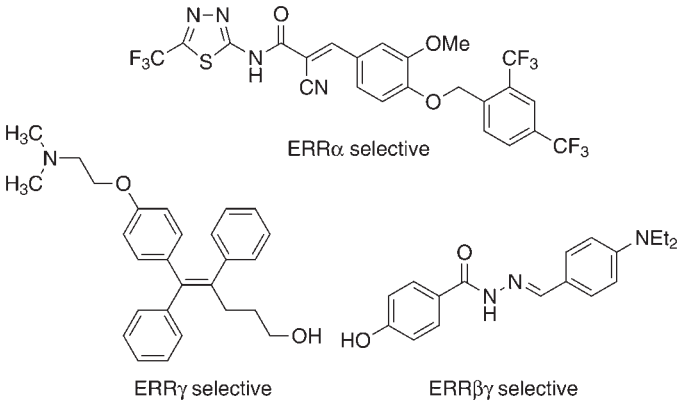


Figure 3.5 Selective ERR ligands.

Overall, the ERR family members do not appear to be true 'ERs', but it is clear that there is some overlap between ER and ERR ligand-binding preferences and the genes regulated by the receptors in tissues such as bone and certain forms of cancer [47, 48].

The knockout mice and selective ligands also suggest that ERR family members are involved in processes distinct from the ER family that could lead to new therapeutic opportunities.

3.6 GPR30

3.6.1 Discovery and Characterization

One of the major challenges facing the ER field right now concerns responses to estrogens that do not seem to arise from the classic transcriptional regulation model of ER action [49, 50]. These responses include the activation of kinase signaling cascades and release of intracellular Ca^{2+}, and can appear in cell lines that do not express ERα or ERβ. This has led to the idea that there are other receptors for estrogens that are not nuclear receptors. One such receptor is an orphan GPCR known as GPR30, which is overexpressed in ER-positive breast cancer cells compared to ER-negative cells [51]. The transient expression of GPR30 in ER-negative MDA-MB-231 breast cancer cells results in estradiol-induced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, downstream effectors of the mitogen-activated protein kinase pathway [52]. The situation was further complicated by a finding based on the use of fluorescently labeled GPR30 and a cell-impermeable conjugate of ethinyl-estradiol that GPR30 appeared to function at the endoplasmic reticulum rather than at the plasma membrane [53].

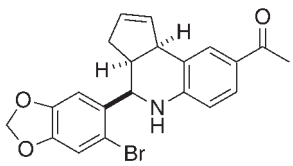
3.6.2 Expression Patterns and Response to Known Drugs

A full expression profile in different tissues has not been reported, but mRNA analysis of a limited number of tissues reveals that GPR30 is expressed in a number of tissues with higher levels of expression seen in breast, placenta and heart [51]. It is also expressed in the endometrium, ovary and vascular endothelium. GPR30's response to known ER modulators has been explored in a limited sense – estradiol, tamoxifen and fulvestrant all bind to GPR30 with binding constants in the nanomolar concentration range, but fulvestrant, an ER antagonist, acts as a GPR30 agonist [53]. As a result, it is possible that GPR30 could be responsible for some of the responses to therapeutic estrogen agents. Some high-affinity ER ligands such as DES have significantly worse binding affinity for GPR30.

3.6.3 Possible Physiological Roles of GPR30

There have been no published reports of the phenotypes of GPR30 knockout mice, so any clues thus far about specific GPR30 functions have come from cell lines with

different ER and GPR30 expression levels and selective ligands. Thus far, potential roles of GPR30 include the stimulation of proliferation in breast, uterine and ovarian cells through activation of c-*fos* expression (reviewed in Ref. [48]). Recently, screening discovered a selective compound for GPR30 known as G-1 (Figure 3.6) [54] with over 1000-fold selectivity for GPR30 compared to ERα and ERβ. Thus far, it has been used to demonstrate that GPR30 can stimulate proliferation in breast and ovarian cancer cell lines and activate calcium mobilization in hypothalamic neurons [55, 56].



G-1

Figure 3.6 Selective GPR30 ligand.

3.6.4 Controversies Over GPR30

Due to its unique proposed localization pattern and the apparent overlap in functions with ERα and ERβ, GPR30 is still a controversial receptor. A number of papers have been published disputing both the localization of GPR30 and the ability of GPR30 to induce certain signal transduction pathways. In the latter dispute, a paper reported that estradiol was unable to stimulate ERK or phosphatidylinositol-3-kinase activation, cAMP generation or Ca^{2+} influx in GPR30-positive, ER-negative SKBR3 cell lines [57], directly contradicting other studies arguing for a role for GPR30 in these responses. The reasons for the apparent discrepancies are still unknown, but it could be that cell context is a key factor and somehow each lab's cell lines differ enough to give different results.

As to the dispute over localization of GPR30, GPR30's presence has been reported on the plasma membrane of CA2 pyramidal neuronal cells and in cells transfected with GPR30 [58, 59]. In addition, subcellular fractionation studies with both endogenously and transiently expressing GPR30 cells revealed that, while GPR30 was found in the microsomal fraction, ligand and G-protein binding was only observed in the plasma membrane fraction. In response to these reports, another study arguing for intracellular activity tested a number of cell-permeable and cell-impermeable ligands, and found that the ability to activate GPR30 was dependent on the cell permeability of the ligand [60]. They argued that it was possible for GPR30 to be expressed in small amounts on the plasma membrane, but functional GPR30 was intracellular.

Overall, research into the role of GPR30 in estrogen signaling is only beginning. The development of a selective agonist is an important milestone that should greatly increase the amount of work on the receptor. The development of a selective

antagonist and a GPR30 knockout mouse are still needed to truly understand the effects of GPR30 activity and its therapeutic relevance.

3.7 Membrane ERs

A membrane receptor for estrogens has been proposed since early studies with radiolabeled estradiol indicated high-affinity, saturable binding in the membrane fraction of cell lysates [61]. Identifying the receptor responsible for that binding and proving its relevance has been difficult and controversial. Tools have been developed to selectively target membrane ERs. These molecules have typically been estradiol or tamoxifen analogs modified into cell-impermeable molecules by increasing the polarity of the ligand, as in the case of Q-Tam (Figure 3.7), a quaternary ammonium salt of tamoxifen [62]. Another method is to conjugate the ligand to a cell-impermeable protein such as bovine serum albumin or horseradish peroxidase [63, 64]. The use of the protein estradiol conjugates has been controversial, however [65]. The conjugates usually contain a high amount of unconjugated ligand, exhibit very slow binding kinetics to the receptor and stimulate pathways that are not stimulated by the estradiol ligand alone.

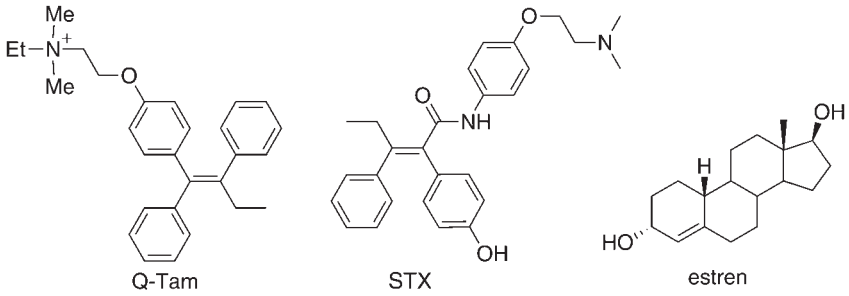


Figure 3.7 Selective estrogen response ligands.

Currently, there is general agreement that some sort of estrogen-binding receptor is present on the plasma membrane, but there is wide disagreement as to the nature of the receptor. The most popular candidate are some forms of the nuclear receptors ERα or ERβ that allow membrane localization, GPR30 or some other type of membrane receptor. There are a number of reports suggesting ER association with the membrane, either due to posttranslational modification or association with another membrane protein such as caveolin [66]. One intriguing response that seems to involve a unique receptor is found in the hypothalamus. Estradiol causes a desensitization of $GABA_B$ and μ-opioid receptors in hypothalamic pro-opiomelanocortin neurons. Further study revealed that this receptor is likely a GPCR unrelated to GPR30 [67]. A diphenylacrylamide ligand known as STX (Figure 3.7) showed no binding affinity for either ERα or ERβ, but mimicked the effects of estradiol in animal studies and blocks weight gain in hypogonadal

female guinea pigs, suggesting that this membrane receptor may be involved in energy homeostasis [68].

3.8 Integrated Estrogen Signaling

One of the main arguments for alternative ERs to ERα and ERβ has been that the signaling transduction pathways rapidly stimulated by estrogens are more associated with receptors that are not nuclear receptors. Over time, however, reports have accumulated that suggest that nuclear receptors can cross-talk with these other signaling pathways. This concept of integrated signaling suggests that ERα and ERβ act through two different types of mechanisms [69]. The first is rapid and involves cross-talk with other signal transduction pathways. The second is slower and involves classic regulation of transcription. Study of the rapid response is complicated by the slow response, so tools that selectively modulate rapid responses are needed. The first reported ligand of this nature was 4-estren-3α,17β-diol (estren; Figure 3.7), which was reported to activate rapid signaling while not affecting classic transcriptional activation [70]. It has also been shown to protect bone loss in hypogonadal female and male mice, suggesting that nongenomic pathways mediate estrogen responses in nonreproductive tissues while the classic pathway mediates estrogen response in reproductive tissues [71]. These findings have been challenged in a number of papers which report transcriptional activity for the estren in both ER and androgen receptor-based assays (reviewed in Ref. [72]). The supporters for the selectivity of estren have countered that the genes expressed after estren treatment are significantly different than those expressed after estradiol treatment [73].

Another method used to selectively target rapid signaling has been to use a ligand that localizes to different regions of the cell. One such approach involved conjugating ethinyl-estradiol analogs to dendrimers [74]. These conjugates could enter the cytoplasm but were too big to enter the nucleus. The estradiol–dendrimer conjugates did not regulate classic ER transcription, but were able to stimulate ERK phosphorylation. Tests on breast cancer cell lines also revealed that the conjugates were unable to stimulate growth, suggesting that proliferation in the breast requires nuclear activity.

3.9 Conclusions

Overall, significant progress has been made into understanding the role of specific receptors in the physiological responses to estrogen. At the current time, it appears that ERα is still the most significant target as it relates to estrogen responses that have been historically considered therapeutically important. The roles of other ERs such as ERβ and GPR30 seem somewhat less important in those tissues targeted for traditional estrogen drug discovery, but more important in tissues not traditionally

thought to be targets of estrogen-based drugs such as the ovary, the behavior centers of the brain and the immune system. In addition, these other ERs can slightly alter the activity of ERα. Considering that cell context is one of the key variables in determining the response of ERα to a drug, these other ERs could be playing an important role in determining that context. Combinations of selective agonists and antagonists are needed to tease apart these sorts of complicated networks.

Five years ago, the biggest challenge in untangling the estrogen web was finding selective ligands for all of the different receptors that could bind to estrogen ligands. Due in large part to the clever work of medicinal chemists, that challenge is almost overcome. There are selective compounds for almost every distinct receptor that can bind to estrogens. With that obstacle close to being cleared, the next big challenge is selectively targeting specific pathways coming from one receptor, as in the case of finding ERα ligands that can specifically target nonclassical over classical signaling. Tools tackling this problem are beginning to be developed, so in 5 more years, perhaps this challenge will seem like it is the easier problem to solve.

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4
Subtype-Selective Estrogens

Gerrit H. Veeneman

4.1
Introduction

4.1.1
Biology of the Estrogen Receptors

The estrogen receptors (ERs) belong to the family of steroid nuclear receptors that act directly on the DNA through specific responsive elements and modulate expression of target genes. Since its discovery in the 1960s, ERα was assumed to be the only ER protein expressed in all estrogen-responsive tissues. In 1996, a second ER was identified in the rat, human and mouse [1–3], and from that time onwards extensive investigations on the biological role of the ER subtypes were performed [4]. ERα is expressed in the uterus, prostate (stroma), ovary (theca cells), bone, breast and various regions in brain, whereas ERβ is present in the colon, prostate (epithelium), ovary (granulosa cells), bone marrow and brain (and other sympathetic ganglia). ERα (ERKO) and ERβ (BERKO) knockout mice have been used extensively to explore the physiological roles of the individual receptors [5]. Despite a large increase in the knowledge on the biological role of the ERs, more questions continue to arise. The reason for this is that signaling of the ERs is a much more complicated process than earlier anticipated. It has become clear that the outcome of gene transcription not only depends on the ER subtype, but also involves many other aspects, including posttranslational modifications such as phosphorylation and acetylation [6–8], and action of the ERs through various responsive elements [9–11]. On top of that a range of splice variants exist [12] and several point-mutated ERs were identified in human patient samples from different disease states [13], all of which may interfere with ER-mediated transcriptional activity. In addition, nongenomic actions have been recognized in which membrane ERs are implicated [14, 15]. Despite this complexity, selective targeting of one of the ERs has been linked to exciting therapeutic opportunities. A panel of subtype-selective ER ligands has become accessible in recent years, which turned out to be highly useful as pioneering compounds to map

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

certain biological effects to the individual ERs. Thus, the biological effects of the ERβ-selective agonists 2,3-bis-(4-hydroxyphenyl)-propionitrile (DPN), 8β-vinyl-estradiol (8βVE2), ERB-041, ERB-196 and SERBA-1 as well as the ERα-selective agonists the 16α,17α-lactone analog of 17βE2 (16α-LE2) and propylpyrazole triol (PPT) were studied utilizing a range of *in vivo* models. The outcome of the various investigations suggest a potential therapeutic opportunity of ERβ agonists to treat inflammatory diseases [16], colon cancer [17], prostate disorders, infertility and cognitive functions in brain, whereas ER antagonists might find therapeutic use in uterine and breast cancer, prostate disorders, infertility, and reproductive functions in brain [18–20].

Notwithstanding the progress made in unraveling the biological role of the ER subtypes and the identification of ER subtype-selective ligands, ultimate evidence of a therapeutic benefit should come from clinically testing sufficiently selective ER ligands in appropriate patient populations. Historically, clinical development of estrogens has met with major difficulties. Thus far, only five nonsteroidal selective ER modulator (SERM)-type estrogens and one steroidal antiestrogen have been marketed (Table 4.1) [21]. Many estrogens have failed during clinical development, mainly in phase III as a consequence of low efficacy or side-effects. The apparent unpredictable aspects associated with clinical development of estrogens reflect the need for robust preclinical models. Several nonsubtype-selective estrogen ligands are in clinical development from which bazedoxifene recently entered the preregistration phase. The most advanced subtype-selective estrogen is ERB-041, which is currently in phase II. ERα-selective SERMs are currently in preclinical development, while no reports on development of ERα agonists or ERβ antagonists have become public.

4.1.2 Interaction of ERα and ERβ with Ligands

An essential interaction between ligand and ER which facilitates high-affinity binding is mediated by a phenolic group (A-ring in 17βE2) that forms a hydrogen-bridge network with Glu353 and Arg394 in ERα, and their counterparts Glu305 and Arg346 in ERβ, and also involves water molecules. In many ligands a secondary hydroxyl is required that can interact with His523 in ERα or His475 in ERβ leading to further stabilization of the ligand–ligand-binding domain (LBD) complex. The need for, and benefit of, this interaction depends on the presence and spatial arrangement of additional substituents that can intermingle with other parts of the LBD. Characteristic for SERM-type ligands is the presence of a basic side-chain (BSC) that interacts with Asp351 in ERα and, although less critical, Asp303 in ERβ. This interaction strongly contributes to the correct positioning of helix 12 (H12) into the antagonist mode. While SERM-type antagonists exhibit a certain degree of partial agonistic activity, these compounds behave as almost full antagonists for ERβ.

There is almost complete homology within the amino acids in ERα and ERβ that are part of or in close proximity to the ligand-binding site. Only two amino acids are different: Met421 in ERα corresponding to Ile373 in ERβ and Leu384 in ERα that is equivalent to Met336 in ERβ. These two amino acid differences within the

Table 4.1 ER modulators in development or marketed.

Compound	Indication	Status	Remarks
Clomiphene	infertility	marketed	*E*/*Z* mixture
Enclomiphene	testosterone deficiency	phase III	
Tamoxifen	breast cancer	marketed	
Toremifene	breast cancer	marketed	
	breast cancer	phase II	combination therapy
	prostate cancer	phase III	
Droloxifene	breast cancer	phase III, cancelled	no improvements with respect to tamoxifen
Idoxifene	breast cancer	phase III, cancelled	no improvements with respect to tamoxifen
Miproxifene	breast cancer	phase III, cancelled	
Ospemifene	osteoporosis	phase III	
	vaginal atrophy	phase III	
Ormeloxifene	contraception	marketed	marketed in India only
Levormeloxifene	osteoporosis	phase III, cancelled	uterine prolapse (among others)
Raloxifene	osteoporosis	marketed	
	breast cancer	phase III	
	prostate cancer	phase II	
Arzoxifene	breast cancer	phase II, cancelled	low time to progression
	osteoporosis	phase III	
Nafoxidine	breast cancer	phase III, cancelled	liver toxicity
Lasofoxifene	osteoporosis	phase III, nonapprovable	
	vaginal atrophy	phase III, nonapprovable	
Bazedoxifene	osteoporosis	preregistration	
Bazedoxifene/ Premarin	osteoporosis/ vasomotor symptoms	phase III	
Pipendoxifene	osteoporosis	phase II, on hold	backup to bazedoxifene
Fulvestrant	breast cancer	marketed	parenteral administration
Acolbifene	breast cancer	phase II	no further development report
ERB-041	inflammation	phase II	
	endometriosis	phase II	
	Crohn's disease	phase I	
ERB-196	inflammation, sepsis	phase I	moved back into safety studies
SERBA-1	benign prostatic hyperplasia	preclinical	
MF-101	vasomotor symptoms	phase II	plant extract
HMR-3339	osteoporosis	phase IIb, cancelled	adverse effects on endometrial tissue
PSK-3471	osteoporosis	phase II	
TAS-108	breast cancer	phase II	ERα full antagonist

ligand-binding site have a significant effect on the overall volume of the ligand-binding region: 490 Å^3 for ERα and 390 Å^3 for ERβ. These differences suggest that it may be easier to find ERα-selective ligands. This indeed turned out to be the case for SERM-type ER antagonists, which in general show a certain degree of ERα selectivity. Despite the greater volume of the ERα ligand-binding pocket it has proven to be difficult to find nonsteroidal ERα-selective agonists. In fact, it has been easier to find ERβ-selective agonists. Soon after the discovery of ERβ, several flavanoids were identified that exhibit ERβ selectivity by nature. Furthermore, a variety of phenolic low-molecular-weight compounds have been disclosed decorated with substituents at specific positions, such that these interact optimal with the two dissimilar amino acids in ERβ, but at the same time interfere with the corresponding more bulky or less flexible amino acids in ERα. On the other hand, dual-profile estrogens have been identified that exhibit agonistic activity for ERα and antagonistic activity for ERβ. This type of selectivity makes use of the apparent intrinsic preference of H12 in ERβ to adopt an antagonist like position.

Following binding of a ligand ERα and ERβ rapidly form homodimers that bind to an estrogen-responsive element within a promoter region of a target gene. Subsequent recruitment of coactivators initiates formation of the transcription machinery leading to gene expression. ERα and ERβ can also form functional heterodimers. The biological function of such dimers is largely unknown. It has been advocated that through the formation of heterodimers ERβ may modulate the transcriptional activity of ERα. Using a U2OS-ERα/β heterodimer cell line it was shown that both 17βE2 and 4-OH-tamoxifen regulate unique sets of endogenous genes compared with all the genes regulated in U2OS-ER homodimer cell lines [22]. X-ray analysis of the crystals from the LBD of both ERα [23] and ERβ [24] with estrogens and antiestrogens revealed that H12 is part of a hydrophobic groove that can accommodate an LXXLL motif from a coactivator [25]. Both raloxifene and tamoxifen bind in the same hydrophobic cavity as do agonists, but the BSC prevents alignment of H12 over the binding pocket, thereby blocking assembly of the transcription complex. The BSC appears to stabilize the conformation through the interaction of the amine moiety with Asp351 and Asp303 in ERα and ERβ, respectively. Although all SERMs contain at least one hydroxyl group and a BSC, they do not all display the same efficacy or tissue selectivity. Seemingly small conformational differences may lead to differences in H12 conformation and/or stabilization that can result in variations in coactivator binding leading to different biological activities [26, 27]. The number of publications and patents addressing subtype-selective estrogens is rapidly expanding, indicating major interest among the pharmaceutical community in this direction.

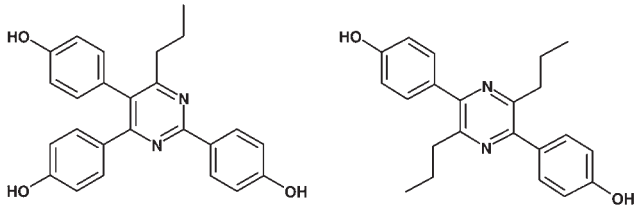
This chapter summarizes the features and status of the various different subtype-selective ER ligands that have been disclosed to date. Apart from ERα- and ERβ-selective agonists and antagonists, full antagonists and dual-profile ligands are also described. Several reviews have appeared in recent years that describe subtype-selective ER ligands [28–31], SERM-type ligands [32–34] and ER full antagonists [35, 36]. For in-depth overviews of the mechanisms involved in ER signaling the reader is referred to excellent reviews [37–39] that have appeared on this topic.

4.2
Subtype-Selective ER Ligands

4.2.1
ERα Agonists

4.2.1.1 Six-Membered Heterocycles

The University of Illinois reported that certain pyrimidines and pyrazines behaved as ERα-selective agonists [40]. Thus, pyrimidine **1** showed 23-fold ERα selectivity. Subtype selectivity appeared to be strongly dependent on the presence of a *p*-hydroxyphenyl group at C4. Replacement of this moiety with an alkyl or phenyl group had no effect on ERα affinity, but completely abolished ERα selectivity. In the pyrazine series ERα selectivity was less pronounced. Nine-fold selectivity was observed for pyrazine **2**, the decoration of which fully resembles pyrimidine analog **1**. Interestingly, **2** shows significantly higher ERα affinity with respect to **1**. The more polar pyridazine scaffold was also examined but compounds derived from this core showed very low receptor affinity.

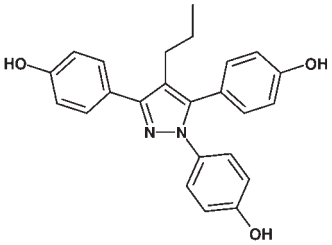


1: $RBA_{ER\alpha}$ = 4.6%; α/β = 23 **2: $RBA_{ER\alpha}$ = 30%; α/β = 9**

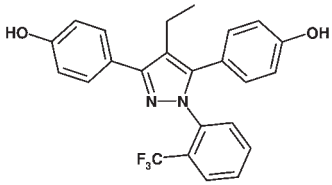
Scheme 4.1 RBA = relative binding affinity (estradiol = 100%).

4.2.1.2 Five-Membered Heterocycles

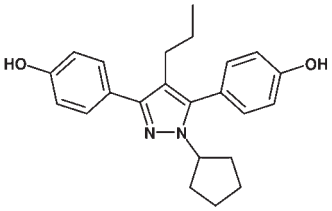
The prospects of five-membered heterocycles as core scaffolds for ER ligands were investigated by the University of Illinois [41, 42]. From a series of imidazoles, thiazoles, oxazoles, isoxazoles and pyrazoles it was found that 1,3,5-tri-(*p*-hydroxyphenyl)-4-alkyl pyrazoles show excellent ERα affinity and very high selectivity [43]. It was further established that both the 4-alkyl and 1-(4-hydroxyphenyl) substituents markedly influenced the affinity and selectivity. Best ERα selectivity was observed for the propyl analog **3** (PPT). Replacement of the 1-aryl group by a 1-cyclopentyl group (**4**) significantly enhanced receptor affinity, but subtype selectivity was completely lost [44]. Closely related pyrazoles were reported by Chiron [45, 46]. Optimization of lead structures from combinatorial libraries provided pyrazole **5**, which showed 60-fold selectivity for ERα. The University of Illinois further investigated thiophenes, pyrroles and furans as subtype-selective ligands for the ERs [47]. From these, certain furans turned out to be high-affinity ligands for ERα. Furan **6**, having a similar spatial orientation of the substituents as PPT, showed higher affinity for ERα. However, ERα selectivity was lower.



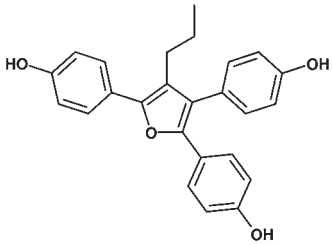
3 (PPT): $RBA_{ER\alpha}$ = 49%; α/β = 410



5: α/β = 60



4: $RBA_{ER\alpha}$ = 74%; α/β = 1

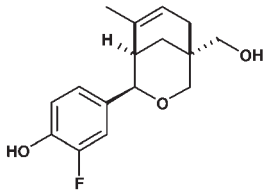


6: $RBA_{ER\alpha}$ = 100%; α/β = 56

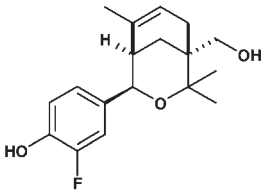
Scheme 4.2

4.2.1.3 Bicyclononene Ring Systems

Estrogen agonists based on an oxabicyclic template [48] were described by Ligand. The enantiomers were separated and several of these compounds displayed potent affinity for the ERs. ER affinity and subtype selectivity was found to be dependent on the nature of the enantiomer and the presence of additional substituents. Thus, the (+)-enantiomer **7** showed selectivity for ERα whereas the (−)-enantiomer **7** was selective for ERβ. A further increase in binding affinity was observed for the 2′,2′-dimethyl analog **8**.



7(+). $ER\alpha_{Ago}$: EC_{50} = 2.6 nM; α/β = 15
7(−). $ER\beta_{Ago}$: EC_{50} = 57 nM; β/α = 4



8 (rac). $ER\alpha_{Ago}$: EC_{50} = 0.6 nM; α/β = 12

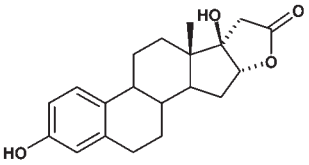
Scheme 4.3 $ER\alpha/\beta_{Ago}$ = transcriptional activity.

4.2.1.4 Steroidal ERα Agonists

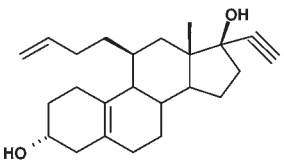
A limited number of steroidal ERα-selective agonists have been described.

Schering reported on the 16α,17α-lactone analog of 17βE2 (**9**; 16α-LE2) [49]. This steroidal estrogen displayed high ERα affinity (RBA = 57%) and 70-fold ERα selectivity. Compound **9** behaved as a full agonist in a transactivation assay and

showed 250-fold ERα selectivity. The 16α,17α-lactone moiety is directed towards Met421 in ERα. The authors reasoned that because the side-chain of this amino acid is more flexible than the side-chain of the equivalent Ile373 in ERβ, larger substituents at the α-face of the ligand which are located at the vicinity of Met421 can be more readily accommodated in ERα. In ovariectomized rat **9** effectively suppressed luteinizing hormone (LH), follicle-stimulating hormone (FSH) and insulin-like growth factor-1 expression.



9 (16α-LE2): $RBA_{ER\alpha}$ = 57%; α/β = 70



10: $RBA_{ER\alpha}$ = 29%; α/β = 290
$RTA_{ER\alpha}$ = 2%; α/β > 50

Scheme 4.4 RTA = relative transcriptional activity (estradiol = is 100%).

Organon disclosed $\Delta^{5,10}$ steroids as ERα-selective agonists [50]. Compound **10** showed 290-fold ERα selectivity in a receptor-binding assay and over 50-fold ERα selectivity in a CHO cell-based transactivation assay.

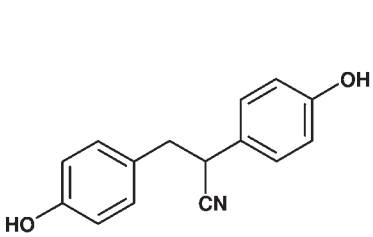
4.2.2 ERβ Agonists

4.2.2.1 Diaryl-Ethylenes, Imidazoles, Isoxazoles and Thiophenes

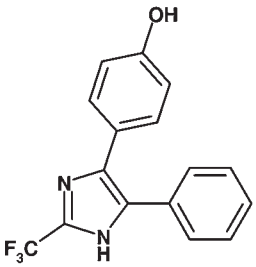
Diarylpropionitriles The University of Illinois reported that small molecules such as DPN (**11**) and related analogs exhibit remarkable selectivity for ERβ [51]. This type of estrogen has been reported earlier, but at that time ERβ was still unknown [52]. Modeling studies with **11** suggest that the nitrile is in close proximity to Met336 in ERβ and the corresponding Leu384 in ERα. The interaction of the nitrile with Met336 in ERβ is likely to be more favorable than the interaction with Leu384 in ERα. Replacement of the nitrile with an ethynyl group increased ERβ affinity, but at the same time decreased ERβ selectivity. Compound **11** has been used quite extensively to clarify the role of ERβ in biological processes.

Imidazoles and Isoxazoles Pfizer reported an imidazole and an isoxazole as ERβ-selective ligands [74]. Imidazole **12** showed 27-fold ERβ selectivity. Isoxazole **13** displayed higher ERβ affinity, but in this case ERβ selectivity was modest.

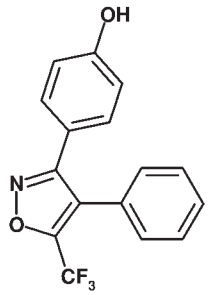
Thiophene-1-Carboxaldehydes Wyeth explored a set of thiophenes as new ligands for the ERs and it turned out that all compounds displayed preference for ERβ [53]. Both phenolic functions were required for ER affinity and it was established that the 1-carboxaldehyde moiety significantly contributed to ERβ selectivity. While replacement of the aldehyde functionality by a nitrile was well tolerated, conversion into an



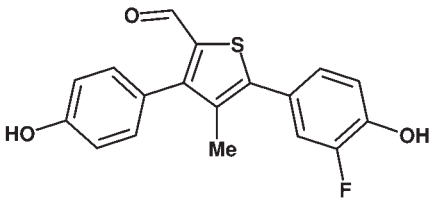
11 (DPN): $RBA_{ER\beta}$ = 18%; β/α = 72



12. $ER\beta_{Bin}$: IC_{50} = 15 nM; β/α = 27



13. $ER\beta_{Bin}$: IC_{50} = 2.4 nM; β/α = 3.5



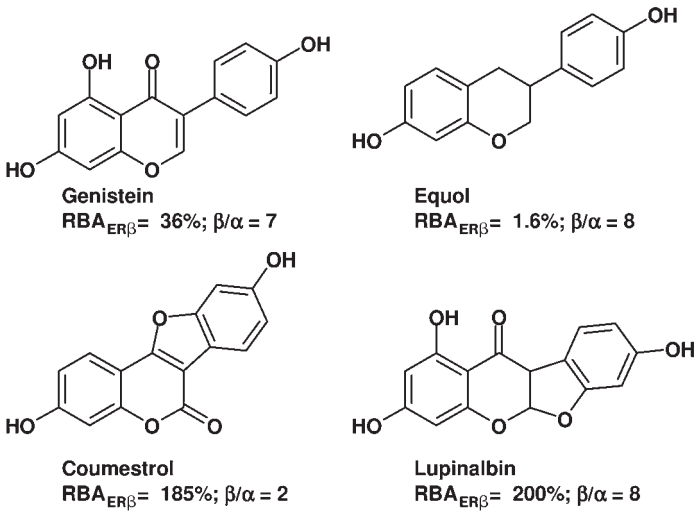
14. $ER\beta_{Bin}$: IC_{50} = 1.8 nM; β/α = 20

Scheme 4.5 ERα/β_{Bin} = receptor binding affinity.

oxime strongly reduced both ERβ affinity and selectivity. Best compound of the series in terms of receptor binding and selectivity was **14**. This compound showed good affinity (IC_{50} = 1.8 nM) and 20-fold ERβ selectivity.

4.2.2.2 Bicyclic 6 + 6 Ring Systems

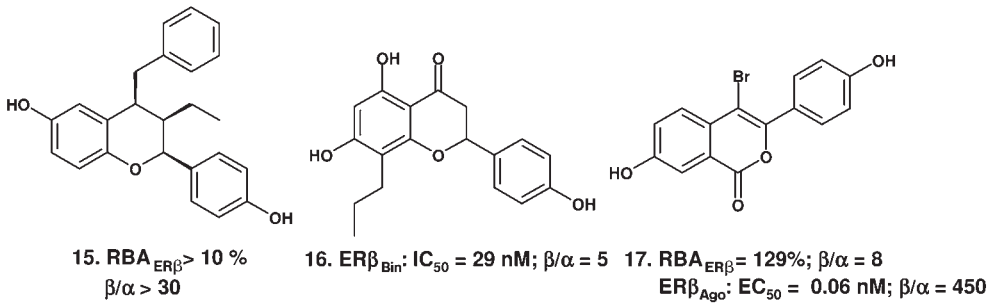
Benzopyranes (a) *Natural flavanoids.* Soon after the discovery of ERβ in 1996 it was reported that certain phytoestrogens express selective affinity towards ERβ [54]. The best-known representative is the isoflavanoid genistein, abundantly present in soy products. This compound shows 7- to 40-fold selectivity for ERβ in a receptor-binding assay [55]. It was reported that genistein behaves as a partial agonist in ERβ-transfected cell lines. Genistein has been cocrystallized with ERβ LBD (1QKM). X-ray analysis revealed that the exocyclic phenol interacts with Glu305/Arg346 and as such mimics the A-ring of 17βE2 [56]. The 7-OH group forms a hydrogen bridge with His475. The observed preference for ERβ is explained by the 4-carbonyl that forms a hydrogen bridge with 5-OH and points to the Ile373 residue. In ERα there is less space available to accommodate the 4-carbonyl/5-OH due to the presence of the more bulky Met421 at the same position. H12 does not adopt the typical agonist position, but lies in an orientation that is more in line with the position of H12 in the crystal structure of raloxifene ERβ LBD. Many other natural flavanoids, including apigenin, lupinalbin, coumestrol, daidzein and equol, have been reported to express selectivity for ERβ. Beneficial health effects have been ascribed to foods containing these flavanoids. In those foodstuffs flavanoids are usually present as *O*-glycosides.



Scheme 4.6

(b) *Chromanes*. 3-Alkyl-4-benzyl-chromanes were disclosed by Organon [57]. Several of these analogs (e.g. **15**) displayed over 30-fold selectivity for ERβ in a cell-based transactivation assay, while the relative binding affinity was over 10% estradiol. It was found that halogen substituents at the 4-benzyl group were better tolerated than methyl or methoxy substituents.

(c) *Naringenin analogs*. 8-Alkylnaringenins were reported as ERβ-selective ligands by Ghent University [58]. ERβ affinity comparable with genistein was shown for compound **16**, but ERβ selectivity was modest – a feature shared with all analogs synthesized within the series.

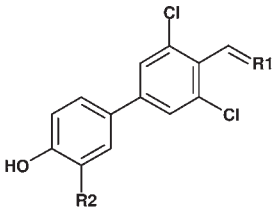


Scheme 4.7

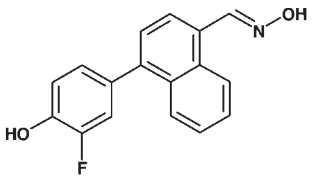
(d) *Isocoumarins*. The University of Illinois investigated isocoumarins as ERβ-selective agonists [59]. The archetype structure already exhibited 80-fold ERβ selectivity albeit binding affinity was modest. Receptor affinity could be increased by attaching halogen or alkyl substituents to the 4-position, but at the cost of ERβ selectivity. Good binding affinity was observed for the 4-Br analog **17**, but ERβ selectivity dropped to 8-fold. Interestingly, using a HEC-1 cell-based transactivation

assay impressive ERβ agonism was observed for analog **17**, exceeding the activity of estradiol. Even higher ERβ selectivity was observed for 4-Cl and 4-Et analogs, but in both cases receptor binding affinity was lower.

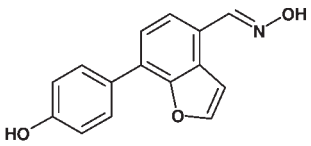
Biphenyls Wyeth reported a series of simple hydroxyl-biphenyls [60]. It was found that biphenyls bearing a 4′-carbaldehyde and one or more substituents *ortho* to the carbaldehyde moiety led to ER ligands (e.g. **18**) with acceptable ERβ affinity and good ERβ selectivity. Attachment of a carbaldehyde oxime moiety gave **19** which showed enhanced ERβ affinity [61]. This result suggests a favorable interaction between oximes and His475 in ERβ. Attachment of fluorine *ortho* to the hydroxyl group had a beneficial effect on ERβ selectivity. Combination of the structure–affinity/selectivity relationship provided compound **20** that showed the best compromise between affinity and selectivity.



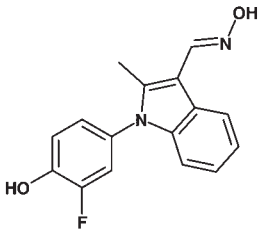
18. R1 = O, R2 = H
$ER\beta_{Bin}$: IC_{50} = 69 nM; β/α = 72
19. R1 = N-OH, R2 = H
$ER\beta_{Bin}$: IC_{50} = 8 nM; β/α = 8
20. R1 = N-OH, R2 = F
$ER\beta_{Bin}$: IC_{50} = 9 nM; β/α = 30



21. $ER\beta_{Bin}$: IC_{50} = 3.8 nM; β/α = 49



22. $ER\beta_{Bin}$: IC_{50} = 10 nM; β/α = 15



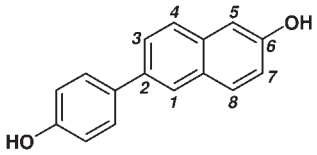
23. $ER\beta_{Bin}$: IC_{50} = 8 nM; β/α = 37

Scheme 4.8

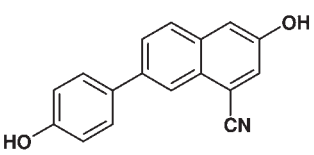
Naphthalenes Wyeth extended its work on biphenyls to 1-phenyl-naphthyls. These investigations resulted in the identification of compound **21** which showed excellent ERβ affinity and 50-fold ERβ selectivity. The investigators further examined replacement of the naphthyl moiety by indoles, benzothiophenes and benzofurans [62]. It was found that many of these analogs still exhibit good ERβ affinity (e.g. benzofurans **22**). However, ERβ selectivity was generally less pronounced. A positive exception was indole **23** which showed 37-fold ERβ selectivity.

A logical next step was the investigations of 2-phenyl-naphthalenes [63]. The archetype structure **24** already showed 13-fold ERβ selectivity. Substituents were incorporated at different positions and it was found that specific substitutions at C4 (e.g. **26**) or C8 (e.g. **25**) resulted in significantly improved ERβ selectivity. Substituent

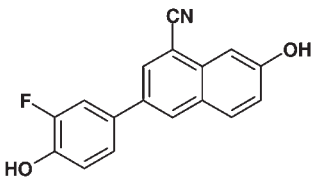
requirements at the C8 position are less stringent than at the 4-position. Again, fluorine substitution *ortho* to the 4′-OH further enhanced ERβ selectivity. X-ray analysis of **26** cocrystallized with ERβ LBD revealed that the exocyclic phenol mimics the A-ring of 17βE2. The 8-CN group is in close proximity to Ile373. It seems that in ERα the 8-CN can be less effectively accommodated due to presence of the more bulky Met421 at the same position.



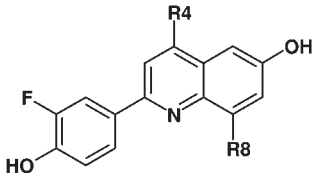
24. $ER\beta_{Bin}$: IC_{50} = 16 nM; β/α = 13



25. $ER\beta_{Bin}$: IC_{50} = 2 nM; β/α = 43



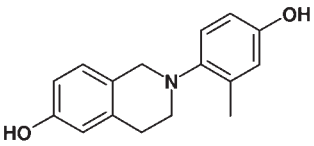
26. $ER\beta_{Bin}$: IC_{50} = 2.7 nM; β/α = 78



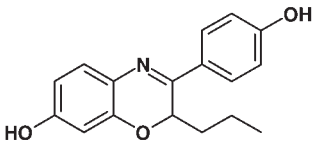
27: R4 = Cl, R8 = Cl
$ER\beta_{Bin}$: IC_{50} = 5.3 nM; β/α = 46

28: R4 = H, R8 =CN
$ER\beta_{Bin}$: IC_{50} = 17 nM; β/α = 32

29: R4 = Cl, R8 = CN
$ER\beta_{Bin}$: IC_{50} = 1.2 nM; β/α = 98



30. $ER\beta_{Bin}$: K_i = 8 nM; β/α = 7



31. $ER\beta_{Bin}$: IC_{50} = 320 nM; β/α = 5
$ER\beta_{Ago}$: EC_{50} = 18 nM; β/α = 95

Scheme 4.9

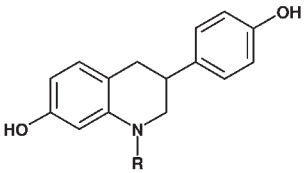
Quinolines and Benzoxazines (a) *Quinolines*. The study with naphthalenes was further extended to quinolines [64]. Similar to observations with the naphthalene series, an ERβ selectivity enhancing effect from substituents at the 4-position was noted. High ERβ selectivity was observed for compound **27**. ERβ selectivity of **28** was somewhat lower, but a synergistic effect was observed for 4,8-disubstituted compound **29**, which not only showed high binding affinity, but also reached 100-fold ERβ selectivity.

(b) *Tetrahydroisoquinolines*. A series of tetrahydroisoquinolines was described by AstraZeneca [65]. Good binding affinity and 7-fold ERβ selectivity was observed for **30**. The compound demonstrated agonistic behavior in a HEK293 transactivation assay. Attachment of a 1-phenyl group did not affect ERβ affinity, but completely abolished ERβ selectivity.

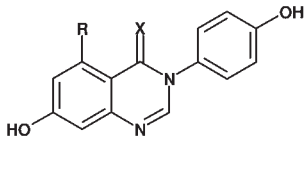
(c) *Benzoxazines*. Bristol-Myers Squibb reported that 3-aryl-benzoxazines expressed ERβ-selective transcriptional activity in a HeLa cell line, stably transfected with ERα and ERβ [66]. Excellent ERβ selectivity (β/α = 95) and high transcriptional activity was noted for **31**. Docking studies revealed that the 2 substituent is located in

the region of Met336 in ERβ and contributes importantly to the ERβ selectivity of 3-aryl-benzoxazines. In ERα there is less room to accommodate this substituent due to the presence of the more bulky Leu384 at the same position. Unexpectedly, ERβ binding studies showed very low binding affinity for **31**, which is hard to explain in view of the high transcriptional activity.

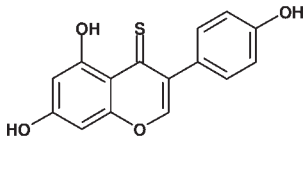
(d) *Tetrahydroquinolines*. Aza analogs of equol were described as ERβ-selective ligands by the Shanghai Institute for Biological Sciences [67]. Ethyl derivative **32** showed the highest ERβ binding affinity, but was 10 times less active than genistein. ERβ selectivity was 17-fold. Methanesulfonamide **33** was less potent, but showed much better ERβ selectivity. ER agonism and antagonism was measured using a yeast two-hybrid assay. It was reported that propyl analog **34** behaved as dual-profile ligand that showed ERα antagonism and ERβ agonism. This is a highly unusual profile in view of the intrinsically less-stable agonist conformation observed for ERβ. Full assessment of the data is not possible as no cellular transactivation data were given.



32: R = Et
$ER\beta_{Bin}$: IC_{50} = 83 nM; β/α = 17
33: R = SO_2Me
$ER\beta_{Bin}$: IC_{50} = 520 nM; β/α = 61
34: R = Propyl
$ER\beta_{Bin}$: IC_{50} = 140 nM; β/α = 18



35: R = OH, X = O
$ER\beta_{Bin}$: IC_{50} = 129 nM; β/α = 62
$ER\beta_{Ago}$: EC_{50} = 76 nM; β/α = 38
36: R = CH_2OH, X = O
$ER\beta_{Bin}$: IC_{50} = 24 nM; β/α = 5
$ER\beta_{Ago}$: EC_{50} = 4474 nM; β/α = ND
37: R = OH, X = S
$ER\beta_{Bin}$: IC_{50} = 47 nM; β/α = 56
$ER\beta_{Ago}$: EC_{50} = 13 nM; β/α = 215



38. $ER\beta_{Bin}$: IC_{50} = 1340 nM; β/α = 42
$ER\beta_{Ago}$: EC_{50} = 14 nM; β/α = 80

Scheme 4.10

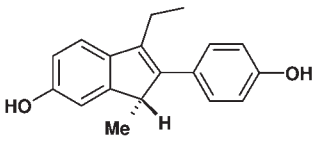
(e) *Quinazolinones*. 3-Arylquinazolinone and 3-arylquinazolinethione derivatives were investigated by Bristol-Myers Squibb [68]. With a few exceptions, receptor binding affinity was low. The most potent analogs identified in the series were **36** and **37**. The latter showed 56-fold ERβ selectivity. In a HeLa cell-based transactivation assay **37** did not show significant transcriptional activity. Furthermore, the thiones (e.g. compound **37**) showed higher ERβ-mediated transcriptional activity than their quinazolinone counterparts (e.g. **35**). This 'thio effect' was also observed for thiogenistein **38** which was 5 times more potent than genistein.

4.2.2.3 Bicyclic [6 + 5]-Ring Systems

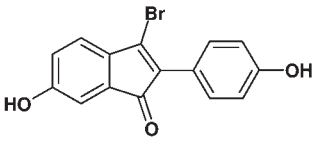
Indenes and Indenones Indenestrol was identified [69] as a metabolite originating from the use of the very potent estrogen diethylstilbesterol (DES). High transcriptional activity and 10-fold ERβ selectivity were observed for the *R*-enantiomer **39**.

The synthesis and biological activity of 2-arylindene-1-ones was reported by Wyeth and stems from their previous work on benzofurans [70]. Due to structural resemblance with genistein it was predicted that this scaffold would afford ERβ selectivity. Both the 6- and 5-OH series were explored. In the 6-OH series it was found that a substituent at the 3-position was required to allow affinity with ERs. Although binding affinity with ERβ was good in several cases, ERβ selectivity was only modest. Best ERβ selectivity was obtained for 3-Br derivative **40**, but at the expense of a lower ERβ affinity. In the 5-OH series the highest ERβ selectivity was observed for the 3-methyl-5,7-dihydroxy analog **41**. This compound showed 10-fold higher affinity for ERβ than 6-OH derivative **40**. Overall, the indenone series did not provide highly selective ERβ ligands.

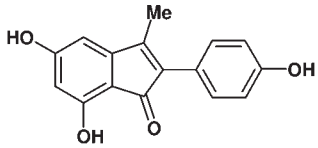
Benzothiophenes 2-Phenyl-benzothiophenes were described as ERβ-selective agonists by Organon [71]. It was found that dibutyryl analog **42** exhibits 15-fold ERβ selectivity in a CHO cell-based transactivation assay. Similar observations were made by Novartis [72]. Receptor binding studies showed 12-fold ERβ selectivity for **43**. Introduction of substituents at C3′ or C4′ reduced ERβ selectivity.



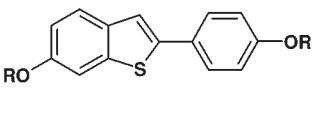
39 (*R*-Indenestrol)
$ER\beta_{Ago}$: EC_{50} = 0.4 nM; β/α = 10



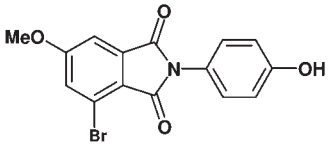
40. $ER\beta_{Bin}$: IC_{50} = 20 nM; β/α = 15



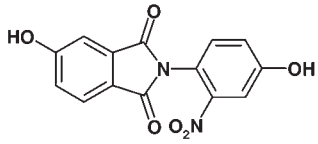
41. $ER\beta_{Bin}$: IC_{50} = 2 nM; β/α = 15



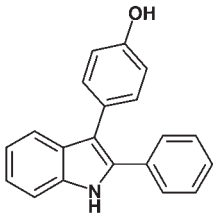
42: R = But
$RTA_{ER\beta}$= 10 %; β/α = 15
43: R = H
$ER\beta_{Bin}$: IC_{50} = 114 nM; β/α = 12



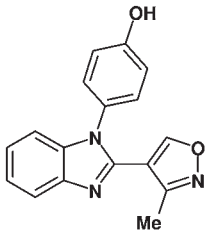
44. $ER\beta_{Bin}$: IC_{50} = 36 nM; β/α = 45



45. $ER\beta_{Bin}$: IC_{50} = 21 nM; β/α = 4



46. $ER\beta_{Bin}$: IC_{50} = 1 nM; β/α = 22



47. $ER\beta_{Bin}$: IC_{50} = 14 nM; β/α = 81

Scheme 4.11

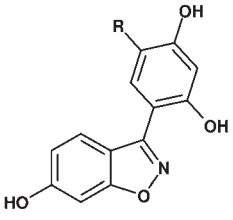
2-Phenyl-Isoindole-1,3-Diones Wyeth investigated isoindoles as ERβ-selective ligands [73]. This type of scaffolds shows structural resemblance with genistein. The docked structure of the archetype isoindole into the ERβ/genistein binding cavity suggested that attachment of a substituent at the 7-position would increase ERβ selectivity by inducing an unfavorable interaction with Met421 in ERα. This indeed turned out to be the case. It was established that the most selective compound from the series (**44**) expressed 45-fold ERβ selectivity. Overall, it was found that isoindoles exhibit modest ER affinity. The most potent compound was **45**, for which an IC_{50} of 21 nM and 4-fold selectivity for ERβ were measured.

Indoles and Benzimidazoles As part of a search towards ERβ-selective ligands Pfizer reported on the estrogenic activity of benzimidazoles and indoles [74]. High-throughput screening (HTS) activities showed 2,3-diphenyl-indole as a weak estrogen that showed 40-fold selectivity for ERβ. Increased potency was obtained by attaching a hydroxyl group to the 3-phenyl group (**46**). In this case, ERβ selectivity was still satisfactory. The importance of the 2,3-diphenyl orientation inspired the investigators to examine various core structures in which the spatial arrangement of the phenyl groups is more or less similar. Good ERβ selectivity was observed for benzimidazole **47** although ERβ affinity was more than 10 times lower. It is noteworthy that the use of a phenyl group instead of the 3-methyl-4-isoxazolyl group dramatically reduced ERβ affinity. AstraZeneca also disclosed a series of benzimidazoles [75]. Although it was claimed that the compounds were ERβ selective, no data supporting this claim were provided.

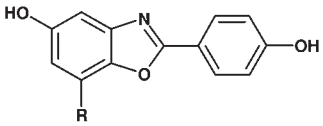
Benzisoxazoles, Benzoxazoles and Benzofurans Benzisoxazole **48** was identified as a potent ERβ ligand by screening of Wyeth's in-house compound collection [76]. X-ray analysis revealed that the molecule adopts a planar orientation in ERβ such that the 6-OH mimics the 3-OH, whereas the 4′-OH represents the 17-OH of 17βE2. The 2′-OH appears to enforce the planar orientation by forming an intramolecular hydrogen bridge with the isoxazole nitrogen. Removal or replacement of the hydroxyl significantly reduced ERβ affinity, but increased ERβ selectivity. Introduction of a bromine at C5′ afforded **49**, which showed the highest ERβ affinity from the series and also showed enhanced ERβ selectivity.

Both AstraZeneca and Wyeth independently reported a series benzoxazoles and benzothiazoles that showed high ERβ selectivity. AstraZeneca disclosed high ERβ selectivity of 7-substituted 5-OH-benzoxazole. From these, 7-bromo analog **50** was identified that showed effective transcriptional activity (HEK293 cells) with an EC_{50} in the subnanomolar range [77]. Excellent ERβ selectivity ($\beta/\alpha = 363$) was also noted. Wyeth also investigated 7-substituted 5-OH-benzoxazoles [78]. High ERβ selectivity was reported but as a result of differences in assay conditions the data cannot directly be compared to the data reported by AstraZeneca. Nevertheless, the trend remained the same. Very high ERβ affinity was reported for the 7-vinyl analog **51** (ERB-041), which showed over 200-fold ERβ selectivity. In 2005, ERB-041 (Prinaberel™) entered phase 2 clinical development as an orally active ERβ agonist, for the potential treatment of inflammatory disorders, including Crohn's disease, rheumatoid

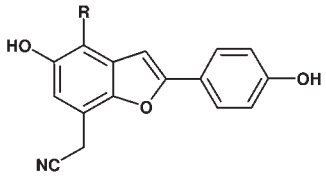
arthritis and endometriosis. X-ray analysis of **51** cocrystallized with ERβ LBD revealed that the vinyl substituent is located in a groove formed by Ile373, Ile376 and Phe377 [79]. In ERα the vinyl substituent is directed towards the more bulky Met421, which can be expected less favorable. 2-Naphthyl-benzoxazoles were also studied [80]. Although ERβ affinity was good for some compounds, ERβ selectivity was much lower.



48. R = H
$ER\beta_{Bin}$: IC_{50} = 3.5 nM; β/α = 8
49. R = Br
$ER\beta_{Bin}$: IC_{50} = 1.8 nM; β/α = 17



50. R = Br (AstraZeneca)
$ER\beta_{Bin}$: IC_{50} = 2 nM; β/α = 78
51. R = Vinyl (Wyeth; ERB041)
$ER\beta_{Bin}$: IC_{50} = 6 nM; β/α = 202

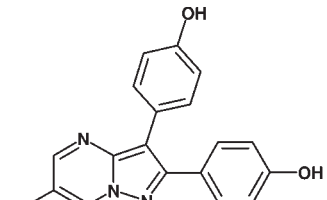


52: R = H
$ER\beta_{Bin}$: IC_{50} = 14 nM; β/α = 80
53: R = Br
$ER\beta_{Bin}$: IC_{50} = 2 nM; β/α = 104

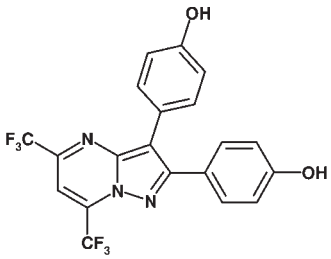
Scheme 4.12

Complementary to the work on benzoxazoles, Wyeth investigated the prospects of 7-substituted benzofurans [81]. Due to the structural similarity between benzoxazoles and benzofurans it is not much of a surprise that the 7-substituted benzofurans showed high ERβ selectivity. ERβ affinity was also quite comparable to the corresponding benzofurans. Best results were obtained with 7-acetonitril analog **52**, which showed 80-fold selectivity for ERβ. Incorporation of an additional bromine at the 4-position (**53**) resulted in improved ERβ affinity and ERβ selectivity.

Pyrazolo[1,5-*a*]pyrimidines The University of Illinois examined the estrogenic properties of the pyrazolo[1,5-*a*]pyrimidine scaffold [82]. The 6-OH-pyrazolo[1,5-*a*]pyrimidine derivatives synthesized (e.g. **54**) showed low affinity for either ERα or ERβ,



54: R = OH
$RBA_{ER\beta}$ = 0.034%; β/α = 8
55: R = H
$RBA_{ER\beta}$ = 0.1%; β/α = 25



56. $RBA_{ER\beta}$ = 1%; β/α = 1.3

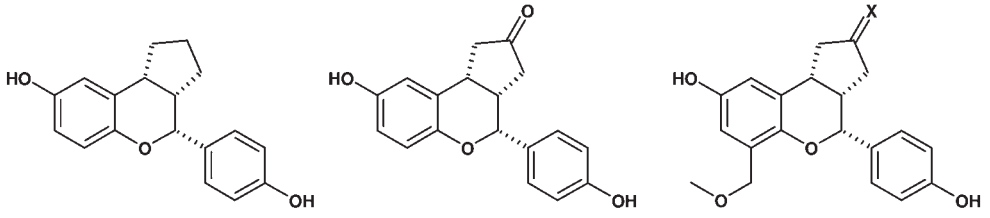
Scheme 4.13

suggesting that the scaffold is too polar to be accommodated effectively in the hydrophobic ligand binding pocket. Interestingly, pyrazolo[1,5-*a*]pyrimidine derivatives without the 6-OH group (**55**) showed a slightly better affinity, suggesting that the 6-OH function does not mimic the phenolic A-ring of 17βE2. Evidence was presented that this function may be performed by the 2-*p*-hydroxyphenyl group. Increased affinity for the ERs was observed for pyrazolo[1,5-*a*]pyrimidine **56** having trifluoromethyl groups attached to the 5- and 7-position. However, no significant subtype selectivity was noted.

4.2.2.4 Tricycles

Tricyclic Benzopyranes Lilly disclosed tricyclic benzopyranes as ERβ-selective agonists [83]. The most selective representative from this series is cyclopentyl derivative **57**. This compound is prepared as a racemate, but was separated into its enantiomers. The most active enantiomer (SERBA-1) is 6–7 times more potent, while the ERβ selectivity is about 2 times higher. Still, the ERβ selectivity of SERBA-1 is only modest (9- to 14-fold).

While SERBA-1 binds to ERβ and ERβ in the same fashion having the 6-OH exposed to the Glu–Arg–H_2O triad and the 4′-OH interacting with His475, its orientation is different. Comparison of the X-ray data of both structures revealed a twist of 180° along the 6-OH/4′-OH axis. Although the 6-OH interacts with His in both structures, it is positioned to different sides of the imidazole residue, suggesting that SERBA-1 not only exploits the differences between two conservative residues, but also makes use of additional conformational flexibility present in the ERs. Repositioning of the 6-hydroxyl to the 5-position did not affect ERβ affinity, but completely abolished ERβ selectivity [84]. In fact, the 5-OH analog showed 2-fold selectivity for ERα.



57 (SERBA-1)
ERβ$_{Bin}$: K_i = 0.17 nM; β/α = 14

58. ERβ$_{Bin}$: K_i = 6.9 nM; β/α = 100

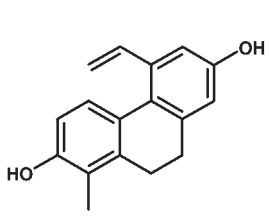
59: X = H_2
ERβ$_{Bin}$: K_i = 0.28 nM; β/α = 43
60: X = F_2
ERβ$_{Bin}$: K_i = 0.53 nM; β/α = 83

Scheme 4.14

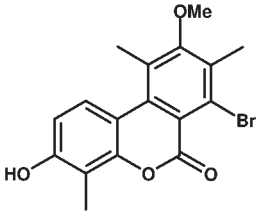
The cocrystal structure of **57** with ERα revealed that the more favorable interaction of the 5-OH with His524 compensates for the shift in the position of the D-ring required for binding with SERBA-1. Changing the ring size of the 3,4-cyclopentyl group to a cyclohexyl or cycloheptyl did not have much impact on either ERβ affinity or selectivity. Attachment of a cyclopentanone (**58**) or cyclohexanone ring to the 3,4-position increased ERβ selectivity to 100- and 40-fold, respectively, but also

seriously reduced ERβ affinity [85]. Attachment of methyl, fluorine or CF_3 substituents to the cyclopentyl ring slightly reduced ERβ affinity while ERβ selectivity was maintained. Analysis of the cocrystal structures of SERBA-1 with ERβ and ERα revealed that in ERα the D-ring is oriented in a more crowded region allowing little space around the 8-position to accommodate substituents. This region differs significantly from the open space around the same position in ERβ in which a twisted orientation of the D-ring is observed [86]. Thus, utilization of this difference may potentially increase ERβ selectivity. Attachment of an 8-methyl group did not affect selectivity or affinity. However, incorporation of a larger and more polar methoxymethyl group (**59**) increased ERβ selectivity to 43-fold, while ERβ affinity was maintained. This result nicely underscores the benefit of having crystal structures available for both ERα and ERβ to rationally improve on receptor affinity and subtype selectivity. In a subsequent report, Lilly disclosed further improvement on ERβ selectivity by combining the A-ring and C-ring structure–activity relationship (SAR) obtained in their previous investigations [87]. Thus, the combination of a 3,4-(3,3-difluoro-cyclopentyl) moiety and an 8-methoxymethyl substituent provided compound **60** which showed 83-fold selectivity for ERβ. X-ray analysis of **60** cocrystallized with ERβ LBD (2QTU) confirmed the predicted binding mode. The difluoromethylene group is directed towards Ile373, while the methoxymethyl is pointed towards Met336.

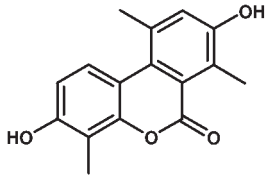
Dihydrophenanthrenes Scientists from Lilly disclosed 6*H*-benzo[*c*]chromen-6-ones as ERβ-selective ligands [88]. The starting point for their efforts was formed by the natural product effusol, an alkylated phenolic 9,10-dihydrophenanthrene isolated from *Juncus effuses* [89], which was identified by screening activities. Effusol bound to ERβ with an IC_{50} of 12 nM and showed 20-fold ERβ selectivity.



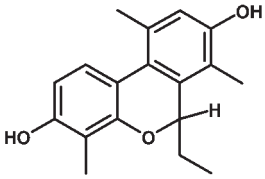
61 (Effusol)
$ER\beta_{Bin}$: IC_{50} = 12 nM; β/α = 20



62. $ER\beta_{Bin}$: IC_{50} = 1.1 nM; β/α = 150



63. $ER\beta_{Bin}$: IC_{50} = 5.7 nM; β/α = 124

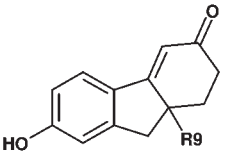


64. $ER\beta_{Bin}$: IC_{50} = 2.3 nM; β/α = 57

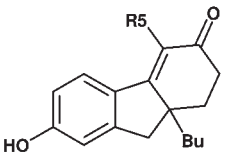
Scheme 4.15

As the dihydrophenanthrene ring system is very sensitive towards oxidation, attention was switched to the structurally analogous 6*H*-benzo[*c*]chromen-6-one scaffold. SERM-type estrogens based on the same scaffold were reported earlier [90], but ER subtype selectivity was not investigated. A wide range of mono- and dihydroxy analogs were prepared having substitutions at various positions. In general, it was observed that increasing the number of small hydrophobic substituents increased both ERβ affinity and selectivity. In the monohydroxy series very high ERβ affinity and excellent ERβ selectivity was observed for **62**. However, it turned out that the majority of the monohydroxy analogs showed large serum binding which resulted in a strongly reduced receptor affinity. In contrasts, the dihydroxy analogs showed much lower serum binding. Analog **63** was among the best compounds from the dihydroxy series in terms of ERβ affinity and ERβ selectivity. Docking experiments with **63** indicate that the core structure of the chromenone spans the length of estradiol while the lactone ring maps the B-ring of the steroid. The high ERβ selectivity is thought to be due to a favorable interaction of Met336 with the aromatic C-ring which is not possible with the corresponding Leu384 in ERα. Replacement of the carbonyl by alkyl groups yielded 6*H*-benzo[*c*]-chromenes. Among the 6-monosubstituted benzochromenes, 6-ethyl analog **64** gave the best ERβ affinity and selectivity.

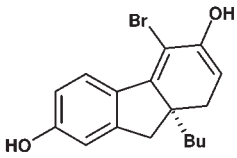
Tetrahydrofluorenones Tetrahydrofluorenones were described as ERβ-selective ligands in a series of joint publications from Karo Bio and Merck [91]. The tetrahydrofluorenone derivative **65** was identified by HTS as a low-affinity ligand but which showed 12-fold ERβ selectivity. Various derivatives were synthesized having variations at the 4- and 9α-positions. The archetype core structure showed very low ERβ affinity. A 9α substituent larger than a methyl was beneficial for ERβ affinity, while ethyl and butyl showed the highest ERβ selectivity. A large impact on ERβ affinity was observed by introducing a substituent at the 4-position. Almost any substituent dramatically increased receptor affinity, while many substituted analogs also showed a much better ERβ selectivity. No data were presented for 4-substituted 9α-*H* analogs. Excellent results were observed for bromo and iodo analogs **66** and **67**. These compounds were also active in a HEK293 reporter cell line stably transfected with ERβ. As the tetrahydrofluorenones contain a chiral center at the 9-position, the enantiomers were separated. It turned out that the eutomer had the 9-*S* configuration. The *S*-enantiomer **68** was 200 times more potent and 7 times more ERβ selective than its *R*-counterpart. X-ray analysis of **68** cocrystallized with ERβ LBD (2GIU) suggests a stabilizing van der Waals interaction of the aromatic surface of the core with Met336 in ERβ, which is not possible in ERα. The butyl group at C9 appears oriented towards Ile373 in ERβ. In ERα there is less space to accommodate the butyl group as this receptor contains the more bulky Met421 at the same position. Although no information is presented about the importance of the 3-keto group, it appears to be positioned in the neighborhood of His474 and possibly interacts with this amino acid through its enol form.



65. $ER\beta_{Bin}$: IC_{50} = 218 nM; β/α = 12



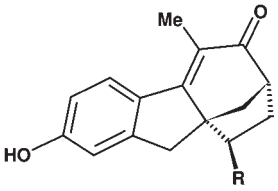
66. R5 = Br
$ER\beta_{Bin}$: IC_{50} = 1.8 nM; β/α = 76
67. R5 = I
$ER\beta_{Bin}$: IC_{50} = 1.3 nM; β/α = 95



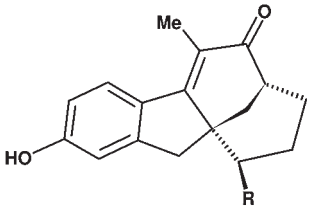
68. $ER\beta_{Bin}$: IC_{50} = 1.5 nM; β/α = 89

Scheme 4.16

As the 9α-butyl group is a key element for receptor binding, the effect of conformationally restricting the butyl group in a ring system was explored. It was found that the 2–9α cyclopentyl bridged system **69** showed potent ERβ affinity and high ERβ selectivity. Improved affinity and selectivity was observed for the exo-propyl substituted derivative **70**. The corresponding 2–9α cyclohexyl-bridged analogs **71** and **72** were somewhat less potent and also less ERβ selective.



69. R5 = H
$ER\beta_{Bin}$: IC_{50} = 12 nM; β/α = 66
70. R5 = Propyl
$ER\beta_{Bin}$: IC_{50} = 1 nM; β/α = 97

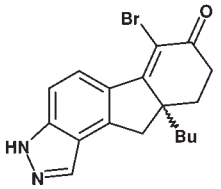


71. R5 = H
$ER\beta_{Bin}$: IC_{50} = 11 nM; β/α = 37
72. R5 = Propyl
$ER\beta_{Bin}$: IC_{50} = 5 nM; β/α = 16

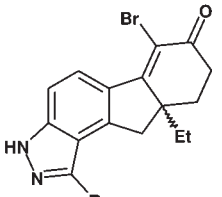
Scheme 4.17

A serious problem associated with the 7-OH-tetrahydrofluoren-3-one derivative **66** was its poor oral bioavailability and high plasma clearance as observed in rats. The poor pharmacokinetic (PK) properties are most likely the result of first-pass glucuronidation of the phenol group, followed by rapid elimination. This problem is not typical for the 7-OH-fluoren-3-one series, but is well documented for numerous low-molecular-weight phenolic compounds, including nonsteroidal estrogens. In some cases first-pass glucuronidation is not a serious problem as the parent phenol may be regenerated by enterohepatic circulation. Nevertheless, minimizing glucuronidation is usually preferred to maintain adequate therapeutic levels. As the phenol is essential for estrogenic activity it cannot easily left out. One way to limit glucuronidation is a prodrug approach. Another very interesting approach to circumvent first-pass liability is the use of phenol bioisosteres that are less likely to act as substrates for intestinal and liver glucuronyl transferases. Various phenol bioisosteres have been documented in the literature, but these have never been applied to estrogens [92].

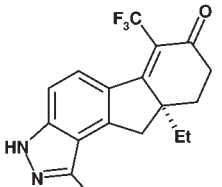
Scientists from Merck explored the use of a fused pyrazole ring as a phenol mimic in their fluorenone system [93]. As it was known that substitution at the 8-position was preferred to substitution at the 6-position, a pyrazole ring was attached to the 7–8 position of the fluorenone core. It was indeed found that the pyrazole ring could effectively replace the phenol with only a slight reduction of ERβ affinity, but with enhanced ERβ selectivity. Substituting the 9α-butyl with an ethyl (compound **74**) increased ERβ affinity. Introduction of a fluorine in the pyrazole ring (compound **75**) further improved ERβ affinity without affecting ERβ selectivity. All compounds mentioned were tested as enantiomeric mixtures. The best compound from the series prepared was the single enantiomer **75**, for which subnanomolar ERβ affinity and 174-fold ERβ selectivity was noted. The transactivation data were in good agreement with the results from the receptor-binding study. For some compounds ERα partial agonism was observed. Most interestingly, all pyrazole compounds tested showed oral bioavailability in rats. These results adequately demonstrate the benefit of the phenol replacement by a pyrazole bioisostere on PK parameters.



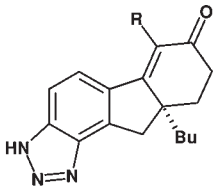
73. $ER\beta_{Bin}$: IC_{50} = 5 nM; β/α = 288



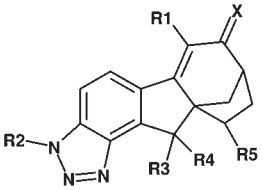
74. R = H
$ER\beta_{Bin}$: IC_{50} = 2.2 nM; β/α = 113
75. R = F
$ER\beta_{Bin}$: IC_{50} = 1.6 nM; β/α = 124



76. $ER\beta_{Bin}$: IC_{50} = 0.3 nM; β/α = 170



77. R = Et
$ER\beta_{Bin}$: IC_{50} = 5.1 nM; β/α = 43
$ER\beta_{Ago}$: EC_{50} = 0.4 nM; β/α = 75
PK (*po*): *F* = 28%
78. R = Br
$ER\beta_{Bin}$: IC_{50} = 5.7 nM; β/α = 333
$ER\beta_{Ago}$: EC_{50} = 5.5 nM; β/α = 94
PK (*po*): *F* = 36%



WO-2006/062876

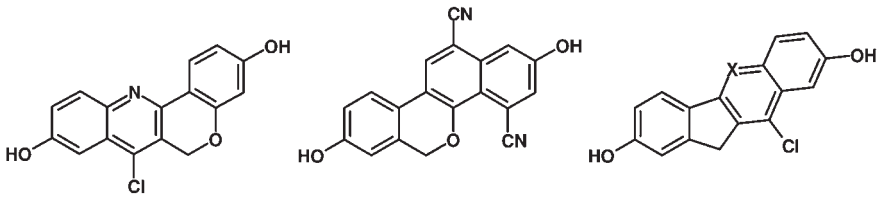
Scheme 4.18

Replacement of the phenol with a triazole was also explored [94]. Several highly potent ERβ-binding ligands were identified as exemplified by analogs **77** and **78**, although, in general, the triazole compounds showed somewhat lower ER affinity as

compared to the corresponding phenols and pyrazoles. However, in many cases ERβ selectivity was higher. Compounds **77** and **78** were both potent ERβ agonists in a HEK-1-based transactivation assay, the former showing an EC_{50} in the subnanomolar range. PK experiments with **77** and **78** indicated reasonable oral bioavailability, much better than corresponding phenols but not as good as their pyrazole counterparts. In a recent patent (WO-2006/062876) triazole-fluorenones were claimed bearing an additional 2–9α ring system [95]. No detailed receptor binding activities were given.

4.2.2.5 Tetracyclics

6*H*-Chromeno[4,3-*b*]quinolines ERβ selectivity of 6*H*-chromeno[4,3-*b*]quinolines was reported by Wyeth [96]. This series was an extension of the earlier reported 2-phenylquinoline and 6-phenyl-naphthalene series [63]. It was anticipated that digitization of the bond connecting the two aryl planes would produce an almost similar alignment as observed for the corresponding phenylquinoline. Substituents were attached to the 7-position as it was shown for the phenylquinoline series that substituents at the corresponding 4-position were essential for ERβ selectivity. It turned out that only Cl, Br or CN substituted chromeno[4,3-*b*]quinolines showed adequate ERβ affinity. ERβ selectivity was modest. These results were in line with the data from the 2-phenylquinoline series, although the latter series showed better ERβ selectivity. Best compound in the chromeno[4,3-*b*]quinoline series was analog **79**. The ERβ-selectivity enhancing effect of the 7-Cl substituent is due to its close proximity to Ile373/Met421 in ERβ and ERα, respectively.



79. $ERβ_{Bin}$: IC_{50} = 3.3 nM; β/α = 27

80. $ERβ_{Bin}$: IC_{50} = 3 nM; β/α = 132

81. X = CH
$RBA_{ERβ}$ > 10%; β/α > 30
82. X = N
$RBA_{ERβ}$ = 2%; β/α > 30

Scheme 4.19

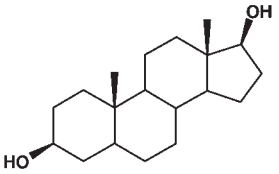
Dibenzochromenes In another study, Wyeth investigated dibenzochromenes as tetracyclic estrogens [97]. Targeting of the ERβ Ile373/Met336 and ERα Met421/Leu384 selectivity region was achieved by introduction of CN substituents at C4 or C12, both of which increased ERβ affinity and ERβ selectivity. The most ERβ selective member of the series was the 4,12-disubstituted compound **80** which showed high affinity and excellent ERβ selectivity (β/α = 130).

Indenoquinolines and Benzofluorenes Organon reported that indenoquinolines and benzofluorenes exhibit ERβ-selective agonistic activity in CHO cells, stably transfected with ERβ and ERα [98]. Attachment of a substituent at C10 was found

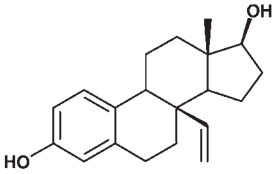
important to obtain adequate levels of ERβ selectivity. Over 30-fold ERβ selectivity was observed for benzofluorene **81**, whereas ERβ affinity was over 10% relative to estradiol. Although the corresponding 10-substituted indeno[1,2-*b*]quinoline **82** was also selective for ERβ, receptor affinity was much lower (RBA = 2%).

4.2.2.6 Steroidal ERβ-Selective Estrogens

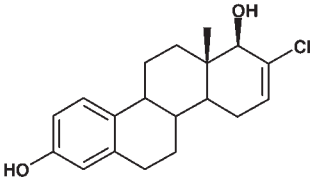
ERα and ERβ show almost equal affinity for the steroidal estrogens 17βE2 and estrone. Various steroids were disclosed that showed preference for ERα. However, over the last few years a number of steroids have been described that express ERβ selectivity. Among the first ERβ-selective steroids identified was 5α-androstane-3β,17β-diol (3β-Adiol). This metabolite of 5α-dihydrotestosterone (DHT) was reported by the Karolinska Institute as the endogenous ERβ ligand in prostate which appears to play an antiproliferative role in prostate epithelium [99]. 3β-Adiol is derived from DHT through the action of 3β-hydroxysteroid dehydrogenase and is inactivated by hydroxylation in a reaction catalyzed by Cyp7B1. In comparison to 17βE2, 3β-Adiol has a lower affinity for ERβ (RBA ~ 10%) and shows 3- to 4-fold selectivity for ERβ [100]. Schering disclosed 8β-substituted steroids as ERβ-selective ligands [49]. Design of these compounds was based on docking experiments which suggested that steroidal estrogens having an 8β substituent would express an unfavorable interaction with Leu384 in ERα, thereby enhancing ERβ selectivity. Indeed, it was found that 8βVE2 expressed high affinity for ERβ and showed 180-fold selectivity for this nuclear receptor. Schering further disclosed a series of 17-chloro-homoestradiol derivatives (e.g. **85**) that were claimed to express selectivity for ERβ [101]. No biological data were provided to support this claim. In another study, Schering reported on 16α-OH analogs of 17βE2 [102]. It was found that compound **86** showed 6-fold selectivity for prostate-derived ER in comparison with uterus-derived ER.



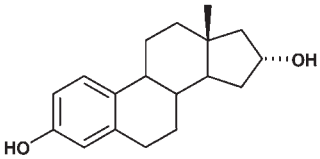
3β-Adiol: ERβ$_{Bin}$: K_i = 1.7 nM; β/α = 3.4



8β-VE2. RBA$_{ERβ}$ = 83%; β/α = 180



85.

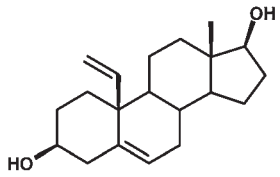


86. RBA$_{ERβ}$ = 50%; β/α = 6

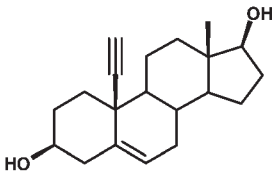
Scheme 4.20

Merck reported that certain 19-substituted androstene diol derivatives exhibited a remarkable degree of ERβ selectivity [103]. However, in many cases the compounds

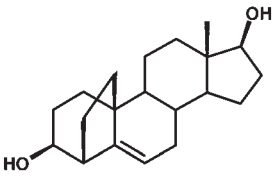
also showed affinity for the androgen receptor (AR). Both ERβ and AR affinity were strongly dependent on the nature of the 19 substituent. Highest ERβ selectivity was observed for vinyl-substituted **86**, but in addition high AR affinity was observed as well. Best compromise between high ERβ affinity/selectivity and low AR affinity was achieved by the 19-acetylene substituted **87**. The ERβ selectivity of **86** and **87** could be explained by the occurrence of an unfavorable interaction between the 19 substituent with Leu384 in ERα. Bridged androstene diol derivatives were also investigated [104]. However, these compounds, as exemplified by **88**, showed lower ERβ affinity and ERβ selectivity.



86. $ER\beta_{Bin}$: IC_{50} = 11 nM; β/α = 212
$ER\beta_{Ago}$: EC_{50} = 2 nM; β/α = 246
AR_{Bin}: EC_{50} = 33 nM
ERβ/AR = 3



87. $ER\beta_{Bin}$: IC_{50} = 17 nM; β/α = 25
$ER\beta_{Ago}$: EC_{50} = 8 nM; β/α = 20
AR_{Bin}: EC_{50} = 1761 nM
ERβ/AR = 104



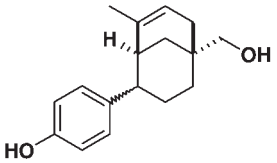
88. $ER\beta_{Bin}$: IC_{50} = 50 nM; β/α = 6
$ER\beta_{Ago}$: EC_{50} = 28 nM; β/α = 2
AR_{Bin}: EC_{50} = 1375 nM
ERβ/AR = 28

Scheme 4.21

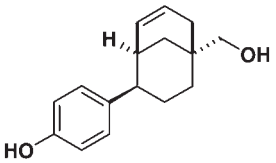
4.2.2.7 Miscellaneous ERβ Ligands

Natural Products Bionovo reported phase II clinical development of MF-101, an ERβ agonist, for the treatment of vasomotor symptoms in pre- and postmenopausal women. Two doses (5 and 10 g) of MF-101 were more effective than placebo at reducing the frequency and severity of hot flashes. MF-101 is an aqueous ethanol extract of 22 herb species and showed selective ERβ recruitment to target genes [105].

Bicyclononene Ring Systems Bayer disclosed ER ligands that are derived from a rigid bicyclo-[3.3.1]-nonene core [106]. This series was derived from the prototype bicyclic ether **89a**, which was identified by HTS. This type of scaffold is unusual in the field of estrogen templates reported thus far. Improved ERβ affinity and ERβ selectivity was observed for the bicyclo-[3.3.1]-nonene derivative **89b**. However, compound **89b** was tested as an enantiomeric mixture making it difficult to draw conclusions on further prospects of this series.



89a: $ER\beta_{Bin}$ = 850 nM; β/α = 4



89b: $ER\beta_{Bin}$ = 75 nM; β/α = 7

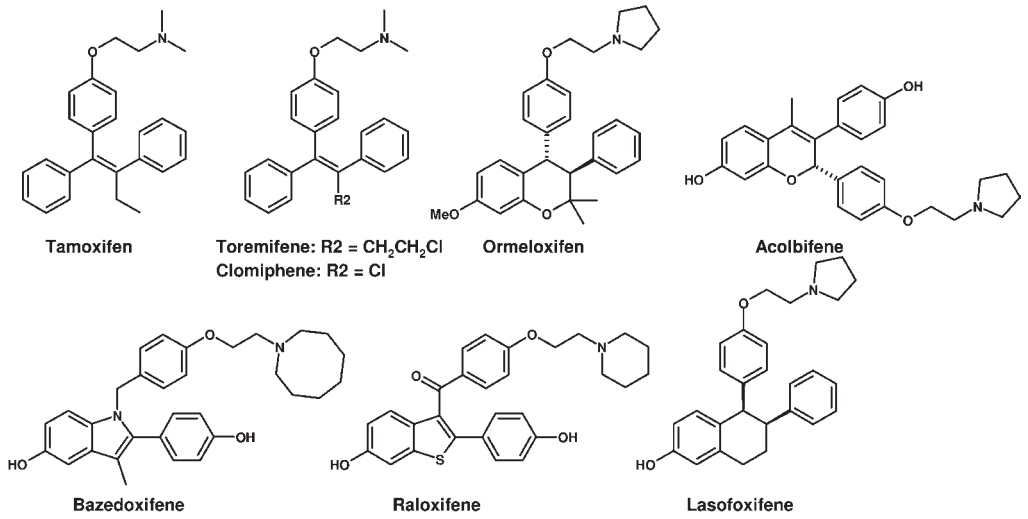
Scheme 4.22

4.2.3 ERα-Selective Antagonists

In this category SERM-type antagonists are described – a category of estrogen ligands that mimic estrogen activity in tissues such as bone and cardiovasculature, while antagonizing estrogen activity in others, including breast, uterine tissue and central nervous system (CNS). SERMs typically behave as partial agonists on ERα and almost full antagonists on ERβ. The triphenylethylene class of estrogens is regarded as the first generation of SERMs. Two compounds from this series, tamoxifen and toremifene, have been marketed for treatment of breast cancer. Clomiphene also belongs to this category, but is used to treat infertility disorders. Raloxifene represents the second-generation SERMs and is used to treat osteoporosis in postmenopausal women. Third-generation SERMs are in clinical development, and include bazedoxifene, lasofoxifene and arzoxifene. All of these compounds are intended to treat osteoporosis with lesser side-effects. Many other SERMs have failed in late-stage clinical development as a result of low efficacy or serious side-effects. It is well established that the beneficial effects of SERMs such as raloxifene on bone metabolism are associated with an increased incidence of hot flashes in a subset of osteoporosis patients. The occurrence of hot flashes in patients treated with SERM-type antiestrogens most likely originates from central ER antagonism leading to deregulation of the thermoregulatory process in the hypothalamus. Furthermore, SERMs may induce vaginal atrophy and increase the risk to develop cardiovascular disorders. Tamoxifen was also reported to induce hot flashes in more than 50% of patients treated for breast cancer. These hot flashes are generally more severe and last longer than hot flashes that develop as a consequence of menopausal transition. More severe is the risk of developing endometrial cancer as a result of continuous endometrial stimulation. The design of SERMs with improved tissue selectivity and devoid of central ER antagonism is a challenge to many scientists active in the ER field. One way to circumvent the problem is to mix an ER agonist with a SERM in such a ratio that central antagonism is counterbalanced while no stimulation of uterine tissue is observed. Wyeth reported on the feasibility of this approach by developing a combination therapy of Premarin™ with the indole-based SERM bazedoxifene [107]. This combination therapy is currently in phase III clinical trials. In recent years a number of antiestrogens have been disclosed for which an improvement on CNS parameters is claimed.

4.2.3.1 Triphenylethylenes

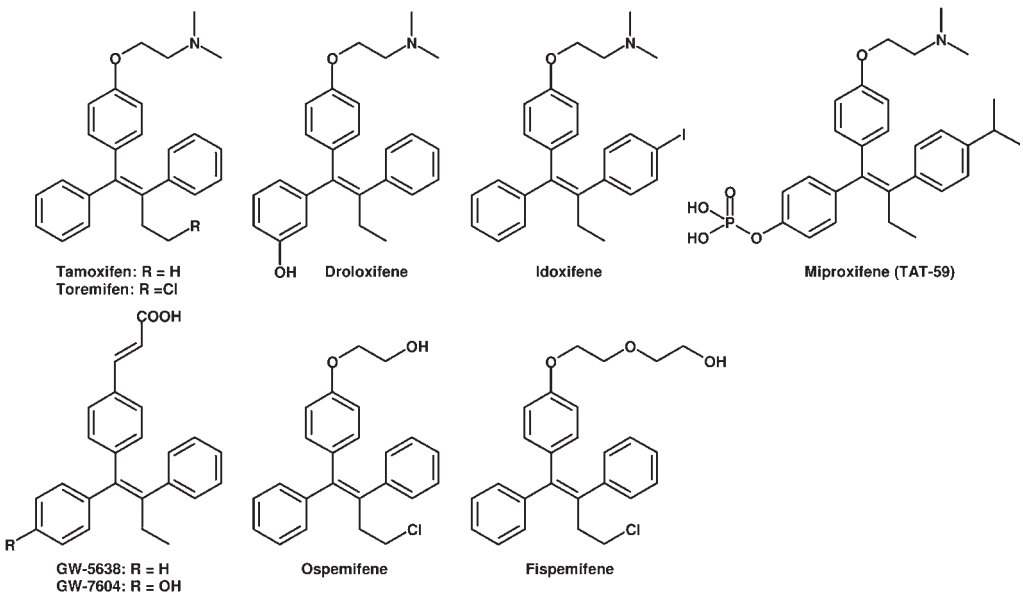
Tamoxifen Variants The earliest triphenylethylene identified as an ER modulator is clomiphene (Clomid™) and was identified by Merrell in 1958. The compound was intended to be used as a contraceptive, based on its contraceptive activity in rat. However, it behaved exactly the opposite in woman, inducing ovulation and improving subfertility. The compound was therefore marketed as a therapy for infertility. The commercial preparation consists of a mixture of enclomiphene and zuclomiphene, the latter acting as an agonist. Yet, even 50 years after it discovery, clomiphene



Scheme 4.23

is still a first-line treatment for infertility disorders. Enclomiphene citrate (Androxal™) is currently in phase III clinical testing (Repros Therapeutics) for the potential oral treatment of testosterone deficiency in men. The best-know representative of the triphenylethylene class and the first marketed SERM is tamoxifen (Nolvadex™). This very successful anticancer drug was identified in 1967 by ICI Pharmaceuticals (now AstraZeneca) and has been around for over 30 years to treat ER-positive breast cancer. Tamoxifen can be considered a prodrug which is rapidly bioactivated to 4-OH-tamoxifen, a metabolite that has a much higher affinity for the ERs, but does not show significant subtype selectivity. Although highly successful as a first-line treatment of ER-positive breast cancer [108], development of tamoxifen resistance is a serious problem. Almost all patients with metastatic disease and as many as 40% of patients receiving adjuvant tamoxifen eventually relapse [109]. Reduced intracellular concentration as a result of decreased influx and increased efflux as well as increased metabolism to agonistic metabolites may alter tamoxifen efficacy. Furthermore, pharmacogenomic changes have its impact on tamoxifen responsiveness. About 20% of the patients who were ER-positive at pretreatment become ER-negative at relapse. In the posttreatment ER positive group, mutations of the ER genes may play a significant role towards development of tamoxifen resistance. About 20% of the patients that have relapsed still respond to aromatase inhibitors or fulvestrant (Faslodex™). The use of tamoxifen is not without side-effects. A common event associated with its use is induction of hot flashes. A more serious adverse effect is stimulation of endometrial tissue. Prolonged treatment (more than 5 years) is associated with a 4-fold risk increase to develop endometrial cancer. This undesired activity is attributed to its partial agonist behavior in endometrial tissue. Whether this effect is induced by tamoxifen itself is unclear as tamoxifen is rapidly converted into at least 20 metabolites some of which behave as agonists. Pure antiestrogens (fulvestrant) and aromatase inhibitors such as

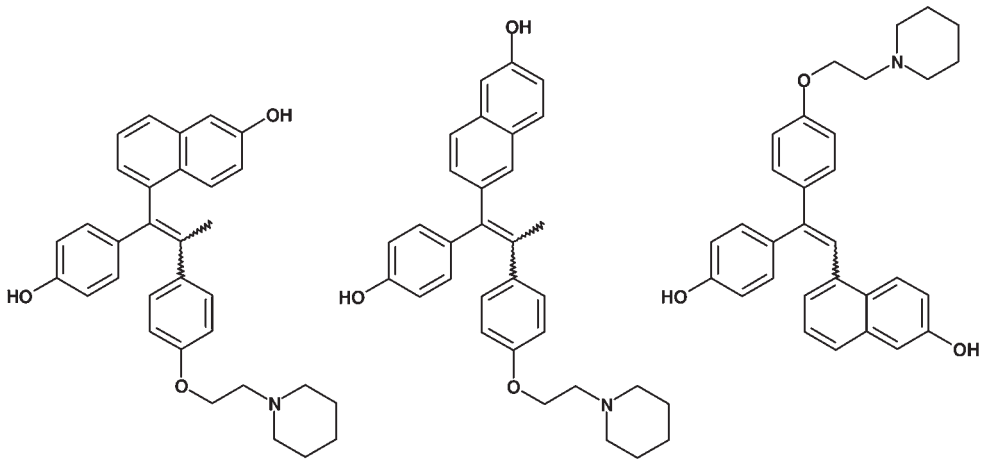
AstraZeneca's anastrozole (Arimidex™), Novartis's letrozole (Femara™), Pfizer's exemestane (Aromasin™) and Intarcia Therapeutics' atamestane may eventually replace tamoxifen entirely as the gold-standard antihormonal therapy for breast cancer. A more recent variant of the triphenylethylene class is toremifene (Fareston™). This compound was developed by Orion and is also on the market as a therapy for breast cancer. Clinical investigations are ongoing to evaluate combination therapy of Toremifene with the steroidal aromatase inhibitor Atamestane™. Additional clinical studies are in progress to evaluate the potential of toremifene to treat prostate cancer [110]. Many other variants, such as droloxifene and idoxifene have failed because of insufficient efficacy. Pfizer halted its development program for droloxifene as interim results from phase III clinical trials had shown that the drug offered no benefit for the treatment of advanced ER-positive breast cancer and osteoporosis beyond existing therapies. Fujisawa came to similar conclusions while developing the compound for osteoporosis in Japan. Lack of efficacy in advanced breast cancer trials forced SmithKline Beecham to terminate further development of its phenylstilbene analog idoxifene. The phosphate prodrug miproxifene (TAT-59) has been under development by Taiho, but was discontinued after phase III trials for breast cancer [111]. A metabolite of toremifene lacking the basic amino group is Ospemifene (Ophena™). This compound is currently in phase III clinical development by QuatRx for treatment of osteoporosis and urogenital atrophy. Recently, recruitment of 827 patients in a phase III study for postmenopausal vaginal syndrome was completed. QuatRx is also developing the oral estrogen antagonist fispemifene (HM-101) for the potential treatment of androgen/testosterone deficiency in elderly men.



Scheme 4.24

Acrylic Acid Variants A distinct set of SERM-type antagonists is made up by the acrylic acid variants of tamoxifen, put forward by researchers at GlaxoSmith-Kline [112]. The tamoxifen analog GW-5638 was found to be less estrogenic than tamoxifen, but exhibits full bone protective activity in the OVX-rat model. GW-7604, the presumed active metabolite of GW-5638, showed a unique peptide recruitment profile when compared to estradiol or 4-OH-tamoxifen against a set of more than 50 cofactor and phage display-derived peptides in a microsphere-based *in vitro* binding assay [113, 114]. GW-7604 did not show significant stimulation in ECC-1 and Ishikawa cells, suggesting this compound may display an improved uterine safety profile [115]. GW-7604 showed satisfactory affinity for both ERs [116]. No ER-subtype selectivity was observed. With an estrogen response element-driven luciferase reporter, both GW-5638 and GW-7604 antagonized an estradiol response. However, with an AP-1-driven luciferase reporter assay it was found that GW-7604 activates transcription with ERβ 50 times more potently at the AP-1 site than with ERα. No clinical development was reported as of 1997. GlaxoSmithKline recently disclosed a series of GW-7604-derived prodrugs [117].

Diphenylnaphthylethylenes Diphenylnaphthyl alkene derivatives (e.g. compounds **90**, **91** and **92**) were recently disclosed by SignalGene [118, 119]. Most of these tamoxifen-related structures showed good affinity for both ERα and ERβ. Although receptor-binding data were presented, no meaningful assessment of ERα/β selectivity can be made as all of the compounds were tested as *E*/*Z* mixtures.



90. ERα$_{Bin}$: K_i = 4.6 nM; α/β = 13 **91. ERα$_{Bin}$: K_i = 1.1 nM; α/β = 1** **92. ERα$_{Bin}$: K_i = 0.9 nM; α/β = 1.5**

Scheme 4.25

4.2.3.2 **Benzothiophenes**

Raloxifene (Evista™) has been developed by Lilly and is considered a second-generation SERM. The compound can be regarded as a planar variant of the

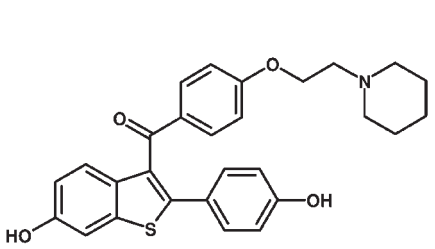
tamoxifen theme. Raloxifene was prepared long before the identification of ERβ and as such was not designed as a subtype-selective ER ligand. Nevertheless, soon after the discovery of ERβ raloxifene was reported to show 20-fold selectivity for ERα. To which extent, if any, this subtype selectivity contributes to its overall *in vivo* profile remains to be established. Raloxifene has been on the market since 1998 as a second-generation SERM to treat osteoporosis. The compound is administered orally at a dose of 60 mg/day. Raloxifene has a very low oral bioavailability and is rapidly glucuronidated, primarily by glucuronyl transferases in the enterocytes which have a preference for the 6-OH position. Its good efficacy on bone parameters has been ascribed to enterohepatic circulation leading to an unexpected long half-live. Unlike tamoxifen, raloxifene does not increase endometrial proliferation or the risk of endometrial cancer. Side-effects include the induction of hot flashes and urogenital atrophy. A clinical study [Continuing Outcomes Relevant to Evista (CORE)] revealed that raloxifene reduced the incidence of invasive ER-positive breast cancer by 60% compared to placebo. Other clinical studies Raloxifene Use for The Heart (RUTH) and Study of Tamoxifen and Raloxifene (STAR)] are in progress to further evaluate the antitumor effects of raloxifene in breast tissue. In addition, preclinical evaluation of raloxifene is ongoing to establish its potential for the treatment of prostate cancer [120]. Not surprisingly, numerous variants of raloxifene have been made [121] some of which are described below.

Hinge Variations A unique feature of raloxifene is the linkage of the BSC through a carbonyl moiety. This so-called hinge allows raloxifene to interact with the ERs in a conformation that is distinct from tamoxifen. Removal of the carbonyl hinge resulted in ER ligand **93** that displayed comparable ER affinity, but markedly increased uterine weight and uterine epithelial height to a level that is comparable to the uterotrophic activity of tamoxifen [122]. Apparently, the coplanar orientation of the side-chain hampers an optimal positioning of the BSC and leads to partial agonism in uterine tissue, a feature shared with tamoxifen. Replacement of the carbonyl by methylene, oxygen, sulfur and nitrogen was also investigated [123]. Examination of these compounds on inhibitory activity in a MCF-7 proliferation assay revealed that methylene, nitrogen and sulfur analogs were 2–5 times more active than raloxifene whereas the oxygen analog was 10-fold more active.

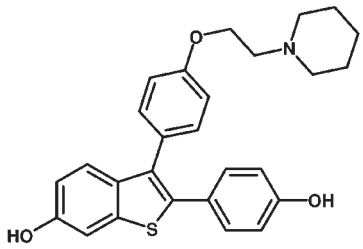
BSC A basic amine moiety is essential for effective interaction with Asp353 in ERα. A variety of amines have been examined by Lilly scientists [122]. It was found that a piperidine was optimal to inhibit ethinyl-estradiol-induced increase of uterine weight in rat. The dimethylamino analog showed partial activity, while the nonbasic cyclohexyl variant did not show significant inhibition. Replacement of the oxygen in the ethoxy chain by nitrogen, methylene or sulfur substitution variants had a minor effect of ER affinity. However, the nitrogen and carbon analogs showed 10- to 50-fold lower inhibitory activity on MCF-7 proliferation, possibly caused by lower cellular permeability.

Piperazine analogs of raloxifene were described by the Shanghai Institute for Biological Sciences [124]. The unsubstituted piperazine **94** showed 19-fold selectivity

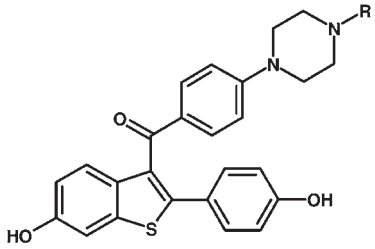
for ERα which is comparable to raloxifene. Various aroyl and alkyl substituents were attached to the piperazine moiety. It was found that ERα affinity and selectivity was significantly increased for *m*-toluoyl (**95**) and isopropyl (**96**) variants. Transactivation data (CV-1 cells) were disclosed for isopropyl variant **96** (Y-134) [125], showing ERα antagonism which was comparable to raloxifene. However, the data indicate that Y-134 shows less ERα partial agonism.



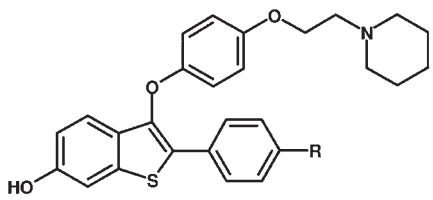
Raloxifene: RBA$_{ER\alpha}$ = 34%; α/β = 20



93. RBA$_{ER\alpha}$ = 29%



94: R = H
ERα$_{Bin}$: IC$_{50}$ = 0.7 nM; α/β = 26
95: R = *m*-toluoyl
ERα$_{Bin}$: IC$_{50}$ = 0.3 nM; α/β = 46
96: R = isopropyl (Y-134)
ERα$_{Bin}$: IC$_{50}$ = 0.3 nM; α/β = 121
ERα$_{Ant}$: IC$_{50}$ = 0.5 nM



Arzoxifene: R = OMe
ERα$_{Bin}$: EC$_{50}$ = 21.5 nM; α/β = 3
97: R = OH (DMA)
ERα$_{Bin}$: EC$_{50}$ = 7.8 nM; α/β = 1.3
98: R = F
ERα$_{Bin}$: EC$_{50}$ = 17 nM; α/β = 1.6
99: R = H
ERα$_{Bin}$: EC$_{50}$ = 11 nM; α/β = 1.6
100: R = SO$_2$Me
ERα$_{Bin}$: K_i = 0.63 nM; α/β = 21

Scheme 4.26

Arzoxifene Arzoxifene is a structural analog of raloxifene in which the carbonyl hinge has been replaced by an oxygen linkage and the 4′-OH function by a 4′-OMe group to reduce first-pass metabolism [126]. Although the intended effect of the 4′-OMe group is clearly to increase the bioavailability of the compound, Arzoxifene appears susceptible to demethylation, yielding the more active dihydroxy compound DMA (**97**) [127]. DMA is over 5 times more potent than raloxifene indicating that the CO → O hinge modification is more favorable. However, DMA plasma levels are highly variable among patients, steady-state concentrations ranging from 0.05 to 2.8 ng/ml for a dose of 50 mg [128]. It was reported that DMA and also raloxifene are sensitive to oxidation, giving rise to cytotoxic metabolites resulting

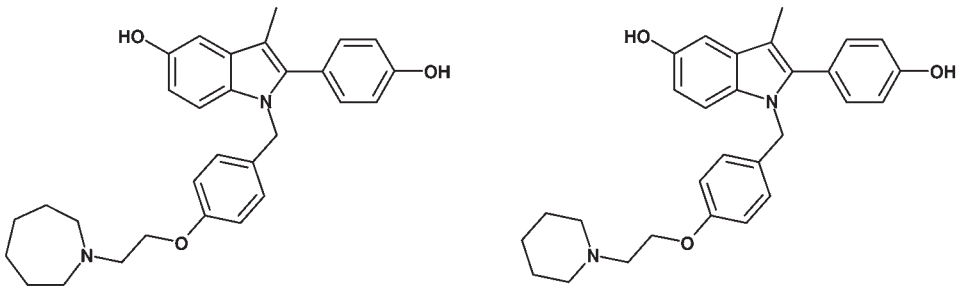
from bioactivation into catechol and electrophilic diquinone methides. These metabolites may lead to glutathione depletion, P450 inhibition and liver protein modification [129]. In addition, DMA gives rise to the formation of more phase I metabolites, including glucuronides, sulfates and glutathione S-transferase adducts. Arzoxifene has entered clinical development for breast cancer [130]. After promising phase II data had been obtained a phase III study was started aiming to compare arzoxifene with tamoxifen in women with metastatic breast cancer. However, this trial was prematurely stopped by Lilly as the first analysis indicated that arzoxifene appeared inferior to tamoxifen with regard to time to progression – one of its primary endpoints. No further breast cancer trials have been scheduled. Arzoxifene is currently being evaluated for the treatment of postmenopausal osteoporosis.

It was further reported [131] that replacement of the 4-OMe group by fluorine provides a more stable arzoxifene analog, exhibiting binding potency to ERα and ERβ comparable to DMA, whereas the antiestrogenic activity was comparable to raloxifene. In comparison with DMA, the 4′-F analog **98** was found to be less sensitive to glucuronide formation as observed in hepatocytes, intestinal microsomes and Caco-2 cells. Furthermore, enhanced stability in microsomal preparations and no glutathione depletion was observed indicating improved stability towards oxidation. In a subsequent study, other 4′-modifications were presented by the University of Illinois with the aim to identify compounds that are less susceptible to oxidative metabolism to quinoids [132]. It was found that the F, Br and SO_2Me substitution variants prepared exhibited good ERα affinity, while redox activity was reduced. In contrast, amine substitution increased redox activity. Interestingly, the methanesulfone analog **100** showed 80-fold ERα selectivity, whereas DMA was not selective. It was predicted that analog **100** may show reduced CNS penetration and hence diminished ovarian stimulation via actions in the hypothalamus. Lilly also investigated compound **100** for the treatment of uterine fibroids [133]. In their study, **100** was 20-fold selective for ERα. However, the activity in the immature rat uterine model was lower than expected, which may be due to low oral absorption and/or higher metabolism.

4.2.3.3 **Indoles**

Bazedoxifene and Pipendoxifene Wyeth is developing the indole-type estrogen bazedoxifene (Viviant™, TSE-424), a SERM for the prevention and treatment of osteoporosis in postmenopausal women [134]. Bazedoxifene originated from a collaboration between Wyeth and Ligand. The compound shows good affinity to ERα ($IC_{50} = 26$ nM), but low ERα selectivity ($\alpha/\beta = 4$). Bazedoxifene is claimed to give less luminal epithelial cell and myometrial hypertrophy in the immature rat uterine model as compared to raloxifene. In the OVX-rat model bazedoxifene was efficacious against bone loss and at a 10 times lower dose as raloxifene. The lower dose to achieve maximal bone sparing activity was considered of relevance to minimize vasomotor symptoms. In the morphine-addicted rat model bazedoxifene did not show antagonism at 0.3 mg/kg. Oral bioavailability in healthy postmenopausal women was 6.2%, which is higher than raloxifene (2%) [135]. Maximum concentration was reached within 1–2 h and elimination half-life was

28 h. 5-OH-glucuronidation was the major metabolic pathway. This metabolic pathway is similar to the equivalent 6-OH glucuronidation observed for raloxifene. In a phase II clinical study bazedoxifene decreased bone turnover rate by 25% at 40 mg/day with no occurrences of endometrial hyperplasia. No phase III data (primary outcome: reduction vertebral fractures) are available as yet. In April 2007, the US Food and Drug Administration (FDA) issued an approvable letter, which contained several conditions to be fulfilled before the new drug application (NDA) could be accepted [136]. Another indole-type SERM that emerged from the Wyeth–Ligand collaboration is the closely related pipendoxifene (ERA-923), which entered development for the potential treatment of breast cancer. Pipendoxifene reached phase II clinical studies involving women with metastatic breast cancer. No further development for this indication was reported as of 2002.

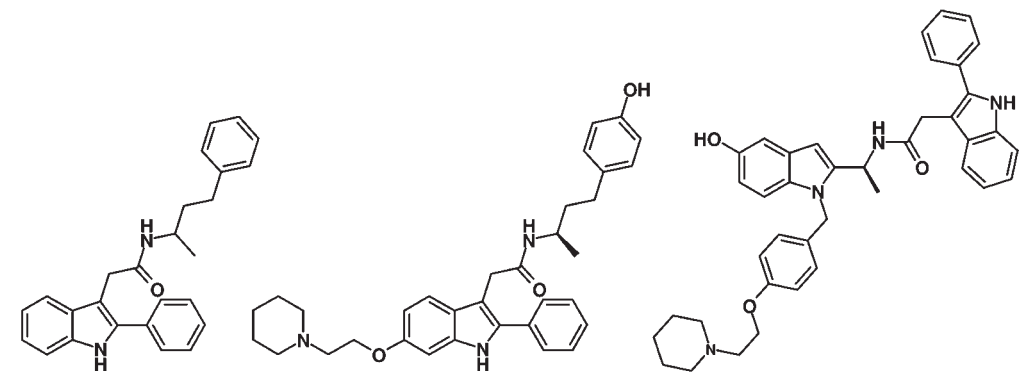


Bazedoxifene
ERα$_{Bin}$: IC$_{50}$ = 26 nM; α/β = 4

Pipendoxifene

Scheme 4.27

2-Aryl-Indoles Scientists at Merck described 2-aryl-indoles as ERα-selective SERMs [137]. HTS uncovered indole **101**, which showed reasonable receptor affinity but very high ERα selectivity. However, **101** expressed agonism in an immature rat

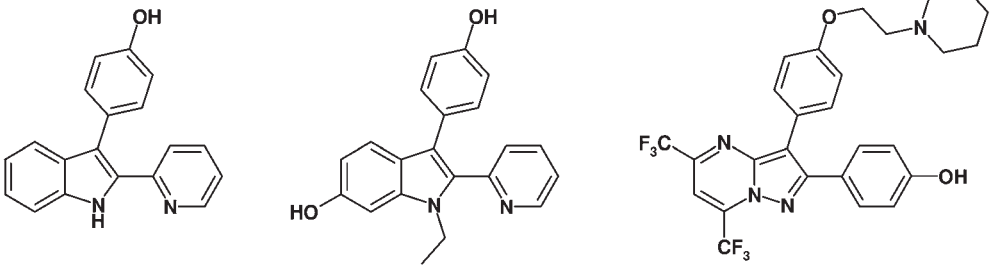


101. ERα$_{Bin}$: IC$_{50}$ = 11 nM; α/β = 445 **102. ERα$_{Bin}$: IC$_{50}$ = 8 nM; α/β = 150** **103. ERα$_{Bin}$: IC$_{50}$ = 2 nM; α/β = 130**

Scheme 4.28

uterine model and was inactive in an MCF-7 proliferation assay. The side-chain attached to this compound is quite unusual and deviates significantly from the traditional side-chains attached to SERM-type scaffolds, indicating that surprises still can be found. Modifications and preparation of hybrid molecules led to the identification of **102** and **103**. Again, the compounds exhibit a remarkable degree of ERα affinity and ERα selectivity. Compound **102** did not inhibit 17βE2-mediated uterine weight increase and did not inhibit MCF-7 proliferation. However, compound **103** showed good *in vivo* efficacy on inhibition of uterine weight increase and inhibited MCF-7 proliferation. As predicted, X-ray analysis of **102**, cocrystallized with ERα HBD confirmed the interaction of the BSC with Asp351.

2-Pyridin-Indoles 2-Pyridin-2-yl-1*H*-indole derivatives were described by the University of Athens [138]. It was aimed for to identify fluorescent probes as tools to characterize cellular binding sites of ERs. It was found that several members of this family (e.g. **104** and **105**) exhibit reasonable good binding activities. Whether these compounds behave as agonists or antagonists is currently unknown. No data on subtype selectivity were disclosed.



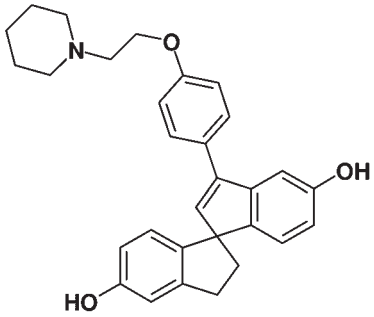
104. $RBA_{ER\alpha}$= 10.4% **105.** $RBA_{ER\alpha}$= 14.3% **106.** $RBA_{ER\alpha}$= 2.8 %; α/β = 4

Scheme 4.29

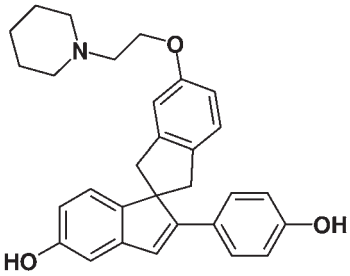
Pyrazolopyrimidines Transformation of the agonist into a SERM-type estrogen was attempted by attaching BSCs to the 2- or 3-*p*-hydroxyphenyl group [139]. Modest ER affinity and 4-fold ERβ selectivity was observed for analog **106**. Compound **106** antagonized 17βE2-induced transcriptional activity in HEC-1 cells.

4.2.3.4 Spiroindenes

Dainippon Pharmaceuticals reported on the identification of spiroindenes that behave as SERM-type estrogens [140]. Interestingly, **107** (OS-689) expressed estradiol-like effects in an OVX-rat tail skin temperature model for human hot flash and in a morphine dependent OVX-rat model. These findings suggest that **107** holds promise for the development of a SERM-type ligand that is devoid of central antagonism. Spiroindenes and spiroindenediones were reported by Merck [141]. Good receptor binding affinity but no significant ER subtype selectivity was observed for analog **108**. No further data were given.



107 (OS-689). ERα $_{Bin}$: K_i = 9 nM

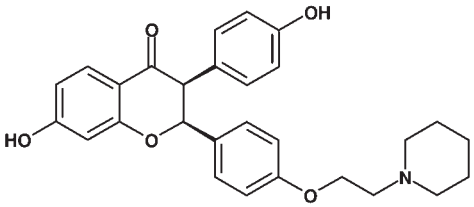


108. ERα $_{Bin}$: IC_{50} = 1 nM; α/β = 2

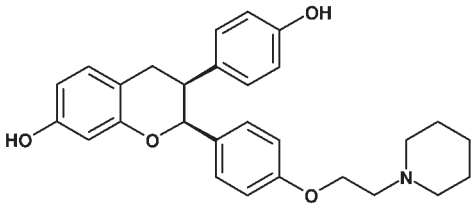
Scheme 4.30

4.2.3.5 Flavanoids

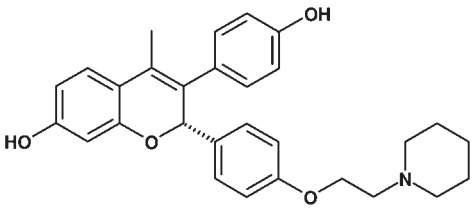
Flavanones Merck reported a series of SERM-type *cis*- and *trans*-flavanones. Although the core flavanone structure is known to express ERβ-selective agonism, attachment of a BSC switched the profile towards ERα-selective antagonism. The *cis* isomers showed higher ERα selectivity than the corresponding *trans* isomers. Compound **109** expressed 66-fold selectivity for ERα and effectively inhibited uterine weight increase in the immature rat model. It was put forward that the keto group in **109** may face steric and electrostatic repulsion from Met336 in ERβ. In agreement with this it was shown that removal of the 4-keto moiety (**110**) completely abolished ERα selectivity, although ERα affinity was significantly increased.



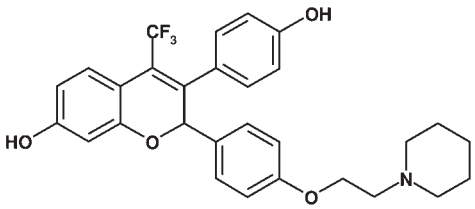
109. ERα $_{Bin}$: IC_{50} = 31 nM; α/β = 66



110. ERα $_{Bin}$: IC_{50} = 6.7 nM; α/β = 1



Acolbifene



111

Scheme 4.31

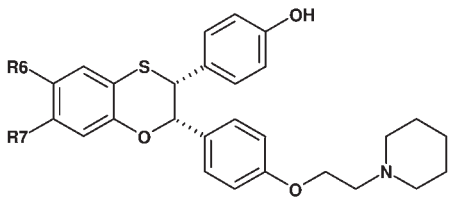
Acolbifene The best-studied flavanoid-type ER antagonist is acolbifene (EM-652), identified by Universite Laval in Quebec. This ER ligand shows very high binding affinity, but did not display significant ER subtype selectivity. Although most if not all SERMs display some degree of partial agonism, acolbifene is claimed to behave as a pure antagonist, a property shared by fulvestrant and related compounds. For the latter group ER downregulation has been described as the mechanism of action, but for acolbifene no mechanistic explanation is available. Development has been concentrated on its dipivaloyl prodrug Sch-57050 (EM-800). Sch-57050 has reached phase III clinical trials for breast cancer in a collaboration between Endorecherche and Schering-Plough. Further development has been discontinued. Schering disclosed closely related 4-fluoroalkyl-2*H*-benzopyrans as ER antagonists (e.g. **111**) [142]. No biological data were given.

Benzoxathiins In a series of 14 papers, Merck reported on the exploration of flavanoid-type SERMS leading to the benzoxathiin class of ERα-selective ligands. As mentioned earlier, compound **110** resulting from removal of the carbonyl in **109** showed enhanced receptor affinity, but at the cost of ERα selectivity. However, it was found that replacement of the methylene in **110** by sulfur preserved ERα affinity, but strongly reduced ERβ affinity resulting in a significantly increased ERα selectivity [143]. Thus, benzoxathiin **112** showed 28-fold ERα selectivity. Again, the *cis*-orientation of the 2,3-aryl groups was found essential. This result is in agreement with the hypothesis that incorporation of the larger, more polar group such as carbonyl or sulfur brings about an unfavorable steric and electronic interaction with Met354 in ERβ, which is not the case with Leu384 in ERα. Further ERα selectivity enhancement was obtained by relocating the 7-OH to the 6-position to give **113**. Interestingly, compound **113** had a much better oral bioavailability than 7-OH derivative **112**. Enantiomeric separation of racemic **112** gave the eutomer **113D** and distomer **113L**. The *cis*-2*S*,3*R* enantiomer **113D** not only showed a much higher ERα affinity and selectivity, it also showed a better oral bioavailability. The postulated binding mode was confirmed by X-ray analysis of the crystal structure of **113D** with ERα LBD. An extensive investigation towards the SAR of the benzoxathiin structure was performed. Thus, replacement of the 3-*p*-hydroxyphenyl group [144] and exploration of substituents effects at the 5, 7 and 8 position [145] and at the 2-aryl [146] was performed, but did not lead to improved molecular properties with respect to **113D**. In the cause of the investigations it was found that lead structure **113** was sensitive towards oxidative metabolism with subsequent formation of protein adducts. A reactive iminium ion resulting from oxidation of the piperidine side-chain was considered a major contributor to adduct formation. Therefore, Merck introduced a cyanide adduct assay as a surrogate measure of protein adduct formation. An in-depth study was performed to the BSC [147, 148]. Many amine variants were explored. It was found that pyrrolidines were less sensitive towards formation of adducts as a consequence of oxidative metabolism, but all of these compounds were deselected because of undesirable uterine profiles.

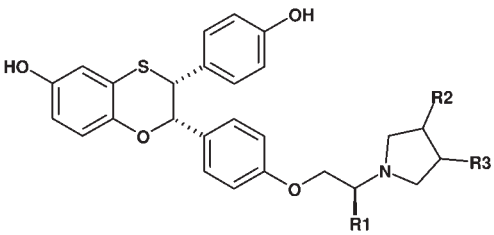
In a further study the prospects of a series of alkylated pyrrolidine side-chains was explored [149]. All compounds prepared showed good binding affinity and ERα

selectivity. It was found that the size, position and stereochemistry of the alkyl substituent had a major impact on the biological activity in uterine tissue. Whereas the 3*S*-methyl substituted analog exhibited partial agonism in uterine tissue, the 3*R*-methyl analog **114** behaved as a full antagonist. A similar positive exception was 2*S*,3*R*-dimethyl analog **115**. This result could be explained by X-ray analysis. The disposition of the methyl groups in **115** allows a more favorable hydrophobic interaction of the pyrrolidine with the H12 residues L536 and L539 as compared to the isomeric 2*R*,3*S* analog. As a result of the less favorable interactions in the 2*R*,3*S* analog, H12 is less effectively stabilized into the antagonist conformation, leading to partial agonism. Incorporation of substituents in the ethyl chain resulted in worsening of the uterine profile. However, a positive exception was **116** which behaved as a full antagonist. Combination of ethyl chain and pyrrolidine methylation led to the **117** [150]. This compound showed 40-fold ERα selectivity and full ERα antagonism in the immature rat uterine weight assay. Attachment of fluorine at C5 (**118**) further increased receptor affinity and ER antagonism on MCF-7 proliferation, but reduced ERα selectivity to only 7-fold.

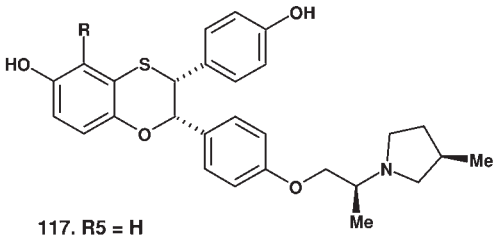
From the benzoxathiins reported thus far, **117** exhibits good prospects for further development. The optimized BSC was also tested on other SERM-type ligands, including raloxifene, bazedoxifene and lasofoxifene [151]. Although some increase in ER affinity was observed, no improvement in the rat uterine profile was observed. This result indicates that the optimized side-chain from the benzoxathiins series is not easily transferable to other estrogen ligands.



112. R6 = H, R7 = OH
$ER\alpha_{Bin}$: IC_{50} = 1.6 nM; α/β = 28
113D. R6 = OH, R7 = H
$ER\alpha_{Bin}$: IC_{50} = 0.8 nM; α/β = 56



114. R1 = R2 = H, R3 = *R*-Me
$ER\alpha_{Bin}$: IC_{50} = 1.6 nM; α/β = 54
115. R1 = H R2 = *S*-Me, R3 = *R*-Me
$ER\alpha_{Bin}$: IC_{50} = 0.5 nM; α/β = 74
116. R1 = Me, R2 = R3 = H
$ER\alpha_{Bin}$: IC_{50} = 1.3 nM; α/β = 35



117. R5 = H
$ER\alpha_{Bin}$: IC_{50} = 1.3 nM; α/β = 40
118. R5 = F
$ER\alpha_{Bin}$: IC_{50} = 0.8 nM; α/β = 7

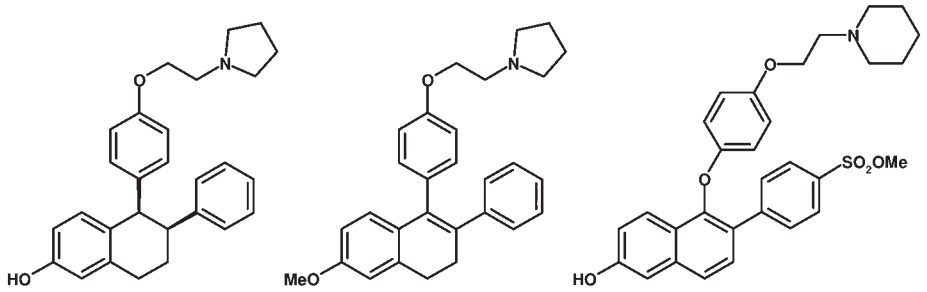
Scheme 4.32

4.2.3.6 1,2-Diphenyl-Tetraline-Type ER Ligands

Tetralin Analogs: Lasofoxifene The most advanced representative from this category is lasofoxifene (Oporia™, CP-336,156) developed by Pfizer, under license from Ligand [152–154]. This saturated 1*R*,2*S* form of Nafoxidine is a potent SERM-type antagonist, binds to ERα and ERβ with high affinity [155] ($ER\alpha_{Bin}$: $IC_{50} = 0.5$ nM; $ER\beta_{Bin}$: $IC_{50} \sim 1.2$ nM), but is devoid of subtype selectivity. Although the *trans* isomer expressed almost equal ERα affinity, the oral bioavailability of lasofoxifene was much better ($F = 60$ versus 11%) and also much better than raloxifene ($F = 2\%$) [156]. The markedly improved oral bioavailability of lasofoxifene is attributed to resistance to intestinal wall glucuronidation, as evidenced by the low levels of the glucuronide conjugate of lasofoxifene detected in the portal vein after oral dosing. The nonplanar conformation of lasofoxifene is considered a major contributing factor as it was reported earlier that planar phenols are more readily glucuronidated as their nonplanar counterparts. Lasofoxifene entered phase III clinical trials for osteoporosis, but in 2004 a nonapprovable letter was issued by the FDA for undisclosed reasons. Pfizer announced to resubmit a NDA at the end of 2007. Furthermore, lasofoxifene has been under clinical evaluation for treatment of urogenital atrophy, but again a nonapprovable letter was issued by the FDA in 2006 for this indication.

Naphthalenes Lilly described 2-phenyl-naphthalenes decorated with an arzoxifene-type BSC as novel SERM-type ligands for the potential treatment of leiomyomas (uterine fibroids) [157]. Since uterine fibroids show strong estrogen dependence, treatment with ER antagonists might be a viable alternative to gonadotropin-releasing hormone agonists. However, accumulation of ER antagonists into brain may result into inhibition of the hypothalamic–pituitary–ovarian (HPO) axis that, as a consequence of raising LH and FSH levels, is considered to increase peripheral 17βE2 levels. Elevated 17βE2 levels may compromise the fibroid growth-inhibitory efficacy of ER antagonists and may also lead to hyperstimulation of the ovary. The authors reasoned that preferred compounds should show little impact on the HPO axis. Polar surface area (PSA) and *c*log*P* were considered critical molecular descriptors to predict brain penetration. A variety of analogs decorated with polar and less polar substituents was made. Uterine wet weight was used as a measure of peripheral ER antagonism while serum 17βE2 levels were used to assess central ER antagonism. Some correlation between molecular descriptors and brain penetration was noted. Compound **119** showed a favorable brain to plasma ratio of 1 : 6, and also showed a good balance between *in vivo* efficacy and circulating 17βE2 levels and could be of interest for further development. Benzothiophene analogs with polar substituents were also investigated.

Napthylenes Nafoxidine is one of the earliest discovered SERM-type ER ligands. This ER ligand was discovered by Upjohn in the early 1960s and has been subjected to extensive clinical evaluation. However, the compound turned out to be far too toxic for general clinical use [158].



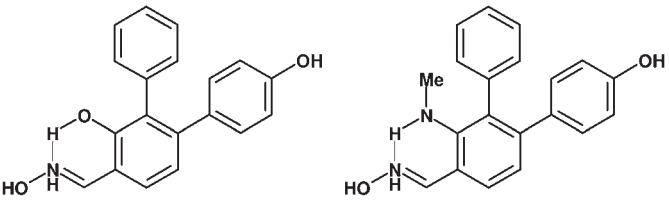
Lasofoxifene
ERα Bin : IC50 = 0.5 nM; α/β = 2.4

Nafoxidine

119. ERα Bin : Ki = 0.47 nM; α/β = 3
ERα Ant : IC50 = 10.7 nM

Scheme 4.33

Anthranylaldoximes Estrogenic activity of the diarylsalicylaldoxime core was studied at the University of Illinois [159]. As these structures form a six-membered pseudocycle by intramolecular hydrogen bonding between the phenol and the adjacent oxime these compounds are of interest as the pseudocycle may function as a bioisosteric replacement of the phenolic A-ring of estradiol. Although the receptor binding activity was modest, proof of concept was obtained. No selectivity for either ER was noted. An increase in binding affinity was observed by attaching a *p*-hydroxyl to one of the phenyl groups (**120**), but simultaneous attachment of hydroxyl groups to both phenyl groups abrogated binding affinity. Replacement of the oxygen by a nitrogen further increased ERα binding affinity [160]. Interestingly, these aniline aldoximes showed 5-fold selectivity for ERα. The most potent analog in this series is aldoxime **121**.



120. RBA ERα = 2.6%

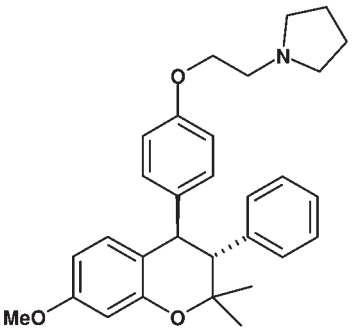
121. RBA ERα = 5.4%; α/β = 5

Scheme 4.34

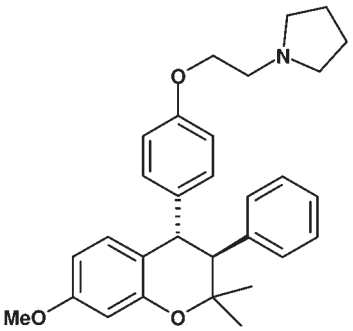
4.2.3.7 Oxygen and Sulfur Variants

Ormeloxifene and Levormeloxifene D/L-Ormeloxifene (Centchroman) was developed by the Central Drug Research Institute and is marketed in India as of 1988 as a once-a-week contraceptive under the trade name Saheli™ [161]. The L-enantiomer shows higher ER binding affinity (RBA = 16%) with respect to the corresponding D-enantiomer (RBA = 2%) and has a 33-fold higher antiimplantation activity. No ER subtype selectivity has been reported. The major metabolite of Centchroman is

the 7-desmethyl derivative (DMC), resulting from oxidative removal of the methoxy group. DMC has a higher affinity to ER and is probably the active entity [162]. Ormeloxifene is also marketed as a treatment for dysfunctional uterine bleeding in the same country. The L-enantiomer levormeloxifene has been under investigation by Novo Nordisk. The compound reached phase III clinical trials for osteoporosis, but development was discontinued in 1998 due to substantially higher occurrences of urinary incontinence and utero-vaginal prolapse in the levormeloxifene treatment population, compared to placebo [163].



Ormeloxifene (Centchroman)



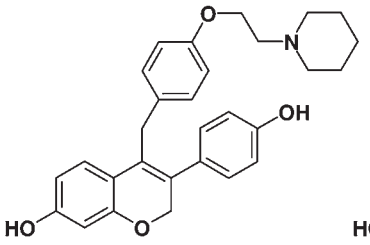
Levormeloxifene

Scheme 4.35

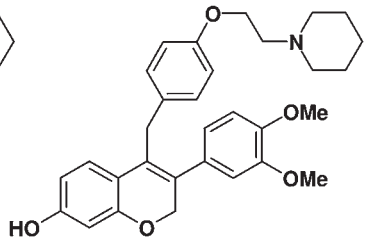
Chromenes Chiesi reported that the flavene **122** (CHF-4056) expressed 2- to 3-fold ERα selectivity [164]. Replacement of the 4′-OH by 3′,4′-dimethoxy substituents (**123**) slightly increased ERα affinity and also raised ERα selectivity to 22-fold. It was advocated that the 3′-OMe group cannot be accommodated properly in ERβ because Phe377 has a different orientation than the corresponding Phe425 in ERα. Although the compounds did not increase uterine weight gain, they only partial antagonized ethinyl-estradiol-induced weight increase.

Novo reported *cis*-diaryl-chromanes as high-affinity partial agonists for the ER [165]. From this series, **124** (NNC 45-0781) was selected for further development. This compound exhibits good ERα affinity ($IC_{50} = 2$ nM). No data on ERβ affinity were reported. Substituent effects at the 3-aryl were explored. It was found that *m*-OH- and *m*-Me-substituted analogs **125** and **126** greatly improved receptor binding affinity. However, *in vivo* comparison of **125** with **124** revealed the latter to express a much higher antiresorptive activity in an OVX-rat model. This deviation is most likely caused by poor oral bioavailability of **125**. Compound **124** has been selected for clinical development, but no progress has been reported as of 2002.

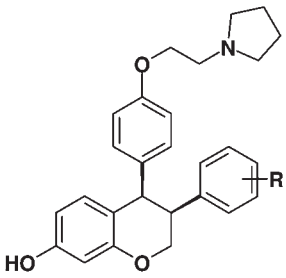
Benzopyranone derivatives were described by Celgene [166]. In this study, the effect of the BSC with and without a methylene hinge was investigated. Furthermore, SAR of the aminoalkoxy chain was studied. It was found that compounds having a methylene hinge showed higher ERα affinities and were more active in U2OS and MCF-7 cell-based assays. Furthermore, the traditional aminoethoxy chain was found



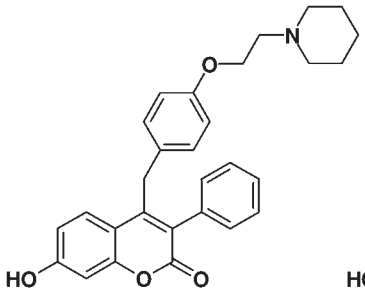
122 (CHF-4056):
$ER\alpha_{Bin}$: pK_i = 9.68; α/β = 2.5



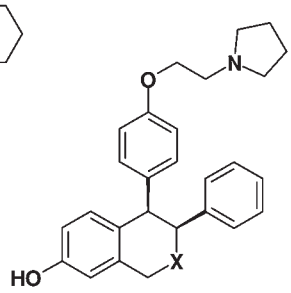
123. $ER\alpha_{Bin}$: pK_i = 10.15; α/β = 22



124 (NNC-45-0781): R = H
$ER\alpha_{Bin}$: IC_{50} = 2 nM
125: R = *m*-OH
$ER\alpha_{Bin}$: IC_{50} = 0.3 nM
126: R = *m*-Me
$ER\alpha_{Bin}$: IC_{50} = 0.04 nM



127 (SP-500263): $RBA_{ER\alpha}$ = 85%



128. X = O
$ER\alpha_{Bin}$: IC_{50} = 2.4 nM; α/β = 1.5
129. X = S
$ER\alpha_{Bin}$: IC_{50} = 0.6 nM; α/β = 3.5

Scheme 4.36

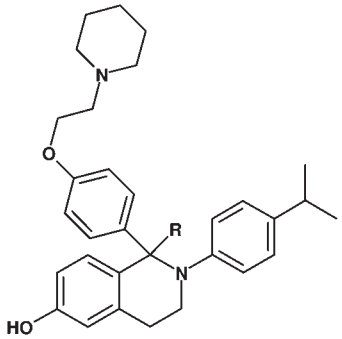
to be optimal. The best compound from the series was **127** (SP-500263). This compound not only showed high ERα binding affinity, but also effectively inhibited IL-6 release in U2OS cells and blocked MCF-7 proliferation. No ERβ binding data were reported. No information on further (pre)clinical development of **127** is available.

Isochromanes and Isothiochromanes Isochromane and isothiochromane analogs of lasofoxifene were investigated by Merck [167]. The compounds (e.g. **128** and **129**) showed high affinity to the ERs, but no significant subtype selectivity was observed. Although the compounds potently inhibited MCF-7 proliferation, partial agonism in the immature rat uterine weight assay was noted, rendering the compounds less suitable for further development.

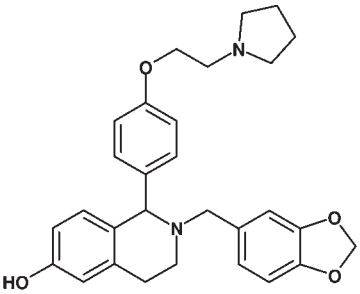
4.2.3.8 Nitrogen Variants

Tetrahydroquinolines and Tetrahydroisoquinolines Various tetrahydroquinoline and tetrahydroisoquinoline variants of lasofoxifene have been investigated. Novartis reported ERα-selective tetrahydroisoquinolines. The archetype structure showed good affinity and 16-fold selectivity for ERα. Introduction of a methyl group at C1 slightly lowered ERα selectivity, but increased transcriptional activity in HeLa cells

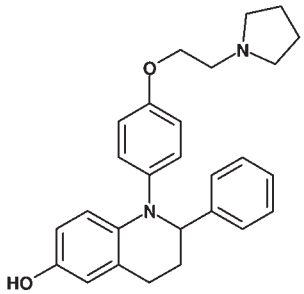
and inhibitory activity on MCF-7 proliferation. ERα selectivity could be raised to 50-fold by introducing an isopropyl group at the 4′-position (**130**). Apparently, ERβ does not readily accommodate the 4′-isopropyl group. Compound **130** showed acceptable oral bioavailability (F = 23%). Better oral bioavailability (F = 56%), but lower ERα selectivity was observed for a 4′-F analog. No *in vivo* data on tissue selective effects are available.



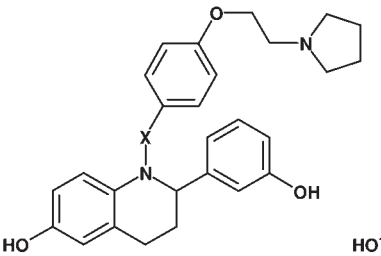
130. $ER\alpha_{Bin}$: IC_{50} = 71 nM; α/β = 52



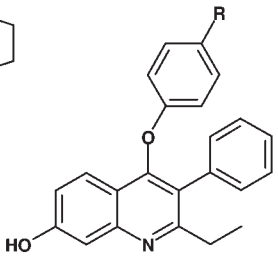
131. $ER\alpha_{Bin}$: IC_{50} = 9.2 nM; α/β = 24



132. $ER\alpha_{Bin}$: IC_{50} = 0.9 nM; α/β = 4



133. X = CO
$ER\alpha_{Bin}$: IC_{50} = 37 nM; α/β = > 27
134. X = CH_2
$ER\alpha_{Bin}$: IC_{50} = 0.6 nM; α/β = 9



135. R = CH=CHCONHiPr
$ER\alpha_{Bin}$: K_i = 10 nM; α/β ~ 1
136. R = $OCH_2CH_2NMe_2$
$ER\alpha_{Bin}$: K_i = 10 nM; α/β ~ 1

Scheme 4.37

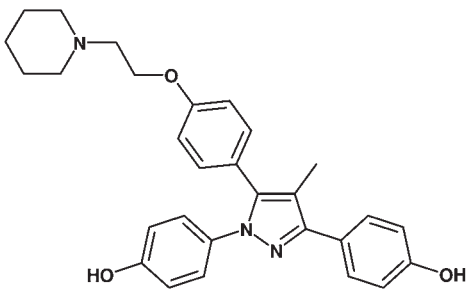
Other tetrahydroisoquinolines were reported by Pfizer [168]. Overall, ER affinity was 10-fold lower than observed for lasofoxifene. Some analogs showed preference for ERα. In particular, compound **131** showed 24-fold ERα selectivity.

The tetrahydroquinoline scaffold was investigated by Lilly [169]. Good ERα affinity and 4-fold ERα selectivity was observed for compound **132**. However, poor MCF-7-inhibitory activity was noted. Introduction of a methylene hinge in the BSC (**134**) did not affect ERα affinity, but slightly increased ERα selectivity. A further increase to 27-fold ERα selectivity was observed when using a carbonyl hinge (**133**), but in this case ERα affinity was 6-fold lower. No improvement in MCF-7 inhibitory activity was observed, indicative for problematic cellular uptake or stability. For this reason, the tetrahydroquinoline series do not look promising.

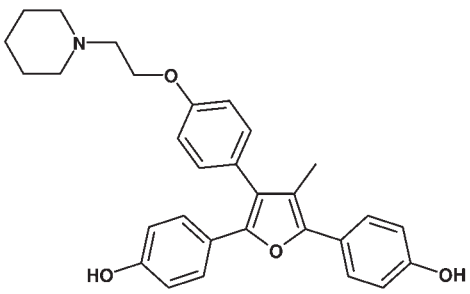
Using peptide interaction profiling, GlaxoSmithKline identified quinoline-based ligands for the ER that induced minimal Ishikawa cell stimulation [170]. The peptide interaction profile of **135** was distinct from that of 4-OH-tamoxifen and 17βE2, but comparable to the acrylic acid-containing tamoxifen analog GW-7604. The latter compound also shows minimal stimulation of Ishikawa cells. Apparently, the biological effect of estrogens decorated with an acrylic acid-based side-chain is independent from the scaffold. When the acrylamide side-chain in **135** was replaced by the classical aminoethoxy side-chain (**136**) the peptide interaction profile became similar to 4-OH-tamoxifen. Both compounds stimulated Ishikawa cell activity, which confirms that the peptide interaction profile has predictive power. Both compounds **135** and **136** showed proper binding affinity with ERα, but neither compound showed ER subtype selectivity.

4.2.3.9 Five- and Six-Membered Heterocycles

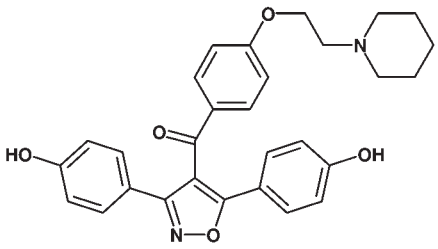
Pyrazoles and Isoxazoles Consistent with the finding that certain pyrazoles exhibit ERα-selective agonism, the University of Illinois reported that attachment of a BSC to these pyrazoles provided ERα-selective antagonists [171, 172]. Thus, 220-fold ERα selectivity was observed for pyrazole **137** (methyl-piperidino-pyrazole). It was found that the combination of a methyl group at C4 and piperidine as part of the BSC were key elements that direct ERα selectivity. Although larger alkyl groups had little effect



137 (MPP): $RBA_{ER\alpha}$= 11%; α/β = 220



138: $RBA_{ER\alpha}$= 32%; α/β = 67



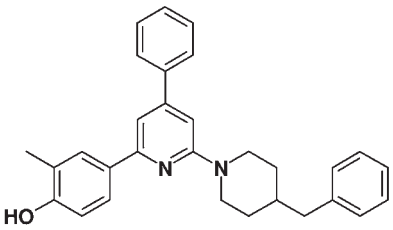
139. $ER\alpha_{Ant}$: IC_{50} = 40 nM; α/β > 50

Scheme 4.38

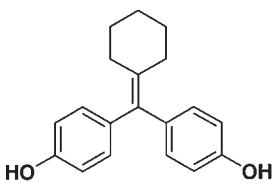
on ERα binding affinity, ERα selectivity was severely compromised. Replacement of the piperidine by other amines also had a negative effect on ERα selectivity, but in some cases ERα selectivity could be enhanced by replacing 4-methyl by 4-ethyl. Translation of the methyl/piperidine key elements to the furan scaffold also provided an ERα-selective antagonist [173]. Although ERα selectivity of **138** was somewhat less, ERα affinity was much higher. The antifertility potential of furans was described earlier, but at that time no subtype selectivity was disclosed [174]. Chiron reported that 2,5-diarylisoxazoles decorated with a BSC at the 4-position provided ERα antagonists [175, 176]. Compound **139** blocked transcriptional activity in CHO cells ($IC_{50} = 40$ nM) and proliferation of MCF-7 cells ($IC_{50} = 46$ nM).

Diarylpyridines 2-Amino-4,6-diarylpyridines were disclosed as novel ligands for ERs by GlaxoSmithKline [177]. In general, ER affinity was low, which is not surprising as the core is rather polar when compared to ligands known to bind to ERs with high affinity. However, one analog (**140**) was identified which showed proper ERα affinity and, in addition, 5-fold ERα selectivity. It was found that the *m*-methyl group and, in particular, the bulky benzyl-piperidine group contributed importantly to the affinity for ERα. Compound **140** is also known as 232802. The compound has entered phase I clinical trials in 2005 for the treatment of menopausal symptoms, including osteoporosis. So far no further development has been reported.

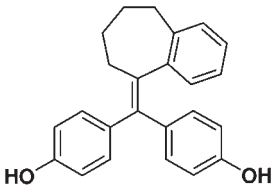
1,1-Diarylalkylidenes Cyclofenil (**141**) has been used as an alternative to Clomid™ to induce ovulation [178]. The compound shows high affinity for both ERs. The University of Illinois described a series of triarylethylidene bisphenol variants of cyclofenil [179]. High ER affinity was observed, but no significant ER subtype selectivity was noted. From the compounds prepared analog **142** showed the highest ERα affinity and minor selectivity for ERα.



140 (232802): $ER\alpha_{Bin}$: K_i = 20 nM; α/β = 5



141 (Cyclofenil): $RBA_{ER\alpha}$ = 152%; α/β = 0.6



142. $RBA_{ER\alpha}$ = 166%; α/β = 2.6

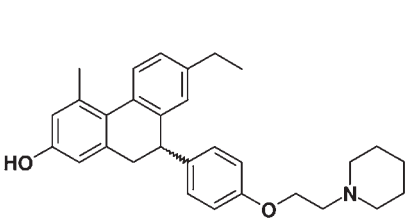
Scheme 4.39

4.2.3.10 Tri- and Tetracyclic SERMs

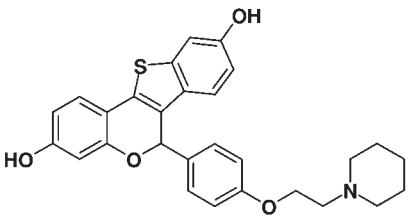
Phenantrenes SignalGene reported on phenantrenes as SERM-type ligands for the ERs [180]. Most of the phenantrenes disclosed showed very low affinity for either ER, but dihydrophenantrene analog **143** showed reasonable ERα affinity ($K_i = 9$ nM) and 22-fold ERα selectivity.

Tetracyclic Benzothiophenes and Benzanthracenes Lilly disclosed conformationally restricted raloxifene and benzopyrane analogs [181]. Both series yielded tetracyclic SERM-type estrogens that showed good affinity for ERα. Benzothiophene **144** and benzopyrane **145** effectively inhibited MCF-7 proliferation. Compound **145** (LY-357489) showed high efficacy on bone protection and cholesterol metabolism at doses as low at 10 mg/kg/day. No development has been reported.

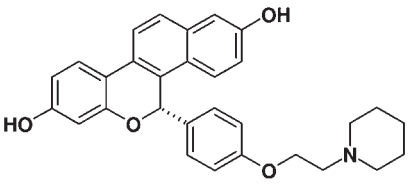
In another report, *N*-aryl-phenanthridine analogs were described [182]. Compound **146** exhibited good ERα affinity. Preliminary *in vivo* studies indicated that **146** behaved as a partial agonist in the uterus. In a more recent study, Lilly reported on the tetracyclic 5*H*-oxachrysen-2-ol LSN-2120310 (**147**) which is an antagonist in cell lines for breast cancer (MCF-7) and uterine cancer (Ishikawa) [183]. LSN-2120310 shows high binding affinity for both ERα and ERβ. The enantiomer **147-*S*** shows 10-fold selectivity for ERα, but affinity for ERα is 20-fold lower. The compound lowers cholesterol and maintains bone mineral density in an OVX-rat model. It is claimed that LSN-2120310 hold promise as a treatment of hot flashes because the compound showed good efficacy in a morphine dependent rat model of hot flash. Unfortunately, no comparative data for other SERMs and no pharmacokinetic data were presented in this study, making it difficult to fully assess its therapeutic potential. Tetracyclic chromene derivatives were described as SERMs by Johnson & Johnson [184]. While these compounds exhibit the typical SERM effects on bone metabolism, the added value claimed was their usefulness to alleviate hot flashes and to increase vaginal fluidity in postmenopausal women. The scientists reasoned that the ideal SERM should have a conformation in the C/D region similar to that of natural 17βE2. For this reason the bis-benzopyrane **148** was prepared. As this compound was found to be instable upon storage in solution it was stored as the dipivaloyl ester. ERα receptor binding of the *S*-enantiomer was 3 times higher than raloxifene, while the *R*-enantiomer showed 30-fold less affinity. Compound **148-*S*** did not show stimulatory activity in MCF7 cells. In the OVX-rat model the compound behaved comparable to raloxifene on bone metabolism, uterine weight, epithelial cell height and serum total cholesterol levels upon oral administration. However, **148-*S*** increased the production of vaginal fluid in the OVX-rat model, while raloxifene showed no effect. The morphine addiction model was used to assess the potential for treatment of hot flashes. Thus, following morphine withdrawal compound **148-*S*** prevented the increase in tail temperature, whereas raloxifene had no effect. Although the benefit of a resemblance with 17βE2 in the C/D region has not been proven, the outcome of the investigations suggests therapeutic promise for SERM-type compounds in alleviating climacteric symptoms in postmenopausal women.



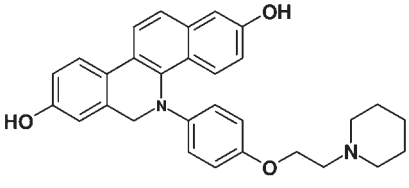
143: ERα $_{Bin}$: K_i = 9 nM; α/β = 22



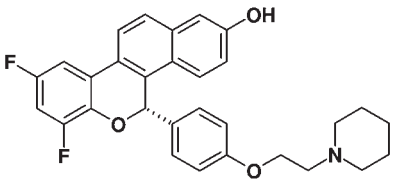
144: RBA $_{ERα}$ = 23%



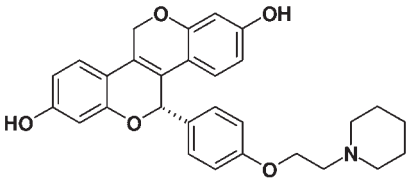
145: RBA $_{ERα}$ = 22%



146: RBA $_{ERα}$ = 24%



147-*R* (LSN-2120310)
ERα $_{Bin}$: K_i = 0.3 nM; α/β = 1.7
147-*S* ERα $_{Bin}$: K_i = 5.8 nM; α/β = 9



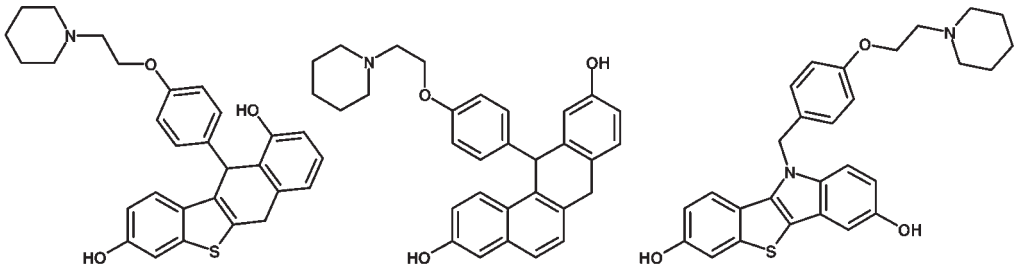
148-*S*: ERα $_{Bin}$: IC_{50} = 0.61 nM; α/β = 2

Scheme 4.40

Lilly also disclosed two series of conformationally restricted benzanthracene derivatives [185]. These tetracyclic compounds showed high ERα affinity, but subtype selectivity was modest. Thus, benzothiophene analog **149** expressed 5-fold preference for ERα and similar ERα selectivity was noted for benzanthracene analog **150**. The latter compound showed high MCF-7 antagonistic activity, whereas analog **149** was 20-fold less active.

The Shanghai Institute for Biological Sciences disclosed the estrogenic activity of SERM-type benzothieno[3,2-*b*]indole derivatives [186]. Various amines were examined. From the series prepared, piperidine-substituted analog **151** displayed the highest ERα affinity. No significant subtype selectivity was noted. Transactivation data were reported showing that **151** is slightly less active than raloxifene.

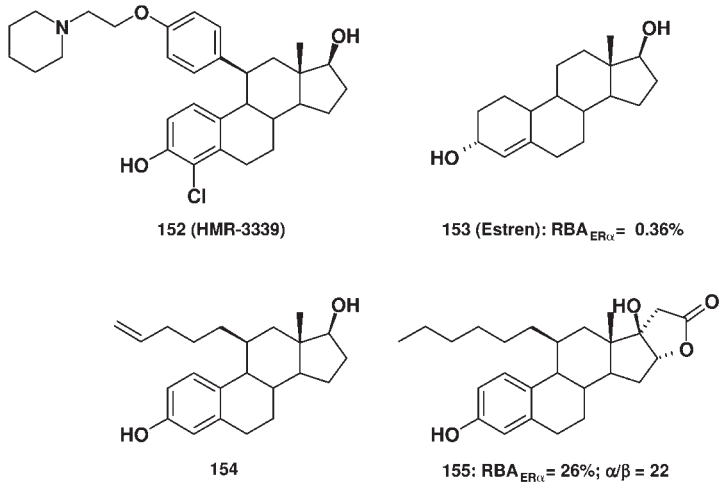
Steroidal SERM-Type Antagonists Several steroidal ER antagonists have been described in the literature. It was established that attachment of a side-chain to the 11- or 7-position of 17βE2 or 17β-ethinyl-estradiol converts the molecules from agonists into antagonists. Aventis Pharma (formerly Hoechst Marion Roussel) disclosed 17βE2 and 17β-ethinyl-estradiol analogs having a SERM-type BSC attached to the



149. ERα$_{Bin}$: K_i = 2.6 nM; α/β = 5.4 150. ERα$_{Bin}$: K_i = 0.8 nM; α/β = 5.2 151: ERα$_{Bin}$: IC_{50} = 1.5 nM; α/β = 2.7

Scheme 4.41

11-position. The 4-chloro analog **152** (HMR-3339) reached phase IIb clinical development [187], but was cancelled due to adverse effects on endometrial tissue [188]. Aventis announced development of another steroidal SERM (PSK-3471) which originates from its spin-off company ProSkelia. The structure of PSK-3471 has not been disclosed as yet, but suggestions were made that the compound might be **153** (estren), a prohormone that activates the AR to regulate both androgenic and estrogenic transcriptional responses by osteoblasts [189]. However, a recent study in which PSK-3471 was compared to α- and β-estren showed that unlike PSK-3471, the estrens induced reproductive organ hypertrophy in both male and female mice,



152 (HMR-3339) 153 (Estren): RBA$_{ER\alpha}$= 0.36%

154 155: RBA$_{ER\alpha}$= 26%; α/β = 22

Scheme 4.42

and enhanced MCF-7 cell proliferation *in vitro* [190]. Organon disclosed a series of 11β-alkyl-17βE2 derivatives [191]. It was reported that compounds having an alkyl, alkenyl (e.g. **154**) or alkynyl chain of five atoms showed a dual-profile character, and behaved as agonists for ERα and antagonists for ERβ. Scientists from Yale University prepared 11β-carboxylates, ketones, amides, ethers and thionoesters [192]. Whereas

amides showed low receptor affinity, all other compounds bound well to the ERs and behaved as antagonists for both ERα and ERβ when the chain length was five or more atoms. A dual-profile character was not observed for these compounds, suggesting that the steroids decorated with 11β-heteroatom-containing chains bind markedly different from steroids containing 11β-alkyl chains. Schering earlier disclosed the 16α,17α-lactone analog of 17βE2 (**9**; 16α-LE2) as an ERα-selective agonist. Attachment of an 11β chain switched the profile from ERα-selective agonism into ERα-selective antagonism [193]. Good ERα affinity and 22-fold ERα selectivity were reported for 11β-hexyl analog **155**.

4.2.4 ERβ-Selective Antagonists

Although no therapeutic potential for ERβ-selective antagonists has been established, the availability of ERβ antagonists is still highly desirable as tools to further untangle the biological role of ERβ. To date, very few ERβ-selective antagonists have been described.

4.2.4.1 Benzofluorenes

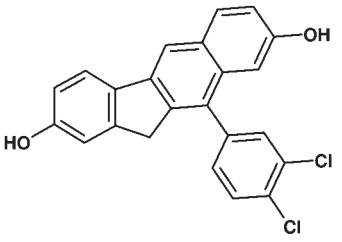
Organon disclosed a series of 10-aryl-benzo[*b*]fluorenes that showed ERβ-selective antagonism in a CHO cell-based transactivation assay [194]. The series originated from benzo[*b*]fluorenes earlier described as ERβ-selective agonists. The introduction of an aryl at C10 (e.g. **156**) appeared to switch the profile into ERβ-selective antagonism, despite the absence of a BSC. Although the compounds behaved as full antagonists for ERβ, they showed partial agonism for ERα. Introduction of a BSC *meta* to the 10-aryl moiety (e.g. **157**) afforded ERβ-selective antagonists that showed high inhibitory activity of estradiol-induced gene transactivation and did not induce ERα partial agonism. Several of these compounds were more active as the steroidal antiestrogen ICI-168.384 and showed over 30-fold ERβ selectivity.

4.2.4.2 Triazines

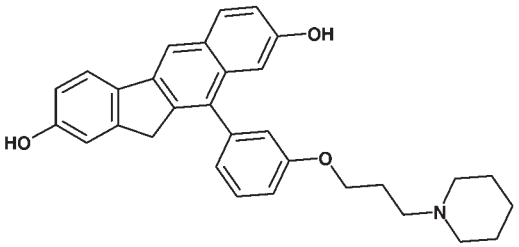
GlaxoSmithKline disclosed 1,3,5-triazines as ERβ-selective antagonists [195]. Twenty-five-fold ERβ selectivity was observed for triazine **158**. X-ray analysis was performed with analog **159**, cocrystallized with ERβ LBD (PDB code: 1NDE). The phenolic ring appeared to mimic the A-ring of estradiol. The *p*-chlorophenyl group was directed towards Ile373. This orientation may explain the ERβ selectivity observed for the triazine series as a similar allocation in ERα is more difficult due to the presence of Met421 at the same position.

4.2.4.3 Steroids

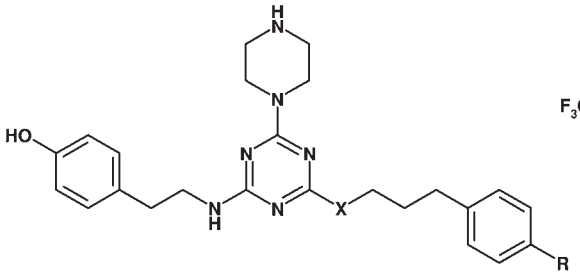
Steroidal ERβ antagonists were described by Schering [196]. Starting from their ERβ agonist 8βVE2 it was possible to switch the profile towards antagonism by introducing an alkyl chain at position 11β. Various alkyl chains containing fluorine or amines were investigated. In a cell-based transactivation assay good inhibition of transcriptional activity was observed for **160** having an 11β-trifluoro-2-hydroxyhexyl chain. In addition, **160** showed 62-fold ERβ selectivity.



156: $RTA_{ER\alpha\ Ant}$ > 40% (ICI-164.384)

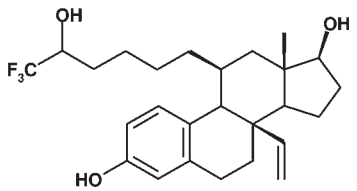


157: $RTA_{ER\alpha\ Ant}$ > 100% (ICI-164.384); β/α > 30



158. X = NH, R = Cl
$ER\alpha_{Bin}$: IC_{50} = 15 nM; α/β = 25

159. X = S, R = F
$ER\alpha_{Bin}$: IC_{50} = 15 nM; α/β = 10

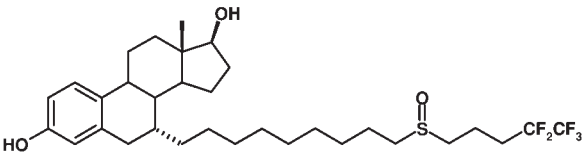


160: $RTA_{ER\alpha\ Ant}$ = 50% (Fulvestrant); β/α = 62

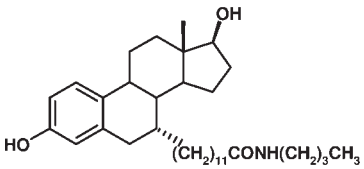
Scheme 4.43

4.2.5
Full Antagonists

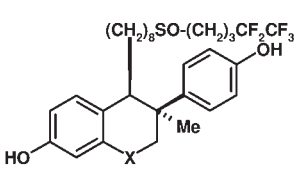
Tamoxifen is known as a partial agonist that suffers from adverse effects due to stimulation of endometrial tissue. Other SERM-type estrogens such as raloxifene, lasofoxifene and bazedoxifene also show partial agonism, but with a much lower intrinsic activity. So-called full antagonists such ICI-182.780, ICI-164.384 and ZM-189.154 are devoid of residual agonism, and do not exhibit any stimulation of uterine tissue. The most advanced full antagonist is ICI-182.780 (fulvestrant, Faslodex™) which was introduced to the market by AstraZeneca in 2002 as a second-line treatment of hormone receptor-positive metastatic breast cancer in postmenopausal women with disease progression following antiestrogen therapy [197–199]. In a phase III study fulvestrant was at least as effective as the aromatase inhibitor anastrozole in patients that do not respond to or relapsed from tamoxifen therapy, but gave fewer side-effects. Fulvestrant is administered as a once a month intramuscular injection and has been reported to induce a lower incidence of hot flashes when compared to tamoxifen. Fulvestrant has multiple effects on ER signaling, including inhibition of dimerization and nuclear localization, and downregulation of cellular ER levels [200]. However, the molecular mechanism through which fulvestrant acts differently from SERM-type estrogens has long been a mystery. In 2001, X-ray



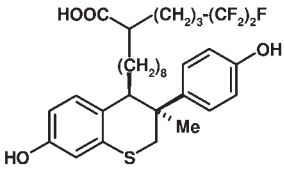
Fulvestrant: RBA$_{ER\alpha}$= 138%



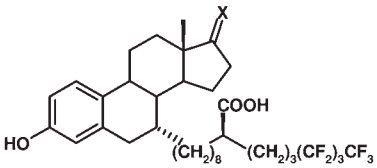
ICI-164.384: RBA$_{ER\alpha}$= 12%



ZM-189,154 (X = CH_2): RBA$_{ER\alpha}$= 169%
161 (X = O): RBA$_{ER\alpha}$= 150%
162 (X = S): RBA$_{ER\alpha}$= 230%



163: RBA$_{ER\alpha}$= 0.8%



164 (X = H,OH): RBA$_{ER\alpha}$= 1.1%
165 (CH4893237; X = O): RBA$_{ER\alpha}$= 1.0%

Scheme 4.44

crystallography data of the closely related full ER antagonist ICI-164.384 in complex with ERα LBD were reported which gave a unique insight into the binding mode of this class of full antagonists. It was found that ICI-164.384 is flipped 180° around the 3OH–17OH axis when compared to the crystal structure of 17βE2 [201]. This flip positions the 7α substituent towards the 11β region such that the long side-chain can exit the binding cavity. The amide side-chain protrudes from the LBD and the hydrophobic terminal chain binds along the coactivator binding site. As a consequence H12 is blocked to adopt both the agonist or AF-2-antagonist positions to the coactivator binding site that appeared from the X-rays from tamoxifen and raloxifene. Thus, the 7α-chain physically prevents productive alignment of H12. No ER subtype selectivity has been noted for any of the 7α-long-chain antagonists.

The long side-chains such as present in fulvestrant have also been applied to other ER scaffolds. Thus, AstraZeneca reported on tetrahydronaphthalene compound ZM-189.154 as an orally active full antiestrogen [202], but this compound did not reach clinical development. The structurally analogous chroman **161** and thiochroman **162** were also described as orally active full antiestrogens by Chugai [203, 204]. ERα receptor binding affinity for chroman **161** was comparable to fulvestrant, whereas affinity for thiochroman **162** was 2-fold higher. A variety of alternative side-chains was also examined. Of particular interest is **163**. While receptor binding activity is 150-fold lower than fulvestrant, **163** was found to exhibit outstanding oral antiestrogenic activity, superior to fulvestrant. It was established that this antiestrogenic activity was attributed to its high oral absorption and high metabolic stability. Attachment of the carboxyl-containing side-chain to the 7α-position of 17βE2 provided the pure antiestrogen **164** [205]. Although the relative binding affinity towards ERα was 100-fold lower than fulvestrant, its *in vivo* antiestrogenic efficacy upon oral administration was much higher. Compound **164** was found to be metabolically unstable and was rapidly

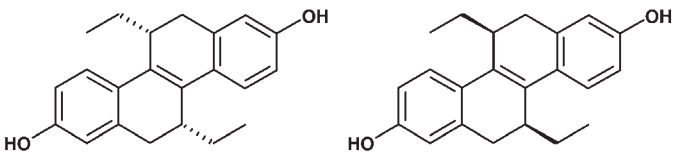
converted to the corresponding 17-keto derivative **165** (CH48932437). This estrone derivative exhibited almost identical receptor binding affinity and *in vivo* antiestrogenic activity. In addition, **165** showed downregulatory effects of ERα in MCF-7 cells and its oral antitumor activity was comparable to the maximum achievable antitumor activity of subcutaneously administered fulvestrant. The enhanced antiestrogenic activity of **165** with respect to orally dosed fulvestrant was attributed to a much better absorption from the intestinal wall and improved metabolic stability.

4.2.6 Dual-Profile Estrogens

In recent years, several ER ligands were reported to behave as full agonists for ERα, but at the same time as full antagonists for ERβ as measured in cellular transactivation assays. These ligands can, therefore, be regarded as 'dual-profile' estrogens. These compounds can be useful to clarify the biological role of the ER subtypes. No therapeutic potential has been ascribed to this category. Thus far, only one ER ligand has been reported that displays a reversed dual-profile character (i.e. agonism on ERβ and antagonism on ERα).

4.2.6.1 ERα Agonist/ERβ Antagonist

The University of Illinois reported that racemic *cis*-diethyl-substituted tetrahydrochrysene (THC) shows 10-fold selectivity for ERβ in a receptor-binding assay [206, 207]. In a HEC-1 cellular assay THC was a full ERα agonist, but did not show transcriptional activation through ERβ. As THC fully suppressed 17βE2-stimulated transcriptional activity via ERβ, the compound acts as an ERβ antagonist and at the same time as an ERα agonist. This profile is characteristic for 'dual-profile' estrogens that apparently express opposite effects on ERα and ERβ in cellular assays. It was found that the two enantiomers displayed different activities. Thus, ***R,R*-166** was an agonist on ERα and fully antagonized 17βE2 activation of ERβ. By contrast, ***S,S*-166** was an agonist on both ERα and ERβ. The *R,R* enantiomer showed a much better affinity for the ERs than the *S,S* enantiomer, but the latter showed a higher ERβ selectivity.



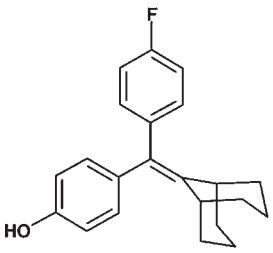
***R,R*-166**
$RBA_{ER\beta}$ = 144%
β/α = 6

***S,S*-166**
$RBA_{ER\beta}$ = 14%
β/α = 16

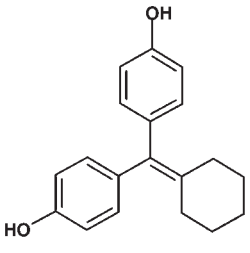
Scheme 4.45

Crystal structures were generated for ***R,R*-166** in complex with the LBDs of ERβ and ERα, and in the presence of a GRIP-1 cofactor peptide [208]. Although the ligand

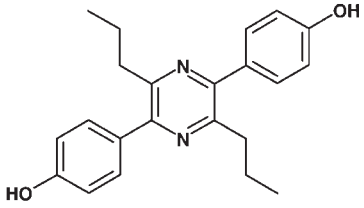
binds in the expected fashion in both ERα and ERβ LBDs, a major difference was observed for the position of H12. In the ERα structure, H12 is aligned over the binding pocket allowing interaction of the LXXLL motif of GRIP-1 with the hydrophobic cofactor recognition cleft, whereas in ERβ the binding of the LXXLL motif of GRIP-1 is blocked because H12 docks into the cofactor recognition groove. The latter orientation of H12 resembles that of raloxifene and tamoxifen. The dual-profile character was also observed for other ER ligands. The University of Illinois reported that 1,1-diarylethylenes expressed high binding affinity for both ERα and ERβ [209]. However, in a HEC-1 transactivation assay, analogs **167** and **168** showed partial agonism on ERα and full antagonism on ERβ. The double bond was found to be essential for the dual-profile behavior. The University of Illinois reported a similar profile for diaryl-pyrazines such as **169** [210]. Although the relative potency was low, **169** showed partial agonism on ERα and nearly full antagonism on ERβ. The 1,3- and 2,3-diarylindenes were reported by the University of California [211]. Aryl and alkyl substitution variants were attached to the 3-position of 2-*p*-hydroxyphenyl-indene. Using ERα and ERβ binding assays, no subtype selectivity was noted. However, in a U2OS cell-based transactivation assay it was found that compounds **170** and **171** showed ERα partial agonism and ERβ full antagonism, and as such behaved as dual-profile ligands.



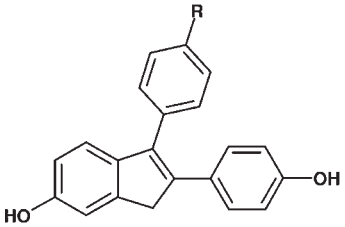
167: $RBA_{ER\beta}$ = 52%; β/α = 1.3
Efficacy in $HEC\text{-}1_{ER\alpha}$: 60%
Efficacy in $HEC\text{-}1_{ER\beta}$: 10%



168: $RBA_{ER\beta}$ = 334%; β/α = 4.9
Efficacy in $HEC\text{-}1_{ER\alpha}$: 25%
Efficacy in $HEC\text{-}1_{ER\beta}$: 2%

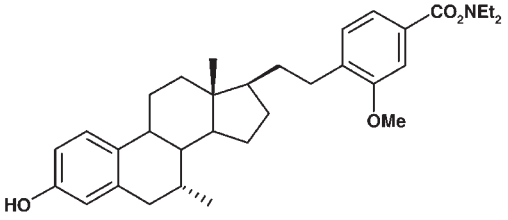


169



170: R = F
$ER\alpha_{Ago}$: K_i = 3.9 nM
$ER\beta_{Ant}$: K_i = 2.8 nM

171: R = Me
$ER\alpha_{Ago}$: K_i = 17 nM
$ER\beta_{Ant}$: K_i = 1.3 nM



TAS-108

Scheme 4.46

4.2.6.2 ERα Antagonist/ERβ Agonist

In contrast to the previous category, compounds that can be classified as ERα antagonist/ERβ agonist dual-profile ligands have been hard to find. In fact, only one compound has been disclosed that may fit in this category. Taiho, under license of SRI, is developing the steroidal full antagonist TAS-108 (SR-16234) for breast cancer. The compound is orally active and is currently in phase II clinical development for breast cancer [212]. TAS-108 shows good binding affinity for both ERα and ERβ ($IC_{50} = 11$ and 5.6 nM), and acts as a full ERα antagonist. In contrast, TAS-108 is a partial agonist for ERβ as the compound was found to induce recruitment of the coactivator TIF-2 to ERβ [213]. This remarkable profile is claimed to be specific for TAS-108 as no other ER antagonist shows a comparable profile. Unlike fulvestrant, TAS-108 does not result in downregulation of ERα and ERβ, but acts through modulation of cofactor recruitment. Whether this ERβ partial agonistic behavior brings additional therapeutic benefit with respect to the nonselective full antagonist fulvestrant remains to be established. Preliminary data suggest that TAS-108 prevents bone loss in the OVX-rat model.

4.3 Conclusions

The current repertoire of marketed synthetic ER modulators consists of six products. From these, three ER modulators are currently marketed as therapies to treat hormone-dependent breast cancer, whereas one ER modulator is on hand to treat vertebral osteoporosis. Many other synthetic estrogen ligands failed during clinical development. The use of these SERM-type ER ligands is not without shortcomings. Apart from the occurrence of therapy resistance, the use of tamoxifen is associated with a high frequency of hot flashes and vaginal atrophy, and increases the risk of developing endometrial cancer. Toremifene and raloxifene are also associated with, and increased incidence of, hot flashes and vaginal atrophy. Progress has been made with the design of SERMs that address undesired CNS antagonism leading to hot flashes. Evidence was presented that introduction of substituents, such as methanesulfonyl groups, increases the polar surface area, which is an important factor that limit blood–brain barrier passage. Also, spiro compounds and tetracyclic ligands were identified that show interesting effects in hot flash models. Fulvestrant fully antagonizes ER-mediated transcriptional activity. Fulvestrant induces hot flash to a lesser extend than other ER modulators, but its effect on bone metabolism and changes in the risk to develop fractures requires further study. The discovery of ERβ fueled the idea that subtype-selective estrogens could be more efficacious and/or minimize the occurrence of side-effects, and may show an enhanced level of tissue selectivity. Although the amino acids surrounding the ligand-binding cavities in both ERα and ERβ are very similar, it has shown to be possible to generate highly subtype-selective ER ligands. In particular, the design of ligands that target the two amino acids that are different have been highly successful and provided a broad range of highly selective ERβ agonists, some of which are orally active. X-ray analysis of a large number of different chemotypes have become available, and provided a much better understanding on the different binding modes

and gave insight how to improve on ERβ selectivity. However, the molecular basis for ERα selectivity is less clear. The larger ligand pocket in ERα is most probably an important factor that directs ERα selectivity. For the benzoxathiin series evidence was presented that the ring sulfur atom brings about an unfavorable interaction with Met354 in ERβ, which is not the case with Leu384 in ERα. Ten years after their discovery, only part of the biological function and interplay of the ERs has been deciphered. From the quadrant of subtype-selective estrogens, preclinical evidence suggests therapeutic potential for selective ERβ agonists. Thus, selective stimulation of ERβ was shown to hold promise as a therapy for inflammatory disorders and prostate hyperplasia, without induction of classical estrogenic effects. Two ERβ agonists entered clinical testing for inflammatory disorders. Other compounds are in preclinical development for benign prostate hyperplasia. Evidence has been generated that ERβ may exhibit antiproliferative activity. ERα selective SERM-type ER ligands have been identified but preclinical data have not yet indicated a therapeutic benefit with respect to nonselective SERMs. ERα-selective agonists and ERβ-selective antagonists were also discovered, but no therapeutic prospects for these categories have emerged as yet. On the whole, the promise of subtype-selective estrogens has partly been fulfilled. However, many questions still need to be answered. A large number of subtype-selective ligands have been disclosed over the past 7 years, some of which exhibit excellent receptor affinities and over 100-fold subtype selectivity. As cross-talk between the signaling pathways of both receptors has been demonstrated, an obvious question is whether ligands exhibiting this level of selectivity have a therapeutic advantage with respect to somewhat less selective ligands. The discovery of ERα-selective partial agonists has received significant attention. However, the identification of subtype-selective full antagonists is still at an early stage. Full antagonists represented by fulvestrant act through various mechanisms. Apart from antagonizing 17βE2-induced transcriptional activity, fulvestrant also blocks receptor dimerization and leads to ER downregulation. This multimechanism activity of fulvestrant is probably the key to its beneficial effects to treat tamoxifen-resistant breast cancer. However, fulvestrant also inhibits ERβ. Although the role of ERβ in breast cancer has not been fully established, there is reason to believe that compounds which do not interfere with the antiproliferative activity of ERβ may be more efficacious. As such, ERα-selective full antagonists could be of interest as a new generation of ER modulators to treat breast cancer. The first examples of such compounds have been reported, but no preclinical information is available as yet. From a scientific point of view, the category of dual-profile ER ligands is very intriguing. In particular, dual-profile ligands exhibiting full antagonism for ERα and agonism for ERβ can be predicted to express a dual antiproliferative activity. However, this category of ER ligands is still very much underrepresented. Only one example has been disclosed, but even this compound has not been sufficiently profiled to confirm it really fits in this grouping.

In the forthcoming years many questions will be answered and will give more insight into the full potential of subtype-selective estrogens. Further design of fine-tuned subtype-selective ligands is mandatory and will undoubtedly widen the therapeutic scope of ER modulators. Future evaluation of the different ligand classes in appropriate animal models may uncover additional clinical indications in which subtype-selective ER modulators offer therapeutic benefits.

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5
Estrogen Receptors as Therapeutic Targets in Breast Cancer

Eric A. Ariazi and V. Craig Jordan

5.1
Introduction

Breast cancer mortality has declined by 24% from 1990 to 2000, likely due to increases in the use of both mammography screening (followed by surgery) and adjuvant therapy, including chemotherapy and antihormonal tamoxifen (Figure 5.1) therapy [1]. Without screening and adjuvant therapy, it is estimated that deaths due to breast cancer would have risen by about 30% from 1975 to 2000. According to the consensus of seven models of reductions in the rate of death from breast cancer, decreases in mortality of 15% (median value) and 19% (median value) are due to mammography screening and adjuvant therapy, respectively. Endocrine therapy alone, most notably tamoxifen, a selective estrogen receptor (ER) modulator (SERM) that blocks estrogen action in breast cancer, is estimated to account for a 9.8% (median value) decrease in breast cancer mortality [1]. Additionally, the recent studies that connect hormone replacement therapy (HRT) with an elevation in breast cancer incidence has resulted in a fall by 8.6% in the annual age-adjusted breast cancer incidence from 2001 to 2004 as women chose to stop long-term HRT [2–4]. Still, the American Cancer Society estimates that in 2007, 178,480 American women will have been diagnosed with new cases of breast cancer and an estimated 40,460 women will have died from the disease, with only lung cancer being responsible for more women's cancer deaths [5]. Worldwide, it is anticipated that in the coming decade, 5 million women will be affected by breast cancer [6]. Clearly, further advances in the development of treatments, particularly ones with fewer undesirable side-effects, are necessary.

Seminal work conducted by Elwood Jensen and reported in 1962 demonstrated that estrogen target tissues, such as the uterus, vagina and pituitary gland retain tritiated 17β-estradiol (E_2) (Figure 5.2) administered subcutaneously to immature rats, while nontarget tissues, such as the kidney, liver and muscle, do not ([7, 8], reviewed in Ref. [9]). This selective retention proved the existence of an ER in the target tissues. The receptor was isolated as an extractable protein from the rat uterus

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

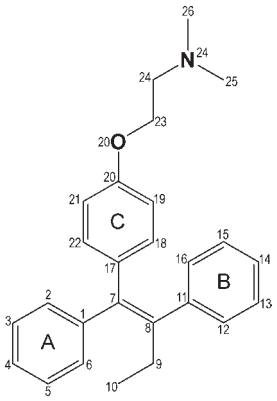

Figure 5.1 Tamoxifen. Atoms are numbered and rings are designated by letters.

by Toft and Gorski in 1966 [10]. Jensen [11] reasoned that if ER was present in a breast tumor, then this would increase the probability of a response to endocrine ablative therapy (oophorectomy, adrenalectomy, hypophysectomy). Indeed, approximately 75% of breast cancers are positive for ER expression, for which the routine testing is used to predict response to antihormonal therapy [12].

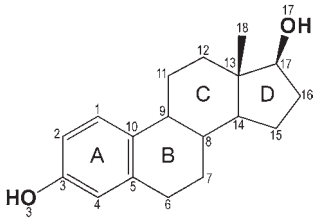

Figure 5.2 E_2. Atoms are numbered and rings are designated by letters.

ER was cloned and sequenced from MCF-7 human breast cancer cells 20 years after its purification in 1986 [13, 14]. This ER has since been renamed ERα (ESR1, NR3A1) (Figure 5.3), due to the cloning of a second ER in 1996, ERβ (ESR2, NR3A2) (Figure 5.3), from a rat prostate cDNA library based on its sequence similarity to ERα [15]. Human ERβ was subsequently cloned from a testis cDNA library [16]. ERs, member of the steroid/thyroid hormone nuclear receptor superfamily, bind estrogens with high affinity and regulate transcription in an estrogen-dependent manner (reviewed in Refs [17–24]). While the classical genomic function of the receptor has been understood for some time, emerging evidence suggests that the receptor participates in a broader range of biological activities, including cross-talk with other signal transduction pathways. ERα is the predominant ER expressed in breast cancer [25, 26] and the clinical significance of ERβ in breast cancer remains unclear (reviewed in Refs [23, 27, 28]). Hence, if not specified, ER refers to ERα.

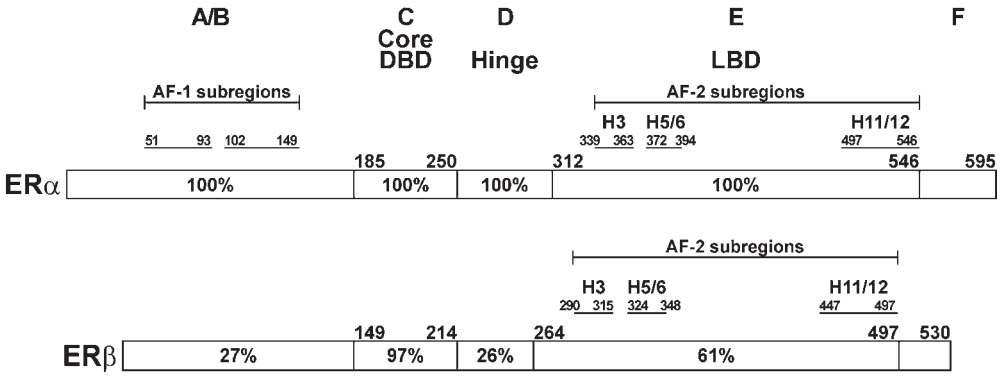

Figure 5.3 Domain structure and sequence identity between ERα and ERβ. Regions were subdivided into the A/B region (N-terminus), core DBD (C region), D region (hinge), LBD (E region) and F region (extra C-terminus). Comparisons were made using the Gap program (Genetics Computer Group). Numbers correspond to amino acids at the ends of the indicated region. Percentages in the bars correspond to the amino acid sequence identities. Open regions indicate no significant sequence identity. AF-1 subregions were defined according to mutant analysis [35]. The core DBD boundaries correspond to the 66 amino acids which define the zinc fingers responsible for sequence-specific DNA interactions. The LBD boundaries correspond to the α-helices H2–H12 and a single hairpin β-sheet defined by the 1ERE crystal structure of human ERα complexed with E_2 [42] and by the 1X7J crystal structure of human ERβ complexed with genistein [46]. The surface of AF-2 corresponds to the coactivator recognition groove formed by H3, H5/6, H11 and H12, which are defined according to ERα and ERβ crystal structures [42, 46].

The functional significance of ER in breast cancer has made it the foremost target, either directly or indirectly, for the development of antihormonal therapies aimed at the prevention and treatment of this disease. SERMs such as tamoxifen have been used to treat breast cancer successfully in the US since the late 1970s [29] and raloxifene (Figure 5.4), a related SERM, has completed testing as a chemopreventive against

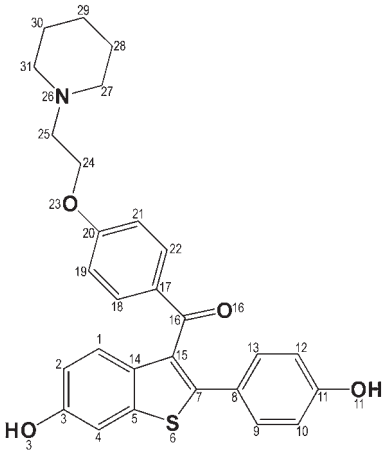

Figure 5.4 Raloxifene. Atoms are numbered.

tamoxifen in the Study of Tamoxifen and Raloxifene (STAR) [30]. We discuss these and the pure antiestrogen fulvestrant currently used for the treatment of breast cancer.

5.2 Biology of ERs

5.2.1 ERα and its Transcriptional Activation

The ERα gene is located on chromosome 6q25.1 and encodes a 595-amino-acid, 66-kDa protein [31] composed of six functional regions (Figure 5.3) [32, 33]. The N-terminal A/B region contains the ligand-independent and functionally minor activating function (AF)-1 domain [34–36]. The C region, or the DNA-binding domain (DBD), consists of a 66-amino-acid motif that forms two structures termed zing fingers that interact with DNA, thereby mediating the receptor's sequence specific binding to estrogen response elements (EREs) found in the promoters of estrogen-responsive genes [32, 33, 37, 38]. The D region, or hinge domain, contains the nuclear localization signal and interacts with heat shock factors. The E region, or ligand-binding domain (LBD), interacts with E_2 (Figure 5.2) as well as a diverse array of other compounds, and overlaps with the ligand-dependent and functionally major transcriptional AF-2 domain [23, 34, 36, 39–41]. The LBD consists of α-helices H2–H12 and a single hairpin β-sheet that, when complexed with E_2, forms a canonical three-layered antiparallel α-helical sandwich structure similar to that observed in other nuclear receptors [42–46]. The C-terminal region of the receptor is termed the F domain and inhibits dimerization of the receptor until it is bound by ligand [47]. In women, ERα is expressed in the mammary gland, uterus, vagina, ovary, bone, brain, cardiovascular system and liver [22].

ER's classic function in response to estrogen binding is genomic. ERα's AF-2 activities are largely regulated by the specific ligand occupying the LBD. In the nucleus, unliganded monomeric ERα exists as a complex with heat shock proteins (HSPs) (reviewed in Ref. [48, 49]). E_2, a hydrophobic molecule, readily diffuses across the plasma and nuclear membranes. Once in the nucleus, E_2 binds the LBD of the ERα–HSP complex, leading to the disassociation of the HSPs. The LBD of the receptor then undergoes a crucial conformational change in which H12 covers the ligand-binding pocket (LBP), and the receptor homodimerizes with another ERα molecule along surfaces in the LBD and DBD (reviewed in Refs [50, 51]). The DBDs of ERα proteins allow the homodimer to interact with EREs in the promoters of E_2-responsive target genes [38]. The consensus palindromic ERE consists of two inverted half-site repeats of AGGTCA separated by 3 nucleotides, to which ERα binds as a dimer with one unit each interacting per half-site. The AF-2 then mediates recruitment of the transcriptional machinery and in a cell-type-dependent fashion interacts synergistically with the AF-1 region, to regulate transcription.

In ERα's LBD [42–44, 52], H2–H11 and the hairpin β-sheet form a 'wedge-shaped' hydrophobic ligand-binding cavity, while H12, in the agonist bound conformation,

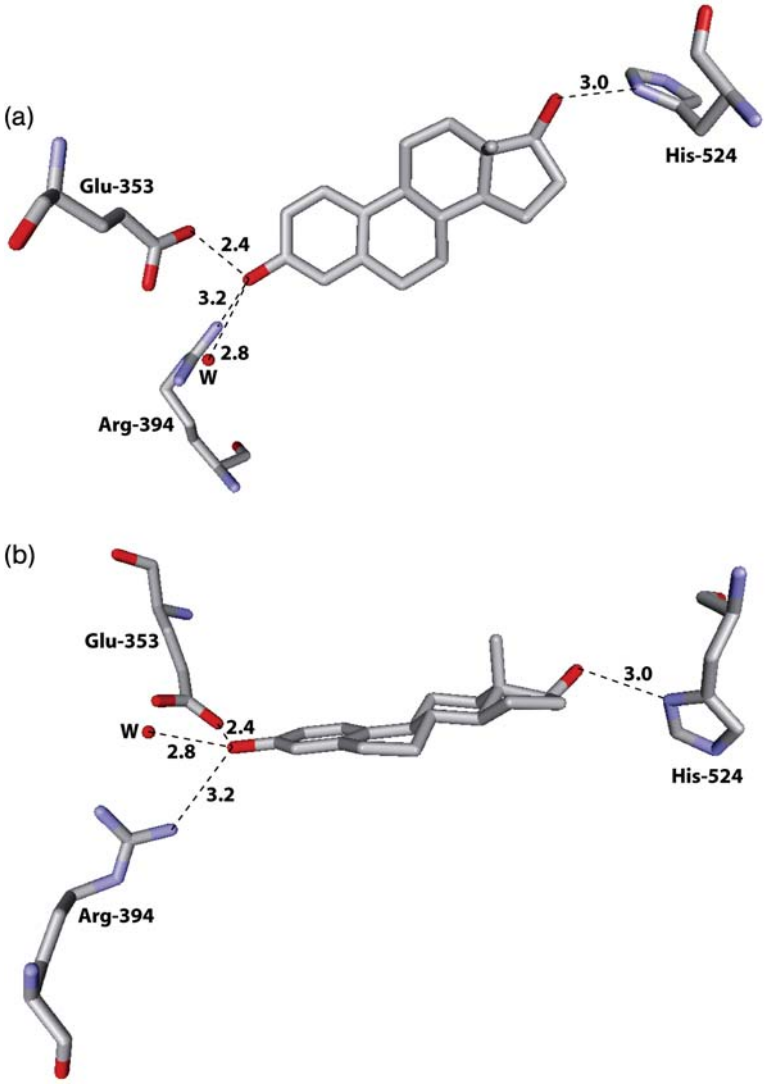

Figure 5.5 Hydrogen-bond interactions between E_2 and ERα. Hydrogen-bond intermolecular interactions of E_2 co-complexed with human ERα LBD using the X-ray crystallographic structure 1GWR at 2.4-Å resolution [42] are shown from a top view (a) and a side view (b). The conformations are visualized using 3D-Mol Viewer (a component of Vector NTI Advance 10.0.1 software; Invitrogen). Hydrogen bonds are indicated by dashed lines and their lengths in angstroms as determined by 3D-Mol Viewer. A highly ordered water molecule stabilized by a hydrogen-bond network is indicated (W). Carbon atoms are shown in gray, oxygen atoms in red and nitrogen atoms in blue.

closes over the cavity filled with E_2 as if a 'lid'. E_2 is aligned in the binding cavity by hydrogen bonds at both ends of the ligand (Figure 5.5); specifically the 3-OH group at the A-ring end of E_2 forms a strong hydrogen-bond network with ERα's Glu353 (in H3) and Arg394 (in H6) as well as an ordered water molecule, while E_2's 17β-OH

group at the D-ring end of the ligand forms hydrogen bonds with ERα's His524 (in H11). Further, hydrophobic van der Waals contacts along the lipophilic rings of E_2, and particularly between Phe404 and E_2's aromatic A-ring, promote a low-energy conformation. Once E_2 binds the receptor, H3, H5/6 and H11 form a groove to which H12 packs closely, orientating its hydrophobic surface towards the ligand and its charged surface away from the body of the LBD. Hence, H12 'seals' the ligand-binding cavity and, together with H3, H5/6 and H11, forms as a highly complementary topology that defines the AF-2 surface (Figure 5.3) by interacting with coactivators to promote transcriptional transactivation [42–44, 52].

Docking of coactivators is mediating by nuclear receptor boxes consisting of an LXXLL-like motif present with the AF-2, in particular H12, and coactivators. Once docked to ERs (Figure 5.6), the core coactivator [*e.g.* steroid receptor coactivator 3 (SRC-3), also known as amplified in breast cancer-1 (AIB1)], or the coactivator that directly interacts with ERs, recruits co-coactivators into a multiprotein complex such as p300/CBP histone acetyltransferase, CARM1 methyltransferase, and ubiquitin ligases UbC and UbL. These coactivator-complex proteins perform subreactions within the DNA–ER–coactivator complex necessary for transcription to proceed such as remodeling chromatin through methylation and acetylation modifications. They also direct their enzymatic activity towards adjacent factors, executing methylations and acetylations which promote dissociation of coactivator-complex components, followed by ubiquitination to selectively target components of the complex to the proteasome for degradation after they have completed their functions, thereby allowing the next cycle of coactivator–receptor–DNA interactions to proceed (reviewed in Ref. [53]). Thus, an orderly yet dynamic sequence of complex assembly and disassembly ensues, culminating in transcription of estrogen-responsive target genes (see Figure 5.6).

ERs also act through a tethered pathway of protein–protein interactions at AP-1 sites [54–56], Sp1 sites [57–61] and NFκB sites [51]. At AP-1 sites, E_2 activates ERα-mediated transcription [54, 56]. Tamoxifen also activates AP-1-regulated transcription via ERα in endometrial cells, but not in breast cells [56]. Thus, tamoxifen's effects on AP-1 activity are cell-type dependent. Regarding ERβ, estrogens do not activate ERβ-mediated transcription via AP-1 elements, but antiestrogens do [54]. Hence, when bound to estrogens, ERα and ERβ display opposing activities at AP-1 elements. However, there exist conflicting reports regarding whether E_2-bound ERα up- or downmodulates AP-1-regulated transcription [56, 62]. It is therefore likely that ERα differentially regulates transcription via AP-1 sites depending on the specific sequence of the AP-1 site, its context (i.e. surrounding sequences) and the cell type.

The ligand-independent activation of ER via the AF-1 domain is closely related to the phosphorylation status of the receptor [63–65]. In particular, phosphorylation of Ser104/Ser106, Ser118 and Ser167 [23] has been identified. In U2OS human osteosarcoma cells; Ser104 and Ser106 are phosphorylated by the cyclin A–cyclin-dependent kinase 2 complex [66]. In multiple cell lines, Ser118 is phosphorylated in response to various treatments, including estrogens and antiestrogens, phorbol-12-myristate 13-acetate (PMA, also known as tetradecanoylphorbol acetate), epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1); mitogen-activated

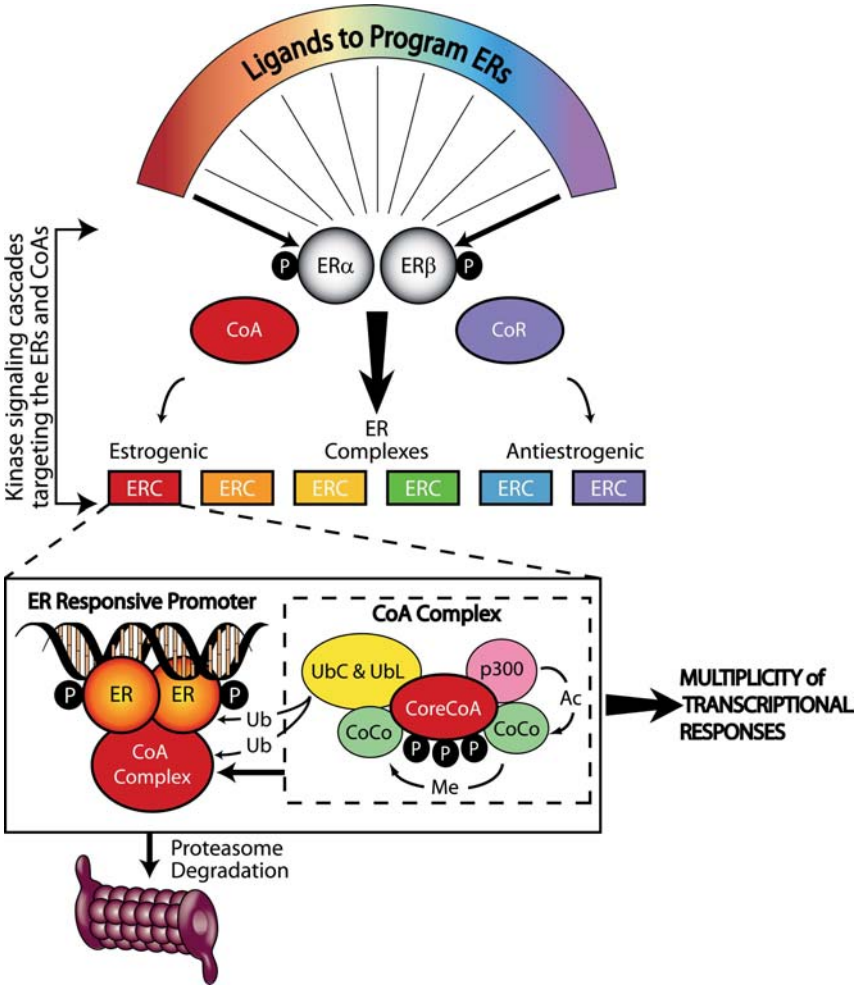

Figure 5.6 Regulation of ER transcriptional activity in a target tissue. The shape of the ligand that binds to the ERs α and β programs the receptor to become an estrogenic or antiestrogenic signal by regulating the balance of coactivators (CoAs) and corepressors (CoRs) that are recruited to the receptors. Further, kinase signaling pathways target ERs and coactivators for phosphorylation, which then influences their ability to form complexes. Hence, a promoter bound by a ligand–ER–coregulator complex (ERC) containing significantly more coactivators than corepressors may be a dominant estrogenic site. However, the regulation of ER action is not simply due to the binding of the ligand–ER–coregulator complex to the promoter of the responsive gene, but a dynamic process of receptor complex assembly and destruction. A core coactivator facilitates assembly of an activated multiprotein complex containing specific co-coactivators (CoCo) that may include p300/CBP histone acetyltransferase, CARM1 methyltransferase, and the ubiquitin-conjugating ligases UbC and UbL. These co-coactivators then acetylate (Ac), methylate (Me) and ubiquitinate (Ub) specific residues in the complex to remodel chromatin and to induce dissociation and destruction of receptor complex components via the proteasome. Thus, a regimented cycle of complex assembly, activation and destruction occurs based on the preprogrammed ER complex. Further, the target tissue is programmed to express a spectrum of responses between full estrogen action and antiestrogen action based on the shape of the ligand and the sophistication of the cell type-specific coregulators.

protein kinase and Cdk7 have been implicated as the kinases responsible [65, 67–71]. Ser167 is phosphorylated in various cell lines by Akt, $pp90^{rsk1}$ and casein kinase II in response to treatment with E_2, EGF or PMA [72–76]. Kinase signaling cascades also target coregulators for phosphorylation, which regulates their ability interact with ERs [77–79]. Overall, posttranslational modification of ER by phosphorylation modulates many facets of its activity including ligand, DNA and coregulator interactions [80].

5.2.2 ERβ

The ERβ gene is located on chromosome 14q23.2 [81] and encodes a 530-amino-acid protein (Figure 5.3) [82]. ERβ's DBD and LBD share the highest degree of amino acid identity, 97 and 61%, respectively, with the corresponding regions of ERα. However, the A/B and D domains only share 27% and 26% amino acid identity, respectively. Consistent with the lack of A/B domain homology between ERα and ERβ, functional studies have indicated that ERβ lacks AF-1 activity [83, 84]. ERβ is expressed in the testis, prostate, ovary, developing uterus, breast, vascular endothelium, smooth muscle, immune system, bone and some neurons (reviewed in Refs [22, 24]). The concentrations of ERα and ERβ vary according to the tissue, and even according to cell type within in a specific tissue. For example, ovaries express ERα and ERβ, but ovarian granulosa cells express exclusively ERβ [81].

Studies employing breast cancer cells demonstrated that ERβ antagonizes the proliferative effects of ERα [28, 85–88]. In MCF-7 cells, ERβ repressed expression of growth-promoting genes c-*myc*, cyclin D1, and cyclin A, while increasing expression of growth-inhibitory genes $p21^{cip1}$ and $p27^{kip1}$, thereby leading to arrest in the G_2 phase of the cell cycle [85]. ERβ exhibits decreased transcriptional activity relative to ERα [83, 84, 89], due to impairment by ERβ's amino terminus [90]. This reduced transcriptional activity of ERβ may inhibit ERα transcriptional activity by competition for EREs and by formation of ERα–ERβ heterodimers [84, 91, 92]. Five isoforms of ERβ exist, with the possibility of unique functions associated with specific isoforms. One of the more characterized isoforms, ERβ2, also termed ERβcx, results from alternative splicing in which 61 amino acids of the C-terminal portion of the LBD and the entire AF-2 is replaced by a unique 26-amino-acid sequence [93]. ERβ2 antagonizes ERα transcriptional activity via EREs and E_2-responsive AP-1 sites, and promotes proteasome-dependent degradation of ERα [94]. ERβ also opposes ERα transcriptional activity at specific promoters through AP-1 sites by altering the recruitment of c-Fos and c-Jun to E_2-responsive AP-1 sites in promoters [95]. For example, ERβ blocks ERα transcriptional activity at the cyclin D1 promoter [96]. However, ERβ does not always inhibit ERα transcriptional activity; genome-wide analysis of MCF-7 cells overexpressing ERβ demonstrated that ERβ enhances or represses distinct subsets of estrogen-regulated genes [97]. Interestingly, ERβ regulated expression of genes which may contribute to suppression of growth (*i.e.* components in the transforming growth factor-β pathway), and genes controlling cell cycle progression and apoptosis [97].

The function of ERβ and its role in breast cancer remains controversial. ERβ is coexpressed with ERα in around 60% of unselected primary breast cancers and in around 50–60% of ERα-negative breast cancers [98–100]. Recent studies have shown that ERβ expression is highest in normal breast and progressively declines through ductal usual-type hyperplasia to ductal carcinoma *in situ* to invasive breast carcinoma [87, 98, 101]. In ductal usual-type hyperplasia, low levels or ERβ compared to ERα predicted progression to invasive breast carcinoma [102]. In malignant disease, high ERβ protein levels predicted improved disease-free and overall survival in patients treated with adjuvant tamoxifen [103], particularly in tamoxifen-treated patients with ERα-negative disease [104]. Other studies found that coexpression of ERβ and ERα mRNA associated with node-positive disease [105], that increased ERβ mRNA levels associated with tamoxifen resistance [106] or that ERβ mRNA expression did not add significant value to predicting response to neoadjuvant SERM therapy [107]. Considering the potential for prognostic value of specific ERβ isoforms, ERβ2 protein levels associated with favorable response to endocrine therapy [108]. The splicing variant ERβ2 has also been observed to correlate with progesterone receptor (PR) negativity in ERα-positive breast cancer, possibly as a result of repressing ERα-mediated induction of PR expression [109]. If expression of ERβ, or of specific ERβ isoforms, proves to be an important prognostic factor for breast cancer, breast tumors will need to be tested routinely for ERβ in the same way that they are now tested for ERα [110, 111].

In summary, the role of ERβ in breast pathobiology remains unclear. However, the majority of recent studies suggests that ERβ expression has a potential growth-inhibitory effect on normal and neoplastic breast cells and could represent a favorable prognostic factor in breast cancer (reviewed in Ref. [23, 27, 28]). A better understanding of the role of ERβ in breast and possibly other forms of cancer could elucidate ways of exploiting it as a therapeutic target using ERβ selective ligands. ERα- and ERβ-selective ligands are addressed elsewhere in this book.

5.3
Therapeutic Basis for Targeting ER

Blocking estrogen synthesis using aromatase inhibitors (AIs) is therapeutically successful for the adjuvant treatment of breast cancer and is considered to be superior to adjuvant tamoxifen treatment with fewer side-effects, such as endometrial cancers, hysterectomies and blood clots [112–115]. There are two classes of agents to prevent the CYP19 aromatase enzyme from synthesizing estrogen: competitive inhibitors (*e.g.* letrozole or anastrozole) and suicide inhibitors (*e.g.* exemestane) [116]. This indirect method of targeting the tumor ER is unfortunately too large a topic to cover adequately in this chapter so the interested reader is referred to the clinical and translational articles mentioned above for further information. We have chosen instead to focus our chapter on compounds that target ER directly. The nonsteroidal compounds that bind to ER and modulate the signal transduction pathway at different target sites around the body are called SERMs. In contrast,

steroidal compounds that bind to the ER and cause rapid destruction of the complex are called 'pure antiestrogens' as they exhibit no estrogen-like actions at sites around the body.

The administration of estrogenic compounds as HRT ameliorates many of the symptoms of menopause, including hot flashes and night sweats, in addition to reducing the risk of colon cancer and osteoporosis/fractures (reviewed in Ref. [117]). Unfortunately, HRT also increases the risk of Alzheimer's disease, strokes, blood clots, breast cancer and reduced cognitive function. This combination of effects in multiple tissues illustrates the importance of selectivity in the modulation of ER for the treatment of breast cancer. SERMs, such as tamoxifen, its active metabolites and raloxifene, function as partial antagonists depending on tissue and promoter context. For example, both tamoxifen and raloxifene function as antagonists in mammary tissues, and as agonists in bone, brain and cardiovascular tissues (reviewed in Ref. [118]). The effects of the two SERMs differ in uterine tissue where raloxifene exhibits antagonistic activity, but tamoxifen exhibits partial agonistic activity thought to be associated with an increased risk of endometrial cancer [119–123]. An ideal SERM would decrease the incidence of osteoporosis, coronary heart disease, hot flashes and breast cancer without increasing the risk of blood clots and endometrial cancer (reviewed in Ref. [117]). We will provide a basic background of the current direct utility of the two pioneering SERMs tamoxifen and raloxifene, discuss in detail the putative mechanism of action of SERMs, consider progress with new SERMs, and close with an examination of pure antiestrogens.

5.4 SERMs

5.4.1 Origins of SERMs

It is important to remember that tamoxifen was discovered in a fertility control program at a time in the late 1950s/early 1960s when interest by the pharmaceutical industry was focused on contraception [124, 125]. Similarly, raloxifene, then known as keoxifene, was designed to be a potential therapy for the treatment of breast cancer, but it failed in that application during the 1980s [126]. By coincidence, both 'nonsteroidal antiestrogens' [127] were subsequently reinvented for use as their current clinical applications: tamoxifen as a targeted, long-term breast cancer therapy and chemopreventive, and raloxifene as a target-tissue-specific modulator of estrogenic and antiestrogenic actions. Raloxifene was found to prevent bone loss in ovariectomized rats [128], while the same doses prevented rat mammary carcinogenesis [129]. It was clear that the drug group, now referred to as SERMs could switch on and switch off estrogen target sites around the body. It was also apparent that based on the fact that tamoxifen and raloxifene could potentially maintain bone density, but prevent breast cancer, that the drugs could be used to prevent osteoporosis in postmenopausal women and prevent breast cancer as a

beneficial side-effect [130]. Since raloxifene did not appear to have the same stimulating effects as tamoxifen in the rodent uterus and in human endometrial cancer [131, 132], it was the obvious candidate for development by the pharmaceutical industry. Others would follow and these compounds will be discussed later in this chapter. It also became pertinent to consider the mechanism of action of SERMs because the application of these compounds grew broader than just targeting ER in breast cancer. The recognition of target site-specific effects of SERMs naturally initiated an investigation of their molecular mechanisms of action because the traditional model of estrogen action with estrogens binding to ER to initiate responses and 'antiestrogens' blocking estrogen-induced events was no longer consistent with the facts. The SERMs became new pharmacology tools to explore the target site-specific actions of ER.

5.4.2
Currently Approved SERMs Tamoxifen and Raloxifene

5.4.2.1 Tamoxifen

Tamoxifen (Nolvadex®; ICI 46,474; Figure 5.1) was reported in 1967 as a possible contraceptive, but Arthur Walpole of the ICI Pharmaceuticals Division had the foresight to include in its patent application a use for the 'control of hormone-dependent tumors' despite the fact that no studies had, at that time, been completed [133, 134]. During the 1970s, tamoxifen was reinvented as a drug targeted to ER, and used strategically as a long-term adjuvant therapy for the treatment and prevention of breast cancer. In the US, tamoxifen received Food and Drug Administration (FDA) approval as an adjuvant treatment for node-positive breast cancer in postmenopausal women with chemotherapy in 1985 and alone in 1986. FDA approval for its use for the treatment of ER-positive advanced breast cancer in premenopausal women came in 1989, and approval as an adjuvant treatment for node-positive ER-positive breast cancer in pre- and postmenopausal women came in 1990 [118]. Tamoxifen is also the first drug FDA-approved for chemoprevention of breast cancer incidence in high-risk pre- and postmenopausal women [135]. Tamoxifen was considered the standard of care for the treatment of ER-positive breast cancer as recommended by the 2000 US National Institutes of Health Consensus Development Conference and the 2001 St Gallen Consensus Panel [136], and is credited with saving the lives of 400,000 breast cancer patients while maintaining bone mineral density.

A 5-year course of tamoxifen treatment provides protection superior to 1–2 years of treatment. Currently, 5 years of adjuvant tamoxifen is recommended to be optimal, since extending treatment beyond 5 years provides no further improvement [137, 138]. There are reports of tamoxifen-stimulated tumor growth occurring during the treatment of advanced (metastatic stage IV) breast cancer [139, 140], but there is currently no evidence that extending tamoxifen beyond 5 years of adjuvant therapy increases the risk of tumor recurrence. Critically important, the protective effects of tamoxifen on breast cancer recurrence and mortality are persistent long after tamoxifen therapy is stopped. A meta-analysis of 15 years of follow-up of 10,386 women shows that 5 years of adjuvant tamoxifen in ER-positive disease versus not

almost halves the annual recurrence rate (recurrence rate ratio = 0.59, SE = 0.03) and decreases mortality by a third (death rate ratio = 0.66, SE = 0.04). These decreased rate ratios translate into 15-year gains of 11.8 (1.3) and 9.2% (SE = 1.2) in recurrence and mortality, respectively [141]. However, tamoxifen is not an ideal SERM because it increases the incidence of hot flashes, vaginal discharge, blood clots and endometrial cancer (reviewed in Refs [118, 136, 142, 143]). After 10 years of clinical reporting (1989–1999), it appears that tamoxifen causes a 3- to 4-fold increase in endometrial cancer in postmenopausal patients, although it should be noted that the absolute risk of developing such cancer is low. However, there is no association between tamoxifen use and endometrial cancer risk in premenopausal women [135, 144, 145], probably because menstrual cycles persist during tamoxifen treatment for the majority of women. Thus, current practice outside the clinical trial setting with tamoxifen is a 5-year course of treatment with regular monitoring for endometrial cancer [136].

In the 1960s, it was observed that the *E* and *Z* isomers of substituted triphenylethylenes exerted opposing biological activities [146]. Tamoxifen (ICI 46,474) is the *Z* isomer of *p*-dimethyaminoethoxy-1,2-diphenylbut-1-ene (Figure 5.1) and is an antiestrogen in the rat [134, 146]. In contrast, ICI 47,699 (Figure 5.7) was confirmed as the *E* isomer by X-ray crystallography and is an estrogen [147, 148].

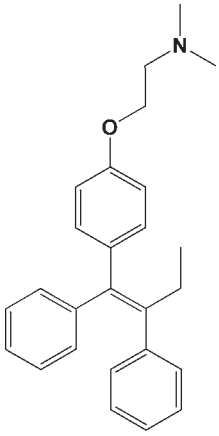

Figure 5.7 ICI 47,699.

Tamoxifen is metabolized to its major metabolite the weak antiestrogen *N*-desmethyltamoxifen (Figure 5.8) by cytochrome P450 3A4/5 (CYP3A4/5) enzymes, and to the minor yet potent antiestrogen 4-OH-tamoxifen (Figure 5.9) by CYP2D6 and other P450s, as well as to other minor metabolites [127, 149–154]. The abundant metabolite *N*-desmethyltamoxifen undergoes secondary metabolism by CYP2D6 into the major secondary metabolite 4-OH-*N*-desmethyltamoxifen, or endoxifen (Figure 5.10) [149, 155], and by the CYP3A subfamily to additional secondary metabolites [149, 155]. Also, 4-OH-tamoxifen undergoes secondary metabolism by the CYP3A subfamily to endoxifen [149]. For decades, 4-OH-tamoxifen was presumed to be the most important active metabolite of tamoxifen,

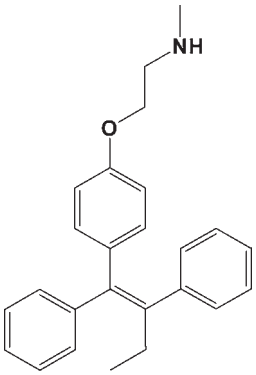

Figure 5.8 *N*-Desmethyltamoxifen.

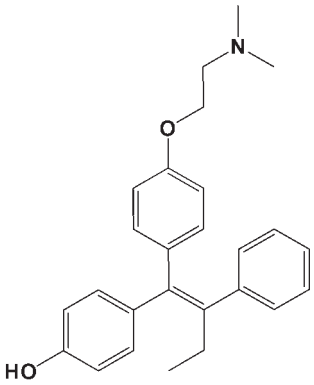

Figure 5.9 4-OH-tamoxifen.

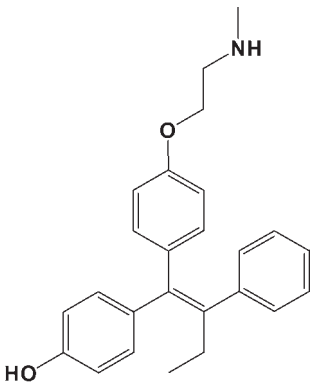

Figure 5.10 Endoxifen.

because tamoxifen has a low binding affinity for ER, but 4-OH-tamoxifen exhibits a high binding affinity for ER equivalent to that of E_2. Further, 4-OH-tamoxifen is a potent antiestrogen in the rat [151, 156]. Thus, tamoxifen is a prodrug that is converted to the active metabolite 4-OH-tamoxifen *in vivo* [151, 157]. However, recent evidence shows that endoxifen is also a potent metabolite: both 4-OH-tamoxifen and endoxifen bind ERα and ERβ with similar affinity, and inhibit E_2-induced proliferation of human breast cancer cells with similar potency [158]. Also, 4-OH-tamoxifen and endoxifen regulate global gene expression similarly [159]. Since the average plasma concentration of endoxifen is 5- to 10-fold higher than that of 4-OH-tamoxifen in breast cancer patients administered tamoxifen chronically [160], endoxifen is the major active metabolite, yet the combined concentrations of both endoxifen and 4-OH-tamoxifen likely determine the total antiestrogenic activity of tamoxifen *in vivo*.

Plasma concentrations of endoxifen are significantly influenced by the CYP2D6 genotype, which exists as wild-type (functional alleles) or variant alleles that exhibit reduced functionality or are nonfunctional [161]. The CYP2D6 genotype homozygous for a nonfunctional allele (*4/*4) results in low endoxifen levels [162], and predicts a higher risk of disease relapse and lower incidence of hot flashes [163, 164]. These studies suggest that hot flashes, a side-effect thought to be due to the antiestrogenic actions of tamoxifen, may be an indirect measure of CYP2D6 activity. In support of this hypothesis, a recent study showed that hot flashes compared to no hot flashes served as an independent predictor of tamoxifen efficacy by associating with relapse-free survival [165]. CYP2D6 can be potently inhibited by paroroxetine, a selective serotonin reuptake inhibitor, which is often prescribed to individuals taking tamoxifen to alleviate hot flashes. Indeed, patient's coadministered paroxetine with tamoxifen exhibit lower levels of endoxifen than patients not coadministered paroxetine [155, 166]. Further, in a prospective clinical trial involving breast cancer patients chronically treated with tamoxifen, individuals exhibiting: (i) low endoxifen/*N*-desmethyltamoxifen ratios associated with the CYP2D6 genotype lacking any functional allele, (ii) intermediate endoxifen/*N*-desmethyltamoxifen ratios with at least one functional allele and (iii) high endoxifen/*N*-desmethyltamoxifen ratios with two or more functional alleles [162] . Therefore, endoxifen plasma levels, affected by the CYP2D6 genotype and CYP2D6 inhibitors, may impact response to tamoxifen therapy [161].

Tamoxifen treatment increases a number of estrogen induced circulating proteins, such as sex hormone-binding globulin [167, 168] and antithrombin III [168, 169], and alters the plasma protein profile [170, 171]. Additionally tamoxifen has an estrogen-like action to reduce luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in postmenopausal women [172]. Tamoxifen has a consistent ability to decrease low-density lipoprotein (LDL) cholesterol, but unlike estrogen does not cause an increase in high-density lipoprotein (HDL) cholesterol [169, 173–177]. Although tamoxifen was originally classified as an antiestrogen, the drug does not predispose women to coronary heart disease [178–180]. Most studies find that tamoxifen does not protect against coronary heart disease, but the finding may be because only clinical trials with small numbers of patients at risk have been examined. Only retrospective results from the Scottish adjuvant tamoxifen trial of

5 years of adjuvant tamoxifen showed a decrease in fatal myocardial infarction [181, 182]. Tamoxifen has not been tested prospectively for the prevention of coronary heart disease in high-risk women. Tamoxifen maintains bone density in postmenopausal women [183–186] and causes a slight decrease in bone density in premenopausal women [187]. The drug has not been tested prospectively as a preventive for osteoporosis, but a nonsignificant decrease in hip, wrist and spinal fractures has been noted as a secondary endpoint in the National Surgical Adjuvant Breast and Bowel Project chemoprevention trial [135]. Interestingly enough, tamoxifen produces significantly fewer fractures compared to AIs when used as an adjuvant therapy in postmenopausal women [188–190]. Although tamoxifen could be classified as a partial agonist in most estrogen-like parameters, the reduced estrogenicity is not reflected in a reduction in the incidence of blood clots relative to HRT [135, 191].

Tamoxifen had been used for more than a decade for the treatment of breast cancer (in Europe since the early 1970s and in the US since 1978) without the reporting of serious side-effects [127]. However, by the end of the 1980s, with the expanded use of tamoxifen as a long-term adjuvant therapy in node-negative breast cancer [192] and the proposed use of tamoxifen for chemoprevention in high-risk women [193], there was a requirement to reexamine the toxicology of tamoxifen in greater detail. Tamoxifen was found to initiate hepatocellular carcinoma in rats by a non-ER-mediated mechanism [194–198]. This finding was a major concern and naturally was linked to an increased incidence of endometrial cancer and two cases of hepatocellular carcinoma noted in women taking tamoxifen [122, 199, 200]. The laboratory finding of carcinogenicity, so late in the drugs' development, occurred because the rat had not previously been used to evaluate the long-term toxicology of tamoxifen prior to introduction as a breast cancer treatment. This was not a requirement. It was equally true that if tamoxifen had been tested and found to be carcinogenic, then adjuvant endocrine therapy, AIs, SERMs and raloxifene would not have been pursued without proof of principle that tamoxifen was a SERM and saved lives [199]. Needless to say, tamoxifen-induced liver carcinogenicity was thoroughly investigated. In rats administered tamoxifen, DNA adducts accumulated in the liver and were identified as α-OH-tamoxifen (Figure 5.11) covalently linked to the oxocyclic amino group of

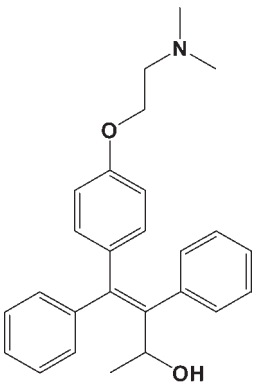

Figure 5.11 α-OH-tamoxifen.

deoxyguanosine [201–205]. Although DNA adducts were readily identified in rat and mouse hepatocytes (90 and 15 adducts per 108 nucleotides, respectively), DNA adducts were not detected in human hepatocytes following tamoxifen treatment [204]. Similarly, the pattern of DNA adducts found in the rat liver was not found in the liver obtained from patients treated with tamoxifen [206]. Overall, it appears that specific metabolic pathways in rat liver predispose that species to liver carcinogenesis [207]. α-OH-tamoxifen is a poorer substrate for human sulfotransferase (which is apparently necessary for adduct formation [208]) than the rat form of the enzyme. Conversely, glucuronidation, which would detoxify α-OH-tamoxifen, predominates in human hepatocytes [209]. This area of drug evaluation is extremely important for understanding the relevance of species related toxicity to clinical practice. Phillips has reviewed the genotoxicity of tamoxifen [210]. In his conclusion he raises the concept of whether tamoxifen is a genotoxic carcinogen in the rat, but a nongenotoxic carcinogen in humans. This may make tamoxifen unique.

5.4.2.2 **Raloxifene**

The SERM raloxifene (Evista®, previously keoxifene and LY156758; Figure 5.4) is a failed breast cancer therapeutic since it showed either no activity or modest activity as a breast cancer therapy [211, 212]. Hence, drug development of raloxifene as an antitumor agent was abandoned in the late 1980s. These data are consistent with the laboratory finding that raloxifene is less effective than tamoxifen in animal models of breast cancer [129, 131]. The fact that raloxifene has extremely poor (2%) bioavailability because of rapid first-pass phase II metabolism [213] suggests that long-acting agents are required for the treatment of breast cancer. However, the recognition of selective ER modulation and the possibility of developing multifunctional medicines [117, 128, 133] has resulted in the successful development of raloxifene to treat and prevent osteoporosis [214]. The successful development of raloxifene is a direct result of a novel finding that nonsteroidal antiestrogens can maintain bone density [128, 215, 216], but may not increase the risk of breast cancer like HRT [3, 4, 217]. Hence, raloxifene was approved in 1997 by the FDA for the prevention of osteoporosis in postmenopausal women.

As a SERM, raloxifene exerts partial estrogen-like action at specific target tissues. Estrogens are known to prevent bone mineral density loss; likewise, raloxifene has been conclusively shown to prevent bone loss and reduce the risk of vertebral fractures [218]. Preliminary studies in 251 normal postmenopausal women randomized into groups taking placebo, raloxifene (200 mg daily), raloxifene (600 mg daily) or Premarin™ (0.625 mg daily) show decreases in serum alkaline phosphatase, serum osteocalcin, urinary pyridinoline and urinary calcium excretion with raloxifene that were no different than with estrogen [219]. However, the doses of raloxifene were far higher than the 60 mg daily currently recommended for the prevention and treatment of osteoporosis. Evaluation of raloxifene (60 mg daily) on bone remodeling in early postmenopausal women, using calcium tracer kinetic methods, found that although remodeling suppression was greater for estrogen, the remodeling balance was the same for the two agents [220]. These results are consistent with the finding that raloxifene increases bone density by $2.4 \pm 0.4\%$ in the lumber spine and

2.4 ± 0.4% for the total hip [221]. Raloxifene has also been shown to decrease spine fractures by 40%, although there was no significant decrease in hip fractures [222].

Raloxifene received a rigorous evaluation in the human uterus. In women prescreened to ensure the absence of preexisting endometrial abnormalities, raloxifene did not show an increase in endometrial thickness [221, 223–225]. Data from postmenopausal women showed that raloxifene was not associated with vaginal bleeding or an increased endometrial thickness [221, 226]. To date, raloxifene has not been associated with an elevated risk of endometrial cancer, but laboratory studies have demonstrated the drug will support the growth of a tamoxifen stimulated endometrial cancer transplanted into athymic mice [227, 228]. However, the growth response of human endometrial carcinoma to raloxifene under laboratory conditions was not as much as that of tamoxifen or toremifene [120].

On the basis of the hypothesis that raloxifene could reduce the incidence of breast cancer as a beneficial side-effect of the prevention of osteoporosis [215], the placebo-controlled trials with raloxifene have been monitored for changes in breast cancer incidence. One of the first studies to show this was the Multiple Outcomes of Raloxifene Evaluation (MORE) trial [226]. In this study, 7,704 postmenopausal women (mean age of 66.5 years) with osteoporosis were randomized to receive one or two daily oral doses of raloxifene (60 mg) or placebo. Raloxifene decreased the relative risk (RR) of invasive breast cancer by 76% [RR = 0.24; 95% confidence interval (CI) = 0.13–0.44] during 3 years of treatment with raloxifene. Raloxifene treatment resulted in a increase in the risk for venous thromboembolism (RR = 3.1; 95% CI = 1.5–6.2), but no increase in the risk of endometrial cancer was observed (RR = 0.8; 95% CI = 0.2–2.7) [226]. Subsequently, women who had been enrolled in the MORE trial were enrolled in the Continuing Outcomes Relevant to Evista (CORE) trial, where 3,510 women who had received either dose of raloxifene in the MORE trial were assigned to receive 60 mg of raloxifene and 1,703 women who had been assigned to receive placebo in the MORE trial continued on placebo. In this second trial, when compared to placebo, raloxifene reduced the 4-year incidences of invasive breast cancer and ER-positive invasive breast cancer by 59% [hazard ratio (HR) = 0.41; 95% CI = 0.24–0.7] and 66% (HR = 0.34; 95% CI = 0.18–0.66), respectively. These differences were not observed in ER-negative invasive breast cancer. When follow-up for both the MORE and CORE trials were combined totaling 8 years, decreases of 66% (HR = 0.34; 95% CI = 0.22–0.50) and 76% (HR = 0.24; 95% CI = 0.15–0.40) in the incidence of invasive and ER-positive breast cancers, respectively, were observed when comparing the raloxifene to the placebo arm. The increase of thromboembolism (RR = 2.17; 95% CI = 0.83–5.70) was confirmed, but no new safety concerns related to raloxifene therapy were identified [229]. Hence, like tamoxifen, raloxifene appears to prevent breast cancer in high-risk women, but unlike tamoxifen, has not been found to increase the incidence of endometrial cancer.

Raloxifene's effects on risk factors for coronary artery disease are similar to those of estrogen, by lowering LDL cholesterol and homocysteine levels. Blood clots with raloxifene also occur at the same frequency as observed with HRT. However unlike estrogens, it does not increase triglyceride, HDL cholesterol or C-reactive protein levels [230]. Therefore, in addition to being evaluated for the prevention of

osteoporosis and breast cancer, raloxifene was evaluated for the reduction of the risk of coronary artery disease in the Raloxifene Use for The Heart (RUTH) trial. This study evaluated whether 60 mg/day of oral raloxifene reduced the risk of coronary events and risk of invasive breast cancer in 10,101 postmenopausal women with documented coronary heart disease or who are at high risk for developing it. Unfortunately, raloxifene had no effect on cardiovascular risk in the RUTH trial, but did serve to further confirm that raloxifene prevents invasive breast cancer and vertebral fracture with no increased risk of endometrial cancer in postmenopausal women [231].

These studies led to the trial that compared raloxifene to tamoxifen in the prevention of breast cancer in women who are at high risk of developing the disease: the STAR trial. This trial enrolled 19,747 postmenopausal women (mean age 58.5 years) at high risk of breast cancer to receive 5 years of either tamoxifen (20 mg daily) or raloxifene (60 mg daily). Raloxifene was demonstrated to be equally as effective as tamoxifen in reducing the incidence of invasive breast cancer (RR = 1.02; 95% CI = 0.82–1.28), while exhibiting a lower risk of thromboembolic events (RR = 0.70; 95% CI = 0.54–0.91) and cataracts than tamoxifen (RR = 0.79; 95% CI = 0.68–0.92) [30]. In contrast to tamoxifen, raloxifene was associated with a nonsignificant higher risk of noninvasive breast cancer (lobular and ductal carcinomas *in situ*). The mechanistic reason for why raloxifene may be less effective against noninvasive breast cancer is unknown. A lower incidence of uterine cancer was associated with raloxifene treatment (23 versus 36 cases in the raloxifene and tamoxifen groups, respectively), but this lower incidence was not statistically significant. However, the incidence of endometrial hyperplasia and hysterectomies was decreased in the raloxifene group compared to the tamoxifen group. Since raloxifene is already approved for prevention of osteoporosis, and it is equally as effective as tamoxifen for the prevention of invasive breast cancer with a lower incidence of side-effects, raloxifene is poised to become a widely prescribed SERM.

5.5 Mechanisms of Action of SERMS

5.5.1 Mechanism of SERM Antiestrogenic Action

Early studies described the interaction between the nonsteroidal antiestrogens and ER in the [^{3}H]E_2 ligand-binding assay [232, 233], but the only conclusion that could be reached was that the compounds did bind to ER and block E_2 binding. There was no ability to describe efficacy at ER without using assays *in vivo* involving rats or mice [134, 146]. The ER assays did, however, help to identify tamoxifen as a prodrug that needed to be coactivated to a 4-OH metabolite to achieve potent antiestrogenic activity [151, 157]. Nevertheless, studies *in vivo* were not able to determine the actual biological activity at the ER. At that time, the assay in the uterus or vagina was really the end result of drug metabolism and individual compounds were difficult to

compare if their pharmacokinetics were different. Only an assay *in vitro* could resolve many of the limitations in understanding the actual drug ER interactions.

Primary cultures of immature rat pituitary gland cells were first used to establish that nonsteroidal antiestrogens were competitive inhibitors of estrogen action, and that metabolic activation was an advantage but not a requirement for antiestrogen action [234]. The assay was used to describe the precise structure function relations of triphenylethylenes related to tamoxifen for the modulation of prolactin synthesis through ER [127, 235], and to propose a precise region (the 'antiestrogenic region') in ER necessary for the interaction of the alkylaminoethyoxy side-chain of antiestrogens which prevents the correct folding of ER to induce full agonist action. These data successfully translated to the study of growth regulation of ER-positive breast cancer cells in culture, once it was discovered that the cells were being grown in media containing fully estrogenic contaminants in the phenol red indicator [236]. Removal of the indicator and growth of cells in charcoal stripped serum (to remove estrogenic steroids) allowed an accurate description of the structure function relationships of estrogens and antiestrogens for the control of breast cancer cell growth [153, 237]. It was proposed that the triphenylethylene's side-chain controlled the subsequent activation of ER interacting with a hypothetical 'antiestrogen region' on ER. Changes in the side-chain length [238] or basicity [239] were predicted to produce a range of complexes with different intrinsic activities that would result in different partial agonist activities [234, 235, 240].

Binding of SERMs such as 4-OH-tamoxifen and raloxifene induces distinct conformations of ERα's LBD different from that of E_2 [42, 44]. In a similar manner as E_2, 4-OH-tamoxifen and raloxifene bind within the same hydrophobic pocket. Also like E_2, the phenolic hydroxyl group of 4-OH-tamoxifen's A-ring (Figure 5.12) and the hydroxyl group of raloxifene's benzothiophene moiety (Figure 5.13) are both positioned near H3 and H6, allowing formation of a hydrogen-bond network with Glu353, Arg394 and an ordered water molecule. However, unlike E_2, the antiestrogenic side-chains of 4-OH-tamoxifen and raloxifene both protrude from the LBP between H3 and H11, making extensive hydrophobic contacts with these helices and interacting with Asp351. Due to the antiestrogenic side-chains of 4-OH-tamoxifen and raloxifene exiting the binding cavity adjacent to Asp351, H12 is displaced and does not cover the LBP. Rather, H12 assumes a conformation that mimics the position of the coactivator's nuclear receptor box motif, and occupies the coactivator recognition groove formed by H3, H4 and H5 [42, 44]. Therefore, the antiestrogenic properties of 4-OH-tamoxifen and raloxifene are due in part to H12 not sealing the LBP and instead acting as an 'autoinhibitor' by preventing coactivator recruitment. This structural evidence confirmed earlier hypothetical models of estrogen and antiestrogen action [130], and provides an elegant solution to AF-2 silencing.

5.5.2
Structural-Based Mechanisms of SERM Estrogen-Like Action

Although much progress has been made in our understanding of estrogen and antiestrogen action, there is no unifying theory that has explained the target

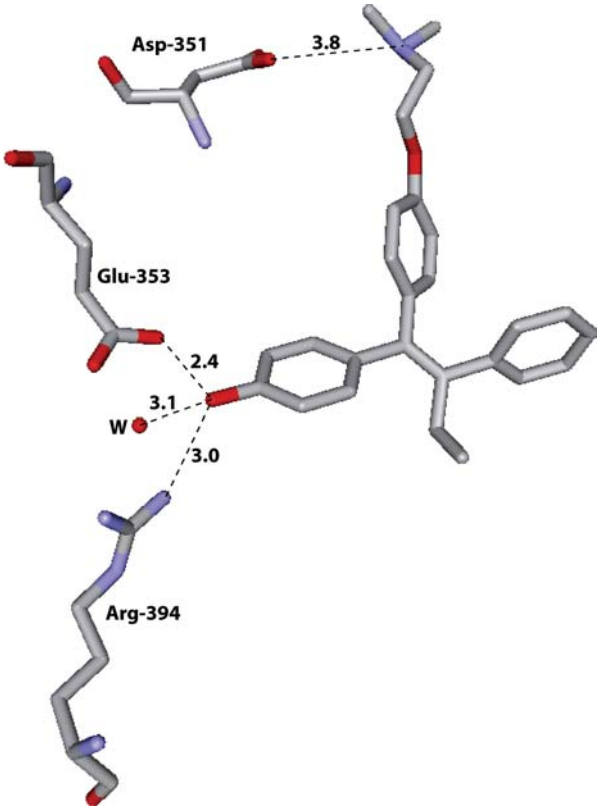

Figure 5.12 Hydrogen-bond interactions between 4-OH-tamoxifen and ERα. Hydrogen-bond intermolecular interactions of 4-OH-tamoxifen cocomplexed with human ERα LBD using the X-ray crystallographic structure 3ERT at 1.9-Å resolution [44] are shown. The conformation was visualized using 3D-Mol Viewer as in Figure 5.5. Hydrogen bonds are indicated by dashed lines and their lengths in angstroms as determined by 3D-Mol Viewer. A highly ordered water molecule stabilized by a hydrogen-bond network is indicated (W). Carbon atoms are shown in gray, oxygen atoms in red and nitrogen atoms in blue.

site-specific actions of SERMs. Despite this deficit, there are opportunities to imagine multiple mechanisms. In other words, there may be different mechanisms at different sites or groups of targets. By way of example, it is intriguing that raloxifene expresses less estrogen-like activity than 4-OH-tamoxifen in breast and uterine cells. The overall architecture induced by the SERMs in the LBD are similar enough that one could conclude that both raloxifene and 4-OH-tamoxifen silence AF-2. However, there are subtle differences between the positioning of the 4-OH-tamoxifen and raloxifene in the LBD that ultimately affect the intrinsic activity of the SERM–ERα complex. These clues now provide a link between the unusual pharmacology of the SERMs and the structure–function relationships of their ERα complexes.

Chambon's group [40] was the first to address the issue of the target site estrogen-like specificity of 4-OH-tamoxifen using recombinant human ER. They reported

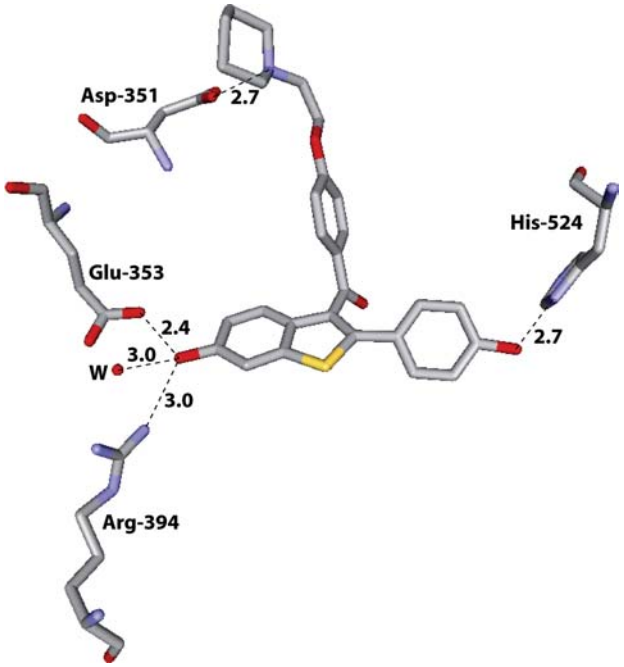

Figure 5.13 Hydrogen-bond interactions between raloxifene and ERα. Hydrogen-bond intermolecular interactions of 4-OH-tamoxifen cocomplexed with human ERα LBD using the X-ray crystallographic structure 1ERR at 2.6-Å resolution [42] are shown. The conformation was visualized using 3D-Mol Viewer as in Figure 5.5. Hydrogen bonds are indicated by dashed lines and their lengths in angstroms as determined by 3D-Mol Viewer. A highly ordered water molecule stabilized by an hydrogen-bond network is indicated (W). Carbon atoms are shown in gray, oxygen atoms in red, nitrogen atoms in blue and sulfur atoms in yellow.

that the estrogen-like actions of 4-OH-tamoxifen were cell-type and promoter-context-dependent, which produced ligand-independent activity of the AF-1 domain. This in turn, they stated, could explain the target site-specific estrogen-like actions observed with tamoxifen in animals and human tissue [127, 128]. In contrast, a pure antiestrogen (zero intrinsic activity) had no estrogen-like actions in model systems *in vitro* or *in vivo* [40]. However, the fact that a pure antiestrogen could produce complete antiestrogenic activity by also silencing AF-1 suggested that AF-1 activity could be ligand-specific, at least under controlled conditions.

A naturally occurring mutation of ER's amino acid 351 has provided valuable evidence that has led to demonstrating a precise interaction of amino acid 351 and the antiestrogenic side-chain of a SERM, which in some way allosterically communicates with AF-1. The Asp351 → Tyr mutation was found in an unusual tamoxifen-stimulated breast cancer tumor model [241]. Incidentally, this natural mutation is not responsible for tamoxifen-induced drug resistance in patients. Asp351 is not involved in either the AF-1 or AF-2 regions, yet its mutation to Tyr converts the more antiestrogenic raloxifene to become an estrogen-like compound. This was shown by measuring effects on expression of a relevant gene target *in situ*, transforming growth

factor-α, in ER-negative MDA-MB-231 cells stably transfected with wild-type and mutant ERα [242, 243]. It is important to note that this system also shows that 4-OH-tamoxifen displays estrogenic-like activity without the need for the Asp351 Tyr mutation, underscoring that proper cellular context, such as in an ER-negative breast cancer cell type, also plays a role in SERM-mediated estrogenic activity. Subsequent X-ray crystallography studies demonstrated that the antiestrogenic side-chains of tamoxifen and raloxifene both exit the crystal structure of ERα adjacent to Asp351. However, the raloxifene piperidine ring nitrogen recognizes Asp351 through a hydrogen bond around 1 Å shorter than that of 4-OH-tamoxifen's dimethylamino group (compare Figure 5.13 to Figure 5.12) [42, 44]. It has been hypothesized that raloxifene's piperidine ring both pushes H12 away (silencing AF-2 [42]) and shields or neutralizes the charge distribution around Asp351 more than that of 4-OH-tamoxifen's diethylamino group. Since 4-OH-tamoxifen interacts with Asp351 only weakly (3.8 Å distance; Figure 5.12), Asp351 instead takes on a position closer to H12. Conversely, raloxifene interacts more strongly with Asp351, thereby preventing Asp351 from positioning itself adjacent to H12. Thus, in the Asp → Tyr mutation, the distance between raloxifene's piperidine ring and amino acid 351 was increased, reflecting a weaker interaction, and instead, Tyr351 interacts with H12, which consequently prevents binding of corepressors and allows estrogen-like activity to manifest [244]. Additional amino acid substitutions lend further evidence in favor of this model. An Asp → Glu mutation also results in an increase in the distance between raloxifene's piperidine ring nitrogen and amino acid 351 from 2.7 Å in the wild-type ER (Figure 5.13) to 3.5–5 Å in the mutant ER. This increased distance translated into an increase in estrogen-like action of the raloxifene–ERα complex [245]. Removal of the charge at amino acid 351 with an Asp → Phe substitution results in a loss of raloxifene's estrogen-like properties [245]. The critical role of the intimate relationship between the antiestrogenic side-chain of raloxifene and Asp351 is confirmed with the raloxifene derivative R1H where the nitrogen in the piperidine ring of raloxifene is replaced by a carbon forming a cyclohexane ring. The ligand loses antiestrogenic properties and is a full agonist [121, 245]. Conversely, in the case of tamoxifen, an Asp → Gly351 mutation results in a decreased distance between tamoxifen's side-chain and amino acid 351, leading to a tamoxifen–ERα (Asp351 → Gly) complex that has lost estrogen- like activity while retaining antiestrogenic properties [246, 247]. The Asp → Gly mutation also decreases the affinity of raloxifene for ERα, thereby illustrating the important role of the interaction of its piperidine side-chain and Asp351.

McDonnell's group [248] has used a phage display technique to identify two separate coactivator binding sites responsible for the expression of the estrogen-like effects of the E_2– or tamoxifen–ER complex. The coactivator binding site on the E_2–ER complex could be the previously described AF-2 region, but the novel site on the tamoxifen–ER complex could be the same as the transactivation site on ER referred to as AF-2b [246, 249]. The AF-2b site is more complex than the AF-2a site previously noted [250], which extends from amino acid 324 to amino acid 351. This is because acidic amino acids on H12 also play an essential regulatory role in the estrogen-like action of tamoxifen. The triple mutation Asp538 → Ala/Glu542 → Ala/Asp545 → Ala in H12 reduces the intrinsic activity of the tamoxifen–ER complex [246, 249],

thus indicating that the expression of SERM estrogen-like actions requires interaction between amino acid 351 and H12, which together may define the occult SERM-induced transactivation site AF-2b. Further, the full expression of SERM estrogen-like actions requires synergistic allosteric interaction between AF-1 and AF-2b comprising amino acid 351 and H12. However, until the whole ligand–receptor complex has been crystallized, it is not possible to visualize the relationship between AF-1 and AF-2.

5.5.3
Coregulator-Based Mechanisms of SERM Estrogen-Like Action

Formation of an occult AF-2b transactivation domain induced by a SERM does not fully explain its tissue-specific estrogenic activity. Other components in the ER signal transduction pathway, particularly coregulators that complex with the receptor, are crucial in determining cell-type-dependent properties of a SERM. It is reasonable to ask, how does the ligand program the receptor complex to interact with other proteins? X-ray crystallography of the LBDs of ER liganded with either estrogens or antiestrogens demonstrates the potential of ligands to promote coactivator binding or prevent coactivator binding based on the shape of the estrogen or antiestrogen-ER complex [42, 44]. Evidence has accumulated that the broad spectrum of ligands that bind to ER can create a broad range of ER complexes that are either fully estrogenic or antiestrogenic at a particular target site [251]. Thus, a mechanistic model of estrogen action and antiestrogen action has emerged based on the shape of the ligand that programs the complex to adopt a particular shape that ultimately interacts with coactivators or corepressors in target cells to determine the estrogenic or antiestrogenic response respectively (Figure 5.6).

It is more than a decade since the first steroid receptor coactivator (SRC-1) was described [252]. Not surprisingly, the coactivator model of steroid hormone action has now become enhanced into multiple layers of complexity, thereby amplifying the molecular mechanisms of modulation. It appears that coactivators are not simply protein partners that connect one site to another in a complex [253]. The coactivators actively participate in modifying the activity of the complex. Post-translational modification of coactivators via multiple kinase pathways initiated by cell surface growth factor receptors (*e.g.* EGF receptor, IGF 1 receptor and ErbB2, also known as HER2) can result in a dynamic model of steroid hormone action. The core coactivator (*e.g.* SRC-3; Figure 5.6) first recruits a specific set of co-coactivators, *e.g.* p300 and ubiquitin-conjugating ligases, under the direction of numerous protein remodelers (*e.g.* the peptidyl-prolyl isomerase Pin1, heat shock proteins and proteasome ATPases) to form a multiprotein coactivator complex that interacts with the phosphorylated ER at the specific gene promoter site [253]. Most importantly, the proteins assembled into the coactivator complex have individual enzymatic activities to acetylate or methylate adjacent proteins. This results in the dissociation of the complex and simultaneous tagging with activated ubiquitin. The activated ubiquitin is transferred to the ubiquitin-conjugating enzyme that interacts with ubiquitin ligase which has already identified its protein

target. Multiple cycles of the reaction can polyubiquitinate a substrate (*i.e.* ER or a coactivator), or dependent on the ubiquitin–ubiquitin linkage, can be either activated further (K63 linkage), or degraded by the 26S proteasome (K48 linkage) [254].

Thus, for effective gene transcription, programmed and targeted by the shape and phosphorylation status of ER and coactivators, a dynamic and cyclic process of transcription complex assembly [255] and destruction of transcription complexes by the proteasome is required. Estrogen– and SERM–receptor complexes have differing accumulation patterns in the target cell nucleus [151, 256] primarily because the relative rates of destruction of the complexes are different [251].

These fundamental mechanisms [253, 257] in physiology can also be applied to the potential development of drug resistance to tamoxifen in breast cancer. Model systems have demonstrated the conversion of the tamoxifen–ER complex from an antiestrogenic signal to an estrogenic signal in an environment enhanced for phosphorylation by overexpression of the ErbB2 (HER2) cell surface receptor and an increase in SRC-3 coactivator accumulation [258, 259]. However, the enhanced level of coactivator and its enhanced phosphorylation state derived from an activated ErbB2 phosphorylation pattern will enhance the estrogen-like activity of tamoxifen at ER. Clearly, issues of SERM action at target tissues and the eventual development of drug resistance in breast cancer will converge over extended duration of SERM use.

5.6 Additional SERMs

5.6.1 Clomiphene

In addition to tamoxifen [125, 134], clomiphene (originally chloramiphene or MRL-41; Figure 5.14) [260] resulted from a search for contraceptives in laboratory models, but in clinical trials unexpectedly induced ovulation in subfertile women [261]. As a

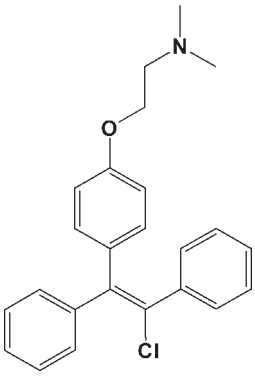

Figure 5.14 Clomiphene.

result of these and additional clinical findings, clomiphene [262–266] and initially tamoxifen [267, 268] were approved as profertility drugs for the induction of ovulation.

5.6.2 Toremifene (Fareston®)

Toremifene (Fareston®, chlorotamoxifen; Figure 5.15) has been thoroughly investigated in the laboratory [269–272] and has antitumor activity in carcinogen-induced rat mammary cancer, but is less potent than tamoxifen [272–274]. Toremifene has been tested extensively in phase I–III clinical trials [275–278] and has been approved for use in postmenopausal women with metastatic breast cancer [279]. As predicted from the reduced potency in animal studies, the dose required for activity is 60 mg of toremifene daily (tamoxifen is used at 20 mg daily). The side-effects are similar to those of tamoxifen and, as with tamoxifen, the responses are observed in ER-positive tumors. However, because adjuvant therapy with tamoxifen is standard throughout the world, issues of cross-resistance of tamoxifen and toremifene are important considerations for the use of toremifene in recurrent breast cancer. Laboratory studies by Osborne *et al.* [280] have demonstrated that toremifene-stimulated tumors can develop from MCF-7 breast cancer cells transplanted into athymic mice. Toremifene is cross-resistant with tamoxifen in tamoxifen-stimulated breast cancer in the laboratory [281]. Similarly, cross-over clinical trials demonstrate that there is little possibility of a second response to toremifene after tamoxifen failure [282, 283].

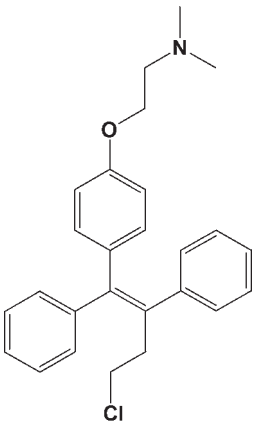

Figure 5.15 Toremifene.

The interesting property of toremifene is the reduced liver carcinogenicity in the rat [284, 285]. Toremifene produces fewer DNA adducts than tamoxifen [284]; however, there are reports of DNA damage [286] and the drug can still act as an

estrogen-like tumor promoter in the rat [197]. The lower potential to produce DNA adducts probably reflects an inability of toremifene to produce the α-OH metabolite observed with tamoxifen (α-OH-toremifene). The chlorine of toremifene would sterically prevent α-hydroxylation. Additionally, even if toremifene could be metabolized to α-OH-toremifene to significant levels, rats treated intraperitoneally with α-OH-toremifene showed a large reduction by 39-fold in hepatic DNA adduct formation compared to tamoxifen [287]. This low level of DNA adducts generated by α-OH-toremifene may be due to its limited esterification and/or the poor reactivity of its sulfated and activated form α-sulfoxytoremifene [287].

Issues of the incidence of endometrial cancer during toremifene therapy are controversial. Toremifene can support the growth of tamoxifen-stimulated endometrial cancers in athymic mice [228], so it would not be unreasonable to predict a modest rise in endometrial cancer in patients treated long-term with adjuvant toremifene. The general pharmacology of toremifene in the uterus is the same as that of tamoxifen [288]. However, an analysis of side-effects in adjuvant studies shows no increases in endometrial cancer with toremifene [289].

5.6.3 Idoxifene

Idoxifene (Figure 5.16) is a metabolically stable analog of tamoxifen synthesized to avoid the toxicity reported with tamoxifen in rat liver [290–292]. Substitution of halogens at the 4-position of tamoxifen is known to reduce the antiestrogenic potency by preventing the conversion of the parent drug to 4-OH-tamoxifen [157]. Additionally, it was argued that by reduction of demethylation, liver toxicity would be reduced because increased local levels of formaldehyde would not occur [291, 292]. Unfortunately, the increased metabolic stability also increases toxicity, since the drug cannot easily be detoxified. Idoxifene accumulates such that high parent drug levels are observed which can cause death in mice at doses that are safe for tamoxifen [281].

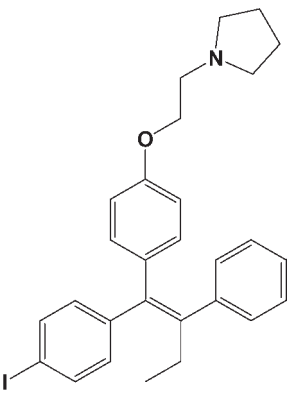

Figure 5.16 Idoxifene.

Idoxifene inhibits the growth of carcinogen-induced rat mammary tumors [293] and MCF-7 tumors grown in athymic mice [294, 295]. When compared to tamoxifen, idoxifene appears to have more antagonistic and less agonistic effects on ER in laboratory studies. Also, idoxifene has been reported to develop acquired antiestrogen resistance more slowly than tamoxifen [294]. However, there appears to be cross-resistance in laboratory models of tamoxifen-stimulated growth [281].

Idoxifene has been evaluated as a breast cancer treatment for postmenopausal patients [296, 297]. In one study, 321 postmenopausal patients with unknown receptor status or hormone receptor-positive metastatic breast cancer were randomized to receive either tamoxifen or idoxifene as first-line endocrine therapy for their advanced disease. Complete plus partial response rates were 9 and 13% for tamoxifen and idoxifene, respectively. The median time to progression was slightly higher for idoxifene (140 versus 166 days), but these differences were not statistically significant. Morbidity was similar for both groups. The authors concluded that in postmenopausal women with metastatic breast cancer idoxifene had similar efficacy and toxicity to tamoxifen [298]. However, idoxifene has not been developed further because of concerns about uterine prolapse [299]. This side-effect is not seen with tamoxifen.

5.6.4
Droloxifene

Droloxifene (Figure 5.17), or 3-OH-tamoxifen, is a mimic of the tamoxifen metabolite 3,4-di-OH-tamoxifen that has weak estrogenic properties in the mouse and weak antiestrogenic actions [151, 300]. Droloxifene has antitumor activity in laboratory animals [301], and does not form DNA adducts under laboratory conditions or produce liver tumors in rats [301, 302]. Droloxifene maintains bone density in rats [303, 304], but clinical trials for the prevention of osteoporosis have not been reported. Droloxifene also reduces LDL cholesterol and lipoprotein(a) to a greater degree than conjugated estrogen in postmenopausal women [305]. However, like tamoxifen and raloxifene, droloxifene does not increase HDL cholesterol. Droloxifene also dramatically reduces fibrinogen.

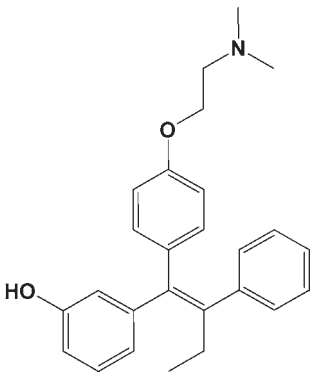

Figure 5.17 Droloxifene.

These data led to the extensive clinical testing of droloxifene in stage IV breast cancer [306]. In a phase III trial for treatment of ER- and/or PR-positive advanced breast cancer, droloxifene was found to be significantly less effective than tamoxifen overall [307]. As might be anticipated for an agent that has rapid clearance because it is rapidly conjugated by phase II metabolizing enzymes [308, 309], doses of 60 mg daily were used in its clinical trials, and may explain why droloxifene was inferior to tamoxifen. Its further clinical development has therefore been stopped.

5.6.5 Ospemifene

Ospemifene (deaminohydroxytoremifene, FC-1271a; Figure 5.18), is related to metabolite Y formed by the deamination of tamoxifen [234]. Metabolite Y has a very low binding affinity for ER [234, 239] and has weak antiestrogenic properties compared with tamoxifen. Ospemifene is a known metabolite of toremifene (4-chlorotoremifene) but unlike tamoxifen, there is little carcinogenic potential in animals [310]. It is possible that the large chlorine atom on the 4-position of toremifene and ospemifene reduces α-hydroxylation to the ultimate carcinogen related to α-OH-tamoxifen. Ospemifene has very weak estrogenic and antiestrogenic properties *in vivo* [311], but demonstrates SERM activity in bone and lowers cholesterol. The compound is proposed to be used as a preventative for osteoporosis. A phase II trial demonstrated that ospemifene decreased bone resorption markers and increased bone formation markers in postmenopausal women as well as did raloxifene with one difference; the highest dose of ospemifene evaluated (90 mg) increased the bone formation marker procollagen type I N propeptide significantly more than raloxifene [312]. Interestingly enough, unlike raloxifene, ospemifene has a strong estrogen-like action in the vagina, but neither ospemifene nor raloxifene affect endometrial histology [313, 314]. Overall, the goal of developing a bone-specific agent is reasonable, but the key to commercial success will be the prospective demonstration of the prevention of breast and endometrial cancer as beneficial side-effects. This remains a possibility based on prevention studies completed in the laboratory [315, 316].

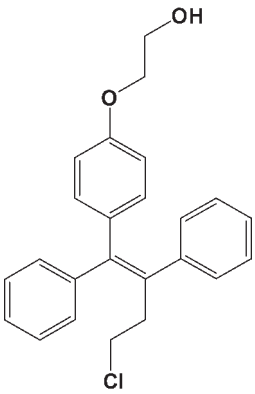

Figure 5.18 Ospemifene.

5.6.6
GW5638 and GW7604

The search for other SERMs which act on ER with a mechanism distinct from tamoxifen and raloxifene has led to the identification of GW5638 (Figure 5.19), a structural analog of tamoxifen, except GW5638 contains an acrylate side-chain that replaces the dimethylaminoethoxy side-chain in tamoxifen. Like tamoxifen, GW5638 is metabolized to its hydroxylated derivative, GW7604 (Figure 5.20), analogous to 4-OH-tamoxifen. GW5638 behaves as an antiestrogen in breast, shows minimal uterotrophic activity in ovariectomized rats, yet protects against bone loss and decreases serum cholesterol [317, 318]. Transcriptional luciferase reporter gene studies have shown that GW5638 inhibits the agonistic activity of E_2, tamoxifen and raloxifene, and derepresses the antagonist activity of the pure antiestrogen fulvestrant [318]. GW5638 inhibits the growth of E_2-dependent MCF-7 breast tumors in athymic mice [319], and is better able than raloxifene to block E_2-induced growth of endometrial tumors [320]. Extensive clinical trials would be appropriate if further laboratory data are obtained.

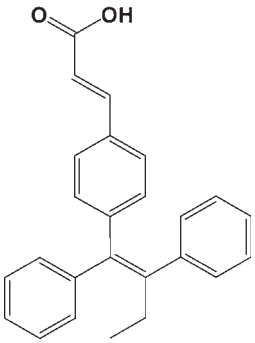

Figure 5.19 GW5638.

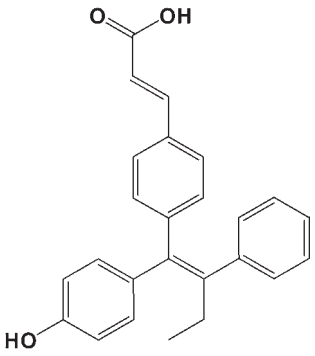

Figure 5.20 GW7604.

GW5638 also shows promise for development as a second-line agent of advanced breast cancer; in contrast to raloxifene, GW5638 effectively blocks the growth of tamoxifen-resistant breast tumors in athymic mice [319, 320]. Thus, the mechanism of antitumor action of GW5638 is different from that of tamoxifen as it downregulates ER protein levels [321]. These characteristics indicate that GW5638 could be further developed as second-line therapy for the treatment of advanced breast cancer and could prove beneficial in the adjuvant and preventive settings.

GW5638 induces a unique conformational change in ER. Using phage display, synthetic peptides which interact with GW5638-bound ER were identified that did not interact with tamoxifen-, raloxifene- or fulvestrant-bound ER [319], indicating that the conformation of GW5638-bound ER is unique relative to the other antiestrogen-bound ER complexes. In MDA-MB-231 cells stably transfected with wild-type ER, 4-OH-tamoxifen acts as an agonist, but GW7604 acts as an antagonist. However, in MDA-MB-231 cells stably transfected with a mutant ER containing Asp351 → Tyr, GW7604 acts as an agonist [322]. Thus, Asp351 serves as a molecular switch, determining the mode by which GW5638 acts. Molecular modeling has indicated that the dimethylamino moiety of 4-OH-tamoxifen weakly interacts with Asp351, but the acrylate side-chain of GW7604 would be deprotonated at physiologic pH and therefore repulse Asp351 [322]. In contrast, the crystal structure of GW5638 complexed with ER [321] suggests that because the acrylate side-chain of GW5638 is buried by hydrophobic residues, the acrylate side-chain would be protonated instead of deprotonated, and allow formation of hydrogen bonds between it and Asp351. Noteworthy, the ER–GW5638 crystal was formed at acidic pH. Also according to the crystal structure, when 4-OH-tamoxifen complexes with ERα, ERα's H12 takes on the position otherwise occupied by the coactivator in a wedge formed by H3, H4 and H5. However, the acrylate side-chain of GW5638 forms water-mediated hydrogen bonds with Leu536 and Tyr537 located at the N-terminus of H12, drawing this portion of H12 closer to the ligand. These hydrogen bonds then cause an approximate 50° difference in the orientation of H12 between the GW5638–ERα and the 4-OH-tamoxifen–ERα structures. This rotation of H12 leads to exposure of hydrophobic residues in H12 to the protein exterior, and hence, increases surface hydrophobicity. E_2 and fulvestrant bound to ER also increase the surface hydrophobicity of ER, which is linked to decreased protein stability. Similarly, GW5638 leads to decreased ER protein levels, likely by increasing ER surface hydrophobicity [321] and a hyperubiquitinated ER complex [251], but does not cause as much degradation of ER as does fulvestrant. A recent structure activity study of GW7604 confirmed that the acrylate side-chain was critical for the downregulation of ER levels in MCF-7 breast cancer cells [323]. In contrast, 4-OH-tamoxifen does not increase ER surface hydrophobicity and hence allows for a more stable ER protein. Therefore, GW5638 and GW7604 may exert more antiestrogenic activity than tamoxifen and raloxifene.

5.6.7
Lasofoxifene

Lasofoxifene (CP-336156; Figure 5.21) is a diaryltetrahydronaphthalene derivative [324] that has been reported to have high binding affinity for ER and have

potent activity in preserving bone density in the rat [325, 326]. Lasofoxifene also exerts potential cardioprotective effects of estrogen, but lacks estrogen's endometrial cancer risks [327]. The structure of CP-336156 is reminiscent of the putative antiestrogenic metabolite of nafoxidine [328] that failed to become a breast cancer drug because of unacceptable side-effects [329]. There are two diastereometric salts. CP-336156 is the *l* enantiomer that has 20 times the binding affinity of the *d* enantiomer. Studies demonstrated that the *l* enantiomer had twice the bioavailability of the *d* enantiomer. The authors ascribed the difference to enantioselective glucuronidation of the *d* isomer [324]. An evaluation of CP-336156 in the prevention and treatment of *N*-nitroso-*N*-methylurea (NMU)-induced rat mammary tumors showed activity similar to that of tamoxifen [330]. It is currently being evaluated in a worldwide phase III trial to determine if it can reduce the risk of vertebral fractures, breast cancer and cardiovascular disease in postmenopausal women [327].

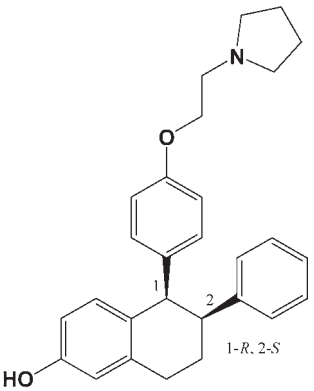

Figure 5.21 Lasofoxifene.

5.6.8
Levormeloxifene

Levormeloxifene (Figure 5.22) is the *l* enantiomer of the racemic chromane ormeloxifene (centchroman), which was marketed in India as a birth control pill [331]. Levormeloxifene was intended for prevention of osteoporosis and coronary artery atherosclerosis. In rabbits, it lowered plasma cholesterol and reduced atherosclerosis to a similar extent as estrogen in rabbits, without estrogenic effects on uterine tissue [331]. In ovariectomized cynomolgus monkeys, levormeloxifene prevented increases in serum markers of bone turnover induced by the ovariectomy and inhibited loss of lumbar spine bone mineral density compared to placebo [332]. The beneficial effects of levormeloxifene translated to the clinic [333], it decreased LDL cholesterol more than HRT without changing HDL cholesterol compared to placebo in healthy postmenopausal women. Levormeloxifene also increased bone mineral density in the spine, total hip and total body, and decreased bone turnover markers. However, unlike in rabbits, levormeloxifene increased endometrial

thickness. It was later shown in rats [334] and pigs [335] that levormeloxifene exerted estrogenic effects on the uterus. In other clinical studies in healthy postmenopausal women, beneficial effects on bone and LDL cholesterol were seen [336, 337], but in one study an additional estrogen-like effect of levormeloxifene was also found on the hypothalamic–pituitary axis since 50% reductions in FSH and LH were observed [336]. Levormeloxifene exhibited rapid absorption with slow elimination (plasma half-life of approximately 1 week in postmenopausal women) [336, 338], which led to small fluctuations in steady-state plasma concentration and drug accumulation. The slow elimination of levormeloxifene was consistent with the inability to determine a minimal effective dose of the drug in clinical trials and, importantly, may also help explain the noted increase in endometrial thickness associated with the compound. Endometrial safety of levormeloxifene was explored by monitoring closely its effects on endometrial thickness and the serum level of an endometrial secretory protein, placental protein 14, in healthy postmenopausal women compared to raloxifene or placebo [339]. At all doses used, levormeloxifene induced large increases in endometrial thickness and placental protein 14 compared to raloxifene. Concurrently, a large multicenter phase III study to evaluate levormeloxifene on osteoporosis was halted after 10 months because of multiple adverse gynecologic and other events, including increased endometrial thickness, enlarged uterus, uterovaginal prolapse and urinary incontinence [340, 341]. Hence, further clinical development of levormeloxifene has been stopped.

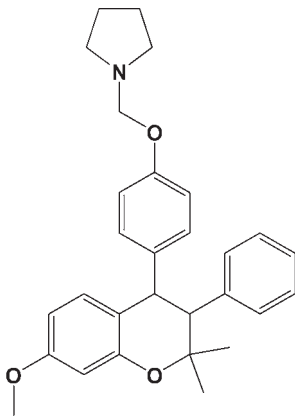

Figure 5.22 Levormeloxifene.

5.6.9 CHF 4227

CHF 4227 (Figure 5.23) is a benzopyran derivative that binds ERα and ERβ with high affinity, and exhibits an improved SERM profile. In rats, CHF 4227 inhibited estrogenic effects on uterine weight gain about 25-fold more potently than raloxifene, while also preventing ovariectomized induced bone mineral density loss and an

improved serum lipid profile comparable to the estrogen, whereas raloxifene was less effective and about 100-fold less potent [342]. Importantly, CHF 4227 also prevented 7,12-dimethylbenzanthracene (DMBA)-induced mammary carcinomas [342]. A separate preclinical study confirmed the beneficial effects of CHF 4227 on bone with no uterotropic effects and also noted that lasofoxifene was less effective than CHF 4227 in the preservation of trabecular microarchitecture [343]. In a clinical trial designed to evaluate tolerability, safety and its pharmacological profile [344], CHF 4227 did not cause any increases in endometrial thickness or placental protein 14, or any vaginal bleeding. CHF 4227 decreased total and LDL cholesterol. It also decreased bone resorption markers. Like levormeloxifene, CHF 4227 showed a long elimination half-life, but unlike levormeloxifene, CHF 4227 was slowly absorbed [344]. Hence, CHF 4227 shows promise as a therapeutic since it appears efficacious, was well tolerated, and seems to exhibit an improved safety profile compared to levormeloxifene.

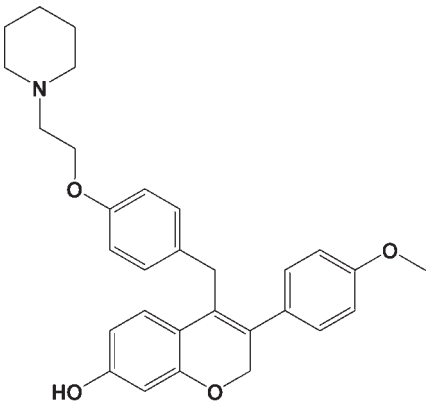

Figure 5.23 CHF 4227.

5.6.10
EM-800 and Acolbifene

EM-800 (Figure 5.24) is a chromene prodrug [324] of the active agent EM-652 (SCH57068) that is now called acolbifene (Figure 5.25). The agent is routinely drawn to show the similarity of side-chain position to the pure antiestrogen fulvestrant (Figure 5.30); however, the compound is a SERM. The advantage with EM-800 and acolbifene is that they are both pure (*S*) enantiomers. Resolution of the active (*S*) enantiomer from the less active (*R*) enantiomers EM776 and EM-651 confers higher binding affinity for ER. A comparison of the potent benzopyran described by Sharma *et al*. [345] (referred to as EM312 by Gautier *et al*. [324]) with acolbifene on the proliferation of ZR-75-1 and T47D cells shows that acolbifene is 9 and 28 times more potent, respectively [324].

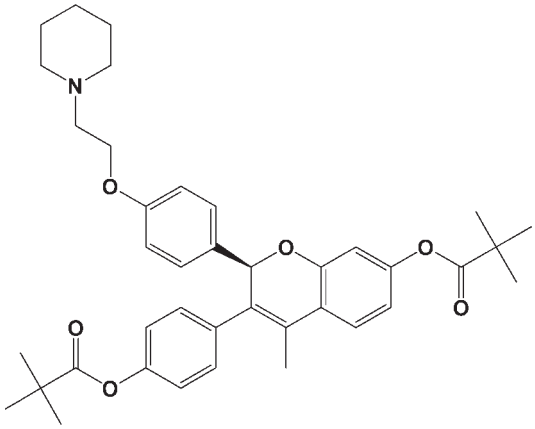

Figure 5.24 EM-800.

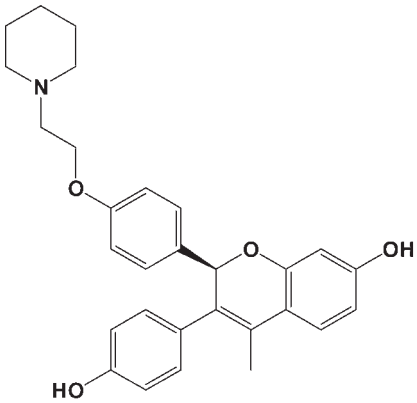

Figure 5.25 Acolbifene.

The compound EM-800 and its active metabolite acolbifene are both orally active agents with virtually no uterotropic activity [324]. EM-800 is an active antitumor agent in the rat DMBA model [346, 347], and long-term studies in the mouse show clear-cut antiestrogenic activity [348] with little or no estrogenic activity compared with either tamoxifen or toremifene [349, 350]. The drug is extremely potent against breast and endometrial cancer cells in culture [351, 352] and prevents the growth of estrogen stimulated tumor xenografts in athymic mice [353]. However, unlike fulvestrant, which has an expected negative effect on bone density [354], EM-800 does not decrease bone density in the rat [346].

Acolbifene is misclassified as an orally active pure antiestrogen [91, 349, 355] and as such could be tested as a second-line therapy following tamoxifen failure. The antiestrogenic side-chain of acolbifene would seem to be too short for optimal pure antiestrogen activity [356]. On the basis of the structural similarity of acolbifene with other benzopyrans and raloxifene analogs one would predict that acolbifene would be a SERM. A recent report demonstrates that acolbifene and

raloxifene both have the antiestrogen side-chain interacting with amino acid 351 in ER [357]. The Asp351 → Tyr ER mutant converts both acolbifene and raloxifene to an estrogenic complex, whereas fulvestrant does not. On the basis of these data, there is potential that acolbifene may fail as a second-line therapy after drug resistance to tamoxifen develops. In a phase II study to determine the efficacy and safety of EM-800 [358], 43 postmenopausal women with advanced breast cancer who had progression of disease while on tamoxifen were randomized to receive two different doses of EM-800. An objective response was seen in 12% of patients with one patient experiencing a complete response. Hence, acolbifene's cross-resistance with tamoxifen is incomplete, yet applying this agent in first-line therapy before antihormonal resistance develops would seem to be more appropriate.

5.6.11
Arzoxifene

The benzothiophene arzoxifene (LY353381; Figure 5.26) is the same molecule as raloxifene except for replacement of the ketone group with a methoxy group. The methoxy modification results in decreased metabolic elimination of arzoxifene and improved bioavailability over raloxifene [359]. Arzoxifene displays exceptionally high affinity for ER in comparison to other SERMs. *In vitro* and *in vivo* studies have demonstrated that arzoxifene exhibits a 10-fold increase in antiestrogen potency, and it does not promote uterine growth [359]. Arzoxifene is partially cross-resistant with tamoxifen in models of drug-resistant breast and endometrial cancer [322, 360].

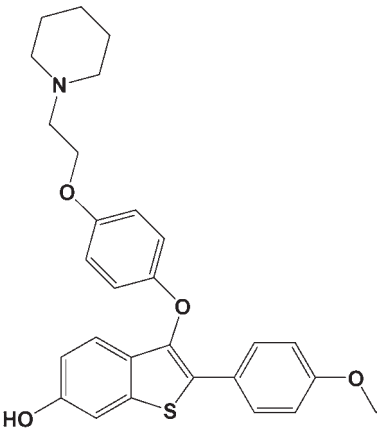

Figure 5.26 Arzoxifene.

In phase II clinical trials of women with advanced breast [361, 362] and endometrial [363] cancer, arzoxifene proved to be marginally effective (response rates 10–30%) with minimal toxicity. However, its main role may be in the prevention of breast cancer as with raloxifene [364]. Arzoxifene has been shown to be superior to

raloxifene as a chemopreventive in rat mammary carcinogenesis [69]. Therefore, due to arzoxifene's improved pharmacokinetic profile over raloxifene, arzoxifene should be evaluated in a chemoprevention trial to determine whether it would decrease the risk of invasive breast cancer more than raloxifene.

5.6.12 Bazedoxifene

Structurally similar to raloxifene, bazedoxifene (TSE-424; Figure 5.27) is under development as a SERM with improved tissue selectivity for use in the prevention and treatment of osteoporosis in postmenopausal women and in combination with HRT [365]. Bazedoxifene binds to ERα with a similar affinity as that of raloxifene, blocks E_2-induced growth of MCF-7 cells in culture, increases bone mineral density and compressive bone strength in ovariectomized rats, yet promotes less uterotropic effects than raloxifene in an immature rat uterine model, and when coadministered with raloxifene, reduces raloxifene-induced cellular hypertrophy [366]. Endometrial effects of escalating doses of bazedoxifene have been clinically evaluated in postmenopausal women [367]. Doses of bazedoxifene from 2.5 to 20 mg/day resulted in no significant changes in endometrial thickness or amenorrhea rates compared to placebo. Interestingly, increased doses of 30 and 40 mg/day bazedoxifene were significantly associated with both decreased endometrial thickness and uterine bleeding. This apparent antagonism in the endometrium is a unique characteristic of this SERM. Bazedoxifene is currently being evaluated in international phase III clinical trials with combined enrollment goals of 9,000 women for the prevention and treatment of postmenopausal osteoporosis [368]. Also like raloxifene, it may be worthwhile to evaluate bazedoxifene for use in breast cancer prevention.

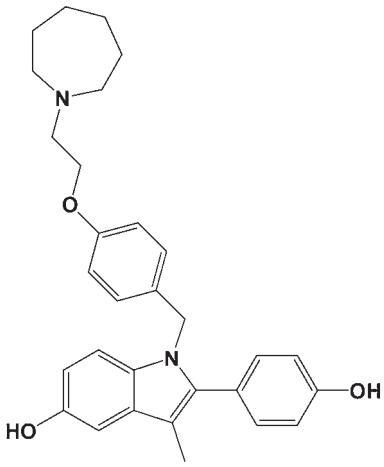

Figure 5.27 Bazedoxifene.

5.6.13
HMR 3339

Unlike the other SERMs, HMR 3339 (Figure 5.28) is a steroid. It is under clinical development for prevention of osteoporosis and coronary heart disease. In rats, HMR 3339 completely prevented bone mineral density loss and even increased it following ovariectomy. Further, HMR 3339 also increased bone mechanical strength at multiple sites following ovariectomy. HMR 3339's increases in bone mineral density and bone strength were more pronounced than raloxifene on cortical bone [369]. In healthy postmenopausal women, HMR 3339 promoted an antiatherogenic lipid profile by reducing total and LDL cholesterol, and decreased homocysteine levels, while not influencing HDL cholesterol and lipoprotein(a), whereas raloxifene showed similar effects but did not reduce homocysteine levels [370]. In a separate clinical study exploring effects of HMR 3339 on markers of coagulation and fibrinolysis in healthy postmenopausal women [371], HMR 3339 at the highest dosage (50 mg daily) reduced antithrombin, protein C, and fibrinogen compared to placebo. At the lowest dosage (2.5 mg daily), HMR 3339 showed beneficial effects on some markers of fibrinolysis by decreasing tissue-type plasminogen activator, plasmin–α_2-antiplasmin complex and D-dimer compared to placebo. HMR 3339 also reduced the fibrinolysis inhibitor pro-carboxypeptidase U [372] and showed a dose-dependent reduction in C-reactive protein [373]. Additional beneficial effects of HMR 3339 on the cardiovasculature in postmenopausal women have been found by observing that it caused a dose-dependent decrease in the nitric oxide synthase inhibitor asymmetric dimethylarginine, whereas raloxifene did not [374]. Hence, HMR 3339 shows promise for protection of coronary heart disease, and perhaps osteoporosis.

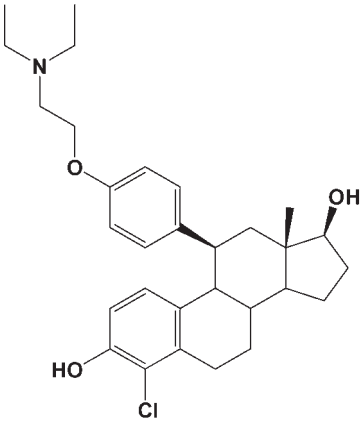

Figure 5.28 HMR 3339.

5.7
Pure Antiestrogens

By definition, a compound that is a pure or complete antiestrogen in all laboratory tests is unlikely to be selectively active in humans. To produce antiestrogen action at all sites, pure antiestrogens have a unique mechanism of action. The compounds have no intrinsic activity by preventing the formation of a transcription complex at target genes and the ligand enhances the ability of the ER complex to be destroyed. The use of pure antiestrogens for the adjuvant treatment of breast cancer is appealing if the benefits in lives saved are not confounded by increases in osteoporosis and coronary heart disease. Although pure antiestrogens were first described by Wakeling and Bowler [375] almost 20 years ago, there is remarkably little information about adverse effects of these drugs on bones and lipids. Drug development has been slow. The concern about increased risk of osteoporosis and coronary heart disease, as well as problems with drug delivery, has encouraged the development of aromatase inhibition as an alternative strategy for 'antiestrogen action' without the endometrial complications observed with tamoxifen. Nevertheless, there is clearly a strategic role for the pure antiestrogen fulvestrant (Figure 5.30) in the treatment of advanced breast cancer [376, 377] when the patient may or may not have received 5 years of adjuvant tamoxifen. Additionally, a pure antiestrogen could find a role in the adjuvant treatment of high-risk (four or more lymph node-positive) breast cancer. It is clear, however, that the application of a pure antiestrogen will compete with the established methods of estrogen deprivation with AIs (postmenopausal) or LH-releasing hormone superagonists (premenopausal) which would cause a medical oophorectomy [378, 379].

5.7.1
ICI 164,384 and Fulvestrant

The first-generation pure antiestrogen ICI 164,384 (Figure 5.29) is a 7α-substituted derivative of E_2 that has no detectable estrogen-like properties *in vivo* or *in vitro* [375, 380]. The compound was identified in a search for drugs that do not possess the estrogen-like effects of tamoxifen and that would, as a result, be more effective antitumor agents.

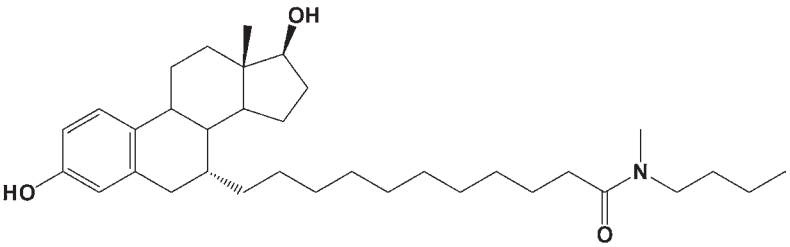

Figure 5.29 ICI 164,384.

Originally, the inspiration to substitute E_2 at the 7α-position came from the observation that ER could be purified on resin columns derivatized with E_2 through

a 7α-carbon chain linker of 10 atoms [381]. Structure activity relationships at the 7-carbon position clearly demonstrated that 7β substitutions are ineffective at producing antiestrogenic activity and the length of the carbon chain determines optimal activity for 7α substitutions [356].

Fulvestrant (Faslodex®, ICI 182,780; Figure 5.30) is a second-generation pure antiestrogen that is more potent than ICI 164,384 [382] and has fluorine atoms at the terminus of the 7α side-chain to retard metabolism to estrogen.

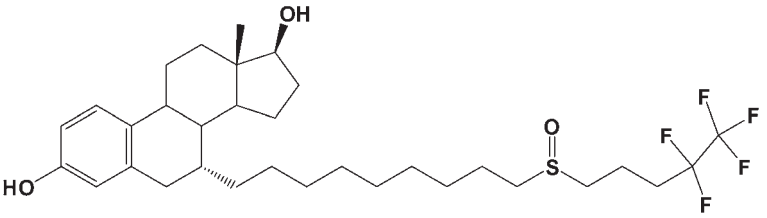

Figure 5.30 Fulvestrant.

Although the pure antiestrogen ER complex exerts no agonist activity there is another dimension to the mechanism of the pure antiestrogens that appears to be unique. Initially, it was believed that pure antiestrogens prevent the dimerizations of receptor complexes, thereby preventing the binding to EREs [383]. Clearly, if receptor complexes do not bind to any ERE, then no gene can be activated and the compound would be 'a pure antiestrogen'. However, investigators [383, 384] have subsequently demonstrated that the pure antiestrogen ER complex does bind to EREs, but both AF-1 and AF-2 are inactivated. What appears to be unique about pure antiestrogens is the observation that they provoke the rapid destruction of ER in breast cancer cells in culture [385], mouse uterus [386], and breast tumors *in situ* [387]. ER is synthesized in the cytoplasm and transported to the nucleus where it functions as a transcription factor. Once a pure antiestrogen binds to the newly synthesized receptor in the cytoplasm, transport of the ER complex to the nucleus is impaired [388]. Further, fulvestrant binding to ER induces increased surface hydrophobicity [321] and an abnormal conformation that leads to accelerated ubiquitination and shuttling of ER to the proteasome for degradation [389]. Although normal target cells could be affected in the long term, the loss of ER in a breast tumor cell will immediately prevent cell survival and result in tumor regression.

Crystal structures of ER complexed with pure antiestrogens would provide a wealth of mechanistic information, but generating these crystals has proved challenging. However, the crystal structure of the rat ERβ LBD and ICI 164,384 has been resolved, but due to internal disorder, the crystal had required treatment with *p*-chloromercuribenzenesulfonic acid [390]. This results in a distorted homodimer structure. There are several similarities and differences of the crystal structure of ICI 164,384 with ERβ when compared to that observed with raloxifene in ERα or ERβ [42, 45]. The bulky *para* substituted phenyl side-chains of raloxifene and 4-OH-tamoxifen occupy a narrow channel in ER, pushing H12 aside to silence AF-2. ICI 164,384 adopts a similar binding mode by flipping 180° about its longest

hydroxyl to hydroxyl axis (Figure 5.31). In this conformation, the 7α-substituted side-chain can exit the binding cavity. This molecular solution has been suggested previously to describe the antiestrogenic activity of the 11β-substituted estrogen RU 39,411 and ICI 164,384 [391]. The unique aspect of the X-ray crystallography is the finding that the long hydrophobic side-chain prevents the binding of H12 to the surface of the LBD. Although the side-chain exits the binding pocket in a manner identical to that observed with raloxifene, the side-chain is bent by 90° at its fifth carbon and interacts with the indole face of Trp290. The antiestrogenic side-chain is 6 Å longer than the side-chain of raloxifene so that it extends deep into the groove between H3 and H5. As a result, H12 cannot dock on the surface of the LBD [390]. This unique structure presumably results in the premature destruction of the complex by the proteasome.

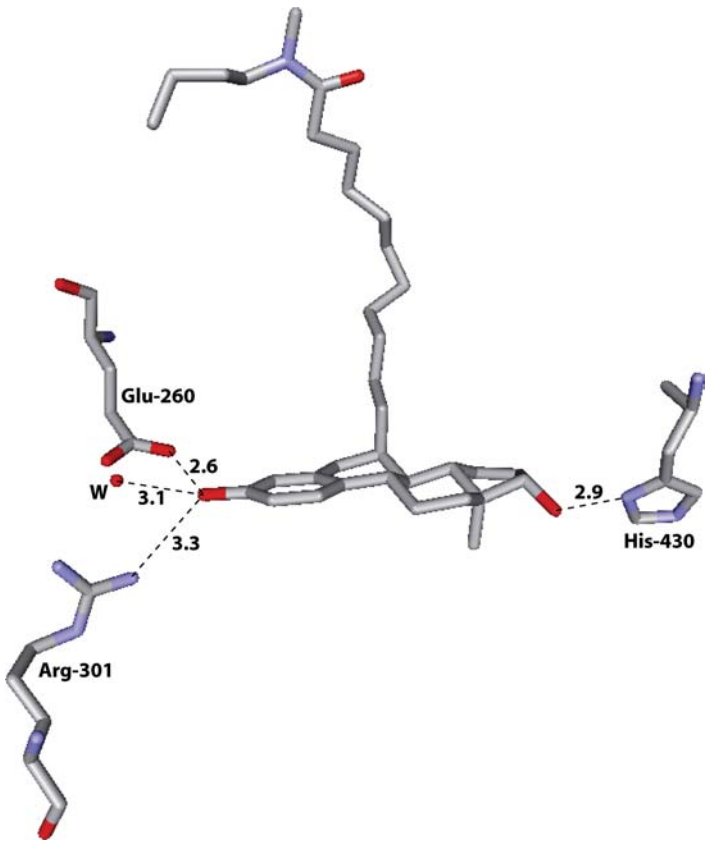

Figure 5.31 Hydrogen-bond interactions between ICI 164,384 and ERβ. Hydrogen-bond intermolecular interactions of ICI 164,384 cocomplexed with rat ERβ LBD using the X-ray crystallographic structure 1HJ1 at 2.3-Å resolution [390] are shown. The conformation was visualized using 3D-Mol Viewer as in Figure 5.5. Hydrogen bonds are indicated by dashed lines and their lengths in angstroms as determined by 3D-Mol Viewer. A highly ordered water molecule stabilized by a hydrogen-bond network is indicated (W). Carbon atoms are shown in gray, oxygen atoms in red and nitrogen atoms in blue.

Fulvestrant has been compared to tamoxifen for the treatment of advanced breast cancer in postmenopausal women. In a study of 587 patients, there was no significant difference between fulvestrant and tamoxifen at a median follow-up of 14.5 months for the primary endpoint of time to progression (median 6.8 versus 8.3 months, respectively); however, the tamoxifen group showed slightly better results. Hence, fulvestrant displayed similar efficacy to tamoxifen and was well tolerated [392].

The steroidal pure antiestrogens ICI 164,384 and fulvestrant are not cross-resistant with tamoxifen in laboratory models of tamoxifen-stimulated breast [227, 393], and endometrial cancer [132] grown in athymic mice. However, drug resistance to fulvestrant does occur in cell culture [394, 395]. Fulvestrant is active as a second-line agent, following tamoxifen failure for the treatment of advanced breast cancer [376, 377]. Two large multicenter randomized trials showed fulvestrant (250 mg, once monthly via intramuscular injection) to be as effective as the AI anastrozole for the treatment of postmenopausal women with hormone receptor-positive advanced breast cancer progressing on prior endocrine therapy. In one of the studies, 451 patients were randomized to receive fulvestrant or anastrozole. At a median follow-up time of 14.4 months, fulvestrant was as effective as anastrozole with a median time to progression of 5.5 months for fulvestrant and 5.1 months for anastrozole, and objective response rates of 20.7 and 15.7%, respectively [376]. In the other study, 400 advanced breast cancer patients whose disease had progressed on prior endocrine treatment were randomized to receive fulvestrant or anastrozole. Fulvestrant was as effective as anastrozole in terms of time to progression (median 5.4 months for fulvestrant versus 3.4 months for anastrozole) and objective response rates were 17.5% with both groups [377]. In both studies, the treatments were well tolerated [377]. Hence, the drug is approved in the US and Great Britain as a second-line therapy for advanced breast cancer.

The destruction of ER and the removal of ER signal transduction by fulvestrant in antihormonal-resistant breast cancer were anticipated to be very effective, given its impressive results in the laboratory. However, response rates seen in these clinical trials have not been as good as initially anticipated, with only two in five tamoxifen-resistant patients responding to fulvestrant. One of the reasons for this may be the fact that plasma concentration levels achieved in clinical studies with the usual dose of 250 mg by intramuscular injection given once a month are significantly lower than those achieved in the media of most cell culture studies (100–1000 nM). After a single intramuscular dose of long-acting fulvestrant the mean minimum and maximum plasma concentrations achieved are 2.6 (4.3) and 8.2 ng/ml (13.5 nM), respectively (reviewed in Ref. [396]), and although a 2- to 3-fold accumulation with continuous dosing of fulvestrant has been observed, the mean concentrations after 6 months of the same dosing are only 6.1 ng/ml (10.05 nM) [396], which still remains significantly lower than that used in cell culture studies.

Although fulvestrant has not proven to be better than other therapies, it clearly represents an additional treatment option for women with breast cancer whose disease fails to respond to other therapies. Currently, its use as a third-line therapy results in a 28% rate of stable disease with a partial response in this group of patients. In addition, its high tolerability profile and novel mode of action offer the potential for

its use in combination with other therapeutic regimens. Indeed, a number of trials are currently evaluating the role of fulvestrant in association with other agents such as AIs, trastuzumab (HER2-targeted antibody), lapatinib (dual EGFR and HER2 tyrosine kinase inhibitor) and other agents [397, 398].

5.7.2 Additional Pure Antiestrogens

5.7.2.1 ZK-703 and ZK-253

Fulvestrant has proven to be as effective as the AI anastrozole in patients who relapsed during treatment with tamoxifen [376]; however, as described earlier it has very low bioavailability. To avoid this problem, the novel pure antiestrogenic compounds ZK-703 (Figure 5.32) and ZK-253 (Figure 5.33) have been developed [399]. Both are administered subcutaneously; however, ZK-253, a structurally optimized form of ZK-703, is orally bioavailable and retains its antiproliferative activity when administered via this route in *in vivo* xenograft breast cancer models. In these models, both ZK-703 (subcutaneous administration) and ZK-253 (oral administration) inhibited tumor growth better than either tamoxifen or fulvestrant. In MCF-7 xenograft tumors, subcutaneous ZK-703 led to very low levels of ER protein compared to controls. In rats, ZK-703 has shown greater oral bioavailability than in mice; hence, antitumor activity of ZK-703 and ZK-253 were evaluated in the DMBA- and NMU-induced rat mammary tumor models via oral administration [399]. ZK-703 and ZK-253 caused a nearly complete (greater than 80%) inhibition of DMBA-induced tumor growth and ZK-703 caused a 75% inhibition of NMU-induced tumor growth. Importantly, ZK-703 and ZK-253 still effectively inhibited growth of tamoxifen-resistant MCF-7 xenograft tumors, whereas fulvestrant was only moderately effective. Also, ZK-703 and ZK-253 led to lower ER protein levels than fulvestrant in this model. Thus, tamoxifen-resistant MCF-7 xenograft tumors are not cross-resistant to ZK-703 and ZK-253 [399]. These preclinical studies suggest that these compounds, similar to fulvestrant, may have a role in the treatment of breast cancer. The fact that ZK-253 appears to be active after oral administration may prove very important when these compounds are compared with fulvestrant. Clinical studies to determine the safety and efficacy of these compounds in humans are warranted.

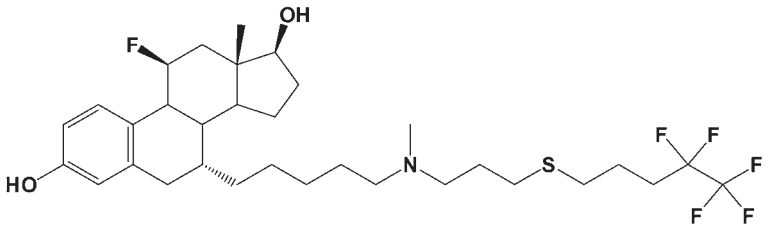

Figure 5.32 ZK-703.

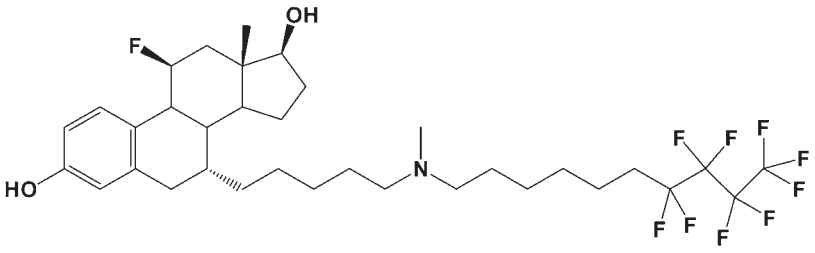

Figure 5.33 ZK-253.

5.7.2.2 **RU 58668**

The discovery of ICI 164,384 and fulvestrant stimulated a search for other potential agents. The compound RU 58668 (Figure 5.34) is substituted at the 11β position with a side-chain of comparable length and physical chemistry as that used for fulvestrant [400, 401]. RU 58668 causes a protein synthesis dependent paralysis of ER in the particulate fraction of the cytoplasm that depends entirely on an intact LBD [402]. Indeed, the authors [402] suggest that antiestrogens that block ER nuclear localization will behave as pure antiestrogens *in vivo*. It has been reported to exert improved antiproliferative activities relative to fulvestrant in *in vitro* [400] and *in vivo* MCF-7 models [401]. RU 58668 also blocks E_2-induced increases in uterine weights without any uterotrophic effects by itself [400]. Like fulvestrant, RU 58668 leads to greatly enhanced proteasome-mediated degradation of ER [403, 404]. Hence, RU 58668 represents an alternative for pure antiestrogen therapy of breast cancer.

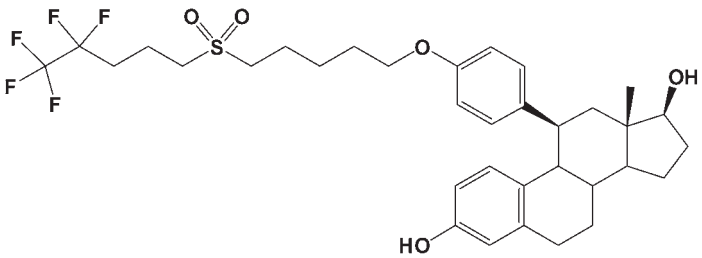

Figure 5.34 RU 58668.

5.7.2.3 **TAS-108**

TAS-108 (SR 16234; Figure 5.35) is a steroidal compound that acts as a high-affinity pure antagonist of ERα. Its mechanisms of action are different than SERMs and fulvestrant. TAS-108 recruits corepressors to ERα without affecting AF-1 activity or DNA-binding [405]. Unlike the SERMs 4-OH-tamoxifen and raloxifene, which activate transcription of the Asp351 to Tyr mutant of ERα (ERα Asp351 → Tyr) isolated from a tamoxifen-resistant xenograft MCF-7 tumor [245, 249, 406], TAS-108 fails to activate transcription mediated by this ERα mutant [405]. Unlike fulvestrant, TAS-108 does show partial agonistic activity for ERβ; TAS-108 promotes recruitment of the coactivator SRC-2 to the AF-2 of ERβ [407]. This partial ERβ agonism may

explain why this drug does not cause increased loss of bone density. However, TAS-108 shows only a minimal uterotrophic effect in ovariectomized rats [407] and no uterotrophic effects in humans by trans-vaginal ultrasound evaluation [408]. Preclinical studies have shown that this agent exerts antitumor effects in DMBA-induced rat mammary tumors and in xenograft MCF-7 breast tumors [407]. In contrast to fulvestrant, which is administered clinically by intramuscular injection, TAS-108 is administered orally. In a phase I study, TAS-108 was administered at 40 mg/day, and dose escalation to 160 mg/day was well tolerated, showing only grade 1–2 toxicities (nausea, vomiting, hot flashes, headache, weakness and fatigue) and no maximum tolerated dose. A circulating mean C_{max} of 2.8–21.0 ng/ml (5.5–41.5 nM) was achieved. Moreover, evidence of antitumor activity was observed in this phase I study [408]. Currently, a phase II study is evaluating this drug and phase III studies are being planned [409].

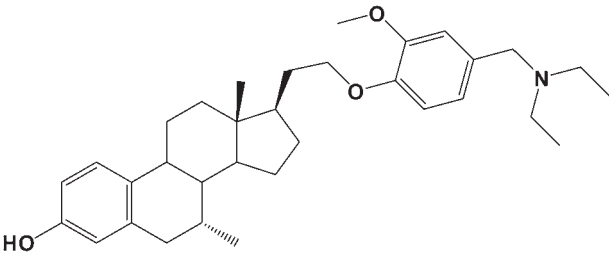

Figure 5.35 TAS-108.

5.8 Conclusions

Over the past 30 years, endocrine therapy has been proven to be the most advantageous targeted therapy for ER-positive breast cancer while sparing the patient from the debilitating toxicities of chemotherapy. Five years of adjuvant tamoxifen therapy for all stages of ER-positive breast cancer, node-positive and -negative, confers a clear-cut survival advantage. Tamoxifen is also used as a preventive in both pre- and postmenopausal women at high risk for breast cancer. Tamoxifen has clearly been successful and serves as the prototype for the development of newer SERMs such as raloxifene that lack tamoxifen's undesirable utertrophic side-effect. Numerous SERMs are currently under development not only for use in breast cancer, but also for use in osteoporosis and coronary heart disease. The pure antiestrogen fulvestrant has now been established as a second-line therapy to inhibit the growth of breast tumors after tamoxifen failure by targeting ER for ubiquitin-mediated degradation. New pure antiestrogens are under development which exhibit improved oral bioavailability over fulvestrant. A possible mechanism of antihormonal resistance includes cross-talk among growth factor receptor signaling, the ER and a change in the balance of coactivators to corepressors recruited to ER. The next challenge is to

build on the success of antihormonal therapy and identify further targets to enhance antitumor activity.

Acknowledgments

Dr. Jordan is supported by the Department of Defense Breast Program under award number BC050277 Center of Excellence (Views and opinions of, and endorsements by the author(s) do not reflect those of the US Army or the Department of Defense), SPORE in Breast Cancer CA 89018, R01 GM067156, FCCC Core Grant NIH P30 CA006927, the Avon Foundation and the Weg Fund of Fox Chase Cancer Center.

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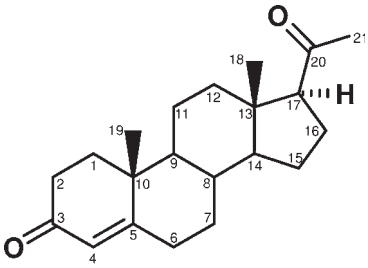
6
Progesterone Receptor: Overview of Modern Steroidal Progestins and Developments in the Field of Nonsteroidal Selective Progesterone Receptor Modulators

Klaus Schöllkopf and Norbert Schmees

6.1
Introduction

Progesterone is the natural hormone that is essential for the initiation and maintenance of pregnancy. Progesterone is also a central intermediate in the biosynthesis of androgens, estrogens and corticosteroids (Scheme 6.1).



progesterone (**1**)

Scheme 6.1

Compounds that produce the same biological effects as progesterone are called progestins [1]. These effects are mediated by the progesterone receptor (PR), which is an important drug target due to its prominent role in the female reproductive cycle.

The ripening follicle secretes estradiol, this increase leads to the surge of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) produced in the pituitary gland. This leads to ovulation and the follicle changes into the corpus luteum, which produces large amounts of progesterone. The increase of the serum levels of progesterone induce the transformation of the proliferated endometrium to prepare for implantation of the blastocyst. If implantation occurs, the blastocyst produces human chorionic gonadotropin (hCG), a hormone that plays

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

an important role in maintaining the corpus luteum and the continuous production of progesterone. These increased levels of progesterone lead, via negative feedback to the hypothalamus and the pituitary gland, to the suppression of the gonadotropins and as a consequence to the lack of ovulation during pregnancy. If there is no implantation at the end of the menstrual cycle the progesterone levels fall, and menstruation and endometrial repair occur.

This antiovulatory effect of progesterone is the pharmacological basis for the oral contraceptives which all contain progestins.

In this chapter the biological function of PR, its structural properties and therapeutic indications for progestins will be described. Since steroidal progestins have now been in clinical use for several decades, the focus of this chapter will be more on the current developments, particularly in the area of nonsteroidal progestins mainly with a medicinal chemistry perspective.

Several aspects of this chapter are also covered in recently published review articles [2–4].

6.2 Biology of PR

6.2.1 Functional Aspects of PR

PR is, like the other classic steroid hormone receptors (Figure 6.1), a member of the nuclear hormone receptor superfamily of transcription factors. Within this group of the steroid hormone receptors the biological functions and respective ligands were discovered before the identification of the receptor proteins.

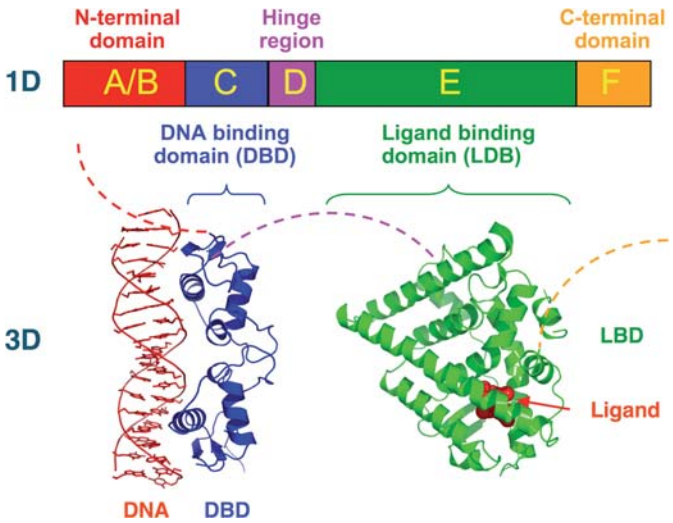


Figure 6.1 Structure of nuclear hormone receptors.

These receptors are multidomain proteins which consist of functionally independent subunits. The N-terminal domain contains transcriptional activating motifs and the DNA-binding domain (DBD) consists of two, highly conserved, cysteine-rich zinc finger motifs. The flexible hinge region is followed by the ligand-binding domain (LBD) which also contains transcriptional activating motifs [5].

In the absence of a ligand, PR is associated with a complex of heat shock proteins as a monomer in an inactivated state in the cytosol. When a progestin binds to the receptor, the overall conformation changes. Heat shock proteins are liberated, and the receptor dimerizes, enters into the cell nucleus and binds to selective progesterone-responsive elements (PREs) on the DNA to induce transcriptional activity.

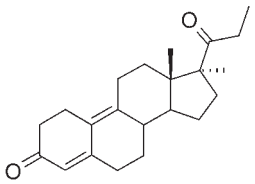
The PR is quite unique among the other members of the steroid hormone receptors. It is expressed in two isoforms in chicken, rats and humans [6]. The full-length isoform contains 933 amino acids and is called PR-B. PR-A is completely identical but lacks 164 amino acids in the N-terminal part. *In vitro* studies with transfected cells showed that PR-A had little transactivation activity by itself but inhibited PR-B activity [7]. The different activities of the two isoforms seem to be related to the differential recruitment of coregulators [8]. *In vivo* studies with mice lacking one of the two isoforms revealed that PR-A and PR-B show distinct but partially overlapping responses to estrogens and progestins. The activation of PR-A is necessary and sufficient for the establishment and maintenance of pregnancy, but has a lower efficacy on mammary gland responses than activation of PR-B. PR-B activation is, however, not sufficient to influence female fertility, but leads to proliferation in the mammary gland and the uterus. These observations of tissue-specific activities of PR isoforms support the hypothesis that isoform-specific modulators might have a different side-effect profile than the known progestins [9].

In addition to PR-mediated transcriptional activities, progesterone also triggers intracellular phosphorylation cascades of the Src/Ras/mitogen-activated protein kinase pathway via PR [10, 11]. Also independent of the transcriptional activities of the receptor are rapid nongenomic effects of progesterone like modulation of sperm acrosome reaction [12], prevention of preterm labor [13] and *Xenopus* oocyte maturation [14].

Some of these effects appear to be mediated by G-protein-coupled proteins.

6.2.2
Test Systems and their Use

The characterization of progestins usually starts with the determination of the binding affinity to PR by a standard displacement experiment. Traditionally these binding studies were performed with cytosol preparations of uteri of estrogen-primed rabbits. These preparations are incubated with the test compound in different concentrations and a radiolabeled reference, mostly tritiated progesterone or R5020 (promegestone **2**) (Scheme 6.2).



R 5020, promegestone (**2**)

Scheme 6.2

After equilibration, the bound reference progestin is separated from the unbound portion and quantitated. From the displacement curve the IC_{50} of the test compound is calculated and usually reported as relative binding affinity (RBA) with an RBA value of 100% for progesterone. Another standard reporting format for the binding affinity is the competition factor (KF) as a ratio of the IC_{50} values of the test compound and the reference progestin [15]. Since the successful cloning of human PR, the recombinant human LBD of PR has replaced the rabbit receptor in this assay.

Binding assays allow no distinction between agonists and antagonists, and therefore functional *in vitro* assays were developed based on mammalian cell lines, such as CHO, CV-1 or COS which contain PR. They are transiently or stably transfected with a hormone-responsive promoter such as the mouse mamma tumor virus (MMTV) promoter, linked to a suitable reporter gene. This receptor gene encodes for enzymes such as luciferase (Luc) or chloramphenicol acetyltransferase (CAT) that can easily be quantitated [16]. Addition of a progestin to the transfected cells triggers a cascade of events, starting with the binding to the receptor, activation of the hormone-sensitive promoter and production of the reporter enzyme. With these artificial transactivation assays, the test compounds can be classified either as agonists if they induce transcription or as antagonists if they block the effect of a reference progestin.

The binding assays as well as the transactivation assays can be adapted to high-throughput screening formats, and allow a suitable determination of relative affinities and efficacies even for large libraries.

Several animal models for the *in vivo* characterization of progestins were established at the beginning of the research in this field and are still established standards to evaluate the biological effects of new compounds [17].

Progesterone induces characteristic morphological changes of the endometrium after estrogen-induced proliferation of this tissue. This effect is the basis of the endometrium transformation assay, in which immature rabbits are primed with estradiol for several days and are subsequently treated with a progestin, either by oral application or by subcutaneous injection. The proliferating response of the uterus is examined by histological analysis and quantitated with the McPhail index on a scale from 1 to 4, with 4 as maximal effect. The assay is very sensitive and specific, even if the rabbit expresses only the B isoform of PR.

For the maintenance of pregnancy in rodents the presence of progesterone produced by the corpus luteum is essential. Removal of the ovaries (ovariectomization)

leads to termination of pregnancy. In the pregnancy maintenance test, pregnant mice or rats are ovariectomized and treated with a progestin subcutaneously. The effect of the compounds is easily measured by examination of the uteri of the animals.

6.3
Structure of the LBD of PR

PR as a member of the steroid hormone receptor family is closely related to three other members of this group: androgen receptor (AR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). Up to now, no X-ray crystal structures of the complete receptors have been described. However, several structures of the DBDs and specific LBDs have been published. In 1998, Williams and Sigler published the first structure of the PR LBD complexed with progesterone [18]. The main motif for the recognition of the 3-keto group of progesterone is a specific arrangement of Gln725, which is conserved in all steroid receptors binding 3-keto steroids, a constitutive water molecule and Arg766, which is also conserved in all steroid receptors. Several hydrophobic interactions with the steroid framework add to the overall binding, but surprisingly no strong hydrogen-bond interactions with the 20-keto group were detected. The two possible hydrogen-bond donors close to the 20-keto group, Asp719 and Thr894, are at 3.6–3.8 Å distance. It seems that Thr894, which is also conserved in all receptors binding 20-keto group-containing steroids, plays the more important role in progesterone binding. X-ray structures with additional ligands confirm the structure of the binding region for the 3-keto group and indicate that in position 17 or 20 of the steroid the receptor can accommodate also larger substituents [19–21]. See Figure 6.2.

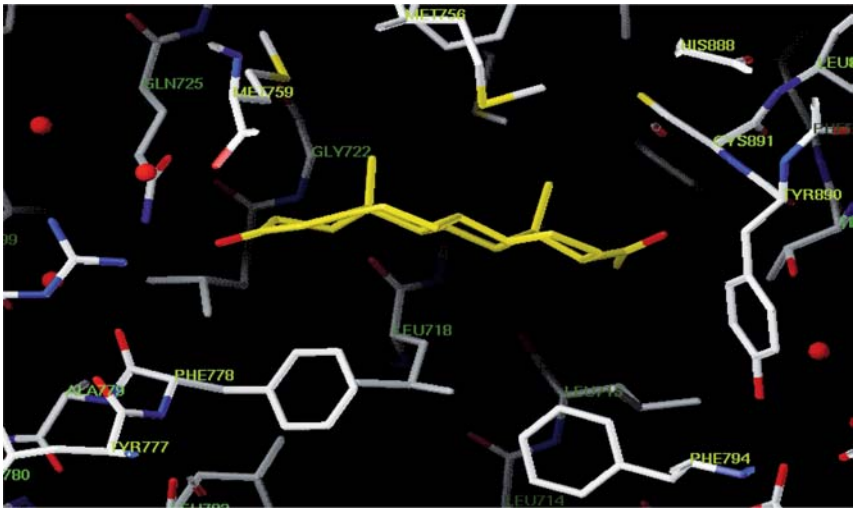


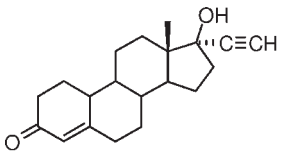
Figure 6.2 Progesterone in the LBD of PR.

Although the LBDs of the steroid receptors share only a low to medium sequence identity, the overall fold of the proteins is very similar and consists of a unique 'helical sandwich' arrangement. The progesterone LBD contains 10 α-helices arranged in the same way and, as in the other receptors, the orientation of helix 12 differs whether an agonist or an antagonist is bound.

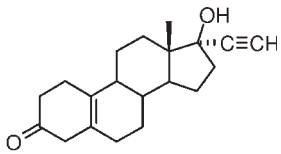
6.4 Steroidal Progestins

The development and the properties of steroidal progestins have been described in several review articles [2, 22–24], therefore this chapter will give only a brief overview about the historical development in this field and will focus on the so-called 'new progestins' [25].

Progesterone exhibits weak antiandrogenic and antimineralocorticoidal activities besides its main activities mediated by PR. It was isolated from pig ovaries and crystallized in pure form by several research groups in 1934 [26–29]. By this time it was already known that it prevented ovulation and could therefore be used for fertility control. Considerable synthetic efforts provided access to larger quantities of progesterone starting from naturally occurring steroids such as stigmasterol or diosgenin. However, progesterone is almost inactive after oral application due to its absorption and stability properties. One of the major breakthroughs in the development of progestins was the finding by Inhoffen and Hohlweg that 17α-ethinyl-testosterone or ethisterone exhibits progestogenic activity after oral application and a largely diminished androgenic potency compared to the parent molecule testosterone [30]. Further optimization led to the 19-nor-testosterone derivatives norethisterone (**3**) and norethynodrel (**4**), which were subsequently the first progestins to be approved in combination with an estrogen as oral contraceptives (Scheme 6.3).



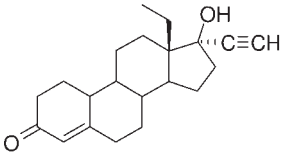
norethisterone (**3**)



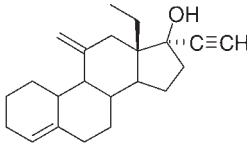
norethynodrel (**4**)

Scheme 6.3

Derivatives of 19-nor-testosterone represent one of the two main structural classes of steroidal progestins. Levonorgestrel (**5**), desogestrel (**6**) and gestoden (**7**) still retain a weak androgenic activity, and they share the 13-ethyl group that increases the progestogenic potency compared to the corresponding natural 13-methyl series. All progestins of the 13-ethyl series are prepared via total synthesis (Scheme 6.4).



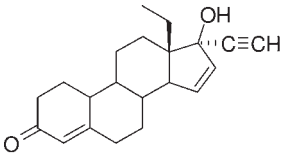
levonorgestrel (**5**)



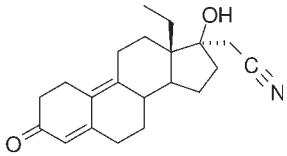
desogestrel (**6**)

Scheme 6.4

Dienogest (**8**) is one of the newer progestins in this series. The 17α-cyanomethyl group and the 9,10-double bond are structural features that differ from the aforementioned progestins. In contrast to most of the other 19-nor-testosterone derivatives, dienogest has an antiandrogenic activity [31]. This effect seems to have a favorable influence on the lipid metabolism of women treated with dienogest in combination with ethinylestradiol compared to a levonorgestrel/ethinylestradiol combination (Scheme 6.5) [32].



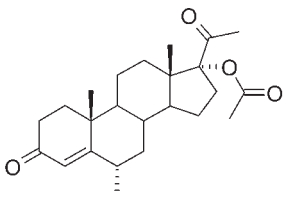
gestodene (**7**)



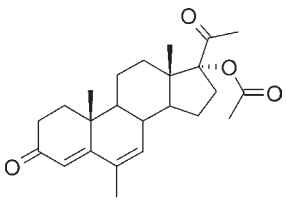
dienogest (**8**)

Scheme 6.5

The second structural class of steroidal progestins consists of pregnane derivatives like medroxyprogesterone acetate (MPA) (**9**), megestrol acetate (**10**) or cyproterone acetate (**11**). The introduction of a 17α-acetoxy group and different substituents in the 6-position of the parent molecule progesterone leads to an increase in metabolic stability and to orally active compounds. Cyproterone acetate was the first progestin with a potent antiandrogenic effect and it is still in use for the treatment of prostate cancer (Schemes 6.6 and 6.7).



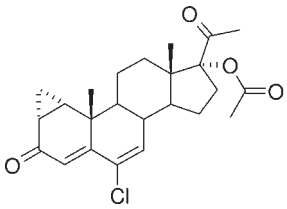
medroxyprogesterone acetate (**9**)



megestrol acetate (**10**)

Scheme 6.6

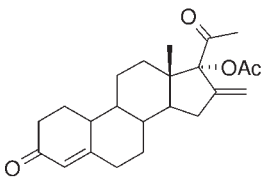
Nestorone (**12**) is a member of the group of 19-nor-pregnane derivatives. It was developed by the Population Council and is one of the most potent progestins. Its profile at other steroid receptors is quite neutral. However, nesterone is not orally active, thus its use is limited to transdermal applications or implant devices.



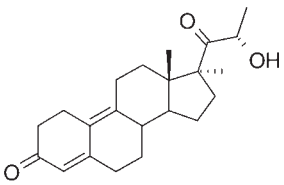
cyproterone acetate (**11**)

Scheme 6.7

Promegestone (**2**) belongs to the same structural class and its 21-hydroxy derivative trimegestone (**13**) is a very potent and pure progestin regarding its activities mediated by other steroid hormone receptors. The 17α-methyl group and the 9,10-double bond are structural elements in this series that are required for oral bioavailability. Acyloins such as in trimegestone are usually quite labile stereochemically. Therefore the development of trimegestone to an approved product for hormone replacement therapy is a remarkable achievement (Scheme 6.8).



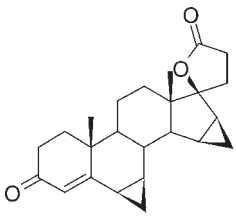
nestorone (**12**)



trimegestone (**13**)

Scheme 6.8

Drospirenone (**14**) is derived from the aldosterone antagonist spirolactone. The lactone ring in position 17 differs from all other progestins, and together with the two cyclopropyl rings in position 6,7 and 15,16 it adds to the unique profile of drospirenone. Apart from being a progestin, drospirenone is a very potent antimineralocorticoid and it also shows antiandrogenic activity [31, 33–36]. This pharmacodynamic profile is quite similar to the natural hormone progesterone (Scheme 6.9).



drospirenone (**14**)

Scheme 6.9

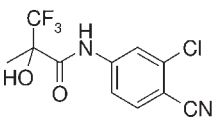
The antimineralocorticoidal activity of drospirenone is considered as a main benefit. It counteracts the salt and water retention effects of ethinylestradiol, the standard estrogen component in oral contraceptives [33].

6.5
Nonsteroidal Progestins

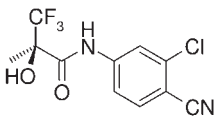
The discovery of nonsteroidal compounds with progestogenic activity, which at first sight have structurally nothing in common with the steroids, initiated new activities in the field of progestins with the aim to find new compounds with marked differences in their pharmacological profile.

6.5.1
Flutamide Analogs

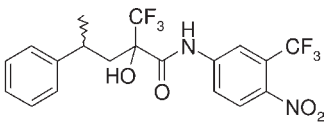
During optimization of the flutamide scaffold towards new nonsteroidal antiandrogen ligands, ICI reported the finding of nonsteroidal compounds to act on PR [37–39]. In this program, PR activity seemed to be only a cross-reactivity of their compounds. In first disclosures, no structure–activity relationship (SAR)-relevant data were presented. A later investigation of **15** (ZM 150271) and its eutomer **16** (ZM 172406) showed an antiprogestinic efficacy comparable to the standard antiprogestin mifepristone (RU-486; Roussel Uclaf) after oral administration in rats and monkeys at a dose of about 10 mg/kg. On the other hand, **17** (ZM 182345) showed agonistic activity on PR in the range of progesterone in animal models. Most compounds still exhibit significant activity at the AR. Some *in vivo* effects where not only explained by progestational activity, but by additional androgenic activity as well (Scheme 6.10).



15 (ZM 150271)



16 (ZM 172406)



17 (ZM 182345)

Scheme 6.10

6.5.2
Tetrahydropyridazines

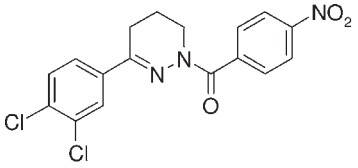
The tetrahydropyridazine [40–44] scaffold was discovered by screening efforts and its initial SAR was explored by a small library of analogs. The selection in the optimization focused on binding affinity against radiolabeled standard ligands at PR isolated from rabbit uterus. The introduction of electron withdrawing groups at the corresponding *para* and *meta* positions of the phenyl rings, such as in **18**, resulted in substantial gain in binding affinity ($IC_{50} = 100$ nM). The central part of the scaffold was described to be sensitive towards conformational changes by, for example, introduction of an additional double bond into the ring system or by changing the carbonyl moiety to a sulfonamide as in **19**. These changes resulted in a loss of binding affinity ($IC_{50} > 1000$ nM) in the initial series. The change of the carbonyl to the thiocarbonyl group or the shift of the carbonyl into the ring system was well

tolerated and the binding affinity was retained. As an extension, bridging between the tetrahydropyridazine ring and the directly attached phenyl ring resulted in an additional series of compounds with reported binding IC_{50}s of up to 0.5 nM (**20**). In the small set of compounds presented, the ring size and the nature of the bridge was modified and all compounds showed promising binding affinities ($IC_{50} \leq 100$ nM).

The screening was performed against different sources of PRs. PR isolated from human bone tissue was the most sensitive for this class of compounds. In contrast to marketed steroidal PR agonists, these compounds showed significant differences in the binding to other sources of PRs like T47D human breast carcinoma cells or receptor preparations from rabbit uterus. It was concluded that this effect could eventually lead to tissue-specific PR ligands.

Some compounds of this scaffold were investigated more intensively. In a comparative binding assay against other steroid hormone receptors [AR, GR and estrogen receptor (ER)], **21** (RWJ-26819) showed a higher selectivity for PR compared to R5020 (**2**). In a MMTV–CAT reporter gene transactivation assay, lead compounds such as **21** or **22** (RWJ 60130) had a 100- to 1000-fold reduced potency compared to R5020. Compound **21** was characterized *in vivo* in the endometrial transformation test in rabbits by McPhail indexing and in the ovulation inhibition model in cynomolgus monkey. In both *in vivo* models, **21** was about 1000-fold less active than R5020.

The compound class was further optimized towards antagonistic activity. A ring contraction to the pyrazoline scaffold and decoration of this ring with several substituents resulted in a series of compounds exemplified by **23**. This displayed weak antagonistic activity in the MMTV reporter gene transactivation assay in CV-1 cells compared to mifepristone (RU-486) (Scheme 6.11).



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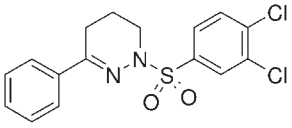

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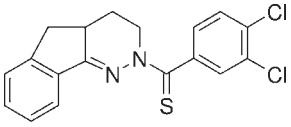

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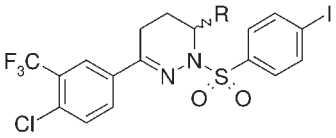
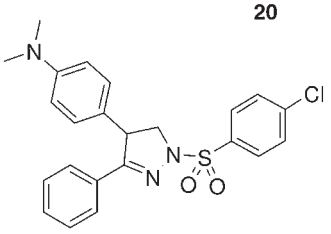


R = H: **21**
R = CH_3: **22**

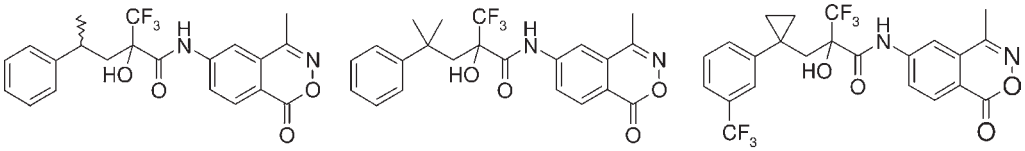


23

Scheme 6.11

6.5.3
Acylanilides

Related to the ICI structure **17** (ZM 182345), the acylanilide motif [45, 46] has been further optimized especially at the two aryl rings. The nitroaryl ring was replaced by a bicyclic methylbenzoxazinone system and the binding of **24** improved to a KF of 0.1 (ZM 182345 KF = 17; progesterone = 1). Furthermore, an additional methyl group at the central pentane chain eliminated the second chiral center and retained binding affinity in **25** with a KF = 0.55. Compounds such as **26** were characterized *in vivo*, and their activity in the maintenance of pregnancy test in rats was nearly an order of magnitude higher compared to R5020 and they had an activity in the ovulation inhibition assay comparable to R5020 in rats. The compounds showed a dissociation in efficacy between PR-A and PR-B which might result in tissue-specific effects (Scheme 6.12).



24 **25** **26**

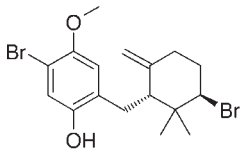
Scheme 6.12

6.5.4
Cyclocymopols

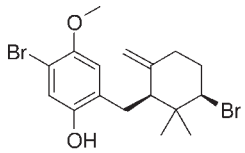
The cyclocymopol ligands **27** (LG 100127) and **28** (LG 100128) [47–49] were isolated as a 4:1 mixture of diastereoisomers from extracts of *Cymopolia barbata* alga collected in the Florida Keys. The difference between these compounds is the configurational change at one stereogenic center switching activity from agonism (**28**) to antagonism (**27**). Compared to its inactive biogenic relative cymopol (**29**), these two structures possess a cyclohexyl ring structure that seems to be the important key to gain activity for PR. A further optimization of these lead compounds on the aryl part as well as on the cyclohexyl part showed some flexibility in the substitution pattern. Most compounds were synthesized in the (*R*)-series in analogy to **27** and were antagonistic as well. In **30** the one bromine atom was eliminated and instead a nonpolar methyl group was added at the adjacent position to deliver a compound of comparable activity to **27**. Most other compounds synthesized were less potent but antagonistic as well.

The screening for the binding affinity showed K_i values reaching those of onapristone (ZK 98299, $K_i = 18$ nM at hPR-A), a steroidal reference antiprogestin similar to RU-486. This structural class of compounds has only weak binding to other steroid hormone receptors and is therefore highly dissociated. Activity of the cyclocymopol compounds has been evaluated in a cotransfected MMTV–Luc reporter gene transactivation assay in CV-1 cells. Antagonistic activity was compared to

mifepristone (RU-486) and onapristone (ZK 98299) and the best compounds were about 100–1000-fold less active in this assay. *In vivo* characterization of **31** in the decidualization assay in mice at a dose of 5 mg/kg showed a comparable efficacy to RU-486 at a dose of 0.1 mg/kg (Scheme 6.13).



27



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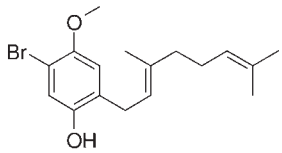

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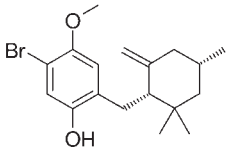

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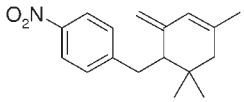


31

Scheme 6.13

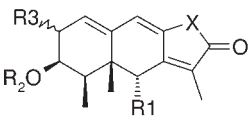
6.5.5
Tetrahydronaphthofuranones

As a different structural class derived from natural secondary metabolites, **32** (PF1092A), **33** (PF1092B) and **34** (PF1092C) [50–56] were isolated from cell cultures of *Penicillium oblatum*. The tricyclic tetrahydronaphthofuranone core ring system possess four stereogenic centers and the exact configuration has been proven by three-dimensional nuclear magnetic resonance, X-ray analysis and total synthesis.

The activity at PR has been shown in a binding assay against radiolabeled progesterone. The compounds inhibited the binding competitively, with **32** being the most active compound with an IC_{50} of about 10 nM. Minor variation of the compounds showed that position R1 can be changed from hydrogen to methyl without losing too much binding affinity (**35**). An acylation at R2 is important and several acyl groups improved binding affinity strongly. Position R3 was investigated with regard to stereochemistry and substitution, and compounds with the *trans*-configuration have the highest binding affinities. An important feature for more chemically stable test compounds was the replacement of the oxygen at the X position by an *N*-methyl group resulting in the tetrahydrobenzindolone ring system of **36**.

The initial assay was binding to PR and the compounds were compared to progesterone (RBA = 100) as standard. The most active compounds exceeded the relative binding affinity by a factor of about 2. Replacement of the acetate by cyclopropanoyl (**36**) or furanoyl resulted in RBAs of 230 and 91, respectively. Carbamates at this position resulted in RBAs of up to 188.

The compounds were investigated with regard to their agonistic/antagonistic profile by transactivation assay in T47D cells with the Luc reporter gene as the read out. The compounds show mixed agonistic/antagonistic profiles with a high tendency for antagonism. Their antagonistic potency has not been disclosed (Scheme 6.14).



	32	33	34	35	36
X	O	O	O	O	NCH_3
R1	H	H	H	CH_3	H
R2	Ac	H	H	Ac	
R3	OH	OAc	OH	H	OCH_3

Scheme 6.14

6.5.6
Tanaproget and Related Structures

One of the most intensively investigated pharmacophores is the bicyclic ring structure core carrying an aryl group at the 6-(5-)position (**37**) [57–67]. Several companies were highly engaged in this area and eventually highly active candidates were identified. Compound **38** can be seen as a starting point. Electron-withdrawing substituents at the *meta* position of the aryl group connected at the 6-position of the scaffold delivered a first active series of antagonistic compounds such as **39**. This compound has a binding affinity of $IC_{50} = 10$ nM and a potency of 30 nM (82% efficacy) in a transactivation assay in CV-1 cells. Transactivation at other nuclear hormone receptors was only observed at doses higher than 1000 nM. The compound was investigated in an *in vivo* blastocyst implantation assay and showed 100% efficacy at an oral dose of 2.5 mg/kg. This effect was comparable to clinically established onapristone and was the first example of proven antiprogestinic activity of a nonsteroidal compound after oral administration. A study showed that the phenyl substituent can be replaced by some 5-ring heterocycles, such as a thiophene or a furan, to reach comparable activity, as well as selectivity, against the other steroidal hormone receptors GR, AR, MR and ER.

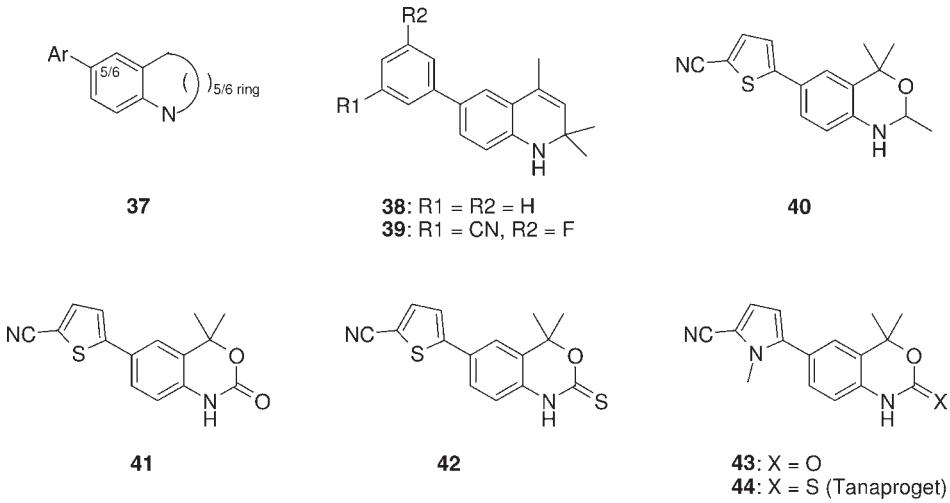
'Scaffold-hopping' from the dihydroquinoline system to the benzoxazine system switched activity from antagonism to agonism. The substitution pattern at the 6-aryl group was comparable to the findings at the initial scaffold. *Meta* substituents like cyano, fluoro or chloro improved binding and activity. The 2-position of the 1,3-benzoxazine scaffold was modulated sterically and electronically. One single methyl group showed a 10-fold stronger binding compared to the double methyl substitution. Compound **40** showed a binding affinity of $IC_{50} = 11.5$ nM and agonistic activity in the alkaline phosphatase assay with an $EC_{50} = 0.35$ nM (compared to the reference MPA $EC_{50} = 0.12$ nM). Changing the 2-position to a carbonyl group reversed the activity of the structure from agonism to antagonism. Although **41** is agonistically active in the alkaline phosphatase assay with an $EC_{50} = 61$ nM, in a cotransfected

PRE–Luc transactivation assay in CV1 cells it was antagonistic with an IC_{50} of 6.7 nM. This antagonistic effect was confirmed in an *in vivo* decidualization assay.

The activity of compound **41** was reversed to agonism by changing the carbonyl group into a thiocarbonyl group (**42**). Compound **42** was agonistic *in vitro* with a potency in the alkaline phosphatase assay of 0.7 nM.

A different way to change compound **41** from antagonism to strong agonism was the exchange of the 5-cyano-thiophen-2-yl substituent into the 5-cyano-1-methyl-pyrrol-2-yl substituent in position 6 (**43**). Compound **43** was agonistic in the PR alkaline phosphate assay with a potency of $EC_{50} = 1.1$ nM. Its activity at the other hormone steroid receptors was very low thus leading to a highly dissociated class of new nonsteroidal PR agonists.

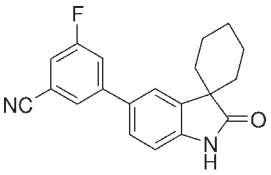
A combination of all the effects rendering agonistic activity resulted in the discovery of tanaproget (**44**). The compound was clinically investigated until phase III, but its development was stopped at this stage (Scheme 6.15).



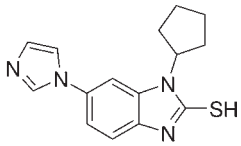
Scheme 6.15

The bicyclic benzoxazine-2-thione core of tanaproget was replaced by several other systems like an indolone or a 2-thioloindole (**45**), resulting in activity in the same dose range like MPA in a decidualization assay.

The benzimidazole-2-thiol core delivered a further series of active agonistic compounds. The nitrogen in the ring had to be substituted by a lipophilic aliphatic group of tailored size. An optimum was achieved with branched alkyl groups like *iso*-propyl or cyclopentyl. The scope of the aryl substituent, here at the 5-position, was extended to a series of imidazole analogs. Some extensions like further nitrogen or methyl groups, benzannulation and de-aromatization were investigated. Compound **46** from this series achieved a strong binding affinity ($IC_{50} = 4$ nM) and selectivity against other nuclear hormone receptors that was better than the reference compounds progesterone and MPA. Its potency in a MMTV–Luc reporter gene



45



46

Scheme 6.16

transactivation assay in T47D cells was at least 8-fold weaker than progesterone and 40-fold weaker than MPA. However, the maximum efficacy was comparable for **46** and the reference compounds. Compound **46** was further investigated in an *in vivo* model to inhibit estrogen induced uterine growth in immature rats. An effect was shown at significantly higher doses than MPA, but a clear response was observed.

In a recent study, the bicyclic core of tanaproget was changed to a *p*-(5-cyano-pyrrol-2-yl) substituted aniline scaffold that was decorated at the aniline by combinatorial chemistry approaches. Most promising compounds carried a small sulfonamide at the aniline and competitive receptor binding with an $IC_{50} = 1.5$ nM has been reported (Scheme 6.16).

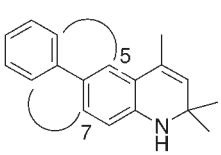
6.5.7
Chromenoquinolines

As an extension of the pharmacophore **37**, a second connection of the bicyclic core with its 6-aryl substituent opened a new class of chromenoquinolines [68–74] with activity at PR. Connection to the 7-position as well as connection to the 5-position have been synthesized (**47**).

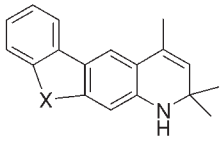
The connection to the 7-position can be exemplified by two of the most active compounds **48** and **49**. The screening for the appropriate ring size and nature of spacer is essential for this tetracyclic scaffold. Compound **48** with the methylene bridge was reported to have competitive binding activity of $K_i = 3.5$ nM at hPR-A and an antagonistic activity of 10 nM (35% efficacy). The NH bridge (**49**) was weaker in binding affinity with $K_i = 113$ nM, the potency was 59 nM, reaching an efficacy of 80% compared to mifepristone (RU-486, 100%).

The connection to the 5-position delivered a further class of compounds that only shows minor similarity to the steroidal backbone at the first glance. Several modifications of this core were investigated such as the nature of the bridge, substitution pattern on the bridge R, and further substituents at the positions R1 and R2 (**50**). A whole series of alkyl- and alkoxy-R substituents showed how easily this scaffold can be turned from an agonist to an antagonist by little steric variation. Compounds **51** and **52** are examples of this phenomenon: **51** is substituted by a methyl R-group, shows a binding affinity of $K_i = 16$ nM and an antagonistic activity of 57 nM (73% efficacy) with nearly no agonism; **52** is substituted by a *n*-propyl R-group, shows a binding affinity of $K_i = 1.5$ nM and an agonistic activity of 14 nM (104% efficacy) without substantial antagonism.

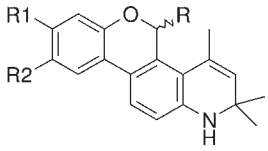
Introduction of aryl groups at the 5-position of the chromenoquinoline system resulted in a series of compounds with a strong binding affinity to PR (K_i up to 0.4 nM). Many compounds showed an agonistic profile with little or no partial antagonistic activity. Compounds such as **53** or **54** show a potency of about 13 nM and an efficacy of 77–96%, being only slightly less active than the natural ligand progesterone. However, the dissociation against GR and AR was not that high compared to some of the other compound classes. Compound **53** showed substantial transactivation on GR ($IC_{50} = 300$ nM; 97% efficacy) and **54** had an activity at GR of $IC_{50} = 500$ nM (96% efficacy) and an IC_{50} at AR of 315 nM (90% efficacy). A further improvement was the change from the phenyl to the benzylidene substituent in position 5. This group eliminates the chirality and the substitution pattern of the system was improved to yield strong agonistic compounds like **55** with a transactivation potency of 5.7 nM (166% efficacy) and a binding affinity comparable to MPA. One compound showed comparable *in vivo* inhibitory activity like MPA in the estrone-induced uterine wet weight assay in ovariectomized rats (Scheme 6.17).



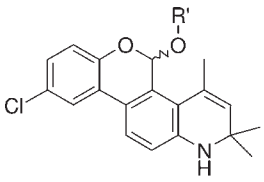
47



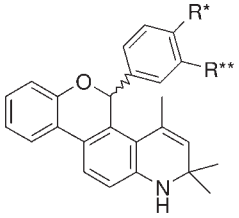
48: X = CH_2
49: X = NH



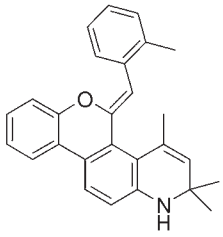
50



51: R' = CH_3 (anta)
52: R' = n-Pr (ago)



53: R* = Cl, R** = H
54: R* = H, R** = CF_3



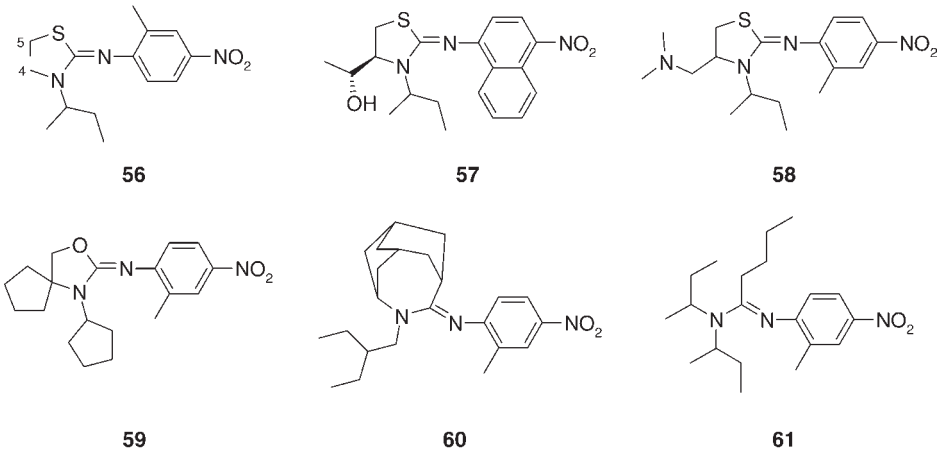
55

Scheme 6.17

6.5.8 Iminothiazolidines

A further structural class is represented by compounds of the type **56** [75, 76]. These compounds have only been characterized by their inhibitory effect on the binding of radioactively labeled progesterone at a standard concentration of 200 nM. A key component in this series is the acceptor group (e.g. NO_2, CN or Cl) at the aryl group

para to the connected imino thiazolidinone ring. A further alkyl group on this phenyl ring, preferentially a methyl group in meta position to the acceptor group seemed to be tolerated as well. The thiazolidinone ring structure has been modified intensively and lipophilic substitution at various positions seem to be preferred. The nitrogen atom in the thiazolidinone ring has to be substituted to avoid a hydrogen donor at this position. Cyclic or small branched alkanes at this nitrogen offer a good affinity towards PR. Substitution at the 4 and 5 position of the thiazolidinone showed, that the 4-position has more flexibility towards variation. At this position, a polar OH group was tolerated in selected positions in **57**. Amines were hardly tolerated; compound **58** is only a weak inhibitor of progesterone binding. The thiazolidinone ring itself has been modified to the ring expanded thiazinone and to the oxazolinone **59** without loss of activity by using comparable substitution patterns. Even the change of this ring system to a polycyclic amine such as **60** or to noncyclic amidines such as **61** delivered compounds with good activity in the reported test system (Scheme 6.18).



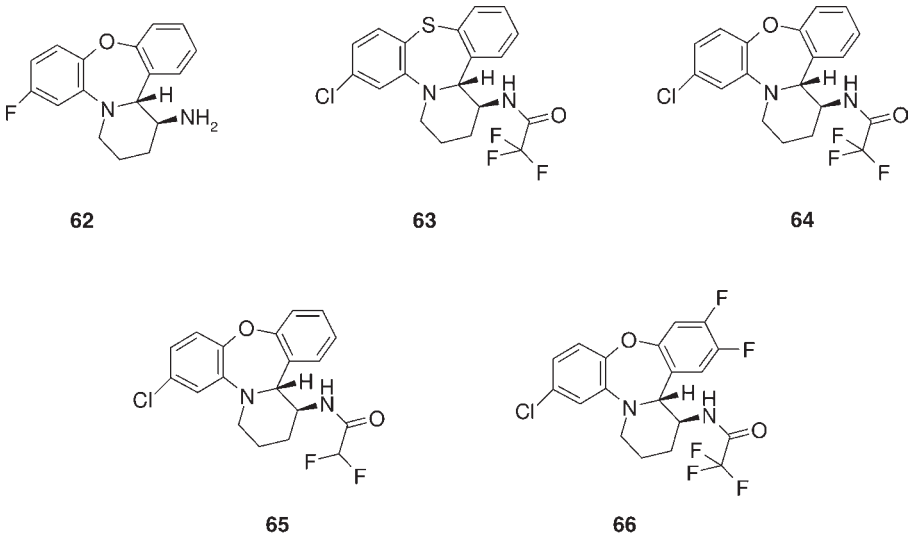
Scheme 6.18

6.5.9
Tetrahydro-1[H]-dibenzo[*b*,*f*]pyrido[1,2-*d*][1,4]oxazepines

A more complex structure class with activity at PR is represented by the tetrahydro-1[*H*]-dibenzo[*b*, *f*]pyrido[1,2-*d*][1,4]oxazepine scaffold (**62**) [77]. This scaffold was modified mainly at the two aromatic systems and the 1-amino functionality. In addition, the central oxazepine ring has been modified to a thiazepine (**63**), a diazepine and an azepane ring in some examples. Comparable decorations of the whole system delivered active compounds with all these modified central ring systems. The acylation of the 1-amino function, especially with the trifluoroacetyl group (**64**), resulted in enhanced activity compared to free amino or alkyl substitution. Trifluorothioacetate was essential for agonism of the compounds, while thioureas

rendered activity antagonistic. In the list of compounds published to date, different activity profiles have been described. Some compounds show only weak agonistic activity in a MMTV–Luc reporter gene transactivation assay in CHO cells. Some compounds with an agonistic activity between 100 and 10 nM show some weak (100–10 nM) to minor (10 000–100 nM) antagonistic activity as well. Compound **65** is more active as an antagonist with an anti PR activity below 10 nM. In this compound the trifluoroacetate of **64** is only replaced by the difluoroacetate!

A set of more than 20 compounds shows agonistic activity below 10 nM with no antagonistic activity. Substitution at the 6- and 7-position of the system with chlorine, bromine or cyano showed a promising activity, with chlorine being the most prominent atom decorating the 7-position. More polar groups such as amino or substituted amino groups resulted in weaker activity. Substitution at the second aromatic ring with fluorine (**66**) or bromine was tolerated as well (Scheme 6.19).



Scheme 6.19

6.6 Conclusions and Outlook

The development of steroidal progestins led to a variety of products with different pharmacological profiles. The use of progestins in combination with estrogens as oral contraceptives has had and still today has an enormous impact on the reproductive behavior in developed countries. This is also reflected in the global market volume of oral contraceptives, which was $5.35 billion in 2005 and is expected to grow to $7.55 billion in 2011. Additional indications for the use of progestins are

menopause management, also in combination with estrogens, and the treatment of endometriosis and myoma. Certain hormone-dependent cancers can also be treated with progestins.

Compared to steroidal compounds, the field of nonsteroidal progestins is still young. Some compounds have already been under clinical evaluation, but as yet none has reached the market. These new nonsteroidal chemical entities differ strongly in structure to the steroids. Therefore, it is hoped, that potentially different profiles with regard to pharmacodynamic and pharmacokinetic behavior may be obtained. At this stage it is already surprising to see such a broad variety of different structural scaffolds being able to mimic the effect of steroids. It will be of high interest to observe how these new classes will develop and if a nonsteroid might become a leading product in medical treatment in the future.

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7
Progesterone Receptor Antagonists

Irving M. Spitz

7.1
Introduction

The hormone progesterone was discovered in 1934. This was followed by the synthesis of synthetic progesterone analogs known as progestins. This step proved crucial in the development of oral contraceptives. The description of the first progesterone receptor (PR) antagonist, RU-486 (mifepristone), occurred in 1981 [1]. Since then, numerous additional compounds have been synthesized which display progesterone antagonist or mixed agonist/antagonist activity. Collectively PR agonists, PR antagonists (PAs) and the mixed agonists/antagonists are referred to as PR ligands. Nonsteroidal PAs and mixed agonists/antiagonists have also been developed, some of which are in an early stage of clinical development [2].

7.2
Chemistry

Mifepristone was synthesized by scientists at Roussel UCLAF [1], and is similar in structure to progesterone and glucocorticoids, but lacks the C19-methyl group and the 2-carbon side chain at C17 and has a conjugated C9–C10 double bond (Figure 7.1). It is a derivative of norethindrone with a 4-dimethylaminophenyl group at the 11β-position which is responsible for its antagonistic activity. The 1-propynyl chain at the 17α-position accounts for its high binding affinity to PR.

In the mid-1980s, Schering synthesized onapristone or ZK 98299 [(11β-(4-dimethylaminophenyl)-17α-hydroxy-17β-(3-hydropropinyl)-13α-methyl-4,9-gonadiene-3-one], which was structurally very similar to mifepristone [3] (Figure 7.1). Pharmacological studies showed that onapristone was a potent PA [4]. Clinical trials commenced but were discontinued because of liver toxicity [5]. This was not observed in any *in vivo* or *in vitro* preclinical study.

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

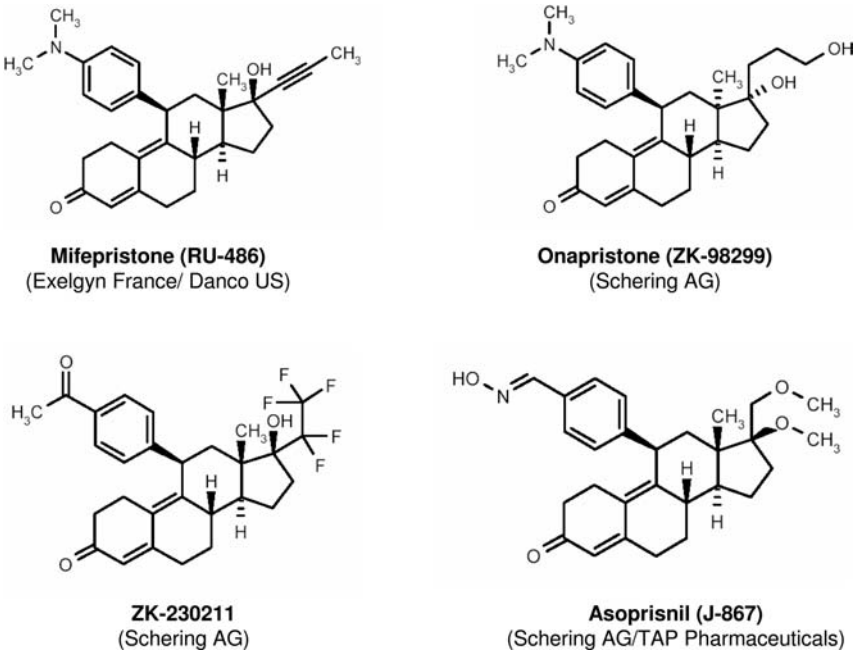

Figure 7.1 Structural configuration of mifepristone (RU-486), onapristone (ZK 98299), ZK 230211 and asoprisnil (J 867).

Schering subsequently developed ZK 230211 [11β-(4-Acetylphenyl)-17β-hydroxy-17α-(1,1,2,2,2-pentafluoroethyl)-4,9-estra-dien-3-one] [6] (Figure 7.1). Encouraging preclinical results have been published in monkeys [7] and to date only one clinical trial has been reported in abstract form showing that ZK 230211 is released from an intrauterine releasing system [8].

EnTec and Jenapharm synthesized the J compounds which are substituted hydrophobic oximes [9–11]. One of these, J867 or asoprisnil [benzaldehyde-4-[(11β,17β)-17-methoxy-17-(methoxymethyl)-3-oxoestra-4,9-dien-11-yl]-1-oxime], is now being clinically developed by TAP Pharmaceuticals and Schering (Figure 7.1).

Organon have developed Org 31710 and Org 33628 (Figure 7.2). Org 31710 has the same substituent at position 11 but differs at the 17-position, where it contains a spiro-ether group. In the B-ring, it has a 6β-methyl group. Org 33628 has a acetophenone group instead of a dimethylaminophenyl group and is combined with a methylene-furan substituent at position 17 [12].

CDB 2914 [17α-acetoxy-11β-(4-*N*,*N*-dimethylaminophenyl)-19-norpregna-4,9-diene-3,20-dione] and CDB 4124 or Proellex® [17α-acetoxy-11β-[4-(*N*,*N*-dimethylamino)phenyl]-21-methoxy-19-norpregna-4,9-diene-3,20-dione] were developed by the Research Triangle Institute (Figure 7.2) under a contract with the National Institute of Child Health and Human Development. CDB 2914 (also known as VA 2914, HRP 2000 or RTI 3021-012) was licensed to HRA Pharma [13] and Proellex® to Repros Therapeutics.

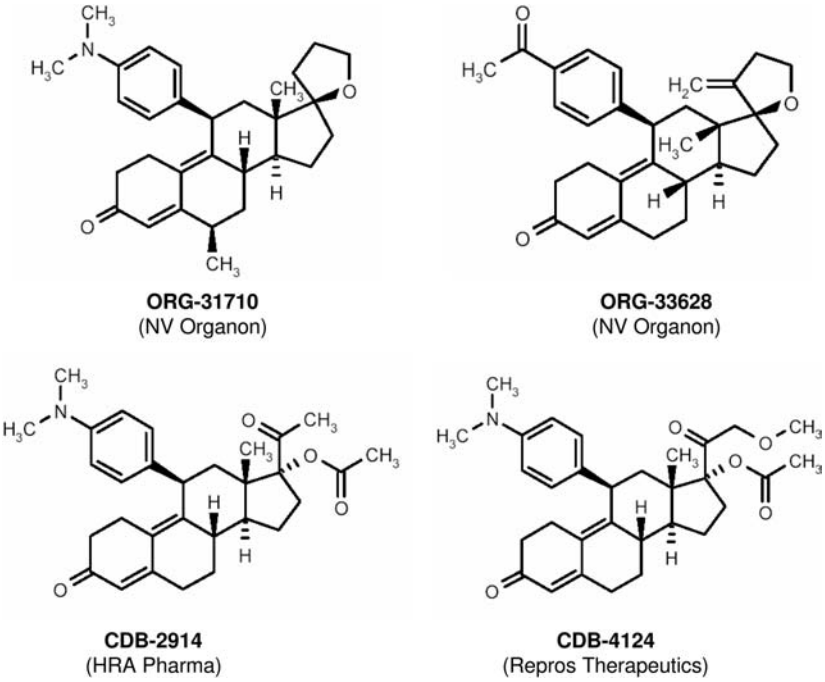

Figure 7.2 Structural configuration of Org 33628, Org 31710, CDB 2914 and Proellex® (CDB 4124).

7.3
Progesterone Receptor (PR)

The actions of progesterone as well as PAs in target tissues are mediated by PR which belongs to a family of nuclear receptors [14]. This family includes receptors for the steroid hormones [glucocorticoid receptor (GR), mineralocorticoid receptor (MR), androgen receptor (AR), estrogen receptor (ER) and vitamin D receptor (VDR)] as well as for thyroid hormones and retinoids. These receptors are ligand-activated transcription factors with domains for DNA binding, hormone binding and trans-activation. PR exists as three separate isoforms, PR-A, PR-B and PR-C, which are expressed from a single gene by alternate promoter usage [14, 15]. The structural configuration of the PR-A and PR-B isoforms is similar although the latter contains an N-terminal fragment of 164 amino acids which is absent from the PR-A isoform. PR-B contains three transcription activating domains while PR-A contains only two [14]. PR-C is an N-terminally truncated form lacking the DNA-binding domain (DBD) and is restricted primarily to the cytosolic compartment of the cell [16]. PR-C increases markedly at term in both the human and the mouse. Although it does not bind to DNA, it binds to PR-B and progesterone, sequestering the latter away from PR-B [17].

PR-A and PR-B have similar DNA-binding activities, but they have distinct functional activities that depend on the cell type and context of the target gene

promoter. In general, PR-B is a much stronger activator than PR-A. Under certain conditions, PR-A is inactive as a transcription factor, but functions as a ligand-dependent transdominant repressor of other steroid receptors including PR-B, and ER, AR, MR and GR. PR-A can act in this repressor mode in response to binding of either PR agonists or antagonists [14]. Studies carried out in PR-A and PR-B knockout mice have shown that PR-A is essential for fertility, ovulation and uterine receptivity, and mediates the antiproliferative effect of progesterone in the estradiol-primed endometrium; PR-B mediates development and differentiation of the mammary gland [18].

The 42-amino-acid sequence at the extreme C-terminal region of the hormone-binding domain of PR is required for the receptor to bind to progesterone; the PA mifepristone, on the other hand, binds to a site situated further towards the N-terminal region of the hormone-binding domain [19]. Deletion of these 42 amino acids causes loss of ability to bind to progesterone or other PR agonists, but binding to PAs is retained. Of importance is the fact that PAs can now activate this mutant receptor. This technology had been utilized and forms the basis for GeneSwitch® (see below).

7.4 Mechanism of Action

Following binding of progesterone (or progestins), PAs or mixed agonists/antagonists to the ligand-binding domain (LBD) of the PR, there is a loss of heat shock proteins and an alteration in its conformation; it undergoes dimerization which converts it from a non-DNA-binding form to one which will bind DNA. The dimerized receptor then binds to progesterone response elements (PREs) located in the promoter region of target genes.

In the presence of progesterone or a pure progesterone agonist (Figure 7.3a) there is interaction with coactivators such as steroid receptor coactivator and cAMP response element binding protein, and this multiprotein complex activates the general transcriptional machinery and increases the expression of target genes. This occurs through various processes which include nuclear receptor and coregulator phosphorylation, regulation of coregulator function by ubiquitinylation or sumolyation and coactivator methylation [20].

In the presence of a PA (e.g. mifepristone; Figure 7.3a) receptors adopt an inactive conformation and preferentially interact with corepressors such as nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), resulting in loss of transcriptional activity [21]. The mixed agonist/antagonist asoprisnil partially recruits coactivators and strongly recruits corepressors [22]. This explains its mixed agonist and antagonist action (Figure 7.3b).

Asoprisnil also displays mixed agonist/antagonist activity in the McPhail assay (Figure 7.4a and b), a test which is based on the degree of progestational activity in an estrogen-primed immature rabbit [10]. In this model (Figure 7.4a and b),

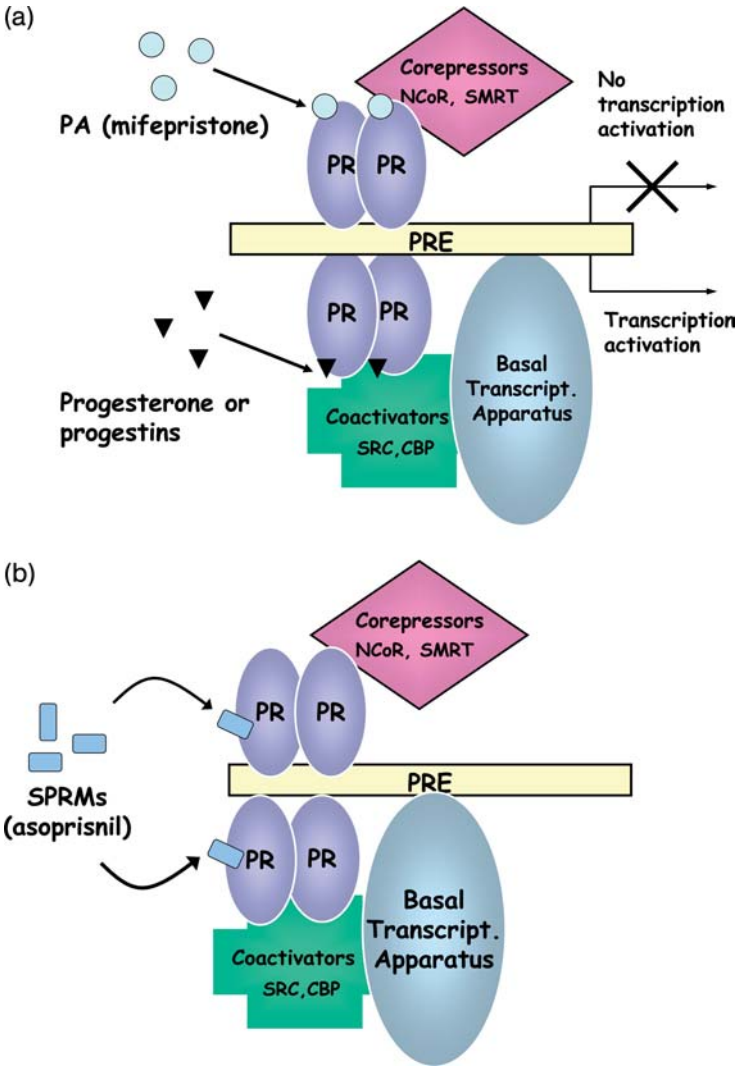

Figure 7.3 Mechanism of action of progesterone, PAs and SPRMs. Binding of progesterone (a) to the inactive receptor complex induces a conformational change, which leads to heat shock protein dissociation, receptor dimerization, DNA binding, and recruitment of coactivators to facilitate communication with the PRE. PAs (a) induce an altered conformation in PR that is transcriptionally inactive. This is caused by PR recruitment of corepressors. The SPRM asoprisnil strongly recruits corepressors and weakly recruits coactivators (b). This explains its mixed agonist/antagonist activity. See text for details. Reproduced with permission from Thomson Scientific from Spitz, I.M. (2006) Progesterone receptor antagonists. *Current Opinion in Investigational Drugs*, **7**, 882–890.

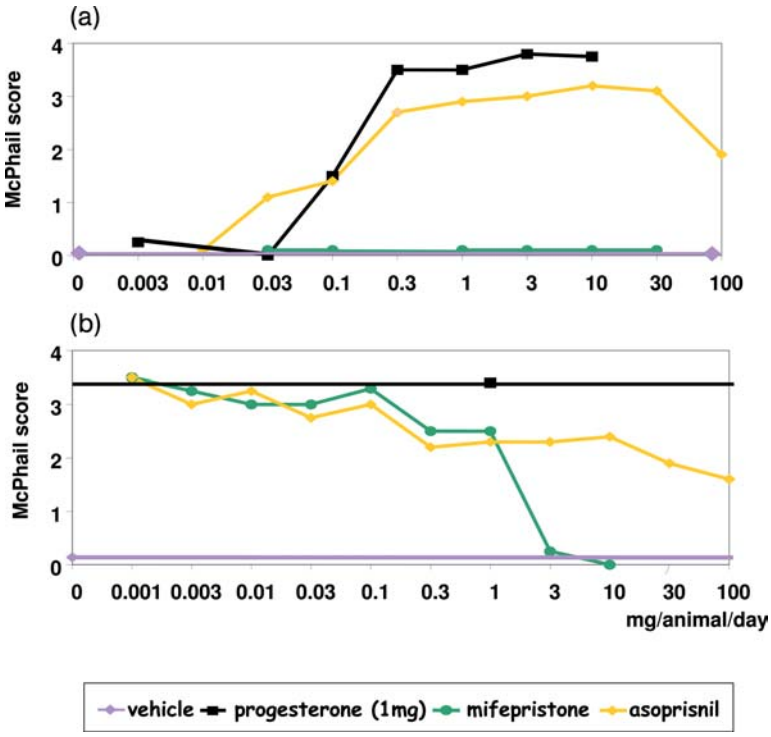

Figure 7.4 McPhail test. Immature rabbits were primed for six consecutive days with estradiol benzoate (5 mg/animal/day, subcutaneously). Mifepristone and asoprisnil were then administered subcutaneously for 7 consecutive days; 24 h after the last injection, the uteri were collected for morphological evaluation. The assessment of stimulatory/inhibitory effects was performed according to the McPhail score (0 = no progestagenic activity, 4 = maximum progestagenic activity). (a) Agonistic activity. In this experiment, the animals were only treated with the test compound. (b) Antagonistic activity. The rabbits were treated with mifepristone and asoprisnil in the presence of progesterone (1 mg/animal/day). Modified from [10].

mifepristone behaves as a pure PA [1]. Thus asoprisnil can be distinguished from pure PR agonists and antagonists based both on its ability to recruit coactivators as well as corepressors, and its response to the McPhail test. Compounds with these mixed agonist/antagonist properties have been defined as SPRMs.

From a pharmacological, biochemical and clinical perspective, SPRMs represent a class of PR ligands that exert clinically relevant, tissue selective, mixed progesterone agonist and antagonist effects, which may be full or partial, on various progesterone target tissues in an *in vivo* situation depending on the biological action studied. Thus, the net biological response to an exogenous PR ligand depends on the tissue, ratio of PR isoforms and coactivators/corepressors, the presence of estradiol and progesterone as well as other factors.

7.5
Biological Effects of PAs and SPRMs

7.5.1
Early Effects

Single-dose administration of the PA mifepristone produces effects in the endometrium, myometrium, decidua and cervix. If implantation has occurred, the inhibition of transcription by the mifepristone–PR complex results in downregulation of progesterone-dependent genes with decidual necrosis and detachment of products of conception [23]. Mifepristone also acts on endometrial blood vessels, causing vascular damage that further compromises the embryo [24]. It also directly promotes uterine contractions by increasing myometrial cell excitability establishing gap junctions between cells and influx of calcium [25].

Mifepristone increases the myometrial response and sensitivity to exogenous prostaglandins which augments uterine contractions [26]. It also increases the release of prostaglandins by decidual cells and induces an accumulation of prostaglandins by inhibiting prostaglandin dehydrogenase, the progesterone-dependent enzyme which metabolizes the active prostaglandins, PGE2 and PGF2α [27, 28].

In the cervix, progesterone suppresses nitric oxide (NO) production [29]. In contrast, mifepristone stimulates NO release, and the expression of inducible NO synthase in the human cervix in early pregnancy, suggesting that this is the mechanism whereby mifepristone initiates cervical ripening [30].

7.5.2
Late Effects

Many PAs and SPRMs display antiproliferative effects in the nonhuman primate endometrium where they suppress estrogen-dependent endometrial proliferation and mitotic activity, secretory activity, and reduce endometrial thickness and wet weight [31, 32]. This antiproliferative effect has been described as noncompetitive [31].

Several explanations have been given to account for this antiproliferative effect. It is accompanied by an increase in ER and PR [33], suggesting that the endometrial antiproliferative effect is due to progesterone antagonism. In addition to the increase in ER and PR, administration of PAs and SPRMs is also associated with an increase in AR [34–36]. Since androgens suppress estrogen-induced endometrial proliferation [37], the increase in AR consequent to PAs could also produce these unexpected antiproliferative effects. Further evidence of the role played by androgens in this antiproliferative effect is the observation that the pure antiandrogen, flutamide, blocks the antiproliferative effects of the PAs ZK137316 and ZK230211 in the endometrium [38]. Flutamide also blocked the hyalinizing degeneration of the spiral arteries induced by PAs [38].

This effect on the AR thus appears to be the most likely mechanism explaining the antiproliferative effect although it may also be related to the fact that the PR-A isoform

inhibits ER gene transcription induced by progestins and PAs [39]. Other potential explanations include reduced endometrial blood supply due to atrophy of spiral arteries [32, 40], blockade of P-dependent growth factors such as keratinocyte growth factor [41], inhibition of angiogenesis via suppression of β fibroblast growth factor [42] or vascular endothelial growth factor (VEGF) [43] and cell cycle block at G_2/M interphase [44]. Since mifepristone has antioxidant properties, it has been suggested that this may also possibly explain its antiproliferative effect [45]. In addition it had been shown that mifepristone induces apoptosis and inhibits cell growth by arresting cell cycle progression at the S phase of the cell cycle [46].

In both women and in nonhuman primates, administration of PAs or SPRMs is associated with a reduction of menstrual bleeding or even amenorrhea. This could be a consequence of this antiproliferative effect although it may also be due to direct effects on endometrial vasculature and may be independent of endometrial atrophy. Low doses of mifepristone (2 or 5 mg daily) upregulate GR in the endometrial glandular nuclei and surface (luminal) epithelium as well as reducing stromal VEGF protein expression. This may also contribute to the amenorrhea [47].

The PA mifepristone also delays or inhibits ovulation, which may produce amenorrhea [48, 49]. Amenorrhea may be a consequence of an effect at the level of the ovary, pituitary or hypothalamus. The SPRM asoprisnil, in contrast, is not so effective in inhibiting ovulation [50]. The amenorrhea consequent to PAs and SPRMs occurs with levels of estradiol in the range of the early follicular phase of the menstrual cycle [48–50]. As a result of the antiproliferative effect and the amenorrhea, PAs and SPRMs have been advocated in the treatment of uterine myoma, endometriosis and dysfunctional uterine bleeding.

7.5.3
Differences in Action between PAs and SPRMs

In the 50-day pregnant guinea pig, asoprisnil demonstrated reduced abortifacient activity as compared to mifepristone [10]. Asoprisnil was also significantly less potent than mifepristone and another PA, onapristone, in inducing labor and preterm parturition in the pregnant guinea pig [9]. As discussed above, mifepristone is very potent in inducing cervical dilatation; this property is not shared by asoprisnil (Chwalisz, unpublished observations).

This selective effect of SPRMs may also extend to different compartments within the same tissue. A study compared the effect of the PA ZK 230211, the SPRM J1042 and a placebo on the endometrium in intact cynomolgus monkeys [51]. Although both compounds exhibited antiproliferative effect, with J1042, the glandular epithelium was columnar and secretory with no marked degenerative changes in the spiral arteries. This is typical of a partial agonist action. In contrast, with ZK 230211, there were marked degenerative changes in the glands in addition to periarteriolar degeneration. This is in keeping with its progesterone antagonist action [51].

7.6 Clinical Applications

Unfortunately, due to political, legal and religious controversies, a very negative public image has been created around mifepristone in light of its abortifacient properties. This has considerably slowed clinical development of mifepristone, in particular, but also other recently developed PAs and SPRMs. Of the numerous compounds that have been synthesized, few have reached the stage of clinical development. Newer PAs and SPRMs will almost certainly never be developed for pregnancy termination, but rather for other long-term applications.

Since progesterone is essential for the initiation and maintenance of pregnancy, SPRMs with a mixed agonist/antagonist action are less likely to produce abortion. Hence, there will be less misuse potential and black market use for these drugs. However, in view of its agonist activity, long-term administration of a SPRM may be associated with unwanted pregnancies.

Mifepristone remains the gold standard since it has been in clinical use as single doses for pregnancy termination for almost a quarter of a century and considerable experience has been accumulated. Nevertheless very few long-term, randomized placebo-controlled studies have been reported. PAs and SPRMs which are undergoing clinical trials are shown in Table 7.1.

Table 7.1 PAs and SPRMs in clinical development.

Compound	Company	Clinical application
Mifepristone (RU-486)	Mifegyne®: Exelgyn (France); Mifeprex®: Danco (USA)	early termination of pregnancy[a], cervical dilatation before surgical termination of pregnancy[a], labor induction in fetal death *in utero*[a], emergency contraception[b], contraception, myomas, endometriosis, antiglucocorticoid applications
Asoprisnil (J867)	TAP Pharmaceuticals (USA); Schering (Germany)	myomas, endometriosis, dysfunctional uterine bleeding
CDB/VA 2914	HRA Pharma (France); Population Council (USA)	myomas, emergency contraception
Proellex® (CDB 4124)	Repros Therapeutics I (USA)	myomas, endometriosis
Org 33628, Org 31710	Organon (Netherlands)	treatment of dysfunctional uterine bleeding during progestin-only contraception

[a] Approved indications.
[b] Only approved in China.

7.6.1 Short-Term Administration

7.6.1.1 Pregnancy Termination

Mifepristone is highly effective in terminating early pregnancy when followed by the administration of a prostaglandin. The prostaglandin most commonly used is misoprostol, a synthetic PGE_1 derivative which is administered orally although it may also be given by the vaginal or sublingual route [52]. Since this method has been used clinically for 25 years and is registered for early pregnancy termination in 31 countries [53], it will not be reviewed in detail. However, a life-threatening side effect, the development of severe fatal infections due to the anaerobic Gram-positive bacillus, *Clostridium sordellii*, has been observed in the USA and this deserves comment. To date, there have been five definite deaths due to *C. sordellii* toxic shock syndrome in previously healthy women after abortions induced with 200 mg oral mifepristone and 800 μg of vaginal misoprostol [54, 55].

The precise reason for this sudden occurrence is unknown. It is possible that pregnancy, childbirth or abortion may predispose a small number of women to acquire *C. sordellii* in the vaginal tract [54]. It is unlikely that the antiglucocorticoid effect is responsible for this serious infection since the increase in glucocorticoids secondary to mifepristone obviates any effect of glucocorticoid antagonism [56]. Furthermore, the glucocorticoid blockade is only transient [57] and, in humans, a short course of high doses of mifepristone (10 mg/kg/day) does not appear to result in any measurable alteration of immune function [56, 58]. Finally, no effects on the immune system have been reported during continuous long-term administration of mifepristone in a dose of 200 mg daily for up to 12 years [59].

A more plausible explanation is that the *C. sordellii* infection is introduced via the dilated cervix [60]. This may be facilitated by the acidic pH of the vaginal tract [54]. Both mifepristone and the prostaglandin analog, misoprostol, induce cervical dilatation. It is probably the vaginal administration of misoprostol rather than mifepristone which precipitates the infection. In all the fatal cases of *C. sordellii* reported to date, vaginal misoprostol was used. The woman herself usually inserted the oral tablet into the vagina, often in her home, under nonsterile conditions. In Europe misoprostol is usually administered by the oral route; when the vaginal route is selected, it is administered by the medical attendant is a clinic or hospital setting. In Europe, only two fatalities, unrelated to infection, have been documented in over 1 500 000 doses.

In the USA, the Food and Drug Administration (FDA) has only approved misoprostol administered by the oral route. Since the rate of pregnancy termination is higher with vaginal as compared to oral misoprostol [52], many investigators administer oral misoprostol tablets by the vaginal route. In view of these fatal cases, the FDA has issued a public Health Advisory Statement related to sepsis and medical termination of pregnancy and has revised the Black Box Warning accordingly. (http://www.fda.gov/cder/drug/infopage/mifepristone/default.htm). In addition, Planned Parenthood of America, the largest provider of medical abortion, no longer recommends the vaginal route of administration.

Prior to undergoing medical termination of pregnancy, women must be informed about this rare complication. If a vaginal infection is present, antibiotics are indicated. Ideally, all women should be screened for infection and treated before termination of pregnancy. However, in practice, this is rarely done. In Europe, routine prophylactic antibiotic treatment is not uncommon [53].

7.6.1.2 Other Short-Term Indications

Mifepristone together with prostaglandins may also be used for late first- and second-trimester terminations of pregnancy [52]. Since mifepristone softens and dilates the cervix, it can induce labor following intrauterine fetal death [61]. For this indication, it has been used successfully alone or together with misoprostol [62].

7.6.1.3 Emergency Contraception

Single doses of mifepristone of 10, 25, 50 or 600 mg appear to be effective as emergency contraceptives when used up to 120 h after unprotected intercourse [63, 64]. Side-effects are often less than observed with other methods although many women using mifepristone had a delay in the expected onset of their next menstrual period. This menstrual delay is a dose dependent phenomenon and is greater with mifepristone doses of 600 and 100 mg than doses of 25 or 10 mg [63–65].

A recently published study showed that CDB 2914 administered in a single dose of 50 mg up to 72 h following unprotected intercourse was as effective as mifepristone as an emergency contraceptive [66]. It should be pointed out that levonorgestrel administered as a single dose of 1.5 mg or two doses of 0.75 mg administered at 12-h intervals is as efficacious as mifepristone or CDB 2914 as an emergency contraceptive and is effective up to 120 h after unprotected intercourse.

Although mifepristone has also been used in the treatment of early fetal demise and for labor induction, these conditions may also be treated with misoprostol, which is inexpensive and has been shown to be equally effective [67]. Vaginal misoprostol alone has also been used without mifepristone in cases of fetal death [68].

7.6.2 Long-Term Administration

7.6.2.1 Treatment of Uterine Myoma

A review of six clinical trials involving 166 women treated with mifepristone administered in doses of 5–50 mg/day for 3–6 months resulted in reductions in myoma volumes of 26–74%, with rates of amenorrhea of 63–100%. The prevalence and severity of dysmenorrhea, menorrhagia and pelvic pressure was reduced [69].

In one of these studies, treatment was extended for up to 1 year in 40 premenopausal women with large symptomatic myoma who received mifepristone in doses of 5 or 10 mg daily [70, 71]. Mean uterine volumes decreased by 48% in both groups by 6 months and to 53% at 1 year. None of the above trials conducted with mifepristone was placebo-controlled.

Recently, a randomized double-blind placebo-controlled 26-week study was performed in 42 women with symptomatic myoma. Women randomized to mifepristone

received 5 mg daily and showed an improvement in leiomyoma-specific quality of life compared with the placebo group. The adjusted uterine size was reduced by 47% [72].

A large double-blind, randomized, placebo-controlled 3-month study has also been performed with asoprisnil [73]. Doses of 5, 10 and 25 mg were administered to 98 women with myoma. The placebo group comprised 31 patients. Asoprisnil suppressed uterine bleeding in 28, 64 and 83% of subjects at 5, 10 and 25 mg, respectively, and reduced myoma and uterine volumes. Mean percent decrease from baseline leiomyoma volumes was significant at 25 mg compared with placebo after 4 and 8 weeks of treatment; by week 12 leiomyoma volume was reduced by 36%. There was a significant reduction in bloating with the two highest doses and in pelvic pressure with 25 mg by week 12 [73].

A small clinical study comprising 30 women was conducted with Proellex® and has appeared in the manufacturer's web site, but to date not in a peer-reviewed journal (http://www.reprosrx.com). Doses of 12.5, 25 and 50 mg were compared to 3.75 mg of the gonadotropin-releasing hormone (GnRH) superagonist, Lupron®. There was also a placebo group. The response was similar to what had been reported with mifepristone and asoprisnil.

These studies indicate that PAs and SPRMs both have the ability to decrease myoma size. A crucial question is what happens to the myoma following cessation of treatment? To date, no study has adequately assessed this important issue.

7.6.2.2 **Treatment of Endometriosis**

Three small clinical trials comprising a total of 22 patients have been reported using three dose schedules of mifepristone (5 or 50 mg/day for 6 months or 100 mg/day for 3 months) [74–76]. With all schedules, there was an improvement in symptoms and with the 50-mg dose, there was a 55% mean regression of visible endometriosis after 6 months of treatment [74–76]. Only one multicenter, placebo-controlled double-blind, parallel group study was conducted with asoprisnil administered in doses of 5, 10 and 25 mg to a total of 130 women. The study has been reported in abstract form [77]. All three doses significantly reduced the dysmenorrhea as well as nonmenstrual pain as compared to the placebo. No observations have been reported on the progress of the disease following cessation of treatment.

The amenorrhea consequent to PAs and SPRMs occurs in the presence of early to mid follicular phase levels of estradiol. Severe hypoestrogenism is observed in women with endometriosis and uterine myoma treated medically with long-acting GnRH analogs and there is significant loss of bone mineral density [78]; in contrast, bone mineral density is maintained after 6 months of treatment of patients with myoma with mifepristone [79]. The lack of hypoestrogenism represents a significant benefit of PAs and SPRMs.

7.6.2.3 **Contraceptive Potential**

Mifepristone as well as other PAs have contraceptive potential and some encouraging pilot studies have been conducted in unprotected women who relied exclusively on mifepristone for their contraception. Ovulation is inhibited with daily doses of mifepristone of 2 mg or higher. A total of 40 sexually active women in Shanghai and 10 women in Edinburgh were randomly assigned to receive

mifepristone 2 or 5 mg daily for 120 days. No pregnancies occurred in the 200 months of exposure days [80].

A sequential regimen using mifepristone (10 mg) and the progestin nomegestrol acetate (5 mg) was studied in 30 women for 12 consecutive cycles without pill-free days. Mifepristone was administered for 15 days and this was immediately followed by the progestin for 13 days. One pregnancy occurred in 359 woman-months of exposure. This study has been published in abstract form [81, 82].

Other strategies are based on the observation that mifepristone can retard endometrial development. In two studies, a total of 53 women received 200 mg mifepristone 48 h after the luteinizing hormone (LH) surge and there were only three pregnancies in a total of 335 cycles [83, 84]. In view of its complexity, a method dependent on the precise timing of the LH surge does not represent a practical approach to contraception.

Weekly administration of mifepristone in doses as high as 25 mg do not consistently inhibit ovulation [85]. However, this regimen does interfere with normal endometrial development. No pregnancies occurred when once weekly administration of mifepristone (25 and 50 mg) was given for 6 months to a total of 76 women. There were a total of 456 women-months of use [86].

7.6.2.4 Reduction of Bleeding in Progestin-Only Contraception

The main problem with the administration of progestin-only methods of contraception is the irregular bleeding pattern. As it has the ability to induce amenorrhea, intermittent mifepristone administration has been used to reduce the occurrence of bleeding irregularities induced by progestin-only contraceptive methods. Double-blind, randomized, placebo-controlled trials showed improved bleeding patterns in women using levonorgestrel-releasing subdermal contraceptive implants [87, 88] as well as in new depot medroxy progesterone (DMPA) users [89]. Mifepristone with ethinyl estradiol also improved bleeding patterns in etonogestrel (Implanon)-releasing subdermal implant users [90].

Placebo-controlled studies with Org 33628 and Org 31710, compounds developed by Organon, have also shown a decrease in the amount of bleeding in women taking continuous desogestrel (75 μg). Despite the reduction in bleeding, several women had evidence of ovulation [91]. The implication is that the addition of Org 31710 and Org 33628 in the doses tested antagonizes the action of desogestrel permitting ovulation. Since the studies were conducted in protected women, it is not possible to ascertain if pregnancies would have occurred.

7.7 Other Potential Obstetrical and Gynecological Applications

7.7.1 Potential Use in *In Vitro* Fertilization Programs

Since it delays the LH surge and ovulation, mifepristone may have application in superovulation induction programs by inhibiting a premature LH surge and/or

premature luteinization [92, 93]. Mifepristone also arrests endometrial maturation and there is an absence of upregulation of some progesterone-dependent genes that are important for endometrial receptivity [93]. By retarding endometrial maturation, mifepristone thus 'shifts' the implantation window [94]. This may possibly lead to a better synchronization of embryonic and endometrial maturation, and to increased pregnancy rates [95]. It should also be noted that mifepristone crosses the blood–follicle barrier and follicular fluid levels are similar to those in blood 34 h after a 100-mg dose [96].

7.7.2
Ovarian and Uterine Carcinoma

Mifepristone has also been shown to be of use in ovarian carcinoma [97] and an encouraging report had been published on its beneficial effect in a woman with a uterine leiomyosarcoma which stained positively for PR [98]. Further studies are warranted.

7.7.3
Dysfunctional Uterine Bleeding

Since both PAs and SPRMs induce amenorrhea, they may be indicated in the treatment of this disorder. A single study has been reported in abstract form showing encouraging results with asoprisnil [99].

7.7.4
Hormone Replacement Therapy in Postmenopausal Women

The possibility of using PAs and SPRMs as a form of nonestrogen hormone replacement therapy for postmenopausal women remains unexplored. Studies in oophorectomized monkeys using the PA ZK 230211 have given encouraging results [100].

7.8
Nongynecological Applications

7.8.1
Tumors

Studies in meningioma have yielded conflicting results [101, 102] and there is inconclusive data on its effect in breast carcinoma [5]. In a recent study, it was shown that mammary glands of nulliparous BRCA1- and p53-deficient mice had increased cell proliferation, accumulated lateral branches and underwent extensive alveologenesis and also developed mammary cancer by age 8 months. When these mice were treated with mifepristone, increased cell proliferation was not observed and the

mutant treated mice were still tumor-free at 12 months of age. PR was overexpressed in the mutant mammary epithelial cells because of a defect in degradation [103]. These interesting results suggest that long-term treatment with a PA or SPRM may represent a possible future option for women with a mutated BRCA1 gene [103].

7.8.2 Antiglucocorticoid Applications

In high doses (200 mg daily), mifepristone is a powerful antiglucorticoid. This dose is considerably higher than that required for a progesterone antagonist effect [104]. Mifepristone is effective when administered in adrenocorticotrophic hormone (ACTH)-independent Cushing's syndrome [105]. It cannot be used long-term in Cushing's disease since the increase in ACTH will overcome the glucocorticoid blockade; however, it can be used short-term to prepare the patient for surgery. Mifepristone may also be used in other situations where an antiglucocorticoid effect is required such as in neuropsychiatric disorders including major depression with psychotic features and bipolar disorders [106]. It had also recently been used successfully in chronic central serous chorioretinopathy [107].

7.8.3 The GeneSwitch® (Inovio Biomedical) System for Ligand-Dependent Transgene Expression

This plasmid-based technology enables precise control of expression for specific genes using a small-molecule inducer was first constructed by Wang *et al.* [108] based on the observations of Vegato *et al.* [19] which have been described earlier. The system consists of two genes. The first codes for the GeneSwitch® regulator protein which contains the 42-amino-acid deletion of the C-terminal LBD of human PR which does not bind progesterone, but retains the ability to bind to PAs and to activate transcription [19]. In addition, it contains the DBD from the yeast GAL4 protein as well as a transcriptional activation domain [109]. This gene is activated by low doses of mifepristone and other PAs, but not by any endogenous hormones present in mammalian tissues [109].

The second gene in this system codes for the inducible transgene of interest. The inducible gene contains a promoter that consists of multiple binding sites for the GAL4 DBD linked to a TATA box element. Binding of the PA triggers a conformational change causing the regulator protein to become an activated homodimer which binds to the GAL4 sites in the inducible promoter. This stimulates transcription of the transgene leading to an increased production of the protein product. On removal of the PA, the regulator protein reverts to its inactive state.

Expression of the therapeutic protein is enhanced by electroporation – the application of a small amount of electricity at the site of injection for a few milliseconds. This high-intensity electric field induces temporary and reversible breakdown of the plasma membrane allowing plasmids and other molecules to gain intracellular access [110]. This plasmid-based PA-inducible transgene regulating

system has been used for the expression of alkaline phosphatase, erythropoietin, VEGF and growth hormone [109]. The advantage of the system is that the regulator is only activated with a PA such as mifepristone and terminated with cessation of its administration.

7.8.4
Future Developments

Progression to malignancy in breast and endometrium is often accompanied by disrupted expression of PR-A and PR-B [111, 112]. Development of PR agonists and antagonists selective to the PR-A and PR-B isoforms may prove to be of great clinical benefit in breast and endometrial cancer. Selective PR-A agonists are currently in development [113, 114].

7.9
Side-Effects of Long-Term Administration of PAs and SPRMs

Information is only available for mifepristone since there is very limited clinical experience with other PAs and SPRMs. Owing to its specific action at the PR and GR, serious untoward effects are rare and mifepristone is well tolerated. Antiglucocorticoid effects are usually only seen with doses exceeding 200 mg daily [59]. Chronic administration of mifepristone has been associated with the development of biochemical hypothyroidism which requires observation and follow-up [115].

Endometrial thickening has also been described in up to 20% of women receiving treatment with mifepristone (200 mg daily) for meningioma [59]. Endometrial thickening is not synonymous with endometrial hyperplasia on histology, but may be related to edematous changes in the myometrium and related connective tissue, to cyst formation, to the collection of fluid in the lumen of dilated glands or to the presence of benign stromal thickening with an increase in collagen production in the stromal cells [116–118]. In one patient with Cushing's syndrome who received 400 mg daily administered for 1 year there was marked thickening of the endometrium associated with simple endometrial hyperplasia. This resolved on cessation of therapy [119].

Histological examination of the endometrium in women receiving lower doses of mifepristone has yielded much controversy. In one study, mifepristone in daily doses of 2 or 5 mg decreased endometrial proliferation [118]. On the other hand, in some women, the endometrial histology had been interpreted as showing hyperplasia. In a study already alluded to, doses of mifepristone of 5 or 10 mg were given to 40 women with myoma [71]. Ten of the 36 women who underwent endometrial biopsies were reported to have hyperplasia – an incidence of 28% after 6 months of therapy [71]. When the slides were reviewed by an experienced gynecological pathologist, the incidence of apparent hyperplasia in these patients decreased to 14% [70]. Further re-evaluation of the same slides by a specially constituted panel of expert

gynecological pathologists which convened at the National Institutes of Health in April 2006 concluded that none of these patients had a histological appearance that warranted any concern [120]. This panel of pathologists blindly examined endometrial samples from women who received four different PAs or SPRMs (including mifepristone, asoprisnil and CDB 2914) for up to 3 months [120]. Doubt was even cast as to whether endometrial hyperplasia was present in any of the women who received these drugs [120].

The controversy with regard to interpretation of the endometrial findings relates to the fact that the endometrial changes seen with long-term PAs and SPRMs are unique, and new criteria have to be applied to validly interpret the histology [121]. Since current criteria of endometrial dating cannot be used, a new classification system has been developed by TAP Pharmaceutical Products and Diagnostic Cytology Laboratories in conjunction with a group of expert gynecological pathologists [73, 121].

A detailed morphological assessment of full-thickness endometrium and underlying myometrium after a 3-month treatment with asoprisnil has recently been published [121]. Asoprisnil was administered in doses of 10 and 25 mg. There was also a placebo group. A majority of the asoprisnil-treated women showed endometrial morphology characterized as having a nonphysiologic secretory effect. The endometrial glands were tortuous, similar to what occurs in the normal secretory phase, but there was a rarity of secretory activity or vacuolation. The glands frequently showed cystic dilatation with a paucity of mitotic activity. In this 3-month study, endometrial thickness was not increased in the asoprisnil group as compared to the placebo and the stroma showed the expected compaction without decidual change. Unusual thick-walled muscular arterioles and prominent aggregation of thin-walled vessels were present in endometrial stroma, but not in the myometrium or nonendometrial vascular beds. Mitotic activity was decreased [121].

There are morphological similarities as well as differences between the effects of asoprisnil and those of mifepristone. In both, there is cystic glandular dilation with inactive epithelium and an antiproliferative effect [118, 122]. With mifepristone, the glands show less tortuous morphology and are lined by nonvacuolated inactive epithelium that show few mitoses (Williams, personal communication). This suggests an absence of agonistic and only antagonistic effects. No specific vascular changes have been identified in the endometrium of mifepristone-treated women.

These endometrial appearances are unfamiliar to pathologists and are not seen in any described condition, and reflect the specific effects of these compounds on the endometrium. Despite the paucity of mitoses, cystic glandular dilatation is often associated in the minds of pathologists with simple hyperplasia [121]. Pathologists must be made aware of the potential diagnostic pitfalls of misdiagnosing hyperplasia in women receiving PAs or SPRMs. To date, detailed morphological changes in the endometrium have not been published with CDB 2914, Proellex®, Org 31710 or Org 33628.

The endometrial thickening occasionally observed on ultrasound with long-term administration of mifepristone as well as the unique histological changes reported

with mifepristone and asoprisnil raise questions as to the feasibility of long-term application of PAs and SPRMs. Resolution of these issues will require carefully conducted clinical trials with endometrial biopsies reviewed by expert panels of experienced gynecological pathologists.

7.10 Conclusions

It is now over 25 years since the first progesterone antagonist, mifepristone, was introduced into the clinic. Mifepristone is now firmly established as an option for the medical termination of early pregnancy. Long-term application of PAs and SPRMs has also been shown to have great potential in the treatment of a broad array of other clinical conditions. Mifepristone has been administered continuously in patients with meningioma for over 10 years without serious untoward effects, although the endometrial findings described above need to be resolved before widespread use of these compounds can be advocated [59]. Pathologists examining endometrial biopsy specimens from PA- or SPRM-treated patients need to be aware of these endometrial effects and avoid misclassifying the appearance as simple hyperplasia. At the current stage of development, the only feasible long-term method of use will be intermittent administration for up to 3 months followed by a progestin to induce endometrial sloughing; alternatively, intermittent courses may be given and then followed by a drug-free interval during which time endometrial sloughing will occur. Further courses may then be given using the same schedule.

Acknowledgments

I thank Ms Hadassa Hartman for her invaluable editorial assistance.

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8
Nonsteroidal Tissue-selective Androgen Receptor Modulators

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8.1
Introduction

8.1.1
Androgen Receptor: Structure and Function

8.1.1.1 Androgen Receptor Structure

Androgens play an important role in male physiology due to their essential roles in male sexual differentiation, male puberty changes, maintenance of muscle and bone mass, prostate growth, and spermatogenesis through the action of the androgen receptor (AR) [1]. The AR is mainly expressed in androgen target tissues, such as the prostate, skeletal muscle, liver and central nervous system (CNS), with the highest expression level observed in the prostate, adrenal gland and epididymis [2]. The AR gene has eight exons spanning a 90-kb region on the X chromosome and encodes a protein comprised of 919-amino-acid protein with a molecular weight of 110–114 kDa [3]. It is a member of the nuclear hormone receptor superfamily which is now known to contain 48 members and is divided into three classes. Among these, the class I or steroid receptor subfamily of nuclear hormone receptors is comprised of receptors for androgens (AR), estrogens (ERα/β), glucocorticoid (GR), progesterone (PR) and mineralocorticoid (MR). The various domains and the amino acid sequence homology between the class I members are depicted in Figure 8.1. Among the domains, the N-terminal domain (NTD) is the least conserved and DNA-binding domain (DBD) is the most conserved. The ligand-binding domain (LBD) is involved in ligand binding and receptor dimerization, and contains the ligand-inducible activation function known as AF-2. The X-ray crystal structure of the AR LBD was first solved by Matias *et al.* in 2000 [4] in a complex with the synthetic steroidal agonist R1881. The three-dimensional structure closely resembles that of the other agonist-bound steroid hormone receptor LBD structures containing two β-turns and 11 α-helices arranged in antiparallel in three layers. Different from other steroid

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

hormone receptors, the AR LBD lacks helix 2 (H2), but follows the same helical numbering scheme with H12 in a similar position as the agonist-bound ERα. The NTD contains cell- and promoter-specific transactivation functions that act independent of ligand (often referred to as constitutively active) known as AF-1. To date, the structure and function of this region are least understood. However, deletion of AF-1 leads to more than 75% loss of AR function [5–7].

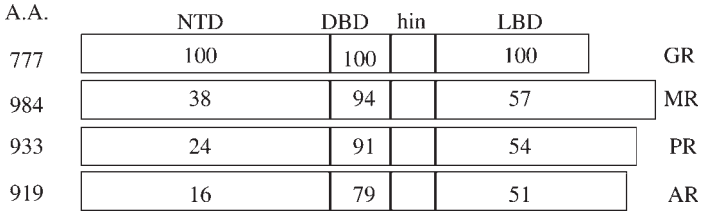


Figure 8.1 Structural homology of class I receptors. GR, MR, PR and AR have a less-conserved NTD, highly conserved DBD, a hinge region (hin) and a moderately conserved LBD. The numbers inside the boxes are the percent homology among this class compared to GR (taken as 100%). The numbers on the left are the number of amino acids (A.A.) that make up each receptor.

Due to the high amino acid sequence homology of the DBD, moderate homology, and similar secondary and tertiary structural features of the LBD, and common chemical features of steroidal ligands, class I receptors are often capable of binding with the steroidal ligands of other class I receptors (i.e. cross-reacting). For example, early studies with AR and ER suggest that the orientation of the steroid in the LBD, with the steroid A-ring in contact with H3 and the D-ring in contact with H11 residues, is likely to be general for all the steroid hormone receptors [8]. Crystallographic evidence and studies of receptor chimera support this conclusion for a wide variety of class I receptors [9–11].

8.1.1.2 **AR Function**

Testosterone is synthesized by Leydig cells in the testes in response to the anterior pituitary hormone, luteinizing hormone (LH), and is the predominant circulating androgen found in the bloodstream of normal healthy males and females. However, free (unbound) plasma concentrations of testosterone are low due to its extensive binding to sex hormone-binding globulin (SHBG) and albumin [12]. Unbound testosterone freely permeates the cell membrane to reach its target, the AR, or can be converted intracellularly into the more potent form dihydrotestosterone (DHT) by the enzyme 5α-reductase in prostate, skin or other selected tissues. In the absence of ligand, AR is located in the cytoplasm [13], maintained in an inactive conformation by heat shock proteins HSP70 and HSP90, and other associated proteins called corepressors. Binding of an agonist ligand promotes a conformational change in the AR that results in dissociation of heat shock proteins, interaction with coregulator proteins, dimerization, phosphorylation and translocation into the nucleus. Agonists ligands incite formation of an activated AF-2 resulting in coactivator recruitment through the

LXXLL motif of the p160 coactivators or interaction of its own NTD through the FXXLF sequence [14–16]. A structural basis for these interactions is now well understood through X-ray crystal structures of the AR LBD complexed to such peptide motifs [16] (Figure 8.2). Translocated AR then binds to the androgen-responsive elements (ARE) on DNA, which are generally characterized by a 6-nucleotide half-site consensus sequence (e.g. 5′-TGTTCT-3′) spaced by 3 random nucleotides and are located in the promoter or enhancer region of AR gene targets where other transcription regulators (including coactivators and corepressors) are recruited for activation of AR-regulated gene expression. This process is known as the classic genomic function of AR, while the nongenomic pathway is characterized by its rapid action and interaction with plasma membrane-associated signaling pathways [17].

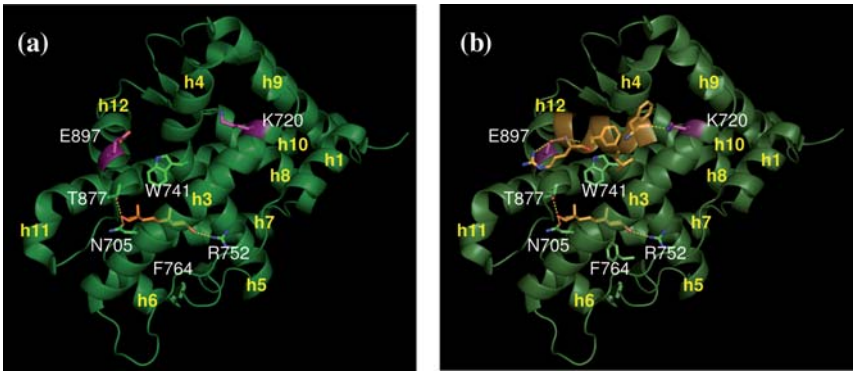


Figure 8.2 Structure of the AR LBD complexed to DHT (a) without coactivator peptide (PDB code 1T7T) and (b) with coactivator peptide (PDB code 1T7R). Notice hydrogen bonds to the 3-keto group of DHT (gold) with R752 of H5 and 17β-hydroxyl group of DHT to N705 and T877 of H3 and H11, respectively. The FxxLF coactivator peptide (gold) binds in a hydrophobic groove located between H3, H4 and H12 forming hydrogen bonds to K720 and E897 (magenta).

8.1.2 Pathogenic Mutations and Polymorphisms of AR

Mutations in AR are known to be present in diseases including androgen-insensitivity syndrome (AIS) and prostate cancer. AIS is an X-linked chromosomal defect that results in resistance to androgens [18]. Mutations in AR known to cause this syndrome are numerous and characterized by the severity of clinical symptoms as complete, partial and mild (CAIS, PAIS and MAIS, respectively). Loss of AR function that leads to AIS can be from point mutations, frameshift mutation, deletions, insertions or abnormal intron splicing [19, 20]. Although point mutations located in the AR DBD are also known, the vast majority of mutations associated with AIS occur in the LBD (Figure 8.3) [4]. For example, mutations to the N705 and R752, which hydrogen bond to the 17β-hydroxyl and 3-keto group of testosterone, respectively, result in CAIS.

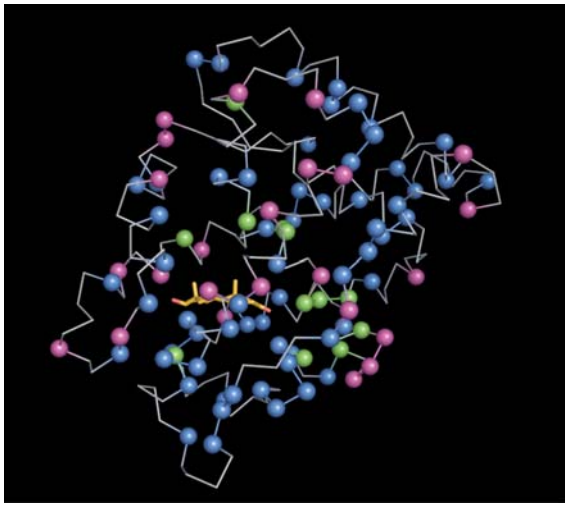


Figure 8.3 Pathogenic mutations in the AR LBD. The location of point mutations in the AR LBD (PDB code 1I37) associated with CAIS (blue), prostate cancer (magenta) and both diseases (green) are depicted as spheres with the AR LBD shown in ribbons in the same orientation as Figure 8.2.

Molecular mechanisms that increase AR functional activity, which include increased levels of AR ligands, increased AR protein levels, activating AR mutations, ligand-independent activation and alteration in coregulator molecules, are thought to be significant contributors to the development of prostate cancer [21]. AR mutation rates in prostate tumors range from 5 to 50% depending on a number of factors, including whether the tissue was from a primary or metastatic site and prior treatment regimens [21, 22]. Most of the mutations identified in prostate cancer are found within the LBD (Figure 8.3), and result in a gain in AR function and/or increased ligand promiscuity [21, 23, 24].

Treatment with AR antagonists (e.g. bicalutamide, hydroxyflutamide and nilutamide) results in mutations that convert these ligands into potent AR agonists resulting in their resistance, a phenomenon known as antiandrogen withdrawal syndrome. A well-known example is the LNCaP cell line, which expresses a T877A mutant AR that causes loss of hormone specificity as well as activation by antiandrogens including cyproterone acetate, nilutamide and hydroxyflutamide. Prostate cancers containing the mutants V715M [25], R726L [26], H874Y [27] and W741L and W741C [22, 28] have shown increased responsiveness to progesterone, estradiol, cortisol and bicalutamide, respectively. X-ray crystallography has elucidated the molecular mechanisms for a number of the above-mentioned changes in AR function and ligand specificity.

Abnormal numbers of CAG repeats in the NTD also are associated with certain diseases. The CAG repeats encode for a polyglutamine tract that begins at amino acid 58 in AR, and normally includes between 11 and 33 residues. Spinobulbar muscular atrophy (Kennedy's disease) is caused by expansion of these repeats leading to lengthening of the polyglutamine tract [29]. Likewise, some studies suggest that shorter

numbers of CAG repeats increases risk for prostate cancer [30]. Other studies have shown mixed results in the number of CAG repeats and risk of prostate cancer [31].

8.1.3
Steroidal Androgen Therapy: Testosterone and Synthetic Analogs

Synthetic steroidal androgens have been developed and marketed, and are collectively referred to as anabolic androgenic steroids (AAS). Unfortunately, all the currently available androgen preparations have severe limitations [32–34]. Most of these are simple structural modifications of the endogenous steroids, testosterone and DHT (Figure 8.4), which improve their pharmacokinetic or pharmacodynamic profiles to some degree. Generally the 3-keto and 17β-OH are retained to enhance AR binding affinity and androgenic activity. All of the currently available AAS are substrates for 5α-reductase or already contain a 5α-reduced steroid A-ring, imparting 'DHT-like' structural properties and pharmacologic activities. The skin and prostate are noteworthy tissues in which DHT is the principle androgen. A common modification, 17α-alkylation, increases oral bioavailability and prolongs the elimination half-life of these ligands. 17α-Methyltestosterone (**1**; indicated for hypogonadism, delayed puberty in males; inoperable metastatic mammary cancer in females) and oxandrolone (**4**; indicated for promoting weight gain due to prolonged catabolism, relief of bone pain in osteoporosis) can be given orally (Figure 8.4). Removal of the 19-methyl group seems to be favorable for separation of androgenic and anabolic activities. For instance, nandrolone (19-nor-testosterone; **3**) and

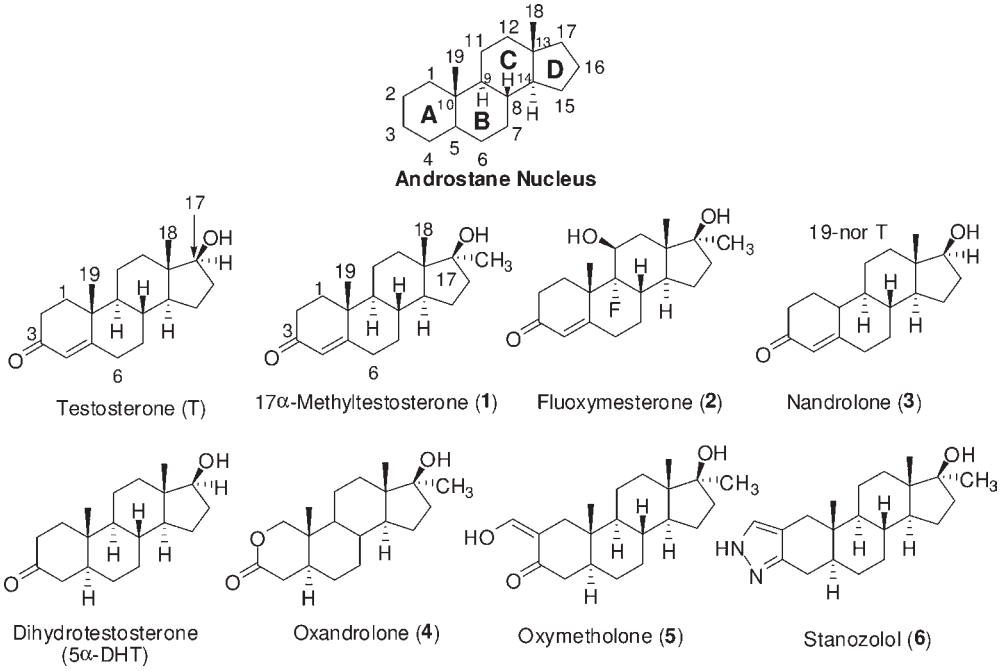


Figure 8.4 Endogenous and synthetic and AR ligands.

nandrolone decanoate were developed as anabolic drugs for the treatment of testosterone deficiency syndromes, wasting disorders and anemia [35] (Figure 8.4). Modification of the A-ring of DHT has been successfully employed such as in oxandrolone (**4**), oxymetholone (**5**) and stanozolol (**6**). Despite the variety of AAS available, none are considered acceptable for long-term therapy [36–38], leaving testosterone transdermal patches and gels as the seldom but most widely used formulations for treatment of androgen deficiency.

Gruenewald and Matsumoto [33] recently reviewed the potential risks of testosterone supplementation in older men. Androgens are known to elicit numerous beneficial effects, including increases in lumbar spine bone mineral density (BMD), prevention of bone loss at the femoral neck, and positive changes in body composition and sexual function [39]. However, concerns related to acne, decreases in high-density lipoprotein (HDL), increases in low-density lipoprotein (LDL), total cholesterol, suppression of pituitary gonadotropins (i.e. LH) [40], elevations in hepatic transaminases [41] and possible influences on incidence of benign prostatic hyperplasia or prostate cancer make the long-term safety and efficacy of steroidal androgen therapy uncertain [32, 42]. Another major concern with molecules related to testosterone is the conversion of such agents to estrogens such as estrone and estradiol, as illustrated in Figure 8.5. Such modifications lead to feminization in a

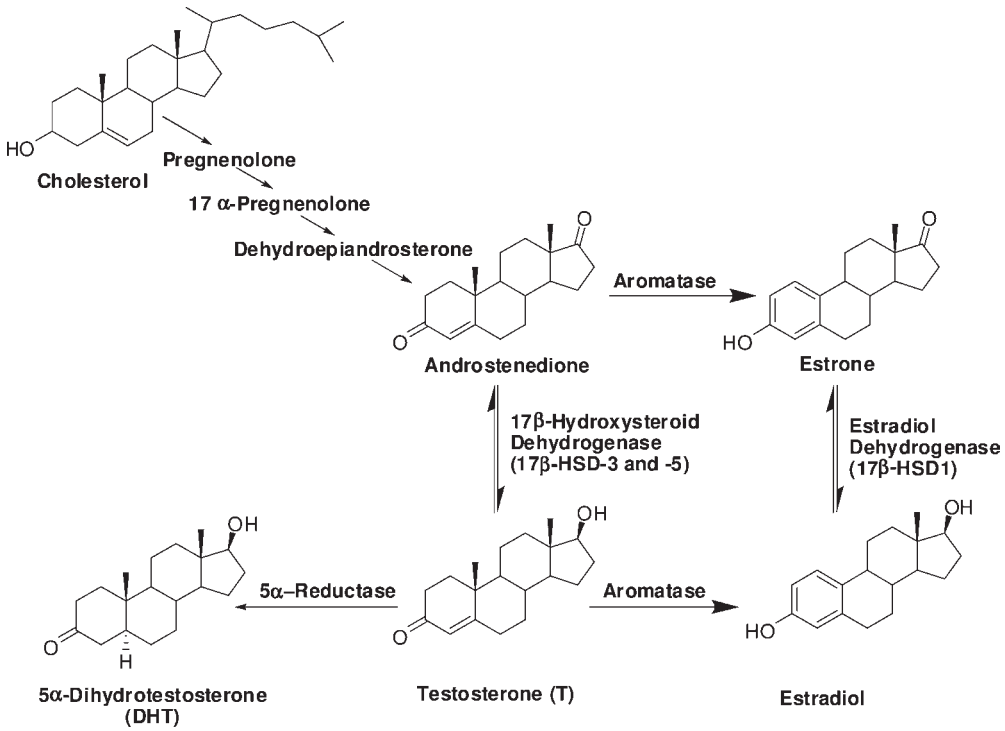


Figure 8.5 Biosynthesis of sex steroids.

male such as gynecomastia and can be a severe problem for men. The development of selective nonsteroidal AR ligands that elicit the beneficial effects of testosterone (e.g. muscle and bone), but avoid the deleterious effects (e.g. skin, prostate and breast), would represent a major step forward in the clinical use of androgens and is now on the horizon.

8.2
Nonsteroidal AR Ligands

8.2.1
Nonsteroidal Antiandrogens

AR antagonists, also referred to as antiandrogens or 'pure' antagonists, are compounds that antagonize the biological responses induced by endogenous or exogenous androgens by competitively inhibiting their binding to AR. Although steroidal antiandrogens such as cyproterone acetate (potent antiandrogen, antiprogestin and antiglucocorticoid) are known, they are no longer used clinically. Nonsteroidal androgen antagonists were first reported in the 1980s. Three anilide derivatives, flutamide (**7**) [43–45], nilutamide (**9**) [46–48] and bicalutamide (**10**) [49–51], were successfully commercialized for the treatment of prostate cancer (Figure 8.6).

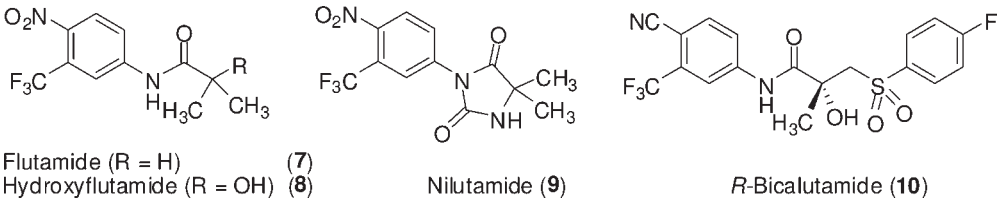


Figure 8.6 Anilide derivatives as antiandrogens.

8.2.1.1 Flutamide and Hydroxyflutamide

Flutamide (**7**) or Eulexin® was the first nonsteroidal androgen antagonist and was launched by Schering-Plough for metastatic prostate cancer in 1983. It was discovered by Neri *et al.* in 1972, originally as a bacteriostatic agent [43]. Unlike steroidal antiandrogens, flutamide is a nonsteroidal pure androgen antagonist that binds reversibly to AR and blocks the action of androgens without cross-activity at other steroid hormone receptors [52]. Flutamide (**7**) is a prodrug and requires hydroxylation *in vivo* on the carbon α to the carbonyl to afford the active metabolite, hydroxyflutamide (**8**) [44, 53].

8.2.1.2 Nilutamide

Bioisosteric replacement of the hydroxyl anilide moiety of hydroxyflutamide (**8**) with a hydantoin analog by Raynard *et al.* in 1977 resulted in nilutamide (**9**) or Nilandron® [48, 54], which was commercialized in 1987 by Aventis Pharma for

the treatment of hormone-dependent prostatic carcinoma. Although widely used, the reduction of the aromatic nitro group and hydrolysis of the imide bond in the imidazolinedione moiety *in vivo* [55] have been associated with hepatotoxicity, especially at the high doses needed for androgen blockage [56, 57].

8.2.1.3 **Bicalutamide**

Bicalutamide (**10**) or Casodex® is considered a second-generation nonsteroidal antiandrogen that was produced by replacing one of the methyl groups in flutamide (**7**) with the 4-fluorophenylsulfonyl moiety. Tucker *et al.* at ICI first reported bicalutamide (**10**) in 1987 [49] and it was launched in 1995 by AstraZeneca. The addition of the B-ring significantly improved binding affinity [2- to 4-fold higher relative to flutamide (**7**) and nilutamide (**9**)] [58]. Although structurally similar to flutamide (**7**), the amide moiety in bicalutamide (**10**) is not a good substrate for enzymatic hydrolysis in humans. Further, replacement of the A-ring nitro group with a cyano group removed a major site for reduction, changing *in vivo* metabolism to primarily glucuronidation thus producing a long half-life of 140 h in humans [44, 53, 56, 57, 59]. As a potent and nonsteroidal pure androgen antagonist, bicalutamide (**10**) causes regression of seminal vesicles (SV), ventral prostate (VP) and muscle [commonly levator ani (LA) in rats] weights [49, 59, 60], a common assay for *in vivo* androgenic or antiandrogenic activity called the Hershberger assay [61]. Cumulatively, these structural modifications decreased hepatotoxicity, increased half-life and afforded much more efficient androgen blockage. Bicalutamide (**10**) has largely replaced flutamide (**7**) and nilutamide (**9**) as the antiandrogen of the first choice for the treatment of prostate cancer [59, 62]. Bicalutamide (**10**) is the most commonly used antiandrogen with sales reaching $1.123 billion in 2005.

Despite the widespread use and success of bicalutamide (**10**) in early-stage prostate cancer, more than half of patients progress to fatal androgen-independent prostate cancer after 1–2 years of antiandrogen treatment. Therefore, novel nonsteroidal pure androgen antagonists, which are targeted to the prostate (i.e. tissue selective) and can bypass the hormone-refractory state, are an unmet medical need. The pursuit of new generation androgen antagonists continues with several groups such as Bristol-Myers Squibb [63, 64], Johnson & Johnson [65, 66], Pfizer [67–69], Endorecherche [70, 71], the University of Delaware [72] and the University of Tennessee Health Science Center (UTHSC) [73–76] actively producing innovative and potent AR antagonists, but to date no third-generation antiandrogens have been approved.

Although the traditional steroidal agonists and nonsteroidal antagonists are used for a narrow spectrum of diseases (owing to the factors discussed in Section 8.2.1), these molecules have served as archetypical scaffolds (generalized structural templates) for the development of many other related AR chemotypes with potentially broad therapeutic application. Over 64 chemically distinct putative AR agonist templates have been reported, including 22 templates with demonstrated *in vivo* tissue-selectivity for anabolic tissues [i.e. selective AR modulators (SARMs)].

8.2.2
Discovery of Nonsteroidal SARMs – Propionanilides (UTHSC/GTx)

The field of SARMs was stimulated by the early discoveries of Drs Miller and Dalton at the UTHSC focusing on chiral synthesis [77] and characterization of electrophilic analogs of bicalutamide as potential irreversible antiandrogens [74]. Dalton *et al.* unexpectedly discovered several nonsteroidal ligands with the ability to fully stimulate *in vitro* AR-dependent transcriptional activation. This unprecedented activity was observed for propionanilides R-3 (**11**) and R-1 (**12**) that differed from hydroxyflutamide and bicalutamide by the addition of halogen to the terminal methyl of hydroxyflutamide and changing the linker sulfone of bicalutamide to a thioether (various *para* B-ring substituents), respectively (Figure 8.7) [78, 79]. (Ligand Pharmaceuticals, a contemporaneous group, also reported *in vitro* agonist activity immediately thereafter [80–82], as will be reviewed below.) Structure–activity relationship (SAR) inquiries explored the *para* A-ring and *para* B-ring positions, thus improving the *in vitro* agonist activity while eliminating reactivity at the *para* B-ring position [see thioacetolutamide (**13**) in Figure 8.7] [79, 83]. Hydroxyflutamide analogs [84] and nonpropionanilide templates were also explored with less success [83].

These thio-ether linked propionanilides seemed very promising, but suffered from a lack of the expected androgenic or anabolic activity *in vivo* due to metabolic oxidation of the thioether to the partial agonist/antagonist sulfoxides or sulfones [85]. Traditional medicinal chemistry design methods were used to eliminate this site of metabolism and enhance the binding affinity by changing the linker heterogroup from thioether to ether. An *in vivo* pilot study in castrated rats demonstrated that these AR ligands were capable of tissue-selective AR-dependent full agonist activity [86, 87]. The ether-linked propionanilides were selective full anabolic agonists with the ability to fully support the weight of the LA muscle but weak partial agonists (or antagonists) in androgenic tissues such as VP and SV [see S-1 (**14**) and S-4 (**15**) in Figure 8.7]. Molecules such as these were termed SARMs in analogy to selective ER modulators (SERMs) where tissue-specific anabolic (bone maintenance) and estrogenic (breast and/or uterine maintenance) activities have been separated.

A potent SARM, S-4 (**15**), was identified which demonstrated rapid and complete oral absorption at low doses and reasonable elimination half-life ($t_{1/2} = 2.6$–5.3 h) in rats, suggesting compounds such as this would be excellent candidates for clinical development [88]. These successes focused our efforts on the diaryl propionanilide class of SARMs of which many have been published [89–92]. Recent studies demonstrated that these SARMs improve skeletal (soleus) muscle strength, increase lean body mass (LBM), reduce body fat, prevent bone loss in rats, in addition to promising pharmacologic activity in animal models of benign prostatic hypertrophy and male fertility [92–95]. Ostarine™ is the most advanced SARM clinical candidate and has demonstrated exciting data from its initial proof-of-concept phase II clinical trial. GTx reported in December 2006 the results of this clinical trial which was

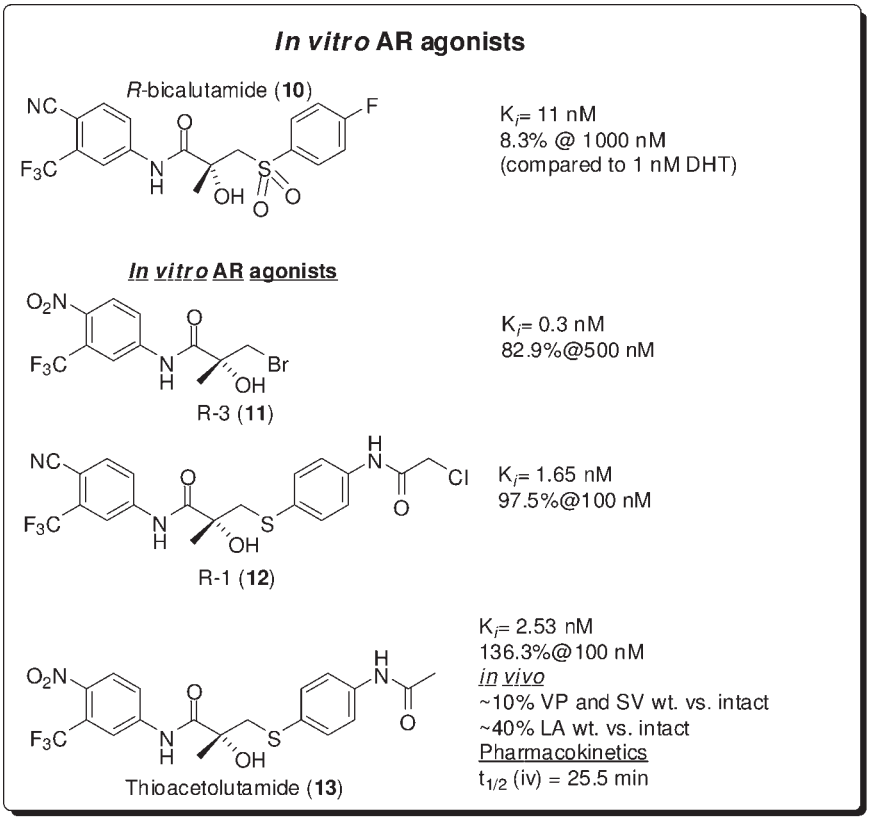


Figure 8.7 Discovery of propionanilide AR agonists *in vitro* and *in vivo*.

a double-blind, randomized, placebo-controlled trial in 60 elderly men and 60 postmenopausal women [96]. Without a prescribed diet or exercise regimen, all subjects treated with Ostarine™ had a dose-dependent increase in total LBM, with a 3-mg/day cohort achieving an increase of 1.3 kg compared to baseline and 1.4 kg

compared to placebo after 3 months of treatment. Treatment of Ostarine™ also resulted in a dose-dependent improvement in functional performance measured by a stair climb test, with the 3-mg/day cohort achieving clinically significant improvement in speed and power. The SARM also demonstrated a favorable safety profile with no serious adverse events reported and no apparent change in measurements for serum prostate-specific antigen (PSA; prostate), sebum production (skin and hair) or serum LH (pituitary) compared to placebo. Interestingly, subjects treated with 3 mg/day of Ostarine™ had on average an 11% decline in fasting blood glucose, a 17% reduction in insulin levels and a 27% reduction in insulin resistance (homeostasis model assessment) as compared to baseline, suggesting that SARMs might have therapeutic potential in diabetics or people at risk for diabetes. Phase I clinical studies with Ostarine™ showed that it was rapidly absorbed after oral administration with a half-life of about 1 day [97]. Additional phase II studies in muscle wasting associated with cancer cachexia began in 2007.

8.2.3 Design and Development of Diverse SARM Chemotypes

8.2.3.1 Orion

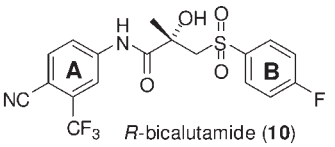
Orion characterized a series of closely-related propionanilide SARMs in which the A-ring *m*-trifluoromethyl group of S-4 (**15**) was converted to methyl or hydroxymethyl [98] (Figure 8.8). Compound **16** (20 mg/kg) demonstrated relatively weak anabolic agonist activity in LA muscle weight compared to 5 mg/kg TP, and relatively strong agonist activity in SV tissue compared to VP tissue. The anabolic activity of **16** appears to be significantly weaker than other propionanilides, such as S-4 (**15**), and several other classes of SARMs to be discussed herein. Nevertheless, Orion reportedly advanced a clinical candidate to phase I clinical trials [99] but no results have been reported, leaving its development status uncertain.

8.2.3.2 Johnson & Johnson and Subsidiaries – Cyclic Bioisosteres of Propionanilides

Johnson & Johnson patented a wide variety of diaryl AR templates in which the propionanilide linker segment has been replaced by a variety of cyclic elements to include thiazolines (**17**) [100], pyrazoles (**18**) [101], imidazolin-2-ones (**19**) [102], diaryl indoles (**20**) [103] and pyrrolopyridines (**21**) [104] (representative examples shown in Figure 8.8). Although this pharmacophoric diversity is impressive, the patents only disclose tissue selectivity in qualitative terms, with no quantitative measures of pharmacologic activity or SAR. The only peer-reviewed publication of diaryl AR ligands reports a number of low-affinity molecules; the most promising of which is an indole (**22**), which binds with 140 nM affinity to the AR [105] (Figure 8.8, top right).

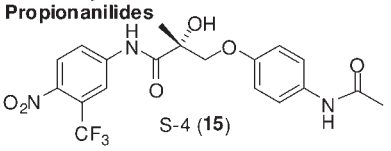
8.2.3.3 GlaxoSmithKline – Diaryl Anilines

GlaxoSmithKline reported a series of compounds which are described as AR modulators, but did not disclose biological activities other than 'favorable' compounds have pIC_{50} (binding) <5 and LA hypertrophy with little prostate stimulation



R-bicalutamide (**10**)

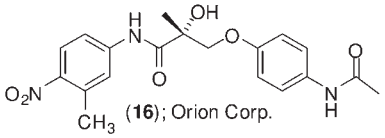
University of Tennessee/GTx, Inc.
Propionanilides



S-4 (**15**)

E_{max} (LA) = 101%; ED_{50} (LA)= 0.14 mg/day
E_{max} (SV) = 28.5%; ED_{50} (SV)= 0.55 mg/day
E_{max} (VP) = 35.2%; ED_{50} (VP)= 0.43 mg/day
partial LH suppression at >0.5 mg/day

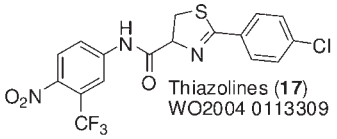
Most Extensively Characterized SARM (Sections 2 to 3)



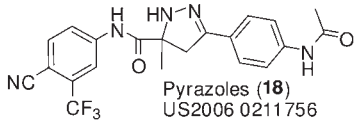
(**16**); Orion Corp.

SARM activity at 20 mg/kg but less potent than T at 5 mg/kg

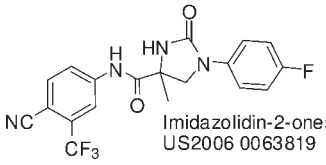
Johnson & Johnson (Patented Diaryl SARMs)



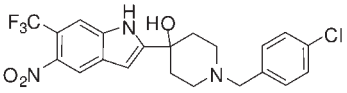
Thiazolines (**17**)
WO2004 0113309



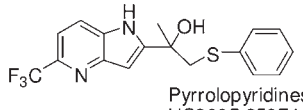
Pyrazoles (**18**)
US2006 0211756



Imidazolidin-2-ones (**19**)
US2006 0063819



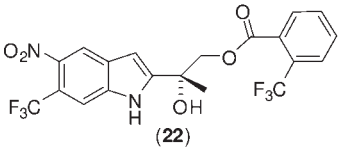
Diaryl Indoles (**20**)
US2005 245485



Pyrrolopyridines (**21**)
US2005 250741

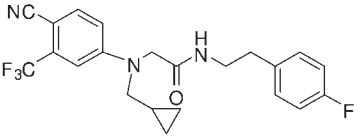
Figure 8.8 Diaryl SARMs.

Johnson & Johnson - Indole
K_i = 140 nM (binding only reported)



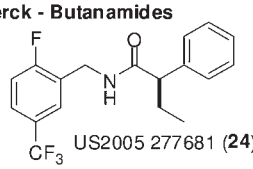
(**22**)

GlaxoSmithKline - Diaryl Anilines

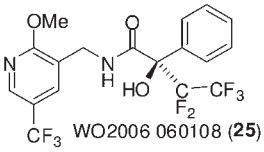


WO2006 044707; (**23**)

Merck - Butanamides

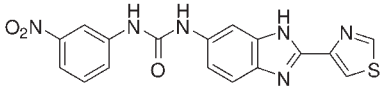


US2005 277681 (**24**)



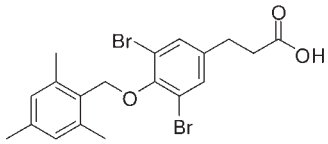
WO2006 060108 (**25**)

Merck - Carbonylamino-benzimidazoles



WO2004 041277 (**26**)

Karo Bio AB - Diaryl propanoic acids



WO2005 094810 (**27**)

(i.e. SARMs) [106]. A representative example (**23**) is given in Figure 8.8. Structural variation included diaryl compounds similar to bicalutamide but separated by three, four, five or six atoms. Often times the anilido nitrogen was tertiary with a substituted aliphatic side-chain. Also, the linker chiral alcohol of propionanilides was replaced by a central amide separate from the aniline.

8.2.3.4 Merck – Diaryl Butanamides and Carbonylamino-benzimidazoles

Merck patented two distinct diaryl SARM templates as shown in Figure 8.8 (**24**–**26**). This series of Merck patents conspicuously lacks any assay to determine activity on muscle and also prominently claims combinations with other potential osteoporosis agents. The diaryl butanamides (representative examples **24** and **25**) closely resemble the propionanilides, but differ by the quarternary carbon being ethyl substituted (making them butanamides) and the insertion of a methylene group between the A-ring and the amide. Points of variation include the A-ring which can be pyridin-4-yl [107], pyridin-3-yl [108] or benzyl [109]; the tertiary carbon alkyl substituent can be methyl or ethyl and perfluorinated or not; also various A- and B-ring substituents and substitution patterns have been explored. The carbonylamino-benzimidazole [110] template has three basic variations: (i) diaryl benzimidazoles with no linker (not shown), (ii) urea + benzimidazole linked triaryl compounds (representative example 26 shown in Figure 8.8) and (iii) amide + benzimidazole linked triaryl compounds (not shown) . Neither lead molecules nor peer-reviewed Merck diaryl SARM articles are known.

8.2.3.5 Miscellaneous (Karo Bio)

A couple of atypical diaryl AR templates were reported in a patent by Karo. This application discloses various diaryl propanoic acid derivatives with binding affinities in the range of 40–10 000 nM which are reported to be antagonists, partial antagonists or partial agonists [111] (representative example **27** in Figure 8.8).

8.2.4 Quinolinone (Pyridone), Coumarin, and Other Fused Ring SARMs

8.2.4.1 Ligand Pharmaceuticals – Bi-, Tri- and Tetracyclic Quinolinones

Ligand Pharmaceuticals was an early leader in the development of nonsteroidal AR ligands with their series of tricyclic quinolinones [80–82, 112, 113]. Ligand Pharmaceuticals has published and patented an extensive array of mono- (discussed below), bi-, tri- and tetracyclic quinolinone templates, some of which demonstrated high affinity and potent tissue-selective anabolic agonist activities. The structural core of this series is a quinolinone (or alternatively pyridone) A-ring which occupies a space in the receptor similar to the steroidal A-ring (discussed below). The earliest members of this class were antagonists such as LG-120907 (**28**) which bound with a K_i value of 26 nM (Figure 8.9) [114] and inhibited testosterone-induced increases in VP and SV tissue weights (ED_{50} [VP] = 18.3 mg/kg; ED_{50} [SV] = 19.2 mg/kg). Changing the A-ring from the quinolinone to a coumarin (representative example **29**) within a 2,2-dimethyl substituted template retained antagonist activity; however,

changing the alkylation pattern from 2,2-dimethyl to 4-ethyl such as in LGD121071 (**30**) produced an early high-affinity, potent AR full agonist in *in vitro* transcriptional studies ($K_i = 17$ nM; *in vitro* $EC_{50} = 4$ nM; 100%) [113]. This α,β-unsaturated quinolinone became a conserved feature in the Ligand Pharmaceuticals agonist series.

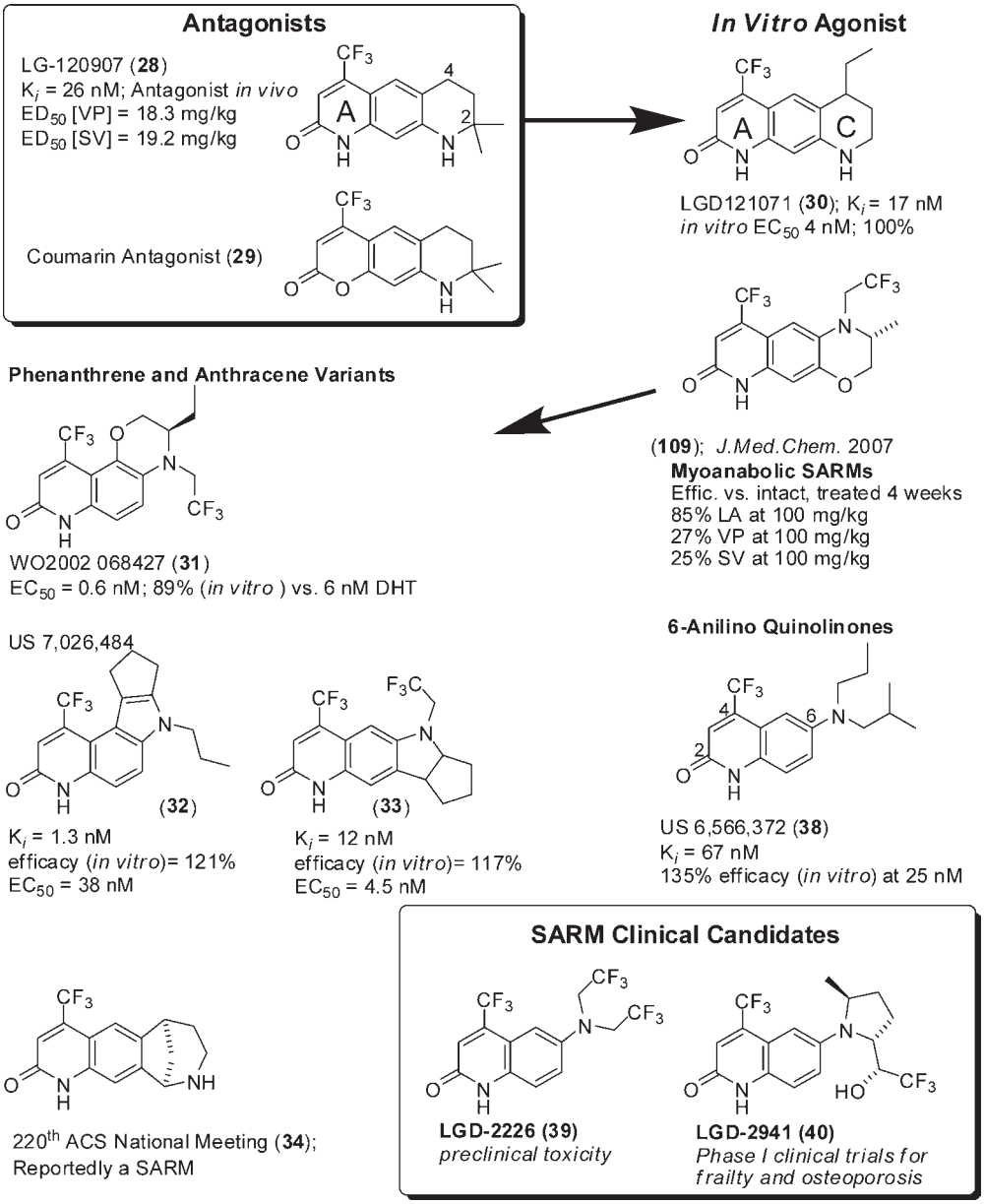


Figure 8.9 Quinolinone (pyridone) fused-ring SARMs from Ligand Pharmaceuticals, Inc.

A few novel phenanthrene-based tricyclic quinolinones were reported as *in vitro* AR agonists [115] (example **31** in Figure 8.9) in transcriptional activation assays with the most potent (**31**; $EC_{50} = 0.6$ nM) showing 89% efficacy as compared to DHT (100% at 6 nM). No *in vivo* data was reported for **32** and **33** [116], and **34** [117] (Figures 8.9 and 8.10). Other anthracene- and phenanthrene-based AR agonists from Ligand Pharmaceuticals were recently reviewed [118, 119] (**35** [120], **36** [121] and **37** in Figure 8.10). They also patented a series of 6-anilino quinolinones in which the aniline was generally disubstituted. The most potent being the *N*-*sec*-butyl, *N*-propyl aniline (**38**) which bound with a K_i of 67 nM and *in vitro* agonist efficacy of 135% at 25 nM. *In vivo* data was not reported [122].

After exploring these numerous SARM templates, Ligand Pharmaceuticals chose LGD2226 (**39**) as its first preclinical lead compound. Although development of LGD2226 was later discontinued due to preclinical toxicity [123], Ligand Pharmaceuticals published characterizations of LGD2226 with regard to the SAR for similar compounds [124, 125], discovery/organic synthesis [126], cocrystal structure with AR [127] (discussed below), myo- and osteoanabolic activity, and maintenance of sexual function in castrated rats [128]. LGD2226 demonstrated myoanabolism weaker than testosterone and osteoanabolism which was shown to increase BMD, improve structure and strength, and positively affect biomarkers. In 2005, Ligand Pharmaceuticals filed an Investigational New Drug (IND) application for LGD2941 (**40**) which is currently in phase I clinical trials for frailty and osteoporosis in collaboration with TAP Pharmaceuticals (an Abbott subsidiary). At the 2006 American Society for Bone and Mineral Research in Philadelphia, Ligand Pharmaceuticals and TAP Pharmaceuticals had several abstracts revealing the structure of LGD2941 and characterizing its osteo- and myoanabolic properties in male rats [129] and male monkeys [130], and osteoanabolic properties in female rats [131].

8.2.4.2 Pfizer – Quinolinone and Coumarin AR Antagonists

Pfizer patented a series of AR ligands which are analogs of the Ligand Pharmaceuticals 6-quinolinone templates. These AR ligands are of three templates: (i) 6-sulfonamide pyridones [132], (ii) benzyl urea pyridones [133] and (iii) benzyl coumarins [134] that appear to be weak affinity (micromolar) antagonists which are illustrated as representative examples **41**, **42** and **43**, respectively, in Figure 8.10. Conspicuously absent from these quinolinones is the aniline moiety, which may explain the low affinity and lack of agonist activity.

8.2.4.3 Merck – Chromeno and Quinolinyl Benzazepines

Merck patented a handful of anthracene-based chromeno and quinolinyl benzazepines (representative example **44** in Figure 8.10). These compounds are reportedly SARMs that were tested by the same panel of assays as for other Merck patents (see above); however, no activity is reported [135].

8.2.4.4 Merck – Azasteroidal SARMs

Merck also patented a variety of steroidal SARMs. These are a variation of the 4-azasteroidal template of finasteride, a 5α-reductase inhibitor. Modifications at

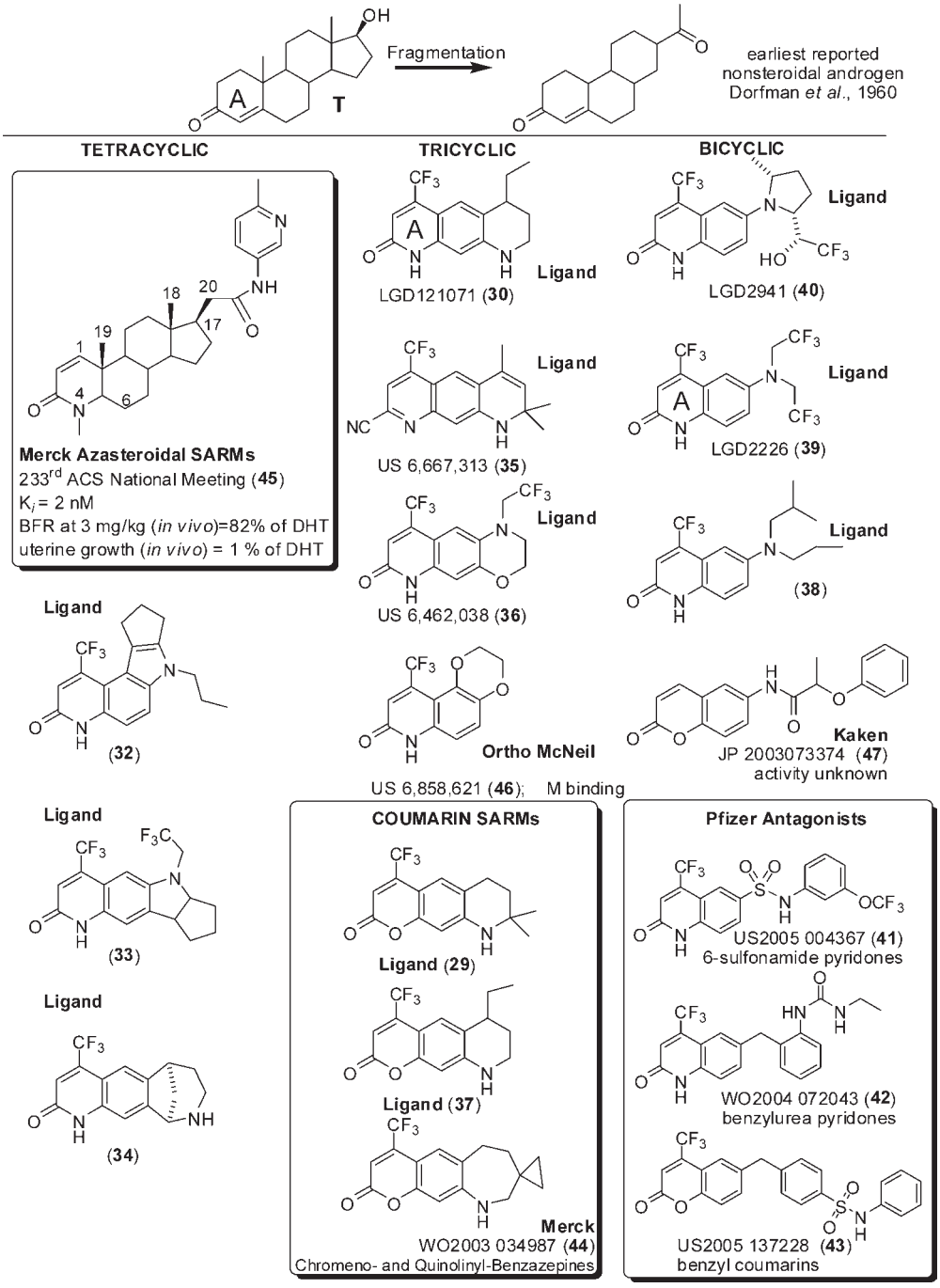


Figure 8.10 Quinolinone (pyridone), coumarin and other fused ring SARMs. Tetra-, tri- and bicyclic AR ligands are organized from left to right.

several positions reportedly produce activity described as tissue selective, with agonist activity in bone and muscle, and antagonist activity in prostate or uterus. Some of the points of variation included fluorination of the A-ring [136], substitution at the 4- [136–146], 6- [137] and 7- [138] positions, and addition of an imidazole ring fused to the 3- and 4-positions [139]. The most conspicuous changes occur at the 17β-position. These substituents are a wide variety of nitrogen-linked aryl groups which includes carboxamides and acetamides [140–143], amines [144], C17 heterocyclics [145], and C20 [146] heterocyclics (see Figure 8.10, top left for numbering). Merck reported data at the 2007 National American Chemical Society Meeting to substantiate their claims of SARM activity in an azasteroidal template. A series of 17-hydroxy-4-azasteroids was analyzed in an *in vitro* transactivation assay. This SAR information was used to select the azasteroid shown in Figure 8.10 which was demonstrated to be osteoanabolic with an *in vivo* bone formation rate of 82% of DHT at 3 mg/kg, but with low virilization potential (only 1% of uterine weight) in a 24 day *in vivo* ovariectomized rat model [147] (**45** in Figure 8.10).

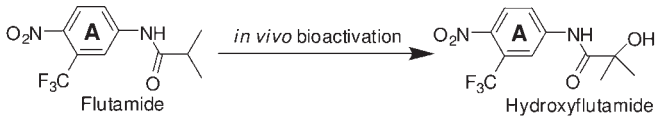
8.2.4.5 Miscellaneous [Ortho McNeil Pharmaceutical (J & J Subsidiary) and Kaken Pharmaceutical]

Ortho-McNeil Pharmaceutical patented a series of compounds which share 2-quinolinyl-fused heterocyclic ring system in both phenanthrene and polyaromatic hydrocarbon fused tricylic configurations (representative example **46** in Figure 8.10). These molecules are reported as AR modulators; however, the only data reported is weak binding (low to mid micromolar) and weak inhibition *in vitro* [148]. Lastly, Kaken Pharmaceutical patented a series of coumarins with both hydroxyflutamide-like and B-ring-containing propionanilines [149] (Japanese language; activity unknown) (representative example **47** in Figure 8.10).

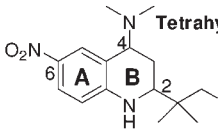
8.2.5 Substituted Aniline and Flutamide Variant SARMs

8.2.5.1 Kaken Pharmaceutical – Tetrahydroquinolines (THQ)

Kaken Pharmaceutical built their compounds around the bicyclic THQ and tricyclic 3,4-cyclopentano THQ scaffolds (Figure 8.11) and disclosed SARs for the binding of AR based on THQ substitution patterns [150]. Substitution at the 2- and 4-positions of the THQ ring with a variety of groups led to the identification of a series of analogs with 7-fold higher affinity than hydroxyflutamide. S-40503 (**48**) was selected for their initial *in vivo* studies. S-40503, when administered for 4 weeks to castrated rats beginning immediately after surgery, exerts androgenic effects, partially restoring the prostate back to a normal level (78 mg/100 g of body weight for S-40503-treated, castrated rats versus 9 mg/100 g of body weight for untreated castrated rats). This compound was also reported to maintain BMD at a comparable level with control animals and is perhaps the best-characterized SARM in bone to date (Figure 8.11). A similar study was performed in ovariectomized female rats. However, a waiting period of 4 weeks was added before they were treated with S-40503 for 8 weeks. DHT was used as a positive control. S-40503 also increased BMD in female ovariectomized



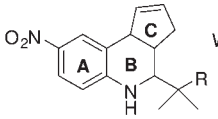
Kaken Pharmaceuticals Co., Ltd.



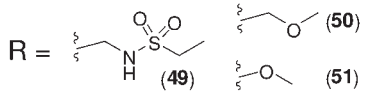
Tetrahydroquinolines (THQ)
US 6,777,427
S-40305 (**48**)

710% RBA relative to hydroxyflutamide

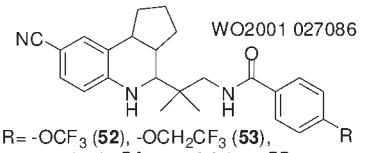
Castrated rats treated immed. for 4 wks
Efficacy [BMD]= 99% of intact at 30 mg/kg
vs. 96% for DHT at 10 mg/kg
VP efficacies = 80% and 155%, respectively
Ovariectomized, 4 wk atrophy, treated 8 wks
S-40503 (**48**) and DHT both had 102% efficacy



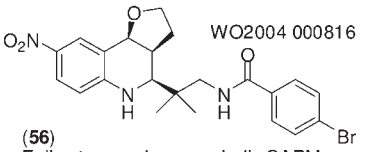
Tricyclic THQ
WO2004 013104



(**49**) **in castrated rats treated immed. for 4 wks**
Efficacy[BMD]= full anabolic activity at 60 mg/kg
VP efficacies = 56% vs. 150% for DHT
(**50**) & (**51**) inhibit T-induced VP growth in castrated rats

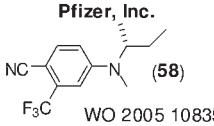


WO2001 027086
R= -OCF_3 (**52**), -OCH_2CF_3 (**53**), -NHCHO (**54**), -$NHCOCH_3$ (**55**)
Full osteo- and myoanabolic SARMs



WO2004 000816
(**56**)
Full osteo- and myoanabolic SARMs

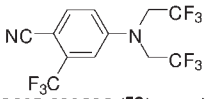
Pfizer, Inc.



WO 2005 108351
K_i = 1.1 nM
Castrated rats:
89% LA and 39% VP at 30 mg/kg compared to DHT (10 mg/kg)

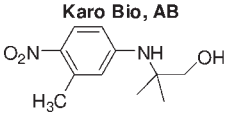
(see also Figure 8.12)

Ligand Pharma., Inc.



WO2005 090282 (**59**); no data

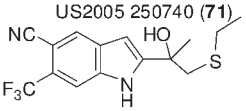
Karo Bio, AB



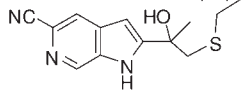
WO2005 042464 (**61**)
in vitro agonists and antagonists

Johnson & Johnson

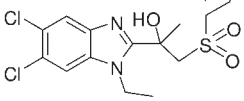
Indoles
US2005 250740 (**71**)

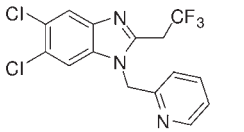


Pyrrolopyridines
US2005 250741 (**72**)



Benzimidazoles
US2006 0116412 (**73**)

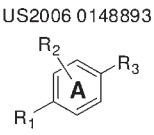




US2006 0116402 (**74**)

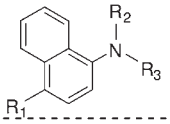
GlaxoSmithKline - Aniline SARMs (no data)

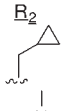
US2006 0148893

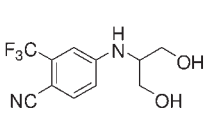


R_1	R_2	R_3	
NO_2	2-Me		(**62**)
CN	2-NO_2		(**63**)

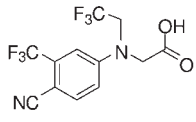
US2006 0142387



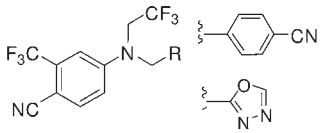
R_1	R_2	R_3	
CN			(**68**)
NO_2			(**69**)



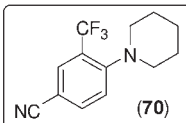
WO2005 000795 (**64**)



WO2005 085185 (**65**)



WO2006 133216 (**66** and **67**)



(**70**)
J. Comb. **9**:107 (2007)

Figure 8.11 Substituted aniline and flutamide variant SARMs.

(i.e. removal of the ovaries which are the primary estrogen-producing gland in females) rats, indicative of osteoanabolic activity, and had the same or better anabolic effects as DHT (Figure 8.11). Hanada *et al*. [151] further characterized the osteoanabolic activity of S-40503 by showing it increased BMD and biomechanical strength of femoral cortical bone compared to estrogen, an antiresorptive agent that does not increase these parameters (not shown). They also demonstrated the expected myoanabolic activity in LA at 30 mg/kg to be greater than intact control, but less than DHT at 10 mg/kg. Unfortunately, when S-40503 was administered for an 8-week period to castrated rats, the prostate weight was restored to the level of the control, illustrating it also showed full androgenic agonist activity. To our knowledge, S-40503 was not advanced to clinical trials, possibly owing to these prostatic effects or due to a lack of oral bioavailability as all experiments were performed with subcutaneous adminstrations.

Kaken Pharmaceutical also explored a variety of tricyclic THQ derivatives similar to some of Ligand's tricyclic quinolinone templates. However, Kaken Pharmaceutical's templates have the nitrogen in the B-ring which is always saturated (Figure 8.11). Early patents demonstrated *in vivo* osteo- and myoanabolic activities for molecules with three distinct C-rings: (i) cyclopentene [152], (ii) cyclopentane [153] and (iii) tetrahydrofuran [118, 154]. An exemplary compound (**49**) of the cyclopentene template (Figure 8.11) when administered at 5 days post-castration at 60 mg/kg for 4 weeks increased VP weights (56 mg/100 g body weight) relative to vehicle-treated, castrated (9 mg/100 g body weight.) but did not approach the positive control of 10 mg/kg DHT (150 mg/100 g) or sham control (104 mg/100 g) demonstrating partial agonist activity in VP. Although BMD for **49** was comparable to DHT and sham controls, the difference between vehicle-treated castrated and sham was small (124 versus 132 mg/cm^2). Compounds **50** and **51** were shown to partially prevent testosterone-induced increases in prostate size in castrated rats treated with testosterone, suggesting that these compounds may be useful in the treatment of prostate cancer (CaP), prostatic premalignancies such as prostatic intraepithelial neoplasia and proliferative inflammatory atrophy [155], and benign prostatic hyperplasia, or other androgenic prostatic maladies.

Compound **52** is an example of the Kaken Pharmaceutical template with the cyclopentane ring fused to the 3,4-positions of the THQ ring system. Compound **52** (30 mg/kg) partially increased VP weight as compared to intact controls (70 versus 94 mg/100 g) but demonstrated full osteoanabolic activity and hypermyoanabolic activity. Compounds **53**–**55** demonstrated full myoanabolic activity, with androgenic effects in the prostate varying from partial to full, demonstrative of the unique characteristics of these and other SARM pharmacophores on a molecule by molecule basis. The tetrahydrofuran variants with oxygen at the 4-THQ position of the THQ nucleus (**56** in Figure 8.11) also demonstrated full myo- and osteoanabolic SARM activity [156].

Despite multiple early and thorough demonstrations of tissue-selective hypermyoanabolic and osteoanabolic activities from several related structural templates, no clinical candidates are known from Kaken Pharmaceutical.

8.2.5.2 Pfizer – Cyclic or Disubstituted Anilines

Pfizer reported a series of high AR affinity (low nanomolar range) cyclic (**57**) or disubstituted (**58**) anilines [157]. Compounds **57** and **58** displayed IC_{50} values of 5.2 and 1.1 nM in binding assays (Figures 8.12 and 8.11), respectively. Compound **57** (10 mg/kg/day, subcutaneously (s.c.)] effectively reduced fat mass (121 versus 121 g sham and 149 g for ovariectomized) and increased LBM (319 versus 299 g sham and 291 g ovariectomized). Similar results were achieved with these compounds in aged (11 months old) rats. Compound **57** increased bone mineral content (11.9 versus 11.9 mg/mm for sham and 11.0 mg/mm for ovariectomized) but had a lesser effect on BMD (602 versus 672 mg/cm^3 for sham and 593 mg/cm^3 for ovariectomized). Compounds **57** and **58** showed tissue selectivity in castrated immature male rats (25 days old). Daily subcutaneous administration (30 mg/kg) of **57** and **58** for 4 days retained 100 and 89% of LA weight, respectively, but only 33 and 39% of VP weight as compared to DHT (10 mg/kg).

Pfizer reported osteoanabolic SARM activity for two molecules (structures unknown), CE-590 [158] and CMP1 [159]. CE-590 is a high-affinity ($IC_{50} = 16$ nM) SARM *in vivo*. An 8-week treatment schedule of CE-590 (30 mg/kg orally, twice per day) significantly decreased prostate weight by 26% in sham rats (acting as an AR antagonist), as compared to 66% increase for DHT-treated sham rats (agonistic activity) [160]. CE-590 completely prevented castration-induced decreases in trabecular content, trabecular density, cortical content, cortical area and cortical thickness and increases in bone resorption and turnover. No SARM clinical candidates are known for Pfizer.

8.2.5.3 Ligand Pharmaceuticals – Disubstituted and Cyclic Anilines

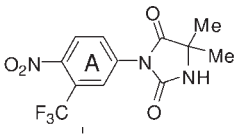
Ligand Pharmaceuticals further extended their earlier SARM portfolio by eliminating the quinolinone A-ring from their bicyclic template, leaving just the aniline. A variety of monoaryl ring systems (phenyl, naphthyl, indole) directly connected to a variety of acyclic (representative example **59** in Figure 8.11) and cyclic (example **60** in Figure 8.12) anilines [161] were reportedly SARMs, but no assays are described and no data reported for these compounds.

8.2.5.4 Karo Bio – Alkanol Substituted 4-Nitroanilines

Karo Bio patented mono-substituted aniline AR ligands which possess some *in vitro* partial agonist activity but included several other examples of low potency full antagonists *in vitro* [162]. Sites of structural variability include the *meta* substituent of the aniline, 2-pyridino A-rings, and a variety of branched alkanol side-chains (representative example **61** in Figure 8.11). No *in vivo* testing or demonstration of tissue selectivity was disclosed. Karo Bio recently entered into a SARMs license agreement with Radius Health [163], suggesting significant *in vivo* activity must be known. No clinical candidates are yet known.

8.2.5.5 GlaxoSmithKline – Disubstituted Anilines

GlaxoSmithKline patents outline a wide variety of disubstituted aniline templates to include *para* nitro/cyano and *ortho*/*meta* electron-withdrawing A-ring substituents on a phenyl A-ring (examples of the structural diversity of this series are given in

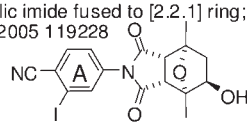


Nilutamide

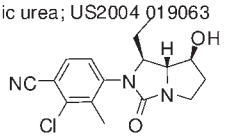
COMPLEX VARIATIONS OF NILUTAMIDE

BMS B-ring Variations

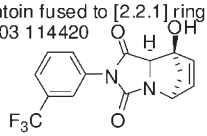
cyclic imide fused to [2.2.1] ring;
US2005 119228



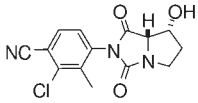
cyclic urea; US2004 019063



hydantoin fused to [2.2.1] ring;
US2003 114420

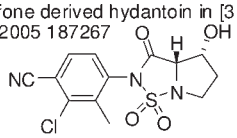


hydantoin in [3.3] ring; US2004 019063

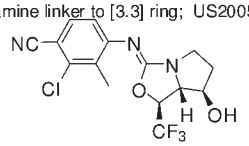


BMS-564929
LA effic. = 125%
ED_{50} = 0.0009 mpk

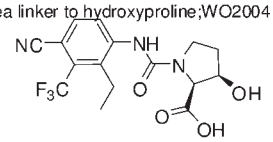
sulfone derived hydantoin in [3.3] ring;
US2005 187267



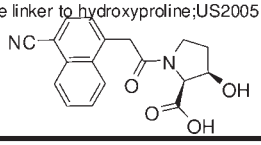
enamine linker to [3.3] ring; US2005 197359



urea linker to hydroxyproline;WO2004 045518

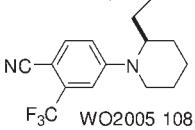


amide linker to hydroxyproline;US2005 059652



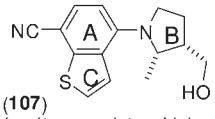
RELATIVELY SIMPLE VARIATIONS OF NILUTAMIDE THEME

Pfizer, Inc.



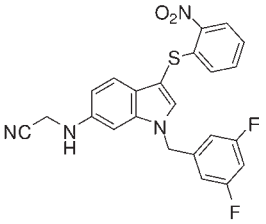
WO2005 108351
K_i = 5.2 nM
ovariectomized rats: 4 days
decreased fat body mass (FBM)
increased lean body mass (LBM)
increased BMD/BMC
castrated rats:
100% LA and 33% VP
compared to 10 mg/kg of DHT
castrated (aged) rats: 8 weeks
decreased FBM
increased LBM,
increased trabecular & total BMD

Takeda Pharma.
WO2004016576
US20060106067



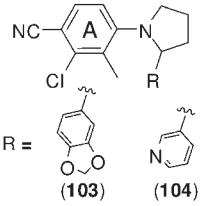
(**107**)
in vitro agonists which are antiproliferative in hormone resistent prostate cancer cell lines

Akzo Nobel NV



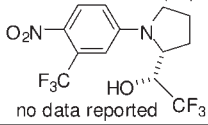
WO2005 102998 (**108**)
transcriptional activation of 40.3% of 100 nM DHT

Eli Lilly and Company, Inc.
WO2006 124447



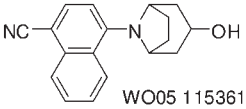
LA Efficacy (% veh. treated)
186% (sc) for (**103**)
164% (po) for (**104**)
at 10 mg/kg per day

Ligand Pharma.
WO2005 090282 (**60**)

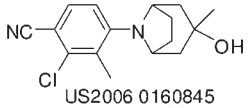


no data reported

Acadia Pharmaceuticals, Inc.



WO05 115361
154BG31 (**105**)



US2006 0160845
198RL26 (**106**)

Potent *in vivo* partial myoanabolic agonists which penetrate the CNS and suppress LH at therapeutic doses.

Figure 8.12 Hydantoin and cyclic aniline SARMs.

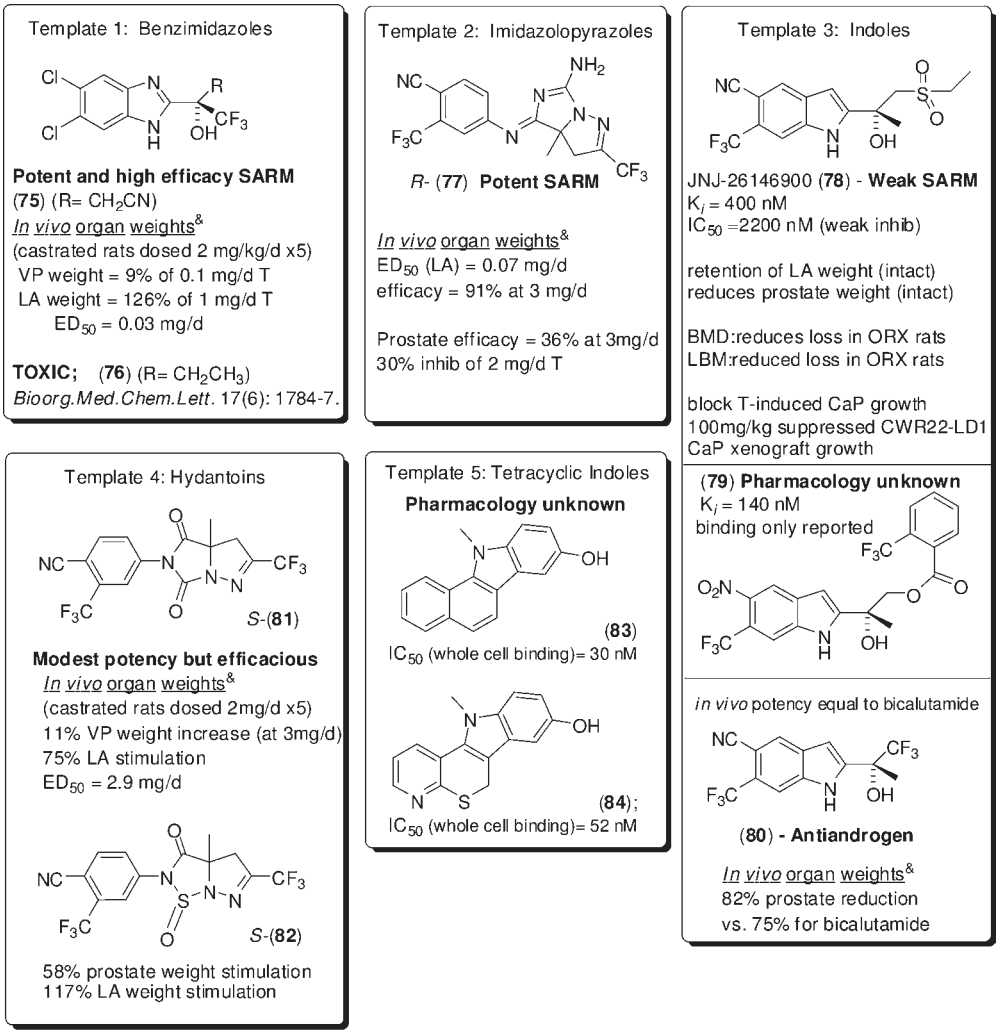
Figure 8.11 ([164] **62** and **63**, [165] **64**, [166] **65**, [167] **66** and **67**, respectively) or, alternatively, *para* nitro/cyano naphthyl A-rings ([168] **68** and **69**). The aniline substituents of these templates include alkyl, haloalkyl, alkenyl, cycloalkyl, alkanol, alkylamino and carboxylate and derivatives (Figure 8.11). The patents describe GR, PR, MR and AR binding affinity and AR–luciferase transactivation *in vitro* assays and *in vivo* studies in castrated rats analyzing VP and SV, and LA and bulbocavernosus (BC) muscles as androgenic and anabolic indicators, respectively. However, the only GlaxoSmithKline disubstituted aniline for which biological data is disclosed is a nilutamide-like cyclic aniline template [169]. A virtual screening-guided combinatorial chemistry approach was used to find AR agonists with various substitutions of the left ring and various replacements of the right ring, as shown, of compound **70**. This yielded 352 submicromolar and 17 subnanomolar AR agonists as measured by a cell-based reporter gene functional assay. GlaxoSmithKline recently disclosed their first *in vivo* SARM characterization. GSK2420A (structure not given) demonstrated an ED_{50} (LA) of 0.026 mg/kg in a 7-day castrated rat model and restored castration-induced LA muscle atrophy in a 28-day treatment. The effects of GSK2420A on the prostate are consistent with partial agonist activity, eliciting 2-fold increase over vehicle versus 7-fold for DHT (3 mg/kg), and decreased prostate weights in intact rats [170].

8.2.5.6 Johnson & Johnson – Benzimidazoles, Imidazolopyrazoles, Indoles, Hydantoins and Tetracyclic Indoles

Johnson & Johnson and its subsidiaries initiated comprehensive SARM and AR antagonist programs, and demonstrated tremendous pharmacophore diversity with templates that arguably span all the broad classes of structural nuclei (bicalutamide-like, nilutamide-like, hydroxyflutamide-like, etc.), referred to as structural archetypes herein, several for which tissue selectivity has been demonstrated. Eight different series of patents were recently published, four of which fall into the hydroxyflutamide-like archetype including two patents on 2-propanol-derived indoles or pyrrolopyridines [171, 172] (Figure 8.11: **71** and **72**) and two patents on benzimidazoles [173, 174] (**73** and **74**). Additionally, Johnson & Johnson recently published AR ligands belonging to five chemically distinct templates (Figure 8.13): (i) benzimidazoles [65, 175, 176], (ii) imidazolopyrazoles [177], (iii) indoles [66, 105, 178], (iv) hydantoins [179] and (v) tetracyclic indoles [180] .

Some of the published benzimidazole compounds were characterized as potent and efficacious myoanabolic SARMs. For instance, **75** is a 2-(2,2,2)-trifluoroethyl-benzimidazole which when dosed at 2 mg/kg supported 126% LA weight (compared to 1 mg/kg testosterone) with an ED_{50} of 0.03 mg/day, but with little stimulation of the prostate [176], demonstrating an activity profile comparable to other hyperanabolic SARMs. Other molecules in the benzimidazole class were recently characterized as AR antagonists [65, 175].

Compound **77** of Template 2 (Figure 8.13), the imidazolopyrazoles, was characterized as a potent ($ED_{50} = 0.07$ mg/day) but relatively less efficacious (91% at 3 mg/kg) myoanabolic SARM and also demonstrated less tissue selectivity than **75**. Several articles characterizing compounds of Template 3 (Figure 8.13), the indoles,



& - Modified Hershberger- test compound + TP (0.1 mg/d) for 5 days in castrated immature rats

Figure 8.13 Peer-reviewed SARMs from Johnson & Johnson and subsidiaries.

were recently published with only JNJ26146900 (**78**) demonstrating tissue-selective activity [178]. JNJ26146900 (**78**) retains LA weight, but reduces prostate weight in intact animals, and partially offsets castration-induced losses in BMD and LBM. JNJ26146900 blocked testosterone-induced prostate cancer growth and demonstrated favorable activity in a prostate cancer xenograft model using CWR22-LD1 prostate cancer cells (Figure 8.13, top right). In a separate publication, indole **79** was a weak binding ($K_i = 140$ nM) AR ligand not characterized as an agonist or antagonist [105] (Figure 8.13, middle right). Indole **80** was characterized as an anti-androgen comparable to Casodex® (bicalutamide) [66] (Figure 8.13, bottom right).

Template 4, the hydantoins, are structurally similar to BMS-564929 (reviewed below), and Johnson & Johnson Template 2, the imidazolopyrazoles. They have SARM activity with relatively weak myoanabolic potency based on **81** ($ED_{50} = 2.9$ mg/day), and variable myoanabolic efficacy [**81**,75%; **82**, (117%) (Figure 8.13). The tetracyclic indoles of Template 5 only have binding affinities reported [**83** and **84** in Figure 8.13]. It is unknown whether these are agonists, antagonists or tissue-selective AR ligands. As seen in Figures 8.8, 8.10 and 8.11 (discussed above), several of the Johnson & Johnson and subsidiary company AR templates have not been characterized in the peer-reviewed literature. Despite significant advances, Johnson & Johnson does not appear to be pursuing clinical development of a SARM at this time.

8.2.6 Hydantoin and Cyclic Aniline SARMs

8.2.6.1 Bristol-Myers Squibb – Hydantoins and Variants Thereof

Bristol-Myers Squibb has a very broad portfolio of AR ligands, many of which are antagonists [63, 64, 181]. Examples of the diversity within the patented AR ligand template portfolio are shown in Figure 8.14 (**85–99**). The A-rings are typically naphthyl or trisubstituted phenyl aniline derivatives. Bristol-Myers Squibb recently reported mutagenicity associated with the naphthyl aniline hydrolytic metabolites and the ability to design out these problems using trisubstituted phenyl A-rings which are *meta* halogen and *ortho* methyl anilines [182]. Separately, Bristol-Myers Squibb explained how to convert certain of their antagonists into agonists with SARMs activity [183]. Figure 8.15 illustrates how Bristol-Myers Squibb obtained potent and selective SARM activity by simplifying the B-ring to a [3.3] bicyclic hydantoin, which has a hydroxyl substituent properly located to interact with N705 (contrast **100a** and **101**). Bristol-Myers Squibb has only published on one of their SARM templates, a combination of the low mutagenicity A-ring and potent agonist conferring B-ring as exemplified by **101** and BMS-564929 (**102**). A recent Bristol-Myers Squibb article characterized their clinical lead compound, BMS-564929 [184, 185], which is a potent and hyperanabolic agonist compared to testosterone in skeletal muscle (LA) with an efficacy of 125% (comparable to other SARMs) and high potency ($ED_{50} = 0.0009$ mg/kg), with hypostimulation of the prostate relative to testosterone ($ED_{50} = 0.14$ mg/kg). As illustrated in Figure 8.15, these experiments in castrated rats demonstrated a 160-fold selectivity for LA compared to prostate which they characterized as 'unprecedented muscle versus prostate selectivity'. However, Bristol-Myers Squibb may have overestimated the selectivity of their compound, as evidenced by irregularities in the dose–response curves, and size of the prostate and LA muscle in castrated rats. Further, the limiting factor for this compound is the 9-fold selectivity between muscle action (i.e. myoanabolic activity) and LH suppression ($ED_{50} = 0.008$ mg/kg). LH suppression may cause side-effects, especially in elderly men, due to suppression of endogenous testosterone, and subsequently estrogen levels, leading to detrimental effects on multiple organs systems including pro-osteoporotic changes in bone. BMS-564929 is reported to be in phase I clinical trials for age-related functional decline.

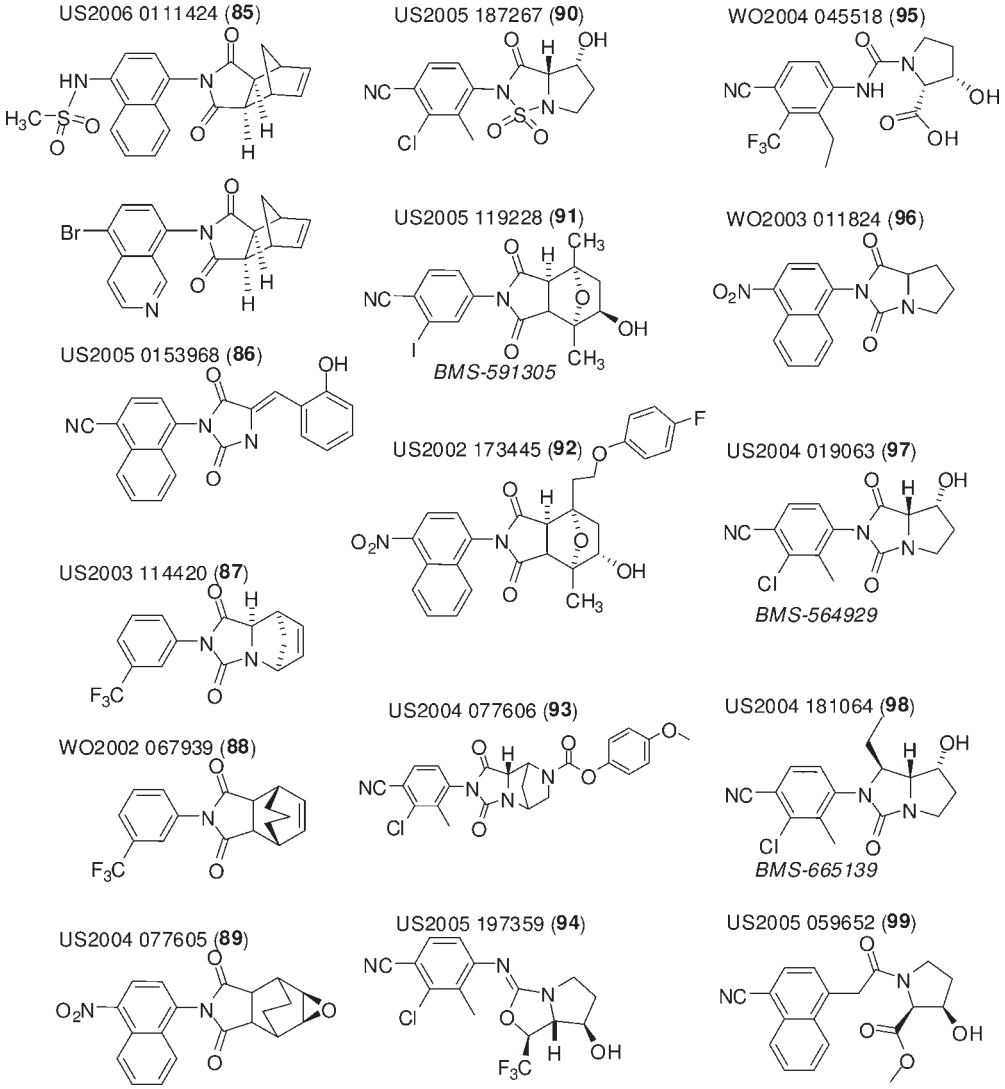


Figure 8.14 Diverse cross-section of patented Bristol-Myers Squibb AR templates.

8.2.6.2 Eli Lilly – Substituted *N*-Arylpyrrolidines

Lilly recently patented a series of substituted *N*-arylpyrrolidines as a SARM template [186]. *In vitro* activity was reported for numerous compounds to achieve low nanomolar AR binding with several potent transcriptional activators that approach full agonist efficacy in C2C12 cells as an indicator of agonist activity in muscle tissue. The *in vivo* activity was reported for two compounds **103** (reportedly commercially available) and **104** that were tested in castrated (at 8 weeks) mice after 8 weeks of wasting (Figure 8.12). Test animals were dosed over a 2-week timeframe at 0.3, 1, 3, 10 and 30 mg/kg per day by mouth [i.e. per os (p.o.)] or s.c. Positive control animals were

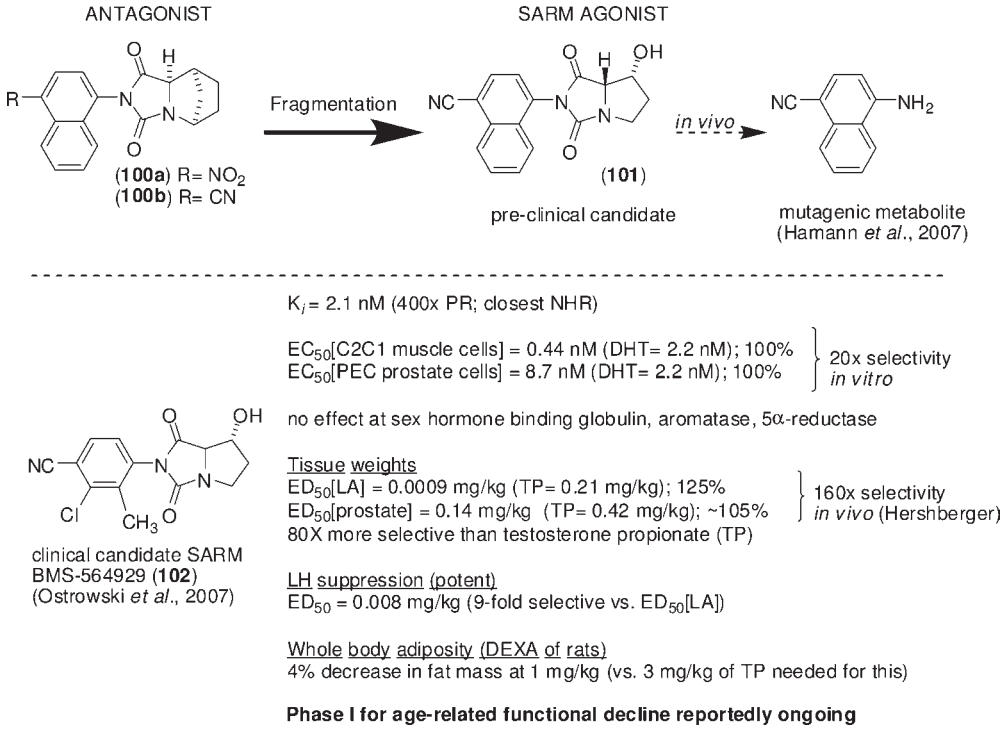


Figure 8.15 Conversion of antagonist to agonist and elimination of mutagenic potential.

dosed at 10 mg/kg daily with testosterone enanthate. LA muscle was used as the indicator of efficacy with percent efficacy (treated versus vehicle treated) for **103** and **104** of 186% (s.c.) and 164% (p.o.) at 10 mg/kg/day, respectively. Unfortunately, percent efficacy versus intact control or testosterone was not reported, making comparisons to other SARMs difficult. Although no data was disclosed, these compounds reportedly did not increase weights of SV or prostate. The most active compounds are highly similar in structure. Apparently *p*-CN, *m*-Cl, *o*-Me substitution of the A-ring [similar to BMS-564929 (**102**)] with an aryl R substituent off an otherwise unsubstituted pyrrolidine produces the most potent *in vitro* agonists some of which also have *in vivo* agonist activity in LA. Pfizer has also reported SARM activity with a similar molecule (**57** in Figure 8.12), an *N*-arylpiperidine described above. Likewise, GlaxoSmithKline has also reported similar compounds with reported *in vitro* agonist activity [169] (Figure 8.11).

8.2.6.3 Acadia Pharmaceuticals – Aminophenyl Derivatives

Acadia reported a novel template for SARMs involving typical A-rings, but the aniline component is a [3.2.1] tricyclic ring system, similar to some of the Bristol-Myers Squibb templates. Acadia reported compounds with modest potency in terms of *in vitro* transcriptional agonist activity (mid to high nanomolar range) with efficacies ranging from 41 to 94% [187]. Compound 154BG31 (**105** in

Figure 8.12) produced significant increases in VP, SV and LA as compared to vehicle. LA weight was approximately 60% at a dose of 30 mg/kg as compared to testosterone propionate (1 mg/kg), whereas VP was approximately 20%. This represents around 3-fold tissue selectivity, but only partial myoanabolic agonism. 154BG31 also fully suppressed LH at a dose of 10 mg/kg, which is in the same range as myoanabolic activity, possibly limiting the utility of these compounds for muscle indications. Compound 198RL26 (**106**) was separately reported to be a high-affinity ligand (79% with an *in vitro* potency of $pEC_{50} = 8.8$) and was selected for *in vivo* experimentation. Like 154BG31, 198RL26 is an *in vivo* partial myoanabolic agonist of similar potency and efficacy, and produced a dose-dependent suppression of plasma LH levels such that a complete reversal was evident at 10 mg/kg, suggesting CNS penetration [188]. Acadia also reported ACP-105 (structure unknown) as a SARM development candidate that has reversed endocrine and bone-related markers of testosterone deficiency in preclinical animal testing, with little effect on the prostrate [189].

8.2.6.4 Miscellaneous (Takeda Pharmaceuticals of North America, Akzo Nobel and Ligand Pharmaceuticals)

Takeda explored the *in vitro* agonist properties of a wide variety of compounds with *N*- (and *O*-) containing unsaturated heterocyclic B-rings (mostly five- or six-membered) with alkyl and/or hydroxyl substitution, in the context of a fused bicyclic A-rings such as naphthalene, benzothiophene and benzofuran [190, 191] (representative example **107** in Figure 8.12). *In vitro* efficacies above 75% at a single 100 nM concentration of test compound were reported including: (i) percent inhibition of binding of tritiated milbolerone ([^{3}H]-MIB), (ii) percent induction of AR-mediated transactivation (i.e. agonist activity) and (iii) percent induction of PSA activity . These data suggest that they found a number of nanomolar range *in vitro* agonists. Akzo Nobel reported a series of indole AR ligands which are predominantly weak AR agonists in *in vitro* transcriptional activation assays (representative example **108** in Figure 8.12). No attempt was made to demonstrate tissue selectivity *in vivo* [192]. Similar compounds were earlier reported in *WO 2005/102998* [193] with the main structural difference being the substitution at the 6-position of the indole ring system with a variety of substituents (O, N, Cl, CF_3, CN, etc.). Ligand Pharmaceuticals also published some cyclic anilines but did not report any biological data (**60** in Figure 8.12).

8.2.7 Summary of the SARM Field (as of May 2007)

SARMs of several structural templates and a variety of potencies are described in the peer-reviewed and patent literature, and the rate of publication is accelerating. Currently there are three groups that have produced clinical candidate SARMs: GTx (Ostarine™ and andarine for cachexia and renal disease in phase II, structures not published), Bristol-Myers Squibb [BMS-564929 (**102**) (Figure 8.15) for age-related functional decline in phase I], and Ligand Pharmaceuticals [LGD2941 (**40**) for frailty and osteoporosis in phase I, and LGD2226 (**39**) which has been discontinued]. Not

surprisingly, these groups have published some of the most detailed characterizations of their SARMs to include S-1 (**14**) and S-4 (**15**) (and many others [87, 90]) from GTx, (**101**) and BMS-564929 (**102**) from Bristol-Myers Squibb, and LGD2226 (**39**), LGD2941(**40**), 16(*R*)e (**109**) [194] and LGD121071 (**30**) (and others such as **35** and **36**) from Ligand Pharmaceuticals. In addition, several other compounds have demonstrated *in vivo* tissue-selective activity including: S-40503 (**48**) and several other patented SARMs **49–56** from Kaken Pharmaceutical; **75**, **77**, JNJ26146900 (**78**), **81** and **82** from Johnson & Johnson; **103** and **104** from Eli Lilly; **57** and **58** from Pfizer; GSK2420A from GlaxoSmithKline; and **45** from Merck. Many more SARMs are present in the patent literature. The salient features of these key molecules include hypermyoanabolic and hyperosteoanabolic efficacy (hyper is defined to be in excess of intact control) at doses associated with decreased prostate size, and little to no suppression of pituitary gonadotropins. Others such as **50** and **51** from Kaken Pharmaceutical and S-1 (**14**) from GTx have demonstrated partial agonist activity in prostate with potential in retarding growth of the prostate, while retaining agonist effects in anabolic tissues. The utility of the various SARMs in patients is yet to be proven, but indeed seems very promising given the multitude *in vivo* preclinical characterizations by many groups, and the auspicious proof of concept results for Ostarine™ in phase II clinical trials (discussed above).

8.3 Molecular Basis for SARM Activities

8.3.1 Archetypical SARM Categories and Their Interactions with AR

A great number of explanations can be posited for the differences between steroidal and nonsteroidal AR ligands in terms of tissue selectivity. Whichever explanation may prove to be accurate, the basis for these biological differences must ultimately derive from variations in the AR–ligand interactions and the distinct AR conformations that result. In this regard it is crucial to understand the binding mode of pan-agonists such as DHT so that it can be contrasted to known or postulated SARM binding modes. Interactions between ligand and the AR that result in a SARM response rather than pure agonist or antagonist responses are not completely understood. However, comparison of bicalutamide and hydroxyflutamide with propionanilide SARMs provides insight as to how such ligands may induce conformational differences in AR upon binding. As replacement of the sulfonyl linkage group of bicalutamide with an ether linkage changes the molecule from an antagonist to a SARM, the antagonist activity of bicalutamide is likely attributable to steric clash of the sulfonyl group with M895 on H12 [10] (not shown). Likewise, the addition of an ether-linked phenyl ring (B-ring) to hydroxyflutamide as in propionamide SARMs provides an additional hydrophilic interaction with H11 by way of H874 that seems to result in increased activity of these ligands [195] (Figure 8.16c.). Pure agonists (i.e. testosterone and DHT)

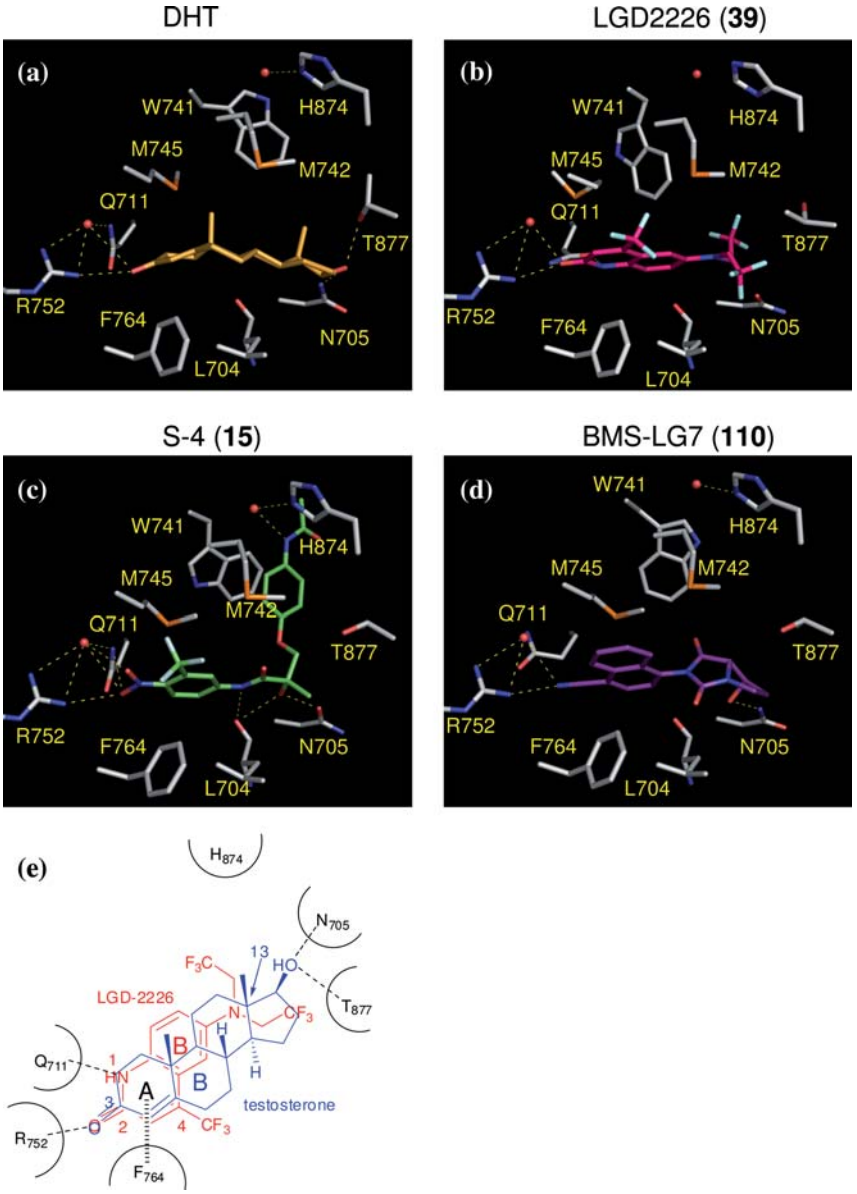


Figure 8.16 Specificity for steroidal and nonsteroidal binding to the AR LBD. (a) DHT (PDB code 1I37, gold), (b) LGD-2226 (**39**, PDB code 2HVC, red), (c) S-4 (**15**, data to be published, green) and (d) BMS-LG7 (**110**, PDB code 2IHQ, purple). Hydrogen bonds shown as yellow dashed lines. (e) Schematic diagram of LGD-2226 binding mode relative to DHT. Oxygen, red; nitrogen, blue; sulfur, yellow; fluorine, cyan; chlorine, green; bromine, brown. Possible hydrogen bonds within 3.5 Å are depicted as dashed lines. Of these ligands, only DHT forms a hydrogen bond to T877. However, note that S-4 (**15**) occupies an additional cavity within the AR and forms a water-mediated hydrogen bond to H874.

demonstrate direct hydrogen bonding to T877 (Figure 8.16a.) of H11, while antagonists and SARMs lack this interaction and instead incorporate hydrophobic groups in this region forming close contacts with this residue [10, 183, 196]. Thus, the increased stabilization of H11 and H12 appears to result in increased levels of agonist drug effect. While ligands that fail to stabilize (i.e. fail to favorably bind to) or even destabilize (i.e. sterically clash with) the agonist positioning of H11 and H12 result in decreased activation.

8.3.1.1 Endogenous Androgens

DHT and testosterone bind to the LBD in much the same manner [197]. The 3-keto group interacts with R752 and a conserved water molecule which is also coordinated with Q711 (Figure 8.16a.). This interaction network is conserved, with some slight variations, throughout the AR ligands discussed here to include all SARMs (Figure 8.16b–d) and putatively antagonists as well, and is likely to be necessary for anchoring the AR ligand. The only other hydrogen-bond donor/acceptor group in DHT and testosterone is the 17β-hydroxyl which acts as a hydrogen-bond donor and acceptor for N705 and T877, respectively. As is important for most steroidal receptors, the shape complementarity between the hydrophobic core template and the receptor drives much of the high affinity seen, whereas the electrostatic interactions help to determine specificity for a particular receptor [4]. The LBD pocket of AR displays a high degree of plasticity [195, 198] as many templates have been successfully employed to achieve high-affinity AR binding (above 64 templates). A smaller subset (above 22) of these high-affinity ligands produces tissue-selective anabolic agonist effects *in vivo* as discussed above. Despite this plasticity for binding to AR, a common observation across different templates is that minor structural variations for a given template can produce dramatic differences in functional and pharmacologic profiles. One case in point would be S-1 (**14**) compared to bicalutamide.

8.3.1.2 Diaryl SARMs

SARMs that resemble bicalutamide were developed by GTx, Orion, Johnson & Johnson, Kaken Pharmaceutical, Merck, Karo and GlaxoSmithKline (Figure 8.8). The crystal structure of an exemplary member of this SARM archetype, S-4 (**15**) (Figure 8.16c) (submitted for publication), serves to explain the necessity of some of the shared pharmacophoric elements. This crystal structure demonstrates that the *para* A-ring electron-withdrawing group (EWG) participates in a polar interaction with the R752/structural water/Q711 triad while the *meta* A-ring halogen/CF_3 makes hydrophobic interactions with the binding pocket. These *para* and *meta* EWG substituents make the A-ring electron deficient, activating it for interaction with the F764 in the A-ring pocket. The anilide NH element of S-4 (**15**) (and postulated for other diaryl SARMs) participates in hydrogen-bond donation to the backbone keto group of L704 and the central tertiary alcohol/NH group participates in hydrogen-bond donation to the N705 side-chain and/or L704 backbone keto groups. The flexibility inherent in the linkers of these compounds allows them to occupy a pocket outside of the testosterone binding pocket with the B-ring interacting with W741 and the *para* B-ring substituent interacting with a

conserved H_2O molecule that is coordinated to, *inter alia*, H874 of H11. It is this interaction which may allow these diaryl SARMs to perturb the coactivator binding site in a manner very distinct from endogenous steroidal androgens such as testosterone or DHT.

8.3.1.3 Substituted Aniline and Flutamide Variant SARMs

SSARMs containing many of the same structural elements as hydroxyflutamide were reported by Karo Bio, Kaken Pharmaceutical, Johnson & Johnson and Pfizer, with Ligand Pharmaceuticals and GlaxoSmithKline showing less resemblance to hydroxyflutamide (Figure 8.11). The crystal structure of R-3 (**11**) (not shown), demonstrates that these SARMs are predicted to bind in much the same way as S-4 (**15**) (*para* A-ring EWG binding to R752, electron-deficient A-ring interacting with F764, amide/aniline as hydrogen-bond donor to L704 keto, and terminal hydrogen-bond donor/acceptor group interacting with N705) except that the B-ring pocket is not occupied. In cases with two terminal hydrogen-bond donor/acceptor groups, interaction with T877 may be possible, and/or extension toward (but generally not reaching) H874.

8.3.1.4 Hydantoin and Cyclic Aniline SARMs

SARMs containing many of the elements of nilutamide were reported by Bristol-Myers Squibb, Ligand Pharmaceuticals, Takeda Pharmaceutical, Akzo Nobel, Acadia, Pfizer and Lilly (Figure 8.12). These SARMs can be subdivided with regard to their AR interactions based on the basicity of the aniline nitrogen of the B-ring. Many of these nitrogen atoms are part of a cyclic amide, imide, urea, hydantoin or related system in which case these nitrogen atoms can not be protonated. The crystal structure of BMS-LG7 (**110**) (Figure 8.16d) demonstrates the binding mode for these planar B-ring compounds in which the L704 keto is no longer a hydrogen-bond acceptor for this heterocyclic nitrogen but rather the plane of the amide bond(s) is rotated compared to R-3 (**11**) and S-4 (**15**). Other compounds resembling nilutamide have a single basic aniline nitrogen that is likely to act as a hydrogen-bond donor to the L704 keto. The terminal alcohol/alkanol group is likely to bind to the N705 as seen for compounds resembling hydroxyflutamide, while the electron-deficient A-ring is likely to bind in a manner similar to that discussed above.

8.3.1.5 Quinolinone (Pyridone), Coumarin and Other Fused Ring SARMs

A series of bi-, tri- and tetracyclic quinolinone or coumarin containing SARMs has been reported by Ligand, Pfizer, Kaken Pharmaceutical, Merck and Ortho-McNeil. The crystal structure of LGD2226 (**39**, PDB code 2HVC [127]) provides insights into how these quinolinone, and by extension coumarin, derivatives may bind to the AR (see Figure 8.16b). The 2-keto of the SARM is superimposable with the 3-keto of the steroid and participates in the same interactions as the A-ring EWGs of the other SARM archetypes (Figure 8.16e). The 1-NH of the quinolinone participates in direct interaction with the Q711 which is not seen for other archetypes discussed. The combined effect of the 2-keto and 1-NH anchors the quinolinone (or pyridone) into the active site. The 4-CF_3 extends in an orientation similar to that seen with the *meta* CF_3/halogens of other archetypes. The B-ring of the quinolinone does not superimpose

directly with the B-ring of the steroid, placing the aniline N approximately in the position of the C13 of the steroid nucleus. The trifluoroethyl side-chains extend across the area occupied by the C/D-ring and 'above' the plane of the steroid into a pocket/channel in the direction of H874. This places the terminal CF3 'above' the plane of the steroid just 2.5 Å from the methyl group of the T877 side-chain. Although the crystal structure does not show this, it could be speculated that the T877 side-chain is actually rotated such that the OH binds with the terminal CF_3 'above' the steroid plane. Likewise the N705 terminal amide could be rotated to form interactions with the other terminal CF_3 (Figure 8.16e).

8.3.2
Multiple Putative Mechanisms for SARM Tissue Selectivity

Many groups have now published favorable *in vivo* SARM characterizations in preclinical models and the proof-of-concept phase II clinical trial of Ostarine™ suggests these to be relevant in humans. However, the molecular basis for the separation of the detrimental androgenic activities (i.e. virilization/prostatic hypertrophy) from the desired anabolic effects remains unknown. The enzyme 5α-reductase offers one simplistic explanation for the phenotypic tissue selectivity of SARMs [199]. The greater ability of 5α-reductase to convert steroidal androgens into more potent metabolites in some (e.g. prostate and skin) tissues as compared to others (e.g. muscle and bone) represents an amplification process that is operative for steroidal ligands, but not nonsteroidal SARMs. The absence of any known nonsteroidal SARMs with equipotent activity in muscle and prostate supports this view. However, a variety of other molecular mechanisms are likely to also contribute to the observed tissue selectivity for SARMs, as evidenced by the ability of some SARMs to elicit only partial agonist activity in some tissues while demonstrating full agonist activity in others. Also, different classes of SARMs may elicit their effects by distinct mechanisms.

Much of what is known about the molecular mechanisms of SARM tissue selectivity has been inferred from research involving SERMs, first discovered 40 years ago. The endogenous ER and AR ligands, estradiol, and testosterone and DHT, respectively, function indiscriminately as agonists in tissues that express their cognate receptors [200–202]. Moreover, unlike estrogen that has two receptor isoforms (ERα/β) androgens have only one receptor. Hence, the tissue selective expression of receptor isoforms or heterodimerization of the receptor is not an issue with AR. Nevertheless, some of the molecular mechanisms of SERMs can be extended to explain the tissue selectivity of SARMs [203] including:

(i) Tissue-specific enzymes that activate or inactivate the ligands [204, 205].
(ii) Ligand-dependent conformation of the receptor [206, 207].
(iii) Tissue-specific coregulator expression [208].
(iv) Tissue-specific coregulator complex formation [209].
(v) Intracellular signaling cascades mediating genomic and nongenomic [210] effects.

As the mechanism for the tissue selectivity of SARMs is not the objective of this chapter, only some of the important aspects are dealt with briefly hereunder.

8.3.2.1 Coregulator Function: Variable Structural Changes Lead to Variable Cofactor Recruitment

Some evidence suggests that the conformational disparity induced by SARMs leads to association and recruitment of different coregulator complexes [211]. Using combinatorial peptide-phage display, McDonnell *et al*. elegantly showed that different ligands induce distinct AR and ER conformations leading to their association with varying coactivator peptides [211, 212]. The SARMs RTI-018 and RTI-001 possessed a spectrum of agonist activities and altered kinetics of response, and these differences were attributed to SARM-mediated structural differences leading to the association of AR with coactivator peptides distinct from DHT [213].

An exquisite study in support of the above conclusion was published recently by Rosenfeld *et al*. [214]. The study was performed in a cell line where bicalutamide was converted to an agonist by activating the interleukin (IL)-8 pathway. As an antagonist, bicalutamide recruited corepressors NCoR and SMRT. As an agonist, in the presence of IL-8, the same ligand recruited steroid receptor coactivators (SRC). This study also demonstrates that of the three LxxL helices (LXDs) in the receptor interacting domain of SRC-1, DHT-mediated action required LXD1 and LXD2, whereas SARM-mediated action required LXD2 and LXD3 [215].

8.3.2.2 Key Coregulators in Testosterone Versus DHT-dependent Tissues

To date more than 200 putative AR-interacting proteins (both coactivators and corepressors) [216, 217], several of them with intrinsic functions such as histone acetyl transferase activity (SRCs, CBP) [203] which facilitates transcriptional activation, histone deacetylase activity (NCoR, SMRT) [203] which suppresses transcriptional activation, and other chromatin-modifying functions (Swi/SNF/BRG) [203], have been identified (Figure 8.17). AR differs from other receptors in its interactions with coregulators. The LBD of AR and other steroid receptors have 12 antiparallel helices that undergo significant rearrangement upon ligand binding creating a shallow hydrophobic pocket containing LxxLL motif (i.e. AF-2) to facilitate association with coactivators [218, 219]. However, in AR most of the coactivators bind to a FxxLF/LxxLL motif in the AF-1 domain and some bind to the LxxLL motifs in the AF-2 domain [14, 217]. Moreover, there are several proteins that coactivate exclusively AR (ARA family of coactivators) and are not shared by other receptors [220, 221].

Figure 8.17 summarizes tissue selectivity as viewed from the coregulator perspective. Ligands induce distinct conformations of the AR leading to the recruitment of a variety of freely available coregulator complexes in various tissues. Depending on the available pool of coregulators, the AR in response to DHT in prostate or SV recruits ARAs, BRG and others (Figure 8.17a), whereas in bone or muscle AR might recruit SRCs, CBP and DRIP/TRAP complex (Figure 8.17b). In response to the antagonists bicalutamide or flutamide, AR recruits corepressors

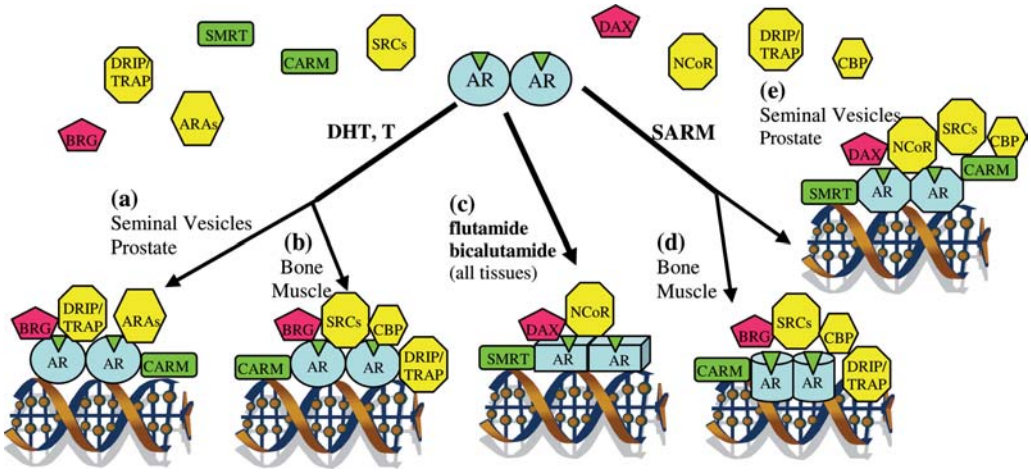


Figure 8.17 Mechanism of SARM action: role of coregulators. In the absence of ligands, AR and the coregulators are not in association with each other. Ligands typically cause dissociation of HSP90 and other cytosolic chaperones and translocation to the nucleus in dimeric form. Different ligands induce various conformational states of AR leading to association with a diverse assortment of coregulators. Depending on the tissue type and the level of available coregulators, AR associates itself with the pool of available proteins. Due to the overwhelming presence of ARAs in prostate (a), AR in the presence of DHT or testosterone preferentially associates with ARAs and chromatin remodelers BRG, DRIP/TRAP and CARM, leading to androgenic effects. However, in bone and muscle, the lack/low expression of ARAs leads to the association of AR with SRCs and CBP (b and d) which leads to the anabolic effects of DHT and SARMs, respectively. In case of antagonists such as bicalutamide and flutamide (c), the altered receptor conformation leads to the association of AR with corepressors such as DAX, NCoR and SMRT, leading to repression of AR function and in turn antiproliferative effects. (e) Role of SARMs in prostate where the altered conformation can lead to association of AR with corepressors (for SARMs that are inhibitory in prostate), coactivators (for SARMs that are androgenic) or steady-state association with both coactivators and corepressors leading to variable effects for different SARMs.

such as NCoR or DAX and does not discriminate the recruitment pattern tissue specifically (Figure 8.17c). SARMs in anabolic tissues such as muscle and bone recruit coactivator complexes similar to DHT (Figure 8.17d). However, in androgenic tissues, depending on agonist or antagonist activity, they recruit coactivator or corepressor complexes, respectively.

Much work needs to be done to ensure that some of the above-mentioned arguments for SERMs hold true for SARMs.

8.3.2.3 Intracellular Signaling Cascades

From ligand sensitization to translation of genes, every single process in cells is dependent on the intracellular levels and activity of various signaling molecules and pathways. These signaling events lead to very critical posttranslational modifications such as phosphorylation, sumoylation, ubiquitination and others that are

important for the function of the receptor. Two major cell signaling pathways have evolved in this regard for SARMs (and SERMs) that may help to explain tissue selectivity.

(i) *Genomic effects of SARMs/SERMs.* Classical, slowly responsive nuclear hormone receptor mechanism of action in which the nuclear hormone receptor acts as a transcriptional regulator, requiring transcription of AR target genes and expression of these target proteins to produce an androgenic response. Intracellular levels of kinases/phosphatases and other pathways mitigate these effects.

(ii) *Non-genomic effects of SARMs/SERMs.* Rapid effects that do not require transcription or translational to generate response. The mechanism and physiological importance of these nongenomic signaling is poorly understood.

Ligands have been shown to adopt different intracellular signaling pathways in different cells to elicit their effects. A classical example is that testosterone signals through inhibition of p38 mitogen-activated protein kinase (MAPK), Notch-1, Notch-2 and Jagged-1 signaling pathways in macrophages, but relies on activation of the phosphatidylinositol-3-kinase–Akt pathway in bone cells [222–224]. However, androgens do not inhibit p38 MAPK in bone cells, corroborating the idea that the same ligand adopts different pathways depending on cell and tissue type to mediate its given physiological response [225]. AR phosphorylation is also affected either ligand dependently or independently by growth factor pathways, leading to divergent physiological responses [226].

Nongenomic effects are important for the bone protective effects of androgens and estrogens [227], whereas genomic effects are critical for the development of sexual organs. Testosterone and androstenedione, but not DHT and synthetic androgen R1881, mediate nongenomic effects to mature *Xenopus* oocytes. Further studies indicated that these nongenomic signaling pathways play a role in ovarian development and the development of polycystic ovarian syndrome [228].

The driving force of future mechanistic studies will be to pursue identification and development of newer SARMs with better tissue selectivity and pharmacology, and to reveal the molecular mechanism for the tissue-selective nature of SARMs.

8.4 SARMs: A Promising Class of Clinical and Preclinical Candidates

Although relatively little is known about how SARMs work at a molecular level, the growing body of evidence supporting their *in vivo* tissue selectivity has stimulated immense interest and speculation regarding the therapeutic potential of SARMs in humans. As demonstrated by our laboratories and other groups, SARMs have numerous advantages over testosterone and AAS. Key amongst these is the ability to elicit a major and therapeutically relevant *in vivo* separation of the anabolic and androgenic activities of the AR as demonstrated in animal models and human clinical trials (see above). Most SARMs reported to date promote bone and muscle growth in

male and female animals regardless of sex hormone status. Concurrent osteoanabolic and myoanabolic activity is thus far unique to AR ligands, and cannot be replicated by existing osteoanabolic (e.g. parathyroid hormone) or myoanabolic (e.g. growth hormone or myostatin inhibitor) therapies.

The vast majority of SARMs that have been reported are nonsteroidal. Notable exceptions are a series of Merck patents (see above) and MENT [229]. Thus, it can be expected that many of the pharmacokinetic (what the body does to the drug; absorption, distribution, metabolism and excretion) and pharmacodynamic (what the drug does to the body; pharmacologic, phenotypic and toxicologic effects) problems inherent to the steroid nucleus may be absent in SARMs. Pharmacokinetic/pharmacodynamic profiles have been published for a number of nonsteroidal SARMs [230].

8.4.1 SARM Pharmacokinetics

The absorption characteristics of exogenously administered testosterone limit its routes of delivery to intramuscular and transdermal for testosterone replacement therapies (TRT). In contrast, multiple groups have reported orally active SARMs (Table 8.1) [88, 128, 179, 183, 184]. Distribution of steroidal agonists to anabolic and androgenic tissues is affected by binding to SHBG which lowers free testosterone or AAS plasma levels, markedly so in the elderly where SHBG levels are higher [231]. SARMs do not appear to bind to SHBG [75, 184]. Testosterone and many steroidal androgens are substrates for aromatase, producing estrogenic metabolites and resulting in feminization in males when large doses are given. Similarly, testosterone (and some AAS members) is subject to reduction to DHT in androgenic targets tissues, possibly worsening androgenic alopecia and increasing prostate cancer liability, and thus limiting its use in the elderly. SARMs have been demonstrated as neither substrates nor inhibitors of aromatase [93, 184] (most SARMs are not aromatizable due to phenyl A-rings) or 5α-reductase [93, 184]. The plasma half-life for testosterone pharmaceutical compositions is variable and unpredictable [232], whereas we and other groups have reported multiple orally active molecules with a wide variety of half-lives, providing flexibility in duration of action. For instance, Kim *et al*. demonstrated a correlation between oral half-life and size of the *para* B-ring halogen within an arylpropionanilide analog series displaying $t_{1/2} = 3.7–17.1$ h [90]. In terms of toxicity, the orally active AAS are characterized by hepatotoxicity and all traditional steroidal agonists have androgenic side-effects, most notably a liability for disorders related to prostate hypertrophy such as benign prostatic hypertrophy or precancerous or malignant adenocarcinomas, but also acne, baldness and behavioral effects. SARMs typically are partial agonists in the prostate [87, 128] and/or full agonists with sufficiently reduced potency in the prostate relative to anabolic tissues to allow separation of AR-dependent anabolic and androgenic activities [184], thus at therapeutic doses, SARMs reduce the size of the prostate in rats (lack of PSA elevation in humans is consistent with this observation). The androgenic effects in skin of increased sebum production, hirsutism, etc., were not observed in clinical trials with Ostarine™ [96]. There is known liability in treating females with steroidal androgens

Table 8.1 Pharmacokinetics and pharmacodynamics of nonsteroidal SARMs vs. traditional steroidal androgens.

		TRT or AAS	SARMs
Pharmacokinetics			
Absorption	bioavailability	topical and intramuscular administration only (TRT) low bioavailability (TRT)	complete oral absorption
Distribution	SHBG binding	lowers bioavailability in elderly	no binding
Metabolism	aromatization	feminization (TRT/some AAS)	no effect
	5α-reductase	excessive androgenicity (TRT)	no effect
Excretion	plasma $t_{1/2}$	variable (TRT)	tunable
Toxicity		hepatotoxicity (AAS)	no major toxicity through phase II
		prostate liability (T/AAS)	shrinks prostate at anabolic doses
Pharmacodynamics			
Androgenicity		liability for cancer, hirsutism, acne	decrease in prostate size, no skin effects
Liability in females		virilization	none known
Nuclear hormone receptor selectivity		some PR, GR, MR, ER reactivity	specificity is achievable
Hypothalmic–pituitary–gonadal axis		major effect (suppresses FSH/LH)	minor effect at high hyperanabolic doses
Myoanabolism		endogenous role	100+% of testosterone in castrated/intact animals
Osteoanabolism		endogenous role	100+% of testosterone in castrated/intact animals
Improved lipid profile		endogenous role	decrease in total cholesterol, triglyceride, LDL, very-LDL; decrease in HDL
Improved body composition		endogenous role	1.3 kg increase in LBM
Performance enhancement		endogenous role	increase in speed/power in stair climb test
Improved metabolism		endogenous role	increase in glucose tolerance; decrease in insulin resistance

such as hirsutism and virilization (deepening voice, masculinization of features); however, this has not been observed for SARMs. Another major issue with androgen therapy is suppression of endogenous steroid synthesis due to CNS penetration and activation of feedback loops, thus suppressing follicle-stimulating hormone (FSH) and LH secretion. This is prevalent for testosterone and AAS therapies, however only observed at high doses for SARMs [87, 128, 184].

8.4.2 SARM Pharmacodynamics

These improved pharmacokinetic and side-effect profiles for SARMs versus steroidal androgens are associated with potent, tissue-selective effects in therapeutic target tissues. Hyperanabolic effects in skeletal muscle have been seen for SARMs in rat for numerous molecules [90, 151, 176, 184] with myoanabolic effects in castrated rats in the range of 100–150% compared to testosterone. These effects have also been seen in human trials as manifested by increases in LBM and improvements in physical performance tests (stair climb time [96]). Osteoanabolic effects have also been demonstrated in rats [95, 128, 151] and monkeys [130] for numerous molecules; however, only limited clinical trials data have been released in human [128].

SARMs should fill a large unmet clinical need in the aging population in terms of preventing or treating sarcopenia, osteoporosis and frailty (weak muscles and bones). The combined ability of SARMs to improve muscle strength and performance, and to restore or prevent weak bones makes SARMs very promising for these patients. This is particularly true in the frail and elderly to prevent debilitating falls and promote more active lifestyles (i.e. decreased morbidity and mortality, and improved quality of life). The selective myoanabolic activity of SARMs represents another unmet clinical need as they may have potential therapeutic uses in persistent catabolic states which result in accelerated loss of lean muscle mass, loss of function and morbidity, as associated with cachexia and wasting disorders that occur in cancer and AIDS patients. Benefits may also redound to a younger population which is increasingly obese through prevention/treatment of metabolic syndrome and diabetes as SARMs have been shown to increase LBM, decrease fat body mass and improve lipid profiles [233]. The unique ability of some SARMs to suppress LH/FSH in intact males while maintaining the necessary anabolic AR activity in the periphery, may also lead to their application in male contraception.

8.4.3 Potential Clinical Uses of SARMs

8.4.3.1 Cancer Cachexia

The potential role of SARMs in the treatment of cancer cachexia and weight loss focuses again on the building of muscle mass. This therapeutic indication stands as

the likely frontrunner for the introduction of a SARM into clinical use. As survival times for many cancers increase through advances in chemotherapy, surgery and radiation therapy, the issue of quality of life becomes more important. In addition to the possibility that increased LBM may confer survival benefits, it is plausible that building muscle mass may increase strength and power and therefore lessen disability by improving physical function [234]. Additionally, hypogonadism and anemia are highly prevalent in this patient population highlighting additional problems addressed by SARMs. GTx initiated a phase IIb trial using Ostarine™ in cancer cachexia patients in May 2007.

8.4.3.2 **Chronic Kidney Disease**

Chronic kidney disease (CKD) patients tend to be elderly and obese, and are at high risk of functional impairment and disability. Over 10% of the elderly population can be categorized as having renal insufficiency [235]. Many patients with compromised renal function or who are treated with hemodialysis or peritoneal dialysis experience involuntary weight loss and hypogonadism [236]. These comorbidities may result from poor nutrition, the inflammatory catabolic state created by renal dysfunction, disturbances of the hypothalamic–pituitary–gonadal axis or combinations thereof. Improved survival rates are found in CKD patients who have a higher BMD or are composed of a higher proportion of LBM (muscle and bone). There are two potential uses of SARMs in this population. First, the ability to build LBM or improve physical performance via a SARM may translate into an improved survival rate. Second, SARMs may provide a large pharmacoeconomic benefit by acting to increase red blood cell production and reduce the need for recombinant erythropoietin therapy [237]. The demonstrated ability of Ostarine™ to reduce insulin resistance further suggests that SARMs may also prove beneficial in CKD due to the high percentage of pre-diabetic and diabetic patients in this group.

8.4.3.3 **Chronic Obstructive Pulmonary Disease (COPD)**

COPD is the third and fourth leading cause of disability and death, respectively, in the US, and costs the American economy over $30 billion per year [238]. Among patients with COPD, 20–40% of these will suffer from muscle wasting [239]. Loss of muscle mass can adversely impact quality of life, exercise tolerance and survival. Like the other conditions discussed above, patients with COPD have a higher prevalence of hypogonadism than their healthier counterparts [240]. Chronic steroid use may exacerbate muscle loss and weakness in this group. Although unproven, treatment of these symptoms with a SARM could offer considerable improvement of quality of life in these patients as well as those who are treated with chronic corticosteroids for rheumatologic or other inflammatory diseases. These are examples of chronic conditions where decades of anabolic therapy may be needed. An anabolic agent that reduces the morbidity and mortality associated with these conditions without unwanted effects would serve a huge unmet need in this patient population.

8.4.3.4 Sarcopenic Disorders

Sarcopenia (the loss of muscle mass) represents perhaps the largest and most compelling of the potential clinical applications for a SARM. Sarcopenia occurs in a variety of settings and is often secondary to physiological, pathological or behavioral problems [118]. Many sarcopenic diseases are characterized by protein catabolism to provide an energy source to replace or augment dietary sources and support pathology-induced hypermetabolic rates. Sarcopenia can result from endocrine abnormalities or neuropathic conditions, such as hypogonadism and hyposomatotropinism (growth hormone is somatotropin), muscular dystrophy, multiple sclerosis or any other of a multitude of diseases that are associated with chronic inflammatory pathology; including traumatic injury, hospitalization, severe burns and/or knee/hip replacement. Perhaps the cause of sarcopenia that is the most rapidly increasing in prevalence is age-related decreases in muscle mass [241]. The definition of sarcopenia is not consistent, but is often taken to be a muscle mass below 2 standard deviations below the mean of a young reference group. Following this definition, there is a linear increase in sarcopenia prevalence following the third decade of life until 65–70 years of age when 13–24% of the population (both genders) is sarcopenic. However, the prevalence increases exponentially for the older population and jumps to more than 50% of people over 80 years of age (both genders) [242]. Sarcopenia is a major cause of frailty and carries with it a 3- to 4-fold increase in the likelihood of disability, as well as increased morbidity and mortality [243]. The effects of ageing on skeletal muscle are unlikely to be halted or reversed. Nonetheless, potential opportunities exist to attenuate muscle wasting, improve muscle function and preserve functional independence. Very few drugs have been developed with sarcopenia specifically in mind [244]. Several drugs used clinically include growth hormone, estrogens, dehydroepiandrosterone and anabolic steroids. Of these, the best evidence for a positive outcome for therapy is growth hormone and testosterone; however, these agents are poorly tolerated by the elderly due to side-effects and prostate cancer liability, respectively [241]. Findings from many studies have indicated that although somatropin therapy increased LBM, it did not improve functional ability and strength in the majority of studies, making its clinical utility questionable [241]. Multiple SARM classes have been demonstrated to be superior to anabolic steroids (i.e. hyperanabolic and tissue selective) in preclinical testing in their ability to support muscle mass as illustrated with the LA [90, 184], soleus [94] and gastrocnemius [131] muscles. The promising effects of the GTx SARM, Ostarine™, in older men (over 60 years of age) and postmenopausal women on lean muscle mass, functional performance and insulin resistance provide an intriguing therapeutic profile for this large and unmet medical need, likely making it a high priority for clinical drug development.

8.4.3.5 Osteoanabolic Agents

The ability of SARMs to increase both muscle and bone strength in animal models suggests that they may provide a unique dual approach to osteoporosis therapy [94, 95, 151]. The process of bone remodeling is ongoing throughout life, and involves the resorption and deposition of bone by osteoclasts and osteoblasts, respectively [245]. The resorption and deposition processes are promoted at the

endocrine level by calcitonin and parathyroid hormones, respectively. The treatment modalities available for osteoporosis include a variety of antiresorptive agents that prevent further breakdown of bone by the body. Thus, therapies such as estrogen replacement, calcitonin, bisphosphonates and SERMs are more effective

Table 8.2 Putative SARM therapeutic applications.

Target organ/ parameter	Disorders	Therapeutic effect
Aging	age-related functional decline	O, M
	andropause	S, L, O, M, P
	cognitive impairment	N
	disability/frailty/hypogonadism	S, L, O, M, P
	exercise tolerance/performance enhancement	O, M, P
Bones/cartilage	arthritic conditions	O
	osteoporosis/osteopenia	O, P
Cachexia/wasting	eating disorders	O, M, PA
	trauma/coma/surgery	O, M, PA
	protein catabolism syndromes: cancer, AIDS, congestive heart failure, chronic obstructive pulmonary disease	O, M, PA
Cancer	AR-dependent: cancer of the prostate, breast cancer, ovarian cancer, etc.	A
Endocrine	hypercortisolemia, hyposomatotropinema	O, M, L
Gynecological	polycystic ovarian syndrome	L
	(post-)menopausal complaints	S, L, O, M, P
	premature ovarian failure	S, L
Hematopoietic	anemia	E
Hypogonadism	androgen deficiency in a male	S, L, O, M, P, N, PA, A
	androgen deficiency in a female	S, L, O, M, P, N, PA, A
Kidney	chronic renal disease	R, E, PA, P, L
Metabolic effects	obesity/insulin resistance/diabetes mellitus	L, M, PA, P, I, G
Muscle	dystrophies, neurodegenerative, neuromuscular junction diseases	M, N
Prostate	prostatitis, prostatic inflammatory atrophy, prostatic intraepithelial neoplasia, cancer of the prostate, androgen-independent prostate cancer, benign prostatic hypertrophy	S, A
Sexual disorders	erectile dysfunction	S
CNS penetrant	male contraception/infertility hypoactive sexual disorders	SG, S, N
Wound healing	adjunct to physical therapy	O, M, P
Miscellaneous	bed sores, burns, connective tissue/muscle damage, fractures, dry eye	O, M, P

A, antiproliferative; E, erythropoietic; G, glucose-lowering; I, insulin sensitizing; L, lipid lowering; M, myoanabolic; N, neuroprotective; O, osteoanabolic; P, physical performance enhancement; PA, protein anabolism; R, renal hypertrophy; S, sexual tissue growth/ maintenance; SG, spermatogenic modulation.

at preventing osteopenic changes than rebuilding bone after osteoporosis has been diagnosed [246]. Currently only one osteogenic therapy is available, teriparatide (Forteo®), which is an N-terminal 34-amino-acid fragment of recombinant human parathyroid hormone. This therapy is only available as a daily injection, is plagued by a black box warning of increased risk of osteosarcoma and is cost-prohibitive in many cases [247]. An important issue from the perspective of developing SARMs as anabolic agents for bone is whether aromatization of androgen is necessary for anabolic action [248]. In humans, androgen-insensitive males that have inactivating AR mutations are frequently osteopenic [249]. In experimental animals, nonaromatizable steroidal androgens significantly prevent the osteopenia that results from castration [250]. DHT also prevents bone loss after ovariectomy in female rats [251] and flutamide (an AR antagonist) induces osteopenia even in female rats with intact ovaries [252]. In addition to this indirect evidence from studies of steroidal androgens, several studies of SARMs have already demonstrated their potential as a treatment for osteoporosis. SARMs significantly increased BMD and bone strength in castrated and ovariectomized rats [95, 128, 151]. SARMs have been shown not only to prevent loss of bone (i.e. treatment begins at time of surgery) in ovariectomized and castrated rats, but also to increase bone strength (i.e. treatment begins after waiting period of sufficient length to allow osteopenic changes) [95, 151]. The pharmacokinetic advantages, selectivity and dual activity of SARMs in muscle and bone suggest that they may indeed become an important new addition to the armamentarium of drugs to treat osteoporosis, particularly in the elderly.

A variety of other therapeutic applications of SARMs can be envisioned; ranging from uses in burn injury, wound healing, male contraception, sexual libido, Alzheimer's disease, mood and cognition (Table 8.2). Clinical trials with SARMs in patients with these diseases within the next 3–5 years will reveal the true value of this promising new class of drugs. Recent progress with SARMs, as discussed in this chapter, indicate that exciting times lay ahead, with the possibility for a great expansion in clinical use of anabolic treatment.

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9
Novel Glucocorticoid Receptor Ligands

Heike Schäcke, Khusru Asadullah, Markus Berger, and Hartmut Rehwinkel

9.1
Introduction

Glucocorticoids (GCs) are among the most effective therapies in the treatment of acute and chronic inflammatory, as well as autoimmune, diseases. They have been applied successfully in the clinical setting for more than half a century. The era of GCs began with the discovery that the adrenal hormone, cortisol, was able to reverse the symptoms and dysfunctions associated with Addison disease [1, 2]. After observing high efficacy in the treatment of rheumatoid arthritis, when cortisol was administered pharmacologically [3, 4], tremendous development of systemic and topical GCs for use in several therapeutic fields was initiated. Early on, interest in more potent compounds was high and consequently such compounds were synthesized; however, with the application of highly potent GCs, especially over long time periods, undesired effects became more apparent. A number of these effects, such as osteoporosis, diabetes, Cushing's syndrome, glaucoma and muscle atrophy, are severe and sometimes irreversible [5, 6]. The incidence of side-effects depends on the potency of the GC being administered, the duration of therapy, the administration route (systemic versus topical), the indication and the individual being treated. Some indications such as dermatological diseases or asthma have also been found to be treatable by topical administration of GCs. This application route has the advantage of strongly reduced systemic side-effects.

Chemical optimization of compounds has been done either to improve activity or to identify prodrugs that overcome suboptimal physicochemical properties of the active compound to enhance its bioavailability. GCs with a predictable and rapid metabolism into inactive metabolites after exerting their pharmacologic effects at the site of disease are classified as soft drugs [7]. GCs that follow the prodrug and the soft drug principle have been designed. They are activated locally by enzymes in lung (e.g. ciclesonide, butixocort 21-propionate) or in skin (methylprednisolone aceponate) and show a low systemic exposure. These GCs locally inhibit proinflammatory cytokines and chemokines at the site of administration potently, whereas they elicit only limited systemic effects [8–10].

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

Although topical GCs usually have less side-effects than systemic GCs, and are successfully used to treat several inflammatory dermatological diseases, their major limitation remains the local side-effects they can cause. Furthermore, severe disease exacerbations often cannot be treated efficiently with compounds that work locally only. In such situations, systemic treatment is mandatory (e.g. in bullous skin diseases). In dermatological indications especially the main drawback of topical GCs is the development of skin atrophy after long-term use [11, 12].

Therefore, there is a great medical need for GC-like compounds that possess both an antiinflammatory/immunomodulatory activity similar to the marketed GCs and a reduced risk of undesired effects.

In the past, different approaches to develop novel GCs or GC-substituting compounds with an improved effect/side-effect ratio have been pursued. Apart from optimization towards local administration, several other strategies have been developed. First, optimization of formulation for systemic treatment leading to a more targeted release of the drug has been followed by some groups [e.g. by using liposomes encapsulating steroids for the treatment of rheumatoid arthritis (I)]. Second, introducing nitric oxide (NO) into the steroid scaffold was established to enhance antiinflammatory activity without increasing the dose or potency of the steroid (II). However, one of the broadest and most promising approaches of the last 10 years is based on optimized GR-ligands, which should address the molecular mechanisms of GR in a very specific way (III).

I. The search for novel formulations is not only restricted to locally administered compounds, but also to GCs that are used for systemic treatments. One example is the encapsulation of prednisolone or dexamethasone in liposomes, e.g. pegylated (PEG) liposomes. In animal studies, pharmacodynamic and pharmacokinetic experiments demonstrated a prolonged and more targeted availability of the drug in comparison to the drug given without liposomes [13–15]. The high affinity of PEG liposomes for macrophages in inflamed tissues makes them attractive GC encapsulation agents. The macrophages do not only serve as target cells, but also play a crucial role in the release of GCs from liposomes and the generation of relatively high and prolonged concentrations of active drug in the synovium. In particular, in indications such as rheumatoid arthritis, this administration form might lead to increased activity of the drug.

II. A second approach to improve the ratio of desired to undesired effects is to combine chemically a known steroidal GR ligand with NO, leading to, for example, nitroxy derivatives of prednisolone. The company NicOx tested NO prednisolone (NCX-1015) and NO hydrocortisone (NCX-1022) with regard to their antiinflammatory activity in animal experiments [16, 17]. In the case of NCX-1022, a phase II study including 40 patients with seborrheic dermatitis was performed and showed promising results regarding efficacy and tolerability of the drug in topical application (NicOx SA, *Media release*, 29 April 2004, available from http://www.nicox.com). In the case of NCX-1015, a phase I study for indication inflammatory bowel disease was conducted, and showed a good local tolerability and no systemic absorption (NicOx SA, *Press release*, 14 February 2002, available from http://www.nicox.com).

III. Major efforts have been made, and promising preclinical results have been achieved, in the search for GR ligands that trigger molecular mechanisms of GR selectively, with the goal of reducing the risk for side-effects while maintaining the therapeutic efficacy of known GCs. To summarize the progress made with this promising approach we will give a brief overview of mechanisms of GR and the working hypothesis used by a number of pharmaceutical companies to search for improved GR ligands. This chapter summarizes reports and reviews published in the field of dissociated GCs by us and by others.

9.2
GR and its Action

GCs function by binding to the cytosolic GR, a member of the nuclear receptor superfamily. Members of this superfamily are ligand-activated transcription factors. The GR consists of a C-terminal ligand-binding domain (LBD), a DNA-binding domain (DBD) with a dimerization interface in the center of the molecule and two transcription activation (AF) domains. One transcription activation domain (AF-1) is located at the N-terminal end of the protein, while the other (AF-2) lies within the LBD and is therefore directly controlled by the ligand [18]. The AF-2 domain is highly conserved among the nuclear receptors, whereas the amino acid composition of AF-1 is diverse [19]. In the absence of ligand binding, cytosolic GR is associated with molecular chaperones, cochaperones, immunophilins and FKPB52 [20–22]. Activation of GR induces translocation of the receptor into the nucleus, where it modulates gene expression either positively (transactivation) or negatively (transrepression). Furthermore, coactivator proteins that interact with AF-1 or AF-2 domains are involved in the transactivation function of ligand-bound GR [23]. See Figure 9.1.

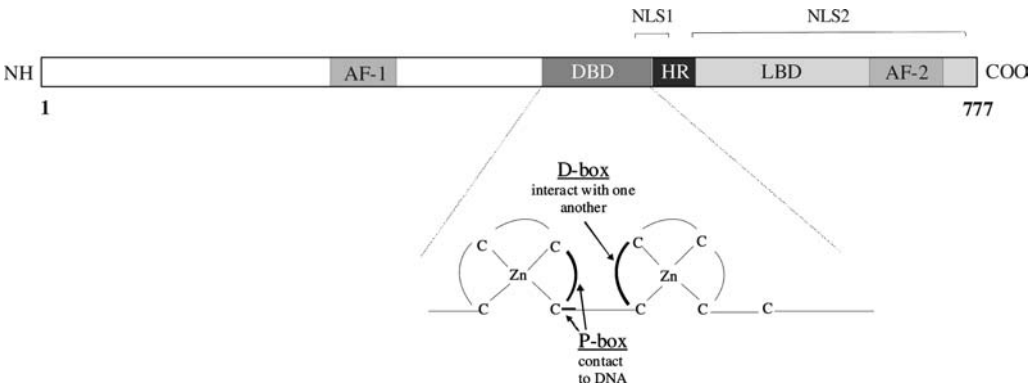


Figure 9.1 Schematic illustration of domain structure of GRα. AF-1 and AF-2 represent the two transcription activation function domains. DBD consists of two zinc fingers, and is responsible for dimerization of the receptor and DNA binding. HR represents the hinge region. LBD is the ligand-binding domain. NLS1 and NLS2 are two nuclear translocation signal sequences. Modified from Ref. [24].

9.2.1
GR and X-ray structures

In 2002, scientists from GlaxoSmithKline published the first crystal structure of human GR (hGR) LBD in a complex with dexamethasone and a coactivator motif [25]. Prior to that finding, the crystal structure of hGR LBD had remained unsolved mainly due to purification problems which were caused by the insufficient solubility of the protein. Bledsoe *et al*. solved this issue by mutating one amino acid in helix 5: F602S. By virtue of this modification expression and solubility were improved without compromising the behavior of the GR. This has been shown in different *in vitro* assays. The publication provided interesting information which might be useful for identifying novel dissociated compounds. The hGR LBD forms a dimerization interface different from that of other nuclear receptors. This interface plays a crucial role in both GR-triggered mechanisms, i.e. transactivation and transrepression. Additional features include a second charge clamp which may play a role in the selective binding of coactivators and a unique distinct ligand-binding pocket that provides a possible explanation for its selectivity toward the GCs [26].

A group led by Kauppi published hGR LBD complexed with both GR antagonist RU 486 and GR agonist dexamethasone [27].

9.2.2
Molecular Mechanisms of GR

9.2.2.1 Transactivation

Positive regulation of gene expression by GR has been shown to be mainly mediated by direct binding of receptor homodimers to specific sequences, GC-responsive elements (GREs), in the promoter or enhancer regions of GC target genes [28]. This interaction is responsible for activating the expression of a number of genes, including those involved in glucose metabolism [29]. Three different types of GREs – simple, composite and tethering – are known [30], suggesting differences in the molecular mechanisms required for the respective types of GREs. Activation of gene transcription by GR via simple and composite GREs is dependent on binding of the activated GR homodimer to DNA directly. At tethering GREs, GR binds to other DNA-bound transcription factors, such as STAT5, to increase transcription activities. The introduction of a point mutation in the dimerization domain of GR was used to demonstrate *in vitro* that dimerization of GR is a prerequisite for transactivation function [31]. In mice carrying a dimerization-deficient GR the induction of several liver-specific genes was strongly reduced [32]. Although the net effect of these response elements is to increase the expression of target genes by GR, different domains of the receptor are required based on the architecture of the elements and on how they are recognized by the receptor. A screen to determine different sets of primary GR targets has shown that a number is still transactivated even in the presence of the dimerization mutant. This indicates that other mechanisms of activation occur, possibly through the AF-1 and AF-2 domains of the receptor [33].

9.2.2.2 Transrepression

Negative regulation by GR (transrepression) occurs via different mechanisms. Regulation via negative GREs that differ in structure and function from positive GREs is well described [34]. Such elements can be found at different positions in promoter regions that interfere either with binding sites of other transcription factors, as is shown in the promoter of pro-opiomelanocortin gene [35], or with the binding site of the basal transcription initiation complex, as described for the osteocalcin promoter [36]. Interestingly, only a few genes are definitely regulated via these negative GREs and, therefore, the proportion of this kind of negative regulation in overall antiinflammatory activities or the induction of undesired effects of GCs is not well understood [23, 34].

The major mechanism of negative regulation by GR is the ability of the receptor to inhibit the activity of other transcription factors by being tethered to these factors. This mechanism is fully functional with dimerization-defective GR [31, 32, 37] and is therefore thought to occur through GR monomers. The transcription factors to which the GR is tethered include activator protein 1 (AP-1), NFκB and interferon regulatory factor-3 [38, 39]. For repressing AP-1 activity in the human collagenase 3 promoter, TIF2/GRIP1, a GR coactivator, is recruited by the receptor to the AP-1 binding site to assist GR in its repressor function. GRIP1 corepressor activity maps to a region distinct from what it uses to function as a coactivator, indicating that different regions of the regulatory protein are necessary for activation and repression by GR. Interestingly, the same GRIP1 corepressor domains are required for GR-mediated repression of NFκB tethering GRE of the human interleukin (IL)-8 gene [40, 41]. In addition, tethering of GR to NFκB inhibits phosphorylation of Ser2 on one of the two sites of the C-terminal domain of RNA polymerase II required for transcriptional initiation [40]. Another report demonstrated that different domains of the GR seem to be responsible for either NFκB or AP-1 interactions. A $GR_{(R488Q)}$ mutant, unable to repress NFκB activity, retained the ability to repress AP-1 activity, while its transactivation activities were unaffected [42]. Another mechanism used to explain the transrepressive function of GR is the binding of GR to Jun N-terminal kinase (JNK), leading to suppression of JNK activity and subsequently to an inhibition of AP-1 [43, 44]. GR is also known to interfere with the extracellular signal-regulated protein kinase (ERK-1/ERK-2) and p38 mitogen-activated protein kinase (MAPK) pathways via an increased expression of the dual-specificity MAPK phosphatase [45–47].

As these transcription factors regulate the expression of proinflammatory genes, their negative regulation by the GR has become a paradigm for the antiinflammatory and immunosuppressive action of GCs.

9.2.2.3 Cofactors

GR as a ligand-activated transcription factor does not function alone, however. To ensure highly coordinated regulation of gene transcription it requires coregulators to modify and remodel chromatin structures. Cofactors can be divided into coactivators and corepressors depending on their function [48, 49]. They do not bind directly to DNA, but they are recruited through protein–protein interactions with AF domains of GR to regulatory sequences and can exert enzymatic activities, e.g. histone

acetylases (HATs) or histone deacetylases. One of the best-described examples of cofactors are CBP/p300 HATs, which function as coactivators for many transcription factors besides GR. A second family of coactivators consists of p160 proteins, including SRC1/NCoA1, TIF2/GRIP1/NCoA3 and pCIP/RAC3/ACTR/AIB1/NCoA3 [50]. Interestingly, it was shown that TIF2/GRIP1 is also able to function as a corepressor of GR at the AP-1 and NFκB 'tethering sites' [41]. Other corepressors, such as nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid receptors (SMRT), have been described as being recruited to nuclear receptors; however, their role in negative regulation of proinflammatory genes is still not well understood. Further studies to investigate GR-dependent cofactor recruitment using dissociated GR ligands, as well as full agonists and/or antagonists, might help to further elucidate the fine tuning of gene expression regulated by GR.

Many research efforts were invested into the study of molecular mechanisms of the antiinflammatory/immunomodulatory activities of GCs. However, they are still not well understood. Behind the transrepression activity, several mechanisms are hidden. The role of cofactors and histone deacetylases, for example, is still under discussion, although much research has been performed in the past [51–53]. Furthermore, there are also transactivation events involved in the therapeutic effects of GCs, as has been shown for MKP-1 [45, 47], lipocortin-1 [54] or very recently for GC-induced leucine zipper (GILZ), to name a few [55]. Nevertheless, repression of many proinflammatory molecules seems to be a major part of the antiinflammatory effects of GCs.

9.3 Selective Modulation of GR Activities

Although the molecular mechanisms of the GR are more diverse than described 10 years ago, the differentiation of positive and negative regulation of gene expression by GR has provided a working model to search for novel synthetic GR ligands with a better therapeutic index compared to classical GCs. This concept is based on studies with a mutant GR, where the exchange of a single amino acid residue in the dimerization domain of the receptor molecule abrogated both the dimerization and transactivation potential of the GR without affecting the ability of the receptor to repress AP-1-regulated genes [31]. Further studies with genetically modified mice carrying this dimerization-deficient GR ($GR^{dim/dim}$) demonstrated a similar phenotype. The expression of a number of genes positively regulated by GR was abolished while negatively regulated genes were not affected [32, 56]. Treatment of $GR^{dim/dim}$ mice with dexamethasone generated in some models of antiinflammatory action of GCs similar antiinflammatory responses as in wild-type animals. For example, inflammatory activity including the induced ear edema following 12-*O*-tetradecanoylphorbol-13-acetate treatment of the skin could be repressed by GCs in wild-type and $GR^{dim/dim}$ mice [56]. However, these mice have not been used in any long-term experiments to find out whether the known side-effects of GCs are eliminated. In a recent study, Tuckermann *et al*. showed that $GR^{dim/dim}$ mice were not protected from

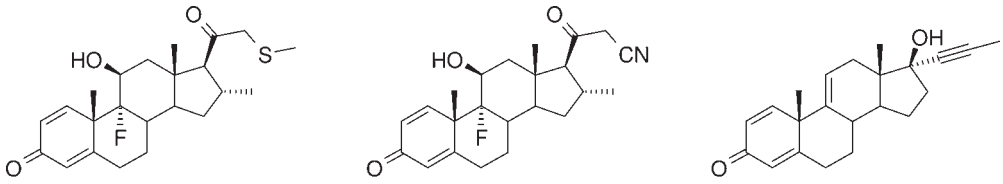
developing GC-dependent osteoporosis after 2 weeks of systemic treatment with prednisolone [57]. This result shows that some, but not all, side-effects might be addressed by a reduced transactivation activity of GR. However, results obtained with $GR^{dim/dim}$ mice might not be translated in a one-to-one relation to the profile of dissociated GR-ligands. It is important to note that while some transactivation function of GR may be destroyed by the dimerization mutation, there are clearly some genes positively regulated by GR that are not affected by this mutation [58].

The ability of GR to function as coactivator in the positive regulation of genes controlled by, for example, STAT5 is not affected by the dimerization and DNA binding properties of the receptor [59]. Thus, GC regulation of growth hormone expression and insulin-like growth factor-1 signaling is not affected by the dimerization mutation [60]. Similarly, there are genes that negatively regulate the immune system that are positively regulated by GR. For example, thymosin β4 sulfoxide [61], GILZ [62], macrophage migration inhibitory factor [63], lipocortin-1 [64] and MKP-1 [45] are some of the known genes that negatively regulate processes in the immune system but are transcriptionally upregulated by GCs. Thus, the transactivation/transrepression model for the screening of antiinflammatory GCs with reduced side-effects, although attractive, has some limitations. Interestingly, several pharmaceutical companies were able to identify novel GR ligands displaying a better therapeutic index *in vivo* by using the hypothesis of dissociation of transrepression from transactivation activities as a starting point in the search for compounds.

9.4 Dissociated GR Ligands

9.4.1 Steroidal Structures

RU 24782, RU 24858 and RU 40066 were the first claimed dissociated GR ligands/modulators (Figure 9.2). These compounds have been described by Roussel-Uclaf (now Sanofi Aventis). The first two structures are characterized by a dexamethasone-like backbone lacking the hydroxyl group in 17 and having a modified side-chain in this position. All three compounds bind with high affinity to GR and show a dissociated profile *in vitro*. They display only 8–35% of the transactivation efficacy shown by dexamethasone while repressing AP-1 and NFκB activity very efficiently. The disillusion came with the first *in vivo* results of RU 24858 [65]. Although as active as prednisolone in a rat asthma model, the compound showed a similar side-effect profile as the classical GC regarding body and thymus weight loss, as well as the induction of osteoporosis. It has been discussed that the origin of these disappointing results is the steroidal structure of these compounds. Perhaps RU 24858 is metabolized *in vivo* into a compound that behaves similar to steroids.



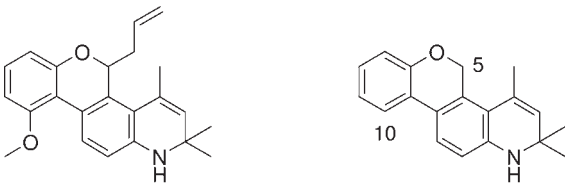
RU 24782 RU 24858 RU 40066

Figure 9.2 Steriodal structures.

9.4.2
Benzopyrano[3,4-*f*]quinolines

AL-438 is a GR ligand/modulator that emerged from the collaboration between Abbott Laboratories and Ligand Pharmaceuticals. This structure is a promising representative of a new chemical class – benzopyrano[3,4-*f*]quinolines (Figure 9.3). Although not a steroid, it binds with high affinity to the GR and is quite selective towards the other nuclear receptors [66]. Some binding affinity has been found with the mineralocorticoid receptor as well. It differentiates between transrepression- and transactivation-mediated effects *in vitro*. While it reduces efficiently the formation of IL-6 and E-selectin, it shows less activity in transactivation assays. This dissociation can be explained by an altered gene regulation profile of AL-438. It seems that the compound activates and represses not the entire array of genes, like the classical steroidal structures, but only a subset. This behavior may be connected with the different recruitment of cofactors dependent on the nature of the ligand. Moreover, this *in vitro* dissociation was observed in the *in vivo* situation as well. Applied in an asthma model in rats, AL-438 is as active as prednisolone. In two other inflammation models, i.e. the carageenan-induced paw edema and Freund's adjuvant-induced arthritis model, AL-438 showed only a slightly reduced potency compared to prednisolone. In contrast to the steroid, it induces less blood glucose (transactivation triggered side-effect), a surrogate marker for the development of diabetes. Moreover, AL-438 antagonizes even the increase in blood glucose levels caused by prednisolone. Additionally, reports have been published showing for AL-438 a diminished potential to induce osteoporosis or, at least, to influence the bone metabolism less than the classical GCs. Thus, it is less potent than dexamethasone and prednisolone regarding the production of osteoprotegerin (OPG) in two human osteoblastic cell lines. Regarding the maximum effect, AL-438 reaches only 40–50% at a concentration of 1 μM. Furthermore, AL-438 is only a poor stimulator of 'receptor activator of NFκB ligand' (RANKL) gene expression [67]. A new publication showed too that AL-438 affects osteoblastic cells less than dexamethasone and prednisolone [68]. The structure–activity relationship of this scaffold has been described [69]. It has been found that this tetracyclic core is suitable as a pharmacophore for several nuclear receptors. The proper substituent in position 10 is essential for, and triggers selectivity toward, GR, whereas the transrepression/transactivation activity is modulated by the substituent in position 5. Depending on the nature of the substituent in position 5, the

resulting compound has full transrepression/transactivation activity comparable with that of prednisolone or it behaves as a full repressor with reduced transactivation activity like a desired dissociated compound.



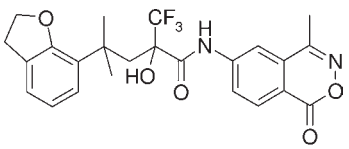
AL-438

Figure 9.3 Benzopyrano[3,4-*f*]quinolines.

LGD-5552, a compound whose structure has not been published yet, binds efficiently to GR and has a dissociated profile *in vitro*. It has been applied in different inflammation models in rodents showing efficacy comparable to that of prednisolone. In contrast to the steroid, it does not increase the arterial blood pressure in a rat hypertension model, and its negative effects on bone resorption, longitudinal mid-femur and periosteal bone formation are less pronounced than after treatment with prednisolone [70].

9.4.3
Methyl Benzoxazinoneamides (MBO-amides)

ZK 216348 another nonsteroidal structure (Figure 9.4) has been made and characterized by scientists from Bayer Schering Pharma AG [71]. The compound is a pentanoic acid 4-methyl-1-oxo-1H-2,3-benzoxazinamide bearing a trifluoromethyl- and a hydroxyl group at the α-carbon and an additional methyl and phenyl group at the ω-end resulting in a compound with two quaternary carbons.



ZK 216348

Figure 9.4 Methyl Benzoxazinoneamides.

The synthesis of ZK 216348 (Figure 9.5) starts from the commercially available dihydrobenzofuran. Deprotonation and carboxylation provides the 7-carboxylic acid which is transformed into its ester. Grignard reaction gives the tertiary alcohol. Treatment with trimethylsilylenolether, followed by saponification, affords the

ketocarboxylic acid which is coupled with the 4-methyl-6-aminobenzoxazin-1-one. Introduction of the trifluoromethyl group with Ruppert's reagent and separation of the enantiomers provides the desired product: ZK 216348, the (+)-enantiomer.

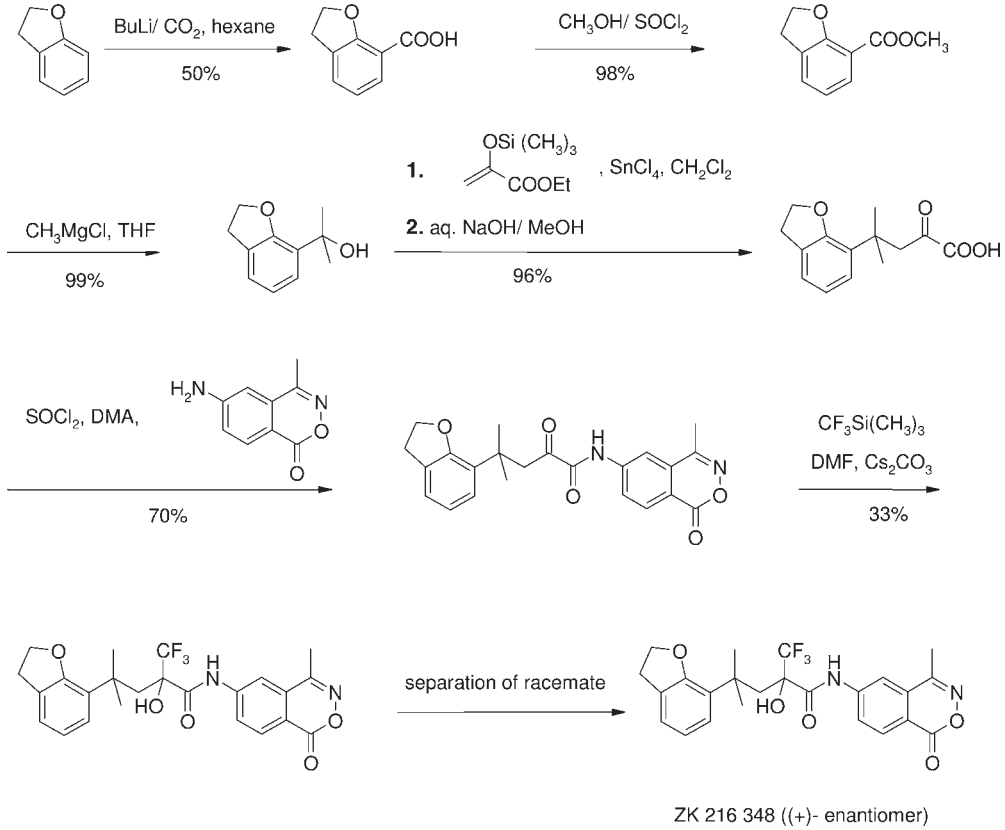


Figure 9.5 Synthesis of ZK 216 348.

ZK 216348 binds to GR and displays a dissociated profile *in vitro*. It inhibits formation of IL-8 (transrepression-mediated effect), and is less efficacious regarding transactivation-triggered effects like the induction of tyrosine aminotransferase (TAT). This dissociation has been observed *in vivo* as well. ZK 216348 inhibits croton oil-induced ear inflammation in mice and rats as efficiently as the standard GC, prednisolone, after subcutaneous and topical application. Compared with the steroid, the compound induces TAT significantly less. Furthermore, it also reduces thymus and spleen weight significantly less after subcutaneous treatment of mice as well as rats. An increase in blood glucose concentration, a transactivation-mediated effect, reflects the risk for developing diabetes. Whereas prednisolone triggers a dose-dependent increase in blood glucose, ZK 216348 does not, even at the highest dose of 30 mg/kg. In addition, it shows less skin atrophy determined by breaking strength and reduced thickness of the skin after long-term topical treatment of nude rats.

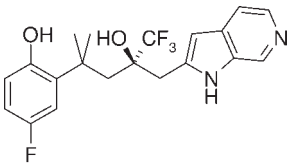
However, regarding suppression of the hypothalamic–pituitary–adrenal axis, a side-effect which is clearly triggered by a transrepression mechanism, ZK 216348 did not show an advantage over prednisolone.

It has been shown for ZK 216348 that it does not affect osteoblastic cells negatively *in vitro*, compared with steroidal GR ligands [67]. The picture is similar to AL-438 regarding the measured parameters: production of OPG and stimulation of RANKL gene expression.

9.4.4
Azaindoles

Compounds that share the same trifluoroethanol pharmacophore, which is present in the class of the methyl benzoxazinoneamides are under evaluation at Boehringer Ingelheim and GlaxoSmithKline. BI 115 is currently the most advanced nonsteroidal GC at Boehringer Ingelheim.

A series of compounds was profiled leading to identification of the 2 connected azaindazole as a valid substitute for the MBO. BI 115 (Figure 9.6) has been described in multiple patents, the earlier of which claim BI 115 to be a GR modulator. BI 115 binds with 2 nM affinity to GR and inhibits IL-6 with an IC_{50} of 6 nM (90% efficacy). Aromatase inhibition was reported to be 10 nM (90% efficacy) (at *SCIpharm*, Edinburgh, 2006 and at the *233rd ACS National Meeting*, Chicago, 2007). This compound was also tested in a collagen-induced arthritis model in mice. The result was described as a better profile in comparison to prednisolone regarding a number of parameters obtained in this model; in particular, metabolic parameters were less affected by BI 115 than by prednisolone.



BI 115; (BI 309)

Figure 9.6 Azaindole.

Efforts have been made to develop a stereoselective synthesis for the trifluoroethanol phamacophore [72].

Boehringer Ingelheim applied a chiral auxiliary for introduction of the trifluoromethyl group in their synthesis of indole and azaindole trifluoropentanols (Figure 9.7). Under tetrabutylammonium fluoride catalysis the trifluoromethylation to the chiral α-ketocarboxylate ester results in a 84 : 16 diastereoselectivity. Further crystallization yields enantiopure material that is saponified to the acid under recovery of the chiral auxiliary. Transformation to the epoxide and construction of the azaindole from alkynyl pyridine [73] give access to the whole class of enantiopure pentanol indoles and azaindoles including BI 115.

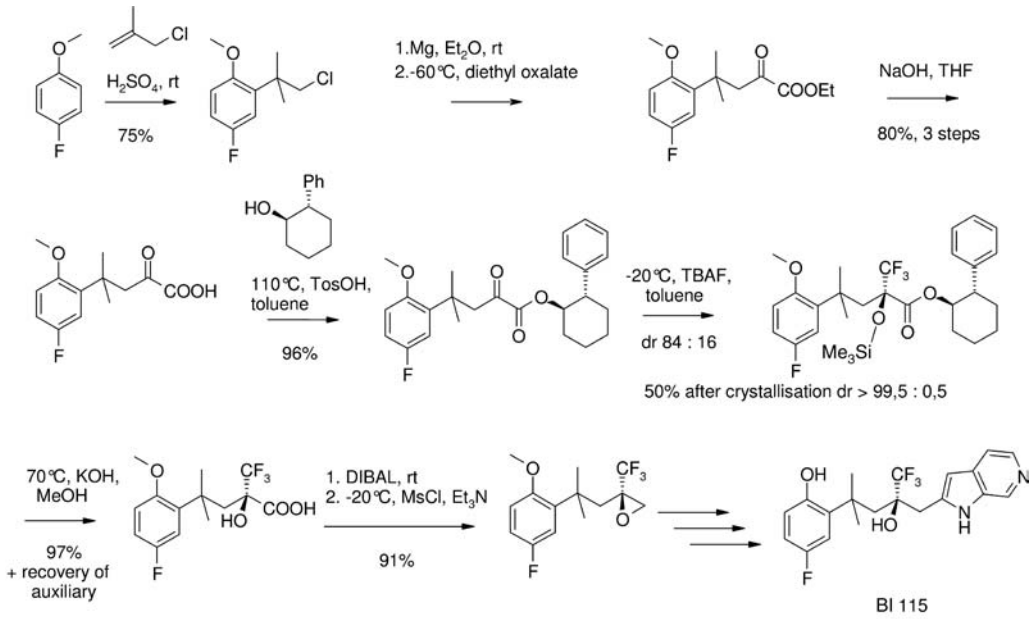


Figure 9.7 Synthesis of BI 115.

9.4.5
Tetrahydronaphthalenes and Phenyl Indazoles

GlaxoSmithKline introduced further derivatizations around the benzylic position of the pentanol methyl benzoxazinoneamides.

The rigidity of the central structural element can be enhanced by bridging the phenyl substituent producing a tetrahydronaphthalene or a benzocycloheptane. Substituents in the saturated part of the tetrahydronaphthalene lead to full GR agonists with increased potency and efficacy.

GlaxoSmithKline reported the benzylic position of their tetrahydronaphthalene methylbenzoxazinoneamides (Figure 9.8) as an agonist trigger in GR binding. Compounds of this class are claimed in two patents (*WO 2006/000398* and *WO 2006/000401*) to exhibit binding below 100 nM to GR. In a functional assay, the depicted cyclopentyl derivative (Figure 9.11) had a pIC 50 of 8.7 (92%) in NFκB inhibition [74].

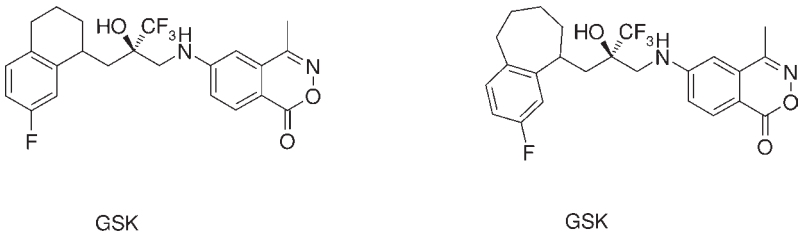


Figure 9.8 GSK structures I.

Synthesis of the tetrahydronaphthalene benzoxazinoneamide series made use of a conceptually different synthetic approach by application of a carbonyl ene reaction that connects the tetrahydronaphthalene ene with the MBO amino pyruvate carbonyl in a more convergent manner (Figure 9.9).

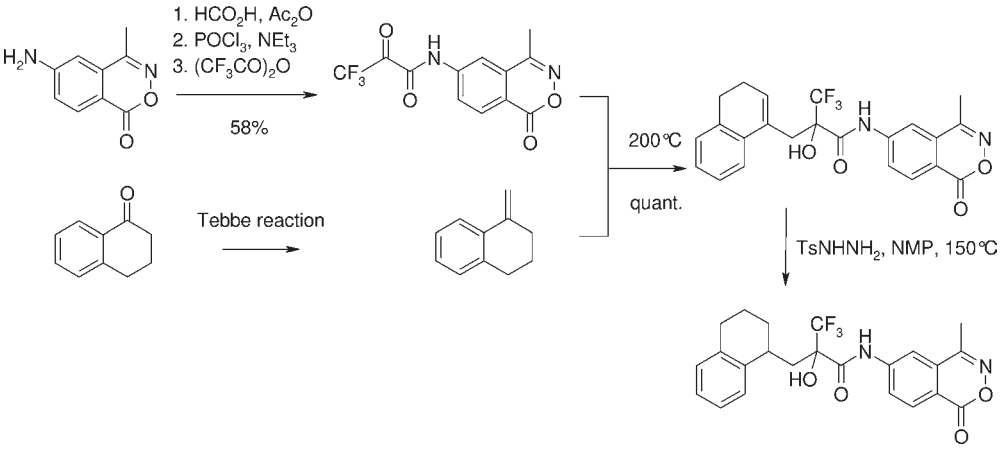
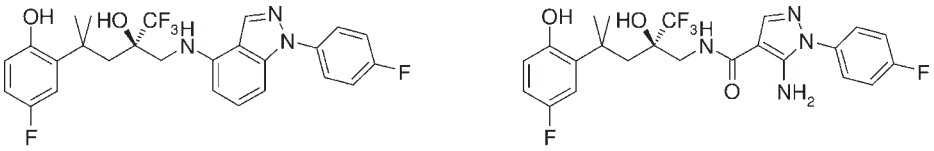


Figure 9.9 Synthesis of tetrahydronaphthalene.

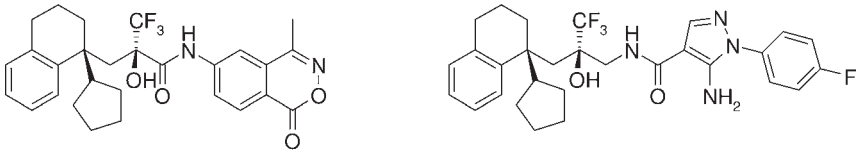
Additional efforts have been made to replace the MBO portion of the molecule (Figure 9.10). These results have been either published in patents (*WO 2006/108699* and *WO 2007/000334*) or disclosed at the *233rd ACS National Meeting* (Chicago, 2007). Amino-bridged phenylindazoles or phenyl amino pyrazole amides can replace the MBO and allow retention of the desired transrepression activity.



GSK GSK

Figure 9.10 GSK structures II.

Further advances in the series are compounds that combine a substituent in the benzylic position of the tetrahydronaphthalene, which triggers the agonist activity with these MBO replacements. In cases of larger substituents at the quaternary center, like cyclopentyl this results in compounds with a dissociated profile; the aryl pyrazole motif is responsible for good GR selectivity (Figure 9.11).

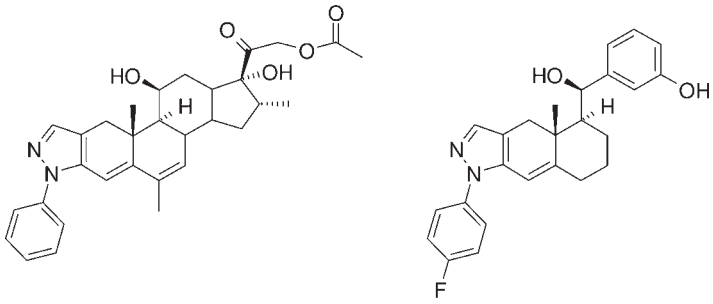


GSK GSK

Figure 9.11 GSK structures III.

9.4.6
Benzo[*f*]indazoles

An additional structural class of newly designed nonsteroidal dissociating GR ligands is represented by benzoindazoles (Figure 9.12).

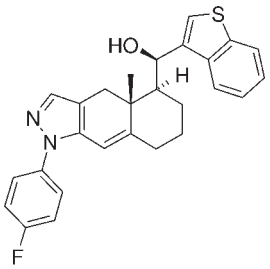


Cortivazol UCSF

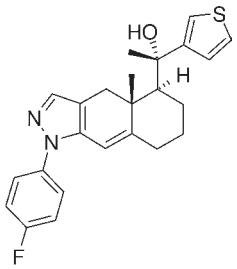
Figure 9.12 Benzo[*f*] imidazoles I.

Merck and the University of California in San Francisco (UCSF) produced independently multiple derivatives of the partially saturated fluorophenyl benzo[*f*] indazole within the last 4 years. The phenylindazole structural element, which lately was used by GlaxoSmithKline, as mentioned in Chapter 9.4.5 of this book to replace the MBO has been known in the field of GCs for four decades. It is present in the steroidal drug Cortivazol, which is known to be a GR-specific agonist. Recent structural analyses of this more bulky GR ligand [75] support the model where ligand-dependent conformational changes in the LBD play a role in GR-mediated gene regulation via modular interaction with the DBD and AF-1. The scaffold includes the steroidal A- and B-ring, and is substituted by α-hydroxyl alkyls or hydroxyl methyl phenyls. These replacements allow more conformational freedom compared with the C- and D-ring portion of the pyrazole corticoids, but keep the important 11-hydroxy functionality of the steroids. The UCSF compounds [76] are GR-selective and display potencies that are in one order of magnitude weaker than dexamethasone.

Further optimization at Merck [77] improved the potency similar to that of prednisolone (Figure 9.13). The depicted benzothiophene derivative shows dissociation *in vitro* and *in vivo*. Inversion of the hydroxyl methyl bridge stereochemistry



Merck & Co.



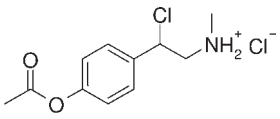
Merck & Co.

Figure 9.13 Benzo[*f*] imidazoles II.

brings the class close to the dexamethasone characteristics in measured transrepression and transactivation parameters.

9.4.7
Natural Product

Compound A, a structure characterized by a research group at the University of Ghent, is the first example of dissociated compounds emerging from natural sources [78]. Compound A, 2-((4-acetoxyphenyl)-2-chloro-*N*-methyl)ethylammonium chloride (Figure 9.14), is a stable analogue of the hydroxyl phenyl aziridine precursor found in the Namibian shrub *Salsola tuberculatiformis* Botschantzev. It has a nonsteroidal structure and binds well to the GR. Haegeman *et al.* could show that Compound A is dissociated by means of distinct *in vitro* assays. This dissociation was also observed *in vivo*. According to Haegemann and his group, Compound A reduces inflammation in a zymosan-induced paw edema model as efficiently as dexamethasone. In contrast, it does not increase blood glucose levels as the steroid does.



Compound A

Figure 9.14 Natural product.

9.5
Conclusions and Perspectives

GCs are among the most successful antiinflammatory and immunosuppressive drugs due to their tremendous clinical efficacy. Their overall clinical value, however, is limited by their substantial side-effect potential. Thus, there is a high medical need

and a significant market for GR ligands that display similar therapeutic activity with reduced side-effects.

The extensive work of many research laboratories in the last 15–20 years on GC effects, the desired and the undesired, led to a better understanding of the underlying molecular mechanisms and consequently to novel therapeutic approaches based on the transactivation/transrepression hypothesis. With this hypothesis, suggesting that a dissociation of molecular action may translate into a dissociation of effects from side-effects, the interest of many pharmaceutical companies was reawakened. Several pharmaceutical companies are currently pursuing such an approach, and most of them are in a preclinical stage of compound identification and development. There is strong competition in this research area between many companies. With the advent of new nonsteroidal structures like AL-438, ZK 216348 and BI-115 it has been possible for the first time to show that a molecular dissociation *in vitro* indeed leads to an improved effect/side-effect profile *in vivo*. All compounds strongly inhibit the activity of proinflammatory proteins (transrepression mechanisms), but affect the transactivation mechanisms differently compared with the classical steroids. This *in vitro* dissociation is reflected in an improved therapeutic index regarding some but not all side-effects connected with the classical steroids. AL-438 antagonizes the induction of blood glucose caused by prednisolone, whereas ZK 216348 does not lead to an increase in blood glucose in rats, in contrast to the steroids. Furthermore, it seems that both compounds have less deteriorating effects regarding osteoblasts and thereby a potential to be less osteoporotic. Taken together, a proof of concept has been achieved in animals already. Whether this will also be observed in humans needs to be investigated. Based on our current knowledge, however, we expect that dissociated GR ligands will move into clinical development now and will eventually lead to new and better drugs with enormous potential.

Along with identification of new compounds has gone research regarding the different GR pathways. Much work has been done in many laboratories to reveal these complex mechanisms. It has turned out that the picture we have had of GR activity is not as simple as anticipated initially. The antiinflammatory activity of GCs is regulated to a major extent by the repression of proinflammatory transcription factors, but not exclusively. Even in discussing transrepression it has become evident that several distinct molecular processes contribute – and the same is true for the mechanisms involved in the development of side-effects. For many of the metabolic effects transactivation seems to be the most prominent mechanism, as has been shown for genes encoding enzymes of gluconeogensis and protein catabolism. However, it seems that not all of the undesired effects can be avoided by the use of dissociated GR ligands. Nevertheless, the progress GR research has made in the last years has led to exciting developments in this fascinating area. Further investigations are necessary to fully illuminate GR action. The dissociated compounds generated so far, together with GR gene mutant mice (e.g. the $GR^{dim/dim}$ mice), are extremely valuable tools for this purpose. One particularly promising area for basic GR research is the role of cofactors. Altogether, these efforts may lead to further novel therapeutic approaches. In summary, new opportunities for an old target have emerged.

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10
1,25-Dihydroxyvitamin D_3 and its Dissociated Analogs as Modulators of Vitamin D Receptor Action

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10.1
Introduction

The biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], commonly known as calcitriol, is a multipotent secosteroid hormone with well-established effects on mineral homeostasis, particularly on calcium and phosphorus, cell proliferation, and bone metabolism [1]. It is therefore a standard therapeutic for the prevention of secondary hyperparathyroidism in patients with vitamin D deficiency due to chronic renal failure. Additionally, its used as an adjuvant for the therapy of osteoporosis. Since the mid-1980s there has been accumulating evidence that $1,25(OH)_2D_3$ exerts remarkable effects on immunocompetent cells [2–4]. Altogether, these findings made calcitriols [$1,25(OH)_2D_3$ and its analogs] therapeutically promising for a wide spectrum of clinical applications including immune diseases.

However, the striking activity of $1,25(OH)_2D_3$ in separate biological systems creates a challenge for its potential therapeutic use. The greatest yet unsolved obstacle for use in immune diseases is its strong calcium-mobilizing effect that can lead to organ calcification and bone resorption. Disturbances of calcium homeostasis (in its extreme leading to potentially life-threatening hypercalcemia) is thus critically minimizing the therapeutic window of $1,25(OH)_2D_3$. Identification of compounds with retained immunosuppressive properties but reduced calcemic activity has therefore sparked an intense research on dissociated vitamin D_3 derivatives [5–18]. Some compounds suitable for topical application to the skin have been approved and are well established for the therapy of psoriasis, although their effects may be caused by inhibition of keratinocytes rather than by immunodulation. The hurdles for a sufficiently dissociated oral bioavailable drug are much higher and no such compound has reached the market yet.

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

10.2 Principles of Cellular and Molecular Action of Vitamin D

$1,25(OH)_2D_3$ is produced by the body, which is therefore not entirely dependent on nutritional supply of $1,25(OH)_2D_3$ or its molecular precursors. Thus, contradicting its traditional name, $1,25(OH)_2D_3$ is not a classical vitamin. Due to its capacity to act as an intercellular messenger, it can rather be considered a hormone.

$1,25(OH)_2D_3$ is synthesized in several steps starting with UV light photolyzing 7-dehydrocholesterol (also referred to as provitamin D_3) in the skin leading to previtamin D_3 (for nomenclature, see Table 10.1). Previtamin D_3 is then thermally isomerized to vitamin D_3 (cholecalciferol), which will be hydroxylated to 25-hydroxyvitamin D_3 (calcidiol) in the liver and eventually to bioactive $1,25(OH)_2D_3$ (calcitriol) in the kidneys [19] (Figure 10.1). However, it is well known that major cellular components of the immune system such as activated macrophages and dendritic cells (DCs) possess 1α-hydroxylase, the key enzyme in $1,25(OH)_2D_3$ synthesis, and are therefore able to synthesize $1,25(OH)_2D_3$ [20, 21].

The regulation of vitamin D metabolism is rather complex, mainly due to the autoregulatory competence of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ inhibits 1α-hydroxylase and induces 24-hydroxylase, which catalyzes the degradation of $1,25(OH)_2D_3$ (reviewed in [22–24]). In addition, $1,25(OH)_2D_3$ regulation is tightly linked to parathyroid hormone (PTH) levels and both are key regulators of calcium homeostasis [25]. The major effects of $1,25(OH)_2D_3$ depend on its genomic activity through binding to the intracellular vitamin D receptor (VDR) [22, 26].

Table 10.1 Nomenclature of vitamin D metabolites.

Recommended name	Other names
Photolyzing 7-dehydrocholesterol	provitamin D_3
Cholecalciferol	vitamin D_3
Ergocalciferol	vitamin D_2
Calcidiol	25-hydroxycholecalciferol, 25-hydroxyvitamin D_3
(1*S*)-Hydroxycalciol	1α-hydroxycholecalciferol, alfacalcidol
(24*R*)-Hydroxycalcidiol	24(*R*),25-dihydroxycholecalciferol
Calcitriol	1,25-dihydroxycholecalciferol, 1,25-dihydroxyvitamin D_3
Ercalcidiol	25-hydroxyergocalciferol
Ercalcitriol	1,25-dihydroxyergocalciferol
(5*E*)-Isocalciol	isovitamin D_3
22,23-Dihydroercalciol or (24*S*)-methylcalciol	vitamin D_4
(6*Z*)-Tacalciol	precalciferol, previtamin D
(24*S*)-Ethylcalciol	vitamin D_5
(22*E*)-(24*R*)-Ethyl-22,23-didehydrocalciol	vitamin D_6

According to the recommendations of International Union of Pure and Applied Chemistry–International Union of Biochemistry Joint Commission on Biochemical Nomenclature [197–202] (http://www.chem.qmul.ac.uk/iupac/misc/D.html, modified).

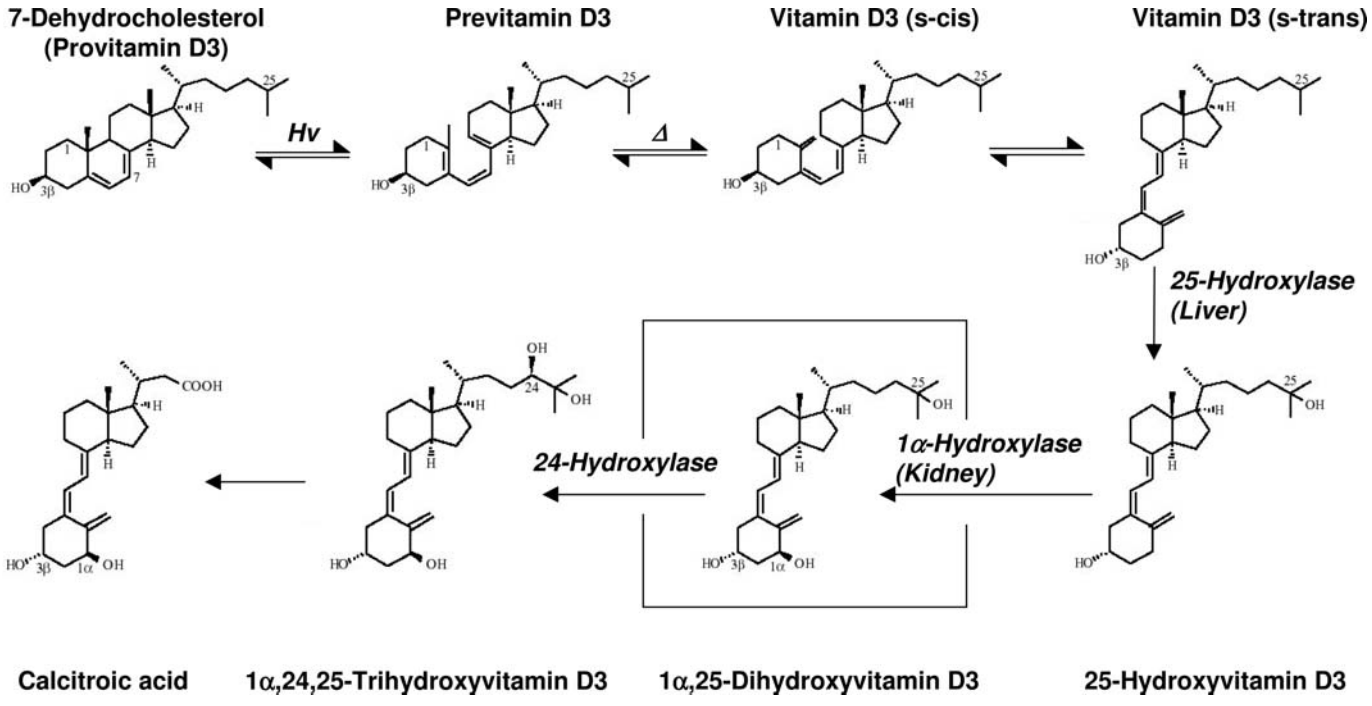


Figure 10.1 Metabolism of $1,25(OH)_2D_3$ (modified from Ref. [25]). Vitamin D_3 is produced in the skin and transported to the liver by binding to vitamin D-binding protein (DBP) where it is converted to $25(OH)D_3$. Again binding to DBD, $25(OH)D_3$ is further transported to the kidneys, where it is converted into the bioactive $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ induces 24-hydroxylase which initiates its catabolism.

VDR belongs to the nuclear steroid/thyroid hormone receptor family. 1,25 $(OH)_2D_3$ binds to cytoplasmic VDR and traffics into the nucleus. There, liganded VDR heterodimerizes with the retinoid X receptor (RXR) and subsequently binds to specific vitamin D-responsive elements (VDRE) within the promoter regions of responsive genes [26–29]. Recruitment of additional coactivator or corepressor complexes enables $1,25(OH)_2D_3$ to eventually exert its effect on the target gene – either by repression or by induction [27]. Transactivation or transrepression primarily depends on the presence of distinct VDRE sequences, which may induce or inhibit gene transcription [30]. Such VDRE are termed positive or negative VDRE, respectively. Inhibition of interleukin (IL)-2 and interferon (IFN)-γ secretion, hallmarks of vitamin D activity in the immune system, is for instance a direct effect of $1,25(OH)_2D_3$ on T cells (by outcompeting NF-AT on the IL-2 promoter and direct binding of the liganded VDR to a VDRE in the promoter of the IFN-γ gene [31, 32]). Transrepression may, however, be mediated through an alternative mechanism. In this case, VDRE binding of liganded VDR may antagonize downstream activities of other transcription factors such as NFκB, NF-AT or AP-1, thereby leading to indirect repression of genes induced by those transcription factors (reviewed in Ref. [26]).

As a consequence, tissue-specific expression of VDR largely determines the biological effects of $1,25(OH)_2D_3$. Not surprisingly, VDR is expressed in tissues involved in calcium regulation such as intestine, bone, parathyroid gland and kidney [26]. In addition, VDR expression has been demonstrated for several tumors, e.g. Kaposi sarcoma, mammary tumor, and prostate and colon cancer [33–37]. Furthermore, the fact that VDR is expressed in the thymus, T [38] and activated B cells [39], as well as in neutrophils [40], monocytes, macrophages and DCs [41, 42], strongly suggests a role for $1,25(OH)_2D_3$ in immune functions (Table 10.2). Interestingly, the highest concentrations of VDR in T cells has been demonstrated in $CD8^+$ T cells [43, 44].

As a consequence, the actions of $1,25(OH)_2D_3$ clearly exceed enhanced intestinal calcium absorption. $1,25(OH)_2D_3$ is a potent inducer of osteoclastogenesis [45]. Therefore, opposed to its beneficial effect on bone density observed in the treatment of osteoporosis, $1,25(OH)_2D_3$ may potentially also negatively affect bone architecture. In addition, $1,25(OH)_2D_3$ exerts significant antiproliferative activities – a characteristic that may explain some of the beneficial effects of $1,25(OH)_2D_3$ and some of its analogs on psoriatic patients where the pathologic hyperproliferation of keratinocytes may be reduced [46, 47]. Moreover, a huge body of literature on vitamin D in the field of oncology reflects the intense efforts to exploit this trait for cancer treatment. Downregulation of proliferation-associated epidermal growth factor receptor, Ki-67, c-myc and cytokeratin K16 are indicative of ceased proliferation induced by 1,25 $(OH)_2D_3$ [48]. Additional pathways for growth inhibition and apoptosis are conceivable as the $1,25(OH)_2D_3$-induced cytokine 'receptor activator of NFκB ligand' (RANKL) exerts proapoptotic effects [49].

$1,25(OH)_2D_3$ is known to mediate proapoptotic effects; however, depending on circumstantial influences, antiapoptotic activity has also been reported. This may happen indirectly through calbindin $D28_k$ which is responsive to $1,25(OH)_2D_3$ and

Table 10.2 Immune cells as targets for $1,25(OH)_2D_3$ and analogs: summary of the most typical effects of $1,25(OH)_2D_3$ and its agonists on immune cells.

Target cell population	Effects mediated by $1,25(OH)_2D_3$ (or analogs)	References
APCs	induced differentiation of promyeloic HL-60 cells to mature monocytes (induction of CD14 and CD11b)	[51–55]
	macrophage phenotype induced in monocytic U937 cells (upregulation of Fcγ receptor, increased phagocytosis and antibody dependent cellular cytotoxicity	[56, 57]
	inhibition/reversal of maturation of monocyte-derived DCs (continued or reinduced expression of CD14, impaired upregulation or loss of CD1a)	[58, 59, 61–65]
	sustained secretion of CCL18 (indicative of immature DCs)	[61]
	induction of 'tolerogenic' DCs that are able to induce T_{reg} cells	[66, 67]
	downregulation of CD40 (required for B cell activation)	[64, 66, 70]
	downregulation of B7 (CD80, CD86) expression on DCs and macrophages	[66, 70, 72, 203]
	induction of CTLA-4 and downmodulation of CD40L on $CD4^+$ T cells	[70]
	elevation of IL-10 production in DCs	[70, 77]
	strong synergism with other immunosuppressive drugs (e.g. dexamethasone) leads to enforced reduction of MHC class II, CD80, CD86, CD40, CD54, IL-12, reduced transcription of transcription factors RelB and c-rel, reduced transcription of RANTES, CCR2, CCR5, CCR7 and induction of T cell inhibitory Ig-like receptor ILT-4 and IL-10 on DCs	[77, 81]
	downregulation of MHC class II molecule expression on APCs (relevant for antigen presentation to T cells and subsequent activation of T cells)	[62, 65, 70, 82]
	abrogation of IL-12 p70 release of DCs by vitamin D_3	[63, 78]
	inhibition of proinflammatory cytokines, e.g. IL-1α, IL-1β and TNF-α in monocytes and macrophages	[83, 204, 205]
	downregulation of monocytic CD4 expression	[206]
	modulation of adhesion molecules expression (e.g. ICAM-1)	[84–86]
	effects on macrophage phagocytosis possible	[89, 90]

(*Continued*)

Table 10.2 (*Continued*)

Target cell population	Effects mediated by $1,25(OH)_2D_3$ (or analogs)	References
T cells	VDR expression in thymus and T cells (particular high in CD8 T cells)	[38, 43, 44]
	inhibition of mitogen-triggered T cell proliferation	[94]
	inhibition of antigen-specific T cell activation	[96, 97]
	reduced secretion of IL-2 by T_{reg} cells	[2, 83, 98–100]
	Downregulation of FasL expression of T cells	[105]
	Gene modulation (e.g. IL-2Rβ, IL-20) in CD4 T cells (gene chip analysis)	[38]
	IFN-γ synthesis is inhibited in mitogen-activated PBMCs	[120]
	suppression of T_h1 and induction of T_h2 T cell response by regulation of T_h2 cytokines *in vitro* and *in vivo*	[38, 121, 126, 127]
	IL-4, IL-5 and IL-10 release is enhanced, and IFN-γ is abolished in murine T cells	[125]
	suppression of IL-4 release during the process of deviation of naive $CD62L^+CD4^+$ T cells	[130]
	inhibition of IL-12, IFN-γ and IL-2 release	[78]
	reduced secretion of IL-1, IL-6, the chemokine IL-8 and TNF-α	[205]
	induction of IL-10 producing murine T_{reg} cells (in combination with dexamethasone)	[118]
	augmentation of IL-10 release	[124, 183]
B cells	VDR expression in B cells	[39]
	inhibition of IgG and IgM production in pokeweed mitogen-stimulated PBMCs	[71, 95]
	suppression of IgE secretion	[39]
NK cells	downregulation of IFN-γ, reduced cytotoxicity	[108, 109]

See text for abbreviations.

may protect from tumor necrosis factor (TNF)-α- and glucocorticoid-induced apoptosis or by inhibiting free radical formation [23, 50].

In summary, $1,25(OH)_2D_3$ is an effective inducer or repressor of many genes involved in cell maturation, differentiation, activation, proliferation and death of a multiplicity of cells.

10.3 Mechanisms of Vitamin D_3-Mediated Immunomodulation

10.3.1 Effects of $1,25(OH)_2D_3$ on Professional Antigen-Presenting Cells

Antigen-presenting cells (APCs) are of crucial importance for the generation and regulation of adoptive immune responses, and are ultimately responsible for the activity of many types of immunocytes, predominantly T cells.

First clues on the effects of VDR ligands on myelocytic cells in general emerged from the knowledge of constitutive expression of VDR in monocytes. The promyelocytic leukemia cell line HL-60 as well as bone marrow-derived cells develop into CD14 and CD11b expressing mature monocytes under the influence of $1,25(OH)_2D_3$ [51–55], whereas the monocytic cell line U937 was further driven towards a macrophage phenotype, upregulation of Fcγ receptor expression, increased phagocytic activity and antibody-dependent cellular cytotoxicity [56, 57].

For the understanding of the immunomodulatory effects of $1,25(OH)_2D_3$ it is of particular interest that $1,25(OH)_2D_3$ effectively inhibits or even reverses the maturation of DCs [58–60] (Figure 10.2). Monocyte-derived DCs continue to express monocyte-typical differentiation marker CD14 [61–64]. $1,25(OH)_2D_3$ may even reinduce CD14 on immature DCs after 6 days of incubation [65]. Also indicative of arrested differentiation, upregulation of the early DC marker CD1a is impaired in monocyte derived DCs [62, 64] and continuous exposure of immature DCs to $1,25(OH)_2D_3$ results in the loss of CD1a [65]. $1,25(OH)_2D_3$ thereby prevents DCs from becoming inducers of T cell activation, but turns them into 'tolerogenic DCs' – DCs that are inducers of T regulatory cells (T_{regs}) [66, 67]. Interestingly, this seems to apply only for DCs of the myeloid line but not for plasmacytoid DCs [68, 69].

Downregulation of the costimulatory molecule CD40 that is essential for mounting immunoglobulin (Ig) secretion of B cells is common on DCs exposed to $1,25(OH)_2D_3$ *in vitro* [64–66, 70]. This may explain the decrease of IgG and IgM in pokeweed mitogen-stimulated peripheral blood mononuclear cell (PBMC) cultures treated with $1,25(OH)_2D_3$ [71].

The costimulatory molecules CD80 and CD86 expressed by APCs are additional targets for $1,25(OH)_2D_3$ and other agonist VDR ligands [66, 70, 72, 73]. The lack of these molecules on DC surfaces is most likely essential for the immunosuppressive character of $1,25(OH)_2D_3$ since naïve T cells that undergo T cell receptor–major histocompatibility complex (MHC) interaction without receiving a second signal via CD80/CD86–CD28 ligation are rendered anergic or tolerant [69].

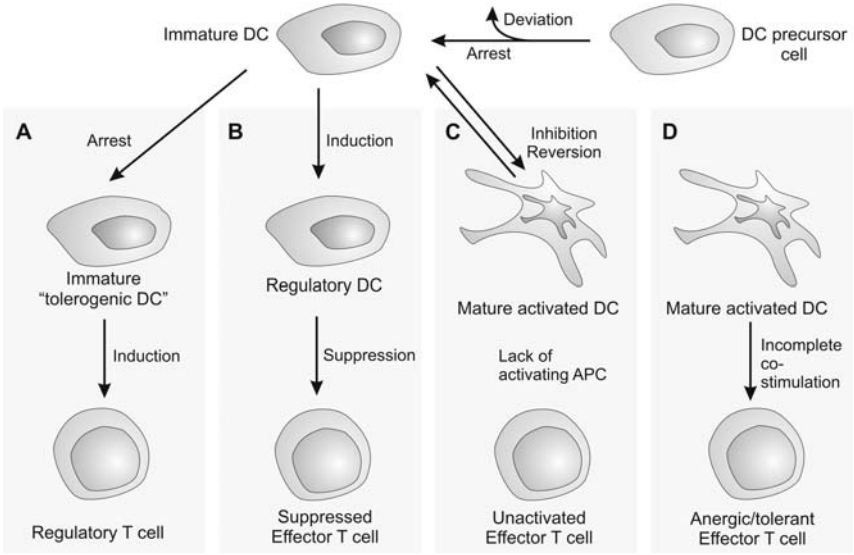


Figure 10.2 The multiplicity of potential 1,25 $(OH)_2D_3$-effects on professional APCs influences T cell function. 1,25 $(OH)_2D_3$ prevents differentiation of DC precursor cells into immature DCs. Arrested maturation of DCs leads to interaction of immature ('tolerogenic') DCs with T cells. This may induce T_{regs} with immunosuppressive capacities (a). Differentiation of immature DCs into 'regulatory DCs' that directly suppress the effector function of T cells mainly via secretion of IL-10 (b). Inhibited differentiation of immature DCs into fully functional proinflammatory DCs or redirection of mature DCs into an immature-like state prevents naïve T cells from antigen-specific activation (c). Mature DCs providing incomplete costimulatory signals induce anergic or tolerant T cells (d).

Retained differentiation may, however, not be the only way in which 1,25$(OH)_2D_3$ affects DCs. Even mature DCs treated with 1,25$(OH)_2D_3$ are able to disrupt the exchange of costimulatory signals with T cells as shown by Penna *et al*. Thus, DCs induce upregulation of CD152 (CTLA-4) on CD4 T cells, a negative regulator of T cell activation, and downregulation of CD154 (CD40L), another important costimulatory molecule [70]. Blockade of both pathways, i.e. CD152–CD80/CD86 and CD154–CD40, effectively inhibits autoimmunity and allograft-rejection [74–76] (Figure 10.3).

Augmented CD40-induced secretion of IL-10 in DCs through 1,25$(OH)_2D_3$ represents an additional mechanism by which immune tolerance may be elicited [70]. Thus, 1,25$(OH)_2D_3$ may induce a DC phenotype termed 'regulatory DCs'. Their functional properties resemble those of immature DCs regarding the inhibition of primary mixed lymphocyte reactions (MLRs) [77], but are phenotypically immature and regulatory DCs differ strongly concerning IL-10 production. DC-derived IL-10 is the critical component for the T cell-inhibitory effect of 1,25 $(OH)_2D_3$ (Figure 10.2).

A key feature of 1,25$(OH)_2D_3$ affected DCs is downmodulation of MHC class II molecules such as human leukocyte antigen (HLA-DR) which leads to reduced or

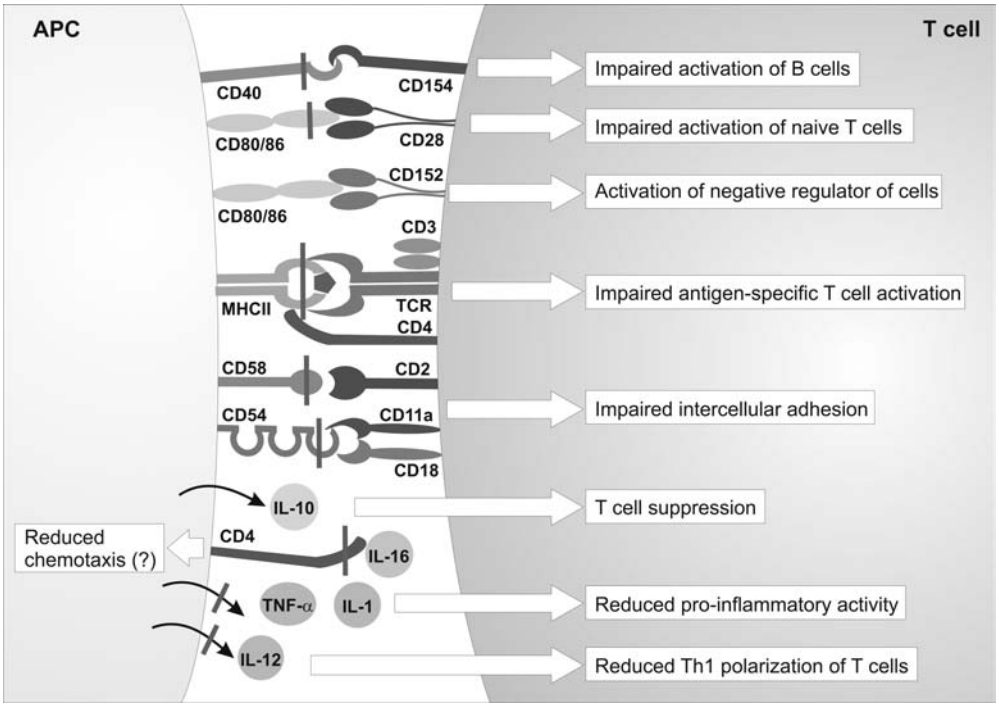


Figure 10.3 Major effects of $1,25(OH)_2D_3$ at the T cell synapse involve reduced antigen presentation, reduced intercellular adhesion, reduced costimulatory activity, reduced secretion of proinflammatory cytokines and increased secretion of antiinflammatory cytokines leading to reduced T cell and B cell activation.

abolished antigen presentation to T cells and subsequent activation of these cells [62, 65, 70]. In addition, there is a common abrogation of IL-12 p70 [63, 78] which is due to VDR-dependent repression of the NFκB-binding site in the IL-12 p40 promoter [70]. Since IL-12 is critically relevant for the induction of T helper (T_h) 1 cell responses, IL-12 repression might predominantly contribute to the beneficial effects of $1,25(OH)_2D_3$ analogs in T_h1-mediated diseases. Notably, IL-12 and IL-23 share p40 as a common subunit. IL-23 is known to be an inducer of T_h17 cells – another highly proinflammatory and pathologically relevant T cell subset characterized by the production of IL-17 [79]. Since p40 is susceptible to inhibition through VDR agonists, dampening effects of $1,25(OH)_2D_3$ on this important cell population are manifest and have recently been demonstrated [80].

Several *in vitro* and *in vivo* investigations revealed synergistic effects of $1,25(OH)_2D_3$ with other immunosuppressive drugs on DCs. $1,25(OH)_2D_3$ and dexamethasone synergize regarding a variety of important features, e.g. by reducing expression of MHC class II, CD80, CD86, CD40, CD54 and IL-12, they reduce transcription of the NFκB-linked transcription factors RelB and c-rel, reduce

transcription of the chemokine RANTES, the chemokine receptors CCR2, CCR5 and CCR7, but increase transcription of macrophage inflammatory protein-1α and monocyte chemoattractant protein-1 [81]. A powerful induction of regulatory DCs can be achieved through $1,25(OH)_2D_3$ in combination with dexamethasone [77]. Such DCs express the inhibitory Ig-like receptor ILT-4, secrete large amounts of IL-10 and are supposed to be potently immunosuppressive. Thus, rather than replacing established potent drugs like glucocorticoids, $1,25(OH)_2D_3$ or its analogs may prove to be particularly beneficial when applied in combination with them. Coadministration might allow significantly reduced dosages of drugs that are otherwise accompanied by partly severe side-effects. In fact, a combination product of the VDR agonist calcipotriol and the glucocorticoid betamethasone dipropionate has recently been launched for topical treatment of psoriasis.

$1,25(OH)_2D_3$-mediated effects on other myelocytic cells resemble those on DCs: $1,25(OH)_2D_3$ prevents upregulation of HLA-DR on monocytes activated by lipopolysaccharide (LPS), IFN-γ, IL-1 TNF-α or adherence to plastic. These effects are similar to those induced by the known immunosuppressive cytokines IL-10 and transforming growth factor (TGF)-β. $1,25(OH)_2D_3$ largely inflicts the ability of macrophages to produce proinflammatory cytokines (e.g. IL-1α, IL-1β and TNF-α) [82, 83]. Reduced activation of monocytes and macrophages will further contribute to impaired T cell function [83]. As in DCs, expression of CD80 and CD86 is $1,25(OH)_2D_3$-sensitive, too, and central to the APC function of macrophages.

$1,25(OH)_2D_3$ may affect monocyte–lymphocyte interaction and leukocyte migration, e.g. by modulation of cell adhesion molecules such as the CD11a (LFA-1), its interaction partner CD54 [intercellular cell adhesion molecule (ICAM-1)], and the β_2-integrins CD11b and CD18 [84–86]. In fact, *in vivo*, migration and homing properties of monocytes are influenced by VDR ligands as shown in experimental autoimmune encephalomyelitis (EAE), a rodent model for multiple sclerosis (MS), where decreased numbers of macrophages are found in the central nervous system (CNS) of $1,25(OH)_2D_3$-treated mice [87]. $1,25(OH)_2D_3$ signals T cells to express CCR10, which enables them to migrate to the skin-specific chemokine CCL27 secreted by keratinocytes of the epidermis, indicating direct effects on T cell homing to the skin. In contrast, $1,25(OH)_2D_3$ suppresses the gut-homing receptors $\alpha_4\beta_7$ and CCR9 [88].

Conflicting data exist on whether VDR ligands reduce or increase phagocytic properties of macrophages [89, 90]. Effects of $1,25(OH)_2D_3$ on various immunocyte populations are summarized in Table 10.2.

Although $1,25(OH)_2D_3$ obviously acts primarily by dampening on APCs, newer results indicate an active immunoprotective and especially antimicrobial role of $1,25(OH)_2D_3$ [91]. Activation of pathogen associated molecular patterns on macrophages such as Toll-like receptor (TLR) 2 leads to VDR expression and to local synthesis of $1,25(OH)_2D_3$. This in turn induced expression of cathelicidin, an antimicrobial peptide of the skin [92]. Vice versa, VDR ligation may increase TLR2 expression on keratinocytes and thereby complement expression of cathelicidin [93].

10.3.2
Effects of $1,25(OH)_2D_3$ on T Cells, B Cells and Natural Killer Cells

Apart from myelocytes, substantial research has been carried out regarding the effects of calcitriols on T cells, B cells and natural killer (NK) cells. $1,25(OH)_2D_3$-induced specific changes on lymphocytes were first observed in *in vitro* experiments more than two decades ago. $1,25(OH)_2D_3$ suppressed mitogen-induced proliferation of PBMCs from a healthy individual, indicating sensitivity of T cells to $1,25(OH)_2D_3$ [94]. It reduced the production of IgG and IgM, reflecting suppressed B cell and/or T_h cell function [95]. $1,25(OH)_2D_3$-induced antigen-specific inhibition of T cell proliferation lead to postulate the presence of VDR in T cells [96, 97] (Figure 10.4). The impeded proliferation of T cells was soon attributed to a reduced secretion of IL-2 from T cells treated with $1,25(OH)_2D_3$ [2, 83, 98–100], but notably not to suppressed expression of IL-2 receptor (IL-2R) α-chain (CD25) [101]. Unaltered expression of CD25, an early cell cycle G_1 event, contrasting inhibited expression of transferrin receptor which happens to occur in the late G_1 stage, pinpointed the proliferative arrest of T cells onto the transition from the early G_1 to the late G_1 phase [102]. The suppressive effects of $1,25(OH)_2D_3$ can be partly abolished by addition of abundant IL-2 [103]. The role of IL-2 was further

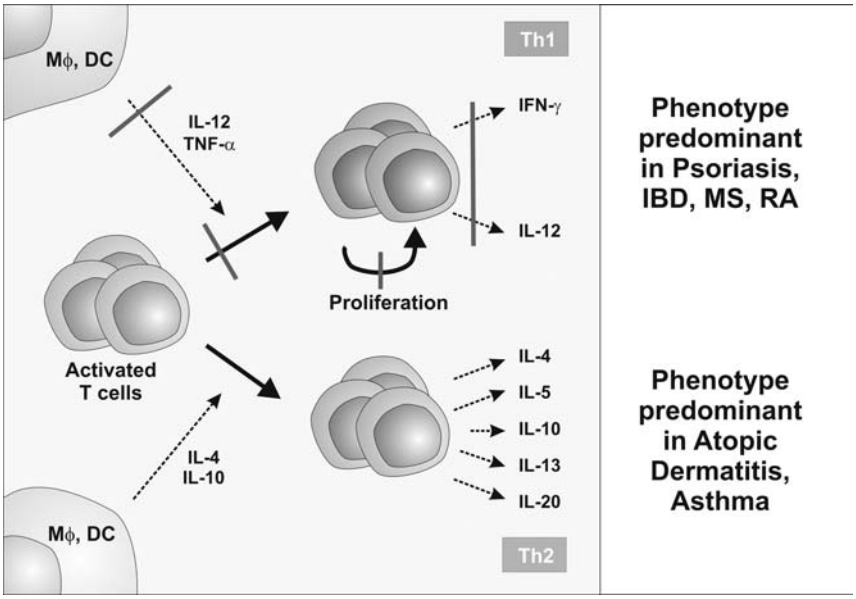


Figure 10.4 Effects of $1,25(OH)_2D_3$ on T_h1/T_h2 polarization. $1,25(OH)_2D_3$ abolishes production of T_h1-polarizing cytokines in APCs, inhibits the generation of T_h1-type T cells and might support production of T_h2-type cytokines. This scenario provides a rationale for the treatment of T_h1-dominated diseases with $1,25(OH)_2D_3$ or its analogs.

underscored through IL-2 knockout mice, where colitis reminiscent of human ulcerative colitis occurs spontaneously. In these mice, colitis was clinically refractory to treatment with $1,25(OH)_2D_3$ [104]. Clearly, $1,25(OH)_2D_3$-induced effects on T cells are likely to be at least partially mediated through diminished IL-2 expression.

Further effector functions of T cells are targeted by $1,25(OH)_2D_3$: CD178 (FasL) is downregulated on $1,25(OH)_2D_3$ treated T cells thereby conferring protection from activation-induced cell death [105].

An adoptive cell transfer model showed that the absence of activated ($CD44^{hi}$ $CD45RB^{lo}$) T cells in the CNS is characteristic for $1,25(OH)_2D_3$-treated mice which are protected against EAE in rodents [106]. The same study provided evidence that Rag1-null mutant mice, i.e. mice that are unable to produce functional T and B cell receptors, are not protected against EAE by $1,25(OH)_2D_3$. Interestingly though, $CD8^+$ T cells expressing relatively large amounts of VDR do not appear to be critical for $1,25(OH)_2D_3$-mediated immunosuppression. This became evident since EAE that can effectively be inhibited by administration of $1,25(OH)_2D_3$ is likewise suppressed in $CD8\alpha^{-/-}$ mice [107].

T cells can also be directly targeted by $1,25(OH)_2D_3$ as indicated by a gene chip array showing a large number of genes in purified $CD4^+$ T cells are up- or downregulated by $1,25(OH)_2D_3$ [38]. Among those were IL-2Rβ, a component of the IL-2R and IL-20. IL-20 is an IL-10-like cytokine implicated in psoriasis. As outlined below, production of IFN-γ by T cells is highly sensitive to $1,25(OH)_2D_3$, providing a rationale for the treatment of T_h1-mediated diseases. However, T cells are not the only source of IFN-γ since NK cells are equally potent to secrete this cytokine. $1,25(OH)_2D_3$-induced inhibition of IFN-γ secretion occurs in NK cells, too, which is paralleled by inhibition of cytotoxic NK activity [108, 109].

10.3.3
Effects of $1,25(OH)_2D_3$ on T_{reg} Cells

T_{regs} have attracted significant interest in recent years [110–112], but their nature is not fully elucidated yet. However, it is clear that a variety of different phenotypes of T_{regs} exists, and that a major T_{reg} population is characterized by the coexpression of CD4 and CD25 [112, 113]. In vitamin D-related research, it was soon discussed whether T_{regs} are important targets of $1,25(OH)_2$ and whether their induction is triggered by $1,25(OH)_2D_3$ [67]. $1,25(OH)_2D_3$ effectively induced $CD4^+CD25^+$ T cells in a transplantation model leading to accumulation of CTLA-4 expressing T_{regs} in the graft and eventual induction of full tolerance to mismatched mouse pancreatic islet allografts [66]. Moreover, graft acceptance was even spread to allogeneic spleen cells and heart grafts, and was transferable to syngeneic recipients through transfer of $CD4^+CD25^+$ cells of tolerized mice [66].

An analog of $1,25(OH)_2D_3$ (Ro 26-2198) in monotherapy significantly expanded the number of $CD4^+CD25^+$ T cells in treated nonobese diabetic (NOD) mice and was effective in suppression of autoimmune diabetes [114]. Again, $CD4^+CD25^+$ T cells accumulated in the pancreatic lymph nodes. Such cells were able to inhibit the

autoreactive T cells in this disease, inhibited LPS-induced production of IL-12 and IFN-γ and halted infiltration of T_h1-type cells into the pancreatic islets [114].

IL-2 knockout mice develop an inflammatory bowel disease (IBD)-like type of colitis that is resistant to $1,25(OH)_2D_3$ treatment [104]. Interestingly, IL-2 knockout mice do not only lack IL-2, but are also unable to generate $CD4^+CD25^+$ T_{reg}s [115–117]. Thus, the observed IL-2 dependency of $1,25(OH)_2D_3$ action may in fact be a T_{reg} dependency.

Combination of $1,25(OH)_2D_3$ and dexamethasone *in vitro* leads to differentiation of naïve murine $CD4^+$ T cells into antiinflammatory or regulatory cells whose primary trait is strong production of IL-10. These cells potently suppress EAE when adoptively transferred into mice before the induction of EAE [118]. Notably, for the generation of this type of T_{regs}, APCs are dispensable.

10.3.4
$1,25(OH)_2D_3$ and the T_h1/T_h2 Paradigm

A well-known mechanism of immunomodulation is represented by immune deviation, i.e. halting of T_h1-mediated autoimmune disease through induction of T_h2-type T cells [119]. Clearly, $1,25(OH)_2D_3$ inhibits IFN-γ production of PBMCs from normal individuals [120]. Suppression of IFN-γ is mediated through direct interaction of the liganded VDR/RXR heterodimer with the promoter region of the IFN-γ gene [32].

$1,25(OH)_2D_3$ potently inhibits synthesis of IL-12p40 by binding to the p40-κB sequence and downregulation of NFκB activation [78]. IL-12 itself is a critical inducer of IFN-γ in T_h1 cells and suppressor of IL-4 in T_h2 cells.

$1,25(OH)_2D_3$ increases the T_h2-polarizing transcription factor GATA-3 in T_h0 cells [38] and upregulates the T_h2 cytokines in normal human PBMCs [121] as well as in murine bone marrow-derived DCs [122, 123]. Consistently, $1,25(OH)_2D_3$ significantly inhibits the production of IL-2, IFN-γ and IL-12 in human PBMCs [124]. At the same time $1,25(OH)_2D_3$ induces the production of IL-10 and IL-5, but not IL-4.

In cell culture experiments with murine cells, IL-4, IL-5 and IL-10 production was enhanced, whereas $1,25(OH)_2D_3$ abolished IFN-γ production in antigen-specific reactions [125]. This was accompanied by increased expression of the T_h2-specific transcription factors GATA-3 and c-maf. The T_h2 bias was observed even in the absence of APCs, indicating a direct effect on the T cells. Administration of $1,25(OH)_2D_3$ into mice led to increased transcription of IL-4 and TGF-β1 [126]. IL-4 and IL-13 were also increased in a mouse model of pulmonary eosinophilic inflammation, a T_h2-mediated airway inflammation model [127]. In line with the T_h1/T_h2 paradigm, IL-4 increased with concomitant decrease of IFN-γ also in the NOD mouse model. $1,25(OH)_2D_3$-treated NOD mice were prevented from T_h1-mediated type-1 diabetes after immunization and challenge with a GAD65 peptide [128]. Consistently, protective effects of $1,25(OH)_2D_3$ against T_h1-mediated EAE and allograft rejection were reduced in IL-4 knockout mice [129]. A pronounced inhibition of T_h1-type responses was observed when $1,25(OH)_2D_3$ was applied in combination with dexamethasone [121].

Yet, whether $1,25(OH)_2D_3$ is an inducer of T_h2 T cell responses remains controversial because $1,25(OH)_2D_3$ may also suppress IL-4 if present during the process of *in vitro* polarization of naïve $CD62L^+$ $CD4^+$ T cells [130]. This may explain why $1,25(OH)_2D_3$ does not exclusively inhibit T_h1-mediated diseases but also has beneficial effects in a T_h2-dependent allergic asthma model (our own unpublished data). Moreover, $1,25(OH)_2D_3$ and its analog EB1089 are potent suppressors of IgE secretion from B cells activated with anti-CD40 and IL-4 [39]. Also, T_h1, T_h2 as well as T_h0 T cells are clearly susceptible to $1,25(OH)_2D_3$, since it inhibits phorbol myristate acetate (PMA)/ionomycin-induced proliferation of all three types of purified $CD4^+$ T cells [38].

Although $1,25(OH)_2D_3$ is a potent inhibitor of T_h1-dominated EAE and IFN-γ secretion is clearly inhibited *in vitro*, 1,25-(OH)2 D_3 failed to suppress T_h1 cells *in vivo* using a myelin basic protein induction model of EAE [106]. At the same time, whatsoever, no upregulation of IL-4 transcription was observed in animals protected from EAE through $1,25(OH)_2D_3$ [106]. This study speaks against both putative immunoregulatory mechanisms in $1,25(OH)_2D_3$ action, inhibition of T_h1 responses as well as induction of T_h2 responses. The immunomodulatory effects of $1,25(OH)_2D_3$ can therefore not entirely be attributed to and explained by effects on the T_h1/T_h2 cytokine balance. However, the majority of reports consistently demonstrate that T_h1 T cells are relatively more susceptible to $1,25(OH)_2D_3$ treatment. This may also explain the clinical efficacy of $1,25(OH)_2D_3$ predominantly in the treatment of T_h1-mediated diseases as outlined below.

10.4 Effects of $1,25(OH)_2D_3$ *In Vivo* and Clinical Implications

10.4.1 $1,25(OH)_2D_3$ in Animal Models of Immune-Mediated Diseases

In EAE $1,25(OH)_2D_3$ is clearly therapeutically effective since it entirely protects mice from disease and it may block disease progression [107, 131]. Using VDR agonists in a chronic relapsing EAE model, protection from relapses was achieved when VDR agonists were administered at disease onset or after the first peak of the disease [132]. Similar results were found in rat EAE and were associated with an inhibition of CD4 (on APCs rather than on T cells), MHC class II, CD11b/c and NOS II expression in relevant areas of the CNS [133, 134]. $1,25(OH)_2D_3$ did not protect VDR-deficient mice against EAE [107] showing the VDR dependence and specificity of the effect.

Clear disease protection through $1,25(OH)_2D_3$ has been shown in the NOD mouse model of type I diabetes since vitamin D deficiency almost doubles the incidence of diabetes in female mice to 88% and induces diabetes in almost 50% of male mice. Diabetes was prevented by dietary administration of 50 ng of $1,25(OH)_2D_3$/day [135, 136]. A variety of additional studies in the NOD mouse model led to the $1,25(OH)_2D_3$-sensitive role of T_h1-type T cells and $CD4^+CD25^+$ T_{regs} (see

above) [114, 128, 137]. 1,25(OH)$_2$D$_3$ has similarly successful been applied in IL-10 knockout mice. Such mice spontaneously develop an IBD-like syndrome when untreated. Under vitamin D deficiency the disease accelerates, whereas its progression is blocked and the symptoms ameliorate when treated with 1,25-(OH)$_2$-cholecalciferol [138]. In contrast, no therapeutic effect has been observed on IBD in the IL-2 knockout model, illuminating the importance of the IL-2 gene as a target for 1,25(OH)$_2$D$_3$ [104]. Support for a potential beneficial role of 1,25(OH)$_2$D$_3$ in IBD is provided by the finding that gastrointestinal inflammation is enhanced in IL-10/VDR double-knockout mice and in VDR$^{-/-}$ mice given dextran sodium sulfate [139, 140]. Similarly, transfer of colitogenic CD4$^+$/CD45RB^{hi} T cells from VDR knockout mice induces comparably more severe colitis than wild-type equivalents [139]. Therapeutic effects have in contrast been observed in trinitrobenzene sulfonic acid colitis mice when the 1,25(OH)$_2$D$_3$ analog ZK 156979 was administered [141].

Models of rheumatoid arthritis (RA) were object to 1,25(OH)$_2$D$_3$ treatment, providing additional support for the high potential of the hormone for the treatment of T$_h$1-mediated diseases. As an example, in rat adjuvant arthritis 1,25(OH)$_2$D$_3$ improved the inhibiting effect of cyclosporine A on arthritis onset as well as on arthritis aggravation [142]. The incidence and severity of type II collagen-induced arthritis was reduced in rats or mice orally treated with 25-(OH)$_2$-D$_3$, the 1,25 (OH)$_2$D$_3$ analog MC 1288 or 1,25(OH)$_2$D$_3$ [143–145]. 1,25(OH)$_2$D$_3$ has further proven to be therapeutically effective in animal models of autoimmune thyroiditis [146], allogeneic transplantation [66, 147, 148], rat Heymann nephritis [149] and autoimmune prostatitis [80]. Results from 1,25(OH)$_2$D$_3$-related studies in animal models are summarized in Table 10.3.

10.4.2
1,25(OH)$_2$D$_3$-Induced Immunomodulation in Man

1,25(OH)$_2$D$_3$ and its precursor 1α-OH-D$_3$ (alfacalcidol) are approved and widely used to treat or prevent secondary hyperparathyroidism in patients with chronic renal failure undergoing hemodialysis and to treat osteoporosis. It was therefore of interest to directly test whether immune parameters were altered after administration of 1,25 (OH)$_2$D$_3$ in these patients. A variety of effects have been described to occur *in vivo*, such as increased numbers of CD4$^+$ T cells [150, 151], restored lymphocyte proliferation rates to tuberculin or to mitogens [150, 152], restored (increased) production of IL-2 [153], decrease of serum IL-1β by about 50%, reduced intradialytic TNF-α increase [154, 155], reduction of IL-6 [155] and increased cytotoxic activity of NK cells [156, 157]. As *in vitro*, moderately reduced HLA-DR expression on monocytes from 1,25(OH)$_2$D$_3$-treated hemodialysis patients was found already at day 2 after an oral pulse of 5 μg of 1,25(OH)$_2$D$_3$ [158].

Results from clinical studies were, however, partly unexpected regarding the large and virtually uniform *in vitro* findings. No significant changes of immune parameters (CD2, CD19, CD4 and CD8) were noted in a study where alfacalcidol was administered to hemodialyzed patients over 6 months; only NK cell numbers were increased

Table 10.3 $1,25(OH)_2D_3$-sensitive animal models of human diseases: effects of $1,25(OH)_2D_3$ and derivatives.

Animal model	Human disease equivalent	Vitamin D_3-mediated effects, observations, proposed mechanisms	References
Murine EAE	MS	protection from EAE, halted progression of established EAE; $CD8^+$ T cells are dispensable	[107, 131, 132]
		decreased numbers of macrophages in the CNS	[87]
		absence of $CD44^{hi}CD45RB^{lo}$ T cells in the CNS but not in lymph nodes of $1,25(OH)_2D_3$-treated EAE-protected mice suggests local tissue-specific effects of $1,25(OH)_2D_3$ reducing activated T cells; Rag1-null mutant mice (unable to produce functional T and B cell receptors) are not protected against EAE by $1,25(OH)_2D_3$, consistent with a critical role of T cells; $1,25(OH)_2D_3$ did not inhibit T_h1 cell IFN-γ production or promote T_h2 cell genesis or IL-4 production	[106]
		$CD8^+$ T cells are not critical for $1,25(OH)_2D_3$-mediated immunosuppression	[107]
		protection against T_h1-mediated EAE and allograft rejection is reduced in IL-4 knockout mice, supporting a role of T_h1/T_h2 bias in effective $1,25(OH)_2D_3$ treatment	[129]
		adoptive transfer of IL-10-producing T_{regs} suppress EAE induction	[118]
Rat EAE	MS	decrease of $CD4^+$ non-T cells, decrease of MHC class II, CD11b/c, and NOS II expression in relevant areas of the CNS; role of $1,25(OH)_2D_3$-sensitive APCs in target organ is suggested	[133, 134]
NOD mice	type I diabetes	$1,25(OH)_2D_3$ and analogs protect from diabetes	[207–209]
		protective effects of $1,25(OH)_2D_3$; vitamin D deficiency increases incidence of diabetes	[135, 136]
		combination therapy of a $1,25(OH)_2D_3$ leads to normal blood glucose levels and pronounced analog with IFN-γ prolongation of syngeneic islet graft survival; effects were associated with increased transcription of IL-10 in the grafts	[137]
		arrested progression of type 1 diabetes through treatment with a $1,25(OH)_2D_3$ analog correlates with inhibition of IL-12 production, blocked infiltration of T_h1 cells in pancreatic islets and increase of $CD4^+CD25^+$ T_{regs}	[114]

		prolonged graft survival in syngeneic islet transplantation under combination treatment with 1,25(OH)$_2$D$_3$ analog (KH1060) and cyclosporine associated with suppressed IL-12 and T_h1 cytokine expression and increased IL-4 levels	[210]
		treatment with analogs of 1,25(OH)$_2$D$_3$ in combination with cyclosporine A effectively treats established diabetes	[211]
		autoantigen-specific T_h1/T_h2 shift.	[128]
		higher incidence of diabetes when held on a vitamin D-deficient diet early in life; higher IL-1 expression in islets of vitamin D-deficient mice, aberrantly low IL-1 and IL-6, high IL-15 in peritoneal macrophages; thymus and lymph nodes contained less $CD4^+CD62L^+$ T_{reg} cells; importance of preventing vitamin D deficiency in early childhood is implied.	[212]
IL-10 knockout mice	IBD	vitamin D deficiency accelerates inflammation, progression is blocked and symptoms ameliorate when treated with 1,25(OH)$_2$-cholecalciferol	[138]
IL-10/VDR-deficient mice	IBD	rectal bleeding, accelerated IBD, 100% mortality by 8 weeks of age; symptoms adoptively transferable by splenocytes; suggests that critical vitamin D effects on the immune system are mediated through VDR signaling	[139]
IL-2 knockout mice	IBD	no therapeutic effect of 1,25(OH)$_2$D$_3$; IL-2 supposed to be essential for 1,25 (OH)$_2$D$_3$ efficacy	[104]
Adjuvant arthritis (rats)	RA	synergism with cyclosporine A, delayed disease onset, inhibited aggravation	[142]
Type II collagen-induced arthritis (rats and mice)	RA	reduced incidence and severity under treatment with 1,25(OH)$_2$D$_3$, 1α-OH-D$_3$ (precursor of 1,25(OH)$_2$D$_3$) and additional analogs	[143–145]
Borrelia burgdorferi-induced arthritis	Lyme disease	acute arthritic lesions were minimized or prevented by 1,25(OH)$_2$D$_3$ diet fed to mice	[145]
CBA mouse model of autoimmune thyroiditis	Grave's disease Hashimoto's thyroiditis	synergism with cyclosporine A, lower incidence of thyroid pathology, milder disease	[146]

(*Continued*)

Table 10.3 (*Continued*)

Animal model	Human disease equivalent	Vitamin D_3-mediated effects, observations, proposed mechanisms	References
Pancreatic islet and heart transplantation	graft rejection	tolerance induction to mouse islet allografts; acceptance of donor-type heart grafts; synergism with mycophenolate mofetil, modulated APC function, induction of tolerogenic DCs; $CD4^+$ transfer from tolerant but not naive mice prevents donor-type islet graft rejection; downregulation of CD40, CD80 and CD86 on APCs; DCs from tolerant mice secrete 10-fold less IL-12 than DCs from rejecting mice; abrogated $CD4^+$ T cell IFN-γ production; impaired development of IFN-γ-producing T cells; increase of $CD4^+CD25^+CD152^+$ T_{regs} in spleen and transplant-draining lymph node	[66, 147]
Allo-/xenogeneic transplantation	graft rejection	cyclosporine A, but not tacrolimus, has strong additive effect with calcitriol on acute rat lung allograft survival and xenogeneic islets in nonobese diabetic mice	[148, 213]
Heymann nephritis (Lewis rats)	chronic glomerulo-nephritis	$1,25(OH)_2D_3$ reduced proteinuria	[149]
Murine model of pulmonary eosinophilic inflammation	airway inflammation	augmentation of allergen-induced T cell proliferation, increased T_h2 cytokine (IL-4 and IL-13) and IgE production; local inflammatory response ameliorated with impaired recruitment of eosinophils and inferior levels of IL-5; vitamin D could sustain T_h2 response, leading to increased prevalence of allergy, but promising beneficial effects in airway eosinophilia	[127]

to a certain degree [159]. In a human phase Ib study where calcidiol (25-OH-D$_3$) was administered to cancer patients, HLA-DR expression on PBMCs paradoxically showed a tendency to increase; the same was true for the number of CD25$^+$ cells. Other parameters such as IL-12p70 and CD34$^+$ cells developed highly inconsistently [160].

Gannage-Yared observed that administration of cholecalciferol (vitamin D$_3$) in combination with calcium to healthy postmenopausal women did not change serum IL-6 or TNF-α although the levels of the bioactive metabolite 1,25(OH)$_2$D$_3$ were significantly increased [161].

In vitro, 1,25(OH)$_2$D$_3$ potently inhibits upregulation of activation markers after stimulation with mitogens or proinflammatory stimuli (see above). Most patient-related data were therefore also generated from *in vitro* stimulated cells of patients that were previously treated with 1,25(OH)$_2$D$_3$. However, even under those conditions, results were variable or contradicted evidence from experiments where cells were directly exposed to 1,25(OH)$_2$D$_3$ *in vitro*. Thus, the proliferative capacity of T cells from 1,25(OH)$_2$D$_3$-treated hemodialysis patients to mitogens or conventional antigens was found unchanged [150, 162]. IL-1 and IL-6 were transiently increased in one study after stimulation with PMA or LPS [163], but decreased according to others [154, 164].

Weak translation of *in vitro* findings into the *in vivo* situation may be due to a variety of factors, e.g. shorter times of exposure of cells to 1,25(OH)$_2$D$_3$ *in vivo* compared to incubation experiments *in vitro*. Furthermore, the stability of the compound may differ *in vitro* and *in vivo*. Importantly, the tissue distribution of 1,25(OH)$_2$D$_3$ is far from homogenous *in vivo*. Thus, it may well be that peripheral blood – the compartment that is usually analyzed regarding effects of 1,25(OH)$_2$D$_3$ – is not fully appropriate for that purpose. In addition, *ex vivo* data from previously 1,25 (OH)$_2$D$_3$-deficient patients, such as patients with chronic renal failure, cannot easily be extrapolated on patients with other types of diseases (e.g. chronic inflammation) or healthy volunteers.

The hallmark of 1,25(OH)$_2$D$_3$ as a therapeutic is its efficacy in treatment or prevention of diseases. Due to the immunomodulatory effects of 1,25(OH)$_2$D$_3$ on T$_h$1-type T cells, T$_h$1-mediated immune diseases such as RA, MS, IBD, systemic lupus erythematosus, psoriasis, psoriatic arthritis and type I diabetes are indications for a potential use of vitamin D compounds (Table 10.4).

Supporting this concept, a Finnish birth-cohort study showed that food supplementation with vitamin D in infancy reduced the risk for type I diabetes [165]. Two studies found an inverse association of use of vitamin D supplements or general vitamin D intake and MS or RA [166, 167], suggesting that high vitamin D supply leads to a 30–40% reduction of the relative risk for the respective disease.

In psoriatic arthritis, oral application of 1,25(OH)$_2$D$_3$ to patients with active disease in a 6-month open-label study improved arthritis to some degree [168]. Alfacalcidol treatment of 140 patients with RA induced slight improvement of RA symptoms, although effects on immunological parameters have again not been clearly identified [169]. More drastic clinical effects have been observed in another 3-month

Table 10.4 Systemic vitamin D_3 treatment of immune-mediated human diseases: therapeutic effects of $1,25(OH)_2D_3$ and derivatives.

Indication	Finding	References
RA	alfacalcidol oral treatment of patients caused some degree of arthritis improvement (4-month double-blind, placebo controlled trial; 140 patients)	[169]
	treatment of RA patients with oral alfacalcidol (3-month open-label trial, 19 patients) resulted in clear improvement of disease symptoms and complete remission in a considerable number of patients	[170]
Psoriatic arthritis	oral application of calcitriol to patients (6-month open-label trial, 10 patients) resulted in arthritis improvement	[168]
Psoriasis	treatment of psoriasis patients (6-month open trial, 21 patients) with oral alfacalcidol resulted in moderate to marked improvement of disease	[214]
	treatment of patients (6-month open trial, two patients) with oral calcitriol improved psoriatic lesions	[173]
	oral treatment of psoriasis patients with calcitriol (3-month open trial, 14 patients) resulted in more than 50% clearing in the majority of patients	[174]
	treatment with oral calcitriol (6-month open label trial, eight patients) caused mild to moderate improvement of psoriatic symptoms	[176]
	oral application of calcitriol (6-month open trial, 85 patients) resulted in the majority of patients in improvement of clinical assessment	[177]
Scleroderma	treatment of linear scleroderma patients with oral calcitriol (3-month open trial, seven patients) resulted in good to excellent improvement of skin lesions	[178]

open-label trial on patients with acute RA applying alfacalcidol. In this trial, the majority of patients experienced clear improvement of disease with a considerable number of patients showing complete remission [170].

Positive effects of orally administered calcitriols were initially noted concerning the cutaneous symptoms of psoriasis in a patient who was orally treated with $1,25(OH)_2D_3$ for osteoporosis [171]. This finding led to a series of clinical trials with systemic $1,25(OH)_2D_3$, $1,25(OH)_2D_3$ precursors or $1,25(OH)_2D_3$ analogs in psoriasis. The majority of these studies demonstrated good and partially excellent clinical efficacy of calcitriols in this disease [172–177]. In linear scleroderma, another dermatological indication, systemic application of $1,25(OH)_2D_3$ lead to beneficial effects in five of seven pediatric patients treated [178].

A general limitation for systemic application of $1,25(OH)_2D_3$ is its calcemic side-effects. Therefore, topical application, e.g. by calcipotriol, a significantly less calcemic analog of $1,25(OH)_2D_3$, is used for the treatment of psoriasis so far [47, 179–182]. The

beneficial effect of topical vitamin D in psoriasis is thought to be primarily due to its antiproliferative capacity that inhibits keratinocyte hyperproliferation but also to its various antiinflammatory actions, i.e. inhibited production of IFN-γ, IL-6, IL-8 and TNF-α [183]. Success rates are remarkably good since 60–70% of patients improve under this therapy, 26% going into complete remission [184]. Thus, a relatively large amount of information on clinical effects of $1,25(OH)_2D_3$ has been gained from this disease [26, 47, 179, 185, 186].

Data are also available for MS patients, where supplementation with $25(OH)_2D_3$ increased serum levels of antiinflammatory cytokine TGF-β after 6 months of treatment, whereas no or little effect was observed on TNF-α, IL-13, IFN-γ and IL-2 [187]. No information was given on the clinical presentation of these patients after treatment. In type I diabetes, a long-term study proved dietary vitamin D supplementation was clinically beneficial in terms of reduced risk of the disease [165]. Heart transplant recipients that were treated with low-dose $1,25(OH)_2D_3$ aiming at reduction of bone loss required significantly less cyclosporin for prevention of organ rejection suggesting a potentially beneficial immunosuppressive role of $1,25(OH)_2D_3$ in transplantational medicine [188].

10.5 Dissociated Vitamin D Derivatives

There has been a great effort to identify vitamin D analogs sharing immunosuppressive properties with calcitriol, but lacking calcemic activity. Such dissociated derivatives would have a significantly more favorable therapeutic profile than calcitriol and would be suited for systemic immunomodulatory therapy. Consequently, several pharmaceutical companies aimed to identify such compounds and various systemically active vitamin D analogs have been reported [7, 189, 190]. The molecular interaction of VDR ligands with the receptor is of significant complexity not only due to the heterodimerization of the liganded VDR, but also due to the multiplicity of cofactors (coactivator and corepressors) that contribute to ligand-induced gene activation or repression. Thus, the mechanisms that contribute to dissociation can be manifold and minor structural changes of a given VDR ligand can lead to drastic changes of pharmacological properties. On the molecular level, differential induction or repression of target genes due to modified coactivator/corepressor recruitment may be as relevant as pharmacokinetic properties (e.g. tissue distribution, half-life, protein binding, etc.). Although our understanding of VDR-associated molecular signaling mechanisms has tremendously improved in recent years, prediction and rational design of dissociated VDR agonists is still very difficult. Consequently, the vast majority of low calcemic analogs were rather identified by systematic modification of the calcitriol backbone, and subsequent profiling of such compounds in pharmacological *in vitro* and *in vivo* assays using iterative optimization processes.

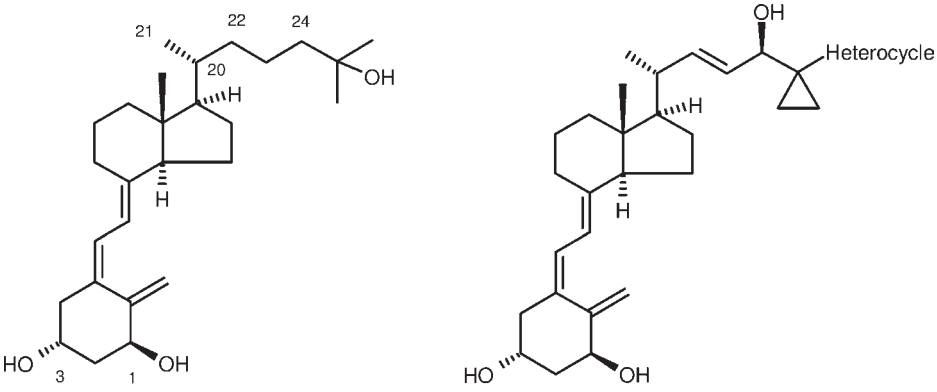
In the following, a novel class of vitamin D analogs identified at Bayer Schering Pharma is portrayed with its unique pharmacological profile.

10.6
Calcitriol Derivatives with Heterocyclic Units in the Side-Chain

Structural modifications of the calcitriol side-chain lead to changes in the biological activity profile of the respective compound series [191]. Introduction of the 22,23-double bond, transposition of the 25-OH group to the 24-position and connection of the terminal methyl groups of the side-chain afforded the so far clinically most relevant calcitriol analog, MC 903, from the Danish pharma manufacturer Leo Pharmaceutical Products [192]. MC 903 has been successfully applied for the topical treatment of psoriasis [193] for a rather long time.

In recent years, extensive manipulations of the calcitriol side-chain have been investigated. At Schering heterocyclic rings have been added to the MC 903 motif for the first time. As for many drug classes such variations might positively influence pharmacodynamic and pharmacokinetic behavior (Scheme 10.1).

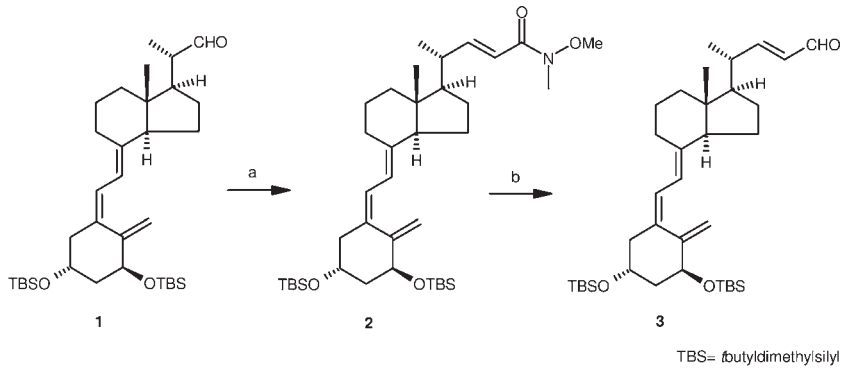
Since a synthetic route to heterocyclic calcitriol derivatives was still lacking, explorative research had to be carried out. The vitamin D aldehyde **1** was prepared according to the Leo protocol [192] followed by a photosensitized isomerization of the triene unit in seven steps (Scheme 10.2). Wittig–Horner olefination selectively yielded the *E*-double bond configuration of the Weinreb amide **2**. Reduction with diisobutylaluminum hydride gave the unsaturated aldehyde **3** without any evidence for overreduction. This aldehyde could serve as a valuable intermediate for further elaboration of the side-chain [194].



Scheme 10.1

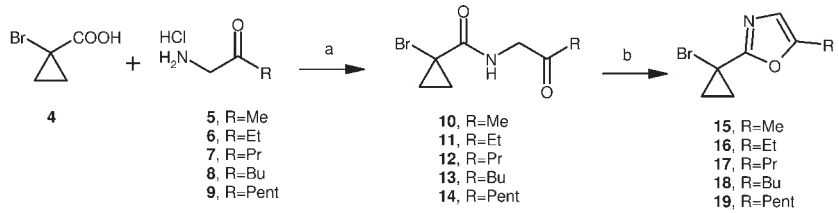
Starting from readily available 2-bromocylopropanecarboxylic acid **4**, a number of amides with different side-chain lengths (**10**–**14**) were effectively formed (Scheme 10.3). Under rather harsh condition using concentrated sulfuric acid the substituted oxazole building blocks (**15**–**19**) were synthesized in good yields.

After bromo-lithium exchange at low temperature the heterocyclic moieties were coupled with the aldehyde **3** giving access to the corresponding bissilyl protected



a) Ph_3P=CH-CO-N(OMe)Me, toluene, 110 °C, 82%; (b) diisobutylaluminum hydride, THF, -78 °C, 92%

Scheme 10.2



a) DCC, NEt_3, CH_2Cl_2, RT, 76%, 74%, 82%, 79%, 89%; b) H_2SO_4 (conc.), 60 °C, 59%, 67%, 55%, 63%, 60%

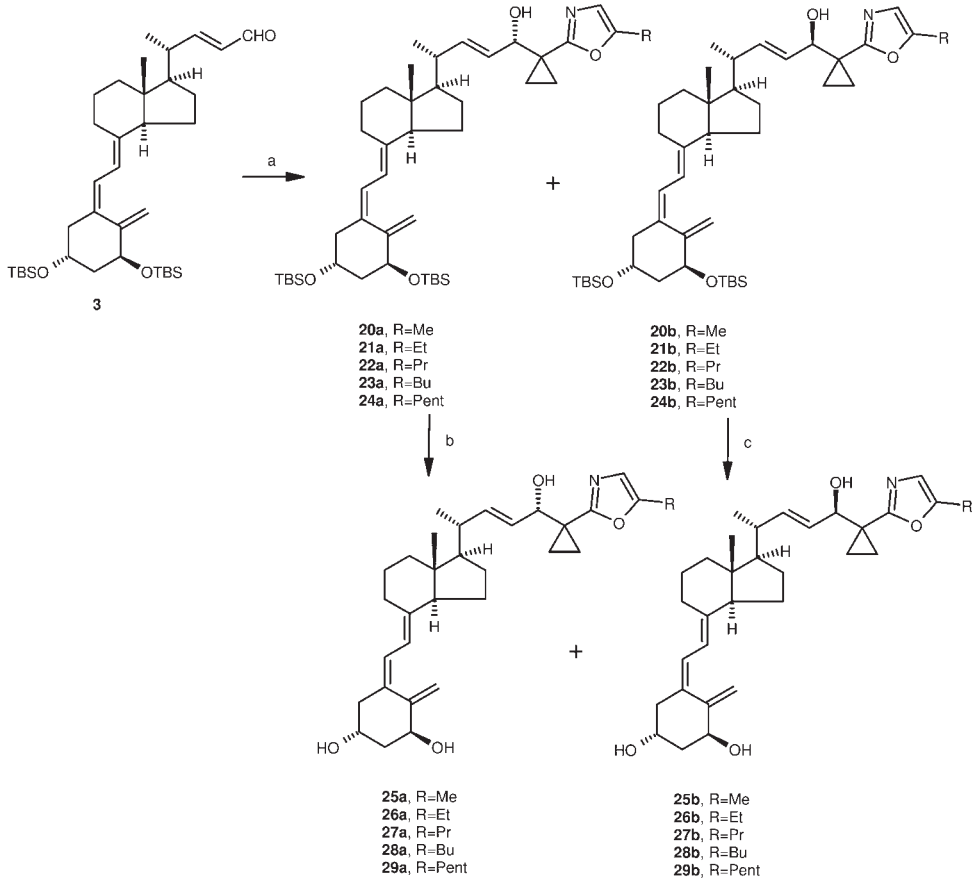
Scheme 10.3

derivatives **20a/b-24a/b** (Scheme 10.4). The C24 diastereomeric alcohols could be separated by flash chromatography and were subsequently subjected to fluoride mediated cleavage of the silyl protecting groups. Finally, the heterocyclic calcitriol analogs **25a/b–29a/b** could be obtained in reasonable overall yields.

The biological effects of these novel oxazole calcitriol derivatives have been assessed at Schering. Initially it turned out that the 24-OH stereochemistry is crucial for biological activity. As the 24α-OH analogs were only very weakly active (data not shown), their respective 24β-OH counterparts exerted substantial activity in all systems investigated.

The relative binding affinities for the VDR were slightly reduced as compared to the natural hormone calcitriol. Thus, the side-chain modifications interfere only marginally with VDR binding (Table 10.5) [8].

The induction of differentiation of HL-60 cells is a prominent assay to evaluate agonistic calcitriol behavior with certain relevance for cancer treatment. In this setting the first calcitriol antagonist, ZK 159222, has been identified by Schering researchers [195]. The methyl (**25b**), ethyl (**26b**) and pentyl (**29b**) oxazole analogs showed dramatically reduced agonistic potency in this assay. On the other hand, the



a) *t*BuLi, **15**, **16**, **17**, **18** or **19**, diethyl ether, -78 °C, chromatography, 42%/38%, 39%/38%, 45%/44%, 32%/34%, 40%/38%; b) TBAF, THF, RT, 56%, 45%, 63%, 59%; c) TBAF, THF, RT, 49%, 51%, 59%, 55%

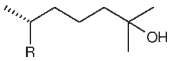
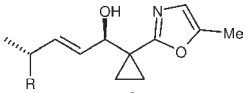
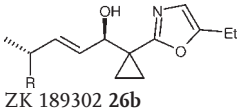
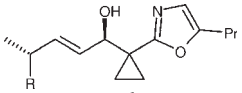
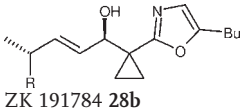
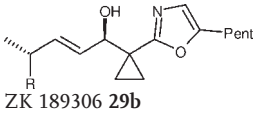
Scheme 10.4

propyl (**27b**) and butyl (**28b**) oxazole derivatives proved to be antagonists. Obviously, the length of the side-chain crucially influences the biological profile balancing between agonistic and antagonistic behavior.

The immunomodulatory activity of calcitriol derivatives can be investigated in PBMCs, giving hints for the application of such compounds in the treatment of immune disorders. All oxazole calcitriols possessing the 24β-OH situation showed strong agonistic potency in this cell system. Overall, the butyl compound **28b** was detected as the most potent analog in this assay, almost reaching the potency level of calcitriol.

As calcitriol is not suitable for any systemic therapy due to the ability to induce hypercalciuria and hypercalcemia in the therapeutically relevant dosage, research was focused on the search of dissociated calcitriols with significantly reduced calcitropic potency. At Schering the induction of hypercalcemia was routinely investigated after administration of the compounds to mice and rats.

Table 10.5

	RBA (%)	DR HL-60	DR PBMC	DR Ca
 Calcitriol	100	1.0	1.0	1
 ZK 189300 **25b**	50	56	12	≫5000
 ZK 189302 **26b**	33	110	9.4	≫300
 ZK 189304 **27b**	50	antagonist	12	≫300
 ZK 191784 **28b**	33	antagonist	1.5	≫500
 ZK 189306 **29b**	25	285	34	≫300

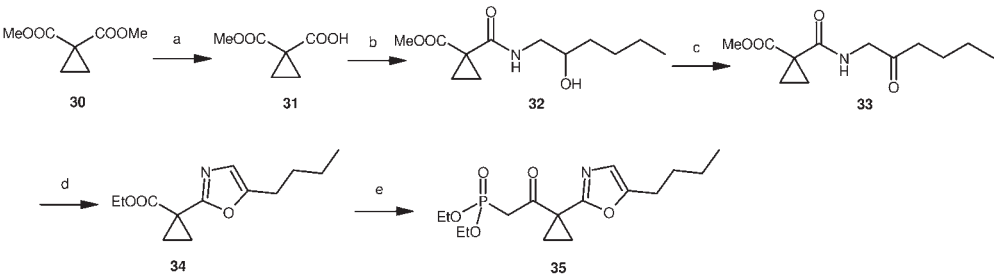
R = bissilylated vitamin D skeleton.
RBA (relative binding affinity) = IC_{50} calcitriol/IC_{50} analog × 100.
DR (dose ratio) HL-60/PBMC = IC_{50} analog/IC_{50} calcitriol.
DR (dose ratio) Ca = dose analog equipotent to 2 µg/kg calcitriol/2 µg/kg calcitriol.

In the rat model of hypercalcemia all oxazole compounds were substantially less active than calcitriol, offering the chance to establish a therapeutic window *in vivo*.

In the course of the Schering calcitriol program a variety of other heterocyclic derivatives were synthesized and investigated. Apart from the oxazole series, thiazole calcitriols were identified as interesting substructures as well.

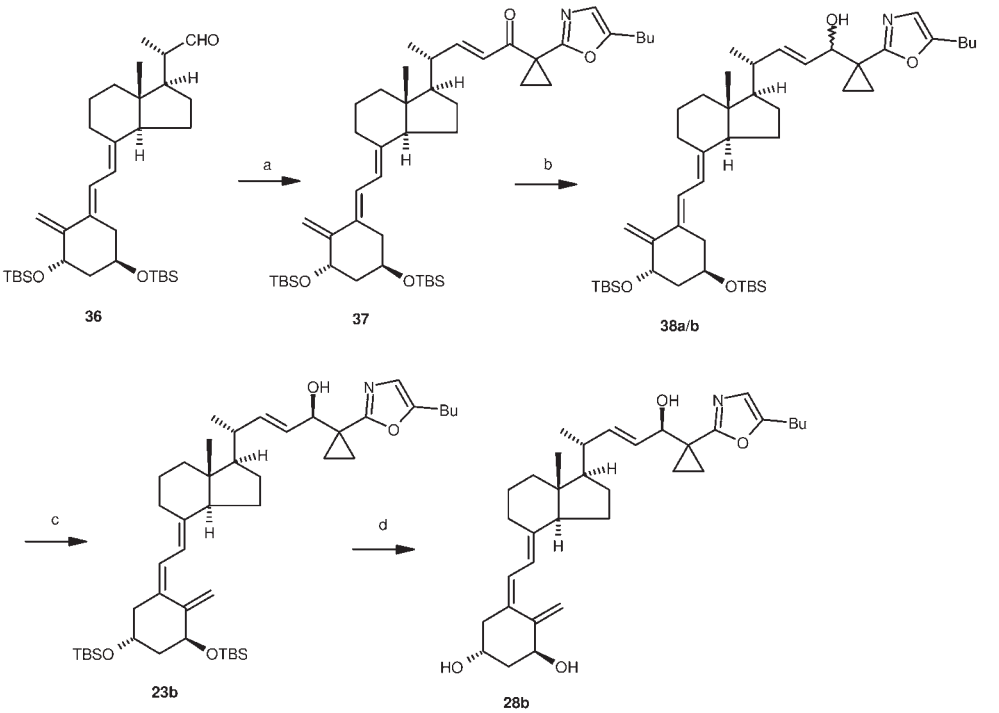
Overall, the profile of the heterocyclic calcitriols is dependent on the nature of the heterocycle, the position of substitution and the length of the side-chain. Analysis of the obtained data led to the conclusion that butyl oxazole **28b** (ZK 191784) displayed the most promising partial agonistic profile which could potentially lead to tissue-selective activity. This compound was therefore selected to be characterized more intensively *in vitro* and *in vivo*.

Since rather large amounts of the compound were required, a more efficient synthetic approach providing a higher yield of the final product was newly developed at Chemical Process Development at Schering (Schemes 10.5 and 10.6) [196].



a) NaOH, CH_3OH, RT, 24 h, 93%; b) 1. *N*-hydroxysuccinimide, DIC, CH_2Cl_2, -10 °C – RT, 4 h, 2. 1-amino-2-hexanol [9], THF, RT, 100%; c) Oxalyl chloride, DMSO, CH_2Cl_2, -40 °C, 100%; d) H_2SO_4, CH_2Cl_2, 40 °C, 14 h, 72%, e) HMDS, dimethyl methylphosphonate, THF, -50 °C, 2 h, 90%

Scheme 10.5



a) NaH, **35**, THF, 50 °C, 20 h, 90%; b) $CeCl_3$ (heptahydrate), $NaBH_4$, THF/water, 5 °C, 1 h, chromatography, 54%; c) hν, anthracene, CH_2Cl_2, 10 °C, 30 min, 100%; d) TBAF, THF, RT, 7 h, 75%

Scheme 10.6

Commercially available dimethyl cyclopropylmalonate **30** was converted to mono ester **31** by careful saponification using sodium hydroxide. Coupling with 1-amino-2-hexanol afforded the hydroxyamide **32** which was oxidized to ketone **33** under Swern conditions. Condensation to oxazole **34** was effectively achieved by utilizing a two-phase system consisting of dichloromethane and sulfuric acid. Reaction with deprotonated dimethyl methylphosphonate gave the β-ketophosphonate **35** in an excellent overall yield.

Wadsworth–Emmons reaction with the Leo vitamin D aldehyde **36** [2] led to the enone **37** which was reduced with Luche's reagent to the diastereomeric mixture of alcohols **38a/b**. The desired 24β-OH derivative **38b** could be cleanly isolated by chromatography. The 24α-OH diastereomer was reoxidized to ketone **37** and again reduced to alcohols **38a/b** in order to increase the overall amount of the valuable 24β-OH analog. Photosensitized isomerization of the triene system afforded alcohol **23b** with the natural triene geometry. Deprotection of the bissilyl ether gave access to the oxazole calcitriol **28b** (ZK 191784) in a significantly improved overall yield. The new synthetic sequence is nicely suited for the production of large batches of oxazole calcitriol **28b**.

10.6.1
Immunomodulatory Properties of Calcitriol Derivative ZK 191784

ZK 191784 is characterized by an artificial side-chain consisting of a 26,27-cyclo situation and a 5-butyloxazole unit, and is one representative of the oxazole series synthesized at Bayer Schering Pharma. This compound has been pharmacologically profiled in-depth, and shows prominent immunomodulatory activity *in vitro* and *in vivo* in rodents. Most remarkably, ZK 191784 exerts maximum therapeutic efficacy also in nonhypercalcemic doses, thus leaving a unique therapeutic window [8]. The *in vitro* profile of ZK 191784 differs to some extent from calcitriol. Binding affinity of this vitamin D analog to the VDR is 3-fold lower than for calcitriol (not shown). Also, other than calcitriol, this compound failed to induce vitamin D-induced differentiation of human promyelocytic leukemia cell line HL-60 to monocytes, but was capable of antagonizing the action of calcitriol (Figure 10.5a). In modulation of immune assays, e.g. lymphocyte proliferation (MLR with human PBMCs), cytokine release (e.g. TNF-α) or surface marker expression (e.g. MHC II) this oxazole analog is active but with lower efficacy and potency than calcitriol (Figure 10.5b for MLR, additional data published in Zügel *et al.* 2002 [8]). The *in vitro* profile of ZK 191784 can be described as partial agonistic.

Our data also point to differences between *in vivo* activity of these two compounds in a T cell-dependent allergic contact dermatitis model in mice (Figure 10.6). Only ZK 191784 showed dissociation of induction of immunosuppressive effects from hypercalcemia over a considerable dose range after oral application. In contrast, calcitriol doses that efficaciously inhibited edema induced hypercalciuria and hypercalcemia. By reducing the dose of calcitriol below 0.1 μg/kg/day in order to limit disturbance of calcium homeostasis, the immunosuppressive activity also diminished. Thus, ZK

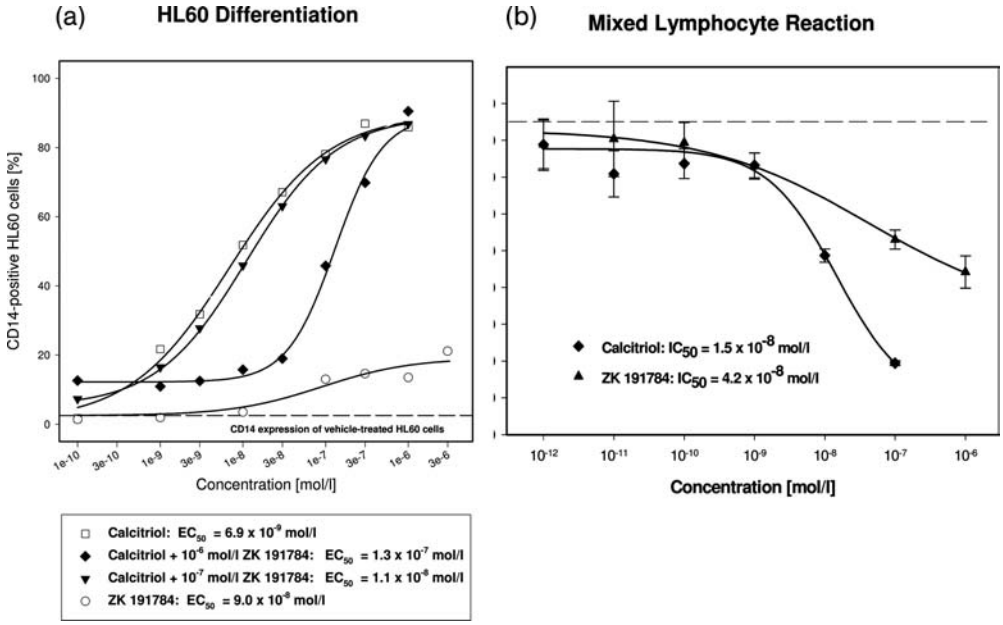


Figure 10.5 Biological activity of ZK 191784 *in vitro*. (a) Inhibition of calcitriol-induced differentiation of HL-60 cells. ZK 191784 was added to cultured HL-60 cells in two doses to increasing concentrations of calcitriol. After 5 days, CD14 expression was analyzed by fluorescence-activated cell sorting. EC_{50} = concentration of ZK 191784 that induced 50% of the cells to be $CD14^+$. (b) Inhibition of MLR. Human PBMCs were stimulated with inactivated PBMCs from an unrelated donor and cultured in the presence of increasing doses of calcitriol or ZK 191784. On day 5 cultures were labeled with [methyl-^{3}H]thymidine for 6-h [^{3}H] thymidine incorporation measured by liquid scintillation counts. (Adapted from Ref. [8] with permission from Macmillan Publishers.)

191784 represents a novel calcitriol analog with clear dissociation of immunosuppressive and hypercalcemic activity and a reasonable therapeutic window.

Although the relevance of the HL-60 differentiation by vitamin D compounds for prediction of *in vivo* activity is unclear, the antagonist effects of ZK 191784 on HL-60 cells may reflect a different tissue selectivity of the partial agonist ZK 191784 compared to calcitriol. The distinct structure of VDRs ligated with agonist and antagonist and the interaction with different coactivators or corepressors in each tissue may be involved in the tissue-selective action of a partial agonist. Although experimental proof is missing, such molecular mechanisms may explain the dissociation of immunosuppressive activity and hypercalcemia of ZK 191784 and other derivatives of the oxazole series.

Our data demonstrate that specific side-chain modifications can yield novel vitamin D derivatives with unique properties. ZK 191784 is a first representative of a novel class of immunosuppressive vitamin D analogs with a considerable therapeutic window. Vitamin D compounds with a dissociated profile may represent

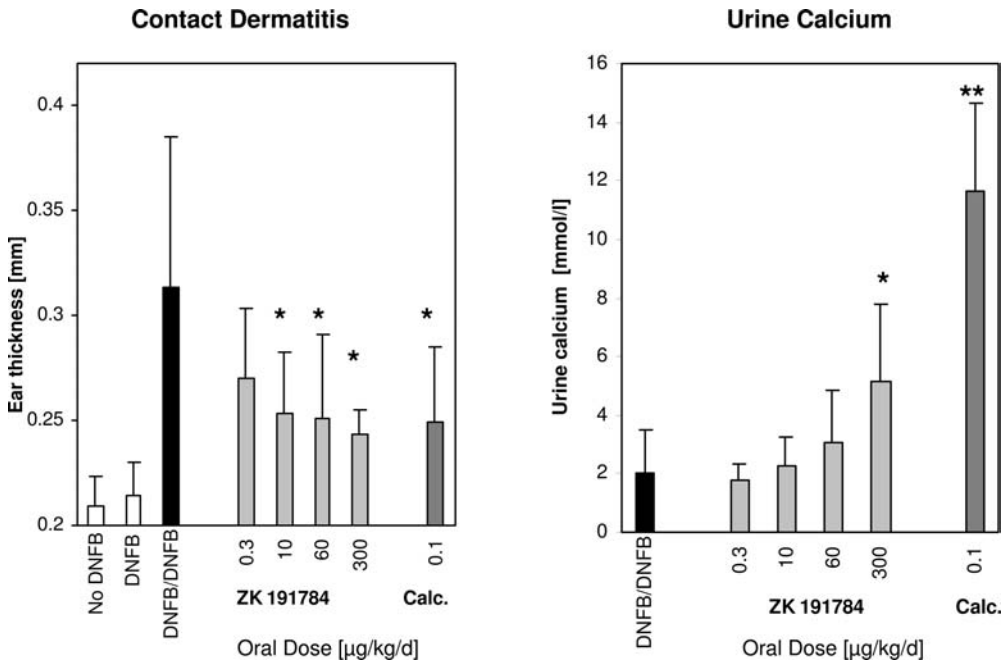


Figure 10.6 Biological activity of ZK 191784 *in vivo*. Inhibition of dinitrofluorobenzene (DNFB)-induced contact dermatitis in mice and effects on calcium homeostasis. NMRI mice were topically sensitized with DNFB (or vehicle, as controls) at the shaved abdomen. On day 5 all mice were challenged with DNFB on both sides of one ear. Mice were treated with compounds orally once daily for 5 days around the sensitization phase. On day 6 ear thickness was determined. Urine samples were collected 24 h after the fourth treatment and analyzed for calcium levels by flame photometry. Statistical significance $^{*}P < 0.05$, $^{**}P < 0.001$. (Adapted from Ref. [8] with permission from Macmillan Publishers.)

promising therapeutic agents for systemic treatment of chronic diseases such as psoriasis, RA, IBD or graft rejection.

10.7 Conclusions

The effects of $1,25(OH)_2D_3$ are extremely pleiotropic and include effects on the immune system. $1,25(OH)_2D_3$ seems to exert its immunomodulatory effects primarily on APCs. It has a clear impact on the generation and differentiation of DCs, their activation, maturation and subsequent migration into the lymph nodes. T_{regs} may selectively be stimulated by interaction with $1,25(OH)_2D_3$-induced immature DCs or $1,25(OH)_2D_3$ may also convert mature DCs into regulatory DCs with the ability to tolerize T cells (e.g. via secretion of IL-10). Thus, the T cell can be seen as a second line target of $1,25(OH)_2D_3$ strongly guided by the specific state of the APCs.

Work of the last two decades has accumulated evidence that $1,25(OH)_2D_3$ is effective (in prevention and/or therapy) not only in animal models of inflammation, but also in human inflammatory diseases. Epidemiologists have gathered data indicating that sufficient supply with vitamin D may significantly lower the risk for development of autoimmune diseases, such as MS, RA and diabetes. In this line, clinical trials demonstrated that established RA and psoriasis are both responsive to treatment with $1,25(OH)_2D_3$. The major obstacle for systemic utilization of $1,25(OH)_2D_3$ in the clinic – its potent calcemic activity (with the risk of calcium mobilization, bone resorption, etc.) – is not entirely solved yet. Striving to overcome this problem, industrial and academic investigators are generating an $1,25(OH)_2D_3$ analogs with dissociated calcemic and immunomodulatory properties. Whereas some of these compounds show promising results in *in vitro* investigations and animal experiments, the concept of dissociation needs to be proven in man. Identification of such compounds will eventually lead to novel, highly effective immunomodulatory drugs.

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11
Peroxisome Proliferator-Activated Receptor γ Modulation for the Treatment of Type 2 Diabetes

Alan M. Warshawsky and Anne Reifel Miller

11.1
Introduction

11.1.1
The Nuclear Receptor Superfamily Includes Peroxisome Proliferator-Activated Receptors

11.1.1.1 Nuclear Receptor Family Members

The nuclear receptor superfamily is composed of approximately 65 distinct genes identified throughout the animal kingdom, including 48 receptors in man that serve to maintain normal physiological functions. Included in the superfamily are the receptors for steroids, lipophilic vitamins, bile acids, retinoids and various fatty acids [1]. The receptors are commonly categorized into subfamilies based on sequence homology (Table 11.1). The seven subfamilies include receptors with homology to the thyroid hormone receptor (TR), retinoid X receptor (RXR), estrogen receptor (ER), nerve growth factor, steroidogenic factor and germ cell nuclear factor. The final subfamily includes four receptors with no sequence homology to any of the other receptors and thus is termed the miscellaneous subfamily [2].

Members of the superfamily can also be classified based on characteristics of ligand binding. These subgroups include the classic hormone receptors, orphan receptors and sensor or metabolic receptors. The classic hormone receptors include the glucocorticoid, estrogen, thyroid, retinoic acid and vitamin D receptors. In general, these receptors bind specific, endogenous ligands with high affinity. Ligands have not been identified to date for some receptors such as the apolipoprotein A-I regulatory protein-1 and chicken ovalbumin upstream promoter transcription factor; thus, these receptors are referred to as orphans. Peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXR), farnesol X receptor (FXR), liver receptor homolog-1 and RXR belong to the subgroup known as the sensor receptors. As a result of their role in maintaining glucose and lipid homeostasis, these receptors are also known as 'metabolic' nuclear receptors. In contrast to the classic and orphan receptors, the sensor/metabolic receptors bind with lower affinity to a broad range of

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

Table 11.1 Nuclear receptor classification based on sequence homology.

Subfamily 1: thyroid hormone receptor homology
thyroid hormone receptor (TR) α, β
retinoic acid receptor (RAR) α, β, γ
peroxisome proliferator-activated receptor (PPAR) α, β/δ, γ
rev-erb α, β
retinoid-related orphan receptor (ROR) α, β, γ
liver X receptor (LXR) α, β
farnesoid X receptor (FXR)
vitamin D receptor (VDR)
pregnane X receptor (PXR)
constitutive androstane receptor (CAR)
Subfamily 2: retinoid X receptor homology
hepatocyte nuclear factor-4 (HNF-4) α, γ
retinoid X receptor (RXR) α, β, γ
testicular receptor (TR2, TR4)
human homolog of the *Drosophila* tailless gene (TLX)
photoreceptor-specific nuclear receptor (PNR)
chicken ovalbumin upstream promoter-transcription factor (COUP-TF) 1, 2
ERBA-related 2 (EAR2)
Subfamily 3: estrogen receptor homology
estrogen receptor (ER) α, β
estrogen related receptor (ERR) α, β, γ
glucocorticoid receptor (GR)
mineralocorticoid receptor (MR)
progesterone receptor (PR)
androgen receptor (AR)
Subfamily 4: nerve growth factor homology
nerve growth factor IB (NGFIB)
nuclear receptor related-1 (NURR1)
neuron-derived orphan receptor-1 (NOR1)
Subfamily 5: steroidogenic factor homology
steroidogenic factor-1 (SF1)
liver receptor homolog-1 (LRH1)
Subfamily 6: germ cell nuclear factor homology
germ cell nuclear factor (GCN1)
Subfamily 0: miscellaneous
dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (NR0B1)
short heterodimer partner (SHP)
nuclear receptors with two DNA-binding domains (2DBD-NR)

physiological ligands encompassing compounds from dietary origin. The molecules that bind and activate the PPARs are most likely fatty acid-derived metabolic substrates and intermediates that, by binding and modulating receptor activity and target gene transcription, provide a mechanism for rapid response to changes in metabolic status. Alterations in the levels of fatty acids or fatty acid-derived molecules, resulting from changes in food availability (fasting/feeding/exercise) or pathophysiological

conditions [chronic inflammation, insulin resistance, type 2 diabetes (T2D), atherosclerosis or cancer], signal the appropriate PPAR isotypes to mediate lipid catabolism or storage.

11.1.1.2 Nuclear Receptor Structure

Although members of the nuclear receptor superfamily vary distinctly in their functions, they share a common structure composed of five conserved regions or domains (A–E): the N-terminal A/B domain, a medial DNA-binding domain (DBD) and hinge region, and the C-terminal ligand-binding domain (LDB) as shown in Figure 11.1 [3]. The N-terminal A/B domain is the least conserved among members of the superfamily, with the length of this region varying significantly between receptors. The A/B region contains a weak ligand-independent transcriptional activation function (AF-1) and is often a site for posttranslational modification that can dramatically affect receptor activity. In contrast, the C region is the most highly conserved and contains the DBD with its two zinc finger motifs, the hallmark characteristic of nuclear receptors. The D region functions as a hinge, allowing the more conserved and structured C and E domains to swivel slightly to accommodate multiple conformations. Region E contains the LBD. The ligand-dependent activation function-2 (AF-2) and the receptor dimerization interface are embedded within this region. Binding of ligands to the receptor induces a conformational change within the LBD that initiates a series of events within the receptor resulting in transcriptional activation of specific target genes.

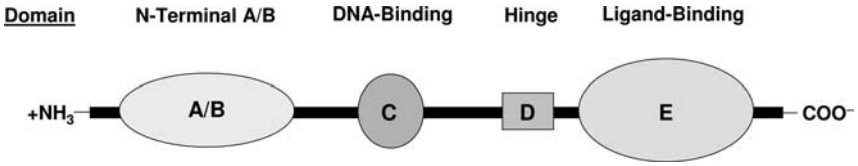

Figure 11.1 The five conserved domains (A–E) that compose the protein structure for nearly all members of the nuclear hormone receptor superfamily.

11.1.2 PPARs

11.1.2.1 PPAR Isotypes

The term PPAR encompasses three distinct receptors: PPARα, PPARδ and PPARγ [2]. Each isotype is encoded by a different gene [4–6] and has been identified in a variety of species [7–9]. These ligand-activated receptors regulate a number of genes involved in nutrient metabolism and energy homeostasis, and thus have served as drug targets for the treatment of metabolic diseases [10–15]. In early studies, the tissue-specific expression patterns of the PPAR isotypes suggested that the receptors had distinct physiological roles. These findings were further supported by the unique phenotypes generated when each receptor was specifically disrupted in mouse gene knockout models.

PPARα expression is seen predominantly in tissues with high rates of fatty acid catabolism, including the liver, heart, kidney, large intestine and skeletal muscle, and in cells of atherosclerotic lesions. Proliferation of peroxisomes in rodents was the first physiological function associated with PPAR activation and thus was used as the name for this newly identified class of nuclear receptors. Over time, this activity was attributed to specific activation of PPARα, and many studies have now confirmed the essential role of PPARα in fatty acid catabolism via peroxisomal and mitochondrial β-oxidation. The roles of PPARα in hepatic lipid metabolism, lipoprotein metabolism and atherosclerosis have been thoroughly described elsewhere [16]. Both endogenous and synthetic ligands to PPARα are known. The naturally occurring ligands are mono- and polyunsaturated fatty acids and various fatty acid-derived eicosanoids. Two synthetic ligands, known commonly as fibrates, are currently marketed for the treatment of dyslipidema: fenofibrate (Tricor®) and gemfibrozil (Lopid®). The primary effects of these molecules are to lower triglyceride (TG) and raise high-density lipoprotein cholesterol (HDL-C). Fibrates may also moderately decrease low-density lipoprotein cholesterol (LDL-C). Several clinical trials have demonstrated that fibrates slow the progression of atherosclerosis, and reduce the risk of cardiovascular morbidity and mortality, particularly in patients with insulin resistance [16].

Expression of PPARδ is ubiquitous and often at levels higher than that seen for PPARα and PPARγ. Although PPARδ has been seen in all tissues examined, the highest levels are observed in the digestive tract and placenta of humans. Due to the broad expression pattern of this PPAR isotype, the physiological role of PPARδ was originally thought to be associated with general housekeeping functions. However, once receptor-specific deficient mice were available to study, it became apparent that PPARδ plays a large role in normal adipose development and lipid homeostasis. In the PPARδ knockout mice, a combination of decreases in both the number and size of adipocytes leads to smaller abdominal and interscapular brown fat depots and a thinner subcutaneous fat layer compared to wild-type mice [17]. As with PPARα, both endogenous and synthetic ligands to PPARδ are known. The naturally occurring ligands are thought to include fatty acids and eicosanoids. Results from recent studies suggest that fatty acids derived from very-LDL-C (VLDL-C) particles activate PPARδ target genes in a receptor-dependent manner. Although no synthetic PPARδ-specific ligands are marketed at present, one molecule is currently in phase II clinical trials for the treatment of dyslipidemia.

PPARγ is most abundantly expressed in white adipose tissue, and at lower levels in skeletal muscle, heart and liver. Homozygous deletion of the PPARγ gene results in embryonic lethality due to defects in the placenta leading to cardiac anomalies. When the placenta defect was corrected by genetic placental reconstitution, only one PPARγ$^{-/-}$ pup survived to term and demonstrated marked abnormalities in lipid deposition and hemorrhages leading to death during the first week of life. Consistent with the tissue expression of PPARγ, the PPARγ-null mouse indicated that the receptor plays essential roles in maintaining normal adipose tissue development [18]. Interestingly, PPARγ has been implicated in both tumor suppression and tumor promotion activities, making the development of pharmaceutical agents to this receptor especially complicated. Unsaturated fatty acids and their derivatives such

as prostaglandin J2 (15-deoxy-$\Delta^{12,14}$-PGJ2) bind and activate PPARγ *in vitro*; however, it is not clear if a bioactive, naturally occurring ligand actually exists *in vivo*. Numerous synthetic ligands to PPARγ have been identified, and two molecules are currently marketed as agents for the treatment of T2D: rosiglitazone (Avandia®) and pioglitazone (Actos®).

11.1.2.2 **PPAR Response Elements**

PPARα, PPARδ and PPARγ each function as a heterodimer with RXR. Research to date suggests that heterodimerization is not dependent on ligand binding to the PPARs. The PPAR–RXR heterodimer initiates transcription of specific target genes through binding to PPAR response elements (PPREs). The PPREs are generally located upstream from the transcriptional start site of a gene and are composed of a direct repeat of the element half site (AGGTCA) with an intervening single nucleotide (n) as shown in Figure 11.2. The PPRE for the PPARs is often referred to as a direct repeat 1 (DR-1) [19, 20]. There is a distinct 'polarity' of the PPAR–RXR heterodimer, with the PPAR occupying the 5′ position and RXR the 3′ position when bound to the DR-1 PPRE.

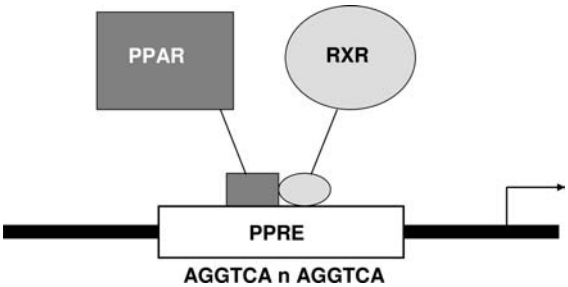

Figure 11.2 PPREs serve as the binding site for the PPAR–RXR heterodimer and are composed of a direct repeat of two element half sites (AGGTCA) spaced by a single nucleotide (n). Binding of the heterodimer to the PPRE initiates transcription of specific target genes.

11.1.2.3 **PPAR Cofactors**

Initiation of transcription for a specific gene requires the coordination of multiple signals through a host of proteins that converge at the gene's promoter. PPAR cofactors are individual proteins that function as either activators or repressors of transcription (Table 11.2). In the absence of a PPAR or RXR ligand, corepressor proteins are bound to the unliganded PPAR–RXR heterodimer and possess enzyme activities that condense DNA chromatin, making the promoter inaccessible to the transcriptional machinery. In contrast, the presence of a ligand promotes the dissociation of corepressor proteins from the PPAR–RXR heterodimer and favors association with coactivator proteins. Coactivator proteins contain enzyme activities (acetylation and methylation) that relax DNA chromatin, making the promoter accessible to binding by the transcriptional machinery leading to the initiation of transcription and

Table 11.2 A partial list of nuclear receptor cofactors.

Coactivators	
steroid receptor coactivator-1, -2 and -3	SRC-1, -2 and -3
transcriptional intermediary factor-2	TIF-2
nuclear receptor coactivator	ACTR
PPARγ, coactivator-1	PGC-1
cAMP response element binding protein (CREB) binding protein	CBP
thyroid receptor accessory proteins	TRAP
vitamin D receptor interacting proteins	DRIP
Corepressors	
nuclear receptor corepressor	NCoR
silencing mediator for retinoid and thyroid hormone receptors	SMART
small unique nuclear receptor corepressor	SUNCoR

expression of the specific gene. Interestingly, PPAR ligands can recruit different cofactors (both repressors and activators) to the promoter of a specific gene, leading to unique and diverse transcriptional profiles. In turn, these unique profiles can contribute to subtle pharmacological differences that result in a successful therapeutic product through the ability to achieve PPAR modulation.

11.1.3 T2D: Prevalence and Pathogenesis

11.1.3.1 Prevalence

Obesity is increasing at epidemic and some say pandemic rates throughout the world, and it contributes to the equally increasing risk of developing T2D. Currently, the number of individuals worldwide with diabetes exceeds that with AIDS, making diabetes one of the most serious health care problems of the developing world. Diabetes currently affects more that 246 million people and this number is expected to increase to 380 million by 2025 according to figures released from the International Diabetes Federation. Each year, 7 million new cases of diabetes are identified. Over 3 million deaths can be attributed directly to diabetes yearly, making diabetes the fourth leading cause of death by disease globally [21]. Diabetes is an expensive disease to treat. Worldwide spending on the care of individuals with diabetes, including direct and indirect costs, is close to $286 billion [22].

11.1.3.2 Pathogenesis

T2D is a serious disease which accounts for greater than 90% of all individuals with diabetes. Type 1 diabetes (T1D) is most frequently diagnosed in children as an autoimmune disease with nearly complete destruction of islet β cells – the insulin-producing cells of the pancreas. Without insulin, these children experience massive elevations in plasma glucose levels; however, they are deprived of the use of glucose as a cellular fuel for normal metabolic functions, since insulin provides the biochemical

signal for transport of glucose across the cell membrane. In contrast, T2D is often referred to as adult-onset diabetes. In past generations, T2D was frequently diagnosed in individuals older than 40 years of age that were often obese with an accompanying sedentary lifestyle. Unfortunately today as obesity and sedentary lifestyles become more prevalent, T2D is seen in young adults and even children [23].

The causes of T2D are 2-fold: insulin resistance and progressive failure of pancreatic β cells to produce adequate amounts of insulin to lower circulating glucose levels. Insulin resistance develops when normal insulin levels are unable to dispose of circulating plasma glucose into target tissues, including skeletal muscle and adipose tissue. As the pancreas produces more insulin to compensate for the excessively high glucose levels due to insulin resistance, the pancreatic β cells eventually become exhausted and no additional insulin is available for secretion. Over time, the pancreatic β cells completely fail and a person with T2D becomes similar to one with T1D. High levels of circulating glucose is the hallmark of diabetes, and can eventually lead to serious complications such as heart disease and strokes, high blood pressure, blindness, kidney and nerve damage, and infections and gum disease. Therefore, it is important to control and treat T2D as early as possible with exercise, a proper diet, oral antidiabetic therapies and eventually with insulin [24].

11.2
Currently Marketed PPARγ Ligands

11.2.1
Actos® (pioglitazone) and Avandia® (rosiglitazone)

11.2.1.1 Historical Overview: Identification and Development

During the 1980s and 1990s, scientists in search of new antidiabetic agents discovered that molecules from the thiazolidinedione (TZD) class lowered glucose levels when administrated to diabetic rodents [25, 26]. These molecules were optimized to improve upon their potency and efficacy through labor intensive and expensive iterations involving compound syntheses followed by extensive testing in rodent models. From these studies, three TZDs were identified and developed as human therapies. Troglitazone (Rezulin®), the first in the TZD class to be launched in the US in 1997, was subsequently withdrawn due to hepatotoxicity. Two other TZDs, rosiglitazone (Avandia®) and pioglitazone (Actos®), have been approved by the US Food and Drug Administration (FDA) as first-line therapies for T2D [26].

As the TZDs were in development for the treatment of T2D, the first PPAR gene was cloned from a murine library [4]. Within a few years of this initial discovery, a *Xenopus laevis* ortholog of PPAR, subsequently termed PPARα and two highly homologous genes, termed PPARβ (also known as PPARδ in mammalian nomenclature) and PPARγ, were cloned [27]. Today, orthologs of all three PPARs have been identified and described in a variety of species [28–30]. The three mammalian PPAR isotypes, which range in size from 441 to 507 amino acids, share similar structural characteristics and mechanisms of action [31, 32] as described above.

Once the association was made that binding of the TZDs to PPARγ resulted in lowered glucose levels in rodent models of T2D and eventually to antidiabetic activity in humans, assays were developed to define the affinity of a ligand for the receptor and to measure the resulting transcriptional activity. These assays provided the tools to conduct high-throughput screens to identify compounds with greater affinity and differential activity at the receptor. Although numerous assays were developed using a variety of formats, the PPAR competitive binding and PPAR cotransfection (CTF) assays were the most influential for the identification and development of future PPAR ligands.

The PPAR competitive binding assay determines the affinity of a ligand for the receptor. Each assay is developed to be isotype specific by using purified PPAR isotype and RXR proteins along with a known high-affinity radiolabeled ligand. Test molecules displace the labeled ligand, and the affinity is reported as an IC_{50} value (the concentration in which 50% of the labeled molecule is displaced) or as a K_i value (the inhibition constant).

The cell-based CTF assay reflects the functional activity that results when a ligand binds to the receptor. The CTF assay is performed in a cell line that has very low levels of the specific PPAR being examined. The specific PPAR isotype and RXR are reconstituted in the cell line using a transfection procedure in which the genes encoding the receptors are transferred into the cells on plasmid DNA carriers. In a parallel transfection procedure, a reporter gene controlled by a PPRE is introduced into the cell. The reporter gene produces a signal when the PPAR–RXR heterodimer is activated by a ligand. The functional activity of a test molecule is characterized by an EC_{50} value (the concentration that produces 50% of the maximum signal) and a percent efficacy (maximum signal compared to that achieved with a control molecule).

11.2.1.2 **Preclinical and Clinical Findings**

Today, pioglitazone and rosiglitazone are commercially available as antidiabetic agents (Figure 11.3). Both pioglitazone and rosiglitazone are potent and efficacious PPARγ agonists with EC_{50} values of 0.58 and 0.043 μM, respectively [33]. Both molecules have similar clinical efficacies in general, demonstrating improvements in insulin sensitivity and an ability to lower fasting plasma glucose by approximately 50–60 mg/dl relative to placebo [34–38]. These changes in fasting plasma glucose are associated with an average 1–2% reduction in glycosylated hemoglobin A1c (HbA1c). In addition to lowering blood glucose, both drugs exert beneficial effects on cardiovascular parameters; lowering blood pressure, rectifying diabetic dyslipidemia, improving fibrinolysis and decreasing the thickness of the carotid artery intima-media [39, 40]. Interestingly, these drugs exhibit different effects on lipid profiles in individuals with T2D suffering from dyslipidemia. Pioglitazone demonstrates a superior impact over rosiglitazone in lowering TG, elevating HDL-C, and improving LDL-C particle size and concentration [41]. Unfortunately, the therapeutic efficacy of the currently marketed TZDs is associated with several adverse effects, including weight gain, edema and plasma volume expansion [42–47]. Congestive heart failure along with pulmonary and macular edemas has been recognized as significant

adverse effects of TZD therapy [48, 49]. Although the mechanism underlying the basis of TZD-induced weight gain remains unclear, several studies suggest that TZD therapy may be associated with an increase in subcutaneous adipose tissue and a concomitant decrease in visceral fat. The improved glycemic control in conjunction with altered fat distribution could explain the observed weight gain [50–52]. The edema-associated weight gain following TZD therapy has been attributed to the ability of these drugs to alter salt balance in the renal collecting duct via activation of PPARγ [53, 54]. Recently, both rosiglitazone and pioglitazone treatment have been associated with decreases in bone formation and bone density in postmenopausal women [55, 56]. The undesirable adverse effects associated with the first-generation TZDs have prompted and intensified efforts in the search for better PPARγ ligands to treat T2D.

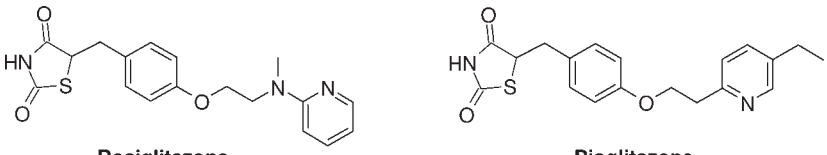

Figure 11.3 Marketed PPARγ ligands.

11.3
Approaches to Improve PPARγ Activity

11.3.1
Structural Considerations

Structural studies with the PPARγ LBD have led to an understanding of the binding affinity, functional activity and PPAR isotype selectivity demonstrated by a ligand. Similar to other nuclear receptors (RAR, TR, ER among others), the PPARγ LBD is comprised of 13 α-helices and four small β-strands arranged in a triple layer or 'sandwich' structure (Figure 11.4a) [57, 58]. In contrast to other nuclear receptors (i) the PPARγ ligand binding site is much larger (volume around 1300 A^3), (ii) a solvent-accessible opening between helix 3 (H3) and the β-strand region allows ligand entry without perturbing the overall receptor conformation, and (iii) the C-terminal H12, known as the activating H2 (AF-2), is positioned close to the LBD rather than away from it in the apo-receptor; only subtle conformational changes in AF-2 are noted upon ligand binding.

The T-shaped binding site spans the region from AF-2 to the β-strands and branches in opposite directions roughly parallel to H3. This cavity is primarily hydrophobic in nature except for the region near AF-2. In this polar area, key hydrogen bonds are established between the acidic headpieces of full agonists and His323 (H5), His449 (H11), Tyr473 (AF-2) and Ser289 (H3) (Figure 11.4b). These interactions stabilize a LBD conformation capable of binding coactivators necessary

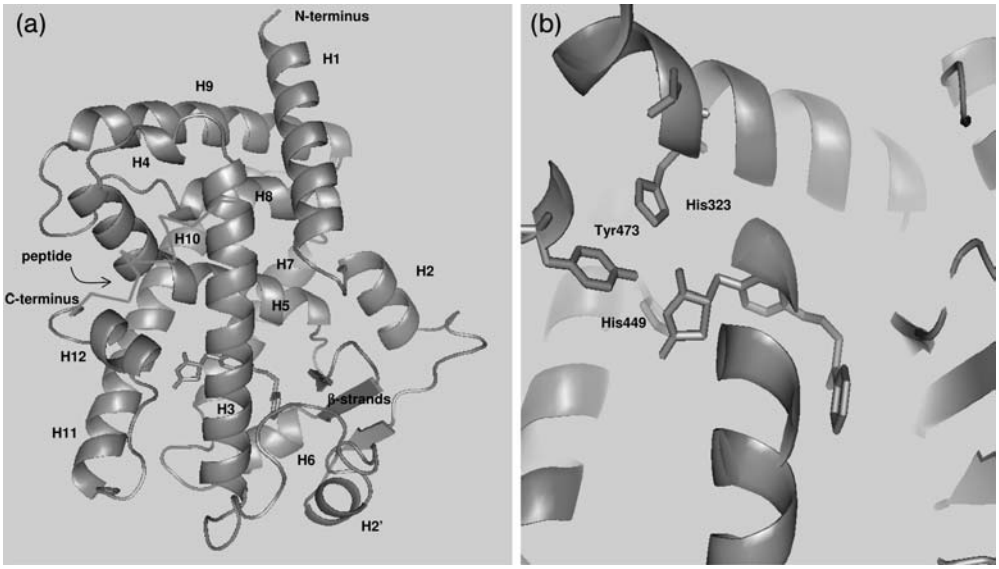

Figure 11.4 (a) Ternary complex of the PPARγ ligand binding domain, rosiglitazone, and a steroid receptor coactivating factor-1 (SRC-1) peptide fragment (PDB code 1ZGY). (b) Extended region showing the Y-shaped conformation of rosiglitazone around H3, and the His and Tyr regions involved in key hydrogen bonding to the TZD functional group.

for transcriptional activation. Polar residues on AF-2 (Glu471) and H3 (Lys301) form a 'charge clamp' that guides cofactor orientation into the newly formed hydrophobic cleft on the surface of the LBD. The spacer ring, linker and tailpiece portions of the ligand make important hydrophobic contacts with the rest of the binding site to impart potency. Additional interactions between the benzophenone substituent adjacent to the carboxylic acid of farglitazar and a pocket formed by H3, H7 and H11 lead to even greater binding affinity (Figure 11.5) [59].

Alternate ligand–receptor binding modes afford different functional activities. Both GW0072 and PA-082 bind in the LBD parallel to H3 near the ligand entry site and make no contacts with AF-2 [60, 61]. These compounds behave as partial agonists and display coactivator recruitment profiles distinct from full agonists. Hydrogen bonding in the AF-2 region is not sufficient to impart full agonism. Isoxazolone 7 forms hydrogen bonds with AF-2 and interacts with the 'benzophenone' pocket [62]. However, this ligand does not extend beyond H3 and was characterized as a partial agonist. While partial agonist pyrazole 1 occupies the ligand-binding site similarly to isoxazolone 7, all interactions are hydrophobic in nature [63]. No hydrogen bonding within the AF-2 domain is noted. Finally, the route to partial agonism is not entirely straightforward. A series of benzoylaminobenzoic acids have been reported to be strong PPARγ agonists even though they do not interact directly with AF-2 [64]. From this series, BVT13 exhibits a binding mode analogous to GW0072 and PA-082.

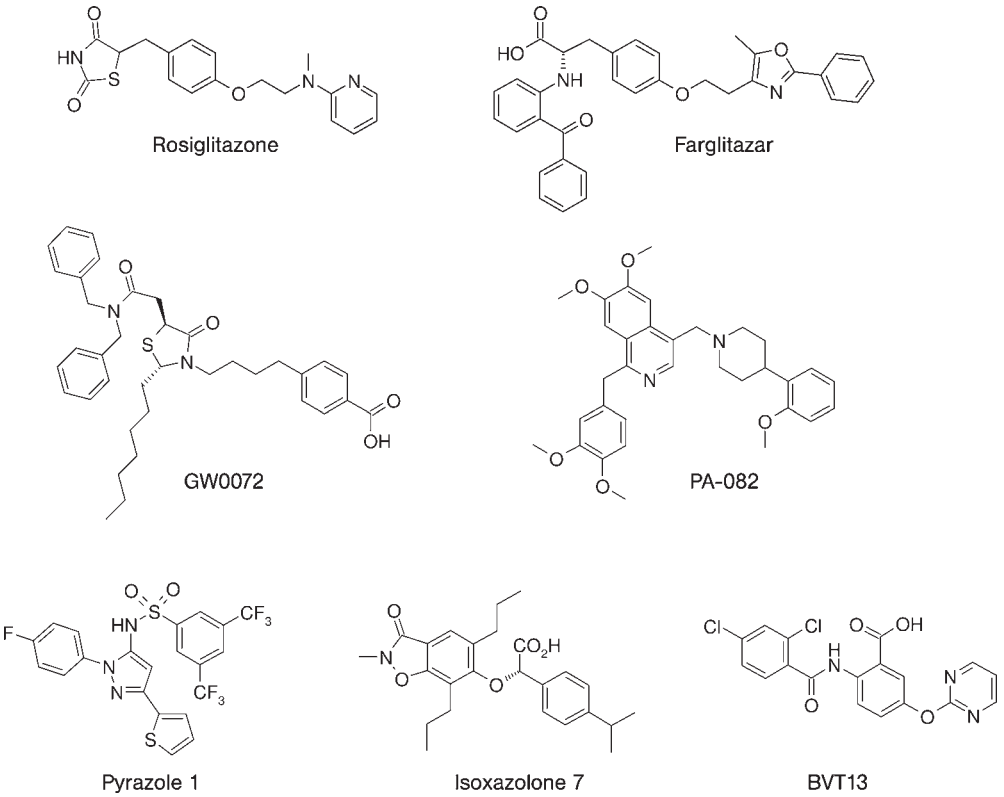

Figure 11.5 Compounds cocrystallized with the PPARγ LDB.

Data from numerous structure–activity relationship (SAR) studies point to the success of being able to develop ligands with various PPAR isotype selectivity profiles. Although highly homologous, the PPAR LBDs exhibit specific differences that convey isotype selectivity [65]. For example, the PPARδ binding site near AF-2 is narrower than PPARγ and PPARα. In this region, hydrogen bonding to His323 in PPARγ is less sterically demanding than to the corresponding Tyr314 residue in PPARα. Comparable influences have been noted for interactions with the tailpiece portions of ligands as well. Thus, the repositioning of ligand or receptor to accommodate the changing steric demands between PPAR isotypes has been a major contributor to PPAR isotype selectivity.

11.3.2 Profile Alterations

11.3.2.1 PPARα/γ Dual Agonists

LSN862 is a novel PPARα/γ dual agonist with a unique *in vitro* profile that leads to improvements on glucose and lipid levels in rodent models of T2D and dyslipidemia (Figure 11.6). LSN862 is a high-affinity PPARγ partial agonist ($K_i = 7.0$ nM,

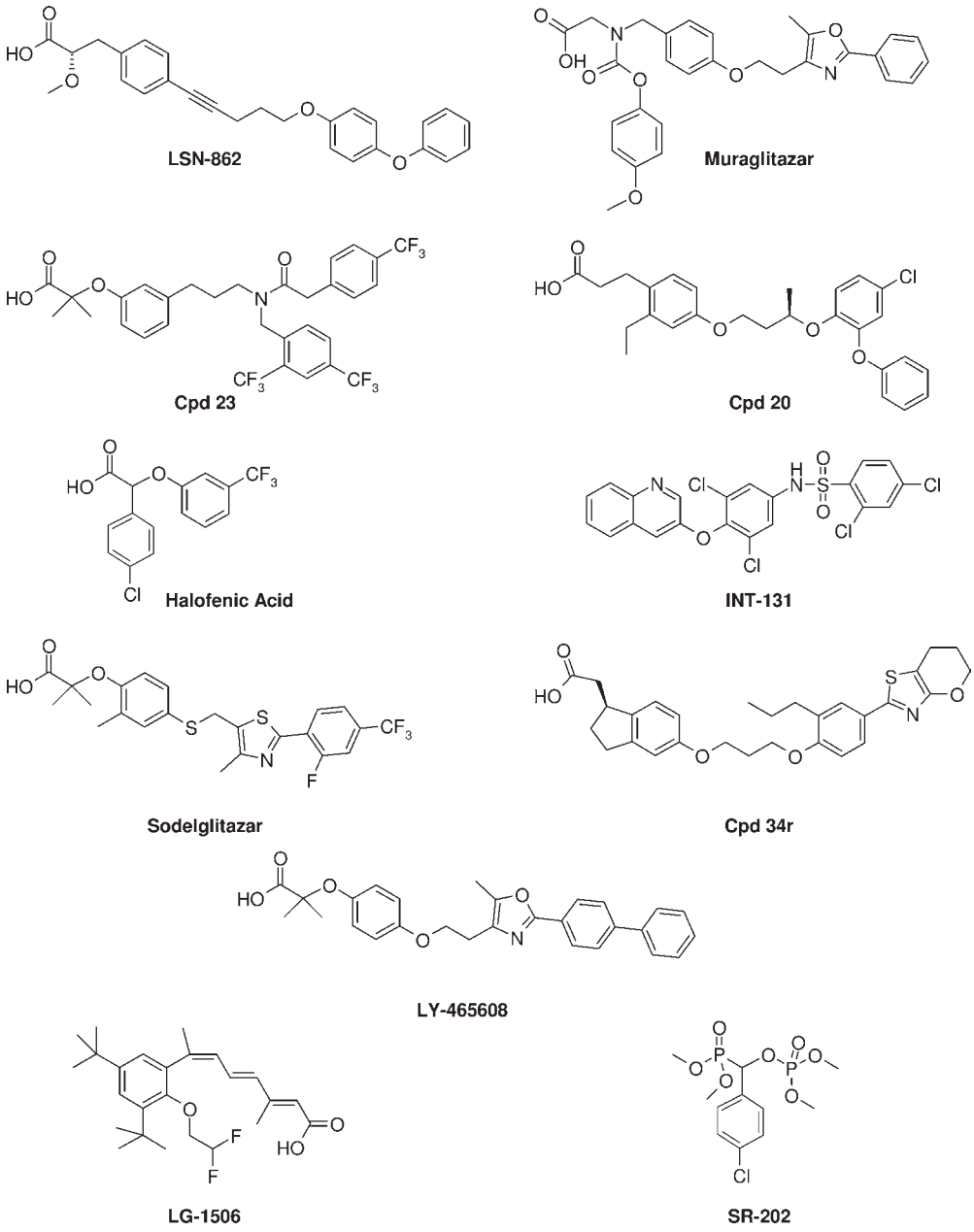

Figure 11.6 Representative ligands for the PPAR–RXR heterodimer.

$EC_{50} = 239$ nM; 77% efficacy) with relatively less but significant PPARα agonist activity ($K_i = 770$ nM; $EC_{50} = 2622$ nM; 35% efficacy). When administered to Zucker Diabetic Fatty rats, LSN862 produces significant glucose and triglyceride lowering. In *db*/*db* mice, another rodent model of T2D, LSN862 demonstrates statistically better

antidiabetic efficacy with an equivalent side-effect profile compared to rosiglitazone. In a humanized dyslipidemic transgenic mouse, LSN862 reduces VLDL-C while rosiglitazone increases VLDL-C. Both LSN862 and rosiglitazone produce maximal increases in HDL-C. These findings illustrate the therapeutic advantages on glucose and lipid levels of a PPARα/γ dual agonist and demonstrate that PPARγ full agonist activity is not necessary to achieve potent and efficacious insulin sensitizing benefits [66].

Muraglitazar (Pargluva®; BMS-298585) is another PPARα/γ dual agonist that improves glucose and lipid levels in preclinical and clinical studies. Muraglitazar is a non-TZD carboxylic acid-based derivative with a slightly PPARγ favored *in vitro* profile (PPARγ $EC_{50} = 110$ nM; PPARα $EC_{50} = 320$ nM) [67]. In preclinical studies muraglitazar improves insulin resistance, mitigates the loss of β cell function, and reduces TG, free fatty acids and total cholesterol [68]. Consistent with these preclinical findings, muraglitazar administration to patients with T2D led to significant reductions in HbA1c and TG with increases in HDL-C [69].

11.3.2.2 PPARγ/δ Dual Agonists

Consistent with the rationale for developing PPARα/γ dual agonists, combining PPARδ and PPARγ agonist activity could result in a therapy that has the expected benefits on glucose levels with additional positive effects on lipid abnormalities commonly seen in individuals with T2D and the metabolic syndrome.

Compound **23** is the lead molecule in a series of PPARγ/δ dual agonists that is dominant towards PPARγ. EC_{50} values from CTF assays are 4, 19 and 620 nM for human PPARγ, PPARδ and PPARα, respectively [70]. When administered orally to diabetic rats, this compound reduces plasma glucose levels and serum TG by 47% and 51%, respectively, and increases HDL-C by 24%.

Another effort identified phenylpropionic acid (**20**) as a potent and selective PPARγ/δ dual agonist (IC_{50}s = 19, 4 and 6932 nM and EC_{50}s = 102, 6 and 2800 nM for human PPARγ, PPARδ and PPARα, respectively) [71]. In rodent studies, this compound improves insulin sensitivity and reduces glucose levels with less weight gain than PPARγ full agonist rosiglitazone. The improved side-effect profile is most likely due to the added PPARδ activity of this dual agonist.

11.3.2.3 Selective PPARγ Modulators and Partial Agonists

INT-131 (formally AMG-131 and T-131) is a selective PPARγ modulator (SPPARM) being developed for the treatment of T2D [72]. Preclinical data presented at scientific conferences reveals that INT-131 has a 20-fold higher affinity than rosiglitazone for PPARγ and displays unique properties in functional assays. INT-131 acts as a partial agonist, recruiting the coactivator DRIP205 and promoting a strong association with the nuclear receptor corepressor NCoR. In addition, a 14-day oral treatment with INT-131 in diabetic rats resulted in a dose-dependent increase in insulin sensitivity. In this model, INT-131 was 3- to 10-fold more potent than rosiglitazone in reducing plasma insulin, glucose, TG, free fatty acids and glucose-induced insulin secretion. Furthermore, INT-131 exerted minimal effects on edema and weight gain even at doses as high as 300 mg/kg/day.

Halofenic acid is a SPPARM that functions primarily as a partial agonist characterized by the differential recruitment of PPAR cofactors, i.e. displacement of corepressors [NCoR and silencing mediator for retinoid and thyroid receptors (SMRT)] and inefficient recruitment of coactivators [cAMP response element binding protein (CREB) binding protein (CBP) and TRAP220] [73]. When dosed orally to diabetic mice and rats as the acetamidoethyl ester, halofenic acid produces insulin sensitization comparable to rosiglitazone without increases in body weight.

PA-082 is a SPPARM partial agonist ($K_i = 0.8\,\mu M$; $EC_{50} = 260$ nM; efficacy = 40% versus rosiglitazone) [61]. When bound to PPARγ, this isoquinoline derivative preferentially recruits PGC1α compared to other cofactors such as TIF2 and SRC3. PA-082 is known to stimulate genes involved in the insulin signaling pathway; however, no preclinical or clinical data demonstrating the antidiabetic activity of this molecule is available presently.

11.3.2.4 PPAR Pan-Agonists

PPAR ligands with the ability to simultaneously activate all three receptor isotypes have the potential to address the adverse metabolic and cardiovascular liabilities associated with T2D, with concomitant reduction of the common side-effects associated with the PPARγ agonists.

Sodelglitazar (GW-677954) is a PPAR pan-agonist in phase II clinical trials for the treatment of T2D [74]. While published data is limited, this molecule is more active on PPARδ compared to PPARα and PPARγ (EC_{50} values of 1.3, 40 and 63 nM, respectively) and is efficacious in diabetic and hyperlipidemic rat models.

Potent PPAR pan-agonists have been discovered recently through SAR studies around a series of indanylacetic acid derivatives [75]. The lead molecule in this series, **34r**, has EC_{50} values of 101, 4.0 and 42 nM in assays that measure PPARα, PPARδ and PPARγ activity, respectively. When tested in dyslipidemic and diabetic rodent models, **34r** was shown to have better therapeutic potential than known PPARγ agonists.

Although LY465608 was previously reported to be a PPARα/γ dual agonist [76], more extensive investigation demonstrated significant PPARδ activity. Thus LY465608 is more appropriately classified as a PPARα/δ dominant pan-agonist (EC_{50}s = 65, 171 and 665 nM for PPARα, PPARδ and PPARγ, respectively) [77]. In rodent models of diabetes and hyperdyslipidema, LY465608 shows potent glucose lowering activity and an ability to significantly improve circulating TG and HDL-C levels [76].

11.3.3 Alternative Approaches

In addition to the PPAR combinations mentioned above, additional approaches have been explored to modulate the PPARγ–RXR heterodimer with the goal of improving glucose and lipid profiles while reducing liabilities, such as weight gain and edema, associated with the conventional PPARγ agonists. These alternatives include

activating the heterodimer through binding to the RXR component and antagonizing PPARγ to achieve anti-diabetic effects.

11.3.3.1 RXR Modulators

LG1506 is a heterodimer-selective RXR modulator that binds specifically to RXR with high affinity and selectively activates RXR–PPARγ and RXR–PPARα, but not RXR–RARα, RXR–LXRα, RXR–LXRβ or RXR–FXRα [78]. In obese insulin-resistant rats, LG1506 functions alone as a potent insulin sensitizer and, in combination, LG1506 enhances the insulin-sensitizing actions of rosiglitazone. Administration of LG1506 to insulin-resistant rodents reduces both body weight gain and food consumption, and blocks PPARγ-induced weight gain when coadministered with rosiglitazone. LG1506 also elevates HDL-C in a rodent model of dyslipidemia.

11.3.3.2 PPARγ Antagonists

SR-202 is a synthetic PPARγ antagonist that inhibits both PPARγ-stimulated recruitment of the coactivator SRC-1 and transcriptional activation of the receptor. In cell culture, SR-202 efficiently antagonizes the classical end points of PPARγ agonist activity including adipocyte differentiation. In contrast to its PPARγ antagonist activity, SR-202 reduces high fat diet-induced insulin resistance and improves insulin sensitivity in diabetic *ob*/*ob* mice [79]. Although these findings are surprising, several investigators have confirmed antidiabetic activity produced by other PPARγ antagonists [80].

11.4 Discussion and Summary

A structurally diverse set of compounds is capable of binding to the PPARγ–RXR heterodimer. The specific nature of these interactions defines the mode of the resulting functional activity – ranging from full agonism to partial agonism to antagonism. Surprisingly, compounds operating through this spectrum of activities can lower glucose and improve insulin in rodents as well as in humans.

These findings remain puzzling and PPARγ ligands need to be examined more thoroughly to elucidate their true *in vivo* receptor modulating abilities. Possibly, receptors in addition to PPARγ are involved in the antidiabetic effects of these molecules. Several recent reports have disclosed that some PPARγ ligands bind to a receptor on mitochondria referred to as mitoNEET and that this receptor is responsible for at least a portion of their antidiabetic activity [81].

Through extensive studies on PPARγ, data have emerged that challenge the well-accepted dogma of receptor pharmacology. While molecules with higher affinity to a receptor are expected to demonstrate greater efficacy in preclinical and clinical studies, numerous examples exist in which PPAR ligands with lower receptor affinity have greater efficacy on glucose lowering. This point is illustrated by comparing the PPARγ affinity of pioglitazone ($EC_{50} = 480$ nM) with rosiglitazone ($EC_{50} = 53$ nM) – both drugs provide nearly equivalent efficacies in large patient populations [34–38].

In addition to the potential development hurdles discussed above, short-term *in vivo* studies with PPARγ ligands do not appear to accurately predict results from longer term toxicology studies. As adverse events with PPARγ ligands may accumulate over time, toxicology studies become lengthy in order to accurately identify potential liabilities.

11.5 Future Direction

The development of better therapies for T2D will benefit greatly from a deeper understanding of the complexities associated with PPARγ ligand–receptor interactions, the resulting cofactor recruitment, and the potential for tissue specific outcomes. A greater understanding of the events that connect ligand–receptor interaction to glucose lowering and potential benefits on lipid parameters is also needed.

In recent years, the difficulties associated with developing better PPARγ modulators for the treatment of T2D have become pervasive. The diverse adverse findings associated with some of the recent molecules tested in preclinical and clinical settings constitute one of the greatest challenges facing this therapeutic approach. These findings range from rare and varied tumors in rodent models to congestive heart failure and the potential for renal impairment in individuals enrolled in late phase clinical trials. Detailed investigations into the origin of these adverse events could help identify a PPARγ activity profile that would eliminate them.

In conclusion, the need for better therapies to combat the approaching obesity and diabetes epidemic justifies the work that is required to solve the current problems associated with the development of PPARγ modulators.

Acknowledgment

The authors wish to thank Dr. Minmin Wang for her help in presenting the PPARγ crystal structure in Figure 4.

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12
Retinoids in Clinical Use

Vincent C. O. Njar

12.1
Introduction

Retinoids are a class of polyisoprenoids that are derived by oxidative cleavage of β-carotenes of plant origin to yield vitamin A (retinol). Dietary sources of vitamin A include eggs, milk, butter and fish-liver oils [1, 2]. Retinoids are essential for embryonic development and play important physiological functions, particularly in the brain and reproductive system, by regulating organogenesis, organ homeostasis, and cell growth, differentiation and apoptosis [1, 2]. Naturally occurring and synthetic retinoids are currently the subject of intense biological interest stimulated by the discovery of retinoid nuclear receptors and the realization of these compounds as nonsteroidal small-molecule hormones [3]. However, it should be stated that before the discovery of the nuclear retinoid receptors, a number of therapeutically useful retinoids were identified [4, 5]. Indeed, most retinoids that are currently used in dermatology and in oncology, such as all-*trans*-retinoic acid (ATRA, tretinoin), 13-*cis*-retinoic acid (13-CRA, isotretinoin), etretinate and acitretin, were discovered by chemical modifications on the basis of vitamin A structure and by biological evaluations in suitable pharmacological models. This chapter presupposes familiarity with the retinoid field in general. For those seeking more background information, many recent and comprehensive reviews are available [2, 6–9]. The purpose here is to provide the reader with an astute knowledge of retinoids that are in clinical use. This chapter mainly is based on perspectives, reviews and abstracts published in the last 14 years up to June 2007. Most of the original articles cited were also consulted.

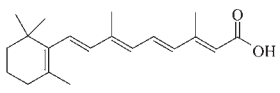
12.2
Mechanism of Action of Retinoids and Retinoic Acids

With current knowledge, the pleiotropic action of retinoic acids and retinoids might be explained mechanistically by the actions of the six known nuclear receptors, the

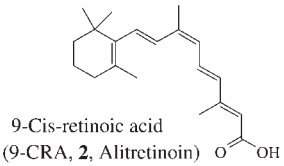
Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

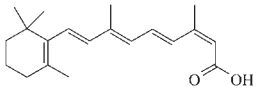
retinoic acid receptors (RARα, β, γ) and the retinoid X receptor (also called rexinoids; RXRα, β, γ) [2, 3, 10, 11]. Each of these receptors are encoded by distinct genes and are members of the steroid/thyroid hormone receptor superfamily. It is also thought that each receptor mediates a set of unique biological functions in certain cell or tissue types. ATRA (Figure 12.1) is the natural ligand of the RARs, while 9-CRA is the ligand for the RXRs and it also has a high affinity for the RARs. The binding of the other ATRA stereoisomers, 11-CRA and 13-CRA, to these receptors is still unclear. However, because of the reported antitumor efficacy of 13-CRA [12–16], it is plausible that 13-CRA is isomerized intracellularly to ATRA or it may act without obvious interaction with the known retinoid receptors. Figure 12.2 depicts the fate of retinoic acids in the cell [17].



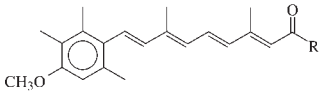
All-trans-retinoic acid
(ATRA, **1**, Tretinoin)



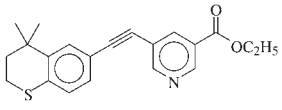
9-Cis-retinoic acid
(9-CRA, **2**, Alitretinoin)



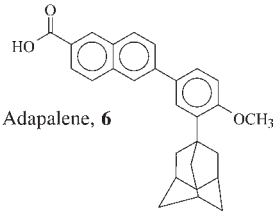
13-Cis-retinoic acid
(13-CRA, **3**, Isoretinoin)



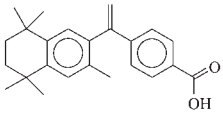
Etretinate, **4a**. R = OCH_3
Acitretin, **4b**. R = OH



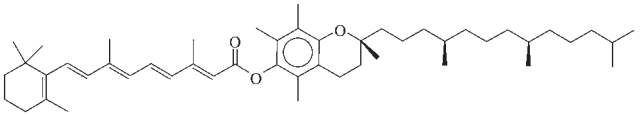
Tazarotene, **5**



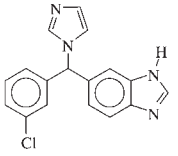
Adapalene, **6**



Bexarotene, **7**



Tocoretinate, **8**



Liarozol (Liazal), **9**

Figure 12.1 Chemical structures of retinoids and related compounds.

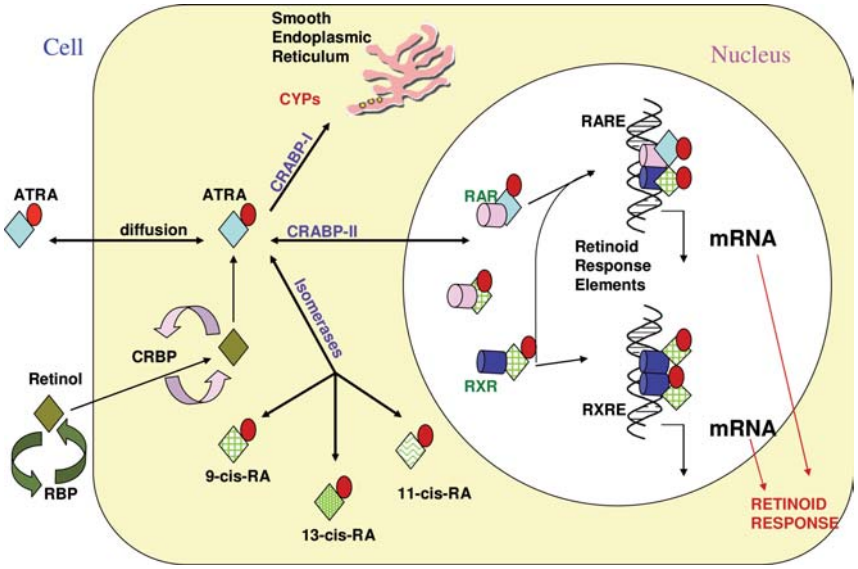


Figure 12.2 Fate of ATRA in the cell. ATRA enters the cell by simple diffusion or by conversion from retinol that has been absorbed from the gastrointestinal tract, bound in circulating form to retinol-binding proteins (RBPs) and rebound intracellularly to cellular retinol-binding proteins (CRBPs). ATRA can be immediately metabolized upon binding to cellular retinoic acid-binding proteins (CRAPBPs) and oxidized by CYPs located in the endoplasmic reticulum. Alternatively, ATRA and its 9-*cis* isomer enter the nucleus and bind to RARs or RXRs, respectively. Upon dimerization of these receptors (i.e. RAR–RXR heterodimer or RXR–RXR homodimer), the activated receptors bind with high affinity to the specific DNA retinoic acid response element (RARE) and effect mRNA transcription. Ultimately, the retinoid response is mediated by primary target genes, by interference with other transcription factors or by control of certain posttranscriptional actions.

Most of the pleiotropc activities of the retinoic acids and other retinoids are elicited by the binding of these agents to the RAR site of RAR–RXR heterodimers. RXRs are the silent partners of the RARs, as the RXR ligands alone are unable to activate the RAR–RXR heterodimers. However, recent studies using RAR- and RXR-selective ligands (agonists, also called rexinoids) have revealed that the RXR ligands allosterically increase the potencies of the RAR ligands [18–20]. Furthermore, RXRs form heterodimers with various nuclear receptors, such as estrogen receptors (ERs), vitamin D_3 receptors (VDRs), thyroid hormone receptors (TRs), peroxisome proliferators-activated receptors (PPARs), liver X receptors (LXRs) and farnesoid X receptors (FXRs). As a result of these unique properties of the RXRs, the RXR ligands are able to modulate the activities of other hormone receptors, in addition to their retinoidal activities [21].

These receptors, as heterodimers (RAR–RXR) or homodimers (RXR–RXR), function as RA-inducible transcriptional regulatory proteins by binding to DNA regions called retinoic acid response elements (RAREs) or retinoid X response elements (RXREs) located within the promoter of target genes. RAREs consist of direct repeats of the consensus half-site sequence AGGTCA separated most

commonly by 5 nucleotides (DR-5), whereas RXREs are typically direct repeats of AGGTCA with 1 nucleotide spacing (DR-1). In the absence of ligand (ATRA or 9-CRA) the apo-heterodimer (RAR–RXR) binds to the RARE in the promoter of the target genes and RAR recruits corepressors such as nuclear receptor corepressors (NCoRs) or/and silencing mediator for retinoid and thyroid receptors (SMRT). These corepressors function by recruiting histone deacetylase complexes (HDACs), causing target gene repression due to compaction of chromatin, making DNA inaccessible to the transcriptional machinery. However, in the presence of ATRA or an agonist, there is a conformational change in the structure of the ligand-binding domain (LBD) that results in destabilization of the corepressor-binding with concomitant recruitment and interaction with coactivators. Some coactivators interact directly with the basal transcriptional machinery to enhance transcriptional activation, while others encode histone acetyl transferase (HAT) activity. HAT acetylates histone proteins, causing the opening of the chromatin and activation of transcription of the associated gene. Other complexes, such as the thyroid receptor-associated protein, are also involved in this process. It should be stated that, whereas the RARα is involved in myeloid leukemias, a growing body of evidence indicates that RARβ is involved in a diverse range of solid tumors [2]. For more details on the mechanism of action of ATRA and other retinoids, reviews by Chambon [10], Altucci and Gronemeyer [2], and Germain *et al*. [22, 23] should be consulted.

12.3
Retinoids and Rexinoids in the Clinic

Although retinoids have shown immense translational potential because of their activities *in vitro* and *in vivo*, their use in the clinic has resulted in limited responses. Part of the problem is that most of the retinoids studied in the clinic (Table 12.1) were stand-alone therapies and not geared towards an optimal therapeutic regimen where they are used in combination with other therapeutic or disease-modifying agents. In addition, most of the promising newer generation of receptor and/or function selective retinoids have not yet been investigated in the clinic [7, 8, 24].

It is now generally believed that retinoids have promising potential for a number of indications, including various dermatological diseases, cancers, ulcer, type 2 diabetes and HIV infection. However, the reality is that these agents are only currently effective in man for the treatment of various dermatological diseases such as acne, psoriasis and other keratinizing dermatoses, and also in the treatment of a few types of cancers. We will focus our attention on retinoic acids and derivative(s), synthetic RAR agonist and antagonist, and then those molecules able to increase the endogenous retinoic acid by inhibiting the cytochrome P450-mediated catabolism of retinoic acid, also known as the retinoic acid metabolism blocking agents (RAMBAs). Specifically, we will discuss the clinical agents (see Figure 12.1), including ATRA (**1**), 9-CRA (**2**), 13-CRA (**3**), etretinate/acitrtine (**4a**/**4b**), tazarotene (**5**), adapalene (**6**), bexarotene (**7**), tocoretinate (**8**) and liarozole (**9**) The recent review by Berrie and Goldhill [6] provides a comprehensive list of retinoids and related compounds in the clinic and in clinical trials.

Table 12.1 Retinoids in the clinic.

Compound (drug name)	Indication	Pharmacology	Clinical status	Company name
ATRA (**1**) (Tretinoin)	acne, photodamage, acute promyelocytic leukemia (AML)	RAR agonist, protein synthesis antagonist, microbial collagenase inhibitor	launched	AP Pharma, Johnson & Johnson, Hoffmann-La Roche, Myland Labs
9-CRA (**2**) (alitretinoin)	psoriasis, Kaposi's sarcoma, AML	RAR and RXR agonist, apoptosis agonist	launched	Ligand
13-CRA (**3**) (isotretinoin)	acne	RAR agonist, protein synthesis antagonist, microbial	launched	Hoffmann-La Roche
Etretinate (**4a**), acitretin (**4b**)	breast cancer, nonsmall cell lung cancer, Kaposi's sarcoma, T cell lymphoma	RXR agonist	launched	Hoffmann-La Roche
Tazarotene (**5**)	acne, psoriasis, cancer	RARα agonist	launched	Allergan
Adapalene (**6**)	acne, psoriasis	RAR agonist, protein synthesis antagonist, microbial collagenase inhibitor	launched	Galderma
Bexarotene (**7**)	psoriasis, keratosis, eczema, head and neck, renal, prostate, ovarian, and colorectal cancers	RXR agonist	launched	Ligand
Tocoretinate (**8**)	ulcer	RAR antagonist, protein synthesis antagonist, microbial collagenase inhibitor	launched	Nisshin Pharma
Liarozol (**9**) (Liazal™)	laminar ithciosis	inhibitor of ATRA catabolism	launched	Johnson & Johnson

12.3.1
ATRA (tretinoin, 1)

Tretinoin (**1**) is an RARα, β and γ agonist and was the first retinoid approved for the treatment of acne and has been in clinical use for almost three decades. It is used as a

monotherapy in patients with noninflammatory comedones, and in combination with other topical or systemic drugs in mild, moderate and severe inflammatory acne [25]. Tretinoin acts by increasing the turnover of follicular epithelial cells and by accelerating the shedding of corneocytes. These processes help normalize keratinization, which leads to drainage of comedones and inhibition of new comedone formation. A major concern with the use of early formulations of tretinoin was excessive skin irritation associated with its hydroalcoholic vehicle and the high concentration of the drug. This side-effect has now been corrected by use of various creams/gels vehicles and with low drug concentrations (e.g. 0.0025, 0.05 and 0.01%) [26]. Topical formulations of ATRA available as cream or gel (Retin-A®) are currently used for treatment of acne, psoriasis and ichthyosis [27].

The best defined among the clinical oncological applications of retinoids is the use of ATRA for treatment of acute promyelocytic leukemia (APL). Oral administration (45 mg/m^2/day p.o.) of this drug to APL patients is currently approved in several countries worldwide. More than 90% of APL patients achieve complete remission with ATRA therapy [28, 29]. The basis for the dramatic efficacy of ATRA against APL is the ability of pharmacological doses of ATRA to overcome the repression of signaling caused by the PML–RARα fusion protein at physiological ATRA concentrations. Restoration of signaling leads to differentiation of APL cells and then to postmaturation apoptosis [30]. Several randomized clinical trials have now defined the utility of ATRA as maintenance therapy [31, 32] and also the benefits of combining ATRA with chemotherapy [33]. The US National Cancer Institute is currently evaluating ATRA as an anticancer agent in phase II trials for brain, head and neck, and prostate caners [6].

12.3.2
9-CRA (alitretinoin, 2)

9-CRA has been detected in humans [34] and was the first RAR–RXR pan-agonist discovered [35–37] and may be classified as a retinoid/rexinoid. It is the only retinoic acid isomer not approved for the common dermatological diseases. However, it has recently been launched in the US as adjuvant topical treatment of AIDS-associated Kaposi's sarcoma [38–41]. This agent is the first RXR ligand to be approved for the treatment of a dermatological disease. In a randomized study with 268 AIDS-associated Kaposi's sarcoma patients, 35% treated with alitretinoin (0.1% gel) had a positive response, compared with 18% treated with vehicle gel irrespective of the number of concurrent antiretroviral therapies [41]. 9-CRA is in clinical trials for the treatment of various cancers, including breast cancer [42], renal cell carcinoma [43, 44] and squamous cell carcinoma [45–47].

12.3.3
13-CRA (isotretinoin, 3)

13-CRA is a metabolite of ATRA [48] that binds poorly to the RARs. Recent studies suggest that 13-CRA is a prodrug, activated in human sebocytes via a selective

intracellular isomerization to high levels of ATRA and subsequent binding to RARs [49]. This agent has been available in topical formulations in Europe since the early 1970s for the treatment of acne. In the US, oral isotretinoin greatly advanced the treatment of severe acne after an important discovery by Peck and Yoder [50]. Isotretinoin gained approval from the US Food and Drug Administration (FDA) for the treatment of resistant nodular acne in 1982 [51]. Numerous clinical studies do not show a fundamental difference between 13-CRA and ATRA [52, 53], although the former is apparently better tolerated and it is the only retinoic acid isomer used in systemic form [54]. On the basis of several clinical trials (reviewed in Ref. [55]), systemic isotretinoin may be considered as an alternative drug in some dermatological diseases unresponsive to conventional therapy. Nevertheless, more randomized clinical trails to determine the role of systemic isotretinoin therapy in dermatological diseases, including skin cancers, other than acne are required. Isotretinoin also represents a potentially useful drug in many dermatological diseases other than acne and also skin cancers, due to its immunomodulatory, antiinflammatory and antitumor activities [55]. It should be noted that isotretinoin is marketed under various tradenames, most commonly Accutane™ (Roche), Amnesteem™ (Mylan), Claravis™ (Barr), Sotret™ (Ranbaxy) or Roaccutane™ (Roche), while topical isotretinoin is most commonly marketed under the tradenames Isotrex™ or Isotrexin™ (Stiefel).

12.3.4
Etretinate (ethyl all-*trans*-3,7-dimethyl-9-(4-methoxy-2,3,6-trimethylphenyl)nona-2,4,6,8-tetraenoate, 4a) and acitretin (all-*trans*-3,7-dimethyl-9-(4-methoxy-2,3,6-trimethylphenyl) nona-2,4,6,8-tetraenoic acid, 4b)

Etretinate (**4a**, Tegison®) is considered as a second-generation retinoid with a characteristic substituted aromatic ring in place of the substituted cyclohexenyl ring in retinoic acids. It was first lunched by Hoffmann-La Roche in the US in 1982 as the first systemic retinoid for psoriasis. Etretinate was replaced by its hydrolyzed metabolite free acid – acitretin (**4b**, Soriatane®) in 1997. Acitretin was found to be clinically as effective as etretinate, but with a much shorter elimination half-life ($t_{1/2} = 120$ days for etretinate and around 2 days for acitretin), advantageous for clinical use. Acitretin does not bind to, but activates, the RARs [56], and it has a high affinity for both cellular retinoic acid-binding proteins I and II (CRABP I and II) [57]. Systemic treatment with acitretin is effective in several disorders of keratinization, due to its action in promoting keratinocytes differentiation in several skin disorders [58]. A review of acitretin as a systemic retinoid for the treatment of psoriasis has recently appeared [59]. Oral acitretin is currently being investigated in several clinical trials for the prevention of skin cancers in solid organ transplant patients [60–62]. Paradoxically, in spite of the similar therapeutic efficacies of acitretin and etretinate, the latter has been reported to succeed in cases where acitretin has failed [63]. In addition, a recent study reported the successful use of etretinate for long-term management of a patient with cutaneous-type adult T cell leukemia/lymphoma [64]. Reports of this nature may warrant the resurgence of etretinate.

12.3.5
Tazarotene (ethyl 6-[2-(4,4-dimethylthiochroman-6-yl)-ethynyl] nicotinate, 5, Tazarac®, Avage® and Zorac®; Allergan)

Tazarotene (**5**) was first approved in 1997 by the FDA for the treatment of acne [65], but it is also currently used for the treatment of plaque psoriasis [66] and photodamage [67]. It is a synthetic acetylinic retinoid that is readily hydrolyzed to its active form, tazarotenic acid, in keratinocytes. Unlike its parent compound, tazarotenic acid has the ability to bind and activate RARβ and RARγ (RARβ > RARγ) with less effect on RARα and no effect on the RXRs [68]. However, because RARβ is not expressed in human keratinocytes, the effect of this drug on the major cell type of the epidermis is clearly attributed to its interaction with RARγ. Through regulation of gene expression in a specific manner, tazarotenic acid modulates abnormal differentiation of keratinocytes, increased keratinocyte proliferation and inflammation [65]. Clinical responses are seen after 2 weeks, with significant clearing after 6–12 weeks of treatment with topical gel or cream formulations of tazarotene [69]. Combination of tazarotene with topical corticosteroids of low potency appears to increase overall therapeutic potential with reduced side-effects, such as local skin irritation, erythema and burning sensation [70]. A review of the use of topical tazarotene in the treatment of plaque psoriasis has recently been published [71].

Following a clinical study which suggested that tazarotene may be effective treatment of cutaneous basal cell carcinoma (BCC) [72], a recent study of 30 patients with small superficial and nodular BCC was conducted to assess the efficacy and mechanism of action of tazarotene (0.1% gel). Overall, 76.7% of treated tumors showed more than 50% regression, while complete healing was observed in 46.7% of all treated BCC. Induction of tazarotene-induced BCC regression was attributed to synergistic RARβ-dependent anti-proliferative and pro-apoptotic activities [73].

12.3.6
Adapalene (6-[3-(1-adamantyl)-4-methoxyphenyl]-2-naphthoic acid, 6, Differin®; Galderma Laboratories)

Adapalene (**6**) is a naphthoic acid derivative with a methoxyphenyl adamant side-chain and is a commonly used antiacne drug [74]. Similar to the action of tazarotene in its hydrolyzed form, tazarotenic acid, adapalene interacts selectively with RARβ and RARγ, and its activity on proliferation and differentiation can be blocked by a RARγ antagonist [75]. In addition, adapalene has antiinflammatory potential due to its anti-AP1 activity [75]. Although its efficacy is similar to that of other retinoids, it has an improved therapeutic ratio due to its better tolerance (reviewed in Ref. [76]).

12.3.7
Bexarotene (4-[1-(5,6,7,8-tetrahydro-3,5,58,8-pentamethyl-2-naphthalenyl)ethenyl] benzoic acid, 7)

Bexarotene (**7**, Targretin®) is a selective RXR agonist (classified as a rexinoid) whose exact mechanism of action in cancer therapy and chemoprevention is poorly

understood [7]. In a multinational phase II–III clinical trials, oral bexarotene (300 mg/m^2/day) showed 55% response rate in patients with refractor advanced stage cutaneous T cell lymphoma (CTCL) [77]. Bexarotene (1% Targretin® gel) is approved for the topical treatment of cutaneous lesions in patients with state 1A and 1B CTCL who have not tolerated other therapies or who have refractory or persistent disease [78, 79]. The ability of bexarotene to activate RXRs and their heterodimer partners results in modulation of gene expression pathways, which ultimately modulate converging signaling pathways responsible for cell differentiation and apoptosis [80]. This multitargeted approach of mediating cell differentiation, apoptosis and proliferation suggests that bexarotene may be particularly active in the treatment of malignancies, especially in combination with chemotherapeutic agents. Thus, following acceptable phase II response rates (25%) in combination with cisplatin and vinorelbine in nonsmall cell lung cancer (NSCLC) [81, 82], oral bexarotene in combination with paclitaxel and carboplatin or vinorelbine is currently being evaluated in multicenter phase III studies in previously untreated patients with NSCLC patients [83]. A recent preclinical study by Yen and Lamph suggests a role of bexarotene in combination with paclitaxel in prevention and overcoming acquired drug resistance in advanced prostate cancer [84].

12.3.8
Tocoretinate [tretinoin tocoferil, (±)-3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-yl (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexene-1-yl)-2,4,6,8-nontetraenoate, 8]

Tocoretinate (**8**) is a unique α-tocopherol ester of ATRA, and has been safely used in Japan for the treatment of decubitus and skin ulcer, acting via stimulation of the proliferation of human skin fibroblasts [85, 86]. The agent is characterized as an RAR antagonist, protein synthesis antagonist and microbial collagenase antagonist. Unexpectedly, although tocoretinate is an α-tocopherol ester of ATRA, it was reported to be stable *in vitro* and *in vivo* [87]. This characteristic of tocoretinate was support from observations that tocoretinate enhances the growth of human skin fibroblasts and stimulates the formation of granulation tissue in ulcers – effects that are different from those of either ATRA or α-tocopherol [87]. Furthermore, toxicity tests in animal models have shown that tocoretinate is at least 150 times less toxic than ATRA [88–90]. Tocoretinate is actively being investigated for the possible treatment and chemoprevention of leukemia [91, 92].

12.3.9
Liarozole (9)

Following extensive studies by researchers at Janssen Research Foundation (now called Johnson & Johnson Pharmaceutical Research & Development) liarozole (**9**, Liazal™) was identified as a modest inhibitor ($IC_{50} = 2.2–6.0\,\mu M$) of ATRA-4-hydroxylase (CYP26) [93–100]. On the other hand, the compound was shown to be a good inhibitor of rat CYP17 ($IC_{50} = 260\,nM$) and a potent inhibitor of CYP19 [93].

Although liarozole has undergone phase III clinical trials for the treatments of patients with metastatic prostate cancer [101] and also phase II trials for the treatment of ER-negative metastatic breast cancer patients [102], its development for these indications have been discontinued [102].

Inappropriate metabolism of ATRA could generate a condition of retinoid deficiency, which is characterized by hyperkeratinization and desquamation as seen in acne, psoriasis, and ichthyosis [58]. Owing to these reasons, liarozole has also been extensively investigated as a potential agent for the treatment of dermatological diseases [98, 103, 104]. Studies in mice revealed that liarozole is able to mimic the antikeratinizing effects of ATRA [98]. In open clinical studies, liarozole was found to be therapeutically effective in patients with psoriasis [105, 106] and with ichthyosis [106]. A double-blind, randomized clinical study involving 20 patients with severe plaque-type psoriasis was conducted; half of the patients were treated with oral liarozole (75 mg, twice daily) and the other half were treated with oral acitretin (25 mg/day) [103]. After 12 weeks of treatment, both groups responded with a similar decrease in the PASI (psoriasis area severity index) score from around 20 to around 10. It is gratifying to state that liarozole was recently (2004) approved in Europe and USA as an orphan drug for the treatment of congenital ichthyosis [6, 106]. Finally, in a most recent (2005) paper, Lucker *et al*. reported that topical liarozole was effective in the treatment of ichthyosis [106]. Liarozole is the first RAMBA approved for clinical use.

12.4 Development of New Retinoids/Rexinoids and the Future for Retinoid-based Therapies

Most of the retinoids that are currently in clinical use are RAR or RXR agonists/antagonists. However, in the desire to generate new retinoids/rexinoids that may exhibit fewer side-effects, the goal of chemists and biologist is to develop RAR- and RXR-specific ligands. The generation of these agents has been made possible by recent progress in crystallographic studies on nuclear receptor LBDs that has enabled useful information of ligand–receptor interactions at the molecular level [107]. Thus, various ligands have been developed by computer-assisted procedures using virtual libraries and/or molecular databases. Studies in this area have recently been reviewed [7] and will not be discussed further in this chapter.

Recent developments in the understanding of gene regulation by nuclear receptors and chromatin organization have increased interest among researchers in the cancer field in the identification of agents that modulate gene expression through chromatin reorganization. Retinoids fit the profile of these agents since they can induce, for example, the expression of a number of tumor/growth suppressor genes, which otherwise are transcriptionally silent in cancer cells. A number of tumor/growth suppressor genes (e.g. RARβ, TIG1, etc.) are epigenetically silenced because of DNA hypermethylation in their promoter regions [108, 109]. As loss of RARβ has been linked to retinoid resistance and RARβ is a tumor suppressor as well as an

intracellular effector of retinoid action, a therapy involving a combination of retinoids and histone deacetylase and/or histone methyltransferase inhibitors may show synergistic efficacy in cancers. As a proof of concept, reversal of transcriptional silencing of RARβ gene and increased growth inhibition has been observed, in the treatment of t(15;17) ATRA-resistant patient with a combination of the HDACi sodium butyrate and ATRA [110]. In addition to HDAC inhibition, reversal of DNA hypermethylation by demethylating agents, 5-aza-2′-deoxycytidine has been shown to restore ATRA-mediated differentiation/growth inhibition in many head and neck squamous cell carcinomas [109]. In addition, there are several studies that document synergistic efficacy in some leukemia and solid tumor cells *in vitro* and *in vivo* [30, 111].

As stated earlier, RXR is a promiscuous dimerization partner for several nuclear receptors, including those related to lipid physiology, such as PPARs, LXRs and FXRs [112]. Since RXR-selective ligands can elicit similar activities to ligands of the heterodimer partner receptors, it is believed that these agents may be useful as antidiabetic and antiobesity agents [113].

Other promising retinoid-related compounds are the atypical or non-classical retinoid, *N*-(4-hydroxyphenyl)retinamide (fenretinide) [114, 115], and the second- and third-generation RAMBAs [17]. As discussions of these promising clinical agents are beyond the scope of this chapter, readers are advised to consult recent reviews on these classes of compounds [17, 114, 115].

12.5 Conclusions

There is now compelling evidence from the number of retinoids in the clinic and in clinical studies (Table 12.1) that these molecules exhibit efficacy in human diseases. The use of ATRA for the treatment of acute promyelocytic leukemia is considered a successful therapy in our view. It is the hope that the application of retinoids, most probably more receptor specific retinoids/rexinoids, in combination with other chemotherapeutic agents, will lead to broad clinical utility in many diseases. We anticipate that the retinoid field will continue to expand as researchers gain more information about new levels of retinoid/rexinoid biology and their relevance to human diseases.

Acknowledgments

The author's research is supported by grants form the US Army, Peer Reviewed Medical Research Program (Grant No: W81XWH-04-1-0101) and NIH/NCI (USA) grant No: CA117991-01. Parts of this chapter draw upon my previous reviews 'Retionoids in clinical use', *Med. Chem.*, **2006**, *2*: 431–438 and 'Retinoic acid metabolism blocking agents (RAMBAs) for treatment of cancer and dermatological diseases', *Bioorg. Med. Chem.*, **2006**, *14*: 4323–4340.

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13
Nuclear Receptors as Targets in Cardiovascular Diseases

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13.1
Introduction

A key characteristic of the nuclear hormone receptors is the multitude of their effector molecules, based on their action as regulators of gene expression. The classical action of nuclear hormone receptors in the human body is control of endocrine, metabolic or inflammatory networks. Hence, the focus of the pharmacological use of natural or synthetic nuclear hormone receptor ligands has been on inflammatory processes, contraception, and glucose and lipid metabolism. It is therefore no surprise that cardiovascular diseases have for a long time not been considered the primary target indication for nuclear hormone receptors, with the important exception of mineralocorticoid receptor (MR, see below). However, it was recognized recently that a number of nuclear hormone receptors or specific subtypes of nuclear hormone receptors have extended ('nonclassical') functions beyond their traditional effects and that modulating these effects may open new options for the treatment of cardiovascular disease. Based on these new data, we believe it is timely to give an overview about nuclear hormone receptors as possible cardiovascular drug targets. Based on their therapeutic potential, we will review three nuclear hormone receptors in detail: MR, peroxisome proliferator-activated receptor (PPAR) α and thyroid hormone receptors (THRs). Other receptors are only briefly discussed, owing to the paucity of data regarding their role in the cardiovascular system. The role of PPARγ activators for the treatment of diabetes and its cardiovascular complications and estrogen receptor (ER) ligands for prevention of atherosclerosis will not be discussed in this chapter, because they are covered in separate chapters.

13.2
MR (NR3C2)

The steroid receptor family is a subclass of nuclear hormone receptors activated by steroid hormones. This nuclear hormone receptor subfamily has six members:

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

glucocorticoid receptor (GR), progesterone receptor (PR), ERα/β, androgen receptor (AR) and MR.

The interaction of MR with its principal ligand aldosterone is crucial for fluid and electrolyte homeostasis since aldosterone-mediated gene expression via MR directly leads to transepithelial sodium transport in the kidney. Apart from these 'classical' mineralocorticoid effects, high aldosterone levels, especially in combination with increased salt intake, inappropriately activate MR leading to a plethora of deleterious effects. In fact, patients with primary aldosteronism (Conn's syndrome), typically caused by an aldosterone-producing adrenal tumor, are characterized by arterial hypertension and accelerated cardiovascular end organ damage, including endothelial dysfunction, vascular inflammation and myocardial fibrosis. However, contrary to previous belief, hyperaldosteronism is not to be confined to the limited population with clinically overt Conn's syndrome, but rather seems to be a disease with a continuous range of aldosterone overactivation relative to physiological renin levels [1]. The prevalence of hyperaldosteronism (including previously unrecognized subclinical forms) is immense. Between 5 and 20% of the patient populations in hypertension or general outpatient clinics have an elevated aldosterone:renin ratio, with an increasing prevalence in patients with refractory hypertension [2–4].

Based on the importance of aldosterone in sodium retention and arterial hypertension, first attempts to synthesize an MR antagonist were started shortly after the characterization of the hormonal activity of aldosterone in 1953. Chemists at Searle succeeded in synthesizing a potent MR antagonist with oral activity called spironolactone (Aldactone®) in 1957 [5, 6]. In fact, spironolactone was the first antihormone that was launched in 1960 as a diuretic for the management of edematous conditions, primary aldosteronism and essential hypertension. Thus, an MR antagonist had been introduced into the clinic only 7 years after characterization of its physiological ligand aldosterone and 27 years before the molecular cloning of its receptor.

Antiandrogenic and progestational symptoms such as gynecomastia, impotence and menstrual irregularities related to the clinical use of spironolactone were observed already at a very early stage [7], and recognized since 1975 as characteristic adverse effects due to the unspecific interactions of spironolactone with the AR and PR [8].

The careful investigation of the efficacy and tolerance of spironolactone in essential hypertension [9] initially supported the idea that a 'second generation of MR antagonists' (i.e. more specific compounds) are desired for chronic treatment of cardiovascular diseases. At least three companies succeeded with the synthesis of steroidal MR antagonists with higher selectivity than spironolactone (Figure 13.1): Ciba-Geigy with 9,11α-epoxyderivatives of spironolactone [10], Roussel-Uclaf with a 7α-alkyl spironolactone [11] and Schering (now Bayer Schering Pharma) with mespirenone [12] and other 17α-pregnane-21,17-carbolactones. Schering synthesized a number of interesting steroids with antimineralocorticoid activity in this period. Mespirenone was selected from these compounds for further development and reached phase II clinical trials, but was discontinued in 1989. Typical for these steroidal compounds was the progestagenic activity despite a significantly reduced

antiandrogenic activity. In retrospect it is therefore no surprise that one of the current most successful nuclear hormone receptor drugs, drospirenone (Schering), was discovered in this period as potent PR agonist and MR antagonist [13].

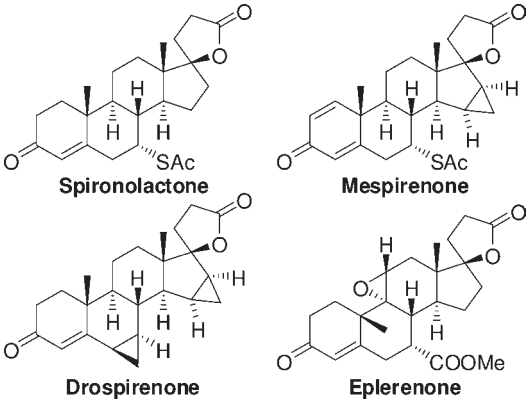

Figure 13.1 Steroidal MR antagonists of the 'first and second generation'.

Drospirenone was developed as a progestin component in oral contraceptives. As such it does not possess the usual water-retaining ability of other contraceptives due to its potent MR antagonistic activity. However, Ciba-Geigy finally won the race for a new clinically used aldosterone antagonist with eplerenone (9,11α-epoxymexrenone) which was selected for a clinical phase I study in 1989 [14]. Later, eplerenone passed from Ciba-Geigy to Searle, Monsanto, Pharmacia and finally to Pfizer who launched the drug in 2002 in the US for the treatment of hypertension. It took therefore 42 years from the approval of the first MR antagonist, spironolactone, to the market introduction of its sole successor, eplerenone (Inspra®), a time frame which is unique in the world of drugs.

Despite its improved selectivity, eplerenone has a relatively low affinity for MR *in vitro* (about 40-fold less potent compared to spironolactone [15]), and a lower potency and efficacy in a human hypertension study in comparison to spironolactone [16]. This study showed that 100 mg eplerenone given once daily or 50 mg twice daily have an efficacy of 50–75% compared to 50 mg spironolactone twice daily. This is important since the maximal approved daily dose of eplerenone for the treatment of hypertension is 100 mg in the US (eplerenone has no approval for this indication in Europe).

Apart from the valuable benefit of aldosterone antagonism in hypertension, MR blockade has been shown to substantially reduce both morbidity and mortality among patients with severe chronic heart failure (CHF) and post-myocardial infarction (MI) in clinical trials [17, 18]. The Randomized Aldactone Evaluation Study (RALES) has shown that 26 mg spironolactone on average per day on top of existing standard therapy [i.e. an angiotensin-converting enzyme (ACE) inhibitor, aspirin and a loop diuretic] given to patients with severe heart failure (New York Heart Association class III or IV, left ventricular ejection fraction $\leq$35%) results in 30%

mortality reduction. This reduction of overall mortality was attributed to both a reduction in mortality from progressive heart failure and a reduction in sudden cardiac death. Painful gynecomastia or breast pain was reported by 10% of the men treated with spironolactone even at the low dose used here [17]. The Eplerenone Post Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) [18] has demonstrated that a mean eplerenone dose of 46 mg daily on top of standard therapy (i.e. ACE inhibitor, β-blocker, aspirin, diuretic) reduces the mortality in patients after acute myocardial infarct by 15%. This reduction was solely attributed to the prevention of sudden cardiac death. Incidences of gynecomastia, impotence or menstrual disturbances with the use of eplerenone were as low as in the placebo (i.e. standard therapy) arm. Unfortunately, there are no clinical studies available comparing spironolactone and eplerenone directly in a heart failure population. Nevertheless, based on the convincing benefit of the two compounds in RALES and EPHESUS, respectively, both spironolactone and eplerenone have been introduced into current guidelines for the treatment of heart failure [19]. Based on their mechanism of action (sodium excretion and potassium retention), all MR antagonists carry the potential to induce hyperkalemia and, hence, arrhythmia. Owing to this inherent risk, the dose range of the MR antagonists is limited, particularly in heart failure patients [20].

Overall, clinical trials have impressively demonstrated that aldosterone is a major contributor to cardiovascular morbidity and mortality in patients with arterial hypertension and heart failure. However, the currently available MR antagonists suffer from two substantial drawbacks that limit their benefit in clinical practice, i.e. lack of selectivity (in the case of spironolactone) and limited efficacy (in the case of eplerenone). Consequently, a 'second race' for new aldosterone antagonists has started, in search of a compound which ideally should combine the potency and efficacy of spironolactone with the selectivity of eplerenone.

Only a limited number of pharmaceutical companies reportedly seem to be working on MR antagonists. There is a trend towards new aldosterone antagonists which possess a nonsteroidal structure because the major adverse effects of the current MR antagonists are related to the interaction with other steroid hormone receptors. These new, nonsteroidal aldosterone antagonists, which arguably can be classified as 'third-generation MR antagonists' [20], are briefly summarized below (Figure 13.2):

Eli Lilly has identified at least three different classes of nonsteroidal MR antagonists represented by compounds **A**, **B** and **C** [21–24]. Neel *et al.* [25] described the *in vitro* activities of potent and selective 3,3-bisaryloxindoles. These compounds exhibit a single-digit nanomolar activity in human MR binding and functional cell-based assays with a 100-fold selectivity with respect to other steroid hormone receptors. Moreover, related 3-benzylindoles were described by Lilly with binding affinities below 500 nM for MR. A third and a fourth compound class discovered as MR antagonists by Eli Lilly are dibenzosuberane and dibenzooxepines, respectively. Some of these compounds are reported to have an IC_{50} below 100 nM in functional cell-based MR assay with modest selectivity over GR.

Exelixis has discovered nonsteroidal MR antagonists of type **E** having an imidazole or a pyrrole core, respectively [26, 27]. The compounds of both structural classes are

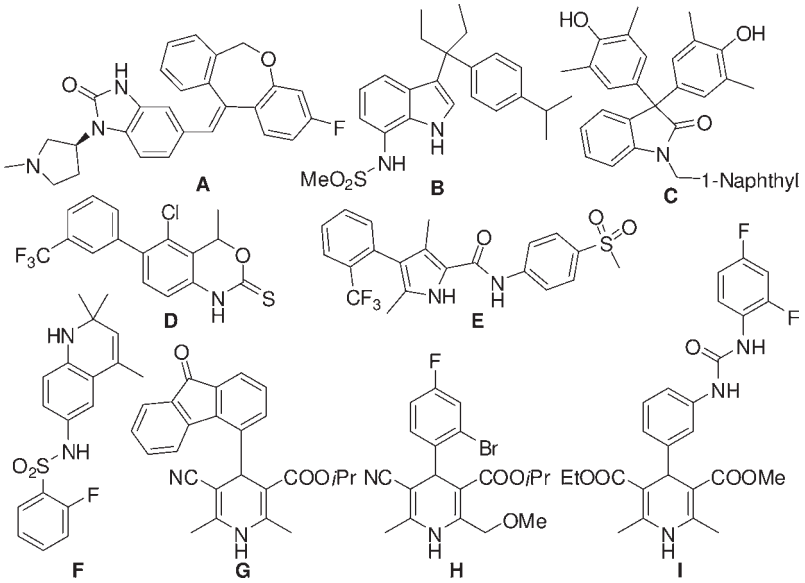

Figure 13.2 'Third-generation' MR antagonists.

generally described as active below 500 nM on MR with more than 10-fold selectivity versus GR, AR, PR and ERα. Exelixis has nominated a potent and selective nonsteroidal MR antagonist, XL550, in its 2005 annual report as a preclinical candidate. XL550 is effective in animal models of hypertension and CHF.

Dainippon Sumitomo has identified benzoxazinethiones and dihydroquinolines, compounds of type **F** with double-digit nanomolar activity on MR [28]. Similar compounds, 6-aryl benzoxazinones or 6-aryl benzoxathiones (**D**), were described by Ligand Pharmaceuticals [29]. Patent applications from Bayer (**G**), Novartis and Takeda (represented by structures **H** and **I**) indicate that these three companies have discovered that compounds belonging to the chemical class of dihydropyridines may act as potent and selective MR antagonists *in vitro* [30–32]. Figure 13.3 visualizes the predicted binding mode of compounds **A** and **D** as representatives of different nonsteroidal chemical classes, within the ligand binding pocket of MR.

Researchers from Bayer were the first to describe flourenyl-substituted dihydropyridines as specific MR antagonists in cell-based assays. Data were presented demonstrating the *in vivo* activity of a new dihydropyridine derivative which reduces blood pressure, prevents end-organ damage and improves survival in preclinical animal models of salt-sensitive hypertension and CHF [33]. The same compound has been shown very recently not only to block wild-type MR, but also the gain-of-function S810L MR mutant which is responsible for early-onset hypertension in men and gestational hypertension in women [34]. This is remarkable since both natural steroidal MR antagonists such as progesterone and synthetic steroidal MR antagonists such as spironolactone paradoxically activate the S810L MR mutant.

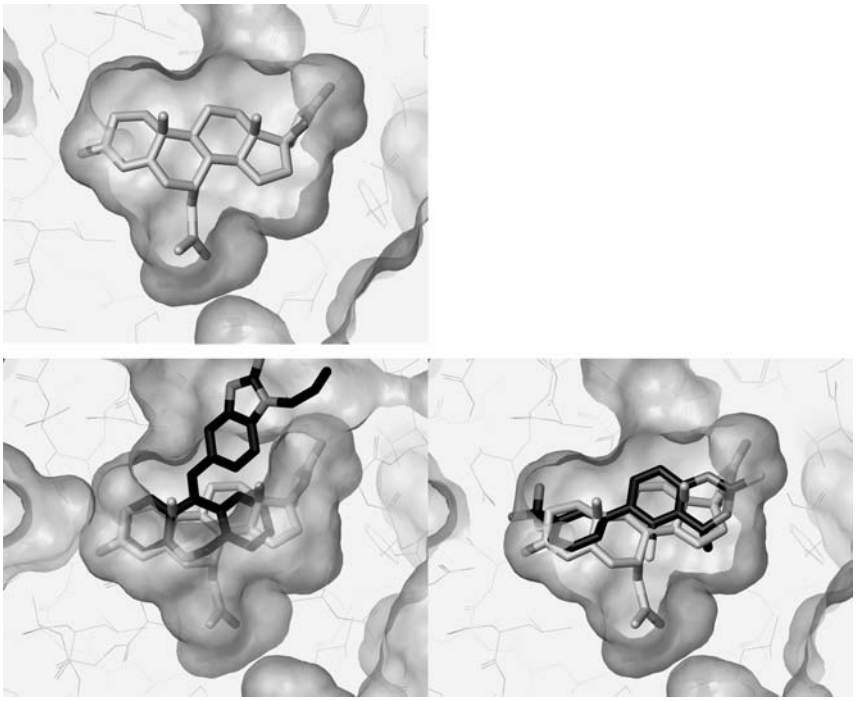

Figure 13.3 Predicted binding mode of several nonsteroidal MR antagonists (in black, **A** and **D** from Figure 13.2) within the ligand-binding pocket of human MR, based on the X-ray structure of the receptor with spironolactone (shown in light grey, upper left) using Glide (version 3.5; Schrödinger LLC) software. Note that helix 12 (H12) in the MR ligand binding domain was omitted. The F-phenyl ring of **A** (lower left) is supposed to mimic the steroidal A-ring of spironolactone, whereas the benzimidazolone moiety is predicted to lead to bulky antagonism by displacing the position of H12. **D** (lower right) contacts Asn770 with the NH group. The *m*-CF3-phenyl ring overlays with the steroidal A-ring.

Thus, several new chemical entities belonging to the third generation of MR antagonists are now under preclinical evaluation which may offer improved treatment options for patients suffering from cardiovascular diseases linked to inappropriately activated MR.

13.3 THRs (NR1A1 and NR1A2)

The thyroid hormones, 3,3′,5-tri-iodo-L-thyronine (T3) and 3,5,3′,5′-tetra-iodo-L-thyronine (T4), are the only known naturally occurring iodine-containing molecules in mammals with biological activity. They are important endocrine signaling hormones that have a profound impact on a number of physiological processes such as lipid metabolism, control of energy expenditure, thermogenesis, growth and development. These effects are mainly caused by direct influences of thyroid

hormones on the transcription of several important genes in skeletal and cardiac muscle, central nervous system (CNS) and liver tissues via binding to their cognate receptors, THRs [35]. THRs can function as monomers, homodimers and also heterodimers with the retinoid X receptor (RXR) which also functions as a heterodimerization partner for several other nuclear hormone receptors. Transcriptional control by THRs occurs in concert with transcriptional coactivators and corepressors on specific DNA sequences termed thyroid response elements (TREs). Since both positive and negative TREs have been identified, several ways of transcriptional modulation exist in the combination with several THR isoforms in ligand bound or unliganded fashion yielding a highly versatile gene regulation system.

In vertebrates, two THR genes are conserved (THRα and THRβ), which are located on different chromosomes. THRα and THRβ seem to regulate both common as well as specific target genes. Both genes yield several different receptor isoforms by alternative splicing and differential promoter usage, but only the THRα1, THRβ1 and THRβ2 subtypes are functional receptors with known physiological relevance. The most widely expressed form of the THR in humans is the THRα1 subtype, especially in the brain and heart where it is the most abundant isoform [36]. Conversely, THRβ1 is expressed later during development and more limited to certain tissues (e.g. the liver). The third functional THR isoform, THRβ2, is more restricted in its expression pattern, and occurs mainly in the pituitary and other cells in the CNS where it plays a key role mediating the negative feedback on the hypothalamic–pituitary–thyroid axis.

The primary origin of thyroid hormones is the thyroid gland that produces predominantly T4. In addition, most T3 (approximately 80% of circulating T3) is produced in extrathyroid tissue by the action of intracellular deiodinases on T4. The naturally occurring hormones do not show any significant preferential binding to a specific receptor subtype. However, tissue distribution and knockout animal studies as well as results with selective ligands reveal specific consequences of different TRH isoform actions for the body [37, 38]. The most interesting tissue-specific effects of the thyroid hormones with respect to the cardiovascular system are their action on the liver and the heart. The beneficial effects of the thyroid hormones or related analogs on plasma cholesterol were originally observed in hypothyroid patients [39]. In the liver, T3 activates several genes involved in cholesterol metabolism via THRβ, such as the low-density lipoprotein (LDL) receptor, apolipoprotein A1 and several lipolytic enzymes, resulting in a decrease in serum cholesterol and triglycerides. In the heart, T3 has positive chronotropic and inotropic effects, and decreases the peripheral resistance. These effects can at least in part be attributed to an increased expression of the pacemaker channel HCN2 [40] and to stimulation of transcription of several other genes which are critically involved in the control of heart rate and contractility, such as myosin heavy chain α and β, sarcoplasmic reticulum calcium ATPases, Na^+/K^+ ATPases and HCN4 [41–43]. Clinical data suggests a potential negative impact of low T3 state on the prognosis of cardiac disease. In a study with 573 cardiac patients (NYHA II–IV) Iervasi *et al.* [44] identified low plasma T3 concentrations as strong predictor of mortality. On the other hand, chronically elevated T3 concentrations may induce cardiac hypertrophy, arrhythmias and heart failure. The pharmacological use

of thyroid receptor agonists has been highlighted by recent observations that metabolic degradation products of thyroid hormones, iodothyronamines (which are generated by the action of aromatic amino acid decarboxylases), can act as ligands for a hitherto uncharacterized G-protein-coupled receptor, trace amine receptor 1 [45]. The most potent agonist identified in this study was 3-iodothyronoamine at an EC_{50} of 14 nM. The compound produced a drop in heart rate and body temperature *in vivo* in mice, and bradycardia as well as a reduction of cardiac output. These effects could be antagonized with the β-adrenergic agonist isoproterenol. However, the relevance of these findings remains unclear since the plasma levels of the thyroid hormone metabolites under physiological conditions are significantly lower than the concentrations needed to evoke the cardiovascular effects under experimental conditions.

Hypothyroidism is associated with an increased cardiac glycogen content and has experimentally been shown to protect against arrhythmia and ischemia-reperfusion damage. Therefore, there is a theoretical basis for a potential benefit of antagonizing cardiac THRα1 activity. On the other hand, owing to the adverse effects of hypothyroidism on lipid metabolism, selective THRβ agonists may potentially be used to treat dyslipidemia and atherosclerosis. In addition, thyroid hormone analog therapy also seems to be an attractive approach in heart failure, especially when it is accompanied by low T3 serum levels. Based on these considerations, a number of synthetic THR ligands have recently been developed to treat cardiovascular diseases. They are described below with respect to their target indications.

Thyroid hormone analogs with bulky 5′ extensions have been designed as THR antagonists with the goal to perturb proper positioning of the C-terminal H12 which is involved in coactivator binding. Examples are depicted in Figure 13.4: NH-3, GC-14, and HY-4 [46–49]. In contrast to GC-14, which is a rather weak THR antagonist, NH-3 exhibits also some *in vivo* activity in a thyroid hormone-induced *X. laevis* metamorphosis assay.

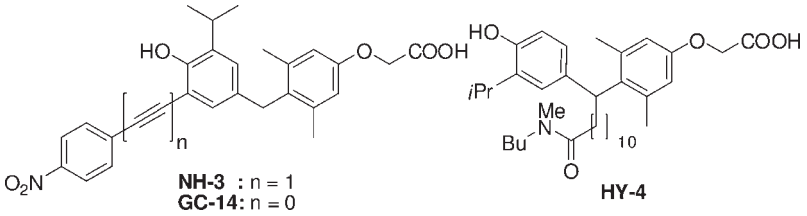

Figure 13.4 THR antagonists.

Debutyldronedarone is the active metabolite of dronedarone, an antiarrhythmic drug developed by Sanofi-Aventis. Apart from its action as a cardiac multichannel blocker, it acts as a THRα and THRβ blocker, with some preference for THRα. In line with these data, dronedarone was shown to mimic the cardioprotective effects of hypothyroidism in rats [50].

3,5-Diiodothyropropionic acid (DITPA; Titan Pharmaceuticals, Figure 13.5) is an orally active analog of thyroid hormone which binds to THRα1 and THRβ1 with equal affinity, but with 100-fold lower affinity compared to T3 [51].

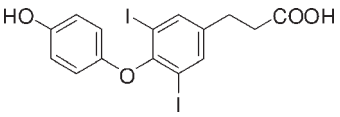

Figure 13.5 DITPA.

DITPA treatment improved left ventricular performance in rat and rabbit post-MI models of heart failure. In a double-blind, placebo controlled, pilot phase II clinical study in 19 patients with NYHA class II or III CHF in 2003, DITPA demonstrated a significant increase in cardiac index, as well as improvements in diastolic function, systemic vascular resistance, and cholesterol and triglyceride levels. In this study, DITPA was well tolerated, with no significant increase in heart rate or significant adverse events. Subsequently, a larger trial was initiated which, however, was discontinued in October 2006, based on a business decision by Titan.

The development of selective THR isoform-specific activators constitutes a considerable challenge, as the amino acid sequences of the inner ligand-binding domains (LBDs) of THRα and THRβ differ by only a single amino acid. A Ser277 in THRα is exchanged for Asn331 in THRβ, which mediates interaction with the polar head groups of THR ligands. Therefore, it is no surprise that mechanisms other than addressing the LBDs of THRα and THRβ are responsible for the selectivity of some of the earlier described thyromimetics purported to possess less side-effects. However, great advancements have recently been made in the design and synthesis of subtype selective THR agonists. Figure 13.6 depicts thyromimetics which have been described to be selective for metabolic activity versus cardiac effects: GC1 [52], CGS 26214 [53] and L-94901 [54].

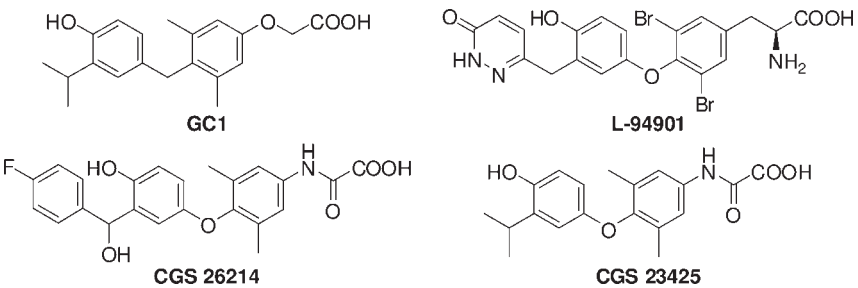

Figure 13.6 Thyromimetics GC1, CGS 26214 (active form), L-94901 and CGS 23425.

In the case of GC1, modest selectivity for THRβ has been described (6.6-fold) which originates from the oxyaceticacid head group [55]. *In vivo*, an increase in selectivity is achieved by accumulation in the liver versus the heart. Ciba-Geigy (now Novartis) discovered the lipid-lowering thyromimetic CGS 26214 devoid of cardiac and thermogenic activity. CGS 26214 was identified based on its ability to access and bind to the nuclear fraction of hepatocytes about 100-fold better compared to that of myocytes in culture. Similar characteristics were reported for L-94901 which was developed by SK&F. It was suggested that the liver selectivity of L-94901 is achieved

via a more effective nuclear transport in hepatocytes compared to pituitary cells [56]. CGS 23425 is described as THRβ-selective thyromimetic which ameliorates hypercholesterolemia by increasing plasma apolipoprotein A1 concentrations and the clearance of LDL cholesterol in the liver. In fact, CGS 23425 does not induce genes encoding contractile proteins, calcium ATPase of sarcoplasmic reticulum and several proteins of mitochondrial oxidative phosphorylation in cultured heart cells which were upregulated by T3 and DITPA further supporting its THRβ selectivity [33]. Therefore, CGS 23425 may be useful for the prevention and reversal of atherosclerosis. Kissei has discovered KAT-681 (*N*-(4-{3-[(4-fluorophenyl)hydroxymethyl]-4-hydroxyphenoxy}-3,5-dimethylphenyl) malonamic acid sodium) and indan analogs [57] which possess both liver selectivity and an up to 60-fold THRβ selectivity leading to cholesterol-lowering activity in animal models. In addition, KAT-681 inhibits the development of preneoplastic lesions in rat livers and may therefore be a candidate for the prevention of hepatocarcinogenesis [58]. Karo Bio is currently investigating at least two selective THRβ agonists in clinical and preclinical studies. KB-5359 was selected as candidate for the treatment of dyslipidemia from animal studies. Another compound, KB-2115 (Figure 13.7), lowered LDL cholesterol of up to 40% in overweight but healthy volunteers in a phase I study.

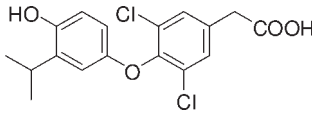

Figure 13.7 KB-2115.

A 3-month, randomized, double-blind, placebo-controlled phase II study was initiated in November 2006 in order to assess the efficacy and safety of the drug in 100 patients with primary hypercholesterolemia.

In summary, several thyromimetics have recently entered advanced preclinical and clinical phases and may offer additional benefits for many patients in the future.

13.4 PPARα (NR1C1)

The PPARs constitute a family of three nuclear receptors that have important roles in physiological lipid and glucose metabolism. Converse, dysregulation of the PPARs has been associated with pathophysiological conditions such as hyperlipidemia, insulin resistance and coronary artery disease. There are three PPAR isoforms – PPARα, PPARγ and PPARδ (also termed PPARβ). All PPARs are expressed in cardiovascular tissues and in addition to their known metabolic actions they exhibit distinct functions. While the expression of PPARα and PPARβ/δ is surprisingly high in the heart, PPARγ expression is very low and does not appear to play an important role in the heart. Since Chapter 11 in this book is dedicated to the well-established role of the PPARs in metabolic diseases, we will focus in this chapter on the role of PPARα in cardiac diseases.

PPARα was the first isoform discovered in the PPAR family in 1990 by Issemann and Green [59]. They identified a heterogeneous group of chemical compounds that activated this receptor which still had an orphan status at that time. Due to their abilities to induce proliferation of the cellular peroxisomes in rodents [60] they called those agents 'peroxisome proliferators'. One of those early compounds investigated was clofibrate, a weak PPARα agonist, and until today no other potential target has been identified for the former orphan drug. Clofibrate as well as feno- and bezafibrate and Wy14643 belong to the class of fibrates that constitutes the most important ligands of PPARα. Originally, the fibrates were identified as lipid-lowering agents before their specific receptors were characterized and identified on a molecular level. The lipid-lowering effects of the fibrates are mainly due to a transcriptional upregulation of lipoprotein lipase and an increase of the β-oxidation of fatty acids in the liver leading to decreased LDL and triglyceride levels, and an increase in high-density lipoprotein (HDL) levels. Endogenous activators of PPARα include fatty acids, oleic acids, linoleic acids, palmitic acids and arachinoic acids.

In addition to its lipid modulation properties, PPARα has become an attractive pharmacological target for the treatment of cardiac diseases. For example, several pieces of evidence directly link alterations in the expression and the activity of PPARα to heart failure. Karbowska *et al.* [61] reported a 54% decrease in the expression of PPARα on protein level in the left ventricle of patients diagnosed with heart failure. In addition, aging is associated with reduced mRNA and protein expression of myocardial PPARα, as well as with a diminished binding to the peroxisome proliferator response element [62]. The mode of action of PPARα agonism for the treatment of heart failure is most likely related to their antifibrotic, antihypertrophic and antiinflammatory properties in the heart. The impact on the regulation of important chemotactic molecules like leukotriene B4, monocyte chemoattractant protein-1 and other inflammatory response markers like endothelin-1, tumor necrosis factor-α or interleukins is well documented in the recent literature [63–65]. The amelioration of heart function by PPARα agonists and furthermore the positive impact on failure development caused by hypertrophy, inflammation and fibrosis has recently been demonstrated in a variety of animal models. For instance, Ichihara *et al.* demonstrated cardioprotective effects of fenofibrate in Dahl salt-sensitive rats, which were associated with significant decreases in mRNA expression of brain natriuretic peptide and atrial natriuretic peptide (ANP), two relevant clinical biomarkers which correlate with the severity of heart failure [66]. The importance of antiinflammatory actions of PPARα activation in heart failure has been highlighted by Ogata *et al.*, who demonstrated an inhibition of NF-κB pathways in rats treated with desoxycorticosterone acetate (DOCA) [67]. Protection from hypertensive end-organ damage has recently been demonstrated in the DOCA salt rat model using fenofibrate [68]. In addition, cardiac function as well as myocardial fibrosis was ameliorated in rats with heart failure following permanent coronary artery occlusion after chronic treatment with a novel PPARα agonist [69].

The original PPARα agonists, which are characterized by a fibrate-like chemical structure, show a rather weak activity on human PPARα in cell-based transactivation

assays, requiring agonist concentrations in the high micromolar range [70]. Therefore, it is not surprising that fibrates are clinically dosed at 300–1200 mg/day to evoke therapeutic benefits for the treatment of dyslipidemia. As shown in Figure 13.8, bezafibrate and Wy14643 exhibit almost a pan-PPAR profile, whereas clofibrate and fenofibrate show at least 10-fold selectivity towards PPARγ.

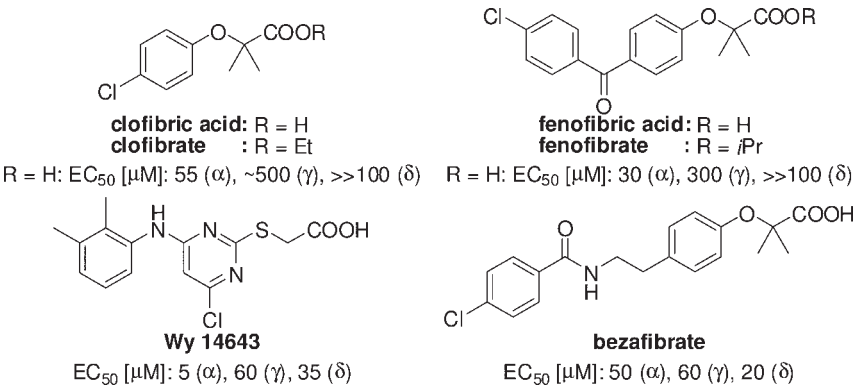

Figure 13.8 Fibrate-like PPARα agonists and their activity on the human receptor isoforms.

Despite its proven benefit in the control of blood glucose, PPARγ agonists have been associated with an increased incidence of myocardial infarction and death from cardiovascular causes [71]. Numerous companies are therefore working actively on specific PPARα modulators, and a number of discovery and preclinical programs have been initiated with the aim of improving potency and selectivity compared to the fibrates. PPARα-selective compounds that are currently under development are shown in Figure 13.9. With the exception of K-111 (for a recent review, see Ref. [72]), which is developed for the treatment of type 2 diabetes mellitus, the development of all known PPARα activators is focused on lipid metabolism.

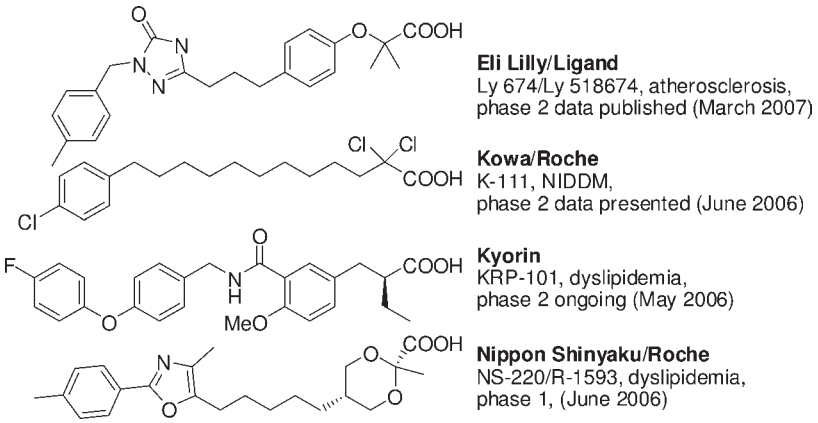

Figure 13.9 PPARα-selective agonists in clinical phases.

In addition to the structures already discussed, Dr Reddy's Laboratories in collaboration with Perlecan Pharma are developing DRF 10945, an oral PPARα agonist, for the potential treatment of dyslipidemia, which is currently in clinical phase 2.

Recently results from phase 2 clinical trials with Ly 674/Ly 518674 in dyslipidemia and atherosclerosis have been published. Despite potency four magnitudes higher than fenofibrate and a greater selectivity, no advantage on lipoproteins compared to fenofibrate have been observed [73]. In the study setup, both Ly 674/Ly 518674, as well as fenofibrate raised safety concerns. Furthermore, no development has been reported by GlaxoSmithKline's GW 590735 that was in development following a similar therapeutic approach. Up to now, only Sanofi-Aventis have reported pursuing a PPARα program for the treatment of chronic heart failure. AVE-8134 is currently in phase II for the treatment of metabolic disorders and in phase I targeting CHF. It will be interesting to see whether selective PPARα agonists will have important contributions in the treatment of heart failure in the future.

13.5
VDR (NR1I1)

Vitamin D plays an essential role in the calcium homeostasis and bone formation in vertebrates. It is either acquired via the diet or from *de novo* synthesis in the skin. Mellanby [74] described the connection between a dietary vitamin and the prevention of a skeletal disorder, rickets, already in 1919. Vitamin D is metabolized in two enzymatic steps to 1α,25-dihydroxyvitamin D_3 (calcitriol) which is the physiological ligand for the VDR. VDR is expressed in a variety of tissues and organs including kidney, heart, liver, vasculature, immune system and brain underlining that vitamin D and its receptor are involved in several fundamental processes. However, its relevance for cardiovascular diseases has only recently become clear and will be briefly summarized in the following. Epidemiological studies documented an interesting inverse relationship among plasma vitamin D and blood pressure in humans. Clinical studies have also shown that vitamin D supplementation reduces blood pressure in hypertensive patients [75, 76]. Moreover, it has been recognized that in patients with severe CHF, endogenous vitamin D levels are below normal [77] and that vitamin D insufficiency leads to ventricular hypertrophy at least in the case of dialysis patients. Accordingly, administration of calcitriol to dialysis patients with secondary hyperparathyroidism (a consequence of vitamin D deficiency) produced reductions in the left ventricular mass index in association with reduced renin, angiotensin II and ANP [78]. Studies in VDR knockout mice provided possible clues for the clinical observations described above in humans. These mice are hypertensive with elevated plasma angiotensin II levels and renal renin expression, and exhibit cardiac hypertrophy [79, 80]. In line with these data, administration of calcitriol reduces renal renin expression in wild-type animals. It was found that ligand-bound VDR is a direct suppressor of the renin gene in renal cells and the ANP gene in cardiomyocytes [81, 82] offering a reasonable rationale for the modulation of blood pressure and left ventricular hypertrophy in patients. Therefore, VDR agonists may

offer therapeutic potential for renal and cardiovascular diseases. Almost all known VDR agonists are analogs of calcitriol. In fact more than 3000 synthetic calcitriol analogs are presently known [83] and it will be interesting to see whether a synthetic VDR agonist will enter clinical trials in the future.

13.6
Retinoic Acid Receptors (NR1B1, 2 and 3) and Retinoid X Receptors (NR2B1, 2 and 3)

Natural retinoids are derivatives of vitamin A. The most important endogenous retinoids are all-*trans*- and 13-*cis*-retinoic acid which is bound by the family of retinoic acid receptors (RARs) consisting of three subtypes, RARα, RARβ and RARγ. RARs form heterodimers with retinoid X receptors (RXRs). RAR and RXR agonists synergize in activating the respective heterodimers but besides some synthetic retinoids, RXRs bind the natural 9-*cis*-retinoic acid. Retinoids regulate a wide variety of biological processes including cellular differentiation and proliferation, growth arrest, and apoptosis. In contrast, aberrant retinoid signaling mechanisms have been linked to cancer and skin disorders. Accordingly, retinoids are used in a variety of chemotherapeutic settings as well as dermatological indications including acne, psoriasis or photoaging. Wagner's group was the first to investigate the effect of all-*trans*-retinoic acid (ATRA) in renal injury and reno-parenchymal hypertension. In a rat model of mesangioproliferative nephritis, ATRA impressively prevented arterial hypertension and renal failure, glomerulosclerosis, interstitial fibrosis, and dramatically reduced proteinuria and macrophage infiltration [84, 85]. In addition, ATRA reduces angiotensin II synthesis and AT1 receptor expression in the kidney, [85], indicating that direct or indirect inhibition of the renin–angiotensin–aldosterone system may account for the observed benefits. The therapeutic potential of activation of the RAR is further illustrated by the fact that even moderate vitamin A deficiencies leads to reduced nephron numbers [86]. It has been suggested that low vitamin A supply to the fetus plays a role in the intrauterine programming of chronic renal disease and hypertension [87]. Investigations of retinoid nuclear receptor isotype-selective ligands revealed that RARα-specific stimulation is sufficient to reduce renal damage and hypertension in rats with established chronic glomerulonephritis [88] indicating the possibility to develop RARα-selective agonists for the treatment of renal diseases. It remains to be seen whether these selective agonists will have a side-effect profile which is acceptable for the use in chronic cardiovascular or renal disease.

13.7
Liver X Receptors (NR1H3 and NR1H2)

The two liver X receptors (LXRs), LXRα and LXRβ, have recently been implied as drug targets for the treatment of cardiovascular diseases. Initially, LXRs were regarded as 'whole-body-cholesterol sensors' activated by physiological concentra-

tions of 25-hydroxycholseterol and 24(*S*),25-epoxycholesterol. Generation of LXRα-deficient mice in which a high cholesterol diet markedly increases LDL cholesterol and decreases HDL cholesterol levels has been crucial for the understanding of the function of this receptor in regulating cholesterol homeostasis. Endogenous or synthetic LXR agonists induce cholesterol efflux and reverse cholesterol transport leading to lowered LDL cholesterol and raised HDL cholesterol levels. From a mechanistic point of view this is mainly achieved by direct activation of genes related to lipid transporters and enzymes involved in bile acid production and fatty acid synthesis by the ligand-bound nuclear hormone receptor LXR. Accordingly, synthetic LXR ligands have been shown to inhibit the development of atherosclerosis in mice [89, 90]. Moreover, it has been shown recently that 24(*S*),25-epoxycholesterol may selectively upregulate expression of macrophage LXR-regulated genes involved in cholesterol efflux without stimulating genes linked to fatty acid synthesis and triglyceride accumulation [91]. LXR also inhibits macrophage-derived inflammation [92]. It is remarkable in this regard that LXR agonists such as T0901317 and GW3965 (Figure 13.10) are capable of repressing the interleukin-1β/interleukin-6-induced release of C-reactive protein from human hepatocytes [93]. CRP is an acute-phase protein and an independent risk factor for future cardiovascular events. Inhibition of this important risk marker by modulating LXR is an interesting new pharmacological approach.

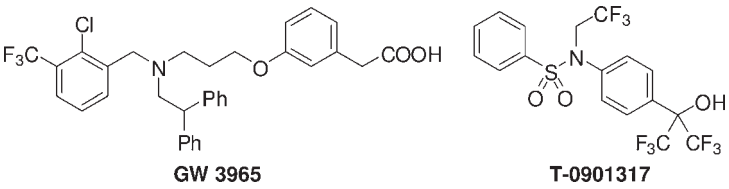

Figure 13.10 LXR pan-agonists.

One of the most surprising recent observations regarding ligand identification of a nuclear hormone receptor was the discovery that the LXRs bind D-glucose and D-glucose-6-phosphate at physiological concentrations [94]. Thus, LXR may act as a transcriptional switch that integrates hepatic glucose metabolism and fatty acid synthesis, and is therefore a promising pharmacological target for the treatment of the metabolic syndrome (or syndrome X) addressing fundamental mechanisms of this syndrome including cholesterol and glucose homeostasis and inflammation.

13.8 Outlook

Beyond the established role of nuclear hormone receptors in the control of endocrine, metabolic and inflammatory processes, an increasing amount of data indicates a significant role of nuclear hormone receptors for the pathophysiology of a variety

of cardiovascular diseases. Following the identification of these previously unrecognized roles, there is substantial activity within the pharmaceutical industry to develop nuclear hormone receptor modulators with the specific aim of treating cardiovascular diseases. We hope that we could draw the reader's attention to some of the intriguing new aspects in this field. It will be fascinating to follow the progress of the many projects and we hope that they will ultimately lead to an improvement of the standard of care of cardiovascular disease.

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14
NR4A Subfamily of Receptors and their Modulators

Henri Mattes

14.1
Introduction

Nuclear receptors comprise a large family of transcription factors that are critical during development and adult physiology. Nuclear receptors include steroid hormone receptors, and receptors for other lipophilic ligands such as vitamin D_3, thyroid hormone and retinoids [1]. Several members of the nuclear receptor superfamily lack identified ligands, however, and are therefore referred to as orphan receptors [2]. NR4A1 (Nur77, TR3, NGFI B, N10, DHR38, NAK-1, TIS1) [3, 4], NR4A2 (Nurr1, HZF-3, RNR-1, TINUR, NOT) [5] and NR4A3 (NOR-1, TEC, MINOR, CHN) [6] constitute the NR4A subfamily of orphan nuclear receptors [7].

The NR4A receptors are immediate/early-response genes and can be induced by a wide range of physiological signals, such as fatty acids, stress, prostaglandins, growth factors, calcium, inflammatory cytokines, peptide hormones, phorbol esters and neurotransmitters [8–17]. Physical stimuli (e.g. magnetic fields, mechanical agitation and membrane depolarization) have also been shown to induce expression of the NR4A receptors [10, 13, 18, 19]. The ability to sense and rapidly respond to changes in the cellular environment thus appears to be a hallmark of this subfamily of orphan nuclear receptors.

14.2
Functions of NR4A Receptor Subfamily

14.2.1
Expression of NR4A1, NR4A2 and NR4A3

The members of the NR4A subfamily are expressed, mostly at low levels, in a wide variety of metabolically demanding and energy-dependent tissues, such as skeletal muscle, adipose tissue, heart, kidney, T cells, liver and brain [20–25]. They are, however, expressed at very high levels in a range of cell types when cells are

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

stimulated by outside signals, including serum, nerve growth factor and receptor engagement. In this regard, the expression pattern of NR4A receptors is reminiscent of immediate/early-response genes such as c-*fos* and c-*jun*. The diversity of signals that induce NR4A receptor expression suggests that the function of these receptors is likely to be highly dependent on cell type and context.

NR4A1 is widely expressed, but primarily in the thymus, osteoblast, liver and pituitary gland [24, 26]. NR4A1 has recently been found in macrophages within human atherosclerotic lesions, suggesting a potential role for NR4A receptors as transcriptional mediators of inflammatory signals during atherogenesis [27].

NR4A2 is mainly expressed within the central nervous system (CNS) during development and in adult mice and rats. Expression is limited to several brain areas including the cortex, ventral midbrain, brain stem and the spinal cord [5, 28]. NR4A2 expression is detected as early as embryonic day 10.5 in the ventral midbrain in developing dopamine (DA) neurons [29, 30] and expression in these cells continues into adulthood.

The tissue expression pattern of NR4A3 is similar to that of NR4A1 [31]. NR4A3 mRNA was detected at low levels in the adult heart and skeletal muscle as well as in the fetal brain. It is expressed at high levels in the pituitary gland. The expression kinetics of NR4A3 are similar to that of NR4A1 in activated T cells, and they appear to play partially redundant roles in T cells and adrenal glands [31, 32].

A detailed analysis of the circadian expression of nuclear receptors in liver, skeletal muscle, white adipose tissue and brown adipose tissue recently revealed that all three members of the NR4A subfamily exhibit circadian-like patterns of expression in these tissues. They follow a unique rhythmic pattern in which their transcripts spike, as a single transient pulse, at Zeitgeber's Time (ZT) 4, followed by a steep decline in the next 4 h. They remain at low levels through the rest of the day. Since ZT 0 is the start of the light cycle, it was speculated that light might serve as an indirect environmental cue that triggers the expression of the NR4A subfamily of receptors in the peripheral tissues [33].

14.2.2 Mechanisms and Roles of NR4A1, NR4A2 and NR4A3

The NR4A subfamily of nuclear receptors activates gene expression in a constitutive ligand-independent manner [34–37]. The DNA-binding site for the NR4A receptors is the octanucleotide 5′-A/TAAAGGTCA [NGFI-B response element (NBRE)] [38–40] where they bind as monomers [37] and homodimers [41, 42]. The transcriptional target of NR4A homodimers, called the Nur-responsive element (NurRE), is an inverted repeat of the NBRE-related octanucleotide, AAAT(G/A)(C/T)CA, and this motif is naturally occurring in the pro-opiomelanocortin gene promoter [42]. NR4A1 and NR4A2 (but not NR4A3) can also bind as heterodimers with the retinoid X receptor (RXR) to mediate retinoid signaling [24, 43]. These RXR heterodimers bind a motif called DR-5, comprised of two direct repeats of the consensus nuclear receptor binding motif separated by 5 nucleotides [43]. Furthermore, heterodimers can also be formed between different members of the subgroup [41].

NR4A1, NR4A2 and NR4A3 have been implicated in cell growth/survival and apoptosis. These seemingly divergent effects have been related to different biochemical mechanisms. The NR4A subfamily can act as transcription factors, whose activation upregulates target genes, leading to cell proliferation and survival [44–46] or to cell apoptosis [47, 48]. They have also the ability to translocate from the nucleus to the cytoplasm, targeting the mitochondria and inducing cytochrome *c* release [49, 50].

Overexpression of NR4A1 was shown to prevent ceramide-induced cell death in a mature B cell lymphoma [46] and caused resistance to retinoic acid treatment in certain human lung cancer cells [51]. Ectopic expression of NR4A1 in mouse embryonic fibroblasts was shown to antagonize tumor necrosis factor-α-induced apoptosis. Moreover, caspase-3 and caspase-8 activities were reduced in cells overexpressing NR4A1, indicating their crucial roles in these effects [44]. Finally, small interfering RNA (siRNA)-mediated silencing of NR4A1 leads to a drastic reduction in cell growth/survival with increased levels of apoptosis in a number of cancer cell lines [45]. On the other hand, overexpression of NR4A1 in transgenic mice resulted in high levels of apoptosis in thymocytes [52] confirming its proapoptotic role *in vivo*.

NR4A1 nullizygous mice have no discernible phenotype and display no abnormalities in hypothalamic regulation, pituitary function, adrenal steroidogenesis [53], and in thymic and peripheral T cell death [54], indicating a functional redundancy among NR4A nuclear receptors.

NR4A2 gene targeting in mice has shown that it is critical for the development of midbrain DA neuron. Although expressed in various regions of the developing neuroaxis, loss of NR4A2 function in nullizygous mice leads to the absence of ventral mesencephalic neurons. [29, 55, 56]. Moreover, these mice die soon after birth due to respiratory failure, likely associated with abnormalities within the brain stem [57]. Histological analyses have also indicated a subtle disorganization of vagus nerve fibers derived from the dorsal motor nucleus of NR4A2-deficient mice [30]. The mechanisms through which NR4A2 mediates its action on midbrain dopaminergic neuron development and/or maturation is still poorly understood.

NR4A3 nullizygous mice were reported to exhibit abnormal ear development and have a defect in gastrulation [58]. NR4A3 is expressed early during embryogenesis, and its suppression leads to embryonic lethality around embryonic day 8.5. The mutant embryos fail to complete gastrulation and display distinct morphological abnormalities, including a decrease in overall size, developmental delay and an accumulation of mesoderm in the primitive streak during gastrulation. [59]. Furthermore, the NR4A3 nullizygous mice are sensitive to excitotoxic glutamate receptor agonists and display increased limbic seizure activity. This phenotype is associated with impaired postnatal hippocampal development, abnormal axon guidance and postnatal death of hippocampal pyramidal neurons [60].

14.2.3
Target Genes of the NR4A Subfamily

To understand the functions of NR4A receptors and the mechanisms whereby they influence developmental and adult biological processes, it is critical to search for

NR4A target genes. Despite the identification of binding sequences for the NR4A receptors and the growing evidence that NR4A receptors mediate the biological responses of a variety of extracellular stimuli, very few direct target genes for NR4A receptors have been identified so far.

NR4A1 has been identified as an important regulator of hormonally regulated gene expression in several endocrine tissues, including the pituitary (pro-opiomelanocortin gene) [42], the ovary (20α-HSD gene) [61], the adrenals (Cyp11B2 gene and hHSD3B2) [62, 63] and testis (hHSD3B2 and Cyp17 gene) [63, 64]. These data suggest a role for NR4A1 in steroidogenic gene expression in all three steroidogenic tissues.

Owing to the high expression levels in specific regions of the brain, the majority of NR4A2 studies examined its role in the central nervous system. NR4A2 plays a role in the transcriptional activation of tyrosine hydroxylase (TH), an enzyme involved in the synthesis of DA [65], as shown in gain-of-function experiments in adult hippocampus-derived progenitor cells. It directly regulates, in a RXR-independent manner, the TH gene via an NBRE located in the TH promoter. Moreover, the Nurr1 nullizygous genotype resulted in the absence of ventral midbrain DA neurons, as shown by a lack of TH expression.

Neuropilin-1 (NRP1) was recently identified as a novel NR4A2 target gene [66]. NRP1 expression was rapidly upregulated by NR4A2 in MN9D cells and *in situ* hybridization analysis showed that NRP1 was coexpressed with NR4A2 in the brain stem dorsal motor nucleus. Importantly, NRP1 expression was downregulated in this area in NR4A2 nullizygous mice. Moreover, two functional NR4A2-binding sites were identified in the NRP1 promoter and NR4A2 was shown to be recruited to these sites in MN9D cells, further supporting that NRP1 is a direct downstream target of NR4A2. Taken together, these findings suggest that NR4A2 might influence the processes of axon guidance and/or angiogenesis via the regulation of NRP1 expression.

Using a dopaminergic cell line in which NR4A2 content was tightly regulated, differential display analysis identified transcripts altered by NR4A2 expression [67], including the mRNA encoding vasoactive intestinal peptide (VIP), a potent multifunctional neuropeptide [68, 69]. NR4A2 was shown to regulate VIP both at the mRNA level and the protein level by direct transactivation of the VIP promoter through NBREs. NR4A2 regulation of VIP was also demonstrated *in vivo*. VIP message is expressed in the developing midbrain, particularly in dopaminergic precursor cells, and a significant reduction of VIP mRNA content is observed in NR4A2 nullizygous embryos compared to wild-type controls. These observations suggest a role of NR4A2 regulated VIP transcription for midbrain dopaminergic neuron development and maturation.

Other functions associated with NR4A2 include regulation of aldosterone synthase in adrenal cortex [62] and aromatase in ovarian granulosa cells [70]. The DA transporter is regulated via a mechanism depending on NR4A2 binding indirectly to a non-NBRE DNA-binding site, probably via interaction with other transcription factors [71, 72]. In addition, NR4A2 is required for embryonic DA neuron expression of amino acid decarboxylase, vesicular monoamine transporter-2, receptor tyrosine kinase Ret and the cyclin-dependent kinase inhibitor $p57^{Kip2}$ [30, 73, 74]. However, it remains unclear if NR4A2 regulation of these genes is direct or indirect.

The orphan nuclear receptor NR4A2 is mainly expressed in the CNS, but is also detected in certain peripheral tissues such as bone. It was recently shown that transfection of NR4A2 in osteoblastic cells increased osteopontin (OPN) mRNA expression [75]. The activation of the OPN promoter was mediated by the monomeric form of NR4A2, required direct binding of NR4A2 to the OPN promoter and was dependent on activation function 1 (AF-1). Two potential NBREs in the rat osteocalcin (Ocn) promoter where recently detected by computer analysis of osteoblastic promoters [76]. Moreover, recombinant and endogenous NR4A2 protein from primary mouse osteoblasts bound to the proximal NBRE-like site, which maps to the cAMP-responsive *cis*-element, in the Ocn promoter as shown by electrophoretic mobility shift assay. Endogenous NR4A2 protein bound to this site as a monomer, because neither RXR nor RXR antibody supershifted the protein–DNA complex. Moreover, NR4A2 protein induced Ocn mRNA in primary rat osteoblasts. In summary, NR4A2 activates the OPN and the Ocn promoters directly in osteoblastic cells, suggesting a role for NR4A2 in the regulation of bone homeostasis.

Using a 'gain-of-function' approach to identify potential target genes for NR4A receptors in macrophages, genes implicated in inflammatory signaling [MARCKS, NFκB-inducing kinase (NIK), inducible IκB kinase (IKKi/IKKε)], cell cycle control (cyclin D2) and apoptosis (cathepsin E) [27] were found to be significantly induced by NR4A receptors. It was furthermore shown that radiolabeled IKKi NBRE oligonucleotide was bound efficiently by *in vitro* translated NR4A1, NR4A2 and NR4A3, and that all three NR4A subfamily members transactivated the IKKi promoter. The ability of NR4A receptors to regulate NIK and IKKi suggests that their induction may potentiate gene activation in response to inflammatory stimuli. It should, however, be mentioned that the murine promoter of IKKi was shown to contain a functional NBRE, whereas the human IKKi promoter does not contain this NBRE.

14.3
Structures of NR4A1 and NR4A2

Nuclear receptors encompass several distinct functional domains. A highly conserved DNA-binding domain, comprising two zinc fingers, is used for specific DNA binding to hormone-response elements localized in the promoters of regulated genes and a somewhat less conserved ligand-binding domain (LBD) is localized in the C-terminal region of nuclear receptors. Extensive functional and structural analyses have revealed how nuclear receptor LBDs bind ligands, and how the LBD can mediate both repression and activation of transcription. Accordingly, in the absence of ligands, many nuclear receptors interact with corepressor proteins that effectively block transcription [77]. Ligand binding induces a conformational transition involving repositioning of the C-terminal amphipathic α-helix (H12) of the canonical LBD structure [78]. Together with other regions of the ligand-activated LBD, H12 forms a well-defined surface [activation function 2 (AF-2)] recognized by coactivator proteins utilizing the alleged LXXLL motifs within amphipathic α-helices for nuclear receptor LBD recognition [79, 80]. These proteins are key components of multiprotein

complexes and mediate nuclear receptor dependent gene activation by inducing histone acetylation and other chromatin modifications [77]. In addition to AF-2, several nuclear receptors contain a less-conserved and a poorly characterized constitutive activation domain (AF-1) within their N-terminal domains. Thus, in the classical model of nuclear receptor regulation, a hydrophobic cleft in the LBD recruits cofactors that function as coactivators or corepressors of transcription. Classically, ligand binding to LBD of nuclear receptors is thought to induce conformational changes in the receptors, resulting in association of the C-terminal AF-2 domain with coactivators. Following these rearrangements, nuclear receptor–ligand complexes contact the basal transcriptional machinery to increase transcriptional activity. However, the NR4A receptors encode unusual and atypical LBDs, which do not function in a classical manner [36].

The elucidation of the NR4A2, DHR38 [*Drosophila* ortholog of mammalian NR4A1 (NR4A4)] and NR4A1 LBD structures using X-ray crystal studies [81–83] has shown that they lack a hydrophobic pocket within their putative LBD. Instead, bulky aromatic or hydrophobic residues occupy the region in the NR4A's LBD that correspond to the ligand-binding pocket in other nuclear receptors. Thus, these residues seem to prevent ligand binding. Modeling studies that revealed the orphan receptors Rev-erb α and β encode LBDs occupied by bulky side-chains [84] led to similar conclusions. Despite these, it has been demonstrated that the LBD of NR4A2 (and, to a lesser extent, NR4A3) can activate transcription. This activation is cell type-dependent, and significant differences have been observed for the transactivation efficiencies of NR4A1, NR4A2 and NR4A3 [85]. Solving these crystal structures also revealed that these receptors are folded in a conformation resembling ligand-activated nuclear receptor LBDs. Thus, the 'active' conformation is independent of ligand binding. This is reminiscent of modeling studies with another nuclear receptor, RORα, that demonstrated the AF-2 domain is locked permanently in the holo-conformation described for other liganded receptors and thus enables ligand-independent recruitment of coactivators [86]. The NR4A LBDs display another distinctive feature. Recent crystal and nuclear magnetic resonance studies identified a hydrophobic patch between H11 and H12 that interacts with cofactors and modulates cell type-dependent transcriptional activity [83, 87]. It was furthermore demonstrated that the H11–H12 regions of NR4A1 and NR4A2 are functionally not exchangeable. Structural and functional comparison of the LBDs of NRA41 and NR4A2 finally suggested that a specifically positioned H12 that contributes directly to a novel coregulator surface determines the cell type-dependent AF-2 transcriptional activities of NRA41 and NR4A2.

Together, these data explain previous difficulties encountered attempting to identify NR4A subfamily ligands and also provide a structural basis for NR4A's constitutive activities.

14.4 Modulators of the NR4A Subfamily

Currently, ligands have been identified for only half of the known nuclear receptors; the remaining receptors, known as orphan nuclear receptors, constitute a promising

area for research and development. The NR4A subfamily of nuclear receptors has been implicated in Parkinson's disease, schizophrenia, manic depression, atherogenesis, Alzheimer's disease, rheumatoid arthritis, cancer and apoptosis. This has led to high interest in the identification of selective low-molecular-weight (LMW) modulators that may help to elucidate the mode of action of the NR4A subfamily.

14.4.1 Indirect LMW Modulators of the NR4A Subfamily

Studies have shown that NR4A1, NR4A2 and NR4A3 are rapidly modulated in different cell types by a variety of small molecules.

The most common saturated and monounsaturated long-chain fatty acids, myristate (C14 : 0), palmitate (C16 : 0) and oleate (C18 : 1, ω9), are very effective in inducing the accumulation of NR4A1 mRNA in the β-cell line INS-1 or in isolated rat islets. A strong accumulation occurred in INS-1 cells at high concentration of palmitate and oleate (0.4–0.5 mM), whereas a modest accumulation was observed at low concentrations of the other fatty acids (0.1–0.2 mM) in the presence of 0.5% bovine serum albumin. On the other hand, the related polyunsaturated fatty acids linoleate (C18 : 2, ω6), linolenate (C18 : 3, ω3), docosahexaenoate (DHA) (C22 : 6, ω3), arachidonate (C20 : 4, ω6), the nonmetabolizable fatty acid analog 2-Br-palmitate (2Br-C16 : 0), short-chain fatty acids decanoate (C10 : 0) and octanoate (C8 : 0) barely changed the expression level of NR4A1 mRNA [14].

A strong induction of NR4A1 and NR4A3 receptor expression was observed with 10 μM 25-hydroxycholesterol or 7β-cholesterol in THP-1 cells [88]. Oxysterols also induced the expression of the NR4A subfamily members in 12-*O*-tetradecanoylphorbol-13-acetate-differentiated THP-1 cells, as well as in primary human monocyte-derived macrophages. Some cell-type specificity in the response to oxysterols was, however, noted. NR4A1 was consistently induced in all cell types while NR4A3 was induced in THP-1 cells but not in human monocyte derived macrophages.

Activation of a number of G-protein-coupled receptors has recently been shown to result in modulation of NR4A receptors. Typical and atypical antipsychotic drugs like haloperidol, raclopride, fluphenazine, chlorpromazine, clozapine, olanzapine, quetiapine and risperidone can induce differential patterns of NR4A receptors [89, 90]. Most antipsychotic drugs tested strongly induced NR4A1 and NR4A3 in the striatal complex (striatum, accumbens) and cortex. They increased NR4A2 mRNA levels in the substantia nigra and ventral tegmental area. Inductions of NR4A2 by antipsychotic drugs are correlated with the expression of DA D2 receptor (D2R) in the striatum and D2 and D3 receptor subtypes in the nucleus accumbens. It was furthermore demonstrated that D2R receptor stimulation, following DA treatment, induced the activation of NR4A2 and NurRE-dependent transcription [91]. NurRE activity was blocked by pertussis toxin and by the mitogen-activated protein kinase kinase inhibitor, PD98059, showing that D2R-mediated activation of extracellular signal-regulated kinase (ERK) is crucial for the DA induced activation of NR4A2. Likewise, expression of the mRNA encoding NR4A3 is also strikingly and transiently induced, in the C2C12 skeletal muscle cell line, by stimulation of the β_2-adrenergic

receptor [92]. This effect was shown to involve activation of the NR4A3 promoter. Expression of NR4A3 mRNA was also increased significantly *in vivo* in mouse skeletal muscle (plantaris) 1 h after treating mice with a β_2-adrenergic receptor agonist [92]. On the contrary, methotrexate (MTX) significantly suppresses expression of NR4A2 *in vivo* in patients with active psoriatic arthritis, who were prescribed low-dose MTX for management of peripheral arthritis. Importantly, reduction in NR4A2 levels correlates with changes in disease activity score [93]. The inhibitory effect of low-dose MTX on NR4A2 activation was shown to be mediated through the adenosine receptor A2A [93].

6-Mercaptopurine (6-MP), which is widely used as an antineoplastic and antiinflammatory drug, has been shown to activate NR4A2 and NR4A3 in an AF-1-dependent manner, independently of direct binding [94, 95]. It was furthermore demonstrated that NR4A3-mediated transcriptional activation is potentiated by the coactivator thyroid hormone receptor-associated protein TRAP220 and that 6-MP modulates the activity of TRAP220 in a dose-dependent manner, with an EC_{50} around 30–50 μM. The effect of 6-MP on NR4A2 transcriptional activity is fully inhibited in the presence of 15 μM adenosine. Taken together, these data suggest that the cellular content of purine nucleotides may modulate both the expression and transcriptional activity of the NR4A subfamily.

Finally, forskolin induced upregulation of NR4A2 protein expression and nuclear translocation in MN9D cells was shown to be mediated primarily through the ERK1/2 pathway [96].

While a growing number of such modulators of activity or expression have been described, none seems selective enough to help in the elucidation of NR4A's mechanism of action or as potential therapy.

14.4.2
Direct LMW Modulators of the NR4A Subfamily

The therapeutic utility of the NR4A subfamily will certainly depend on whether novel assay technologies, in concert with reverse pharmacology and innovative chemistry, can identify selective small-molecule regulators (agonists, antagonists and/or selective modulators) directly interacting with this orphan nuclear receptor family. A growing number of such regulators have been identified and are highlighted below.

14.4.2.1 NR4A1 Activators and Suppressors

3,3′-Diindolylmethane is a metabolite of the phytochemical indole-3-carbinol and both compounds exhibit a broad spectrum of anticancer activities [97, 98]. 1,1-Bis(3′-indolyl)-1-(*p*-substitutedphenyl)methanes (C-DIMs) are synthetic analogs of 3,3′-diindolylmethane, which also exhibit anticancer activity and bind nuclear receptors such as the peroxisome proliferator-activated receptor (PPAR) γ [99, 100]. By screening a panel of structurally diverse C-substituted DIMs using a GAL4–NR4A1/GAL4 reporter gene assay in which a structure-dependent activation of NR4A1 was monitored in cells cotransfected with GAL4–NR4A1 (full-length) chimera or GAL4–NR4A1 (LBD) chimera, along with a reporter vector containing five GAL4

response elements linked to luciferase gene, *p*-trifluoromethyl (DIM-C-pPhCF_3) and two PPAR inactive analogs, *p*-methoxy (DIM-C-pPhOCH_3) and DIM-C-Ph, where found to activate NR4A1-dependent transactivation at 20 μM (Figure 14.1) [48]. Moreover, ligand-induced transcriptional activation was observed with GAL4–NR4A1 (LBD) chimeras in which only the LBD of NR4A1 is expressed. This suggested direct binding of the agonists to the LBD of NR4A1 and activation of NR4A1-mediated transcription. These results may sound surprising given the finding that while NR4A2 shares the general features of the classical ligand-activated nuclear receptors, the LBD of NR4A2 is filled with hydrophobic amino acid residues. The novel hydrophobic interaction surface that has recently been identified [87] could, however, serve not only for coactivator binding, but also as molecular target for NR4A2-activating compounds like those described above.

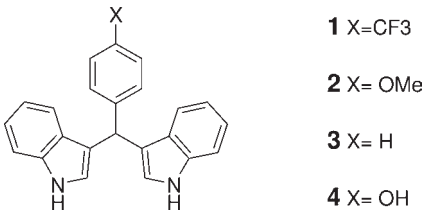


Figure 14.1 NR4A1 activators and suppressors.

The role of NR4A1 in mediating ligand-dependent transactivation was confirmed in studies showing that these responses were inhibited by either siRNA for NR4A1 or DIM-C-pPhOH, which exhibited NR4A1 antagonist activity. Binding of these compounds has also been shown to induce interaction between NR4A1 with a few coactivators like SRC-1, PGC-1 and TRAP220. Finally, these NR4A1 agonists significantly decreased survival of Panc-28 cells *in vitro*, by increasing apoptosis, with respective IC_{50} values for DIM-C-pPhCF_3, DIM-C-pPhOCH_3 and DIM-C-Ph between 1 and 5 μM. At a dose of 25 mg/kg/day every second day, given by oral gavage, DIM-C-pPhOCH_3 significantly inhibited tumor growth and tumor weights *in vivo* in athymic mice bearing Panc-28 cell xenografts. Analysis of tumors from control and treated animals by terminal uridine deoxynucleotidyl transferase dUTP nick end-labeling assay, however, indicated similar levels of apoptosis. This study showed for the first time that ligand-dependent activation of the orphan receptor NR4A1 induces apoptosis in cancer cells, suggesting that NR4A1 agonists may represent a new class of anticancer drugs.

14.4.2.2 **NR4A2 Activators**

Using a similar NR4A2 luciferase reporter gene assay in MN9D cells [102], a number of micromolar activators of NR4A2 (Figure 14.2) were discovered in a combinatorial library of benzimidazoles designed with a focus toward nuclear receptors. The choice of the benzimidazole core structure was based on several criteria: physicochemical properties, modularity and structural overlap with known nuclear receptor ligands. This scaffold which represents a common motif found in many compounds of

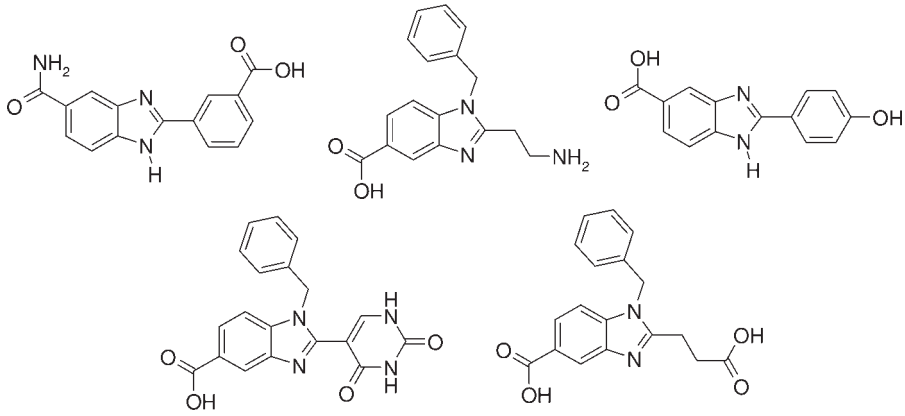
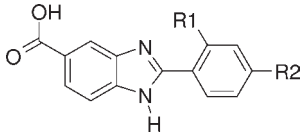


Figure 14.2 Selected NR4A2 activators discovered in the first-generation benzimidazole library.

medicinal interest is part of the core structure of retinoic acid receptor (RAR) antagonists [101]. The trisubstituted benzimidazole 5-carboxamide structure was selected as scaffold based on superposition experiments with a set of known nuclear receptor ligands and on 3D Manual docking experiments of potential ligands into nuclear receptor models [e.g. estrogen receptor (ER), PPAR] which further defined size and shape of potential side-chains. The limited structure–activity relationships gained from the first NR4A2 screening results indicated a clear preference for an acidic function in position 5 of the benzimidazole scaffold. It also highlighted the preference of a hydrophobic spacer linking an electronegative atom to position 2 of the scaffold. A second-generation library of benzimidazole-5-carboxylic acids biased toward NR4A2 was therefore synthesized. Very potent NR4A2 activators (EC_{50} 8–70 nM) (Table 14.1) were identified by screening this small biased library in the above-mentioned reporter gene assay. These compounds increased the luciferase reporter gene activity by at least a factor 2. Specificity of the effect was confirmed by measuring the activity of a second reporter gene lacking the NR4A2 responsive elements in front of the thymidine kinase (TK) minimal promoter [103]. Compound **5** displays very good aqueous solubility (above 0.2 g/l at pH 1 and 6.8). It's pharmacological selectivity was assessed against a large panel of receptors and enzymes for

Table 14.1 Benzimidazoles as NR4A2 activators.



Compounds	R1	R2	EC_{50} (nM)
5	H	Cl	8
6	H	F	24
7	Me	OH	70

which it had no measurable affinity when tested up to 30 μM. Finally, when given as a single 10 μmol/kg dose orally, the compound was still present in plasma (813 nmol/l) and brain (20 nmol/kg) 8 h after dosing. Given its potency, selectivity and properties, this new NR4A2 activator might serve as a useful tool to uncover the pathophysiological role of NR4A2.

Using the same reporter gene assay with NR4A2 overexpressing MN9D cells to screen the corporate compound collection, scientists at Novartis discovered another class of NR4A2 activators – isoxazolo[4,5-*c*]pyridin-4-ones (Figure 14.3) [104]. Specificity of the effect was again confirmed by measuring the activity of a second reporter gene lacking the NR4A2 responsive elements in front of the TK minimal promoter. Examination of the screening data and evaluation of further compounds from the substance collection revealed that three pharmacophoric elements present in the chemical structures were important for submicromolar activity: two aryl substituents linked by a heterocyclic, hydrogen-bond acceptor-containing system, resulting in a rather flat architecture. It was furthermore demonstrated that the amide group could be alkylated without loss of potency and that a variety of substitutions of the 6-phenyl ring were tolerated. Modifications of the 6-phenyl substituent of this class of compounds, with the goal to increase solubility, was reported in more detail. In general, small substituents were well tolerated especially in the 3- and 4-position, whereas larger substituents were tolerated only in the 4-position. Incorporation of larger ether substituents with the potential to increase the aqueous solubility was well tolerated. For instance, the benzylic ether **9** (Figure 14.3), as well as the corresponding methylated derivative **10**, showed a doubling of the basal luciferase activity in the cellular assay with EC_{50}s of 3.5 and 3.9 nM, respectively. Compound **10** also has reasonable aqueous solubility of 20 mg/l. Incorporation of a [1, 3]-dioxolane moiety into the 3,4-positions yielded the most potent compound described, **8**, with an EC_{50} of 0.8 nM. Its solubility was, however, rather low, thereby limiting its further use. In a preliminary pharmacokinetic experiment, **10** was found to have an excellent oral

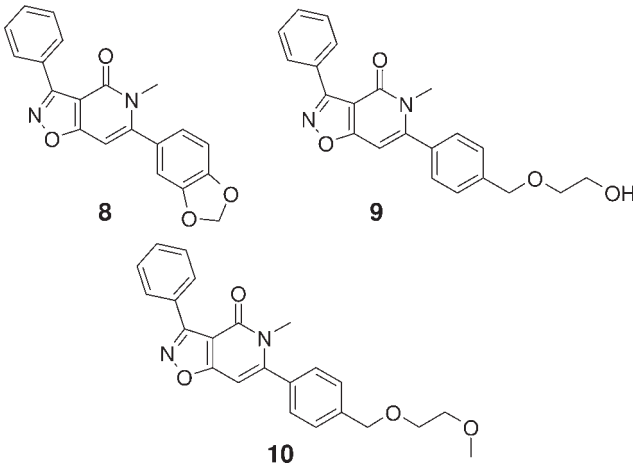


Figure 14.3 Isoxazolo[4,5-*c*]pyridin-4-ones as NR4A2 activators.

bioavailability of 95% in mice, and a rapid and extensive brain uptake, reaching levels more than two orders of magnitude higher than the cellular EC_{50} within 30 min [104].

14.4.2.3 NR4A3 Activators

By screening a large number of bioactive lipid-soluble mediator pathway metabolites contained in the Kyoto Encyclopedia of Genes and Genomes database in a reporter gene assay using GAL4–NR4A3 receptor chimera, prostaglandin (PG) A2 was shown to activate NR4A3-dependent transcription [12]. PGA1 also proved to have similar levels of transactivator activity to PGA2 in the presence of the GAL4–NR4A3 receptor chimera, enhancing the transcriptional activities at above 5 μM. It should, however, be mentioned that the effects of PGA2 on NR4A3-dependent transcription were relatively low compared to those of estradiol on ER and of retinoic acids on RAR. PGA1 and PGA2 did not behave as transactivators for the N-terminal AF-1 domain-deleted construct of NR4A3 in the assay. PGA2 was, moreover, shown to directly bind to NR4A3 in a way requiring the LBD domain of NR4A3. A PGA derivative, 13,14-dihydro-15-keto PGA2, which had no transactivation activity for the NR4A3-dependent transcription, did not bind to the NR4A3 LBD. While the target molecules or receptors for PGA2 have not yet been identified, the observations that the effects of PGA2 (induction of $p21^{Cip1}$, suppression of cyclin D1 and inducibility of cell death) were enhanced in spleen cells derived from NR4A3-overexpressing transgenic mice led to the suggestion that the effects of PGA2 are influenced, at least in part, by the expression levels of NR4A3.

14.4.2.4 Heterodimer Activators

Several lines of evidence suggest that NR4A1 and NR4A2 function as heterodimers with RXR, a receptor for 9-*cis*-retinoic acid, *in vivo*, although endogenous target genes for these heterodimers remain unknown. RXR ligands increase the number of dopaminergic cells and other neurons in the rodent embryonic central nervous system in a process mediated by NR4A2 heterodimers [105]. Suppression of haloperidol-induced dyskinesia in mice treated with RXR ligands, including HX531, required the expression of NR4A1 [106].

By screening a set of known RXR ligands using a reporter gene assay in which activation of NR4A1 was monitored in HEK293 cells transfected with CMX–GAL4–NR4A1, in combination with control CMX vector, CMX–RXRα, CMX–RXRβ or CMX–RXRγ along with a MH100(UAS)x4–TK–Luc reporter vector, the weak RXR agonist HX600 was found to activate NR4A1–RXR heterodimers at submicromolar concentrations (Figure 14.4) [107]. HX600 also activated the heterodimer formed by RXR and NR4A2. Close analogs of HX600, like HX531 and HX603, were weak activators of NR4A1–RXR and HX665 was not effective. Moreover, HX600 was found to activate NR4A1–RXR heterodimers in a selective manner, as the compound did not induce the transactivation of NR4A1, NR4A3–RXR, RARα–RXRα, FXR–RXRα, LXRα–RXRα, LXRβ–RXRα or PPARγ–RXRα. HX600 and HX603 activated the heterodimer formed by NR4A1 and the RXRα AF-2 deletion mutant, although induction was decreased compared to that of the wild-type heterodimer. These ligands had no effect on a NR4A1 AF-2 deletion mutant

transfected alone or in combination with RXRα. These findings suggest that the AF-2 domain of NR4A1, and to a lesser extent of RXRα, is required for activation of the NR4A1–RXR heterodimer by these RXR ligands.

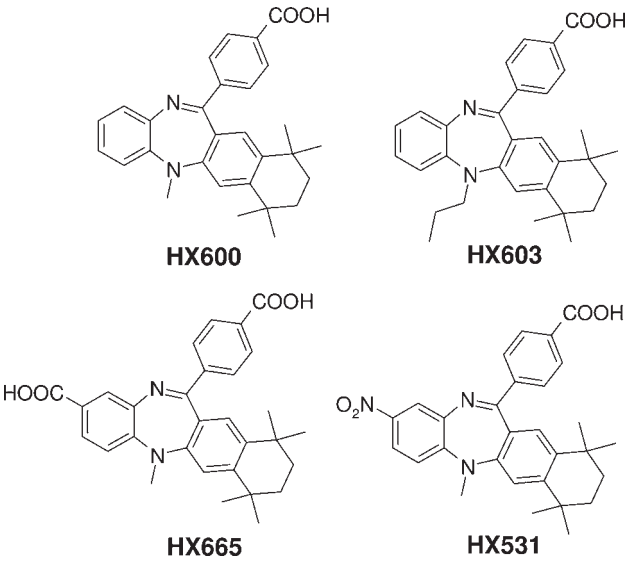


Figure 14.4 NR4A1–RXRα heterodimer activators.

Dibenzodiazepine-derived heterodimer ligands, such as HX600, may therefore represent useful tools for revealing previously unknown physiological roles of the NR4A1–RXR and NR4A2–RXR heterodimers.

14.5 Conclusions and Outlook

It is well known that most nuclear receptors have been implicated in human diseases. The importance of the NR4A subfamily of nuclear receptors as potential therapeutic targets for disease stems from their described implications in Parkinson's disease, schizophrenia, manic depression, atherogenesis, Alzheimer's disease, rheumatoid arthritis, cancer and apoptosis.

Apoptosis in many tumor types, including colon, breast, prostate, lung and gastric cancers, has been shown to involve the NR4A subfamily members. All three members are downregulated in no-transformed HeLaHF revertants, as compared with parental HeLa cells. NR4A1 is implicated in oncogenesis as part of the EWS fusion protein, resulting from chromosomal translocation found in human extra-skeletal myxoid chondrosarcoma tumors. Stable silencing of NR4A2 leads to a reduction in tumorigenicity of PC3 cells. Therefore, specific compounds that down-regulate NR4A-dependent transcriptional activity have the potential to be useful in the treatments of cancers.

Expression of all NR4A subfamily members has recently been identified in human atherosclerotic lesion macrophages. These receptors reduce the uptake of oxidized low-density lipoprotein and the inflammatory responses (i.e. release of proinflammatory cytokines and chemokines) in cultured human macrophages, providing further evidence for a protective role of NR4A receptors in atherogenesis.

NR4A1, as well as NR4A3, play a key role in apoptosis of T lymphocytes and eosinophils. They may therefore be implicated in disorders related to defects of activation-induced apoptosis, including autoimmune diseases and allergic diseases.

The facts that dopaminergic neurons lost in the substantia nigra pars compacta of Parkinson's disease patients depend upon NR4A2 for their development and that mutations in the NR4A2 gene were also found to be associated with familial Parkinson's disease suggest a potential for NR4A2 activators in the treatment of Parkinson's disease.

A number of recent studies demonstrate that despite the lack of a classical LBD, direct modulators for the NR4A subfamily can be identified. This suggests the possibility of pharmacological modulation of NR4A-specific pathways via regulation of receptor-dependent transactivation. Further studies profiling these compounds should allow to reveal previously unknown physiological roles of the NR4A subfamily and unravel their significance as therapeutic targets.

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15
Induction of Drug Metabolism: Role for Nuclear Receptors

Christoph Handschin

15.1
Introduction

Xenobiotics regulate the transcription of many genes in the liver, intestine, kidney and other organs to alter their own metabolism and excretion. The barbiturate phenobarbital (PB) is one of the best studied examples of a drug that potently induces cytochrome P450s (CYPs) and other genes in the liver [1]. The mechanisms that control the induction of detoxifying genes and the receptors recognizing those have remained elusive for many years [2]. This changed in 1998 when the pregnane X receptor [PXR, alternatively called pregnane activated receptor (PAR) or steroid and xenobiotic receptor (SXR); NR1I2] and the constitutive androstane receptor (CAR, alternatively called constitutive active receptor; NR1I3) were discovered to play a role in xenobiotic-mediated induction of CYPs [3–7]. It turned out that PXR and CAR could accommodate all the conundrums associated with induction of CYPs by xenobiotics. First, due to an unusually flexible ligand-binding domain (LBD), PXR and CAR are promiscuous towards many structurally diverse compounds. Second, as heterodimers with the retinoid X receptor (RXR; NR2B1, 2 and 3), PXR and CAR are able to bind to different DNA-binding elements and thus directly activate many genes involved in drug detoxification and excretion. Third, the LBDs of PXR and CAR orthologs are unusually divergent between species, and therefore are activated by different chemicals in a very species-specific manner. A number of review articles provide an up-to-date description of the basic functions of PXR and CAR [8–13]. This chapter is focused on the recent advances in the characterization of PXR and CAR. In particular, it highlights the novel findings about endogenous functions and their clinical implications.

15.2
PXR: Structure and Activation

Of the two xenobiotic-sensing nuclear receptors PXR and CAR, the former appeared to be the more 'conventional' nuclear receptor. Recent data challenge this view, and

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

have provided fascinating new insights into the structure, function and regulation of PXR. It has been obvious from sequence alignments and the first crystal structures of the PXR LBD that this receptor has a very large ligand-binding pocket [14–16]. The ligand-binding pockets of other nuclear receptors, notably the peroxisome proliferator-activated receptors (PPAR; NR1C1, 2 and 3), have similar volumes (reviewed in Ref. [17]). A recent PXR structure with the macrolide antibiotic rifampicin, however, revealed a new strategy by which PXR expands its repertoire of ligand binding [18]. Binding of rifampicin is achieved by structural disordering of part of the ligand-binding pocket in the presence of the ligand [18]. One wall of the LBD remains flexible; nevertheless, rifampicin potently activates human PXR. Thus, the promiscuity of PXR is further enhanced by this structural flexibility [19]. Interaction with a coactivator protein can subsequently stabilize binding of a ligand in the ligand-binding pocket. For example, the synthetic ligand SR12813 is found in three different conformations in the PXR ligand-binding pocket [20]. In the presence of the coactivator SRC-1, SR12813 is restricted to one conformation [20]. Thus, the combined action of ligand and coactivator protein results in stabilization of the conformation of the PXR LBD.

Second, the proposed mode of action of PXR includes heterodimerization with RXR and subsequent binding to DNA. This view has recently been expanded by structural observations that suggest homodimerization of PXR [21]. Conserved tryptophan and tyrosine residues in the PXR LBD form a dimer interface and provide a tryptophan zipper interface unique to nuclear receptors. Site-directed mutagenesis of the aromatic residues eliminates homodimer formation without affecting PXR–RXR interaction, ligand or DNA binding. Homodimerization-deficient PXR mutants are significantly less active and unable to recruit the coactivator SRC-1 [21].

Finally, new findings suggest that PXR shuttles between cytoplasm and nucleus upon activation, and does not exclusively reside in the nucleus as previously suggested. Three different domains, the nuclear localization sequence in the C-terminal region of the DNA-binding domain, the xenobiotic response sequence (XRS) and the activation function 2 (AF-2) in the LBD, are involved in ligand-induced nuclear translocation [22, 23]. In the cytoplasm, PXR is sequestered by the cytoplasmic CAR retention protein (CCRP) [23]. However, the mechanistic details of the effect of ligand binding on PXR localization are still poorly understood.

15.3
CAR: Structure and Activation

Tremendous progress in the understanding the biology of CAR has been made by solving the crystal structure of the LBD [24–26] (reviewed in Ref. [27]). Unique properties of helix 12 (H12)/AF-2 account for the ligand-independent and -dependent function of CAR. This helix is in a more rigid conformation compared to other nuclear receptors. The AF-2 conformation promotes interaction with RXR while also dividing the CAR ligand-binding pocket as a structural barrier. Potent CAR activators, such as the mouse CAR ligand TCPOBOP, can penetrate this barrier and when present in the

ligand-binding pocket, interact with hydrophobic residues in AF-2. Inverse agonists like androstanol disrupt the interaction between AF-2 and H4 of the ligand-binding pocket thereby destabilizing H12/AF-2 and preventing coactivator proteins from binding. Interestingly, the CAR ligand-binding pocket is about half the size of the PXR LBD. Experimental knowledge of the CAR structure and the high accuracy of homology models should now allow the identification of endogenous ligands and the design of chemicals that bind with high affinity [28].

Cytoplasmic–nuclear shuttling has been recognized as an important step in the regulation of CAR activity [29]. Several peptides and amino acids important for CAR localization have been found, including the XRS, a leucine-rich region at the C-terminus and Ser202 [30, 31]. The mechanism by which nuclear translocation of CAR is controlled remains unknown. However, several components of the CAR protein complex in the cytoplasm and the nucleus have been recently identified and the importance of posttranslational modifications in this process appreciated. In the cytoplasm, CAR is retained by binding to the tetratricopeptide CCRP and heat shock protein 90 (Hsp90) [32, 33]. Protein phosphatase 2A activity releases CAR from this complex and allows nuclear translocation, presumably by dephosphorylation of serine 202 [31, 32]. The PPAR-binding protein (alternatively called TRAP220 or MED1) is either required for the actual translocation step or serves as a nuclear retention signal [34]. Similarly, the p160 transcription factor GR-interacting protein 1 (GRIP1) increases nuclear accumulation of CAR by binding to the XRS [35, 36]. Furthermore, in the nucleus, binding of the cohesion protein structural maintenance of chromosome 1 prevents CAR from binding to ocadaic acid-sensitive DNA response elements in the human CYP2B6 promoter [37]. Finally, increased levels of CAR are also found at the cell membrane of mouse livers after PB treatment implying a nongenomic action exerted by CAR [38].

15.4 Detoxification of Drugs and Other Xenobiotics

The key roles for PXR and CAR in hepatic drug metabolism have been established unequivocally [39, 40]. Apart from the liver and the intestine, other tissues have been the focus of recent studies of the molecular mechanisms regulating drug metabolism by CAR and PXR. Most prominently, novel physiological functions of the xenobiotic-sensing nuclear receptors emerged from studies of the brain and the blood–brain barrier. For example, activation of PXR *in vivo* tightens the blood–brain barrier towards methadone [41]. Furthermore, PXR is activated by neurosteroids and nicotine in the brain [42]. Nicotine is metabolized by PXR-regulated CYPs and activation of PXR by nicotine could thus provide an explanation for the development of nicotine tolerance. Activation of PXR by nicotine could also play a role in the induction of drug-metabolizing enzymes in different lung compartments of smokers with likely implications for the metabolism of procarcinogens to carcinogens of tobacco smoke [43]. Therapeutically, activation of PXR in Niemann-Pick C disease might be a novel approach to relieve the cholesterol trafficking defect in Purkinje cells

in the cerebellum [44]. In contrast, CAR expression is reduced in human astrocytoma cells treated with cocaine, which might contribute to the cerebrovascular risks associated with cocaine abuse [45].

Expression of CAR and PXR in peripheral mononuclear cells was tested for correlation with expression of these two receptors in liver and intestine. Unfortunately, the correlation was not sufficient to be able to measure blood cell PXR and CAR as surrogates for drug metabolism activity in the liver and intestine and thereby avoiding detrimental drug–drug interactions [46]. However, expression of the xenobiotic-sensing nuclear receptors and their target genes is of importance in the metabolism of drugs targeting mononuclear blood cells. In particular, PXR regulation of P-glycoprotein is linked to bioavailability and accumulation of HIV protease inhibitors [47].

15.5
PXR and CAR Regulation of Bile Acid, Cholesterol and Bilirubin Metabolism

An early observation about endogenous functions of PXR and CAR has been their implication in hepatic bile acid metabolism [48, 49]. Most of the more recent findings on this topic are summarized in a number of reviews [9, 50–52]. Briefly, CAR and PXR have been shown to play overlapping but not identical roles in hepatic bile acid detoxification [53, 54]. Recent work has also drawn attention to the importance of PXR in cholesterol detoxification in the liver, kidney and intestine [55–59]. Both CAR and PXR are involved in bilirubin metabolism and clearance [60]; activation of these pathways is of potential therapeutic interest in jaundice and chronic arthritis [61].

Studies of the activation of CAR and PXR by bile acid intermediates and metabolites shed light on the evolution of the xenobiotic-sensing nuclear receptors. The highly divergent LBD of PXR might have evolved according the different bile salts encountered in different species [62, 63]. Interestingly, nonmammalian species only have a PXR ortholog and lack genomic information for a second xenobiotic-sensing receptor [64, 65]. Sequence analysis between nuclear receptor orthologs of the N1I subfamily suggests a recent appearance of the CAR gene just prior to the evolution of mammals, presumably by duplication of the PXR gene [66, 67]. It is not clear why mammals have two xenobiotic-sensing nuclear receptors in contrast to nonmammalian species that only have one. Future studies will provide insights into the question about the evolution of CAR in mammals, a constitutively active nuclear receptor that is mainly activated by cytoplasmic–nuclear translocation and not by ligand binding like many other nuclear receptors including PXR.

15.6
Xenobiotic-Sensing Nuclear Receptors in Cancer, Oxidative Stress and Pollution

Many of the xenobiotics that activate PXR and CAR are carcinogenic or tumor promoters. Classically, PB has been recognized to promote tumors in rodent, but not human livers. Recent studies now have shown that at least part of the tumorigenesis

is mediated by CAR, at least in the case of PB [68] (reviewed in Refs [69, 70]). In fact, CAR promotes hepatocyte proliferation and blocks apoptosis [71]. In part, liver tumor promotion is promoted by CAR-mediated alterations of DNA methylation [72]. Moreover, CAR induces the proproliferative transcription factor growth arrest and DNA damage inducible gene 45β (Gadd45β) and negatively influences tumor necrosis factor (TNF)-α signaling [71]. Finally, CAR-mediated upregulation of the antiapoptotic myeloid cell leukemia factor-1 (Mcl-1) and downregulation of the proapoptotic Bcl-2 antagonistic killer (Bak) and Bcl-2-associated X protein (Bax) likely contribute to the decreased apoptosis subsequent to CAR activation [73]. Similarly, PXR exerts antiapoptotic effects by inducing B cell CLL/lymphoma 2 (Bcl-2) and Bcl-2 related protein, long isoform (Bcl-x_L), two crucial apoptosis inhibitors [74]. Furthermore, PXR sensitizes normal and cancerous tissue to oxidative damage, which may contribute to carcinogenesis [75]. The involvement of CAR and PXR in tumor promotion and carcinogenesis is important with regard to therapeutic interventions that aim at activating these receptors. Intriguingly, the xenobiotic-sensing nuclear receptors are also activated by a range of endocrine disrupters and environmental pollutants, and thus might be responsible for the carcinogenic effect of many of these substances [76, 77].

15.7
CAR and PXR in Inflammation

Hepatic drug metabolism is repressed in inflammation. A lot of progress has been made in our understanding of the dysregulations underlying this repression. Expression of PXR and PXR target genes is reduced by inflammatory stimuli, partially mediated by Kupffer cells and reactive oxygen species [78]. In addition, PXR action itself might also be required for repression of some drug metabolizing genes upon inflammatory stimuli in the liver [79], although other studies failed to reproduce these findings [80]. Most strikingly, a cross-talk between PXR and NFκB has been found in the liver and the small intestine [81–83]. This cross-talk potentially explains the reduction of intestinal drug metabolizing genes by inflammatory signals [84, 85]. It is controversial if activation of PXR has a therapeutic effect on inflamed intestinal epithelium in inflammatory bowel disease [86, 87]. Moreover, further studies are required to test the association of the PXR locus with inflammatory bowel disease [88, 89].

15.8
Regulation of Glucose and Lipid Homeostasis by CAR and PXR

A myriad of recent studies concerning the impact of CAR and PXR on glucose and lipid homeostasis emerged in the last few years. John T. Moore and colleagues showed in 2004 that CAR is a regulator of thyroid hormone metabolism under caloric restriction [90]. These results were substantiated by the findings that CAR regulates type 1 deiodinase and thyroid hormone activity in the regenerating liver [91].

Interestingly, CAR knockout animals have increased food intake due to higher expression of neuropeptide Y (NPY) and NPY receptor subtype 1 in the hypothalamus [92]. Thus, CAR was postulated to constitute a first-line defense against metabolic and nutritional stress [93]. However, both CAR and PXR have more direct effect on hepatic lipid and glucose metabolism than just by influencing thyroid hormone actions. First, the xenobiotic-sensing receptors modulate FoxO1 activity and thereby regulate the expression of gluconeogenic genes [94]. Moreover, PXR and CAR are coactivated by the PPARγ coactivator (PGC)-1α [95, 96]. This transcriptional coactivator is a key regulator of hepatic metabolism (reviewed in Refs [97, 98]). Hepatic CAR expression is induced by fasting, presumably by direct activation through PGC-1α [99]. Moreover, CAR and PXR activity in the induction of several target genes depends on coactivation by PGC-1α [99, 100]. In contrast, in liver-specific PGC-1α knockout animals, PB induction of CYPs remains functional [101]. Competition between PXR versus hepatocyte nuclear factor 4α (NR2A1) for recruiting PGC-1α also plays a role in the regulation of hepatic glucose metabolism by xenobiotic-sensing nuclear receptors [95]. In addition to glucose homeostasis, xenobiotic-sensing nuclear receptors have also been found to alter lipid and lipoprotein levels. In the fasted mouse liver, PXR cross-talk with FoxA2 results in alterations of lipid metabolism independent of SREBP-1c [102, 103]. Moreover, PXR directly activates lipogenic gene expression while inhibiting fatty acid oxidation [103]. At the same time, PXR regulates lipoprotein levels, and controls lipoprotein particle assembly and metabolism [104–106].

Assuming that the impact of PXR and CAR activity on glucose and lipid homeostasis is significant, alterations of xenobiotic-sensing nuclear receptors and their target genes in diabetes would be expected. Moreover, activation of PXR and CAR should result in changes in plasma glucose levels and systemic insulin sensitivity. In fact, these observations have been made in different experimental systems, both rodent and human. The levels of the xenobiotic-sensing nuclear receptors and CYPs are changed in mouse models of type 2 diabetes (e.g. see Refs [107, 108]). Moreover, increase in insulin sensitivity after PB treatment has been reported decades ago (e.g. Refs. [109, 110]). Finally, an interesting link between metabolism and xenobiotic-sensing nuclear receptors has been found by the involvement of the AMP-activated protein kinase in sensing PB and subsequently activating CAR in the liver [111–113].

15.9 Conclusions and Perspectives

In the last couple of years, the research focus regarding the xenobiotic-sensing nuclear receptors PXR and CAR shifted from the traditional study of drug metabolism to the discovery of novel functions for these receptors (Figure 15.1). The different areas outlined above are exemplary for the most advanced studies so far. However, indications about the involvement of PXR and CAR in other fields have started to emerge. To name a few, PXR seems to be protective against liver fibrosis by inhibiting hepatic stellate cell transdifferentiation [114–116]. By activation of CYP

epoxygenases, PXR contributes to the regulation of vascular tone during pregnancy [117]. In the bone, cross-talk between PXR and the vitamin D receptor (NR1I1) mediates drug-induced osteomalacia [118, 119]. Finally, CAR has been implicated in the pathogenesis of non-alcoholic steatohepatitis [120]. Thus, our knowledge about PXR and CAR most likely will expand to other areas of human physiology and pathophysiology. The challenge in terms of pharmacological manipulation will be to dissect the pathways that are involved in the different functions in order to specifically target xenobiotic-sensing nuclear receptor-regulated gene programs in certain contexts and tissues. This also requires further studies of CAR and PXR polymorphisms and splicing variants (e.g. see Refs [121, 122]), and should take into account the circadian regulation of the drug metabolizing system [123]. Ultimately, CAR and PXR constitute attractive drug targets for a number of pathologies because of their promiscuity in terms of ligands and in terms of physiological function.

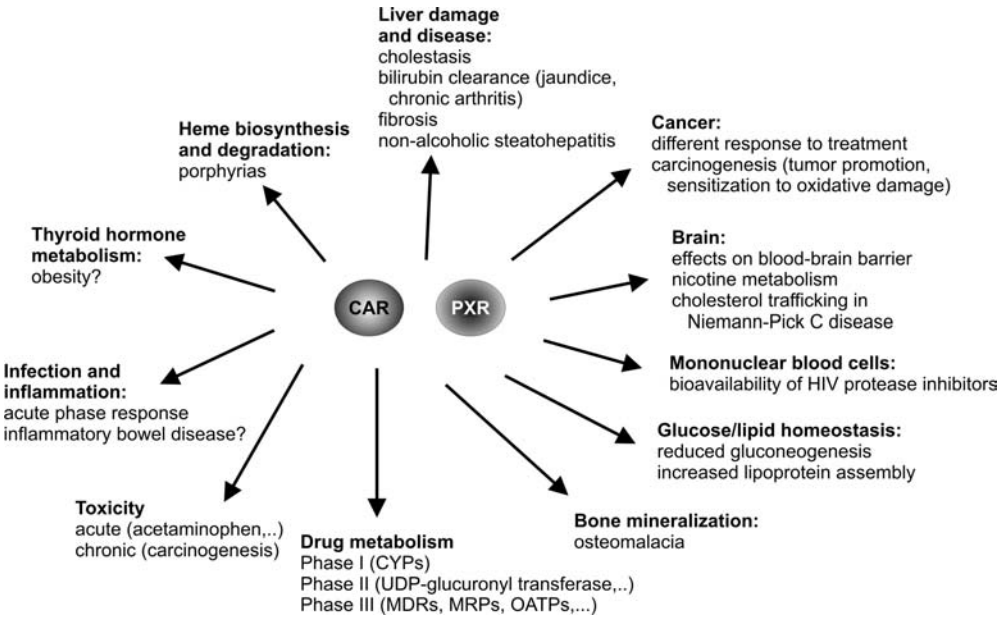

Figure 15.1 Different functions for CAR and PXR in health and disease. In recent years, novel functions for the xenobiotic-sensing nuclear receptors CAR and PXR beyond hepatic drug metabolism have been found. Potential implications of these findings for various physiological and pathophysiological processes are depicted in this figure. See text for details and references.

Acknowledgments

CH is supported by the Swiss National Science Foundation Professorship PP00A-110746 and the University Research Priority Program "Integrative Human Physiology" of the University of Zurich.

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16
Designing Chemical Libraries Directed to Nuclear Receptors

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16.1
Introduction

In the early 1990s, the advent of high-throughput screening (HTS) increased dramatically the capacity for testing compounds. The implementation of this wondrous piece of robotic equipment in the pharmaceutical industry was soon perceived as the technological solution to the relatively poor performance of drug discovery in terms of new chemical entities approved per year. Obviously, an increase in the capacity for testing compounds implied immediately increasing the number of compounds available for testing. Within this scenario, the size of the corporate compound collection was perceived as one of the key aspects for having a successful HTS campaign. Accordingly, the high demand for more compounds generated suddenly a strong need for wide compound synthesis and acquisition activities directed mainly at obtaining optimally diverse screening libraries [1].

However, a review of the performance of HTS in its first years of implementation revealed that the number, diversity and progressibility of the hits identified were below original expectations [2]. It became then clear that augmenting the capacity for testing alone was not sufficient for delivering high-quality leads and that more effort was required in carefully balancing the composition of the screening collections with compounds containing features compatible with the nature of the targets or target classes of corporate interest. Therefore, novel strategies were conceived to design chemical libraries focused to a particular target or directed to entire protein families to enrich corporate collections with a pool of targeted compounds that could complement those selected by diversity means [3]. Some of the main protein families for which chemical libraries have been designed in the last few years include G-protein-coupled receptors (GPCRs) [4, 5], kinases [5], serine proteases [6] and nuclear receptors [7].

The focus of this chapter will be on introducing novel knowledge-based strategies for designing chemical libraries directed to the family of nuclear receptors, with special emphasis on more generic, often overlooked aspects such as the degree of expected family coverage and bias by the compounds in the library. As extensively

Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

covered in previous chapters, nuclear receptors form a family of ligand-activated transcription factors that regulate a variety of biological processes, including lipid and glucose homeostasis, detoxification, cellular differentiation, embryonic development, and orphan physiology. Consistent with these important regulatory roles, mutations in nuclear receptors are associated with many common human diseases such as cancer, diabetes and osteoporosis, and thus they are considered highly relevant protein targets [8]. Furthermore, many nuclear receptors also play an important role in mediating the induction of hepatic cytochrome P450s – a class of enzymes involved in drug metabolism and in the toxification/detoxification of xenochemicals prevalent in the environment. Accordingly, many nuclear receptors are also regarded as potential off-targets [9]. Finally, there are still a number of orphan nuclear receptors involved in novel regulatory systems that impact human health for which ligands have yet to be identified and that are likely to lead to the discovery of new drugs in the near future [10]. The combination of these three aspects makes nuclear receptors a protein family of utmost therapeutic relevance for the pharmaceutical industry and, thus, highlights the need for having access to chemical libraries designed especially to cover the entire family.

16.2 Collecting and Storing Prior Knowledge

The design of targeted chemical libraries is an activity that requires the availability of prior knowledge either on bioactive ligands (ligand-based approaches) or on crystallographic data (target-based approaches) for the different members of the protein family of interest. In this respect, one of the current challenges in biomedical research is to collect, store, organize and connect the increasing amount of data being generated around small molecules, proteins, genes, pathways and diseases [11]. The efficient access to and linkage of all these data will essentially constitute the stepping stone towards the establishment of novel integrative knowledge-based approaches to drug discovery activities, in particular to the design of targeted chemical libraries [12, 13].

A number of initiatives have focused recently on collecting and storing the structures of small molecules for which pharmacological data to a given protein target have been reported in the literature, giving rise to the so-called annotated chemical libraries [14]. Among those, the MDL Drug Data Report (MDDR) includes information on the therapeutic action and biological activity for over 132 000 compounds gathered from patent literature, journals and congresses [15], the WOMBAT database offers current data on 307 700 biological activities for 154 236 molecules annotated to 1320 protein targets reported in medicinal chemistry journals over the last 30 years [16], the AurSCOPE databases offer a collection of chemical libraries containing over 323 000 molecules annotated to over 1 300 000 biological activities related to members of therapeutically relevant protein families covered in over 38 000 publications [17], and the MedChem and Target Inhibitor databases compile around 2 000 000 molecules with biological activity, toxicity and pharmacological information for therapeutically relevant protein families extracted from 20 000

publications [18]. All these commercial databases provide an invaluable source of pharmacological data for ligands that can be ultimately exploited for designing targeted libraries.

In the same spirit, a more modest initiative took place in our laboratory to assemble an annotated chemical library directed to the nuclear receptor family (NRacl) on the basis of public sources of information, mainly reviews and medicinal chemistry journals of the last 13 years [19]. Each entry in NRacl contains information on its topological chemical structure and the connection to nuclear receptors is established through the associated pharmacological data (K_i, IC_{50} and/or EC_{50}), as reported. At this stage, only biologically active compounds (activity below 10 μM) are entered into NRacl. In total, NRacl includes currently 2718 small molecules connected to 29 nuclear receptors, some molecules containing multiple annotations to nuclear receptors.

Since the ability to extract knowledge from annotated chemical libraries will be greatly determined by the way chemical and biological data are stored, when constructing NRacl special emphasis was put on storing both chemical structures and nuclear receptors using appropriate unique identifiers and classification schemes. For chemical structures, we used an in-house proposed Chemical Structure Code (CSC) purely based on topological features of molecules. Accordingly, each molecule in NRacl is identified with a unique hierarchical five-level CSC [19]. The first and second levels are integers specifying, respectively, the number of rings in the largest ring system present in the molecule and the total number of ring systems in the molecule. The third, fourth and fifth levels are a unique seven-character hash code for the molecular framework, scaffold and the complete molecular structure, respectively. On the other hand, for the annotation of ligands to nuclear receptors, we avoided using the receptor names directly, but using instead the more compact and unified three-character code nomenclature system proposed by the Nuclear Receptors Nomenclature Committee [20]. Within this scheme, the first character is a number that designates the subfamily. There are six main subfamilies, assigned to identifiers 1–6. All nuclear receptors in these subfamilies contain a highly conserved zinc finger DNA-binding domain (DBD) and a less conserved ligand-binding domain (LBD). However, some unusual receptors contain only one of the two conserved domains and thus an additional subfamily, assigned to identifier 0, has been included to account for them. The second character is a capital letter specifying the group within the subfamily and the third character is a number identifying the individual nuclear receptor within a group. Globally, this classification scheme of nuclear receptors defines at present seven subfamilies, 25 groups and 73 receptors. The use of hierarchical classification schemes for both molecules and receptors takes the exploitation of family-directed annotated chemical libraries to another level, the added value coming from the fact that complete flexibility exists for extracting knowledge at all levels of those classification schemes. For the sake of clarity, Table 16.1 compiles a list of 36 nuclear receptors for which prior knowledge on both small molecules and/or receptor structures is currently available.

Recent trends in nuclear receptor medicinal chemistry can be analyzed in terms of the number of novel scaffolds representing all small molecules reported to have

Table 16.1 List of nuclear receptors and the number of bioactive ligands (BL) and protein structures (PS) currently available for each of them.

Subfamily	Group	Receptor	Name	Abbreviation	BL	PS
1	A	1	thyroid hormone	TRα	81	3
		2		TRβ	93	12
	B	1	retinoic acid	RARα	107	1
		2		RARβ	112	2
		3		RARγ	118	9
	C	1	peroxisome proliferator-activated	PPARα	134	4
		2		PPARβ	37	8
		3		PPARγ	280	25
	F	1	retinoic acid receptor-related orphan	RORα	0	2
		2		RORβ	0	3
	H	1	ecdysone	ECR	2	3
		2	liver X	LXRβ	4	6
		3		LXRα	30	2
		4	farnesoid X	FXR	33	3
	I	1	vitamin D	VDR	16	18
		2	pregnane X	PXR	1	6
		3	constitutive androstane	CAR	0	4
2	A	1	hepatocyte nuclear factor 4	HNF4α	0	2
		2		HNF4γ	0	1
	B	1	retinoid X	RXRα	135	18
		2		RXRβ	78	2
		3		RXRγ	97	1
		4	ultraspiracle protein	USP	0	5
3	A	1	estrogen	ERα	729	39
		2		ERβ	757	21
	B	1	estrogen receptor-related	ERRα	0	2
		3		ERRγ	9	11
	C	1	glucocorticoid	GR	312	3
		2	mineralocorticoid	MR	14	10
		3	progesterone	PR	380	7
		4	androgen	AR	280	32
4	A	1	nerve growth factor IB	NGFI-B	0	2
		2		NURR1	0	1
		4		DHR38	0	1
5	A	1	steroidogenic factor-1	SF1	0	4
		2	fetoprotein TF	FTF	0	5

affinity under 10 μM for a nuclear receptor over the last 13 years. As can be observed in Figure 16.1, early efforts in 1994 and 1995 focused primarily on synthesizing compounds directed to the groups of retinoic acid receptors (RARs; 1B), retinoic X receptors (RXRs; 2B), thyroid receptors (TRs; 1A), peroxisome proliferator-activated receptors (PPARs; 1C) and estrogen receptors (ERs; 3A). Accordingly, the overall distribution of annotations among all nuclear receptors is a fair reflection of the

historical therapeutic relevance of some of the members of these groups (Table 16.1). In particular, the nuclear receptor containing the largest number of chemical annotations is ERα (3A1), an important target in reproductive medicine and cancer research. Due to its high homology, many compounds binding to ERα are also reported to be active to ERβ, thus justifying the large number of annotations present also for the latter. Another nuclear receptor for which vast information on bioactive ligands is available is PPARγ (1C3), widely recognized as a key regulator in multiple metabolic pathways including fatty acid and carbohydrate metabolism, and thus being considered a relevant target in cardiovascular research. In contrast, the groups of hepatocyte nuclear factor 4 receptors (HNF4s; 2A) and estrogen-related receptors (ERRs; 3B) are among the youngest in terms of medicinal chemistry exploration. This will be the main body of ligand-based information used for designing targeted chemical libraries to nuclear receptors.

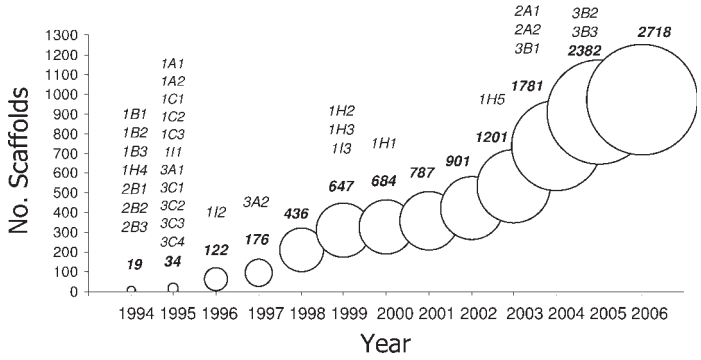


Figure 16.1 Progress over time in the number of novel scaffolds generated from nuclear receptor medicinal chemistry efforts in the last 13 years. The radius of the circles reflects the cumulative number of molecules entered in NRacl (specific numbers are given above each circle, in bold). When a bioactive molecule to a nuclear receptor was first encountered in the literature, its code (in italics) is added above the circle. (Reprinted with permission from [44]. Copyright (2007) Bentham Science Publishers Ltd.)

Apart from pharmacological data on ligands, the other important source of knowledge generated within protein families is the availability of experimentally determined protein structures. Recent advances in high-throughput methods for protein expression and production, nuclear magnetic resonance spectroscopy, and X-ray crystallography have led to a significant rise in the number of protein structures solved [21]. Many of these structures are ultimately deposited and made publicly accessible in the Protein Data Bank (PDB; http://www.pdb.org), currently containing over 51 000 entries and its size continuing to increase annually at an almost exponential rate [22]. In particular, the first structure of a DBD of a nuclear receptor was deposited in the PDB in 1992 [23], whereas the first LBD structure was not deposited until 4 years later [24]. Since then, the number of nuclear receptor structures has grown significantly and, as per 27 July 2007, there were 319 entries in the PDB involving 294 separate PDB files, some of which have more than one NR number associated with them [25] (http://cgl.imim.es/fcp). Of them, 36 entries

correspond to nuclear receptor DBDs covering five subfamilies, 11 groups and 16 receptors. The remaining 283 entries correspond to nuclear receptor LBDs covering a total of six subfamilies, 14 groups and 37 receptors, which provide essential structural information that can be exploited for designing targeted libraries directed to the entire family of nuclear receptors (Table 16.1). To complement Figure 16.1 from a receptor structure viewpoint, the evolution in the number of LBD structures deposited in the PDB over the years is illustrated in Figure 16.2. Overall, beyond the mere increase in the population of structures, it is important to stress again that the general adoption of classification schemes for proteins is an essential aspect for analyzing quantitatively the functional coverage and structural bias of target families in the PDB, and thus ultimately for assessing the applicability of structure-based approaches to targeted chemical library design [26].

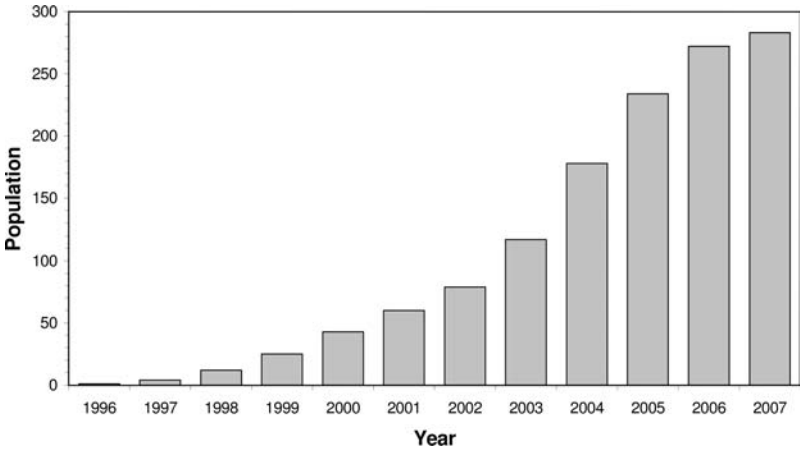


Figure 16.2 Growth in the number of nuclear receptor LBD structures deposited in the PDB.

16.3 Nuclear Receptor Profiling

Beyond having access to prior knowledge, a key aspect of being able to design compounds directed to the protein members of a particular family is the ability to detect that compounds possess the right features arranged in an optimal complementary manner to the protein cavities of interest by properly scoring them on the basis of some predefined metrics. In this respect, the process of scoring and ranking molecules in large chemical libraries according to their likelihood of having affinity for a certain target is generally referred to as virtual screening [27]. The term itself was coined in the late 1990s when computer-based methods reached sufficient maturity to offer an alternative to experimental HTS techniques. In spite of the initial reluctance, over the years the pharmaceutical industry has learned to accept that

virtual screening methods can indeed be an efficient complement to HTS to the point that they have undoubtedly become an integral part of today's lead generation process [12]. It is worth emphasizing again that, in contrast to technology-driven HTS, virtual screening is a knowledge-driven approach that requires structural information either on bioactive ligands for the target of interest or on the target itself. Comparative studies have suggested that information about a target obtained from known bioactive ligands is as valuable as knowledge of the target structures for identifying novel bioactive scaffolds through virtual screening [28]. Therefore, the final choice for a ligand-based or target-based method will ultimately depend on the type and amount of information available without *a priori* having a large impact on performance.

With virtual ligand screening well integrated in the drug discovery process, a wave of new strategies is currently emerging with the aim of exploiting both the ever-increasing amount of information and computational power available to add a biological dimension to traditional single-target virtual screening. In this respect, it has been shown recently that these strategies are capable of estimating the pharmacological profile of molecules on multiple targets and promise to have a strong influence in drug discovery as a means for detecting potential side effects of compounds due to off-target affinities earlier on during the optimization process [29]. As mentioned above, some members of the nuclear receptor family may be considered relevant off-targets to which it is important to avoid affinity to a safe degree. Therefore, not surprisingly, some ligand-based and target-based approaches to nuclear receptor profiling have been recently described in the scientific literature.

From a ligand-based viewpoint, it is worth understanding that the relative success of ligand-based methods depends to a great extent on the use of biologically relevant mathematical representations of molecules. In this respect, a novel set of molecular descriptors called SHED (SHannon Entropy Descriptors) was recently introduced [30]. SHED are derived from distributions of atom-centered feature pairs extracted directly from the topology of molecules stored in standard MDL SD file format. From a SD file, each atom in a molecule is first mapped to a Sybyl atom type. Subsequently, each atom type is assigned to one or more of four atom-centered features, i.e. hydrophobic (H), aromatic (R), acceptor (A) and donor (D). For example, an aliphatic C.3 carbon will be assigned to a hydrophobic feature (H), whereas a protonated N.4h nitrogen will be assigned to both aromatic and donor features (R,D). Then, the shortest path length between atom-centered feature pairs is derived and its occurrence at different path lengths stored to create a feature-pair distribution. A maximum path length of 20 bonds was used. Feature pairs at distances over 20 bonds are accumulated in the last bin. An equivalent distribution is derived for each of the 10 possible feature pairs resulting from all pair combinations of the four features used. In summary, each chemical structure is ultimately represented by a SHED profile composed of 10 real numbers reflecting the particular feature-pair distributions present in the molecule.

These SHED descriptors were recently used to profile *in silico* a chemical library of 2033 molecules against 25 nuclear receptors [31]. As described above, this annotated

chemical library to nuclear receptors (NRacl) was assembled internally in our laboratory from various medicinal chemistry sources [19]. The distribution of annotations contained at that stage in NRacl is visually illustrated in Figure 16.3(a). In the heatmap shown, annotations of molecules (in rows) to nuclear receptors (in columns) are represented as red cells, meaning that the interaction of a particular molecule with a specific nuclear receptor has been positively reported and experimentally quantified in the literature with a pharmacological value below 10 μM. In contrast, green cells indicate current lack of information on the possibility of any interaction between a given molecule and a certain nuclear receptor. The extent of the green area denotes the existence of large information gaps – clearly one of the main limitations of dealing with annotated chemical libraries relying on data extracted directly from public sources of information. This is due to the fact that, because of limited time and resources, molecules are usually not screened systematically through a large panel of protein targets for the sake of obtaining the maximum amount of information possible but solely to the target of interest at that point in time. However, even if they were screened through multiple targets, habitually only a limited amount of data is made available, since publishing large amounts of negative data is often regarded as not informative. These important, yet often overlooked, aspects lead to a situation of data incompleteness within the interaction matrix depicted as a heatmap in Figure 16.3(a).

The information on bioactive ligands contained in NRacl was then used to derive a ligand-based model of each nuclear receptor based on the SHED descriptors defined above. Essentially, the scoring of each compound in a chemical library with respect to a given nuclear receptor is assigned to the minimum value of all Euclidean distances calculated between the SHED profile of the target compound and each one of the SHED profiles describing the molecules annotated to that particular nuclear receptor [31]. The result of applying this process to each one of the 2033 molecules with non-redundant SHED profiles present in NRacl is given in Figure 16.3(b), in which the order of the molecules is exactly the same as the one obtained from the original annotations shown in Figure 16.3(a). In contrast to the binary heatmap illustrated in Figure 16.3(a), in which red was annotated and green was not annotated, Figure 16.3 (b) presents a color gradation between red and green reflecting the value of the minimum SHED Euclidean distance between the SHED profile of each molecule and the set of nonredundant SHED profiles annotated to each nuclear receptor. Taking the annotation threshold of 0.6 as the center of the color scale, distance values close to 0.0 are represented in red, those close to 0.6 are seen as light orange, and as distances increase in magnitude they turn to yellow and finally green at a value of 1.2 and over. There are two main aspects worth mentioning when comparing the heatmaps of Figure 16.3(a and b). On one hand, it is remarkable to notice that the essential pattern observed when plotting the original annotations (Figure 16.3a) is preserved when molecules are processed through the ligand-based descriptor model of nuclear receptors (Figure 16.3b). This result reveals that the remaining molecules in NRacl are to a great extent representative of the molecule being processed after leaving that molecule out, something that can only be achieved if the annotated chemical space has been sufficiently saturated with as many known bioactive molecules as possible.

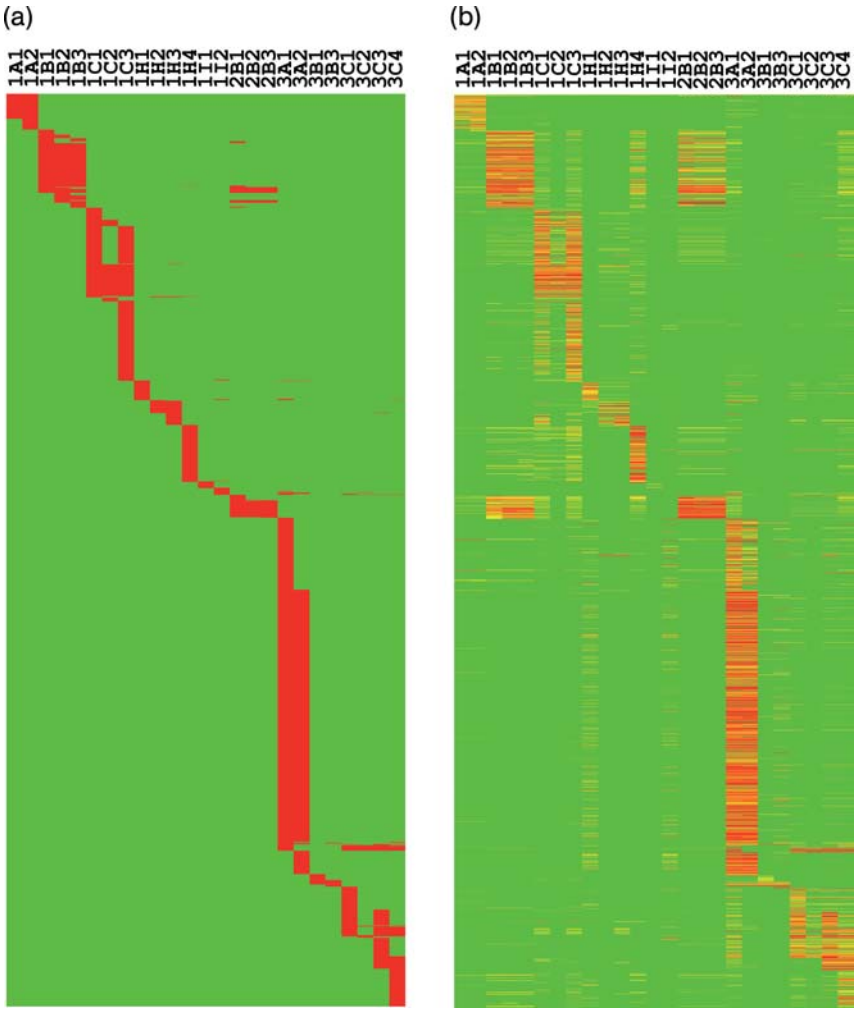


Figure 16.3 Comparison between the heatmap representing all original annotations extracted from bibliographical sources and stored in NRacl (a) and the heatmap reflecting the minimum SHED Euclidean distances between the SHED profile of each molecule and the set of nonredundant SHED profiles annotated to each nuclear receptor (b). Color coding: (a) red is annotated and green not annotated; and (b) red reflects distance values close to 0.0, and as distances increase in magnitude they turn to orange, yellow, and finally green at a value of 1.2 and over. (Reprinted with permission from [31]. Copyright (2006) American Chemical Society.)

On the other hand, despite the clear discrimination between nuclear receptor groups, some correlation patterns between them emerge. The most apparent example is the clear correlation observed in Figure 16.3(b) between RARs (1B1, 1B2 and 1B3) and RXRs (2B1, 2B2 and 2B3) – a result that provides an indication of the potential of this approach for understanding side effects through the identification of off-target affinities.

In contrast, from a target-based viewpoint, it is also important to understand that the relative success of a target-based method will depend to a great extent on the availability of representative experimental crystal structures for all members of the protein family of interest, as the performance of these methods tends to degrade depending on whether a holo (ligand bound), an apo or a model structure of the protein is available [32]. In this respect, the family of nuclear receptors is relatively well covered in terms of structural information available, with representative LBD structures for up to 37 receptors (Table 16.1). Another equally important aspect in target-based methods is the docking procedure used to generate a binding hypothesis of the interaction between the ligand and the protein, which involves conformational sampling and scoring of small molecules into protein cavities [33, 34].

Despite the decent amount of structural information available, applications of target-based methods to nuclear receptor profiling have been so far scarce. Perhaps the most comprehensive work in this respect is the recent systematic virtual screening of a library composed of 78 known active ligands against 19 different structures representative of 13 nuclear receptors [35]. Note that for some nuclear receptors, more than one crystal structure was considered to assess the dependency of the results on the particular conformation of the receptor. As mentioned, each one of the 78 ligands is a known binder to certain nuclear receptors (black bars in figure 1 of Ref. [35]). This information is illustrated in Figure 16.4(a) (the structure-based counterpart of Figure 16.3a), in which annotations of molecules (in rows) to nuclear receptors (in columns) are represented as red cells, meaning that the interaction of that particular ligand with a specific nuclear receptor has been positively reported and experimentally quantified in the literature. Remarkably, Figure 16.4(a) provides a very crisp picture of the interaction of ligands to nuclear receptors, promiscuity being observed only to a limited degree between some of the steroid hormone receptors. However, interpretation of Figure 16.4(a) at this stage needs to be taken with the same level of caution highlighted previously when discussing Figure 16.3(a), since large information gaps exist in these data (represented by green cells) due to the incompleteness of the experimental information.

When this set of 78 nuclear receptor ligands was put into a library of 5000 random compounds and scored, the sensitivity of the method for distinguishing between true binders and nonbinders could be assessed. In order to perform an analysis comparative to the one presented above when using a ligand-based method, an annotation criteria was selected. Accordingly, a ligand was considered annotated to a given nuclear receptor if its docking score was above the score threshold to select 1% of a 5000 random compound database (the horizontal solid black line in figure 1 of Ref. [35]). The resulting heatmap is depicted in Figure 16.4(b). In essence, there are two main aspects worth mentioning when comparing the heatmaps of Figure 16.4(a and b). On one hand, it is remarkable how the target-based method is able to identify the correct nuclear receptor target for the majority of true binders. On the other hand, along the same lines as observed previously in Figure 16.3(b) for a ligand-based method, some stronger correlation patterns between nuclear receptors emerge as a

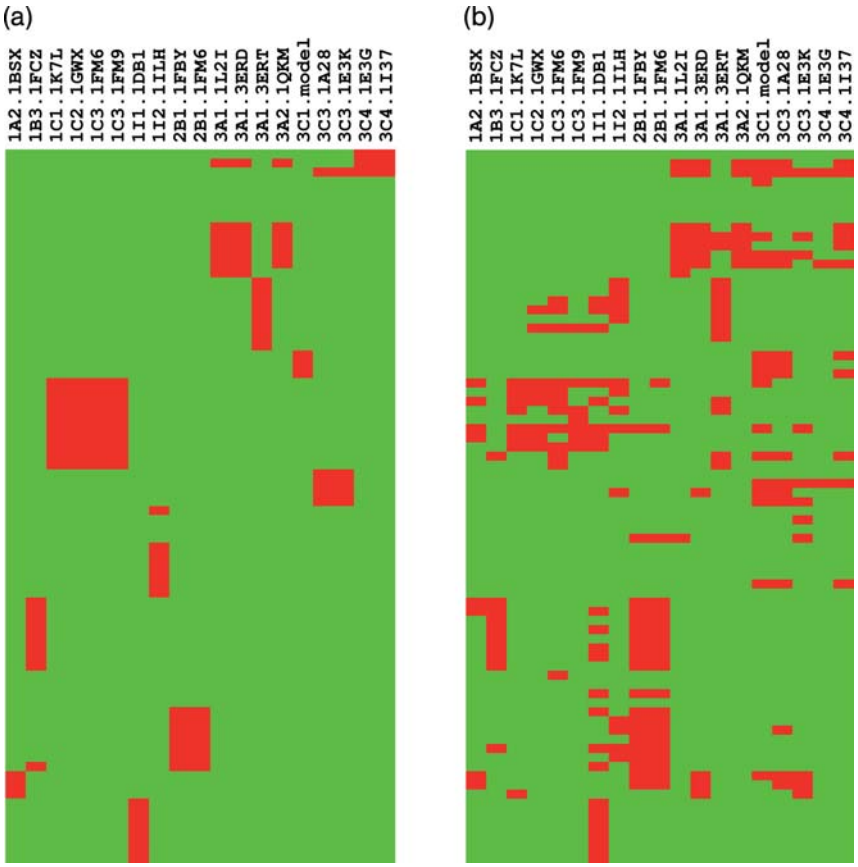


Figure 16.4 Comparison between the heatmap representing all original annotations extracted from bibliographical sources on 78 known nuclear receptor binders (a) and the heatmap reflecting the annotation associated to a docking score being above the score threshold to select 1% of a 5000 random molecule database (b). Color coding: red is annotated and green not annotated. (Information derived from Ref. [35]).

consequence of the extend of the promiscuity profiles predicted. For example, the relatively limited signal shared between steroid hormone receptors in Figure 16.4(a) is transformed in Figure 16.4(b) into a strong promiscuity signal among them. Again, however, the conclusions extracted from Figure 16.4(b) need to be taken with caution, since a full affinity matrix between the 78 ligands and the 13 receptors is not available.

In summary, examples have been provided in which both ligand-based and target-based methods performed decently when profiling compounds against the family of nuclear receptors. Therefore, it is reasonable to say that these methodologies have reached a sufficient level of maturity to be applied sensibly for designing the next generation of chemical libraries directed to entire protein families.

16.4
New Trends in Designing Targeted Libraries

Despite its recognized relevance, it is remarkable to realize that very few reports document recent efforts towards designing chemical libraries particularly directed to the family of nuclear receptors. However, analyses on the characteristics of nuclear receptor ligands have revealed valuable information on the specific molecular properties and topological substructures these ligands possess compared to other family-directed sets of ligands. For example, in terms of molecular descriptors, nuclear receptor drugs seem to be characterized by significantly high mean *c*log*P* values (4.1) and low mean counts of oxygen and nitrogen atoms (3.8) compared to drugs designed for primary targets belonging to other protein families [36]. Similar trends were also found when analyzing sets of hit-to-lead ligand pairs instead of drugs [37]. In this case, a mean *c*log*P* value of 5.0 was found for nuclear receptor ligands, the largest mean *c*log*P* value among all compound entries directed to targets belonging to a list of 11 protein families.

To investigate this aspect further, we took all ligands annotated to nuclear receptors in Wombat having an affinity value (pK_i, pIC_{50} or pEC_{50}) larger that 7.0. This resulted in a list of 2929 molecules containing 3839 annotations to 24 nuclear receptors. Figure 16.5 illustrates the distribution of this set of 2929 nuclear receptor bioactive

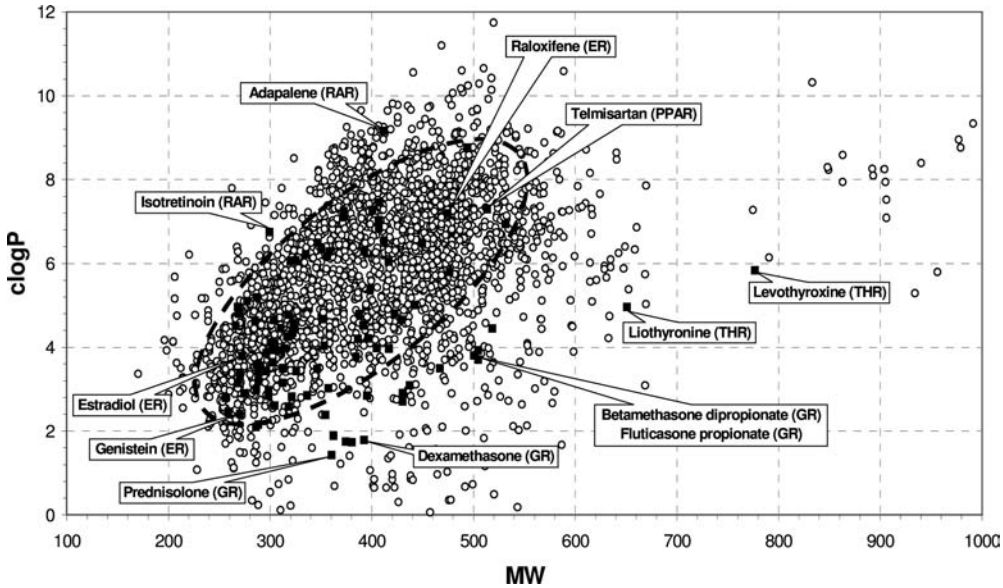


Figure 16.5 Distribution of a set of 2929 nuclear receptor bioactive ligands (white circles) and 135 nuclear receptor drugs (black squares) in the plane defined by the molecular weight (MW) and *c*log*P* descriptors. The dashed region defines the nuclear receptor space.

ligands (white circles), together with a set of 135 nuclear receptor drugs (black squares), in the plane defined by two molecular descriptors related to size and hydrophobicity such as molecular weight and *c*log*P* values. It was observed that 65.4% of all bioactive ligands fail to meet the Lipinski criteria for both molecular weight and *c*log*P* values and thus, under the Rule-of-Five, they would receive an alert as having poor oral bioavailability. One could then conclude that high-affinity ligands for nuclear receptors are intrinsically handicapped for oral bioavailability relative to high-affinity ligands for other protein families. In fact, one could delineate and oval-shaped region within the molecular weight versus *c*log*P* space that would contain the vast majority of both nuclear receptor bioactive ligands and drugs. The south-west of this oval region appears to be populated by the smaller more compact steroid-like drugs, such as ER agonists estradiol and genistein, whereas the north-east side is occupied by larger hydrophobic compounds, such as the ER antagonist raloxifene and the PPAR modulator telmisartan. Outside this region, we find a set of outliers including the iodine-substituted thyroid hormone receptor drugs (levothyroxine and liothyronine), a variety of glucocorticoid receptor modulators (such as betamethasone dipropionate, fluticasone propionate, dexamethasone, and prednisolone) and some RAR ligands (such as isotretinoin and adapalene). Therefore, this region could certainly be used as a fast molecular descriptor filter when designing chemical libraries directed to the nuclear receptor family.

Apart from ranges of molecular descriptor values, another strategy often applied for biasing chemical libraries towards particular protein families is to generate and synthesize compounds around so-called privileged substructures [38]. However, the results obtained in a recent substructure-class analysis of ligand sets from five target families (i.e. GPCRs, nuclear receptors, ligand-gated ion channels, serine proteases and protein kinases) put a question mark on the actual existence of target-family-privileged substructures [39]. For nuclear receptors in particular, the study revealed that nuclear receptor substructure classes were present in 40% of a total of 21 620 GPCR ligands, 30% of a total of 3792 ion channel ligands, 17% of a set of 1079 kinase ligands and 15% of a set of 3015 protease ligands, but, most interestingly, 45% of a set of 10000 random ligands. Altogether, these results are an indication that the nuclear receptor substructure classes generated are in fact nonprivileged substructure classes for the nuclear receptor family and, thus, its use for designing targeted chemical libraries is questionable.

In order to investigate this further, we performed a comparison of the most populated scaffolds in three sets of ligands: the same set of 2929 nuclear receptor bioactive ligands used above (NRactive set), a set of 2900 drugs and drug candidates (Drugs set), and the 'open NCI database' composed of 250 251 compounds (NCI set). The lists of 10 most promiscuous (for NRactive) and 10 most populated (for Drugs and NCI) scaffolds are collected in Table 16.2, the definition of scaffold in this work being equivalent to that of atomic framework given earlier [40]. As can be observed, phenyl emerges as the most promiscuous scaffold among nuclear receptors, with 60 compounds showing high affinity for eight nuclear receptors from five different nuclear receptor groups (1B1, 1B3, 1C1, 1H3, 2B1, 2B2, 2B3 and 3C4). Comparatively, phenyl is also by far the most populated scaffold in both Drugs and NCI sets.

Table 16.2 List of the 10 most promiscuous scaffolds in a set of nuclear receptor bioactive ligands (NRactive) and 10 most populated scaffolds in a set of drugs and drug candidates (Drugs) and in the open NCI database (NCI).

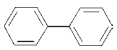
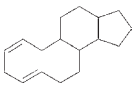
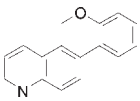
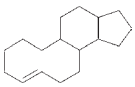
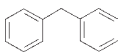
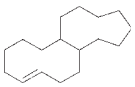
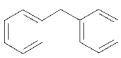
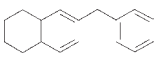
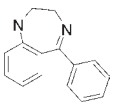
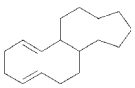
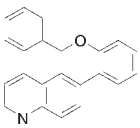
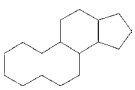
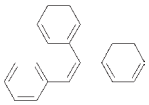
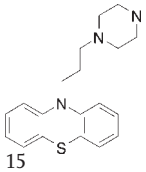
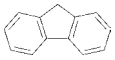
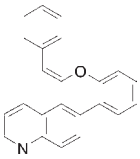
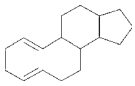
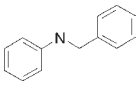
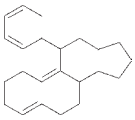
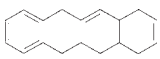
NRactive	Drugs	NCI
		
60 (8)	326	30560
		
43 (6)	45	2810
		
73 (4)	44	2328
		
23 (4)	41	2279
		
20 (4)	21	2013
		
86 (3)	17	1850
		
51 (3)	17	1786

Table 16.2 (*Continued*)

NRactive	Drugs	NCI
 39 (3)	 15	 1448
 39 (3)	 15	 1076
 11 (3)	 15	 1037

Numbers refer to the population of molecules containing each scaffold in the respective sets. The figures in parentheses give the level of nuclear receptor promiscuity associated to scaffolds in the NRactive set.

The second most promiscuous scaffold among nuclear receptors is a biphenyl core. It is present in 43 compounds having high-affinity for six nuclear receptors from four different nuclear receptor groups (1B2, 2B1, 2B2, 2B3, 3A2 and 3C4). A recent study suggested that HTS libraries enriched with biphenyl-containing compounds can be expected to have increased chances of yielding high-affinity ligands for proteins [41]. The results presented here for nuclear receptors, together with the fact that the biphenyl substructure is found also quite frequently in GPCR ligands, would be supportive of the conclusions reached in that study. Going further down the list of most promiscuous scaffolds present in the NRactive set we notice that the remaining scaffolds have promiscuities below 5 and that, for the majority of these scaffolds, their associated nuclear receptors belong to the group of glucocorticoid-like receptors.

Altogether, this scaffold promiscuity analysis leads to two main conclusions. On one hand, it is remarkable to realize that besides phenyl and biphenyl no other scaffold could be identified that could cover vastly this apparently well-conserved protein family, in terms of tertiary structure. Along the lines of a recent study [39], the true existence of privileged scaffolds for the entire nuclear receptor family remains thus unclear and, consequently, the use of nuclear receptor substructure classes for targeted chemical library design is also dubious. On the other hand, also in agreement with a previous work [41], the present results would indicate that enriching chemical libraries with compounds containing a variety of substituent

decorations around the phenyl and biphenyl cores could be a strategy worth considering to deorphanize nuclear receptors. However, it must be stressed at this stage that these conclusions have been derived from information contained in annotated chemical libraries. As highlighted earlier [19], due to limited time and resources, molecules are usually not screened systematically against the complete panel of proteins forming a family for the sake of generating the maximum amount of information but solely to the target(s) of interest, leading to a data completeness issue. Should the molecules contained in the NRactive set be screened against the entire nuclear receptor family, the existence of privileged scaffolds, currently hidden because of lack of information, could potentially be revealed.

Lately, we have seen a new trend in designing targeted chemical libraries in which not only the descriptor profiles and the presence of particular substructures observed in known bioactive compounds is considered, but also the concrete potential coverage of the protein family by the molecules in the chemical library is assessed [31]. This represents adding a biological dimension to the process and that both chemical and biological diversity are included when designing the composition of a targeted chemical library. Using a ligand-based approach to nuclear receptor profiling, Figure 16.3(b) provides an example of a chemical library covering fully all nuclear receptors under consideration. However, it reveals also that the chemical library is clearly biased with compounds potentially being active to the ERs and thus it is far from being optimally diverse in terms of projected nuclear receptor pharmacology. Addressing protein family coverage and bias should become standard procedure when designing targeted chemical libraries.

Finally, a new wave of computationally efficient *in silico* pharmacology methods promises to have the ability to profile large chemical libraries against hundreds of protein targets in a reasonably short period of time. These activities may lead to the identification of potential protein family off-targets, defined as those protein targets against which compounds designed for a particular protein family may have some residual affinity. We have profiled the NRacl chemical library used to generate Figure 16.3(b) against a panel of 674 protein targets covering 411 enzymes, 168 GPCRs, 48 ion channels, 32 nuclear receptors and 15 transporters. Of those, only six targets contained more than 100 annotations from compounds annotated also to any nuclear receptor and only 23 targets had more than 50 annotations. The corresponding heatmap is illustrated in Figure 16.6, in which the family off-target signals (on the left) can be compared against the nuclear receptor profile (on the right) equivalent to Figure 16.3(b). In rank 2 of the off-target list we find cyclooxygenase (COX)-2. Interestingly, using a cavity site-based similarity searching method, a relationship between the PPARγ agonist binding pocket and the COX-2 binding site was recently identified [42]. Also, in rank 14 of the off-target list we can locate angiotensin II type 1 receptor (ATR1). Again, evidence could be found in the literature of clear cross-pharmacology between ATR1 antagonists and activation of PPARγ [43]. Unfortunately, we could not find evidence in the literature relating directly the other off-target names in the list to nuclear receptors. Further investigation is underway in our laboratory.

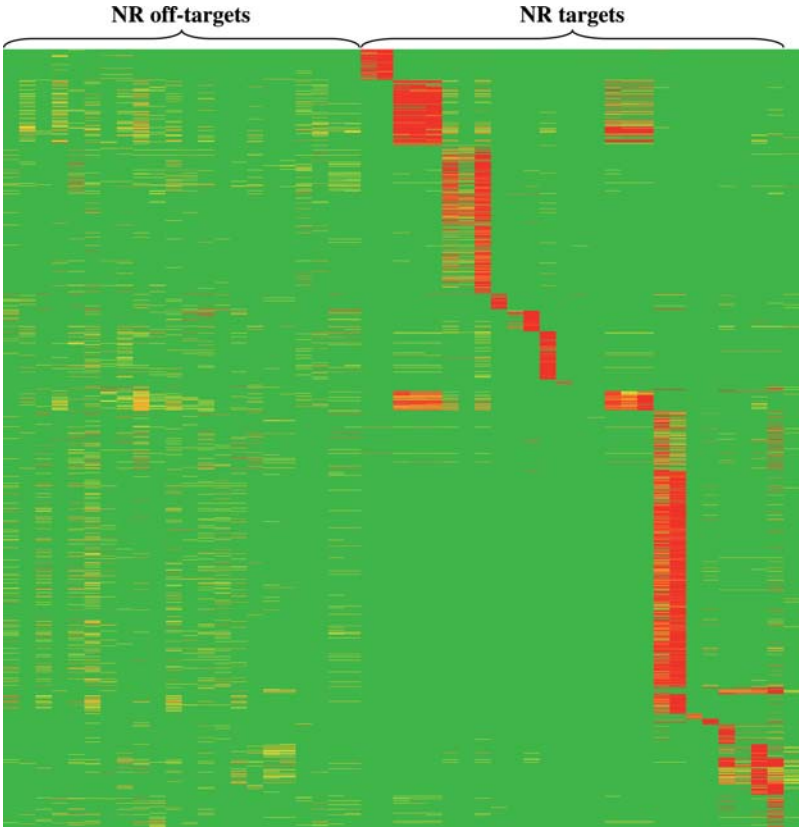


Figure 16.6 Heatmap reflecting the minimum SHED Euclidean distance between SHED profile of each molecule and the set of nonredundant SHED profiles annotated to each one of the 26 nuclear receptor targets and the 23 off-targets identified. Color coding: red reflects distance values close to 0.0, and as distances increase in magnitude they turn to orange, yellow, and finally green at a value of 0.6 and over.

16.5 Conclusions and Outlook

Nuclear receptors are a protein family of utmost importance for pharmaceutical research and thus chemical libraries directed to probe this family exhaustively are required. Lately, a variety of strategies have been applied to designing nuclear receptor chemical libraries. In view of the fresh perspectives novel *in silico* pharmacology methods are offering, it is envisaged that properly addressing coverage and bias during the design process together with the ability to identify potential protein family off-targets would lead to a new generation of high-content chemical libraries directed to nuclear receptors composed of small molecules exposing a rich diversity

of therapeutically relevant pharmacological profiles. However, recent studies are highlighting the need to go beyond the target level when designing chemical libraries and incorporate information at the pathway level [44]. The relative importance of achieving target selectivity when the target has an intrinsic promiscuity at the pathway level may change the way drug discovery is perceived and smoothly shift from target-focused to systems-oriented research.

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Nuclear Receptors as Drug Targets. Edited by Eckhard Ottow and Hilmar Weinmann

ISBN: 978-3-527-31872-8

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