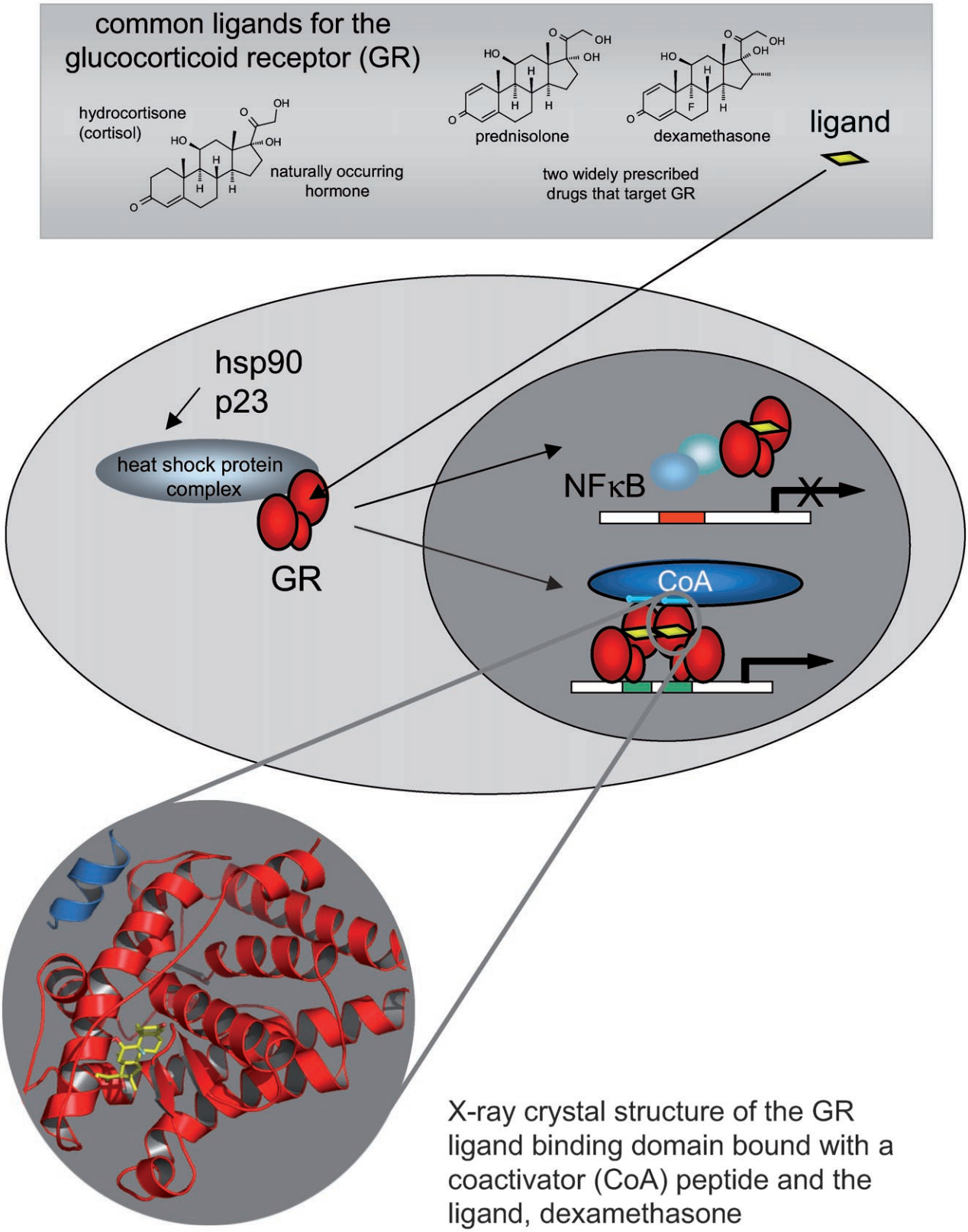
Steroid/Nuclear Receptor Function



 DOI: 10.1002/cmdc.200600006

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The Nuclear Receptor Superfamily and Drug Discovery**

John T. Moore, Jon L. Collins, and Kenneth H. Pearce*[a]

1. Introduction

A central theme that defines the field of endocrinology is the act of controlling activities and processes at distal sites in the body. Signaling molecules, in some cases nonprotein small molecules, traverse the body and ultimately relay their chemically encoded information to a protein receptor at the target tissue. The nuclear hormone receptor (NR) is a classic example of a receiver for such small-molecule chemical messengers. The NR is well-adapted for this type of function because it not only specifically binds the small molecule, but is capable of relaying or transducing a complex set of signals carried along by the properties of the ligand. As reviewed herein, the nature of the information that the ligand-bound NR relays depends on a complex interplay of factors, such as ligand and cell type.

In humans, 48 NR genes have been identified (Figure 1).[1] A unifying feature of the NR superfamily is that each receptor consists of an assembly of functional modules (Figure 2).[2] For the purpose of this review, the module most relevant to current drug-discovery approaches is the C-terminal ligand-binding domain (LBD). The LBD is typically about 250 amino acids in length and contains a key regulatory element, the so-called activation function-2 (AF2) domain, as well as all the recognition elements required for ligand binding (Figure 3 A, left).[3]

The fold of the NR LBD is typically described as three stacked α-helical sheets. The helices that make up the "front" and "back" sheets are aligned parallel to one another. The helices in the middle sheet run across the two outer sheets and only occupy space in the upper portion of the domain (Figure 3 A, right). The space in the lower part of the domain is relatively void of protein, and for most NR LBDs, this creates an internal cavity for small-molecule ligands.

The central region of the typical NR contains the DNA-binding domain (DBD), which is usually about 70 amino acids long, contains two zinc-finger motifs, and is the most highly conserved sequence segment among the NRs. For some NRs, the DBD forms a dimer and binds a DNA response element containing a direct repeat of six base pairs (Figure 3 B).[4] The DBD contains three helices, the first of which docks into the major groove of the DNA recognition site. A second, smaller helix and the loop preceding it create a domain–domain interface. Most NRs have an N-terminal domain, commonly referred to as the activation function-1 (AF1) domain. This module varies greatly in length among receptors and generally contains a ligand-independent transcriptional activation function.

Upon activation by the ligand messenger, NRs typically function as transcription factors where they bind to recognition elements and regulate the expression of target genes. Once complexed with DNA, NRs recruit accessory proteins such as co-activators, co-repressors, and basal transcriptional factors, thus regulating gene transcription (Figure 4). In some cases, genes under the control of a negative response element are down-regulated by an NR; thus NRs are able to act directly as activators or suppressors of gene function. As discussed in this chapter, NR pathway regulation goes beyond direct, DNA-mediated transcriptional regulation. For example, some NRs crosstalk with other important signal-transduction schemes such as nuclear factor κB (NFκB) and activator protein-1 (AP-1)[5] (Figure 4).

NRs have a rich and long-standing history in drug discovery. This can be attributed to features inherent to this class of targets: 1) NRs have been designed by nature to selectively bind "druglike" small molecules and 2) a diverse set of biologically important functions can be regulated through a single ligand-activated receptor (see Table 1 for examples of NR-targeted drugs). Using data compiled for 2003,[6] 34 of the top 200 most prescribed drugs target an NR. Today, drugs that target a given NR account for over 30 billion dollars in pharmaceutical sales and are used in the treatment of numerous debilitating diseases. In light of these facts, the NR field remains an area of intense research with most of the current effort directed toward the improvement of current NR drugs or screening currently unexploited NRs. The purpose of this review is to briefly cover the following general topics as they pertain to the chemical biology of NRs: the history of NR-targeted drug discovery, principles of NR–ligand recognition and protein conformational change, biological pathways controlled by NRs, recent NR drug pursuits, and finally some new technologies and future pharmaceutical prospects for this target class.

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[**] *This review will also appear as a chapter in the book* Chemical Biology—From Small Molecules to Systems Biology and Drug Design *(Eds.: S. Schreiber, T. Kapoor, G. Wess), Wiley-VCH, Weinheim, which is scheduled for publication in October 2006.*

2. A Brief History of NRs in Medicine and Drug Discovery

The first generation of NR drugs was discovered prior to detailed knowledge of the target class. Many clinically useful compounds were initially found by tracking down biological activity from natural extracts. Only later did these bioactive molecules lead scientists to the actual drug target.

John T. Moore received his undergraduate degree in biology from the University of Virginia in 1981 and a PhD in cell and molecular biology from the University of Minnesota in 1985. He carried out his postdoctoral work in the research group of Professor James Fristrom at the University of California, Berkeley until 1987. After several years as an assistant professor of biology at Bard College in Annandale, New York, he joined GlaxoSmithKline in 1992. His current research is focused on the functional analysis of nuclear receptors.

Jon L. Collins received his BS degree in chemistry from the University of North Carolina at Chapel Hill in 1987. In 1988 he moved to Indiana University at Bloomington, where he earned a PhD in organic chemistry in 1992 under the direction of Professor Paul A. Grieco. In 1992, he moved to Harvard University as an American Cancer Society postdoctoral fellow in the research group of Professor Stuart L. Schreiber. He joined GlaxoSmithKline in 1995 and has been involved in research directed toward the identification and optimization of ligands for orphan nuclear receptors.

Kenneth H. Pearce received a BS degree in chemistry from the University of Richmond in 1989. He received a PhD in biological chemistry from the University of North Carolina at Chapel Hill in 1993, where his work involved the biophysics of proteins at membrane surfaces under the direction of professors Nancy Thompson and Richard Hiskey. He then moved to the research group of Dr. James Wells at Genentech to study hormone–receptor interactions and cell surface signal transduction. Following these postdoctoral studies, he joined drug-discovery research at GlaxoSmithKline in 1996 and currently studies aspects of nuclear receptor biochemistry and signaling.

Studies of bioactive fractions from natural extracts containing steroid or thyroid hormones helped to lay the foundation for modern NR-based endocrinology. For example, the study of adrenal gland extracts initiated glucocorticoid receptor (GR) drug discovery, and these tissue extracts were used clinically to correct the manifestations of Addison's disease (glucocorticoid deficiency)[7]. From this early clinical work, a well-defined relationship began to emerge that connected the adrenal extract with maintenance of homeostatic function. For example, it was noted that, in addition to bringing about remission from stress-related diseases, the extracts also suppressed symptoms in patients suffering from inflammatory conditions such as allergy, hay fever, and asthma. At the same time, biochemical characterization of the adrenal gland extracts identified cortisone as an active steroidal component. In 1948, when sufficient quantities of cortisone could be purified, its effects in inflammatory disease were tested directly. Ultimately, total syntheses of cortisone were independently carried out by Woodward and colleagues and a group at Merck,[8,9] thus completing the first-generation evolution of this drug and setting the stage for later syntheses of potent synthetic steroids such as prednisolone and dexamethasone.

A similar history was observed with the first generation of drugs that targeted other steroid receptors. It was known as early as 1916 that ovariectomy could decrease the incidence of mammary cancer in high-incidence strains of mice.[10] Studies of the biological effects of extracts containing estrogenic activity prompted screens for compounds with anti-estrogenic effects, initially for contraception in the 1960s, but later for estrogen-responsive breast cancers. Screens for anti-estrogenic nonsteroidal compounds led to the discovery of ethamoxytriphetol, clomiphene, and then tamoxifen. Tamoxifen ultimately became the gold standard for the endocrine treatment of breast cancer and relatively recently became the first approved cancer chemopreventative agent.

Not surprisingly, the first set of NR genes cloned were from the steroid receptor subgroup for which prior research yielded compounds to aid in the purification of the receptor. The first human NR cloned was the GR, an accomplishment that relied heavily on 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.[11–13] cDNAs representing the full-length coding region of GR provided the first full-length amino acid sequence of an NR. The estrogen receptor (ER) was also cloned around the same time by three research groups using independent strategies.[14–16]

Comparison of emerging NR sequences (from human and other species sources) revealed conserved domains shared between virtually all NRs. The finding that NRs could be isolated without knowledge of their ligand increased the rate at which new NRs could be identified. Initially, oligonucleotides representing conserved NR motifs (such as the highly conserved DBD) were employed as molecular probes to perform low-stringency DNA hybridizations to cDNA libraries. The number of orphan NRs quickly surpassed the number of classical nuclear hormone receptors.[17–19]

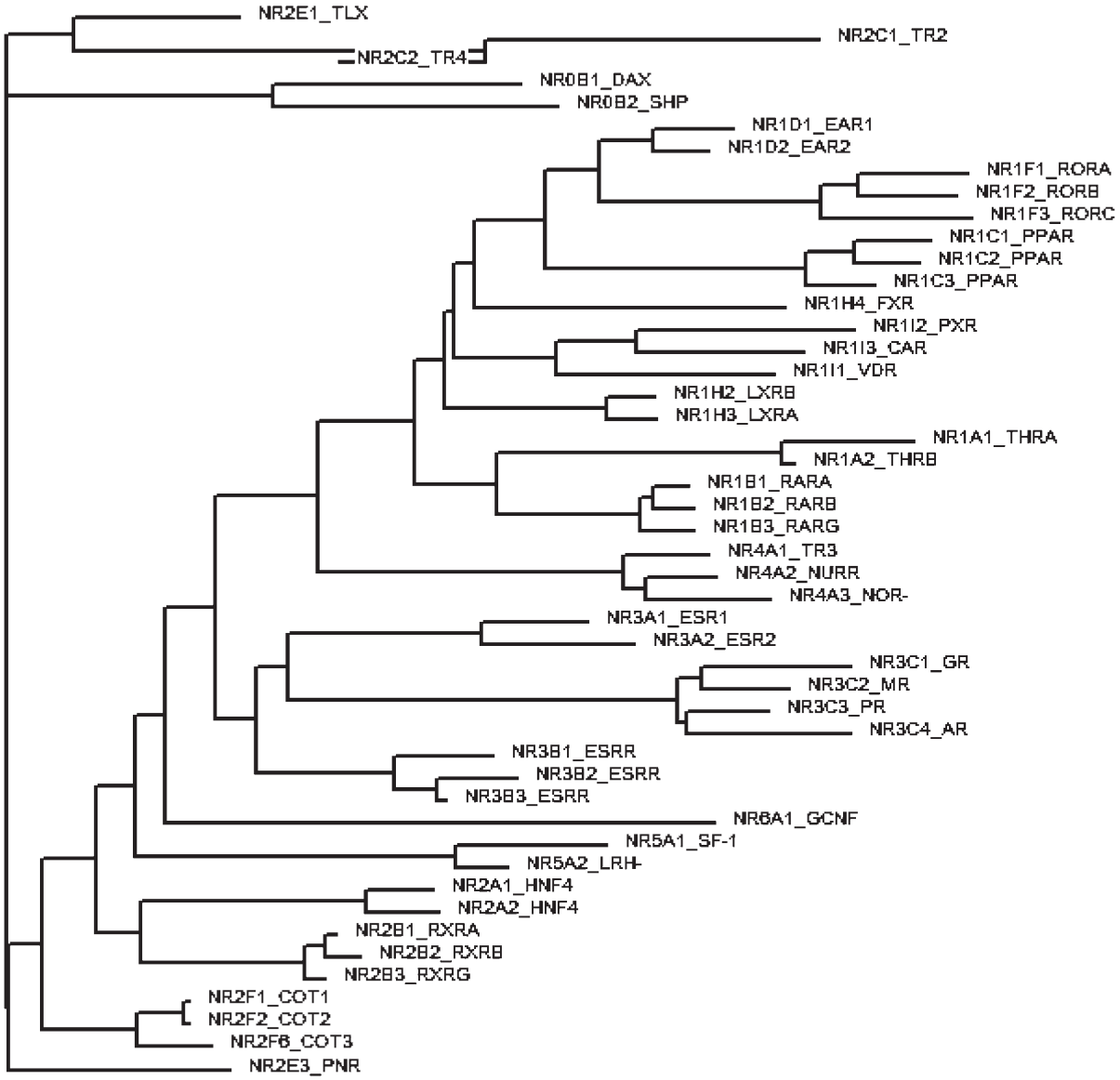


Figure 1. The NR superfamily represented as a phylogeny plot. The 48 identified receptors within the human genome are shown clustered according to amino acid sequence relationships. NRs are named according to the accepted unified nomenclature (see Table 1 for a more detailed description).[1]

By the late 1990s, the chosen method for the identification of new NRs shifted from the laboratory to in silico methods. This advance was made possible by the availability of large databases of randomly generated partial cDNA sequences known as expressed sequence tags (ESTs) and the development of bioinformatic searches and query tools such as BLAST. Two new mammalian NRs were successfully identified through automated searches of EST databases. The pregnane X receptor (PXR) was identified in a public mouse EST database by a high-throughput in silico screen for NR-like sequences,[20] and the photoreceptor cell-specific receptor (PNR) was found in a human EST database.[21] After the isolation of PNR from EST databases, the number of known human NRs totaled 48. The availability of the complete human genome sequence in 2001 confirmed that this set of 48 is the complete NR genome.[22,23]

As new NRs were isolated, new connections between first-generation drugs and their targets were made. For example, thiazolidinediones had previously been discovered through traditional pharmacological methods to show clinical benefit in diabetes; however, the molecular basis for this therapeutic effect remained unclear. By using expression constructs derived from the isolated NR genes, activity screens for each receptor were developed. Under these screens, thiazolidinediones were found to be potent and selective activators of peroxisome-proliferator-activated receptor γ (PPARγ).[24] Once this link was made, the search for a second generation of PPARγ compounds could be initiated 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. Traditionally, ligands were

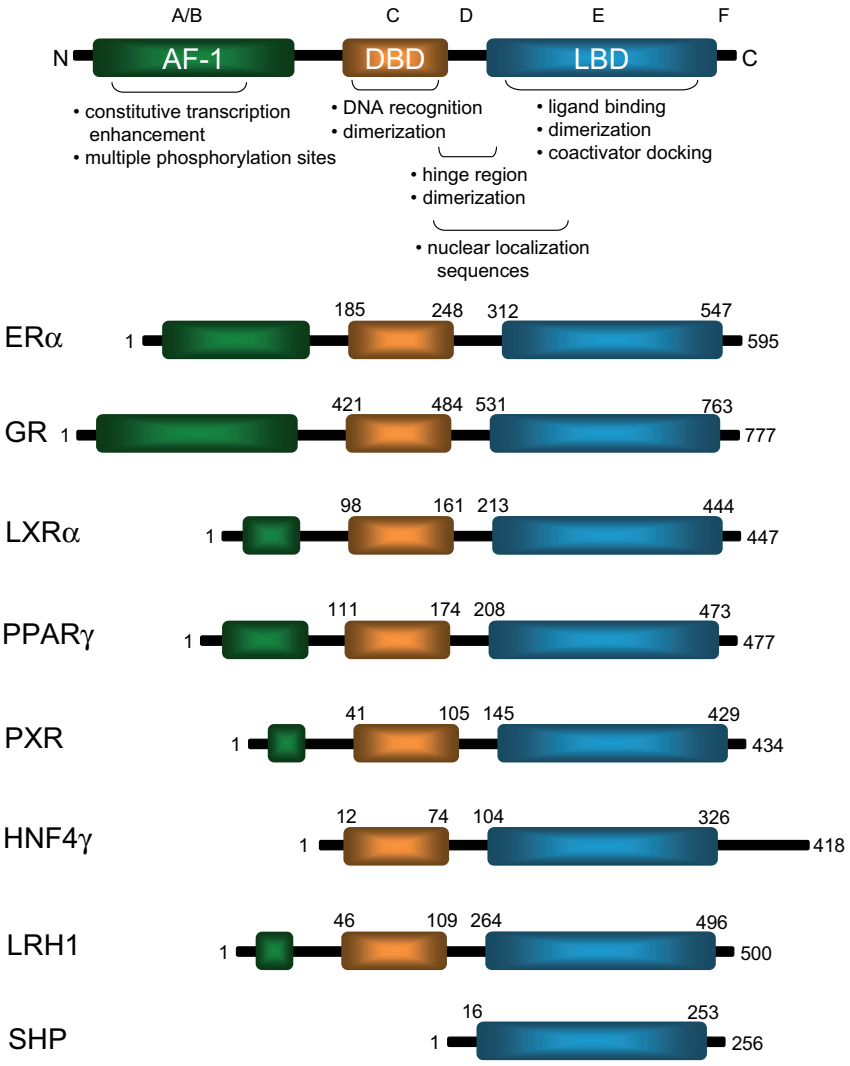


Figure 2. Domain organization of the NRs. The basic structural modules that constitute the NR are shown. The general functions of the respective regions of NRs are noted in the linear schematic at the top. Examples of selected NRs are shown below to demonstrate that most NR LBDs are similar in amino acid length, but the N-terminal region varies among family members. Numbers represent amino acid position.

identified based on their biological effects. When this process is reversed, however, the orphan receptors are used to identify the ligands, which are subsequently used to dissect the biology of the receptors. For example, a reverse endocrinology approach was used to link farnesoid X receptor (FXR) to bile acid ligands. The availability of chemical tools (bile acids and synthetic ligands) for FXR led to experiments that linked FXR to bile acid homeostasis and suggested the possibility that FXR ligands could be beneficial to the treatment of disorders such as cholestatic liver disease.[25]

Among the NR superfamily, a third-generation drug-discovery effort has recently begun. In this phase, screening methods that give information beyond potency and selectivity (for example, selective effects on gene expression) are used to discover compounds with therapeutic advantages over drugs in current use. Strategies that underlie this new drug-discovery effort are the subject of a following discussion on NR modulators.

3. Basic Principles of Ligand–NR Recognition

From a medicinal chemistry perspective, targeting NRs with novel small-molecule ligands is a fairly tractable exercise. As mentioned above, most NRs have a small, enclosed ligand-binding pocket, and a wide variety of druglike, high-affinity molecules can be identified that bind in this pocket. The inherent difficulty of rational drug design for NRs derives from the vast complexity of NR-associated biology. Whereas small molecules that bind the target NR with high affinity can be fairly readily identified, the corresponding functional activity is not always clear or immediately interpretable given the current level of biological understanding (discussed in greater detail below). In this section, the general principles of ligand binding for NRs is discussed.

Steroid receptors: GR, MR, PR, AR, and ER

The ligand-binding pockets of the steroid receptors, which include the GR, the mineralocorticoid receptor (MR), the progesterone receptor (PR), and the androgen receptor (AR), as well as the more divergent ER, have many common features required for binding the natural hormone. At least one crystal structure is available for each of these ligand-binding domains[26–29] (see Figure 3A and Figure 5 for examples of PR and GR, respectively). Typically, about 75% (roughly 17 of 22 residues) of the ligand-binding pocket inner lining consists of hydrophobic residues. Generally, all of the polar residues within the binding pocket (roughly three to five residues) make a hydrogen bond to the natural ligand. In each case, the A ring of the steroid hormone is positioned between helices 3 and 5. The oxosteroid receptors GR, MR, PR, and AR lock the carbonyl group at position 3 on the A ring of the steroid into place with a hydrogen bond "charge clamp" using conserved glutamine and arginine residues on helices 3 and 5, respectively. With ER, coordination of the hydroxyl group at position 3 is made with glutamate and arginine residues at the respective locations. In all cases, the D ring of the steroid points toward helix 10 and the AF2 helix (the C-terminal α helix of the LBD).

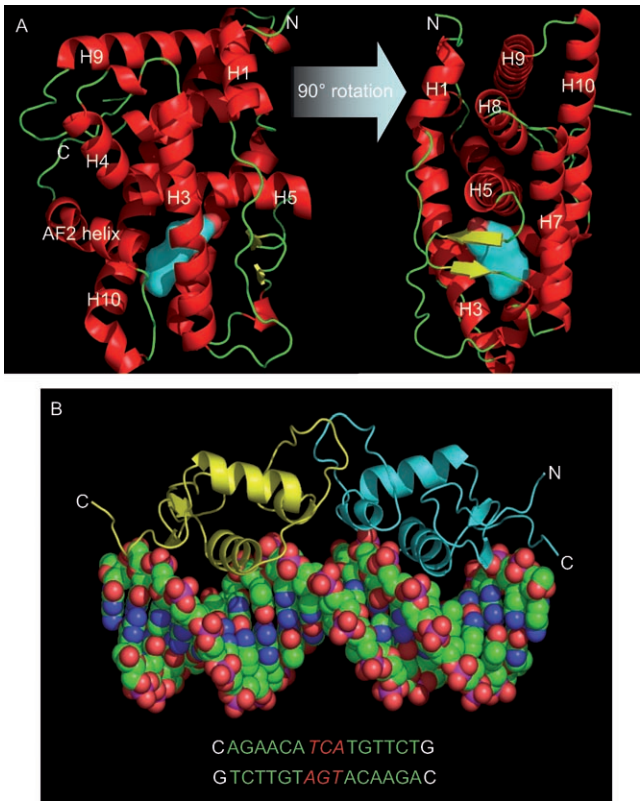


Figure 3. Representative structures of NR functional modules. A) The first NR LBD structure to be solved crystallographically was the apo-RXR LBD.[46] The representative example structure shown is that of the progesterone receptor (PR) bound to its natural ligand, progesterone.[28] This structure, which was the first of the steroid receptors to be solved, shows the basic fold conserved among members of the NR superfamily. The major helices (red) are labeled, the well-conserved small β sheet is shown in yellow, and the random-coil stretches connecting the major structural elements are colored green. The final C-terminal helix is labeled as the activation function-2 (AF2) helix and is described in more detail in the text. The progesterone molecule is shown in space-filling mode and colored by atom type, with carbon atoms in blue and oxygen atoms in red. The domain on the right is rotated 90° to clearly show the three helical layers that constitute the NR LBD fold. B) The first X-ray crystal structure of an NR DBD bound to a DNA response element is shown as a ribbon diagram. This representative structure is the DBD from the glucocorticoid receptor (GR) bound in an antiparallel fashion to its inverted direct-repeat DNA response site.[129] The GR DBD is bound as a homodimer, with the different subunits shown in yellow and blue. The DNA helix is shown in space-filling representation and is colored according to atom type (carbon, green; oxygen, red; nitrogen, blue; phosphorus, magenta). All structure figures were generated by using PyMol (Delano Scientific; www.delanoscientific.com)

In all, the volume of the pocket varies slightly among the receptors when complexed with the respective natural ligand: approximately 420 Å^3 for AR, 450 Å^3 for ERα, 560 Å^3 for PR, 580 Å^3 for MR, and 590 Å^3 for GR. However, the volume of the pocket can change significantly depending on the size and shape of the ligand bound. This dynamic flexibility allows this class of receptors to accept a wide variety of synthetic ligands with numerous shapes and volumes. Interestingly, there are as yet no reports of a crystal structure of an unliganded steroid receptor; therefore, the precise nature of the pocket in the absence of ligand is unknown.

Crystal structures of steroid receptors in complex with synthetic ligands have revealed alternative binding modes relative to that of the natural steroid hormone. To date, ERα and ERβ subtypes[30] have provided the greatest variety of crystal structures with bound synthetic ligands.[31] There are currently several examples of ER in complex with synthetic ligands: diethylstilbestrol (DES), 4-hydroxytamoxifen (OHT),[32] genestein,[33] raloxifene,[34] (*R*,*R*)-5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC),[35] and the pure anti-estrogen ICI 164384.[36] Each of these complexes, either with ERα or ERβ, reveals the conservation of the hydrogen bond clamp with a hydroxy group on the A-ring analogue. The presence of this interaction in each of the structures emphasizes the importance of this hydrogen bond for high-affinity binding. The other commonality between these ligands is that they fill the core of the ligand-binding pocket with hydrophobic groups, each occupying roughly the same volume. One of the key features shared between the structures of OHT, raloxifene, and ICI 164384 is an extended amine or hydrophobic group directed toward the AF2 helix, which causes steric repositioning of this structural element (see Figure 8B and the following discussion).

RXR heterodimer receptors: PPARs, RXR, LXR, and FXR

Unlike the steroid receptors, most of which function as homodimers, members of a second NR class function as heterodimers with the retinoid X receptor (RXR). Importantly, these receptors serve as sensors for metabolites such as fatty acids, oxysterols, and bile acids. The key elements of ligand recognition and receptor activation have been elucidated following structure–function analyses of several receptors in this family, including the PPARs, liver X receptors (LXRs), and FXR.

The X-ray crystal structures of the PPARs, LXRs, and FXR have been determined in various unliganded and liganded states. The volumes of the ligand-binding pockets are larger than the steroid receptors and range from 700–850 Å^3 for FXR/LXRs to 1300 Å^3 for the PPARs. As with the steroid receptors, the size and shape of the ligand-binding pockets can vary depending on the size and shape of the ligand. This plasticity permits the binding of diverse, structurally distinct chemotypes.

The majority of amino acids that line the ligand-binding pockets of these receptors are hydrophobic. However, several key polar amino acids are present which have been shown to be critical for ligand recognition and receptor activation. For the PPARs, an acidic group present in fatty acids is involved in a complex hydrogen-bond network consisting of a tyrosine residue on the AF2 helix and two histidine residues on helices 5 and 10, most of which are conserved between the three PPAR subtypes (Figure 6). Importantly, the direct hydrogen-bonding interaction of the acidic moiety with tyrosine on AF2 stabilizes the helix in an active conformation and initiates transcriptional activation. The requirement for this interaction for transcriptional activation is evidenced by the fact that PPAR ligands (such as GW0072, Figure 9) that lack this hydrogen-bonding interaction show partial agonist or antagonist activity.[37]

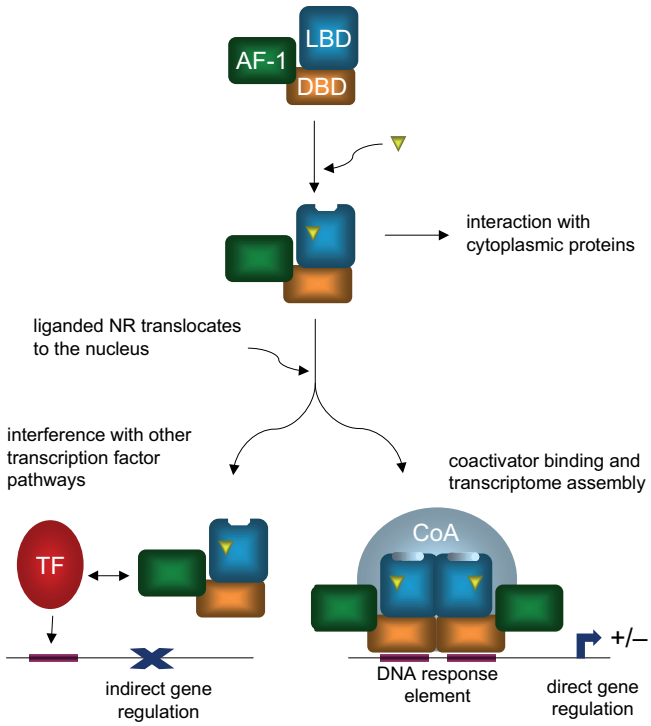


Figure 4. A simplified scheme of the general mechanisms of NR function. Some unliganded NRs, such as the steroid receptors, are present in the cytoplasm in an inactive complex with heat-shock proteins. Ligand binding triggers heat-shock protein uncoupling and transport of the NR to the nucleus. To directly regulate gene transcription, the ligand-bound NR associates with a DNA response element within the promoter of the target gene. In many cases, the NR localizes in the form of a homo- or heterodimer. This complex is able to recruit co-activator (CoA) proteins and other transcriptional components to regulate the expression of target genes. Another mechanism by which ligand-activated NRs can affect gene transcription involves association with other transcription factors (TFs) such as NFκB and AP-1. The precise molecular mechanism of this latter activity remains controversial.

In contrast to the PPARs, the interaction between oxysterols and bile acids with LXR and FXR, respectively, does not occur through a direct interaction with an amino acid on AF2.[38,39] A critical hydrogen-bond interaction is observed between a histidine group on helix 10/11 and either an acceptor oxygen atom on the natural ligand (expoxycholesterol) or a donor oxygen atom on a synthetic ligand (T0901317). This interaction positions the histidine perpendicularly to a tryptophan residue located on the AF2 helix (Figure 7) which, in turn, promotes an electrostatic interaction between these two amino acids. In addition to contributing to ligand binding, this network of interactions connecting ligand to the AF2 helix helps stabilize the receptor in an active confirmation (Figure 7). Notably, hydrophobic interactions between ligand and receptor can also initiate the histidine–tryptophan electrostatic switch.[40] The cumulative data suggests that this histidine–tryptophan interaction is the molecular basis for ligand-dependent activation of the LXRs and FXR. Clearly, a select number of polar amino acids within the binding pockets of PPARs, LXRs, and FXR play important roles in mediating ligand recognition and receptor activation.

"Orphan" receptors: HNF4, CAR, and NGFIB

Whereas the steroid and RXR heterodimer receptors show low transcriptional activity in the basal state, several NRs have been identified which are transcriptionally active in the basal state and are thus referred to as "constitutively active receptors". Structural analyses of two NRs in this class, the hepatocyte nuclear factors 4 (HNF4s)[41] and nerve growth factor induced B (NGFIB),[42] provide insight into two unique mechanisms that give rise to constitutive activity. The X-ray crystal structure of HNF4γ has revealed the presence of expression-host-derived fatty acids in the ligand-binding pocket. A similar observation was made for HNF4α.[43] The fact that these fatty acids are not displaceable led to the proposal that these natural ligands serve as structural cofactors for HNF4. In contrast to HNF4γ, the X-ray crystal structures of NURR1 and DHR38, the mouse and *Drosophila* orthologues of NGFIBβ, respectively, showed the absence of a ligand-binding pocket.[42,44] Instead, several bulky hydrophobic residues fill the space that is normally occupied by ligand, suggesting that the receptor may not be regulated by the classical ligand-based approach. Clearly, determination of the X-ray crystal structures for the remaining orphan NRs will provide insight into the tractability of these targets for drug discovery.

4. Influence of Ligand on NR LBD Conformation

There have been numerous key studies demonstrating that ligand binding does not simply trigger NRs from an off-state to an on-state. In fact, these studies revealed at a molecular level that the activation of an NR by a small-molecule ligand is dramatically more complex than a two-state process. The concept in which ligand alters NR conformation to produce activity profiles pertains mostly to the steroid receptors, PPAR, TR, RXR, RAR (retinoic acid receptor), LXR, and FXR. There is considerable doubt that this concept applies to select "constitutively active" receptors such as HNF4 and NGFIB.

One of the first studies to reveal the conformational effect of ligand involved a protease digestion assay to show that ER ligands could differentially affect the pattern of protease-generated peptides.[45] As suspected from earlier work, this study demonstrated that different ligand classes could affect NR conformation and thus alter the AF2 activity of the receptor.

X-ray crystallographic studies have been predominant in shedding light on how ligands can alter NR conformation. In the late 1990s, two ground-breaking reports on ER showed that ligand can particularly affect the orientation of the C-terminal α helix of the LBD, referred to as the AF2 helix.[32,34] In these studies, the AF2 helix of ER, bound with an agonist ligand such as estradiol or the synthetic diethylstilbestrol, was shown to adopt a position similar to that observed in the original RAR and PPARγ agonist-bound structures[46,47] (Figure 8A). In this active conformation, the AF2 helix spans across helices 3 and 10. This arrangement creates a shallow hydrophobic groove adjacent to the AF2 helix. This pocket accommodates a short helical peptide presented at the surface of a co-activator

Table 1. The human nuclear receptor superfamily and examples of ligands and therapeutic utilities.

Category[a]	Name	Subtypes and Abbreviations	Unified Nomenclature[b]	Natural Ligand	Examples of Therapeutic Ligands (Trade Name)	Therapeutic Relevance[c]
Classic Steroid Receptors	estrogen receptor	ERα ERβ	NR3A1 NR3A2	estradiol, estrogens	tamoxifen, raloxifene (Evista), genestein, diethylstilbestrol, equine estrogens (Premarin)	menopausal symptoms, osteoporosis prevention, breast cancer
	glucocorticoid receptor	GR	NR3C1	cortisol, glucocorticoids	prednisone, dexamethasone, fluticasone propionate (Flovent, Flonase), mometasone furoate (Nasonex), budesonide (Rhinocort/Pulmicort)	inflammatory and immunological diseases, asthma, arthritis, allergic rhinitis, cancer, immune suppressant for transplant
	mineralocorticoid receptor	MR	NR3C2	aldosterone, deoxycorticosterone	spironolactone (Aldactone), eplerenone (Inspra)	hypertension, heart failure
	progesterone receptor	PR	NR3C3	progesterone, progestins	RU486 (Mifepristone)	abortifacient, menstrual control
	androgen receptor	AR	NR3C4	testosterone, androgens	flutamide, bicalutamide (Casodex)	prostate cancer
Classic RXR Heterodimer Receptors	thyroid hormone receptor	TRα TRβ	NR1A1 NR1A2	thyroid hormone	levothyroxine (Synthroid)	thyroid deficiency
	retinoic acid receptor	RARα RARβ RARγ	NR1B1 NR1B2 NR1B3	retinoic acid	isotretinoin (Accutane)	acne
	peroxisome proliferators-activated receptor	PPARα PPARδ PPARγ	NR1C1 NR1C2 NR1C3	fatty acids, eicosanoids	fenofibrate (Tricor; PPARα), thiazolidenediones (Avandia, Actos; PPARγ)	dyslipidemia (PPARα), diabetes and insulin sensitization (PPARγ)
	liver X receptor	LXRα LXRβ	NR1H2 NR1H3	24,25-epoxycholesterol, 24-hydroxycholesterol	—	role in lipid and cholesterol metabolism, atherosclerosis
	farnesoid X receptor	FXR	NR1H4	chenodeoxycholic acid	—	cholesterol maintenance, protect hepatocytes from bile toxicity, cholestasis
	vitamin D receptor	VDR	NR1I1	vitamin D, bile acids	calcitriol (Rocaltrol)	hypocalcemia, osteoporosis, renal failure
	retinoid X receptor	RXRα RXRβ RXRγ	NR2B1 NR2B2 NR2B3	all-*trans* retinoic acid	LG1069 (Targretin)	skin cancer
Xenobiotic Receptors	pregnane X receptor	PXR	NR1I2	xenobiotics	St. John's wort, rifampicin	role in protection from toxic metabolites
	constitutive androstane receptor	CAR	NR1I3	xenobiotics	phenobarbitol	role in protection from toxic metabolites
Orphan Receptor (or Recently Deorphaned)	estrogen-receptor-related receptor	ERRα ERRβ ERRγ	NR3B1 NR3B2 NR3B3	*unknown*	tamoxifen, diethylstilbestrol (ERRγ)	muscle fatty acid metabolism (ERRα)
	RAR-related orphan receptor	RORα RORβ RORγ	NR1F1 NR1F2 NR1F3	cholesterol, cholesterol sulfate	—	role in cerebellum development, maintenance of bone (ROAα), circadian rhythm (RORβ), lymph node organogenesis (RORγ)
	human nuclear factor 4	HNF4α HNF4γ	NR2A1 NR2A2	palmitic acid	—	role in diabetes
	reverse erbA	Rev-erbAα Rev-erbAβ	NR1D1 NR1D2	*unknown*	—	circadian rhythm
	testis receptor	TR2 TR4	NR2C1 NR2C2	*unknown*	—	*unknown*
	tailless-like	TLX	NR2E1	*unknown*	—	role in neuronal development
	photoreceptor-specific nuclear receptor	PNR	NR2E3	*unknown*	—	role in photoreceptor cell differentiation
	chicken ovalbumin upstream promoter–transcription factor	COUP-TFI COUP-TFII COUP-TFIII (Ear2)	NR2F1 NR2F2 NR2F6	*unknown*	—	role in neuronal development (COUP-TFI), vascular development (COUP-TFII)

Table 1. (Continued)

Category[a]	Name	Subtypes and Abbreviations	Unified Nomenclature[b]	Natural Ligand	Examples of Therapeutic Ligands (Trade Name)	Therapeutic Relevance[c]
	NGF-induced factor B	NGFIBα (also NUR77)	NR4A1	*unknown*	—	role in thymocyte apoptosis
	Nur-related factor 1	NGFIBβ (NURR1, NOT1)	NR4A2	*unknown*	—	role in dopaminergic neuron development
	neuron-derived orphan receptor 1	NGFIBγ (NOR1)	NR4A3	*unknown*	—	*unknown*
	steroidogenic factor 1	SF1	NR5A1	phospholipids	—	role in mammalian sexual development
	liver receptor homologous protein 1	LRH1	NR5A2	phospholipids	—	role in lipid homeostasis, cell-cycle control
	germ cell nuclear factor	GCNF	NR6A1	*unknown*	—	role in vertebrate embryogenesis
NR-like, DBD-less Repressors	DSS-AHC critical region on the chromosome gene 1	DAX1	NR0B1	*unknown*	—	role in sex determination and development
	short heterodimer partner	SHP	NR0B2	*unknown*	—	general repressor of NRs, obesity

[a] Each of the 48 human receptors is roughly categorized into several generalized groups. The order descends from the historically more studied, classical receptors (top) to the more recently discovered family members (bottom). [b] Nomenclature from ref. [1]. [c] Description of the biological role of the receptor if ligand is currently not identified.

protein (discussed below). Peptides that bind this region of the activated NR typically contain an LXXLL motif (for which L = leucine and X = any amino acid). This short peptide motif is typically α helical, and the leucine residues are presented on one face of the amphipathic helix. An additional electrostatic interaction between amino acid side chains of the receptor to the peptide backbone are believed to aid the orientation and stability of the interactions.

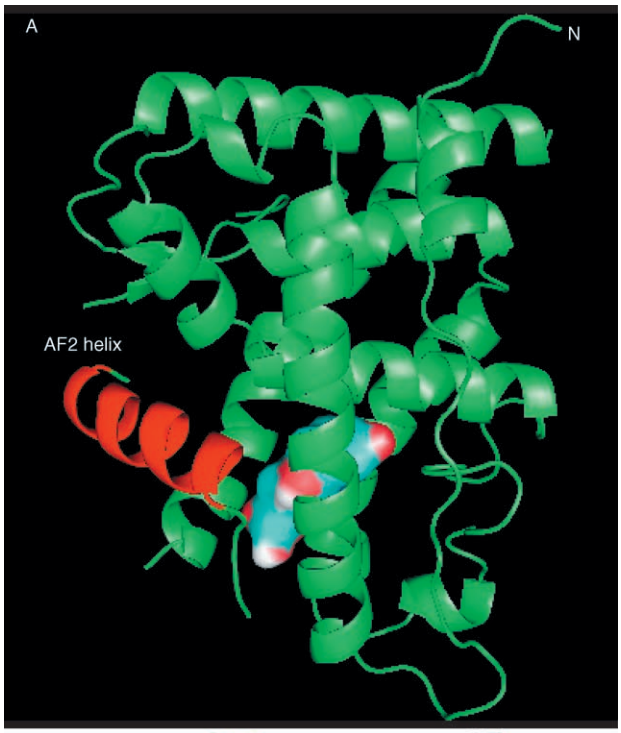
The structures of ER bound with either tamoxifen or raloxifene, both of which are antagonists of AF2 function, strikingly revealed that the AF2 helix could be repositioned from the agonist conformation (Figure 8B). In each of these structures, an amine-containing head group from the ligand protrudes toward the surface of ER to destabilize the active position of the AF2 helix. This shift causes the AF2 helix to rotate approximately 90° from the active position. According to the crystal structures, in the antagonist position, the AF2 helix occupies the co-activator peptide-binding site on the surface of the receptor. These studies highlight the ligand-induced flexibility and plasticity of the NR LBD, particularly with respect to the AF2 helix.

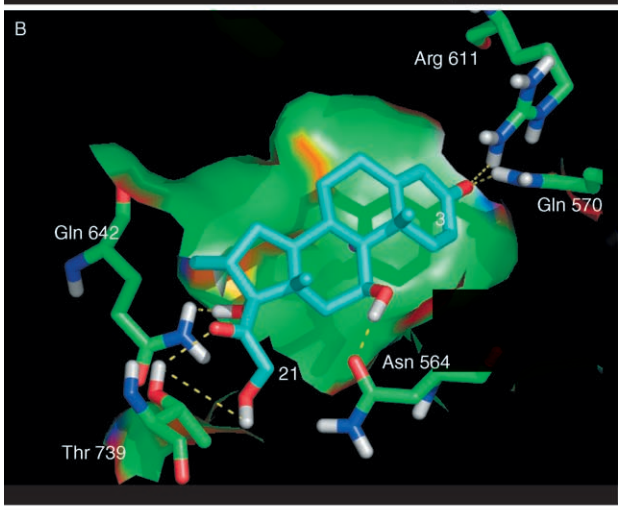
More recent structural studies with the GR LBD further demonstrate how ligand can influence the conformation of the LBD.[26,48] The structure of GR bound with the agonist dexamethasone shows that the AF2 helix is present in an active position to allow co-activator peptide association. Two structures of GR bound with the antagonist ligand RU486 have shown that a protruding dimethylaniline group effectively prevents the AF2 helix from occupying the active position. In one of these structures, the AF2 helix intramolecularly blocks the co-activator site. In the other structure, the AF2 helix extends away from the core of the LBD and associates with an adjacent LBD subunit in the crystal. Again, these studies suggest that the AF2 helix and the loop that precedes it are prone to ligand-induced conformational flexibility.

Two studies of PPAR also demonstrate the ligand-induced conformational aspects of the LBD. As observed in a structure of PPARα in complex with both an antagonist ligand GW6471 (Figure 9) and a peptide motif from a co-repressor (reviewed below), the AF2 helix assumes an alternative location (Figure 8C).[49] In this case, the AF2 helix occupies neither the agonist nor antagonist position (that is, the co-activator groove as observed with ER), but lies adjacent to the co-repressor peptide. Another study with PPARγ using NMR spectroscopy shows that the apo-LBD is a highly flexible module for which over half of the chemical shifts of the backbone atoms are missing.[50] When bound with rosiglitazone, these shifts can be assigned, particularly those for the ligand-binding pocket and the AF2 helix regions. In all, these studies suggest that the physiochemical properties of the NR ligand can dramatically influence conformational dynamics of the LBD, which in turn ultimately governs the downstream signaling aspects of the liganded receptor.

5. The Multitude of Ligand-Induced NR Activities

By virtue of their ability to interact with a repertoire of molecules within the cell, ranging from DNA response elements to protein accessory factors, the NRs represent a target class of





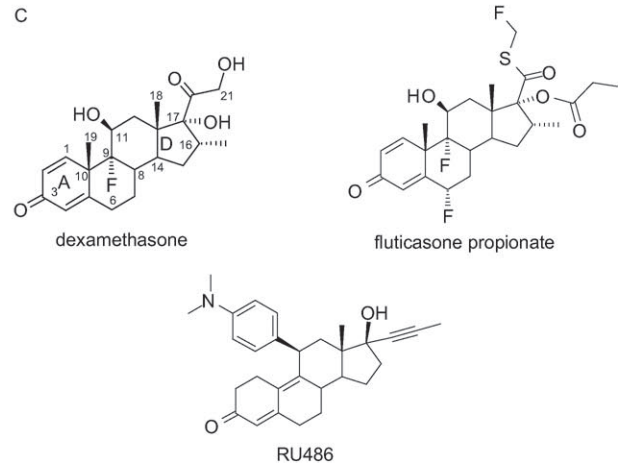


Figure 5. Structure of the GR LBD and features of ligand binding. A) Crystal structure of the GR LBD with the dexamethasone agonist bound.[26] The protein is shown as a ribbon diagram, and the AF2 helix (red), is in the active orientation. Dexamethasone (space-filling model) is shown with carbon atoms in blue, oxygen atoms in red, and hydrogen atoms in white. B) Close-up view of the GR ligand-binding site. The pocket is shown as a cut-away, and the back face represents the hydrophobic nature of the pocket (carbon atoms are colored green). Dexamethasone is shown oriented with the position 3 ketone group of the A ring directed toward the back of the pocket, and the D ring is positioned toward the AF2 helix. Hydrogen bonds with key amino acids within the pocket are shown as dashed yellow lines. C) Representative structures of well-known GR ligands.

complex, multitasking proteins.[51],[52] Most of the NRs were initially considered to be simple ligand-induced transcription factors. However, studies over the past decade have revealed that NRs are much more complicated and serve more than a unified functional purpose. In this section, we highlight some of the types of activities of NRs by citing particular examples.

Gene regulation and the role of activity-enhancing accessory proteins

At various stages in the activity cycle, NRs act in concert with a variety of binding partners. For example, prior to ligand binding, the GR resides in the cytoplasm of the cell in complex with chaperone proteins such as heat-shock protein (hsp) 90 or p23.[53] Ligand association causes dissociation of chaperones and allows the GR to traverse the nuclear envelope. Using amino acids within the DBD, the GR binds to a recognition site on a specific promoter, a site referred to as a glucocorticoid response element (GRE). NR response elements have a general half-site consensus of RGGTCA (for which R = purine); these DNA half-sites are commonly arranged as repeats, either direct or inverted. The precise mechanism by which NRs associate with DNA response elements varies among members of the superfamily. In general, the steroid receptors bind to their respective response elements as homodimers, although GR can form heterodimers with MR and ERα, and ERβ can also bind DNA in heterodimeric form. Several NRs, such as TR, PPARs, LXR, VDR, RAR, and FXR require heterodimerization with RXR. Further, many of the orphan receptors such as LRH-1, SF-1, and NGFIB can bind DNA as a monomer.

The DNA-bound, ligand-activated NR serves as the docking site for a rather large extended family of proteins called co-activators. Binding of a co-activator protein is believed to be one of the key events in initiating transcriptome assembly and subsequent gene transcription. The first co-activator, steroid receptor co-activator-1 (SRC1), was identified in 1995,[54] and since then over 200 such cofactors have been discovered. The wide variety of co-activator functions is a vastly complex field, and a full description of the many co-activator functions is beyond this review; indeed, details have been reviewed previously.[55–57] The focus herein is on one representative, SRC-1, which is a member of the p160 family of co-activators. This family also includes SRC-2 (also called transcription intermediary factor-2

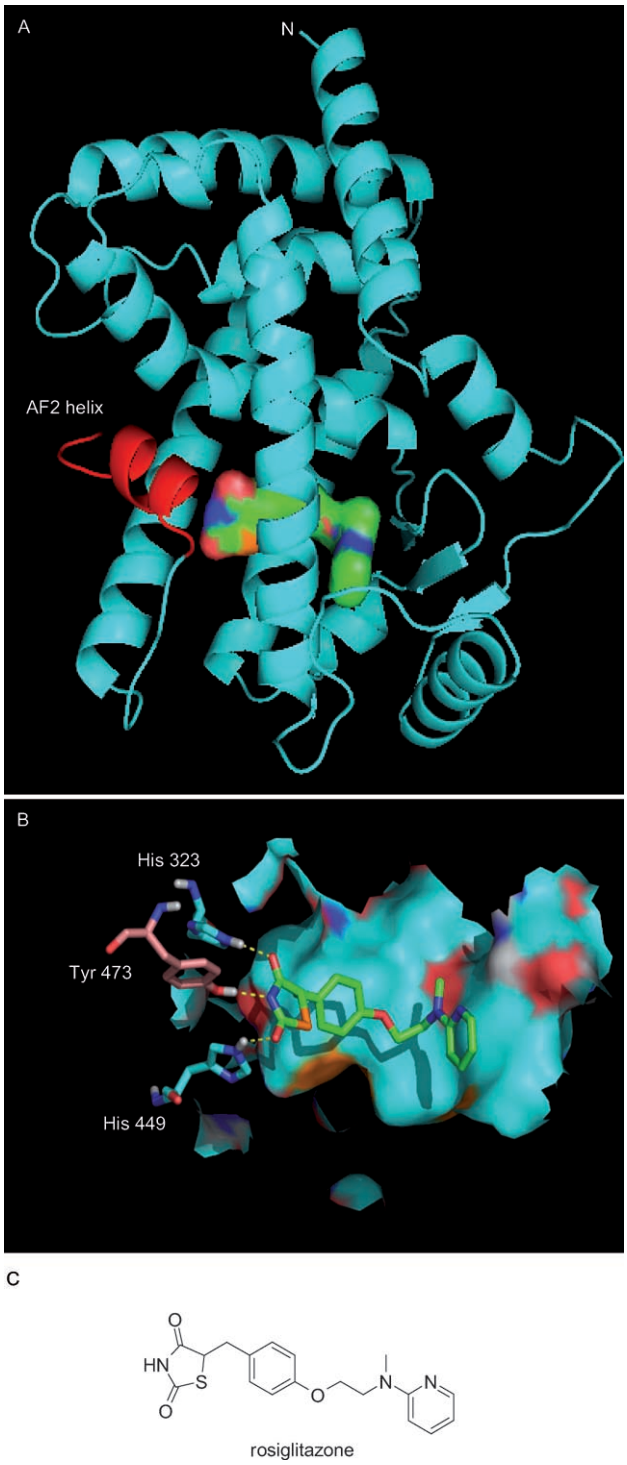


Figure 6. Structure of the PPARγ LBD and features of ligand binding. A) The crystal structure of PPARγ LBD bound with rosiglitazone.[47] The AF2 helix (in red) is in the active position for binding a co-activator peptide LXXLL (not shown). Rosiglitazone (space-filling model) is buried in the receptor, with carbon atoms in green, oxygen atoms in red, and nitrogen atoms in blue. B) Close-up view of the binding site of PPARγ LBD. The front face of the site is clipped away to show the bound rosiglitazone molecule and the hydrophobic back side of the binding pocket. As shown, a tyrosine residue from the AF2 helix of PPARγ makes a hydrogen bond with the thiazolidinedione head group of rosiglitazone. C) Rosiglitazone is a well-known PPARγ ligand.

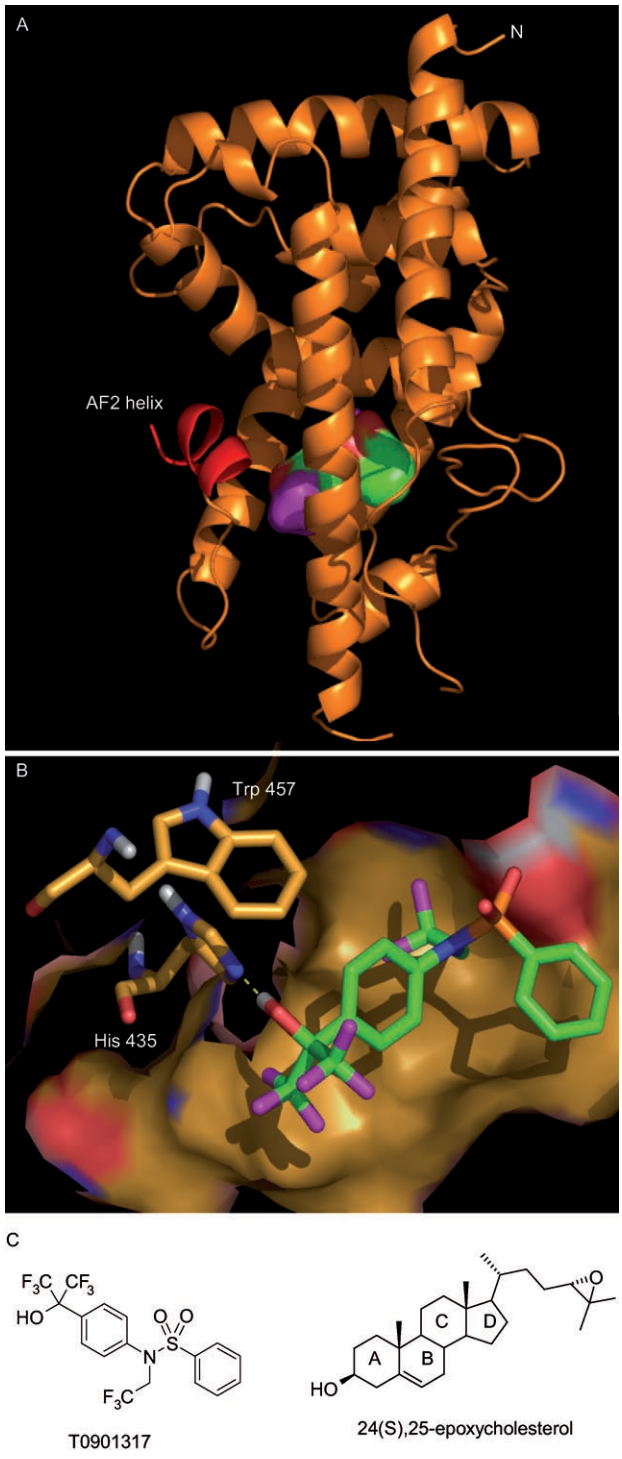


Figure 7. Structure of the LXRβ LBD and features of ligand binding. A) The crystal structure of LXRβ (orange) in complex with the synthetic agonist ligand T0901317 is shown in orange.[39] The AF2 helix (red) assumes the agonist conformation. The ligand (space-filling model) is shown with carbon atoms in green, oxygen atoms in red, nitrogen atoms in blue, and fluorine atoms in magenta. Similar to the orientation of steroids with the steroid receptors, the D ring (or D-ring mimetic in the case of nonsteroidal synthetic molecules) protrudes toward the AF2 helix. B) Close-up view of the ligand-binding pocket of LXRβ. The front half of the receptor is cut away to show the bound ligand and the back face of the pocket. The histidine–tryptophan switch, which is key for ligand-induced activation of LXR, is highlighted. The His-mediated hydrogen bond is indicated with the dashed yellow line. C) Representative structures of well-known LXR ligands.

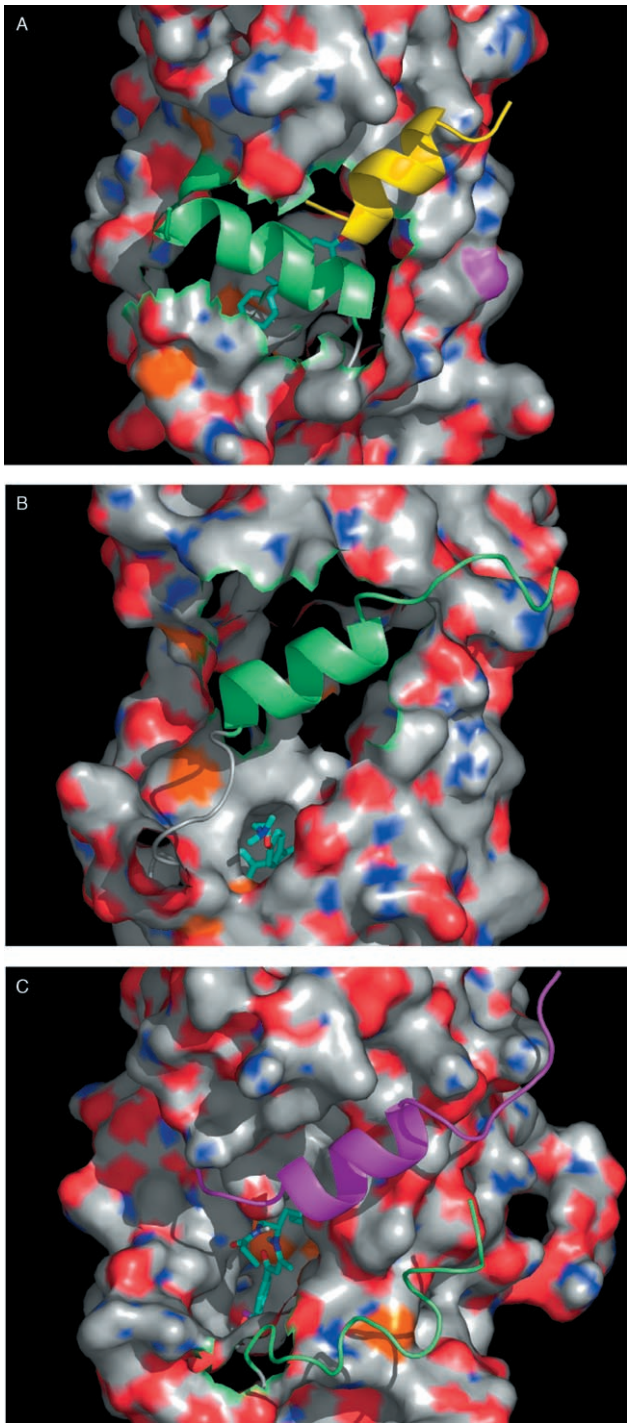


Figure 8. Examples showing the many possible conformations of the AF2 helix. A) ERα with the agonist diethylstilbestrol (DES);[32] B) ERα with the anti-estrogen 4-hydroxytamoxifin (OHT);[32] C) PPARα with the antagonist GW471.[49] Each receptor, oriented in the standard position with helices 1 and 3 in front and slightly off to the right, is shown in space-filling representation. The AF2 helix for each receptor is shown as a green ribbon (or as a green random coil for PPARα). In the DES–ERα structure, the AF2 helix lies across the receptor to help form a binding site for an LXXLL co-activator peptide (yellow). The OHT ligand causes steric interference with the loop preceding the AF2 helix and causes the AF2 helix to reorient, bind within the co-activator cleft, and block LXXLL peptide binding. In the PPARα–GW471 complex, the AF2 helix is perturbed in a way to allow accommodation of a co-repressor peptide (magenta). In this case, the AF2 helix is somewhat unwound and localizes on the receptor in a position different than that observed for other NR LBD structures.

(TIF2)) and SRC-3 (also called ACTR/pCIP/receptor-associated co-activator (RAC3/TRAM-1/amplified in breast cancer 1 (AIB1)).

SRC-1 illustrates many features common among the co-activators. First, it contains several LXXLL motifs, otherwise known as NR boxes.[58,59] As mentioned above, these short α-helical motifs present a hydrophobic surface that is critical for the successful docking of the co-activator protein onto an activated NR. Second, an activation domain within SRC-1 contains an acetyltransferase activity, which acts locally on histones to unravel DNA at the initiation site.[60] Third, SRC-1 is able to aid the recruitment of other nuclear enzymes such as other histone-acetylating proteins. These include cAMP response element binding protein (CBP), p300, and an arginine methylating enzyme called co-activator-associated arginine methyltransferase-1 (CARM1). To initiate gene transcription, the NR–co-activator complex ultimately recruits the chromatin remodeling complex SWI–SNF and the basal transcription-factor-recruiting complex TR-associated protein–vitamin D receptor-interacting protein (TRAP–DRIP), and other basal transcription factors.

Co-repressors and the role of activity-diminishing accessory proteins

Essentially the functional counterpart to co-activators are co-repressor proteins, which bind to many NRs in the absence of ligand and serve to repress basal transcription activity.[61] Co-repressors play a particularly important role for NRs that are found almost exclusively in the nucleus, as opposed to the apo-steroid receptors, which are located in the cytoplasm. Studies involving the nuclear-localized receptors TR and RAR led to the identification of silencing mediators of retinoid and thyroid (SMRT) receptors and the nuclear receptor co-repressor (NCoR).[62,63] Both SMRTs and NCoR recruit histone deacetylases (HDACs), namely HDAC3, which function to reverse the chromatin unwinding mediated by the co-activator-recruited histone acetylases.[64]

Much like the co-activators, which use the LXXLL motif as a docking point, the co-repressors contain an LXXIIXXXL motif referred to as the CoRNR box.[65] The precise nature of the interaction between co-repressors and NRs remained elusive until the solution of the crystal structure between PPARα and a peptide from SMRT. As mentioned previously, this structure shows that the CoRNR box occupies the same general site on PPARα as the co-activator LXXLL motif. However, the CoRNR box is approximately one α-helical turn longer, and the AF2 helix on PPARα is pushed out of position and does not play a role in molecular recognition (Figure 8C). There are several reports showing that NRs occupied by non-agonist ligands, such as ER with raloxifene and GR with RU486, increase co-repressor binding. These results suggest that these types of ligands not only disfavor co-activator binding, but also create a surface on the NR favorable for co-repressor binding.

Interference in NFκB and AP-1 pathways

In addition to interaction with co-activator and co-repressor proteins, NRs have been shown to associate with a variety of

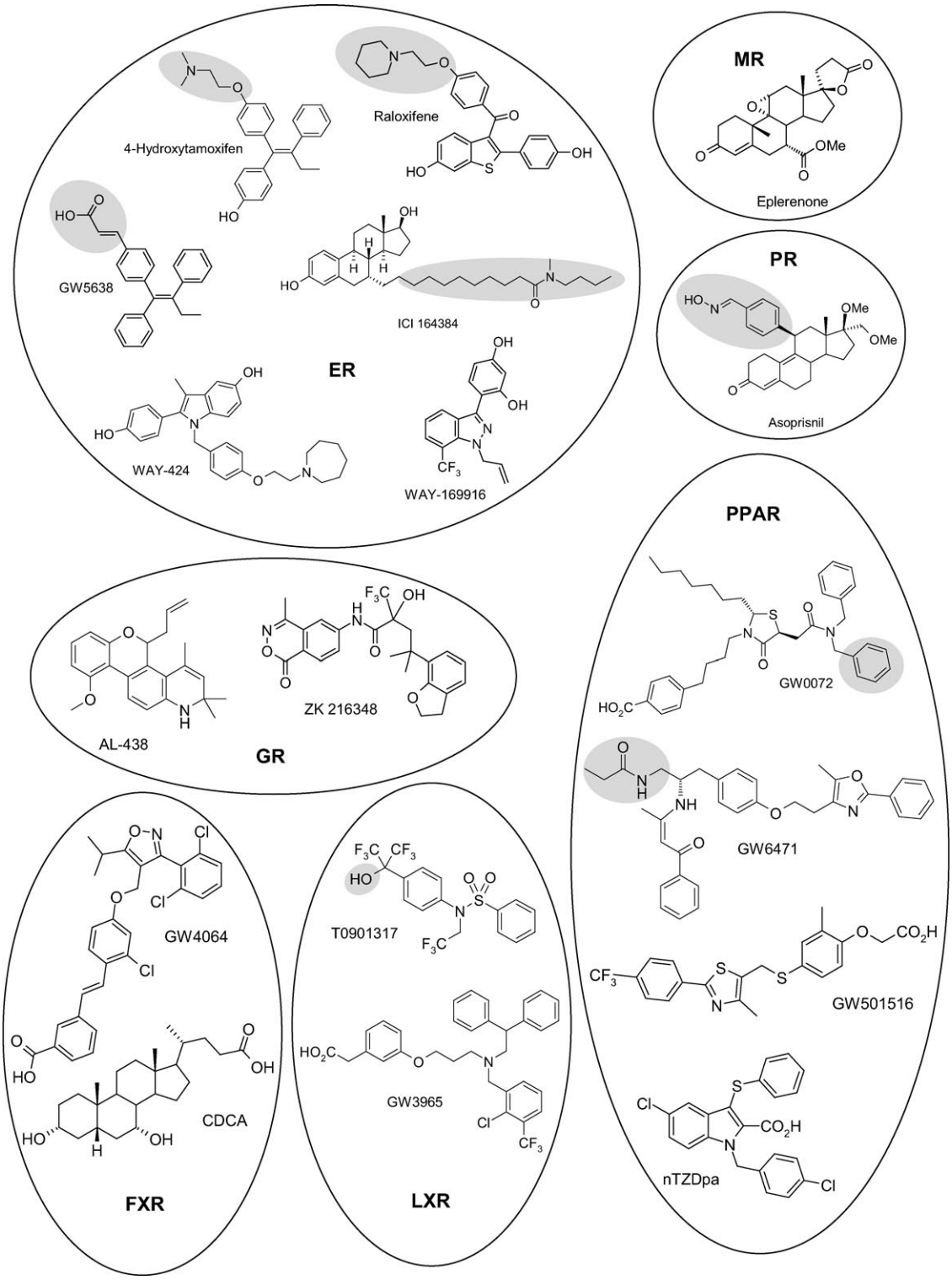


Figure 9. Examples of NR modulating tool compounds and drugs, many of which are referred to and discussed in the text. For some ligands, the region of the molecule that is oriented toward the AF helix (as determined from the crystal structure of the NR–ligand complex) is shaded.

other proteins key to cellular maintenance and function. It has been well documented that several NRs, predominately the steroid receptors but also PPARs, LXR, RXR, and RAR, have the ability to crosstalk with signaling pathways involving the transcription factors NFκB and activator protein-1 (AP-1).[66,67] Activated NRs typically repress the ability of NFκB and/or AP-1 to transcribe their targeted genes. This interference is believed to be the basis for the anti-inflammatory actions of corticosteroids and estrogens.[68,69] There have been several mechanisms proposed for these activities, but a conclusive molecular basis

for them remains elusive. One proposal suggests a direct interaction between the NR and NFκB.[70,71] As both NRs and NFκB require the aid of co-activator proteins such as SRC-1 and CBP, another mechanism is that which involves a "cofactor squelching" event. A third proposal involving GR is one that involves a direct association between the NR and protein kinase A, in which cross-coupling of NFκB and GR occurs in the cytoplasm.[72] Clearly, these studies show that NRs play a complex and integrated role in pathway management beyond the direct DNA-mediated regulation of gene transcription.

Non-nuclear functions and interactions with other cellular proteins

Aside from the vast network of co-activator, co-repressor, and NFκB/AP-1 interactions, another level of complexity in NR functions involves interaction with a wide variety of cellular proteins. In general, these activities are commonly referred to as "nongenomic actions".[73] Full coverage of this arena is beyond the scope of this review, but a few selected examples are highlighted to demonstrate the breadth of complexity that liganded NRs have on adjacent pathways. For example, PR and other steroid receptors have been shown to interact with numerous cytoplasmic kinases, such as c-Src tyrosine kinases, in a ligand-dependent manner.[74,75] GR has been shown to interact with a variety of cellular factors such as SMAD3[76] and JNK.[77] ER has been shown to interact with a variety of factors, such as phosphatidylinositol-3-OH kinase (PI3K).[78] Additionally, NRs are phosphorylation targets, primarily within the AF1 domain, and it has been shown that NR activities can be modulated by phosphorylation state.[79–82]

6. Specific Examples of Recent NR Drugs and Novel Drug Candidates

As mentioned earlier, the NRs have a rather illustrious history in pharmaceutical discovery (Table 1). Once a synthetic ligand has been identified for a receptor, typically through screening and/or structure-guided design efforts, the goal is to chemically alter the properties of the ligand to appropriately modulate the activities of the receptor. Throughout the last decade or so, ligands that display differential activities relative to the natural ligand have been commonly referred to as selective NR modulators (SNuRMs). One of the original demonstrations of this concept involved ER and the two classic selective ER modulators (SERMs), 4-hydroxytamoxifen (OHT) and raloxifene. Essentially, it was found that these SERMs retained tissue-selective agonist activity (such as in bone tissue and on lipid profile for raloxifene), but functioned as antagonists in reproductive tissues.[83,84] Furthermore, even though both molecules were originally considered "antiestrogens", OHT generally shows a trend toward estradiol-like activity in uterine tissue,[84] whereas raloxifene does not. The groundbreaking work around novel ER ligands has opened the door to the identification of novel, tissue-selective synthetic modulators for several of the therapeutically relevant NRs.

In this section, we highlight a few of the more recent pursuits of SNuRMs (Figure 9). The purpose of this brief discussion is to give an overview of the current state of the art for ligand and drug discovery by mentioning a few somewhat recent specific examples. Overall, the present mission in NR drug discovery is to manipulate the receptor with ligand to retain tissue-selective benefits while minimizing unwanted activities (Table 2). These few selected examples cover the basic principles of NR drug discovery, such as identifying small-molecule binders, modifying hits for NR modulation, and use of recent techniques and methodologies.

Selective ER modulators (SERMs)

First reported in the 1970s, tamoxifen was the first synthetic NR small-molecule modulator to show differential tissue effects. The primary reason it has not been widely used to treat menopausal symptoms is the fact that this molecule shows stimulatory effects on the uterus which causes a significant risk for endometrial cancer.[85] However, tamoxifen remains a first-

Table 2. Examples of therapeutic profiles for designer, tissue-selective nuclear receptor modulator ligands.

Receptor	Desired Efficacy with Modulator	Unwanted Activity Decreased with Modulator
estrogen receptor (ERα)	reduce menopausal hot flashes prevent post-menopausal osteoporosis	breast and uterine tissue stimulation
glucocorticoid receptor	reduce inflammatory conditions suppress immune system for transplant	fat redistribution and weight gain increased bone loss diabetes depression/mood effects
mineralocorticoid receptor	reduce hypertension protection against congestive heart failure	hyperkalemia
progesterone receptor	reduce endometriosis	abortive activities
androgen receptor	protection against skeletal muscle atrophy	prostate stimulation
PPARα	improve dyslipidemia	peroxisome proliferation
PPARδ	improve dyslipidemia	*unknown*
PPARγ	glucose lowering	edema and weight gain
liver X receptor (LXRα or LXRβ)	reduce atherosclerosis anti-inflammatory antidiabetic	hypertriglyceridemia
farnesoid X receptor	protection against cholestasis	*unknown*

line treatment for ER-positive breast cancer. A second generation SERM, raloxifene, was originally developed as a tamoxifen follow-up for breast cancer, but it was demonstrated that this molecule has significant osteoporosis protective effects without the endometrial activities relative to tamoxifen.[86] The molecular basis for these ER-modulating activities has been the focal point for a wide body of pharmacological research.[87] One proposed mechanism is the differential effects of SERM-bound ER to promote co-repressor association versus co-activator association.[88,89]

Driving on the theory that ligands can induce specific ER conformations, a series of triphenylethylene ligands for ER were made and screened through a uterine Ishikawa cellular assay.[90] Compounds with the ability to decrease estrogen-mediated Ishikawa cell stimulation were then tested in ovariectomized rats for the ability to protect against loss of bone mineral density. GW5638 was identified with this approach (Figure 9); it was further shown that the compound had antagonist properties on the uterus and agonist activities on bone and the cardiovascular system.[91] A further study has shown that the unique biological properties of GW5638 derive from producing a structural conformation of ER different from the conformations imposed by other SERMs.[92] In addition to this one example, a number of novel SERMs have been identified with a combination of cellular screens, primarily uterine- and breast-cell-based assays.[93,94] These SERMS include idoxifene, lasofoxifene, Wyeth 424, levomeloxifene, and others (Figure 9).

Two new approaches to ER ligand discovery have recently been reported. One involves the use of NFκB-driven reporter assays to discover pathway-selective ligands with the potential to treat inflammatory disorders.[95] Another relatively recent focus for ER-directed drug discovery relates to the fact that there are two subtypes of this receptor, ERα and ERβ, which derive from two separate genes.[30,96] Stimulated by the distinct tissue distribution pattern of these two related receptors, the concept is that new indications, such as inflammation and cancer, can be treated with an ER-selective molecule. Toward this goal, several reports have demonstrated the ability to identify ERβ-selective ligands.[35,97–99]

Selective GR modulators (SGRMs)

A variety of debilitating diseases such as rheumatoid arthritis, inflammatory myopathies, cancers, and various immunological disorders are treated with the classic synthetic glucocorticoids dexamethasone and prednisone. However, chronic treatment with these drugs often leads to serious side-effects such as fat redistribution, diabetes, vascular necrosis, and osteoporosis. There is currently an intense effort to identify new small molecules that are able to differentially modulate GR to retain the beneficial effects of glucocorticoids yet which decrease the incidence of unwanted side-effects.[7]

A key genetic study in which a knock-in mutation of a dimerization-deficient mutant of GR was used has shed some light on the molecular basis for dissociative activity.[100] In essence, this GR^{dim} mutant demonstrated that some of the direct gene-transduction properties of GR can be decreased while other immune-modulating functions of the receptor can be retained. This concept forms one of the principles of selective modulation of GR. Importantly, many of the anti-inflammatory 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 pro-inflammatory cytokines such as interleukins (IL)-1, -2, -6, -8, and TNFα.[68]

There have been several recent reports of ligands that display differential GR activation. Although a complete survey is beyond the scope of this review, a few examples have been selected to demonstrate the concept and the methods used to discover the ligands. Typically, three measures of GR activity were used to identify these ligands: 1) direct GR binding relative to other steroid receptors, 2) a cell-based assay measuring GRE-mediated gene transcription (referred to as "transactivation"), and 3) cell-based assays measuring the ability of GR to regulate NFκB and AP-1-driven genes (referred to as "transrepression").

Several steroid-based compounds have been shown to differentially decrease transactivation with only minimal effects toward transrepression.[101,102] In the nonsteroidal class of GR ligands, a quinoline-based series of compounds, particularly those with an aryl substituent at the C5 position, such as AL-438 (Figure 9), yielded a trend toward a preferred transactivation/transrepression profile in cellular assays. Some of these ligands also showed a more promising therapeutic window for selective in vivo effects.[103,104] In another study, a nonsteroidal GR ligand, ZK 216348, was reported to show significant dissociation of transactivation and transrepression activities.[105] Following a GR-binding assay to identify high-affinity-binding compounds, hits were characterized using 1) an assay measuring a GRE-driven reporter gene (induction of tyrosine aminotransferase), 2) an assay monitoring the decrease of lipopolysaccharide (LPS)-induced IL-8 production from THP-1 cells, and 3) an assay measuring the inhibition of TNFα and IL-12 p70 from LPS-induced peripheral blood mononuclear cells. This linear approach highlighted ZK 216348 as a dissociative molecule. Further work in vivo 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.

Other modulator efforts: PR, MR, AR, PPAR, FXR, and LXR

The concept of selective NR modulation to produce an activity and therapeutic profile distinct from the natural ligand has been applied to numerous other receptors (Figure 9). For example, a modified steroid ligand for PR called asoprinisol has been shown to produce anti-uterotrophic effects with only minimal labor-inducing and breakthrough bleeding effects.[106] A selective MR modulator called eplerenone, a molecule that was discovered decades ago, has recently been approved as a drug for hypertension.[107] This synthetic steroid has improved specificity for MR over related receptors, and functions as a partial antagonist of aldosterone.[108] There is currently an effort to identify a modulator of AR for utility in prostate

cancer and possibly treating the neurological and muscular degenerative symptoms of androgen deficiency.[109–111] One recent example of a tissue-selective AR modulator is LGD2226, which appears to retain some anabolic effects on bone and muscle with diminished proliferative effects on the prostate.[112]

Several groups have shown progress in the development of selective modulators for PPARγ (SPPARMs). The first-generation thiazolidinedione (TZD) class of PPARγ agonists, used pharmacologically as insulin sensitizers, also exhibit dose-limiting liabilities such as hemodilution and edema (Table 2). Initial studies of PPARγ activation by TZDs revealed that these compounds activate through direct interaction with the C-terminal AF2 helix.[47] Structural studies have also revealed PPARγ activators that bind the LBD using non-TZD epitopes such as the partial agonist GW0072.[37] Compounds with distinct binding and/or activation modes represent a potential avenue to discover PPARγ modulators with modified biological activities. Non-TZD-selective PPARγ modulators (for example, nTZDpa compounds) have been found which induce an altered LBD conformation compared with TZDs, as measured by protease protection and NMR spectroscopy.[113] Like GW0072, these compounds function as partial agonists and antagonize the activity of PPARγ full agonists in 3T3L1 adipogenesis assays. Moreover, the nTZDpa compounds demonstrated qualitative differences versus traditional agonists toward gene expression in cell culture (3T3L1 adipocytes) and in vivo (white adipose tissue), as well as toward in vivo physiological responses such as adipose depot size. Thus, further efforts to develop SPPARMs may lead to compounds with improved characteristics relative to compounds presently in clinical use.

Modulator efforts have also begun for NRs that have only been investigated preclinically so far. In studies of both FXR and the LXRs,[114–116] compounds with potential novel biological activity relative to natural ligands are being identified. For example, LXRα/β are regulated in vivo by oxysterols, and this regulation is consistent with the role of the LXRs in cholesterol homeostasis. Animal models using non-subtype-selective LXR tool compounds indicate that in addition to conferring atheroprotective effects, these agonists also promote lipogenesis and triglyceride accumulation in liver. Miao et al. reported that two LXR agonists (T0901317 and GW3965) show differential effects on cofactor recruitment in human hepatoma cell assays.[116] Additionally, these two compounds differ in their in vivo effects on hepatic lipogenesis genes. These studies point toward the promise of developing LXR modulator compounds that possess anti-atherogenic activities with limited hepatic liability. Whether the difference between these compounds reflects tissue versus gene selectivity remains to be elucidated. For both the steroid receptor and nonsteroid receptor modulators, more work is needed to better understand the underlying basis of modulator effects.

Taken together, these examples highlight the 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 NR modulation, and the use of relevant in vivo models for measuring the therapeutic index of effects. Because of the structural and functional similarities within the NR superfamily, lessons learned from one receptor concerning modulation by a designer small molecule can probably be applied to other members of the family.[3,117,118] Overall, with increasing knowledge of NR functions, there is a high probability that novel, safer, and more effective medicines will be the eventual outcome. Important in this pursuit is the use of new technologies to profile ligands; this is the topic of the following and final section.

7. New Approaches to NR Drug Discovery

One of the more recent principles in the field of NR research and drug discovery is the realization that a subset of the myriad functions of NRs can be selectively manipulated by ligand, a general concept referred to as NR modulation. New technologies, including advanced computational methods and peptide interaction methods, are inspiring new strategies for discovering novel NR-modulating drug candidates. Importantly, new technologies allow the profiling of NR ligands at greater speed and in a more physiologically relevant context. Several new approaches to NR modulator discovery are illustrated in this section, drawing on recent work with ERα/ERβ to provide specific examples.

As discussed briefly above, NRs do not act in isolation, but in complex associations with other cellular factors. Cofactor interaction screening exploits the relationship between NR structure and functional activity. If a particular ligand uniquely alters the pattern of cofactor interaction relative to other ligands, it is likely that the differential in vitro profile will translate into a unique gene-expression pattern or physiological outcome in vivo. Peptides representing these interactions can be synthesized based on known interaction motifs or isolated through screening random peptide libraries. In ER modulator discovery, this method has been used to characterize known SERMs and to discover ER ligands with unique properties. Norris et al. applied affinity selection of peptides to identify binding surfaces that are exposed on ERα/β when complexed with different ligands, such as with estradiol or 4-OH tamoxifen.[119] They found that the established SERMs, known to produce distinct biological effects, induced distinct conformational changes in the receptors. The ability of the peptides to discriminate between different ERα/β ligand complexes has enabled screens for subtle differences between ER ligands. Ligand screens have been developed based on NR–peptide interactions using a high-throughput multiplexed technology, which employs fluorescently encoded microspheres.[120,121] Purified NR LBD domains can be used in these screens, and the repertoire of novel NR-interacting cofactors has expanded dramatically in the past few years. To rapidly identify novel interactors, genome-wide screens for binding partners have been carried out in yeast and mammalian-based two-hybrid systems. As mentioned above, over 200 human NR cofactors have been identified. These interactors are important in the era of NR-modulator discovery, as each new cofactor brings the potential to recapitulate a particular cellular interaction and thus pro-

vides the basis for a molecular screen for molecules that uniquely affect the interaction.

Since NRs are transcription factors, monitoring ligand effects on NR target genes is a powerful approach to NR drug discovery. Difficulties and expense in measuring endogenous gene expression has limited this approach in drug-screening methods until recently. Microarray technology has made it possible to assess endogenous gene expression on a genome-wide scale, and this technology has been used to define an unbiased set of NR target genes. For example, multiple groups have used microarray technology to differentiate the functions of ERα and ERβ in estrogen target organs such as bone, breast, and uterus. In one specific set of experiments, human U2OS osteosarcoma cells (which express neither ERα nor ERβ) were stably transfected with human ERα/β to selectively overexpress the receptors in this bone model system.[122] Treatment of the two cell lines with 17β-estradiol resulted in two overlapping but distinct patterns of gene expression. Interestingly, 28% of the estradiol-regulated genes were ERα-cell specific, whereas 11% were ERβ-cell specific. Not only did this work allow the functional dissection of the pathways regulated by two functionally similar receptors, but it has identified unique sets of endogenous target genes for use in ligand-screening assays.

By using a similar system as described above (U2OS cells expressing either ERα or ERβ), Kian Tee and colleagues[123] evaluated the effects of different ER ligands (including the SERMs raloxifene and tamoxifen) on ERα and ERβ target genes. Microarray analysis showed that raloxifene and tamoxifen regulated only 27% of the same genes in both the ERα- and ERβ-containing cells. These results indicate that estrogens and SERMs exert tissue-specific effects by regulating unique sets of target genes through ERα/β. Thus, these specific genes serve as unique identifiers of compound action, and a subset are especially useful for discriminating ER ligands.

Higher-throughput methods to analyze gene expression hold the promise of screening large numbers of compounds in a cellular environment using a cost-effective technology. For example, with advances in glass slide preparations for monitoring transcriptional changes of thousands of genes, a hit from a multi-well cell treatment can be inexpensively assessed over a genome-wide range of genes. With such an analysis, it is possible to observe distinctions between even very closely related chemotypes. A recent study has used gene-expression profiling to characterize breast cancer cells and to identify desired "molecular fingerprints" within the data.[124] Key "biomarkers" can be identified that provide information linked to the phenotypic effect of a compound. With such a screen, knowledge of the target of the compounds (for example, whether a compound has anti-estrogen effects) are not an a priori requirement. One challenge in this type of approach is that vast amounts of data are generated, and bioinformatics analysis becomes a limiting factor. Current advances in gene-expression profiling as a drug-screening method must go hand-in-hand with advances in bioinformatics and data handling.

Changes in the steady-state levels of mRNA do not tell the whole story. Research groups are now involved in the integration of data obtained from mRNA steady-state level analysis with proteomic data. Huber et al. analyzed differences in the gene- and protein-expression pattern of the human breast carcinoma cell line T47D and its derivative T47D-r, which is resistant to the pure anti-estrogen ZM 182780.[125] Microarray analysis was carried out in parallel with a proteomics analysis, in which the total cellular protein content of T47D or T47D-r was separated on 2D gels. Thirty-eight proteins were found to be reproducibly up- or down-regulated more than 2-fold in T47D-r versus T47D in the proteomics analysis. Comparison with differential mRNA analysis revealed that 19 of these were up- or down-regulated in parallel with the corresponding mRNA molecules. For 11 proteins, the corresponding mRNA was not found to be differentially expressed, and for eight proteins an inverse regulation was found at the mRNA level. A general conclusion from such studies is that although the pattern of expression of the two data sets is similar, the disconnected trends emphasize the importance of post-translational mechanisms in cellular development. These types of changes can only be observed through integration of the proteomic and transcriptomic approaches. New higher-throughput methods to carry out proteome variation are making this type of analysis more practical.

The above examples illustrate how NR target genes have been discovered through physical experimentation. In silico approaches are also being developed that increase the speed of NR drug discovery. For example, comprehensive computational approaches can now be carried out to identify NR target genes. NUBIscan represents a new computer algorithm for predicting NR target sequences in regulatory regions of genes.[126] This approach is being combined with methods to quickly validate the target genes predicted by the in silico method. High-throughput, genome-wide chromatin immunoprecipitation methods have been combined with computational methods to identify ER target genes and promoter sequences.[127] Genes identified by computational analysis are not biased by target tissue or expression levels, and thus complement microarray approaches.

In sum, NR drug discovery is moving closer to the realm of being able to profile compounds in a setting closer to the native physiological environment, or in an in vitro environment, with a physiologically comprehensive array of functional partners in a high-throughput fashion.

8. Future Developments and Conclusions for NR Chemical Biology

The human NRs as a structural class are essential for survival and play an integral role in many critical physiological processes such as metabolism, homeostasis, differentiation, growth and development, aging, and reproduction. This family of receptors has a common evolutionary history as evidenced by their sequence relationship and their commonality in cellular function.[128] The myriad functions of NRs are vastly complex, and the pathways they control are intertwined with each other as well as with numerous accessory proteins and partners in function. Even with this inherent complexity, as reviewed brief-

ly above, this family of receptors has had a long and fruitful history for drug discovery. With the advent of high-throughput chemistries, structural biology, novel biochemical methods, and pathway analysis technologies such as differential gene expression and proteomics, there will undoubtedly be new discoveries leading to drugs with improved therapeutic profiles. These NR-modulator efforts should help to better define the ligand-induced activities that produce tissue-selective beneficial effects and minimize unwanted activities. Moreover, there are likely to be advances toward ligand discovery for the remaining orphan receptors. Studies using these tool compounds should lead to target validation and better definition of therapeutic relevance for the remaining orphan NRs. Overall, the future of targeting the NR superfamily with novel synthetic ligands holds tremendous potential and should lead to a variety of safer, more effective medicines for the treatment of a plethora of human diseases.

Acknowledgements

We thank Tim Willson for critically reading this review. We also thank Lakshman Ramamurthy for his kind contribution of the NR superfamily phylogeny plot. Finally, we thank our many GlaxoSmithKline colleagues for helpful discussions and collaborations on NR-related projects.

Keywords: drug design · gene expression · inhibitors · medicinal chemistry · receptors

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Received: January 10, 2006
Published online on April 11, 2006

