Mostafa Z. Badr *Editor*

Nuclear Receptors

The Art and Science of Modulator Design and Discovery

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Editor
Mostafa Z. Badr
School of Pharmacy
University of Missouri-Kansas City
Kansas City, MO, USA

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Preface

As transcription factors, nuclear receptors regulate numerous biological functions. Consequently, altering the activities of these receptors is proposed, and indeed documented, to affect many physiological and pathological conditions in experimental animals and humans.

This book presents a unique perspective in the field of nuclear receptors through an attempt to decipher the thought process scientists go through in their respective research efforts. The chapters of this book are presented in an order to narrate a story, beginning with a lofty goal seeking to conceptualize a treatment for a disease and ending with making available a therapeutic for that disease. Collectively, these chapters shed light on intricate phases involved in designing as well as developing physiological and pharmacological means to modulate the activity of nuclear receptors.

Last but not least, I would like to extend my utmost gratitude to my world-renowned colleagues who agreed to share with the reader their professional expertise and extensive experience acquired through decades of working with nuclear receptors.

Kansas City, MO, USA Mostafa Z.Badr

Contents

About the Editor

Mostafa Z. Badr is a professor emeritus at the University of Missouri-Kansas City, USA. Dr. Badr obtained his bachelor's degree in pharmacy and a master's degree in organic chemistry from the Faculty of Pharmacy, Cairo University, Egypt. Dr. Badr subsequently obtained a Ph.D. degree in pharmacology and toxicology from the University of Louisville, after which he received postdoctoral training in toxicology at the University of North Carolina-Chapel Hill. In 1987, Dr. Badr accepted the position of assistant professor at the University of Missouri-Kansas City School of Pharmacy, where he retired as professor in 2015.Dr. Badr has authored and co-authored over 70 publications in peer-reviewed journals and has published three previous books with Springer Nature. Dr. Badr founded the journal *PPAR Research* and served as its editor-in-chief from 2005 to 2011, and the journal of *Nuclear Receptor Research* and served as its editor-in-chief from 2013 until 2019.

Chapter 1
Molecular Pharmacology of the Youngest Member of the Nuclear Receptor Family: The Mineralocorticoid Receptor

Mario D. Galigniana

Abstract The mineralocorticoid receptor (MR) was the last member of the nuclear receptor superfamily to evolve. It is responsible for the maintenance of the water and salt homeostasis. Like most ligand-activated transcription factors of this superfamily, it is activated by ligand binding. The MR exists as a large heterocomplex assembled with the heat-shock protein of 90-kDa chaperone, Hsp90, and other associated chaperones and cochaperones. The composition of this heterocomplex is affected by the nature of the bound steroid. MR biological responses are also affected by the redox status of the cell or due to protein phosphorylation. In this chapter, the conformational requirements of the steroid to become an optimal MR ligand, the role of the Hsp90-based heterocomplex, and the influence of MR modifications by oxidation and phosphorylation is discussed. These properties are analyzed in the light of the relevance of this nuclear receptor as a key pharmacological target for disorders mostly related to the hydroelectrolytic homeostasis.

Keywords Mineralocorticoid receptor · Aldosterone · Steroid conformation · Heat-shock protein of 90-kDa · Immunophilins · Dynein · Phosphorylation · Glutathion

M. D. Galigniana (✉)
Instituto de Biología y Medicina Experimental de Buenos Aires/CONICET, Buenos Aires, Argentina

Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina
e-mail: mgali@qb.fcen.uba.ar; mgaligniana@conicet.gov.ar

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1.1 An Overview of the MR Physiology

The conserved steroid receptor subfamily is comprised within the nuclear receptor superfamily. They are counted among the first members of the nuclear receptor superfamily to be cloned and structurally characterized [1]. The last two steroid receptors that emerged during evolution are the close-related partners GR [2] and MR [3]. The high homology between these two receptors led to confirm the close kinship that was hypothesized previously due to the cross-talk of their biochemical and pharmacological properties. This is particularly remarkable since the GR is a receptor that can replace MR in some functions. Simply to begin with, the main glucocorticoid ligand, cortisol, shows the same affinity for MR as the natural mineralocorticoid ligand, aldosterone [4]. In view of the higher plasma levels of circulating cortisol (2–3 orders of magnitude higher than those of aldosterone), there is a problem for the specificity of the biological response since a priori, the MR should be permanently activated by cortisol. In other words, the typical response to aldosterone (sodium- and water-retention and potassium and proton elimination) can be triggered by cortisol.

In most epithelial tissues and exceptionally in a few non-epithelial tissues such as vessel walls and nucleus tractus solitarius of the medulla oblongata, the MR is protected from activation by cortisol due to the action of the microsomal enzyme 11βHSD2 (*11β-hydroxysteroid dehydrogenase type-2*), which is co-expressed in the same cells where MR is expressed, and converts cortisol into the receptor-inactive oxidized metabolite cortisone [5]. When the enzyme expression is deficient or blocked by drugs or natural products such as liquorice, this protective mechanism fails, and cortisol is available to bind and activate MR. Consequently, a pseudohyperaldosteronism syndrome is developed, i.e., a paradoxical syndrome of hyperaldosteronism showing hypertension and high levels of sodium retention but also normal or low plasma levels of aldosterone (see [6] for a comprehensive review).

In most non-epithelial cells, remarkably in the brain, the MR is not protected by that enzymatic activity since there is no 11βHSD2 co-expression in these cells. Inversely, in the nervous system, there is a considerable expression of MR coexisting with high levels of GR in the same cell types. Remarkably, the intranuclear distribution of MR and GR in the same hippocampal neurons shows a distinctive individual distribution, i.e., specific speckles that exclusively contain MR or GR, but not colocalization of both of them [7]. This clearly indicates that there are specific nuclear sites capable to specifically recognize each receptor. Does it mean that the biological response is redundant for both receptors? It does not seem to be the case since, for example, salt-intake is still aldosterone-regulated, and it is not a cortisol-dependent phenomenon [8, 9]. MR activation has also been related to behavioral responses, including memory-related events and affection feelings [10]. Interestingly, the MR is also expressed in the granulosa cells of the ovary, one of its proven specific roles being the regulation of progesterone synthesis [11], a steroid with MR antagonistic action. The reasons for the exclusion of alternative ligands

and the specificity for aldosterone action in these tissues are still subject of intensive investigation and speculations.

The most important and relevant pathology associated with the biology of MR is the hypertension syndrome that results from high plasma levels of aldosterone and consequently hypernatremia and water retention. Therefore, patients with primary hyperaldosteronism have higher cardiovascular risk profiles [12] and greater evidence of cardiovascular damage [13]. Furthermore, the MR has also been associated with other pathological situations such as inflammation processes, organ fibrosis, oxidative stress, adipocyte metabolism, and aging [14]. It has been documented that MR activation induces the proliferation of smooth muscle cells in pulmonary arteries, which is a contributing factor for the development of pulmonary arterial hypertension [15]. Retina is also a target of mineralocorticoid action, such that the use of MR antagonists has shown beneficial effects in retinal diseases [16].

1.2 Evolutionary Profile of the MR

It is accepted that the first life forms originated in the sea. Because of this origin, it is thought that the circulating fluids of today living beings resemble the composition of the sea water of some millions of years ago, when life began. However, the composition of the primitive Archean Ocean has been gradually changing since that time. This was the consequence of the permanent precipitation of salts on the seabed and the washing-down of compounds from the land that were deposited in the sea by the erosive action of rivers. Today animals are unquestionably consequence of a slow but constant evolutionary adaptation to that new environment during this long period of time. Despite the biological divergence, the composition of their blood is remarkably alike in ionic composition. This fact suggests that the life conditions should be highly restricted, and it is likely that they remained relatively constant during this evolutionary process. Thus, animal life has been regulated by mechanisms whose main purpose was the maintenance of an inner optimal environment or the continued life of its constitutive cells. The Dobzhansky's aphorism "nothing in biology makes sense except in the light of evolution" [17] is quite appropriate for the case of the MR.

It is reasonable to postulate that when the first life forms abandoned the salty waters of the sea, they must solve an additional difficulty for keeping the osmotic pressure of their blood above that of the surrounding fresh water. Furthermore, when animals moved on to the land from the waters, far-reaching adjustments of their regulatory mechanisms became a mandatory condition simply because the limits of tolerance were even narrower as a consequence of the influence of previously inexistent variables, for example, evaporation and perspiration. This is the point where both the ligand aldosterone and the receptor MR emerged simultaneously during the evolutionary process, i.e., when amphibians jumped from the waters to land. Interestingly, most fish lack both aldosterone and the enzyme responsible for its synthesis [18]. Actually, the main corticosteroid produced by the fish

interrenal tissue is cortisol, which is the steroid that manages not only the metabolism but also the regulation of salt and water balance in these animals.

The biosynthetic pathway of aldosterone provides some insights into the evolution the MR ligands. Aldosterone is at the end of the pathway, i.e., it is the youngest ligand. As a matter of fact, the late developing of the MR along with CYP11B2 (aldosterone synthase) offers a clear example of a co-evolutionary process to preserve the intracellular milieu from environmental changes. Also, the six related steroid receptors expressed in vertebrates—GR, MR, estrogen receptors (ER) α and β, progesterone receptor (PR), and androgen receptor (AR)—evolved thanks to a series of gene duplications from a common ancestral receptor gene [19]. It is regarded that the first steroid receptor was ER, followed by PR. More recently, AR and corticosteroid receptors appeared. There is a common ancestor of the two youngest members of the steroid receptor subfamily, GR and MR—the CR [20]. Like the GR and MR, CR is promiscuous in the sense that it is activated by both mineralocorticoids and glucocorticoids. Descendants of this ancestral receptor are still found in jawless fish, lampreys, and hagfish (along with the expression of ER and PR), which evolved about 530 million years ago and are located at the base of the vertebrate line. These species do not produce neither cortisol nor aldosterone, but 11-deoxycortisol and 11-deoxycorticosterone, which represent their respective biosynthetic precursors and are present at physiologically relevant levels [21]. Recently, it was suggested [22] that 11-deoxycortisol is the main steroid that controls the hydromineral balance in sea lamprey, an organism that represents the most basal osmoregulating vertebrate. GR and MR are derived from that CR in cartilaginous fishes about 450 million years ago, and the consensus is that 11-deoxycorticosterone, corticosterone, and cortisol were all of the ligands for MR before the CYP11B2 enzyme required to make aldosterone from DOC evolved, following the divergence of those two receptors [23, 24].

MR is the largest protein among all human steroid receptors and the last to evolve. Like the other members of the nuclear receptor superfamily, it shows three major functional domains (Fig.1.1a): The N-terminal domain (∼603 amino acids), which has the most variable comparative sequence compared to the other steroid receptors (≤15%). It is classically known as the transactivation domain (TD). The central DNA-binding domain (DBD) shows the highest homology with other members of the subfamily, especially with GR (∼94% identity across the 66 amino acid domain). It contains two Zn-finger protrusions responsible for the recognition of the DNA promoter sequence of the target genes. The C-terminal domain (∼253 amino acids) comprises the ligand-binding domain (LBD) where the steroid binds. Between the LBD and the DBD, there is a "hinge" domain (HD), a region of ∼62 amino acids that it is thought to play a role in receptor dimerization.

There are two possible evolutionary reasons that may lead to that particular intramolecular organization of these receptors. Perhaps different domains showing different origins such that those related to the regulation of metabolism became fused to a DNA-binding motif to generate a novel transcription factor. Alternatively, a multi-domain precursor that at first may mediate a simple signal transduction pathway could have acquired increasingly complex functions during the evolution.

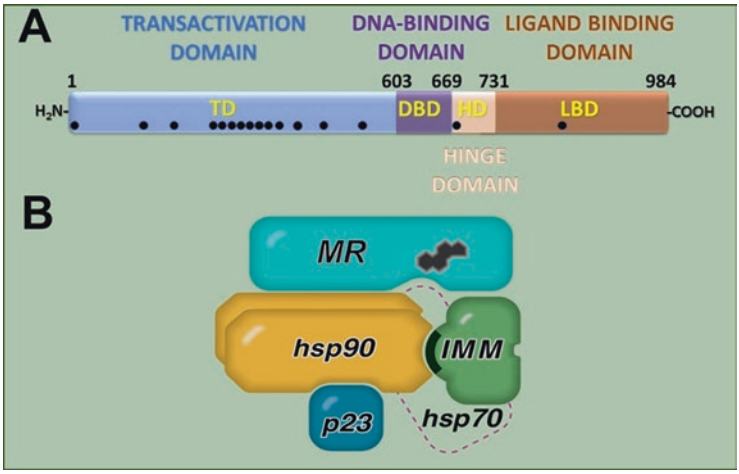

Fig. 1.1 (**a**) Structural domains of human MR. Black dots show potential phosphorylation sites based on the consensus sequence. (Modified from Ref. [92]). These sites correspond to serines in position 8, 129, 183, 250, 255, 259, 262, 274, 283, 299, 311, 361, 424, 543, 703, and 843. (**b**) Mature heterocomplex of MR with the Hsp90-based chaperone machinery. The black crescent of the immunophilin (IMM) represents its TPR domain, and the bay in the Hsp90 dimer represents the TPR-acceptor site

Based on the analysis of protein sequences and the evolutionary trees, the second model is more likely and the one accepted by general consensus. However, regardless of how the organization of nuclear receptors had taken place during evolution, these proteins only stand for a part of the tale; the other significant part corresponds to the steroid. Therefore, the information encrypted in the hormonal response is dictated neither by the steroid nor the receptor exclusively, but it is complementary written in both modules of a multifaceted operational unit. In turn, this functional unit is subject of other kinds of non-hormonal- and non-receptor-dependent regulations such as receptor modifications by post-transcriptional modifications stimulated by ligand binding, association with other proteins that may conduct to trans-repression mechanisms, or the competitive action of metabolizing enzymes that sequester active ligands from the medium making them unavailable for the receptor.

1.3 The Hsp90-Based Heterocomplex

Steroid receptors exist as oligomeric structures with the Hsp90-based chaperone heterocomplex (Fig.1.1b). The assembly of the oligomeric structure has been well characterized for GR [25], PR [26], and MR [27] and appears to be quite representative for the assembly of most Hsp90-client proteins associated to the same oligomeric complex. The chaperone Hsp90 always function as a dimer, such that the

stoichiometry of the mature receptor•$(Hsp90)_2$ complex shows one molecule of Hsp70, one molecule of p23, and a TPR (*tetratricopeptide repeat*)-domain cochaperone bound to the TPR accept or site of the Hsp90 dimer [28–31]. The final heterocomplex depicted in Fig. 1.1b must pass through a maturation cycle in the cell cytosol [32].

Due to the high hydrophobicity the steroid binding cleft of the LBD of MR, this domain is collapsed and consequently unable to bind aldosterone, unless the Hsp90-based heterocomplex is bound to the receptor. When this happens, the steroid binding cleft of the LBD becomes thermodynamically more stable and steroid binding does occur. It is accepted that the minimal composition for the assembly of MR that permits aldosterone binding is the cytosolic complex named "foldosome," which already exists folded in the cytoplasm. It includes $(Hsp90)_2$•Hop•Hsp70/Hsp40•p23. Nonetheless, a step-by-step mechanism primed by binding of Hsp70 to the receptor followed by Hop and Hsp90 binding is also viable [25, 33]. Note that Hsp70 is associated with Hsp40, which is required in sub-stoichiometric quantities to enhance the intrinsic ATPase activity of Hsp70. The foldosome is transferred to the receptor in an ATP-, K^+-, and Mg^{2+}-dependent manner, and the resultant complex is now able to bind steroid.

Although the MR is biologically inactive in the sense that it does not bind hormone, it should be pointed out that the Hsp90-based chaperone system binds to a structure that shows a stable tertiary structure rather than to a denatured protein. The TPR-domain protein Hop (formerly called p60) is important because it brings together Hsp90 and Hsp70, two chaperones that are essential for the complex, but they are incapable to associate by themselves spontaneously. It occurs that Hsp90 dimers are in a dynamic equilibrium between an open (ADP-bound) and closed (ATP-bound) conformation [34]. Hop first stabilizes the open (V-shaped) conformation of the dimer and consequently prevents the intrinsic ATPase activity of Hsp90. Then, the small acidic cochaperone of p23 is recruited to the Hsp90 dimer. This step is critical for two reasons: first because p23 stabilizes the MR•Hsp90 association and, second, because p23 binding favors the release of Hop from the TPR-acceptor site of Hsp90 since the dimer closes its open conformation. This weakens Hop binding [35, 36]. In other words, even though Hop is required for priming the folding of the heterocomplex, it is not present in the final, mature form of the oligomer. Nevertheless, some Hop can always be recovered co-immunoprecipitated with MR, but it merely represents the intermediate complexes.

When Hop is released, the TPR-acceptor site of Hsp90 dimers is empty and can be occupied by other TPR-domain co-chaperone such as a TPR-domain immunophilin. Because there is only one acceptor site per dimer [29, 37], these TPR proteins compete one another for binding to Hsp90 in a mutually exclusive manner [38–40]. The most frequent members of the immunophilin family that can interact with Hsp90 in steroid receptor complexes are FKBP51, FKBP52, CyP40, and PP5 [41–43]. They are also found associated with cytoskeleton shaping the phenotype of the cell [44–46]. In the cases of MR and GR, the presence of CyP40 in the final mature heterocomplex is unusual in biological samples. CyP40 is more frequently found associated with PR and ER [47].

Binding of the Hsp90-based chaperone complex to the MR is mediated by the C-terminal of the hinge region [48]. The dissociation of Hsp90 or its functional disruption by drugs leads to the polyubiquitylation and consequent proteasomal degradation of MR via the ubiquitin-protein ligase CHIP (C-terminus of Hsp70-Interacting Protein) [49]. This E3 ligase is also shared with the GR [50].

1.4 MR Trafficking

In the absence of ligand, the MR is primarily cytoplasmic, and rapidly translocates into the nucleus upon steroid binding [51–53]. For decades, it has been heuristically accepted that Hsp90 anchors MR to cytoplasmic structures, such that its release from the complex was thought to be a requirement to permit the nuclear localization of the receptor. However, it has been proved that the Hsp90•FKBP52 complex is necessary for the active retrotransport of cytoplasmic receptors on cytoskeletal tracks, the motor protein dynein powering this transport (see Fig. 1.2). This model was first demonstrated for the GR [54, 55] and then for the MR [38, 40, 56]. A similar model was also reported for the transcription factor NF-κB, but in this complex Hsp90 in not an interactor and the binding of the immunophilin occurs directly to the p65/RelA [57].

In unstimulated cells, the immunophilin FKBP51 is primarily bound to the MR•Hsp90 complex. Upon steroid binding, FKBP51 is exchanged by FKBP52, an immunophilin that shares 75% similarity with FKBP51 and is capable to interact with the dynein/dynactin motor protein machinery via its PPIase domain (i.e., a domain that has enzymatic activity of peptidylprolyl isomerase). Immunophilins FKBP52, CyP40, and PP5 can associate dynein via their respective PPIase domains, but not FKBP51 [58]. When the PPIase domains of FKBP51 and FKBP52 were exchanged in chimera constructs and assayed in intact cells, the properties of both immunophilins were also exchanged, i.e., FKBP51, but not FKBP52 was capable to favour the retrotransport of GR via dynein [55]. Interestingly, FKBP51 has also been reported as a mitochondrial protein [59, 60] and is also complexed with mitochondrial GR in identical oligomers as that depicted for cytosolic GR in Fig.1.1b.

Because of its biological relevance in the receptor retrotransport, we named the $(Hsp90)_2$•FKBP52•dynein functional unit as "transportosome." The active, transportosome-dependent movement occurs on microtubules filaments [40, 44, 58, 61]. When the MR reaches the nuclear pore complex, the entire transportosome passes intact through the nuclear pore, the chaperones and immunophilins being interacting factors with the nucleoporins and importins of the pore complex [40, 62, 63]. The permeability barrier of the pore is in part due to a sieve structure created by the reversible cross-linking between Phe and Gly (FG)-rich nucleoporin repeats, which create a three-dimensional meshwork with hydrogel-like properties [64]. According to the novel model, nuclear transport receptors overcome the size limit of the sieve and catalyze their own nuclear pore passage by a competitive disruption of adjacent inter-repeat contacts, which transiently opens adjoining meshes. The

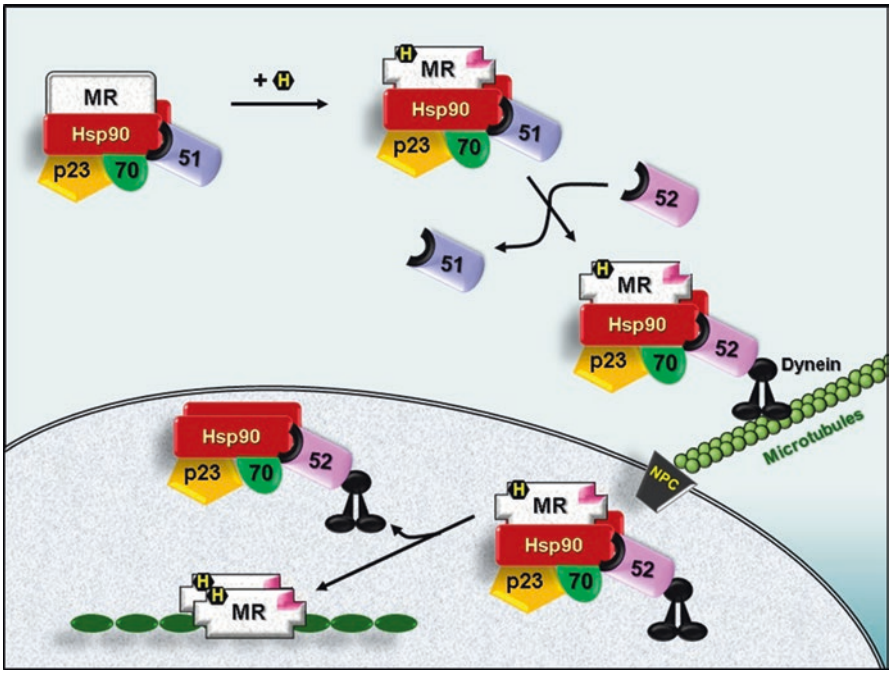

Fig. 1.2 Transportosome model. In the absence of steroid, MR forms cytoplasmic complexes with Hsp90, Hsp70, p23, and FKBP51. Upon hormone (H) binding, MR undergoes a conformational change, and FKBP51 is exchanged by FKBP52, an immunofilin that recruits dynein in its PPIase domain. MR is actively transported to the nucleus, passes intact through the nuclear pore complex (NPC), is "transformed" in the nucleoplasm, and dimerizes and binds to the promoter sequences of target genes. The Hsp90-based heterocomplex can be recycled. The black crescent represents the TPR domain of the immunophilins

chaperone complex would enhance the capability of the MR to overcome the resistance of the meshwork simply by accomplishing its standard role of chaperones. Therefore, the MR•Hsp90 complex dissociates in the nucleus rather than in the cytoplasm as it has always been thought. The receptor dimerizes in the nucleoplasm [65–67] and becomes activated to acquire its main biological role, i.e., to be a transcription factor. In contrast to the classic model of action posited for steroid receptors years ago, all these mechanistic steps are not heuristic and have been experimentally supported for each individual step.

An additional relevance of the presence of FKBP52 associated with the MR is the capability of this immunophilin to anchor the receptor to nuclear matrix structures [63]. Actually, the overexpression of FKBP51 expels MR from the nuclear compartment, perhaps due to competition with FKBP52 for the nuclear anchoring sites [40]. Similar observations and conclusions were also achieved for the role of FKBP52 in the mechanism of action of NF-κB [57, 68].

1.5 Agonist Structure-Activity Relationship

Binding of the cognate ligand to its specific receptor is the primordial first step to trigger cellular events that lead to the final biological effect in the body, i.e., binding of aldosterone to MR have profound effects in the electrolyte balance of the body, plasma osmolarity, blood pressure, heart rate, adiponectins activity, slow wave sleep, salt appetite, interoception, emotionality, etc. Therefore, the proper recognition of both components of the ligand-receptor functional unit is essential. Decades ago, Duax et al. [69–71] summarized the minimal conformational requirements on ring A of steroid hormones for optimal binding to different receptors. According to that study, the optimal conformation for the MR would be a 1α-envelope to a 1α,2β-half-chair containing the 3-keto-4-ene function. Better affinity ratios for the MR were also measured when those substituents that show the tendency to bend the A-ring toward the α face of the steroid molecule were eliminated, for example, for steroids lacking the C_{11}-hydroxy function or the C_{19}-methyl group [72]. An equivalent result is also observed upon introduction of ketalic bridges that flatten the overall structure, for example, in the cases of aldosterone itself and related 18-oxygenated analogues [73].

Although several compounds have been synthesized for all the other members of the steroid receptor family (and many of them have even replaced the natural ligands in many clinical treatments), only one synthetic MR agonist showing no cross-reaction with the other members of the steroid receptor subfamily is currently available to study the agonist mineralocorticoid function—11,19-oxidoprogesterone [74, 75] (see its structure in Fig. 1.3). In vivo assays in rats demonstrated that this

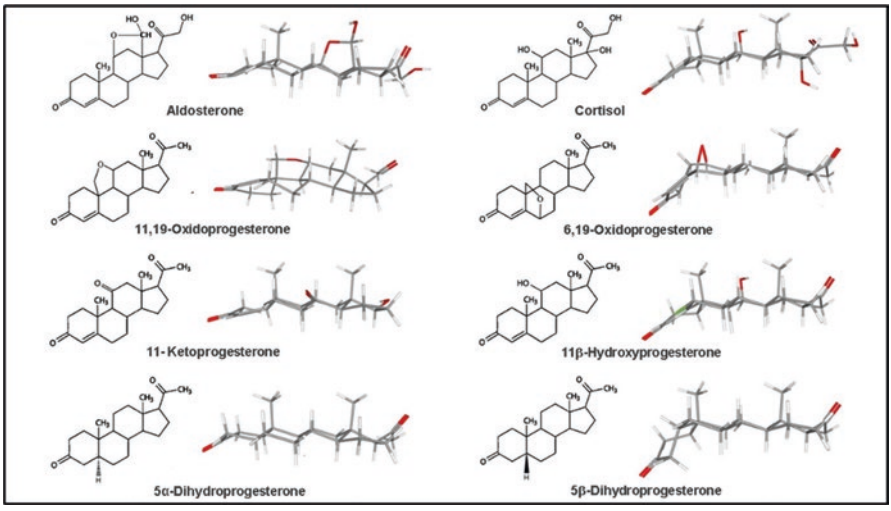

Fig. 1.3 Most stable conformers for some pairs of steroids. Under physiologic conditions, all ligands on the left column exhibit better Na^+-retaining activity and higher relative affinity for MR than the bent partners depicted on the right column

steroid shows identical activity to the naturally occurring mineralocorticoid 11-deoxycorticosterone at low doses and becomes undistinguishable from aldosterone at as low dose as 10μg/100 g [75]. On the other hand, the bent conformers 6,19-oxidoprogesterone and its 21-hydroxylated derivative are devoid a mineralocorticoid effect and show no binding to the MR [75, 76]. Similar observations can be made for other pairs of steroids that share similar or identical functional groups but show different conformational structures (Fig. 1.3 depicts some practical examples). Based on these facts, it has been postulated that the essential requirement of a ligand to become a mineralocorticoid ligand is to possess an overall planarity of the steroidal frame, and its ability to preserve it in vivo is critical to confer a steroid mineralocorticoid activity [75, 77, 78]. The last statement refers to putative chemical modifications the steroid may suffer because of the metabolism or associations with other molecules or proteins that may affect its conformational structure according to its molecular flexibility. Thus, A/BCD angle for progesterone is not greatly different from that of aldosterone (−24.0° vs −21.4°, respectively), but progesterone is a highly flexible steroid, whereas the presence of a hemiketalic ring that involves the C_{18}-aldhehyde in aldosterone (from which the name of the steroid derives) favors that aldosterone can preserve its overall conformational flatness due to the rigidity of the molecule. A similar property is conferred by the presence of the C_{11}-O- C_{19} bridge in the synthetic agonist 11,19-oxidoprogesterone.

An interesting property of the dose-response curves for Na^+-retention is that most agonists exhibit a parabolic shape [74, 75], that is, a maximal antinatriuretic action at certain doses and then, a clear reversion at higher doses, a feature that is less evident for the most active ligands versus the less active steroids. Such a biphasic function of the dose-response curves makes unsuitable the concept of a conventional ED_{50} to quantify properly the entire function of the biological effect. Nevertheless, a good correlation could be observed if the overall function is considered as a second-order polynomial function defined by the equation $y = ax^2 + bx + c$. Thus, the second-order coefficient "a" is a direct measure of the concavity of the polynomials and quantifies the biopharmacological parameters of the dose-response curve. Therefore, the most potent mineralocorticoid action corresponds to the lowest "a" value. Figure 1.4 shows the excellent correlation between this coefficient and the relative affinity of the steroid for the MR (Fig.1.4a) and the overall flatness of the conformers estimated as the angle between the C_3-carbonyl and the middle plane of the D ring (Fig.1.4b).

1.6 MR Antagonism

Aldosterone antagonists that are capable to impair the activation of the MR have a cardinal importance in the treatment of cardiovascular diseases [79]. Consequently, a considerable effort has been made by several laboratories and companies for the development and safe clinical use of synthetic anti-mineralocorticoid steroids, particularly during RALES (Randomized Aldactone Evaluation Study), EPHESUS

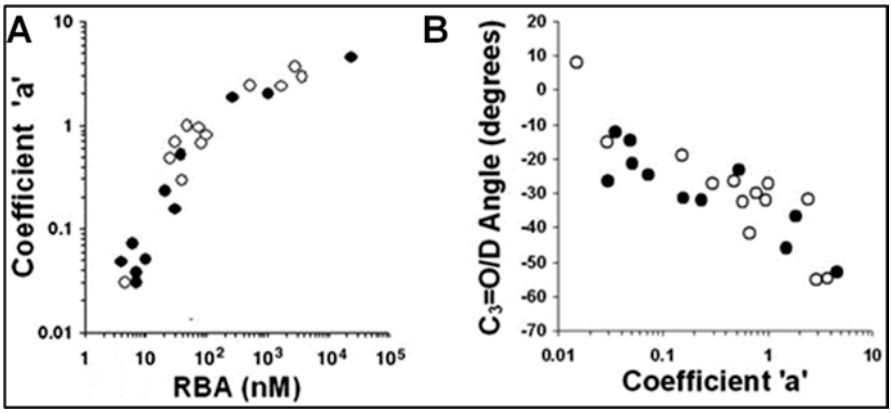

Fig. 1.4 (**a**) Mineralocorticoid response measured as the co-efficient "a" of the parabolic dose-response curves (low "a" means high response, see text). RBA: Relative binding affinity relative to [^{3}H]-aldo-sterone. (●) 21-hydroxyste-roids (○) 21-deoxysteroids. (**b**) Steroid flatness improves the biological effect ('a)

(Eplerenone Post-AMI Heart Failure Efficacy and Survival Study), and EMPHASIS-HF (Eplerenone in Mild Patients Hospitalization And Survival Study in Heart Failure) pioneer past trails, and most recently the ARTS (Arterial Revascularization Therapies Study) series [80, 81].

During the 1960s, spironolactone (Aldactone®) became the first synthetic anti-mineralocorticoid approved for massive human use. This synthetic steroid is indicated in cases of primary hyperaldosteronism, congestive heart failure, edematous conditions, hepatic cirrhosis, and nephrotic syndrome [82], among the most common pathologies. With time, it was also indicated for cases of severe heart failure and hypokalemia when standard alternative treatments were not well tolerated or are ineffective. Eplerenone (Inspra®) was approved in the year 2002. Even though it shows lower pharmacologic potency than spironolactone as an MR antagonist, it has other advantages such as longer half-life and does not generate active metabolites [83].

From the physiologic perspective, it should be pointed out that at the renal level, progesterone behaves as an MR antagonist in most vertebrates [84]. Progesterone shows equivalent affinity to aldosterone for the MR, a property conserved among mammalian species, suggesting the potential existence of unexplored roles for this ligand bound to MR. During pregnancy, progesterone raises plasma levels up to one order of magnitude higher than those of aldosterone, perhaps it is a self-protective mechanism since aldosterone also increases its concentration. Interestingly, it has been reported that a single nucleotide mutation (S810L) in the gene encoding the human MR and creates an MR that responds to progesterone resulting in early-onset hypertension, which is very much exacerbated during pregnancy [85].

The crystal structure of MR associated with aldosterone and antagonists [86] unrevealed key structural characteristics of the MR for the further development of

synthetic antagonists. In combination with mutational studies, it was evidenced that Asn^{770} is essential for MR activation and also that the interactions of Thr^{945} in helix 10 are critical for the activation of the receptor. The crystal structure of MR with the natural antagonist progesterone evidenced that the orientation of the Thr^{945} side chain is somehow vague because of competition between the ligand and intramolecular hydrogen bond acceptors. This decrease of both number of hydrogen bonds and the strength of the effective hydrogen bonds, plus the lack interaction with the Asn^{770} residue is the most likely molecular reason by which progesterone produces a weak activation (if any) of the MR despite the fact that its affinity for the receptor is high.

As a consequence of these studies, various possible antagonistic mechanisms have been postulated for the MR [86], i.e., competitive antagonism where there are no conformational changes induced in the LBD (such as in the case of spironolactone binding); impairment of MR dimerization; ligands whose binding favors MR degradation; and the case of selective ligands (antagonists or agonists) of transrepression, this being a similar situation to that already reported for GR antagonists [87]. As it was stated above, the distinction between an MR antagonist and an MR agonist could be subtle since the substitution of a single amino acid (S810L) can make the difference and transforms not only progesterone, but also the antagonist spironolactone and the endogenous cortisone in strong MR agonists [85, 88].

1.7 MR Regulation by Phosphorylation and Redox Potential

Like most members of the nuclear receptor superfamily, the MR is a phosphoprotein. The first evidence was obtained when hMR was expressed in *Spodoptera frugiperda* cells grown in the presence of ^{32}P [89]. Then, it was demonstrated that rat kidney MR is a phosphoprotein in a physiologic milieu [90, 91]. In that early work, a treatment of native MR with alkaline phosphatase resulted in the loss of aldosterone-binding capacity and dramatic changes of MR hydrodynamic properties in sucrose density gradients, causing a strong shift from the 8.8 S (untransformed, Hsp90-bound) isoform to the 5.1 S, transformed isoform. During the late 1990s, it was postulated that Ser/Thr-phosphatases of the PP1/PP2A family are involved in the mechanism of activation of MR and that this fact enhances its capacity to interact with the promoter sequences of target genes in the DNA [90, 91]. It was also hypothesized that the phosphorylated forms of MR are not beneficial for aldosterone-binding capacity [51, 90]. These early findings were confirmed years later by the Lifton lab [92, 93] in a study where it was demonstrated that phosphorylation at S^{843} in the MR LBD prevents aldosterone binding. In line with this, an MR phosphomimetic mutant (S843E) revealed showed that steroid binding capacity is severely impairs, increasing the dissociation constant by more than 100-fold [93]. This phosphorylated form of the MR was found in intercalated cells of the distal nephron. Importantly, angiotensin II signalling decreases phospho-MR levels, a phenomenon dependent on the activity of a PP1 protein-phosphatase, just as it had

been predicted in the early studies [90, 91]. This increases the aldosterone-dependent biological response of the cells. More recently it was demonstrated that the effect of angiotensin II is due to inhibition of the Ser/Thr-protein-kinase ULK1 (Unc-51 Like-autophagy-activating Kinase 1), which results in decreased MR phosphorylation via mTOR [94].

Phosphorylation of MR is also related to the regulation of the receptor by the redox potential of the cell, glutathione (GSH) being the most prevalent and abundant (mM range) intracellular reducer thiol. GSH is not required in the diet, but synthesized by the sequential actions of two enzymes: Q-glutamyl-cysteine synthetase and GST [94]. GSH is exported continuously and degraded extracellularly. Therefore, in vivo GSH deficiency can achieved with the inhibitor of the enzyme that generate GSH and BSO (L-buthionine-(S,R)-sulfoximine). When adrenalectomized rats were treated with BSO [95], the low redox potential generated exerted drastic and uncompensated inhibition of the MR-dependent response with loss of the mineralocorticoid response (i.e. Na+-retention, kaliuresis, low aldosterone-binding capacity) accompanied by a higher level of receptor phosphorylation. The loss of steroid binding capacity was assigned to the oxidation of essential cysteine groups of the MR but also due to an inefficient synthesis of MR due to failures at the elongation/termination step during the receptor translation, mimicking the observations made with rats along the ageing process. There are several other variables that may affect the MR-dependent response by influencing the redox milieu. For example, the use of drugs that are designed for unrelated applications, but they may affect the redox potential of the cell. It is known that melatonin affects the GR nuclear translocation due to unknown reasons [95], and its influence on the close-related partner MR has not been studied to date.

As is was detailed in the first section, when 11βHSD2 activity is deficient or blocked, its protective mechanism on the MR against cortisol activation fails, such that cortisol activates principal cell MR. In tubular intercalated cells, MR but not 11βHSD2 is expressed. However, the MR is protected from cortisol activation by phosphorylation at S^{843}. When MR becomes dephosphorylated in response to angiotensin II, MR can be stimulated by both steroids, aldosterone or cortisol, but more likely by the latter given its 2–3 orders of magnitude higher of plasma levels compared to aldosterone [11]. In addition to converting cortisol to cortisone, this enzyme also produces NADH from NAD^+. Interestingly, it has been postulated that what appears to hold cortisol-MR complexes inactive is the high levels of NADH generated [96].

In the renal target cells, the enzyme 11βHSD2 can debulk intracellular cortisol by 90%, i.e., to levels ~tenfold those of aldosterone. This implies that when 11βHSD2 is functional, most epithelial MR pool can be still occupied by cortisol, but it is not active. When intracellular redox state changes due to inhibition of 11βHSD2 (no NADH is produced), the increased production of ROS and oxidized glutathione (GSSG) could transform cortisol from an MR antagonist to an MR agonist. Thus, it was reported [96, 97] that when rabbit cardiomyocytes are patch clamped and treated with 10 nM aldosterone, ion-influx is increased tenfold (as expected), whereas 100 nM cortisol shows no effect. When both steroids are added

together, the aldosterone action is 90% antagonized. If 5 mM GSSG is instilled intracellularly (i.e., to mimic the redox state under tissue damage conditions) cortisol becomes an MR agonist and similar effect as that measured with aldosterone alone is observed. Cardiomyocytes do not express 11βHSD2, so MR is "unprotected" and overwhelmingly occupied by cortisol, previously shown not to mimic the effects of aldosterone via MR in neonatal rat cardiomyocytes [98]. In line with these observations, in other study it was also demonstrated that corticosterone action via MR in rat ventricular cardiomyocytes requires an oxidized milieu [99]. Therefore, oxidative stress experienced after a postischemic reperfusion would favor glucocorticoid activation of the MR and the potentiation of the GR response, such that both receptors could contribute to remodelling the functional properties of ventricular cardiomyocytes. This makes them prone to spontaneous contractions and consequently, increasing the deadly risk of ventricular arrhythmias.

Importantly, oxidative stress can be transmitted through a glutathione-S-transferase (GST) "switch" connecting to kinase cascades influencing cell signalling. High ROS can cause a disassociation of the GST•JNK complex, thus activating JNK pathways [100]. PKC can also be activated, a protein-kinase that has been related to phosphorylation of MR [101, 102]. Aldosterone-dependent activation of MR induces the expression of the immunophilin FKBP51 [103], a phenomenon also reported for angiotensin II stimulation of smooth muscle cells via MR where PKC activation is also involved [102]. ROS effects are not entirely surprising since several studies have already shown that many kinases affect their activity upon the onset of oxidative stress such as JNK, p70-S6 kinase, Akt/PKB, PDK1, and SGK, among many other examples [104, 105]. Interestingly, the last two kinases are linked to the MR-dependent response. SGK affects the activation of the epithelial sodium channel and is in turn regulated PDK1 [106].

1.8 Conclusions

The MR and its two most potent physiologic ligands aldosterone and 11-deoxycorticosterone have evolved together under the evolutionary pressure of maintaining the internal homeostatic balance of water and electrolytes in an environment where especially the offer of water is frequently limited. On the other hand, glucocorticoids took on the task of ensuring energy homeostasis. Both receptors, MR and GR, may often work in concert or in counterpoint to meet the constant presence of new and varied environmental challenges. Therefore, the MR/GR ratio of selective activation is critical for normal function of the body. This is particularly relevant for the brain, where the highest concentrations of MR per gram of tissue are expressed. Within the most conventional diseases, hypertension is perhaps the best known when there is unequal or inappropriate MR/GR occupation and activation, as well as it is the case of metabolic syndrome and depressive disorders.

In pathologic situations, it is regarded that MR can be often be occupied by glucocorticoids rather than aldosterone. Moreover, the required concentration of local

ligand necessary to activate the MR could be quite different from their plasma concentrations, especially in the nervous system and other systems where both aldosterone and glucocorticoids could be produced locally in an reduced or increased manner under pathologic situations.

It should be kept in mind that the transactivation activity of the MR is crucially dependent on the nature of the bound ligand. Agonist or antagonist ligands are capable to induce a unique conformational change that drives interactions of the MR with several coregulators and tissue-specific transcriptional factors. Therefore, each ligand•MR complex surely shows distinct and often opposite tissue-specific target genes and therefore distinct downstream biological effects depending on how the steroid has been accommodated in the ligand binding pocket.

The uncovered mechanism of action of the MR to date show a complex picture of multifunctional systems that require additional studies to unravel several poorly understood events such as the protection of the MR against nonspecific activation, by its several binding ligands, or the influence of the cellular context for its activation. Its cognate endogenous ligand aldosterone is a key therapeutic target in hypertension [81, 107] and chronic heart failure [108, 109]. Accumulating data also indicate that MR antagonists can be protective against the chronic kidney disease [109, 110]. After several years of intensive research, the development of new therapeutic approaches and the development of novel cardiovascular drugs is a fact based on studies that began explaining the basics of the molecular mechanism of action of the youngest member of the nuclear receptor superfamily. Nonetheless, more detailed characterization of the molecular mechanisms regulating MR function in the kidney, heart, brain, and other tissues may reveal new targets that might be exploited for therapeutic purposes.

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Chapter 2
A Simple Method for Visual Assessment and Quantification of Altered Subcellular Localization of Nuclear Receptors

Sudhir Kumar, Jyoti Kashyap, Keshav Thakur, and Rakesh K. Tyagi

Abstract All nuclear receptors reside in the nuclear compartment when transcriptionally active. However, their subcellular localization can vary when unliganded, functionally inactive, or dysregulated in diseased conditions. This property of differential localization and inducible nuclear translocation of nuclear receptors has proven to be useful tool in characterizing their transcription functions, discovery of novels drugs, ligands (agonists, antagonists, selective modulators of receptor action, endocrine disruptors, endobiotics, xenobiotics, etc.), partner-mediated interactions and coregulators. To efficiently study these ligand-modulated transcription factors, we describe a simple method for visual assessment and quantification of subcellular localization achieved by these receptors under varying physiological conditions. Contrary to some of the emerging high-end instrument-based assessments, the current method is simple, economical and highly reproducible, giving options to conduct similar studies in less sophisticated settings. It is expected that the current assessment approach will help investigators in their discovery of mechanisms of actions of these receptors in health and disease, and also in defining novel small molecule modulators to overcome physiological perturbations.

Keywords Steroid/thyroid/nuclear receptors · Subcellular localization · Live-cell imaging · Cell quantification · Fluorescence microscopy

2.1 Introduction

Members of steroid/thyroid/nuclear receptor (NRs) superfamily are a group of evolutionarily related, intracellular, and ligand-modulated transcription factors with a total of 48 affiliates identified in the human genome [1–3]. These NRs are now well-established to have vital roles in a multitude of physiological events relating to development, reproduction, metabolism, and homeostasis [4]. Some of these

S. Kumar · J. Kashyap · K. Thakur · R. K. Tyagi (✉)
Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India

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receptors have already been shown to have enormous potential as targets for small molecule modulators in the treatments of health-threatening diseases such as diabetes, osteoporosis, asthma, thyroid disorders, endocrine-related cancers, and several other metabolic diseases [5, 6].

NRs are believed to be nucleocytoplasmic shuttling proteins, and their steady-state localization is a consequence of a fine balance between the operational strengths of their nuclear localization signals and nuclear export signals [7–9]. Under normal physiological conditions, these receptors are routinely observed to be nuclear when transcriptionally active or liganded [10–13]. On the contrary, in unliganded or transcriptionally inactive form, their subcellular localization may vary to a differing extent between nuclear and cytoplasmic compartments [12, 14–16]. Since the early years of their cloning during the 1980s, the exact subcellular localization of NRs remained mostly ambiguous and ridden with controversies, which could be partly attributed to the quality of the antibodies or limitations of detection tools, cell type expressing it or the interspecies differences between these receptors [17]. However, with the arrival of different fluorescent protein probes, a chimera of these receptors could be generated, expressed, and visualized in living cells in differing physiological states [9]. This revolutionized the field in terms of divulging the cellular dynamics, subcellular localization, and mechanisms of actions of these receptors [9, 18]. However, a significant number of these receptors remain incompletely understood in terms of their exact physiological roles and activating ligands [3].

Appropriate localization and dynamics of cellular proteins at the site of its action is crucial to execute a physiological function in a specific biological network [19]. This will also be true for transcription factors in general and NRs in particular. Once synthesized in the cytoplasm, the cellular residency of the factor determines its site-specific interacting partners, its post-translational modifications, as well as its preparedness for its physiological action [20]. In context to receptor-specific diseases, the aberrant localization, deregulation of receptor dynamics behavior, or altered response to the interacting partners/ligands will be reflected mostly in the "loss of function" or sometimes even "gain of function" of NRs or other transcription factors [21]. This may sometimes warrant for a search for novel therapeutic NR ligands or receptor modulators for normal functioning of the receptor. Several reviews have discussed the concepts and strategies for therapeutic restoration of disrupted or altered protein localization to prevent a disease-inflicting event(s) [19, 21].

Normal functional attributes to NRs can be assigned by observing their responses to cognate ligand interactions, receptor-coregulator interactions, or other physiological cues released in a time-bound manner to orchestrate an event [13, 22]. Classically, other than promoter-reporter-based assays, the receptor response to a signaling cue has also been observed using immunological tools for documenting the altered receptor localization, nuclear translocation, or inter-molecular interactions [23]. The latter test assays are exploited for both fixed cells and tissues and, more recently, with live cells imaging using fluorescent probes [9, 17, 18]. These receptor localization and translocation assays based on nuclear import or export

have proven to be exceptionally useful, for understanding the mechanisms of their action. In addition, these assays have the potential to develop into high-throughput screening protocols for novel drug discovery, endocrine-disrupting chemicals, or discovery of unknown physiological ligands for orphan receptors [14, 24–26]. These simple cell-based receptor localization and translocation assays, though not exploited to the utmost potential, have proven to be extremely promising in terms of being direct, fast, reliable, economical, and simple [24, 26–29]. Without the employment of advanced, sophisticated instrumentations, these cell-based assays can be adapted (i) to characterize novel small molecule modulators [24, 30]; (ii) to study the relative potency and kinetics of receptor translocation by diverse ligands [13, 24, 31]; (iii) to study receptor-partner or receptor-protein interactions [8, 32]; (iv) to establish the transcriptional status of the receptor [14, 21, 33]; (v) to determine the receptor-chromatin interactions [34, 35]; and (vi) to examine the regained or lost functional status of the disease-inflicting non-functional receptor [21, 34, 35].

Since cytoplasmic retained inactive NRs have the inherent property to translocate to nuclear compartment when induced by specific ligand(s) or signaling mechanisms, it provides an excellent opportunity for studying the receptor functioning and the underlying signaling mediators (hormones, growth factors, pharmacules, small molecule modulators, endocrine disruptors, dietary or herbal compounds, etc.) [12, 36]. For the purpose of quantification of nuclear translocation or strength of the inducer in inducing the event, the receptor can be classified into five distinct patterns of receptor distribution, as shown in Fig. 2.1 and described in material and methods.

A few other methods are also utilized for subcellular localization and/or quantification purposes of NRs, albeit less frequently. These methods include (i) cell fractionation into nuclear and cytoplasmic fractions followed by western blotting; (ii) bioinformatics tools-based approaches; (iii) immunocytology and immunohistochemistry approaches; and (iv) automated high-throughput single-cell image cytometry [17, 20, 37–41]. Most of these methods however offer limited or specific advantages.

Some challenges in cell selection for quantification purposes may be evident when working with transiently expressed proteins/receptors. More often than not, it will be observed that not all transfected cells in a given population express the ectopic gene to a similar extent or level. A range of gene expression profile, with some cells expressing relatively lower while others expressing higher levels of protein, are routinely observed. A small population of cells may also be observed which exhibit aberrantly high expressions with a fraction of protein/receptor aborted mostly in the cytoplasm. These fewer cells may also appear to be multi-nucleated or have abnormal morphology [42]. Such cells are to be best avoided for quantification and data representation as they do not represent the larger and normal population of the gene expressing cells.

The method discussed above for the assessment and quantification of subcellular localization and translocation of NRs is readily applicable to other nucleocytoplasmic proteins as well and has been in use for a few decades [7, 11, 12, 23, 32, 43–46].

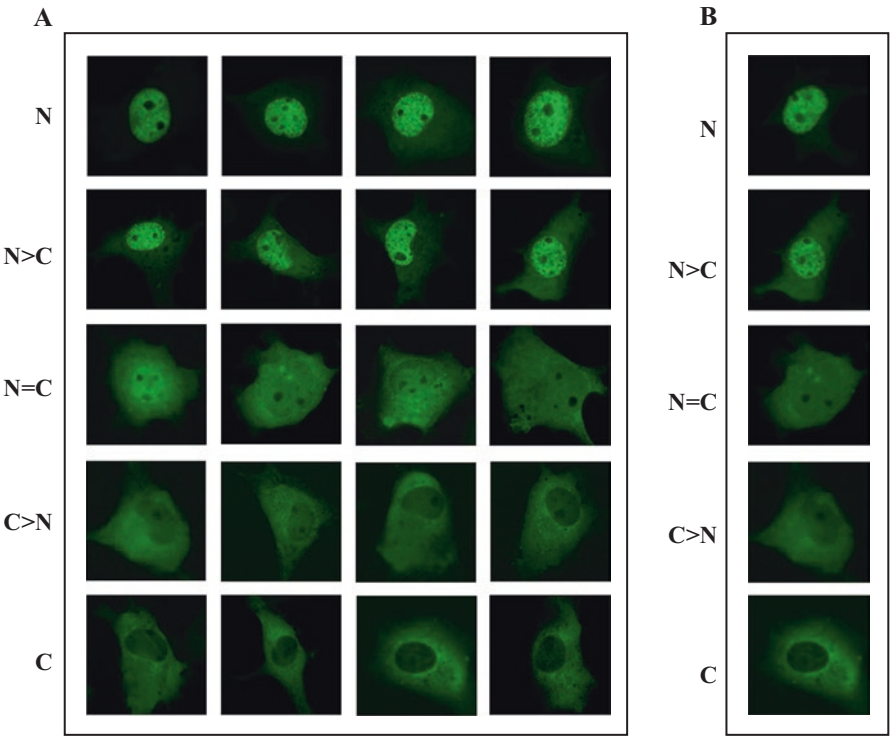

Fig. 2.1 Representation of categories for differential subcellular localization using transiently expressed green fluorescent protein-tagged androgen receptor (GFP-AR). Mammalian COS-1 cells were transiently transfected with GFP-AR chimeric plasmid and allowed to express the receptor protein for 30 hours post-transfection. A total of 100 cells were randomly visualized, counted with a cell counter and simultaneously assigned into any of the five different categories based on receptor distributions profiles, as indicated in the figure. The representative patterns of subcellular localization were recorded using live-cell imaging with fluorescence microscopy. Approach to assign and categorize cells into different groups is suggested (**a**). Single-cell panel on the right (**b**) is shown to represent diversity and category based on receptor localization in a single cell. N nuclear, C cytoplasmic

Despite having good reproducibility, and efficacy between what is visualized and recorded, the method has not been as widely used as would be expected. However, when the scientific literature is surveyed, it is convincingly observed that a large number of major discoveries in the "nuclear receptor biology" have been based on this method of quantification as mentioned earlier for subcellular localization of transcription factors [12, 23, 26–28, 32, 42–45]. However, with the advent of emerging sophisticated instrument-based technologies [47–49], where NR translocation is assessed by high-throughput screening and with a much larger number of small molecule modulators, the classical fundamental approach has also been provided a much-awaited credence as an attractive option. Nonetheless, the classical method presented herein is still superior for routine laboratory studies in terms of it being highly reproducible, economical, and simple.

2.2 Materials and Methods

1. *Mammalian Cell Lines*

Some mammalian cell lines are preferred for gene expression studies as they are highly transfectable with plasmids and include COS-1, COS-7, HEK-293T, CHO cells, and baby hamster kidney (BHK21) cell lines. These cells can be procured from ATCC (USA) or other national or international cell depository. Other cell lines may also be moderately transfectable or difficult to transfect. The success of achieving efficient transfection also depends on the nature and the type of transfection reagent used, the presence of endotoxins in the plasmid preparations, and optimization of the protocol with the specific experimental cell line.

2. *Cell Culture Condition and Media*

The mammalian cells are typically cultured in a humidified incubator at 37 °C with 5% CO_2. The pH value of $NaHCO_3$-buffered culture media depends on the CO_2 content of the incubator's atmosphere. Maintenance of optimum CO_2 in the incubator is necessary to achieve a stable pH of culture media to maintain healthy cells. The media and supplements are chosen according to the specific requirement of cell lines as is generally recommended by the culture provider. Here, we used a "complete DMEM" that was supplemented with 10% FBS and antibiotics-amphotericin for the routine culture procedures. When required for NRs, the 10% FBS was replaced in the media with 5% steroid-stripped serum.

3. *Steroid-Free Media and Cell Culture*

Steroid-free conditions for cell cultures can be easily maintained by culturing the cells in steroid-depleted serum-containing media. Due to the absence of steroids, the steroid receptor family members and a few other receptors exhibit only minimal/basal transcriptional activity in promoter-reporter transcription assays. The receptor is also observed to be localized in the cytoplasm or is shifted toward the cytoplasmic compartment. Both the observations support the ligand-unbound status of the nuclear receptor. The better this ligand-free status of the receptor is achieved, the superior will be the observed results obtained after ligand treatment. A steroid-free serum can be prepared in the laboratory or easily procured from a few commercial sources.

4. *Transfection Reagents*

A few readily available transfection procedures (e.g., calcium phosphate, electroporation, lipofection) are commonly used which may be preferred depending on the experimental cell type being used. In the current examples, the simple and easy lipofection method was used according to the manufacture's protocol. Lipofectamine is a cationic lipid-based transfection reagent which provides an advantage over conventional methods in terms of higher transfection efficiency, reproducibility, and simplicity. A few other variables of lipofection reagents are commercially available,

including but not limited to lipofectamine 3000 (Invitrogen); lipofectamine Plus (BD Biosciences); FuGENE (Roche Applied Science); ESCORT (Merck), etc.

5. *Live Cell Imaging and Image Acquisition*

Generally, the commonly used microscopes for the live cell imaging are equipped with different lenses, appropriate filters, lamps, shutters, camera, and stage. For cell imaging performed here, we have used a Nikon upright fluorescence microscope (model 80i) equipped with water immersion objectives and connected to cooled CCD digital camera model Evolution VF, Media Cybernetics, USA.

Receptor subcellular localization was recorded by visualizing the fluorescence intensity of tagged receptor protein in cellular compartments. As an example, the subcellular localization of androgen and estrogen receptors fused with GFP and treated with and without hormone are quantified and represented in Figs. 2.1, 2.2 and 2.3.

6. *Cell Counter or Blood Cell Counter*

We have observed that a commonly available blood cell counter is best suited to record the five diverse subcellular localization (spread between N and C localization) of NRs or other proteins (Fig. 2.4). When recording a total of 100 cells, the numbers acquired for localization will directly represent values in percent (%). If counted above or below 100, the values will have to be calculated into percentages for convenience of representation.

7. *Cell Quantification for Receptor Localization*

Changing physiological status is central to alterations in dynamic behavior of cellular proteins including nuclear receptors. Therefore, recording and quantifying the altered behavior and localization of cellular proteins or receptors help decoding a cellular event. The current method shown underneath is such one approach to achieve this objective.

The classification for quantification of nuclear receptor distribution includes (i) the extreme ligand-unbound, inactive, and exclusively cytoplasmic receptor (C) and (ii) the other extreme ligand-bound, transcriptionally active, and fully nuclear-shifted receptor (N). Between these two extreme states of localization (C & N), a further distinction into three more categories can also be easily made. When the receptor appears only partly shifted to the nuclear compartment and is still primarily localized in the cytoplasm, it is classified as C > N. If the receptor is uniformly distributed between the nucleus and cytoplasm compartments, it is classified as N=C. However, if the receptor is predominantly shifted into the nuclear compartment, but a significant fraction is observable in the cytoplasm, it is considered as N > C. In this way, the receptor distribution can be visually judged into five different categories, namely, N, N > C, N=C, C > N and C. The Fig. 2.1 depicts a visual example for the classification of diverse possibilities experienced during receptor localization studies. Subsequently, Fig. 2.2 shows the application of this assessment and quantitation using androgen receptor as a representative example. These five categories work best for the receptors which are exclusively cytoplasmic when

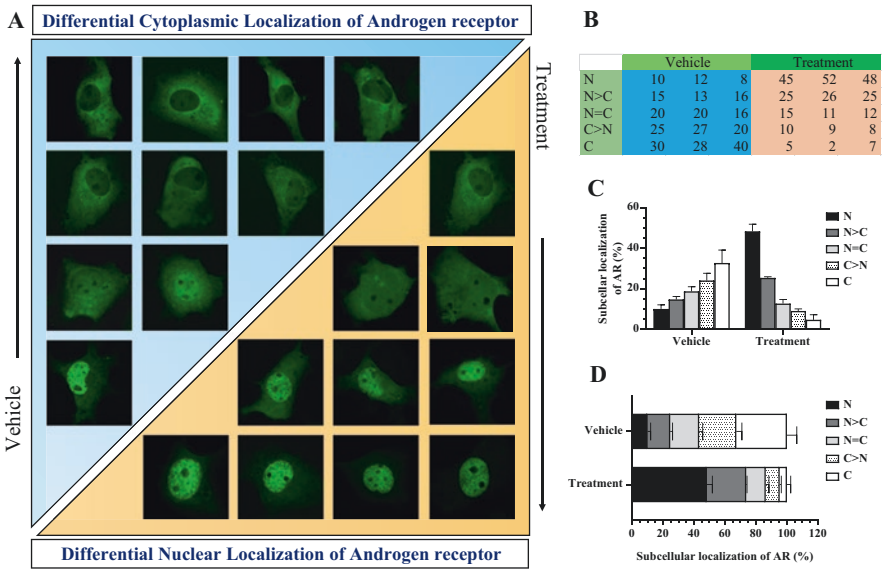

Fig. 2.2 Quantification of differentially expressed GFP-AR receptor before treatment (control vehicle) and after hormone (androgen) treatment. (**a**) Representative population of GFP-AR receptor in the presence and absence of hormone treatment are shown. (**b**) A total of 100 cells for each set of experiments were randomly counted and recorded with a cell counter according to the specified category by receptor subcellular distribution. (**c**, **d**) Graphical representation of subcellular localization (%) of GFP-AR by two different bar graphs is shown. N nuclear, C cytoplasmic

unliganded and transcriptionally inactive (e.g., glucocorticoid receptor, androgen receptor) and translocate almost fully to the nuclear compartment when bound to an activating ligand. For the purpose of counting these five categories, a "manual blood cell counter" is found to be most suitable and efficient.

However, some unliganded inactive NRs (e.g., estrogen receptor, vitamin D receptor) may show a significant population of receptor cells as exclusively nuclear (N) or predominantly nuclear (N > C) and a few other cells as N=C. Similarly, a few other ligand-unbound receptors may exhibit a dominant population of cells distributed uniformly between nuclear and cytoplasmic compartment. Nonetheless, when bound to an activating ligand/agonist, they exhibit a larger cell population with the nuclear-shifted receptor. In such instances, the receptor quantification with N, N > C, N=C for former and N + N > C, N=C, C > N + C for the latter may be more convenient for data collaging and interpretation for receptor localization. Figure 2.3 depicts the concept using estrogen receptor as an example. This brings in objectivity to the real experimental observations when represented graphically. With little experience and clear inclinations, the data profile obtained by these approaches have proven to be surprisingly close between the experimentalist and the blind observers. Counting and categorizing a total of 100 cells per experimental set converts the values conveniently into percent (%) distributions as shown in Figs. 2.2 and 2.3.

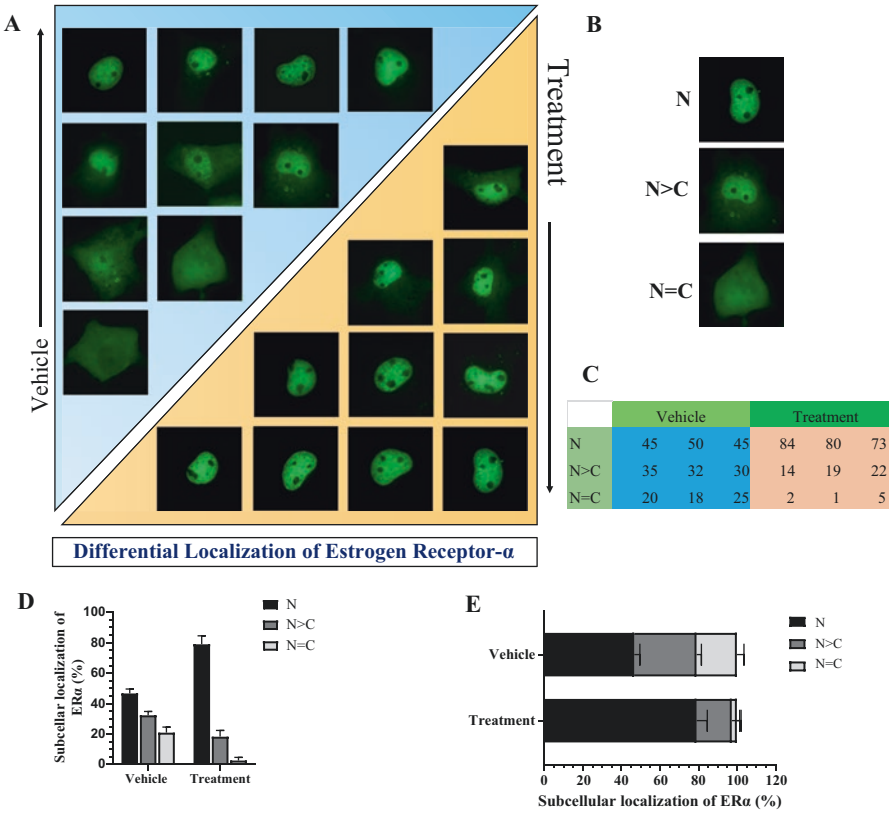

Fig. 2.3 Representation and quantification of categories for differential subcellular localization of transiently expressed green fluorescent protein-tagged estrogen receptor (GFP-ER) before treatment (control vehicle) and after hormone (estrogen) treatment. (**a**) The representative population of GFP-ER receptor-expressing cells in the presence and absence of hormone (estrogen) treatment are shown. Approach to assign and categorize cells into different groups is also suggested here. (**b**) Single-cell panel on the right represents the diversity and category based on receptor localization in single cells. (**c**) A total of 100 cells for each set of experiments were randomly counted and recorded with a cell counter according to the specified category by receptor subcellular distribution. (**d**, **e**) Graphical representation of subcellular localization (%) of GFP-ER by two different bar graphs. N nuclear, C cytoplasmic

8. *Data Acquisition and Image Processing Softwares*

There are numerous image acquisition and image processing software available with, manufacturers (e.g., Image Pro-plus, cellSense dimensions, NIS-Element, ImageJ, Metamorph (Molecular Devices, USA). These softwares are helpful in image acquisition, processing, quantification, compilation, labelling, and improving the presentation of the acquired images. However, appropriate care must be taken to apply the processing parameter on the whole image without the introduction of unwarranted features that were not observable when initially acquired.

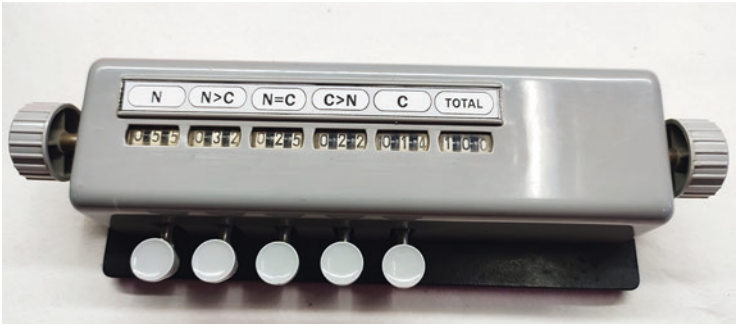

Fig. 2.4 A blood cell counter adapted as manual cell counter for quantification of five cell categories representing different receptor distribution between nucleus and cytoplasm as indicated in the figure. The "total" represents the overall fixed number of cells that the experimentalist intends to count

9. *Data Processing and Analysis*

In each experiment, as per statistical requirement, random 100 cells are quantified in each set according to our scoring strategy as mentioned under "cell quantification for receptor localization." Here, for convenience of representation, we suggest two different bar graphs which can be plotted by using the GraphPad prism or Microsoft office (Figs. 2.2 and 2.3).

2.3 Notes

1. *DNA Transfection: Transient* Versus *Stable Protein Expressions*

Protein trafficking can be studied with the endogenous cellular protein if a cell expresses it in a significant amount. Transcription factors are generally expressed at much lower levels or only moderately in some target tissues/cells. In view of this limitation, transient transfections and expression are methods of general choice. Improved transfection efficiency can be achieved in serum-free and antibiotic-free culture conditions that are introduced at least a day before transfection steps. But transient transfection approaches help to express a cellular protein only transiently, sometimes raising concerns of non-uniform or overexpression of a gene within the same cell population. In this context, stably transfected cells express a protein much uniformly without overexpressing the gene or inviting serious concerns [24]. In all the above cases, wild-type or chimeric fluorescent/non-fluorescent protein tags can be conveniently used to suit one's needs.

2. *NLS-Deleted or RFP-Tagged Nuclear Receptors*

Nuclear translocation studies can be easily conducted with those protein factors that have cytoplasmic localization as a starting point. Not all NRs when unliganded are cytoplasmic or predominantly cytoplasmic [23, 44]. For these reasons, constitutive NLS (nuclear localization signal) of NRs may be deleted for retention in the cytoplasm. Subsequently, the treatment with an activating ligand will help induce nuclear translocation of the receptor. In another approach, the application of a red fluorescent protein (DsRed-express) tagging to some NRs were observed to preferentially shift the nuclear protein towards the cytoplasm side [50]. This cytoplasmic shifted protein expressed in ligand-free conditions could then be subsequently activated to shift into the nuclear compartment [50]. Fluorescent protein-tagged NR constructs can be easily generated, procured as a gift or obtained from a non-profit company Addgene (Watertown, Massachusetts, USA).

3. *Nuclear Translocation: Detection in Fixed Cells* Versus *Live Cell Microscopy*

Several standard cell fixations and permeabilization methods are currently adapted by different laboratories for subcellular localization of a protein by immunocytochemistry analysis. However, some limitations or artifacts may be encountered depending on the cell type, fixative reagents, nature of antigen, and quality of primary and secondary antibodies employed for detection. Currently, the use of chimeras generated with fluorescent proteins tags serve as excellent tools for the standard implementation of receptor investigation and localization by fluorescence microscopy. An excellent review on this central topic has been elaborately compiled by Schnell et al. (2012) [17].

4. *Buffering of Live Cells During Microscopic Analysis*

Not all microscopes are equipped with an inbuilt chamber providing culture conditions, while cells are being examined for imaging and quantitation. Observing the same set of cells for a prolonged period may alter the optimum pH and temperature of the cell media. To stabilize the pH changes, it is advisable to add a calculated amount of sterile HEPES buffer from a 1.0M stock buffer (pH 7.4) to achieve the final concentration of 10 mM.

5. *A Point of Consideration When Viewing Cells for Protein Localization*

An adherent cell manifests different appearance when viewed from top vis-à-vis laterally. Even when a protein is uniformly distributed throughout the cell, it may appear to be more concentrated in the nuclear compartment when viewed from the top. This is due to the fact that the depth of field in the nuclear region is more than the exclusive cytoplasmic regions. This point should be considered when assessing the subcellular localization of a protein distributed in these two cell compartments (Fig. 2.5). In addition, when performing optical sectioning of a cell, the localization of a protein may not be accurately predicted until all the optical sections are considered for the holistic image.

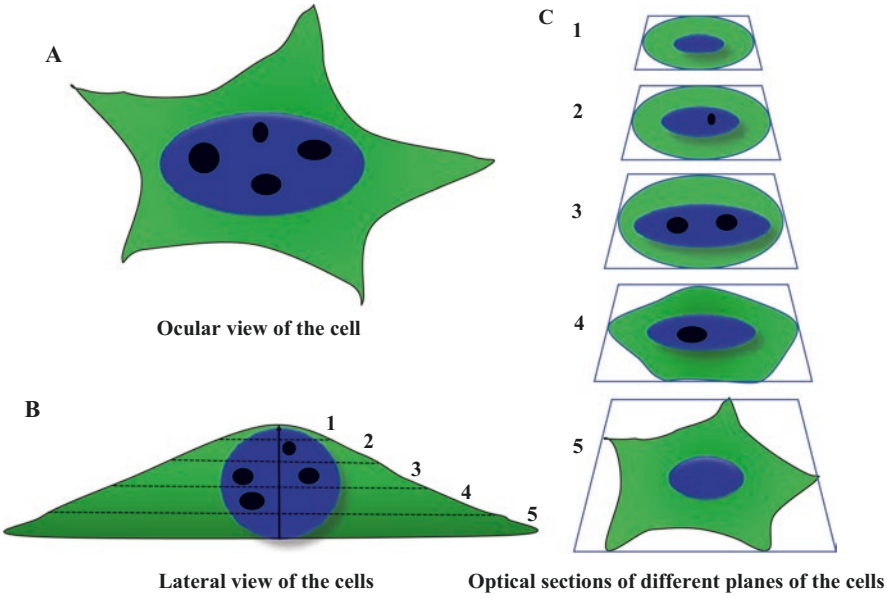

Fig. 2.5 Optical sectioning and ocular viewing of a cell. (**a**) Microscopic top view of the cell. (**b**) Lateral view of a cell showing planes of optical sectioning. (**c**) Optical sections from different planes [1–5] derived from image (**b**) are shown

6. *Miscellaneous Notes*

Standard mammalian culture facility and fluorescence microscope imaging facility will be essential to conduct the studies discussed herein. When imaging fluorescent live cells, it is preferred that water immersion objectives are attached to a dedicated fluorescence microscope. The quality of epifluorescence-based imaging is generally superior by these water immersion objectives.

Hoechst 33258 (0.5 μg/ml) and DAPI (0.1 μg/ml) are commonly used counterstain for viewing cell DNA/nuclei. DAPI is generally used with fixed cells as it is less permeable through cell membrane, while Hoechst is used for the live cells due to its high membrane permeability. Hoechst dye is also less toxic than DAPI and allows staining and visualization of live cells for a prolonged period.

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Disclaimer The cell images shown are prepared exclusively for representation purposes.

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Chapter 3
Multifaceted Effects of Ligand on Nuclear Receptor Mobility

Lizabeth A. Allison and Vincent R. Roggero

Abstract Members of the nuclear receptor superfamily function as both intracellular receptors and transcription factors, modulating transcription of target genes in response to hormone. In the classical model of steroid hormone action, after crossing the plasma membrane hormone binds to cytoplasmic pools of receptor that are then rapidly translocated to the nucleus to facilitate gene transcription. Extensive studies since then have revealed a far more complicated story. The nuclear receptors are remarkably dynamic proteins that can undergo rapid nucleocytoplasmic shuttling in the presence or absence of hormone. They display a diversity of distributions within the cell, showing variation in transport mechanisms between the cytoplasmic and nuclear compartments and in their intranuclear dynamics. This chapter highlights key features of three main categories of intracellular localization into which the nuclear receptors can be roughly sorted: ligand-dependent nuclear accumulation, ligand-dependent intranuclear localization, and ligand-independent trafficking.

Keywords Nuclear receptors · Nucleocytoplasmic shuttling · Intranuclear dynamics · Nuclear import · Nuclear export · Steroid hormones · Thyroid hormone

3.1 Introduction

Members of the nuclear receptor superfamily are both intracellular receptors and transcription factors. As such, they are able to bypass the elaborate second messenger signaling pathways and phosphorylation cascades of cell surface receptors. Their mode of action at first glance may appear a model of simplicity. Yet,

L. A. Allison (✉) · V. R. Roggero
Department of Biology, William & Mary, Williamsburg, VA, USA
e-mail: laalli@wm.edu

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underlying this perceived simplicity is an intricate complexity. In essence, the nuclear receptors act as highly abbreviated signaling pathways. When ligand enters the cell from the surrounding environment, nuclear receptors directly regulate ligand-dependent transcription of target genes linked to a wide array of biological processes, including metabolism, cell proliferation, reproduction, development, and the inflammatory response. Cloning of the glucocorticoid receptor (GR) cDNA in 1985 was followed closely by cloning of cDNAs encoding the estrogen receptor (ER) and the thyroid hormone receptor (TR) [1–7]. The field has since exploded, with 48 members of the nuclear receptor superfamily identified in humans alone [8, 9]. The ligands for nuclear receptors are equally diverse, including endogenous hormones, metabolites, drugs, and xenobiotics (e.g., herbicides and plasticizers). Adding to this complexity, the response to ligand is less straightforward than originally thought, and it is now understood that unliganded receptors play critical roles in both the regulation of gene activation and repression.

Despite their diversity, the nuclear receptors share characteristic structural features which define the superfamily. With the exception of the atypical receptors SHP (small heterodimer partner) and DAX (dosage-sensitive sex-reversal adrenal hypoplasia congenital critical region on the X chromosome gene 1), almost all members of the nuclear receptor superfamily share a common modular domain structure, with five domains, labeled A-E [10, 11] (Fig. 3.1). The A/B domain is a disordered, poorly conserved N-terminal region that houses a ligand-independent transcriptional activation function surface, termed AF-1. The DNA-binding domain (DBD), or C domain, is the most conserved of the domains. The DBD consists of two zinc fingers important for receptor dimerization and DNA binding. The D domain, also referred to as the hinge region, is a short flexible linker with the least sequence and size conservation, although it harbors a conserved nuclear localization signal (NLS) in some receptors [12]. The C-terminal ligand-binding domain (LBD), or E domain, is a structurally conserved domain that commonly contains 11 α-helices and four β-strands that fold into a bundle, forming a hydrophobic ligand-binding pocket. Helix 12 houses the ligand-dependent AF-2 surface, which interacts with transcriptional coregulator proteins. The LBD recognizes specific ligands that are lipophilic molecules, such as steroid hormones, vitamin D, thyroid hormones, and fatty acids. Finally, there is a highly variable C-terminal region, absent in several nuclear receptors, that is sometimes designated as the F domain.

Fig. 3.1 Schematic representation of nuclear receptor domain structure. A typical nuclear receptor contains five structural and functional domains, labeled A-E. Key domain functions are depicted above and below the diagram. NLS, nuclear localization signal; NES, nuclear export signal; AF-1 and AF-2, activation function domain-1 and domain-2, respectively

Nuclear receptors modulate transcription through many distinct mechanisms. In most cases, ligand binding stabilizes an active state, in which nuclear receptors recruit coactivator proteins that then recruit histone modifying enzymes, such as histone acetyltransferases (HATs) that facilitate the opening of chromatin, making it accessible to the transcription machinery [13]. Nuclear receptors also can repress transcription by binding corepressors in the unliganded state, such as histone deacetylases (HDACs) that facilitate the closing of chromatin, blocking the transcription machinery from accessing the DNA. Alternatively, nuclear receptors can interact with "negative" DNA response elements. In this mode of action, the nuclear receptor adopts a conformation distinct from the conformation when bound to a "positive" DNA response element and facilitates corepressor recruitment to block transcription [14, 15]. In order to modulate gene expression, after synthesis in the cytosol, precise targeting to their ultimate destination in the cell is essential for nuclear receptor function.

Categorizing the diversity of nuclear receptors is akin to sorting LEGO® bricks into bins. Should they be sorted by color, shape, or function? How does one deal with the complexity of curvy bricks? If each subtle difference is taken into account a sorting solution might need multiple bins for what overall is a limited number of bricks (Fig. 3.2). Reflecting this conundrum, the nuclear receptor superfamily is not only divided into seven subfamilies based on structural characteristics and the type of ligand [11] but also classified into four mechanistic subtypes, based on dimerization properties and intracellular localization (Table 3.1). One of the hallmarks of

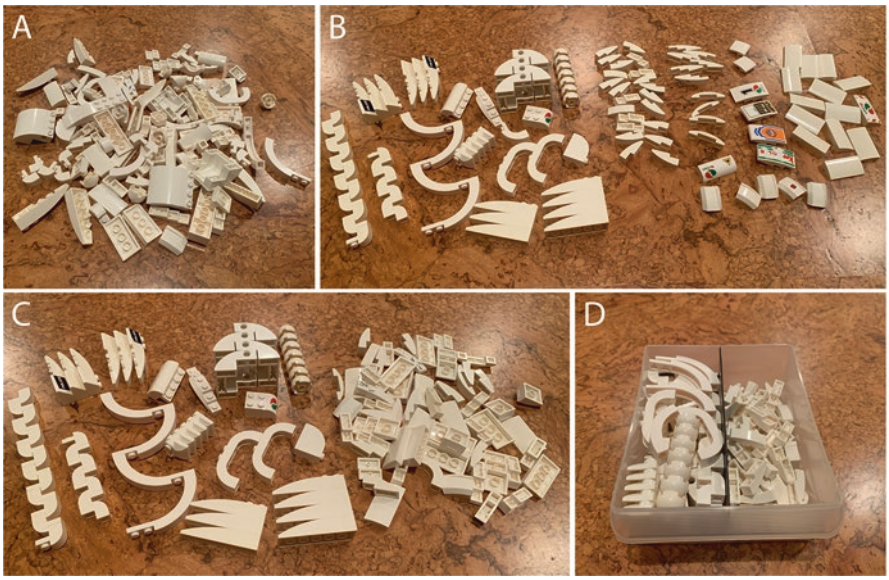

Fig. 3.2 Categorizing nuclear receptors is analogous to sorting LEGO® bricks into bins. (**a**) A one-bin sorting scheme for white, curvy bricks. (**b**, **c**) Alternative sorting schemes based on functional characteristics. (**d**) A two-bin sorting scheme. (Photo courtesy of Andrew C. A. Levine)

Table 3.1 Comparison of nuclear transport mechanisms of select members of the nuclear receptor superfamily

Subfamily	Mechanistic subtype	Nuclear receptor	Ligand	Dimerization	Predominant cellular localization of the unliganded receptor
3C	Type I	Glucocorticoid receptor (GR)	Glucocorticoids	Homodimer	Cytoplasm
3C	Type I	Mineralocorticoid receptor (MR)	Mineralocorticoids (aldosterone) and glucocorticoids	Homodimer	Cytoplasm
3C	Type I	Androgen receptor (AR)	Androgens (testosterone)	Homodimer	Cytoplasm
3A	Type I	Estrogen receptors (ERα, ERβ)	Estrogens	Homodimer	Nucleus
1A	Type II	Thyroid hormone receptors (TRα, TRβ)	Thyroid hormones	Heterodimer	Nucleus
2B	Type II	Retinoid X receptors (RXRα, RXRβ, RXRγ)	9-*cis* retinoic acid	Heterodimer	Nucleus
1B	Type II	Retinoic acid receptors (RARα, RARβ, RARγ)	Retinoic acid	Heterodimer	Nucleus
1I	Type III	Vitamin D receptor (VDR)	1,25-dihydroxy vitamin D3	Heterodimer	Nucleus

eukaryotic cells is the compartmentalization of the genome into the nucleus, a separate membrane-bound organelle. Trafficking of nuclear receptors into and out of the nucleus represents an additional level of transcriptional regulation beyond DNA-protein interactions. This chapter focuses on select members within subfamilies 1, 2, and 3 of the nuclear receptor superfamily that have well-studied nucleocytoplasmic shuttling characteristics and intranuclear dynamics in response to ligand. The following questions are addressed: Are unliganded receptors localized in the cytoplasmic "bin" or the nuclear "bin" within a cell? Are there additional "bins" within the nuclear space? What effect does ligand have on receptor localization?

3.2 Nucleocytoplasmic Shuttling of the Nuclear Receptors

The members of the nuclear receptor superfamily are remarkably dynamic proteins that display a diversity of distributions within the cell and variation in transport mechanisms between the cytoplasmic and nuclear compartments (Table 3.1). In the classical model of steroid hormone action, after crossing the plasma membrane,

hormone binds to cytoplasmic pools of receptor that are then rapidly translocated to the nucleus. Once in the nucleus, the liganded receptor associates with specific DNA regulatory sequences and activates or represses target gene transcription. With the discovery in the early 1980s that unliganded nuclear receptors can be distributed in the cytoplasm and nucleus, it became clear that this simple model did not fit all nuclear receptors [16–21]. In addition, extranuclear signaling pathways have been proposed for some of the nuclear receptors [2], but these nongenomic effects are outside the scope of this chapter.

Regardless of their primary localization at steady-state, it is now clear that members of the nuclear receptor superfamily undergo rapid shuttling between the cytoplasm and the nucleus [22–26]. While the biological significance of shuttling is not always readily apparent, what has emerged is that the fine balance among nuclear import, nuclear retention, and nuclear export of nuclear receptors is a critical control point for modulating hormone-responsive gene expression, and potentially for inducing extranuclear signaling pathways [27, 28]. Given their structural and functional diversity, it should come as no surprise that there is no general mechanism for nucleocytoplasmic shuttling of the nuclear receptors. Instead, there are multiple pathways for both nuclear import and export. How is receptor nuclear localization achieved?

3.2.1 Nuclear Pore Complexes: Gatekeepers of the Nucleus

Nuclear receptors, like other macromolecules, cross the nuclear envelope, the double membrane that surrounds the nucleus, through the nuclear pore complexes (NPCs). The NPCs are intricate protein assemblages of approximately 120 MDa with a central channel that acts as a "gatekeeper" for nuclear entry [29, 30] (Fig. 3.3). This selective barrier, generated by proteins called nucleoporins that contain intrinsically disordered phenylalanine-glycine (FG) repeats, allows diffusion of small molecules and ions, but hinders diffusion of proteins greater than 3 nm in diameter and ~40 kDa in size [31]. Proteins destined for the nucleus, even those as large as 40 nm in diameter, can be rapidly transported through this mesh-like barrier by a temperature- and energy-dependent mechanism. Nuclear transport is mediated by members of the family of evolutionarily conserved karyopherin β-like transport factors, 20 of which have been identified so far in humans. Karyopherins weakly bind to the FG repeats in the cytoplasmic filaments of the NPC and facilitate translocation through the central channel. Karyopherins that import proteins into the nucleus are called importins, whereas those that export proteins back to the cytoplasm are called exportins [32, 33], with each member performing a distinct nuclear import, export, or bidirectional transport function [32, 34–41]. Most nuclear proteins contain nuclear localization signals (NLSs) that direct binding with importins, and they also may contain nuclear export signals (NESs) that direct nuclear export by interacting with exportins [41–44]. Transport is driven by the asymmetrical distribution of the small GTPase Ran in either its GTP or GDP bound state. A high nuclear

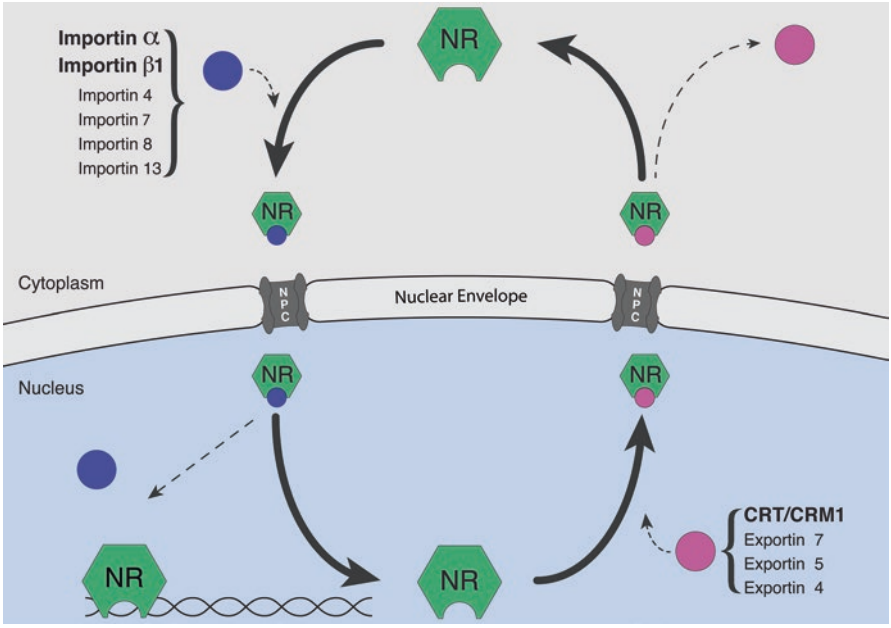

Fig. 3.3 Nuclear import and export pathways of nuclear receptors. Nuclear receptors bind to specific importins in the cytoplasm, as indicated. The NR-importin complex passes through a nuclear pore complex (NPC) embedded in the nuclear envelope into the nucleus, where the complex is disassembled and the NR binds to target genes. The NR exits the nucleus through the NPC in association with specific exportins or calreticulin (CRT)/CRM1. The most commonly used importins and exportins are in bold type

RanGTP concentration is required for dissociation of import complexes in the nucleus and for assembly of export complexes that will exit the nucleus [45].

3.2.2 Nuclear Import Pathways

In the classical import pathway, used by many of the nuclear receptors, importin β1 interacts with one of seven adaptor proteins from the importin α family, which binds directly to a classical lysine-arginine-rich NLS within the protein, while importin β1 interacts with the nucleoporins [46] (Fig. 3.3). Nuclear receptors typically have more than one NLS, as is the case for the estrogen and progesterone receptors where three NLSs have been defined, one in the hinge domain and two in the LBD [47–50]. TRα1 harbors two NLSs, NLS-1 in the hinge domain and NLS-2 in the A/B domain, while TRβ1 only contains NLS-1 [12]. NLS-1 and NLS-2 in TRα1 both interact with the importin α1/β1 heterodimer, while NLS-2 also interacts with importin 7 [27, 51]. As another example, the mineralocorticoid receptor (MR) and the androgen receptor (AR) contain three NLSs: NLS0 in the N-terminal A/B

domain, a bipartite NLS1 in the DBD/hinge region, and a ligand-dependent NLS2 in the LBD [52–55]. NLS1 in MR and AR is recognized by the importin α/β1 heterodimer. Interestingly, importin 7 regulates AR in a counterintuitive way: it binds to AR, masks NLS1, and inhibits nuclear import in the absence of ligand. Androgen binding then induces a switch that promotes exchange of importin 7 for the importin α/β1 heterodimer, which mediates nuclear translocation [56]. In this case, ligand binding causes a conformational change in AR, unmasking both the dimerization domain and the NLS that allows nuclear import [57].

Nuclear entry of nuclear receptors is also mediated by other members of the karyopherin family. For example, GR has two NLSs, NL1 in the hinge domain and NL2 in the LBD [58], both of which are ligand-inducible, and can follow multiple pathways through the NPCs. Importin-α/β1 heterodimers, importin 7, importin 8, and importin 13 have all been shown to mediate nuclear import of GR [59, 60]. In contrast, nuclear import of the vitamin D receptor (VDR) and the retinoid X receptor (RXR) follows another pathway, mediated by importin 4 [61–63].

3.2.3 Nuclear Export Pathways

The predominant pathways for nuclear export of nuclear receptors involve CRM1 (Chromosome Region Maintenance 1), also known as exportin 1, and the calcium-binding protein calreticulin (CRT) [64, 65] (Fig. 3.3). Some nuclear receptors are exported by CRM1, some by CRT, and some by both [66, 67]. CRM1 recognizes the canonical, 8–15 amino acid NES that includes 4–5 hydrophobic residues rich in leucine [68]. Nuclear export signals, in general, have proven to be difficult to fully define as both necessary and sufficient for exit from the nucleus. However, noncanonical sequences in the LBD and DBD of nuclear receptors have been shown to act as NES-like sequences that can be recognized by CRM-1 and other exportins. Illustrating the complexity of nuclear export, rapid GR export is mediated by CRT via binding to a NES in the DBD [64, 65], but it is suggested that there is another slower export pathway mediated by CRM1 [69]. Upon withdrawal of ligand, the GR-DNA complex dissociates and is thought to complex with hsp90 and p23, which facilitate nuclear export [66, 70]. In addition, a “nuclear retention signal” has been identified in the hinge region of GR that overlaps with NLS1 and slows down nuclear export mediated by CRT. In this way, GR transcriptional activity is enhanced by its increased nuclear accumulation [71]. As another example, upon steroid withdrawal, MR export is very slow, remaining present in the nucleus after 16 hours, a time frame in which GR would be exported. Originally this observation suggested that transport of MR might be unidirectional [54]; however, there appears to be a conserved CRM1-independent NES in the LBD, and MR may also use the DBD as an NES [54, 72, 73].

Nuclear export is also mediated by other members of the karyopherin family. For example, although nuclear export of AR is mediated in part by CRT, using the DBD as an NES [64], exportin 5 also can stimulate AR export [74]. Moreover,

exemplifying the interplay between functional domains, a CRM1-independent NES in the LBD that is necessary for AR nuclear export, is dominant over the NLS in the DBD/hinge region in the absence of ligand. When hormone is present, the NES is repressed, and the NLS directs nuclear localization of AR. This ligand-regulated NES is also present in MR and ERα [73], but it also has been proposed that the nuclear export of ERα is mediated via its DBD [64]. Similarly, nuclear export of the retinoic acid receptor α (RARα) and RXR are proposed to be mediated via their DBDs [64]. Highlighting the multiple pathways out of the nucleus, TRα1 export is mediated by multiple NESs, including at least two CRM1-independent NESs in the LBD (NES-H3/H6 and NES-H12) and an unidentified CRM1/CRT-dependent NES [12]. Export can follow multiple pathways, mediated by CRM1/CRT, exportin 4, exportin 5, and exportin 7 [28, 51, 75].

3.2.4 Dynamics of Movement Within the Nucleus

Even within the nucleus, transcriptional events do not occur homogeneously across the nuclear space. Transcription may be limited to defined spatial regions which are not physically separated by membranes, including phase-separated nuclear condensates that concentrate macromolecules, such as RNA polymerase II and mediator subunit 1 (MED1) [76, 77]. Current models propose that dynamic exchange of nuclear receptors and other proteins, including pioneer factors, drives increased chromatin accessibility and transactivation. Intrinsically disordered transactivation domains are thought to play a key role in the assembly of highly dynamic transcription factor signaling hubs [78–82]. Transcription factor mobility is a key determinant in gene expression regulation, corresponding to transcriptional fine-tuning. How is receptor function modulated by its localization and exchange between nuclear subcompartments? Visualizing intranuclear organization in living cells in real-time has offered an intimate glimpse into the trafficking of nuclear receptors across the nuclear landscape, their dynamic and stochastic assembly into transcriptional complexes, and how they reach their recognition sites in a vast array of DNA [83, 84].

Major strides in quantifying the dynamics of protein shuttling kinetics in live cells have been made since the development of fluorescence recovery after photobleaching (FRAP) [85]. FRAP has been instrumental, for example, in our examination of TR nucleocytoplasmic shuttling, where recovery of a completely bleached nucleus in a cell with multiple nuclei occurs within 60–120 minutes [28, 75]. Early on, intranuclear FRAP experiments revealed that most nuclear proteins are highly mobile and the interaction of proteins with chromatin and nuclear compartments is highly dynamic. After photobleaching of a region of interest in the nucleus, many transcription factors exhibit complete recoveries within seconds [86], indicating that they can diffuse throughout the entire nucleus and are immobilized to nuclear structures only transiently [87]. Even such transient interactions, however, are

sufficient to promote large-scale remodeling of chromatin lasting a few hours and correlate with transcriptional activation.

Prior to acting as a transcription factor within the nucleus, however, each type of nuclear receptor begins its journey in the cytoplasm, after synthesis on free, cytosolic ribosomes. Three main intracellular localization "bins" can be used to roughly sort members of the nuclear receptor superfamily according to how their journey unfolds: ligand-dependent nuclear accumulation, ligand-dependent intranuclear localization, and ligand-independent trafficking (Fig. 3.4).

3.3 Ligand-Dependent Nuclear Accumulation of Nuclear Receptors

GR, MR, and AR all primarily localize in the cytoplasm in the absence of ligand [88]. As early as the 1980s to 1990s, however, it was recognized that a small proportion of unliganded receptors could be found in the nucleus [21, 53, 89–94] (Fig. 3.4a). After ligand binding, the cytoplasmic receptors rapidly translocate into the nucleus and accumulate fully within 30–60 minutes. On the other hand, the unliganded progesterone receptor (PR) has a subtype-specific subcellular distribution that spans two "bins," with the A form predominantly in the nucleus and the B form in both the nucleus and cytoplasm [95–98]. In endometrial cancer cells, PR-B is principally cytoplasmic and becomes enriched inside the nucleus in response to ligand [99]. How does this rapid ligand-dependent translocation occur?

Soluble proteins can move by simple diffusion in the cytoplasm, but the crowded cellular environment needs to be taken into account. Protein movement may be impeded by the highly organized cytoskeletal filaments and the close packing of other macromolecules. Is random movement rapid enough for cell signaling? A more direct mechanism for soluble protein movement to the nucleus is directed retrograde movement along the microtubules using molecular motor proteins. In the next sections, the routes to the nucleus employed by GR, MR, and AR will be examined.

3.3.1 Glucocorticoid Receptor Nuclear Accumulation and Intranuclear Dynamics

The glucocorticoid receptor regulates a wide variety of processes, including cell proliferation, metabolic homeostasis, and aspects of development and reproduction and also contributes to the stress, inflammatory, and immune responses [66, 100–102]. In the classical model, nuclear import of ligand-bound GR is initiated by dissociation of the chaperone complex in the cytoplasm (Fig. 3.4a). However, it turns out that both ligand-bound and unbound forms of GR can shuttle between the

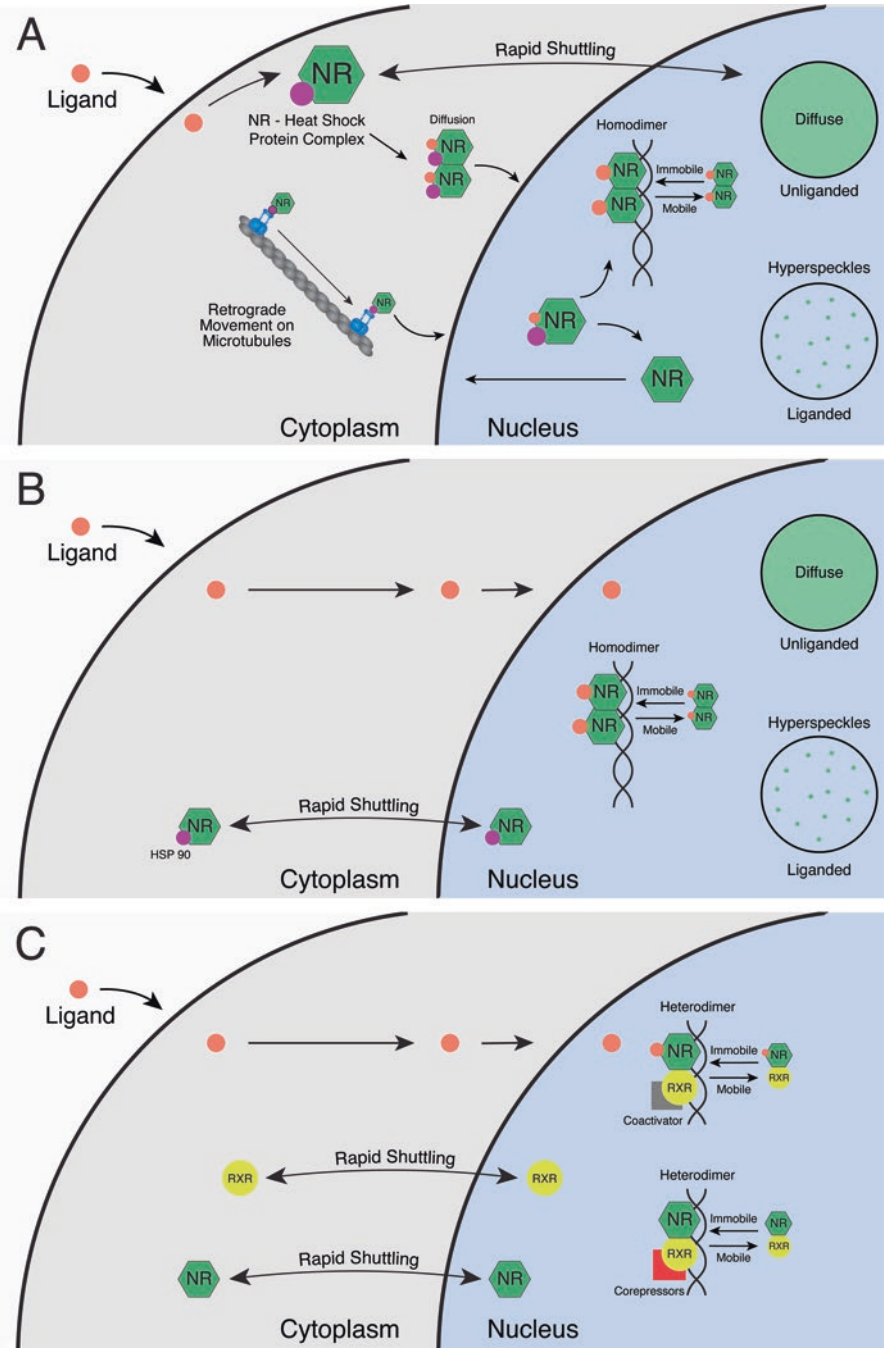

Fig. 3.4 Models of the three main intracellular localization "bins" for nuclear receptors. (**a**) Ligand-dependent nuclear accumulation is exemplified by the glucocorticoid receptor, mineralocorticoid receptor, and androgen receptor. (**b**) Ligand-dependent intranuclear localization is exemplified by the estrogen receptor. (**c**) Ligand-independent trafficking is exemplified by the thyroid hormone receptor and retinoic acid receptor. A detailed description of each model is provided in the text

nucleus and cytoplasm [103]. The distinction is that nuclear *accumulation* is ligand-dependent for nuclear receptors in this "bin," although shuttling is ligand-independent. This distinction suggests that the relative rate of import versus export in the presence or absence of ligand is what determines the distribution pattern of GR.

3.3.1.1 GR Nuclear Accumulation

In its inactive state, GR is unbound by ligand and mainly resides in the cytoplasm in a multimeric chaperone complex composed of heat shock protein 90 (hsp90), hsp70, hsp90-binding protein p23, immunophilins, and other factors [104, 105]. Heat shock proteins are rapidly synthesized by eukaryotic cells in response to stress, although some members of the heat shock protein family are expressed normally within cells and are involved in protein folding, assembly, and transport [106]. This ATP-dependent chaperone complex aids in folding of GR into a conformation with high affinity for ligand. Activation of GR and intranuclear accumulation can be induced by natural glucocorticoids such as cortisol, by synthetic glucocorticoids such as dexamethasone, but also by antagonists of GR-mediated gene expression, such as RU486 [107]. In an interesting twist, Italian Espresso extract has been shown to block the conversion of inactive cortisone to active cortisol by 11β-hydroxysteroid dehydrogenase type 1 (HSD1). This inhibitory effect prevents nuclear accumulation of GR and, as a consequence, prevents transcriptional activation [108]. Consumption of both caffeinated and decaffeinated coffee is associated with reduced risk for the development of type II diabetes [109]. Although it is not clear what compound in coffee is responsible for the observed effects, these findings point to the intriguing possibility of a link between GR mislocalization and pathogenesis.

It was once thought that dissociation of the chaperone complex was the first step in GR nuclear import. However, experimental evidence is consistent with a model in which the chaperone machinery is not left behind in the cytoplasm, but instead is required for rapid and efficient nuclear import of GR and for interaction with the NPC [26]. Hsp90, p23, the immunophilin FKBP52, dynamitin, and dynein together assist in facilitating the retrograde movement of GR along the microtubules en route to the nucleus [110–112]. NLS1 near the DBD/hinge boundary of GR has been shown to mediate rapid nuclear import of GR ($t_{1/2}$ = 4–6 minutes), by retrograde movement along microtubules. In contrast, NLS2 in the LBD mediates import that is much slower and less efficient ($t_{1/2}$ = 45–60 minutes), likely occurring by diffusion [58, 113, 114] (Fig. 3.4a).

3.3.1.2 GR Intranuclear Dynamics

Once in the nucleus, ligand-activated GR functions as a homodimer that binds to positive or negative glucocorticoid response elements (GREs) in target genes, leading to transcription activation or repression, respectively [107]. Alternatively, GR

can act as a monomer that cooperates with other transcription factors, such as AP-1 and NK-κB, to activate or repress transcription, by modulating activity of those transcription factors without direct binding to DNA. Earlier studies have shown that receptor mobility is dependent on ligand [87, 115, 116]. For example, in baby hamster kidney (BHK) fibroblast cells, ligand-activation induces colocalization of mCherry-GR and GFP-NCoA-2 (nuclear receptor coactivator 2) to a large number of intranuclear foci, or "hyperspeckles" [117]. These nuclear foci rely on the integrity of DNA, but the biological relevance of these structures is still under debate. This highly punctate distribution of GR within the nucleus has been shown to occur in the presence of high-affinity ligands, whether agonists or antagonists, suggesting that GR intranuclear distribution depends on affinity-based differences between ligands, as opposed to transcriptional activity alone. In support of this model, although GR localizes to the nucleus in the presence of low-affinity agonists and antagonists, it distributes homogeneously under these conditions [107].

Depending on the technique used, various populations of GR in the nucleus have been described. As an example, an experiment that used single-molecule tracking-based measurements to quantify dwell time and the fraction of GR molecules on target DNA in live cells, showed that approximately half of ligand-bound GR is freely diffusing, while the remaining population is bound to DNA, either for short periods of time (~0.7 seconds) or for longer time periods (~2.3 seconds) [87]. Other studies using simultaneous tracking of GFP-tagged RNA polymerase II revealed that approximately 10% of GR resides at sites with active transcription. The dwell time of GR at these sites was approximately 10 seconds, basically showing the GR-chromatin association is dominated by transient interactions of small populations of approximately 5–10% of the receptor on chromatin for only short periods of times (less than a millisecond) [118–120].

Fluorescence correlation spectroscopy (FCS) studies, which collect information on faster interactions compared with single molecule tracking methods, provide another view of the mobility of ligand-activated GR. FCS data suggest that the diffusing fraction of GR represents 45–65% of the total GFP-GR population, while the remaining population is engaged in shorter-lived (lifetime of 20–60 milliseconds) and longer-lived (lifetime of 200–500 milliseconds) interactions, including association with chromatin targets, DNA-dependent foci, and NCoA-2 nuclear foci. These proportions vary with the amount of ligand and appear to depend on receptor conformation [107]. Another recent study combined use of FRAP, which has better resolution over longer periods of time (i.e., seconds) and single-molecule microscopy, which has better resolution over shorter time periods (i.e., milliseconds), to further distinguish between diffusing and immobile states of GR [121]. In this study, YFP-GR expressed in COS-1 cells was shown to diffuse rapidly through the nucleus at an impressive rate of 3.1 $\mu m^2/s$, likely as a dimer or in complexes with other proteins. In this state, liganded GR can potentially search large areas of the nucleus for GREs. Once it leaves this free diffusion state after >10 seconds, data suggest that GR enters a repetitive switching mode where it is proposed to alternate between slow diffusion (0.5 $\mu m^2/s$) as a result of brief, nonspecific DNA-binding events that include short sections of sliding, hopping, and intersegmental exchange and a short

immobile state (~0.6 seconds) which may represent interactions with DNA mediated through the N-terminal domain or LBD via "tethering" with other protein partners, such as AP-1. Finally, a long immobile state (~2.9 seconds) is proposed to depend entirely on the DNA-binding capacity of the receptor and to represent direct specific DNA binding by GR to a GRE [121].

3.3.2 Mineralocorticoid Receptor Nuclear Accumulation and Intranuclear Dynamics

The mineralocorticoid receptor (MR), also known as the aldosterone receptor, plays a key role in sodium transport in renal cells when bound to aldosterone. However, MRs are found in varying abundance in both epithelial and nonepithelial tissues and have multiple roles in cardiovascular function, immune cell signaling, neuronal fate, and adipocyte differentiation [122, 123]. In addition, MRs have equivalent affinity for the glucocorticoids, cortisol, and corticosterone. Like GR, MR resides primarily in the cytoplasm in the absence of ligand (Fig. 3.4a).

3.3.2.1 MR Nuclear Accumulation

MR is kept in a complex with hsp90 in a high-affinity conformation ready for ligand-binding. When MR binds ligand, the chaperone complex undergoes an immunophilin switch. FKBP51 is replaced by FKBP52, which forms the connecting partner between the MR-hsp90 complex and the microtubule network. Recent studies suggest that hsp90 does not dissociate from MR directly after binding ligand, but is still attached when MR enters the nucleus [124, 125].

3.3.2.2 MR Intranuclear Dynamics

As for GR, rapid dynamics of nuclear import and recruitment of coactivators have been observed for ligand-bound MR [90]. MR binds cortisol with high affinity, about tenfold higher than GR. In the hippocampus, for example, MR is activated at low concentrations of cortisol, whereas GR is only activated at high concentrations. MR has been shown to accumulate in the nucleus faster than GR in the presence of 10^{-9} M cortisol; in contrast, no significant difference was shown in accumulation rate in the presence of 10^{-6} M cortisol [126]. In a comparative FRAP study, liganded GFP-tagged GR and MR both showed a high degree of colocalization and mobility in the nuclei of transiently transfected, cultured hippocampal neurons [126]. The half-time for recovery of GR was longer than that of MR in the presence of 10^{-6} M cortisol (4.5 vs 3.7 seconds) but shorter (2.4 vs 4.2 seconds) in the presence of 10^{-9} M cortisol. Interestingly, inhibition of the proteasome increases the nuclear

accumulation of GR and reduces its mobility within the nucleus, in a similar manner to other steroid receptors such as ER, AR, and PR [127–130]. These findings suggest that there is a direct link between proteasome activity, receptor turnover, and transcriptional regulation.

3.3.3 Androgen Receptor Nuclear Accumulation and Intranuclear Dynamics

Androgen receptor-regulated genes are involved in the development and maintenance of the male phenotype. Like GR and MR, unliganded AR resides primarily in the cytoplasm. Comparable to MR, AR is sequestered in a cytoplasmic complex with hsp90 in a high-affinity conformation that is poised for ligand binding.

3.3.3.1 Androgen Receptor Nuclear Accumulation

Upon androgen binding, the chaperone complex undergoes a switch from the immunophilin FKBP51 to FKBP52, allowing the AR-hsp90 complex to associate with the microtubule network. Dynein has been shown to mediate AR trafficking to the nucleus. Further, treatment with taxane, which binds to microtubules and prevents their disassembly, results in cytoplasmic sequestration of AR, implicating microtubules in the shuttling of AR from the cytoplasm to the nucleus [131]. However, more recent studies using multimodal image correlation spectroscopy (mICS) in both HeLa cells and a prostate cancer cell line (PC3) revealed that the majority of GFP-AR is not bound to microtubules and is likely free to diffuse throughout the cytoplasm. These findings suggest that the probability of AR crossing the nuclear membrane by cytoplasmic diffusion is an important factor in determining AR distribution between the cytoplasm and nucleus, independent of retrograde transport on the microtubules [132].

3.3.3.2 Androgen Receptor Intranuclear Dynamics

After ligand-dependent translocation into the nucleus, within 20–30 minutes, AR forms intranuclear foci, or hyperspeckles [132–135]. Both agonists and antagonists promote nuclear translocation of AR, but only agonists appear to cause GFP-AR to form these discrete foci that colocalize with coactivators [134]. For example, within minutes of addition of agonist to HeLa cells, cytoplasmic GFP-AR was imported into the nucleus, where it displayed a hyperspeckled pattern that also correlated with decreased mobility [136]. Nuclear accumulation of AR was readily apparent after 10 minutes and complete by 30 minutes. In contrast, antagonist-bound AR had a more diffuse intranuclear distribution, consistent with a lack of transcriptional

activity. Further, nuclear import was shown to be slower for antagonist-bound AR, only reaching partial nuclear accumulation after 30 minutes, and import remained incomplete after 90 minutes. In this study, FRAP showed that agonist-bound AR had reduced mobility relative to unliganded or antagonist-bound AR (half-maximal recovery of ~2.5 seconds in the absence of ligand and ~5.5 seconds in the presence of agonist) [136]. Further only agonist-bound CFP-AR was shown to colocalize with YFP-CBP (CREB binding protein), and CBP mobility was comparable to AR mobility.

Notably, amino acid substitutions in the first zinc finger motif of the AR-DBD that are associated with two patients with androgen insensitivity syndrome lead to receptor mislocalization in transfected cells. AR-DBD mutants were shown to initially form large cytoplasmic dots. After the addition of dihydrotestosterone, a proportion of the proteins moved into the nucleus and localized in larger foci compared to wild-type AR [135]. FRAP analysis revealed significantly reduced intranuclear mobility of the mutants compared to wild-type AR. These findings suggest that mislocalization and lower mobility of the mutant ARs may contribute to pathogenesis of androgen insensitivity syndrome.

3.4 Ligand-Dependent Intranuclear Localization

In contrast to GR, MR, and AR, the estrogen receptors, ERα and ERβ, are primarily distributed in the nucleus, even in the absence of ligand, but they still shuttle rapidly between the nucleus and cytoplasm (Fig. 3.4b). Estrogen is mainly secreted by the ovary and maintains female homeostasis by regulating development of secondary sex characteristics, maintenance of the reproductive cycle, sexually dimorphic behaviors, bone metabolism, and cardiovascular and nervous system protection [137]. Unliganded ER, and PR as well, have been shown to associate with hsp90 in both the nucleus and cytoplasm [25, 138]. Outside the nucleus, a small population of ERα localizes to the plasma membrane [139, 140].

In the unliganded state, ERs are diffusely distributed in the nucleoplasm, and FRAP analysis shows that unliganded GFP-ERα is highly mobile [141]. When ligand-bound, shuttling of ERα decreases, promoting further accumulation in the nucleus [24, 142]. Within minutes of exposure to ligand, as visualized by GFP-tagging of ERα and ERβ, there is formation of hyperspeckles, and recruitment of coactivators [130, 141, 143, 144]. Upon ligand binding, ERα and ERβ form homodimers, bind to a specific estrogen-responsive element (ERE), and regulate expression of target genes. During the receptor activation process, transcriptional cofactors are recruited that form a large protein complex which alters chromatin conformation. Ligand activation-associated interaction with nuclear structures, chromatin, and coregulatory proteins restricts ER mobility [141]. In the transcriptionally active state, the ER dwell time on chromatin is on the order of seconds.

The complexity of ligand-specific mobility patterns of YFP-tagged human ERα in living cells has been analyzed in more detail by diffusion-time distribution

analysis (DDA), a method based on fluorescence correlation spectroscopy (FCS) [84]. In the absence of ligand, YFP-ER was shown to be homogenously distributed and upon addition of the agonist 17β-estradiol (E2), the well-documented hyper-speckled pattern appeared. However, even in the absence of ligand, the population of ER is not completely homogeneous, suggested that there are at least two mobility states, affected by ligand and the availability of DNA binding sites. For example, increasing the concentration of E2 results in a gradual shift toward longer diffusion times, and FCS data show that the average time ERα is found in the bound state is longer on an integrated, multicopy prolactin gene array [142].

Recently, live cell imaging has demonstrated that the antagonist fulvestrant dramatically slows the intranuclear mobility of ER, resulting in transcriptional inhibition of ER. For wild-type mNeon-tagged ER, after a 45-minute treatment with the partial agonist 4-OHT, between 70 and 80% of the original fluorescence intensity was recovered within 5 seconds post-bleach during FRAP. In contrast, cells treated with the full antagonist fulvestrant recovered to only ~50% of the original fluorescence level within 5 seconds and to <70% at the end of the 60-second experiment. These findings reinforce the key role of nuclear receptor intranuclear dynamics in gene transactivation and, more specifically, may have implications for developing treatments for ER-positive breast cancer [145].

3.5 Ligand-Independent Trafficking

TR, RAR, RXR, and VDR are also primarily nuclear, like ER, but ligand-dependent hyperspeckles have not been observed [146, 147] (Fig. 3.4c). What has been observed, however, is that mutants of TRα1 and TRβ1 that mimic nonacetylation adopt a granular, mottled appearance in the nucleus, compared to the characteristic smooth, diffuse pattern of wild-type TRα1 and TRβ1 [148]. This altered distribution pattern correlates with reduced intranuclear mobility and decreased transactivation by the nonacetylation mimics. In addition, VDR, RAR, and TR do not interact with hsp90 [149, 150]. However, studies using fluorescent-tagged ligand to track VDR in living cultured cells showed that VDR localizes to the microtubules [151], and studies using human monocytes and microtubule-disrupting agents suggest that nuclear import of ligand-bound VDR requires an intact microtubule network [152].

3.5.1 Thyroid Hormone Receptor Intracellular Trafficking

By mediating thyroid hormone action in numerous tissues, TRs play key physiological roles in the regulation of many aspects of development, growth, and metabolism [153–155]. Their most well-characterized role is as thyroid hormone-dependent transcription factors; TRs bind thyroid hormone response elements (TREs) in the presence or absence of thyroid hormone to facilitate the expression of target genes,

often as heterodimers with the RXR [153–177]. On positive TREs, corepressors, such as nuclear receptor corepressor 1 (NCoR1) and histone deacetylase, are bound to TR in the absence of ligand, leading to repression of target gene expression [178–180]. Upon thyroid hormone binding, TR undergoes a conformational change, resulting in binding of coactivators such as SRC-1, histone acetyltransferase, and MED1, a subunit of the Mediator complex that functionally bridges TR with the general transcription apparatus [181–183]. Interactions of TR with coactivators lead to changes in chromatin structure and the subsequent transcription of the target gene [24, 184–193].

Although they primarily reside in the nucleus, we and others have shown that the TR subtypes, TRα1 and TRβ1, whether ligand-bound or unbound, shuttle rapidly between the nucleus and cytoplasm [22, 194]. In *Xenopus* (amphibian) oocytes, localization of microinjected ^{35}S-labeled TRα1 to the nucleus was enhanced in the presence of hormone [22], and nuclear localization of GFP-tagged TRβ1 in mammalian cells has also been reported to be enhanced in the presence of thyroid hormone, displaying a shift from 60% to 85% nuclear [195]. However, we have not observed any significant differences between TRα1 nucleocytoplasmic shuttling kinetics in the presence or absence of thyroid hormone in mammalian cells [75, 148]. Although ligand binding does not appear to be obligatory for import of TR, interacting partners such as NCoR1 and RXR [24, 194], and MED1 [146] have been implicated in promoting nuclear retention of unliganded TR. For example, when MED1 was overexpressed, there was a striking shift towards a greater nuclear localization of TRβ1 and the oncoprotein v-ErbA, subtypes with cytosolic populations at steady-state. Consistent with a role for MED1 in nuclear retention, the cytosolic TRα1 and TRβ1 population was significantly greater in MED1−/− knockout cells, compared with wild-type MED1+/+ cells [146].

Using FRAP in transfected cells, we have shown that TR is highly dynamic, with a half maximal recovery time ($t_{1/2}$) of less than 1 second, with up to 99% of TR within the mobile fraction [146, 148] (Fig. 3.5). When MED1 was overexpressed, TRβ1 intranuclear mobility was reduced, whereas for TRα1, there was no observable change in its predominantly nuclear distribution pattern or mobility [146]. Further, exposure to thyroid hormone had no significant effect on TRα1 intranuclear dynamics, and although there was a significant difference in overall recovery rate for TRβ1 in the presence of thyroid hormone, there was no significant difference in the mobile and immobile fraction or $t_{1/2}$. In contrast to the minimal impact of ligand on TR trafficking, we have shown the post-translational modification of TR by acetylation significantly alters its shuttling and nuclear mobility. Nonacetylation correlates with nuclear retention, while acetylation promotes cytosolic localization of TR [148]. In addition, FRAP analysis showed wild-type intranuclear dynamics of the TR acetylation mimic TR, whereas the nonacetylation mimic had significantly reduced mobility and transcriptional activity. Taken together, these findings suggest that post-translational modification of TR may make a greater contribution to TR intracellular trafficking than ligand.

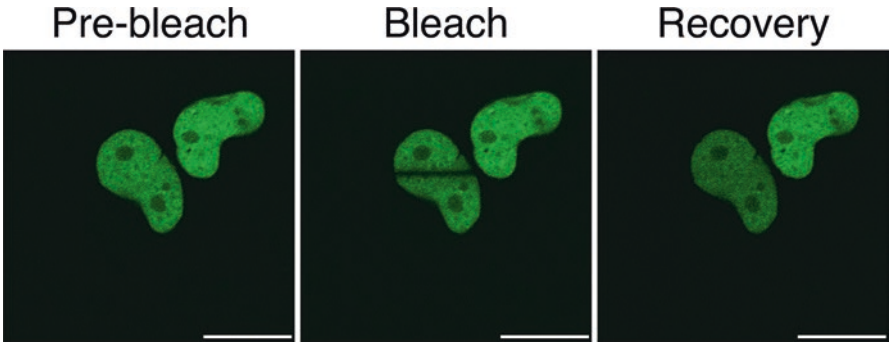

Fig. 3.5 Rapid intranuclear dynamics of the thyroid hormone receptor visualized by fluorescence recovery after photobleaching (FRAP). HeLa cells were transfected with an expression plasmid encoding GFP-TRα1. Strip-FRAP was conducted using a stimulation laser bleaching line near the middle of a nucleus. Confocal microscopy images show examples of a nucleus prior to bleach (pre-bleach), directly after beaching was terminated (Bleach), and at the end of the recovery period (Recovery). Scale bar 10 μm

3.5.2 Retinoic Acid Receptor Intracellular Trafficking

Study of the diffusion of GFP-RARα in the nucleus by fluorescence correlation spectroscopy in HeLa cells revealed two distinct species with different mobilities in the absence of ligand. The fast component, corresponding to dimers, or other smaller complexes, has a diffusion coefficient of 1.8–6.0 μm^2/second and was interpreted to represent freely diffusing receptors or DNA-scanning receptors with short residence times on DNA. The slow component has a diffusion coefficient of 0.05–0.10 μm^2/sec and was interpreted to represent the fraction of transcriptionally competent, ligand-bound RAR that has a longer residence time on chromatin [196]. More recently a combination of Single Plane Illumination Microscopy (SPIM) and fluorescence (cross-) correlation spectroscopy (F[C]CS) was used to simultaneously map the molecular proximity and co-mobility of RAR and RXR and to study their dimerization and DNA-binding behavior [197]. Data from this study showed that RAR agonist enhances RAR-RXR mobility and heterodimerization and that their chromatin binding and heterodimerization are positively correlated.

3.6 Retinoid X Receptor and Vitamin D Receptor Intracellular Trafficking: A Bin of Their Own?

In the scheme presented in this chapter, which intracellular localization “bin” best fits RXR and VDR is not clear-cut, in part because the effect of ligand on intracellular trafficking may be cell-type specific and because of the interaction between RXR and VDR during the transport process. As noted earlier, RXRα plays a critical

role in DNA binding and transcriptional activity through heterodimerization with other nuclear receptors, including TR, RAR, and VDR.

1α,25-dihydroxyvitamin D_3 ($1,25[OH]_2D_3$), the hormonally active metabolite of vitamin D, affects bone and mineral homeostasis and immunomodulation and promotes cellular differentiation [198]. Early on, studies showed that binding of $1,25[OH]_2D_3$ to VDR in the cytoplasm of COS-7 kidney cells stimulates heterodimerization of VDR with RXR and the redistribution of the VDR-RXR-hormone complex to the nucleus [151]. The VDR/RXR heterodimer then binds to vitamin D response elements of target genes resulting in activation or repression of transcription via interaction with cofactors and the basal transcription machinery. VDR and RXRα have also been reported to be both cytoplasmic and nuclear in the absence of ligand in transformed human keratinocytes, with ligand addition significantly increasing nuclear accumulation of both endogenous and fluorescent protein-tagged receptors [147]. In Caco-2 cells (intestinal cell line), however, unliganded VDR is about 60% nuclear, and $1,25[OH]_2D_3$ treatment was not shown to lead to further nuclear accumulation of VDR. Instead, FRAP experiments suggested that an increased rate of nuclear import in the presence of ligand was balanced by nuclear export of VDR [199].

It is still debatable as to whether RXR and VDR move independently or as a complex through the NPC; however, some current models propose that nuclear import of VDR/RXR is controlled by RXR and regulated by vitamin D [147, 200]. Phosphorylation of RXR at serine-260 has been shown to disrupt its nuclear localization, interaction with VDR, intranuclear trafficking, and binding to chromatin of the VDR-RXR complex [147]. These data suggest that RXR dominates the activity of the heterodimer; but this differs from another study suggesting that nuclear accumulation of RXR-VDR is mediated predominantly by VDR. In this latter study, RXR and VDR were shown to translocate into the nucleus by distinct pathways, with RXR import mediated directly by importin β1 and VDR by the adapter importin α [63]. RXR was shown to be predominantly nuclear in the absence of ligand, with nuclear localization modestly enhanced by its ligand, 9-cis-retinoic acid. VDR nuclear import also was enhanced by $1,25[OH]_2D_3$; however, RXR-VDR dimerization recruited the heterodimers to importin α, and nuclear import of the heterodimers occurred in response to $1,25[OH]_2D_3$. In other words, in this case, nuclear import of RXR-VDR heterodimers was mediated preferentially by VDR and was controlled by the VDR ligand.

Adding to the complexity of RXR-VDR trafficking, a recent study provides evidence that agonist binding directs dynamic competition among nuclear receptors for heterodimerization with RXR [201]. Results from a three-color imaging system that detected changes in heterodimerization between RXRα and one of its partners in the presence of another competing partner showed dynamic competition. In the absence of agonist treatment, there was a hierarchy of affinities between RXRα and its partners, with RARα having greater affinity than VDR. Upon agonist treatment, RXRα is thought to favor the liganded partner.

3.7 Conclusions

The nuclear receptor superfamily is the focus of intensive research that aims to develop therapeutics for diseases such as diabetes, cancer, and inflammatory disorders, as well as for protection against environmental endocrine disruptors [202, 203]. Understanding of nuclear receptor movement has progressed from the simple classical model of steroid hormone signaling to complex trafficking pathways for both liganded and unliganded receptors. Exploration of the machinery that drives nucleocytoplasmic shuttling and intranuclear mobility should provide insights into the complex nature of nuclear receptor-mediated transcriptional regulation. With regard to development of novel therapeutic approaches, for drug design, it will be important to consider the overall consequences of both synthetic agonists and antagonists on nuclear receptor trafficking. Selectively inhibiting nucleocytoplasmic trafficking of specific proteins has garnered much interest but remains a challenge [204]. On the other hand, live cell imaging technologies combined with high-throughput systems may enable drug screens for small molecule regulators of nuclear receptor dynamics [145].

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Chapter 4
Chemical Considerations in Discovery of Receptor Modulators

Anush Abelian and Adeboye Adejare

Abstract Nuclear receptor drug discovery has been an area of increased interest in recent years. These receptors are attractive pharmacotherapeutic targets due to their omnipresent role in gene transcription that controls several biological processes, including cell proliferation, reproductive functions, and metabolism. Nuclear receptor modulators are unique intracellular messengers in that they must possess certain chemical structure characteristics and/or physicochemical properties in order to be transported or pass through the cell and/or nuclear membranes to reach the receptor in the nucleus. Receptor modulation is inherently contingent upon ligand-receptor binding and, by extension, ligand-receptor interactions. These interactions are based on intermolecular bonding forces, including hydrogen bonds, hydrophobic bonds, and other interactions. Stereochemical considerations and steric effects have also been shown to influence these interactions. Ligand-receptor binding can be either strengthened or weakened by modifying the chemical functional groups based on structure-activity relationship studies. In this chapter, we will explore various chemical fundamentals of ligand-receptor binding and probe the chemical considerations needed in drug discovery for nuclear receptors.

Keywords Ligand-receptor interactions · Ligand-receptor binding · Intermolecular binding forces · Functional groups · Stereochemistry · Receptor modulator

A. Abelian
Department of Chemistry & Biochemistry, Misher College of Arts and Sciences, University of the Sciences, Philadelphia, PA, USA

A. Adejare (✉)
Department of Chemistry & Biochemistry, Misher College of Arts and Sciences, University of the Sciences, Philadelphia, PA, USA

Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, University of the Sciences, Philadelphia, PA, USA
e-mail: a.adejar@usciences.edu

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4.1 Introduction

The prototypical approach for receptor modulator development prompts scientists to first identify the receptor of interest in the pathological signal transduction and then develop a ligand to modulate it. Usually, the ligand is designed to bind to the ligand binding pocket (LBP) in the ligand binding domain (LBD) of the receptor. However, alternate sites in the LBD are potentially viable targets as well and are currently being investigated in intracellular receptor pharmacology [1]. General drug discovery principles and applications can be found in other publications of the authors [2–5].

A ligand will only bind to the LBP if it is chemically and structurally able to interact with it. Receptors are generally proteins, so naturally, ligand design will be based upon the specific amino acid residues that the LBP is comprised of. In particular, the amino acid residues of the binding regions are fundamental in dictating receptor-ligand interactions. The different chemical composition of these residues give rise to variations in shape, size, charge, and spatial orientation, all of which help determine ligand binding [6].

For nuclear receptors in particular, the ligand must pass through the cell membrane in order to bind to the transport protein in the cytosol which will deliver it to the nucleus or be able to cross both cell and nuclear membranes in order to reach the receptor. So, it must be lipid-soluble. Nuclear receptors are relatively similar in structure and follow similar signal transduction pathways. The key components of a nuclear receptor include the N-terminal domain (activation function 1 or AF-1), the DNA binding domain (DBD), the hinge region, the LBD, and the C-terminal domain (activation function 2 or AF-2). The DNA binding region contains nine cysteine residues, eight of which bind two zinc ions, called the zinc finger domain. The zinc finger domain stabilizes and contributes to the conformational changes of the DNA binding region. These regions recognize specific nucleotide sequences. Once the ligand has crossed the cell and nuclear membranes, with or without the assistance of a transporter protein, it binds to the ligand binding site of the receptor in an induced fit manner which causes the receptor to morph in shape. This conformational change causes ligand-receptor complex dimerization. The dimer then binds to a co-activator protein, and then this complex binds to a specific DNA region of the cell. Since the dimer contains two receptors and two DNA binding regions, the complex recognizes two sequences of nucleotides that are base pairs in the DNA that are close together. Binding to the DNA either prompts or inhibits the start of transcription and protein synthesis. Overall, many nuclear receptors are implicated in various disease states and are therefore viable targets for the development of pharmacotherapeutics. Nuclear receptor ligands may act in an agonistic, partial agonistic, or antagonistic manner due to the gene and/or tissue. As a result, these divergent factors must be taken into consideration during modulator design. In fact, desire for increased selectivity in modulation of nuclear receptors is a clear trend and perhaps the most influential factor in the design and discovery of nuclear receptor modulators [7–9].

4.2 Intermolecular Binding Forces Drive Ligand Action

Arguably, the most important factor in determining ligand binding specificity and selectivity are intermolecular forces. These forces help the ligand to cross the cell and nuclear membranes as well as draw a ligand to the receptor. They vary in nature and bond strength and are dependent upon the functional groups that constitute the ligand as well as the receptor binding site. Ionic bonds, also called electrostatic bonds, are the strongest of all of the intermolecular forces and occur between atoms that carry opposite charges. A positively charged ammonium ion of a ligand will interact with a negatively charged carboxylate ion of aspartic or glutamic acid on the receptor. This bond becomes stronger as the distance between the two charged species become shorter. Ionic bonding is stronger in a nonpolar environment, a characteristic of binding sites in most proteins. This feature can be used as a strategy for receptor modulation. Raloxifene (Fig. 4.1), for example, is an antagonist at the estrogen receptor. It contains a side chain with a positively charged protonated amine group that interacts with the negatively charged carboxylate moiety of the aspartate residue in the binding pocket. This interaction causes the side chain to protrude from the binding pocket and sterically prevents the protein folding, co-activator binding, and signal transduction pathway that normally follows with binding of the agonist estradiol [10].

Hydrogen bonds and Van der Waals interactions are also particularly significant for nuclear receptors, since the ligand must pass through the hydrophobic cell membrane in order to reach the receptor. For example, estradiol (Fig. 4.2) contains a hydrophobic skeleton, which aids in passing through the hydrophobic membranes to reach the estrogen receptor. The phenolic hydroxyl group hydrogen on estradiol can interact with the carboxylic acid on the glutamate residue, and the oxygen on the phenolic hydroxyl group interacts with the amines of the arginine residue in the binding pocket. Additionally, the aliphatic hydroxyl group on estradiol can interact with the amines of the histidine residue in the binding pocket.

Dipole-dipole and ion-dipole interactions, which can be attractive or repulsive forces, are also intermolecular forces found in ligand-receptor binding. All of these forces must be considered synergistically in ligand design. Ligands are drawn to the

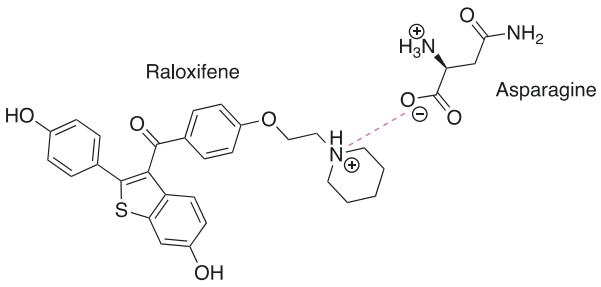

Fig. 4.1 Raloxifene's protonated amine group can have ionic interactions with the asparagine residue in the LBP of the estrogen receptor

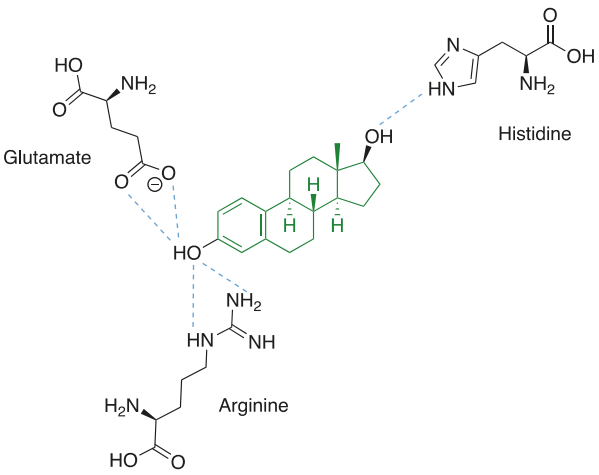

Fig. 4.2 Estradiol can hydrogen bond with the glutamate, arginine, and histidine residues in the LBP of the estrogen receptor. The hydrophobic backbone is shown in green

binding pocket as a result of the presence of multiple different types of these forces. The small but critical differences between agonists and antagonists, e.g., estradiol and raloxifene, illustrate the significance of intermolecular forces in nuclear receptor drug discovery. Going even further, some ligands can show a mixed agonist/antagonist binding profile depending on the tissue site of the receptor. For example, tamoxifen acts as an antagonist of the estrogen receptors in breast tissue but agonizes estrogen receptors in endometrial tissue thus leading to adverse effects. This phenomenon is most likely due to the difference in expression of co-activator proteins in different tissues [7], which is another factor that must be considered during ligand design [11].

4.3 Sterics and Hydrophobicity in Ligand Binding

In addition to intermolecular forces, the size and shape of both ligands and receptors influence their binding. Even if a ligand contains functionalities that bind with the amino acid residues of the binding pocket, the shape of the ligand could sterically hinder it from binding properly. However, with nuclear receptors, the effects of induced fit need to be considered. In this case, although a compound may appear too large compared to the endogenous ligand, an induced fit could allow the compound to bind. One example of this phenomenon is the binding of cortisol, the endogenous ligand, compared to synthetic deacylcortivazol at the glucocorticoid receptor binding pocket. Compared to cortisol, deacylcortivazol contains a phenylpyrazol group (Fig. 4.3) which renders it too bulky to fit into the binding site the same way. It instead binds in an altered induced fit that involves hydrophobic interactions between a new channel in the binding pocket and the phenylpyrazol group [8, 12].

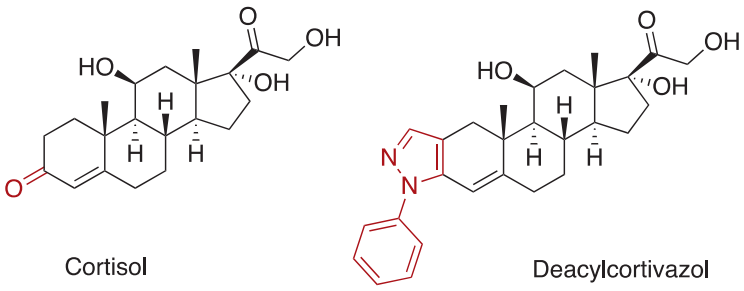

Fig. 4.3 Deacylcortivazol contains the phenylpyrazol, compared to cortisol, which only bears a ketone in that position. (Adapted from [8])

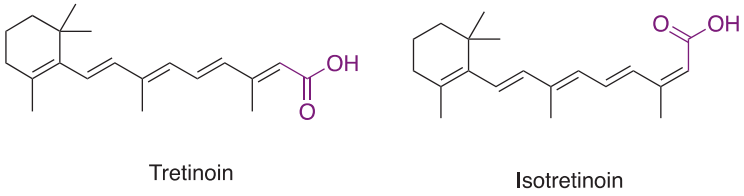

Fig. 4.4 Tretinoin is a direct retinoic acid receptor modulator, compared to isotretinoin, which acts as a prodrug for the retinoic acid receptor

4.4 Stereochemical Considerations

The stereochemistry of a ligand can influence its binding and therefore its physicochemical properties. Stereochemical isomers include geometric (cis/trans or E/Z), optical (enantiomers and diastereomers), and conformational (staggered, Gauche and eclipsed) isomerism. Consider the vitamin A derivatives tretinoin and isotretinoin (Fig. 4.4). Tretinoin is all-*trans*-retinoic acid and is used topically for skin disorders, whereas isotretinoin is 13-*cis*-retinoic acid and is used orally for skin disorders and cancer. All-*trans*-retinoic acid binds directly to the retinoic acid receptor, whereas isotretinoin is taken orally as a prodrug to produce metabolites that bind to the retinoic acid receptor. Isotretinoin itself does not bind directly to the retinoic acid receptor. The only chemical difference between the two ligands is the geometric isomerization of the carboxylic acid moiety on carbon 2 [13].

Moreover, there are multiple studies suggesting stereoselectivity of estrogen receptors. Estrogen receptor alpha, for example, exhibits stereoselectivity in both ligand binding and transactivation for a number of structural analogs and metabolites of diethylstilbestrol, a synthetic estrogen (Fig. 4.5). There is also evidence to suggest a difference between the *R* and *S* enantiomers of indenestrol A, a diethylstilbestrol metabolite, in transcription activator potency *in vitro*. The *S* isomer was shown to have higher binding affinity and transactivation potency compared to the *R* isomer [14].

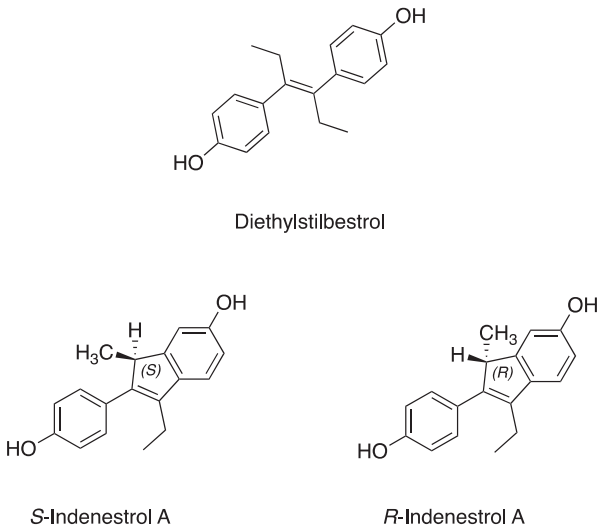

Fig. 4.5 Diethylstilbestrol metabolites exhibit difference in stereoisomer potency *in vitro*

4.5 Molecular Dynamics as a Tool for Modulator Design

Ligand-LBD binding can be observed through x-ray crystallography or cryo-electron microscopy. However, these interactions can be effectively predicted through computational methods in order to facilitate ligand design by predicting ligand potency. Additionally, molecular simulations have indicated that conformational changes of the receptor occur upon ligand binding and release. Several simulations have supported the hypothesis that the quaternary state of nuclear receptors is significant in determining the prevalence of a particular pathway, indicating that nuclear receptor dimerization is integral in ligand dissociation. With this in mind, molecular docking can be approached using specific conformations of the nuclear receptor that ligands may interact with preferentially [15, 16]. Several agonists, antagonists, and inverse agonists have been explored using molecular dynamics [17, 18].

4.6 Case Study: A Holistic Approach to Liver X Receptor Modulator Design

The central concepts of drug design are typically ubiquitous for any receptor of interest. The challenges lie in probing pharmacodynamics and pharmacokinetics of the designed ligand with respect to the target receptor, as well as off-target receptor interactions. However, there are several factors to consider in ligand design, especially with intracellular receptors. Therefore, we will take a hollistic approach to

summarize and exemplify key considerations in nuclear receptor drug design through illustrating the simplified design of a liver X receptor (LXR) modulator.

The LXR is implicated in lipid metabolism, and its transcriptional activation is stimulated through increased cellular cholesterol levels. These receptors are pivotal regulators of cholesterol homeostasis and are also implicated in fatty acid and glucose metabolism. There are two known isoforms of the LXR: LXRα and LXRβ. LXRα is found primarily in the liver, adipose tissue, macrophages, and intestines, whereas LXRβ is found systemically throughout the body, [19, 20]. LXRα and LXRβ are ligand-dependent and heterodimerize with retinoid X receptors (RXRs). These dimers bind to LXR response elements in DNA promoters and subsequently regulate expression of several genes via transactivation [20]. Murine models have shown differential actions of LXRα and LXRβ, and both have been investigated as potential therapeutic targets for metabolic, cardiovascular, and even neurodegenerative disease states [20]. We will focus our case study on the suppression of liver LXR activity as a potential pharmacotherapeutic approach for treatment of hepatic disease states, such as cirrhosis and hepatosteatosis.

LXR agonists have the potential to reverse cholesterol transport and impede inflammation and fatty plaques in the arteries. Although several LXR agonists have been designed and investigated, none are in use clinically as a result of lipogenesis side effects, such as hepatic steatosis and elevated triglyceride levels. Because agonism induces these effects, we can reasonably predict that antagonism and inverse agonism of the LXR receptors will inhibit or even reverse these effects, respectively [18, 20].

As existing data permits, an effective first step to modulator discovery is elucidation of the structures of the endogenous ligands. Oxysterols, which are derivatives of cholesterol (22-(R)-hydroxycholesterol, and cholestenoic acid), have been classified as the endogenous ligands of LXR [21, 22], (Fig. 4.6). Next, the interactions between the endogenous ligands and the LBP of the LXR can be predicted. More effectively, if the crystallographic data exists, various ligand-LBP interactions can be observed and better explored.

Several X-ray crystal structures for LXR-ligand complexes have been resolved and are available in the Brookhaven Protein Data Bank (PDB). Analagous to other nuclear receptors, the LXR contains an N-terminal activation domain that is ligand-independent, a zinc finger DNA binding domain, a hinge region, the LBD containing the LBP, and a C-terminal domain [23]. The amino acid residues constituting the LBP give it an overall hydrophobic character, but some residues contain polar moieties that interact with the ligand. Several ligand-receptor interactions (Fig. 4.7) have been shown to be pervasive across all of the existing crystal structures. Hydrogen bonding is critical for receptor activation, particularly between the ligand and histidine residues in both the LXRα and LXRβ. This interaction has been shown to stabilize the histidine-tryptophan switch, holding the receptor in its active conformation [24] and allowing co-activator interactions. Upon binding of an agonist to the LBP of LXR, corepressors are released and AF-2 undergoes a conformational change that allows coactivators to bind, permitting gene transcription [25]. As a result, ligands that have been designed to bind tightly to the LXR and prevent the

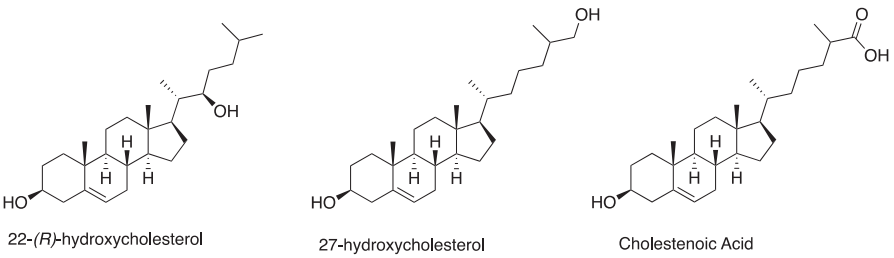

Fig. 4.6 Examples of oxysterols, which are the endogenous ligands of the LXR

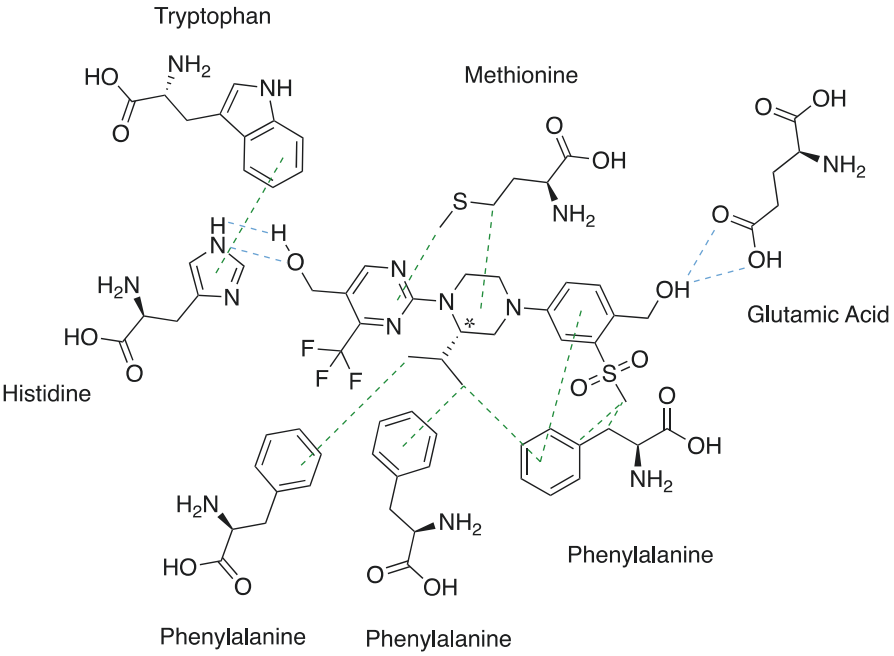

Fig. 4.7 A simplified schematic of the LBP of LXRβ and interactions of the agonist {2-[(2R)-4-[4-(hydroxymethyl)-3-(methylsulfonyl)phenyl]-2-(propan-2-yl)piperazin-1-yl]-4-(trifluoromethyl)pyrimidin-5-yl}methanol with key amino acid residues. Hydrogen bonding is shown in blue and aliphatic interactions are shown in green. (Adapted from [18])

helix from undergoing this conformational change have been hypothesized to act as antagonists that can downregulate gene transcription [18].

Although the hydrogen bonding is critical for receptor activation, several ligand-receptor interactions can be observed in Fig. 4.7 which show the potent agonist VTP-766 in a simplified schematic of the LXRβ LBP. Most of these interactions are aliphatic in character. This observation is common for nuclear receptor ligands, since they require hydrophobic characteristics in order to permeate the cell and/or nuclear membranes. Binding strength variations modify receptor activity profiles

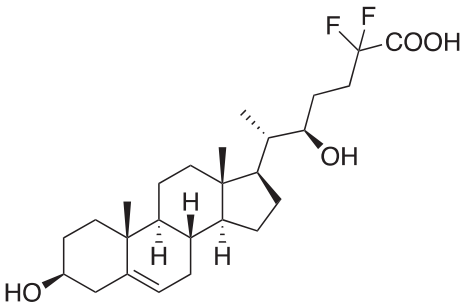

Fig. 4.8 25,25-difluoro-27-norcholestenoic acid, a difluorinated oxysterol analog that acts as an inverse agonist at the LXRβ receptor

among agonists, antagonists, and inverse agonists. One strategy to approach this phenomenon is to alter critical bonding groups with bioisosteres. For example, replacement of the two hydroxymethyl groups on VTP-766 with fluorine and chlorine showed less potency, which is likely due to the decrease in the strength of hydrogen bond interactions (although fluorine can still form loose bonds with hydrogen). Both the *R* and *S* isomers of VTP-766 were investigated, and the *R* isomer was found to bind more potently than the *S* [18, 26].

Interestingly, analogues of the endogenous LXR agonist oxysterol have been developed as inverse agonists (Fig. 4.8). 25,25-difluoro-27-norcholestenoic acid, for example, shows *in vitro* inverse agonist activity in vivo and shows a disruption in the histidine-tryptophan interaction required for receptor activation through molecular docking. These minor modifications of the cholestenoic acid side chain can induce conformational changes in the C-terminal domain of the LXR, thereby altering its activity profile from an agonist to an inverse agonist [27].

4.7 Conclusions

The chemical principles of drug design and discovery are ubiquitous, but not absolute, especially considering nuclear receptors. The key components discussed here, including intermolecular forces, steric considerations, and stereochemistry are paramount in ligand-receptor interactions. Contemporary investigations of nuclear receptor ligands involve non-LBP targets, such as AF2, in the effort to modulate signal transduction cascades without inducing adverse effects that occur with traditional LBP ligands. Different signal transduction pathways that influence gene expression, as well as metabolism, must be considered in a holistic manner in order to effectively progress nuclear receptor modulator design and discovery.

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Chapter 5
Structure-Based Design of Estrogen-Related Receptors Modulators

Shaimaa S. Goher and Bahaa Elgendy

Abstract Estrogen-related receptors (ERRs) are members of the nuclear hormone receptor (NR) superfamily. The ERR subfamily comprise three members, ERRα, ERRβ, and ERRγ. They are closely related to the estrogen receptors (ERα and ERβ), but unlike ER receptors, ERRs have constitutive activity and can function in the absence of ligands. The ERRs are orphan receptors because no natural ligands have been identified for any of the three ERR isoforms. Although ERRs are structurally related to ERs and share sequence similarity with these receptors, they do not bind with estrogens. ERRs are expressed mostly in all tissues that have been examined to date with variation of the level and type of isoform existed in a particular tissue. ERRs play an essential role in many physiological processes, and they are potential therapeutic targets in many disease areas such as Alzheimer's disease, cancer, diabetes, and other metabolic diseases. In this chapter, we mainly focus on the structure and function of ERRs, and the medicinal chemistry efforts to design modulators of these receptors. We put great emphasis on the structure-based design of ERR modulators, which we believe is an essential tool to advance the drug discovery in this particular research area.

Keywords Estrogen-related receptors · Structure-based design · Agonists · Inverse agonists · Alzheimer's disease

S. S. Goher
Chemistry Department, Faculty of Science, Benha University, Benha, Egypt

Nanotechnology Research Centre (NTRC), The British University in Egypt (BUE), Cairo, Egypt

B. Elgendy (✉)
Chemistry Department, Faculty of Science, Benha University, Benha, Egypt

Department of Pharmaceutical and Administrative Sciences, St. Louis College of Pharmacy, University of Health Sciences and Pharmacy, St. Louis, MO, USA

Center for Clinical Pharmacology, Washington University School of Medicine and St. Louis College of Pharmacy, St. Louis, MO, USA
e-mail: bahaa.elgendy@uhsp.edu; belgendy@wustl.edu

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5.1 Introduction

Estrogen-related receptors (ERRs) were the first orphan receptors to be identified among the nuclear receptor (NRs) superfamily, but their endogenous ligand is yet to be identified [1]. ERRs are constitutively active receptors that belong to class III of the steroid hormone receptor subfamily (estrogen receptor-like) [2]. There are three known isoforms of the ERRs, namely, ERRα, ERRβ, and ERRγ. ERRα (NR3B1, ESRRA gene) and ERRβ (NR3B2, ESRRB gene) were the first estrogen-related receptors to be discovered, while ERRγ (NR3B3, ESRRG gene) was the latest [1, 3]. Both ERRα and ERRβ were initially named hERR1 (human estrogen-related receptor 1; renamed as hERRα) and hERR2 (human estrogen-related receptor 2; renamed hERRβ), respectively [1, 4]. The gene encoding ERRγ was isolated in 1998 from the critical region of Usher Syndrome Type IIa at 1q41 [5]. One year later, ERRγ was identified functionally because it was shown to interact with its transcriptional coactivator glucocorticoid receptor interacting protein 1 (GRIP1) both in mammalian cells and in yeast using yeast two-hybrid system [6].

ERRs are expressed mostly in all tissues that have been examined to date with variation of the level and type of isoform existed in a particular tissue. ERRα has ubiquitous expression and is highly expressed in metabolically active tissues such as the heart, kidney, intestinal tract, brown adipose tissue (BAD), and skeletal muscles and functions as a key regulator of energy metabolism [1, 7–9]. It is also highly expressed in prostate especially in prostate stromal cells, cerebellum, and hippocampus and with lower expression level in the liver, lungs, and spleen [1]. ERRα is also expressed throughout the adipocyte differentiation program and in bone-derived macrophages activated by interferon γ (INF-γ) or lipopolysaccharide [10–12]. ERRβ and ERRγ are important in early embryonic development, and their expression is restricted to metabolically active and highly vascularized tissues such as the heart, kidney, brain, and skeletal muscle [1, 3, 5]. ERRγ is predominantly expressed in various tissues associated with central nervous system, circadian clock, brain stem, and spinal cord. The three ERR isoforms are widely distributed in the tissues related to CNS [5, 8, 12, 13].

ERRs play essential role in many physiological processes, and they are potential therapeutic targets in many disease areas such as breast cancer, diabetes, and other metabolic diseases [14–16].

ERRs share common target genes with ERs such as lactoferrin, osteopontin, and pS2 [17–19]. Moreover, ERRα was found to regulate the aromatase gene involved in estrogen synthesis through encoding the enzyme that catalyzes the conversion of androgens to estrogens, and this suggested that ERRs might modulate the estrogenic response [20].

Similar to ERα, ERRα and ERRβ bind to and regulate transcription through classical estrogen response element (ERE) and the SF-1 response element (SFRE) [18]. On the other hand, DNA binding and transcriptional activity of ERβ is limited to the ERE. The binding motif was designated as (TCA**AGGTCA**) and called ERR

response elements (ERREs), which was identified using an unbiased binding site selection approach.

ERRs can bind not only as monomers and homodimers but also as a heterodimer of two ERR isoforms [21–24]. In contrast, the ERs bind as homodimers to the classical ER response element (ERE) designated as 5-AGGTCANNNTGACCT-3 separated by three nucleotides N, and that is why their function is distinct from ERRs [3, 7, 19, 25]. The DNA binding data of both ERs and ERRs suggests a crosstalk between the two receptors, which in return suggest that these receptors may control overlapping regulatory pathways [26]. Despite ERRs being constitutively active and independent of exogenous ligands, their activity is highly dependent on the presence of coregulator proteins in the cell that are either coactivators or corepressors [27–30]. For instance, ERRs are activated by peroxisome proliferator activated receptor γ (PPARγ)-coactivator 1 α (PGC-1α) and PGC-1β. Therefore, PGC-1α and PGC-1β were often considered as protein ligands for ERRs. On the other hand, ERRα was found to act as a repressor to the PGC-1α transcriptional activity suggesting a novel transcriptional mechanism [9]. Steroid receptor coactivator (SRC-1) and glucocorticoid receptor-interacting proteins (GRIP-1) were also found to bind as coactivators to ERRs, while the small heterodimer partner (SHP) and the small heterodimer partner interacting leucine zipper protein (SMILE) can act as corepressors for ERRs [6, 28, 29]. These protein-protein interactions and the unique binding mechanism of ERR and coactivators or corepressors as protein ligands provided the evidence for the significant constitutive activity of ERRs in absence of exogenous ligands (Fig. 5.1) [6, 27, 28, 31].

ERRs play an important role in the regulation of several physiological processes [9, 26, 30, 32–35]. For example, they were identified as key regulators of several glucose transporters (e.g., SLC2A1, SLC2A2, SLC2A4, and SLC2A12, also known as GLUT1, GLUT2, GLUT4, and GLUT12, respectively) (Fig. 5.2) [36]. ERRS

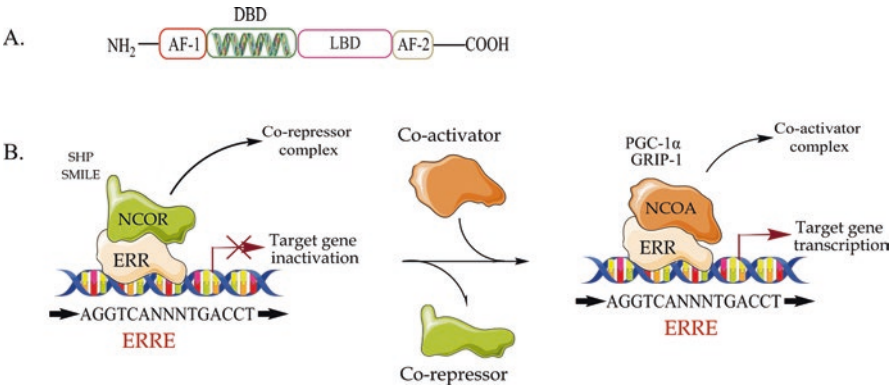


Fig. 5.1 Structural features and activation of ERRs: A. NRs domains. B. Activation mechanism of ERRs

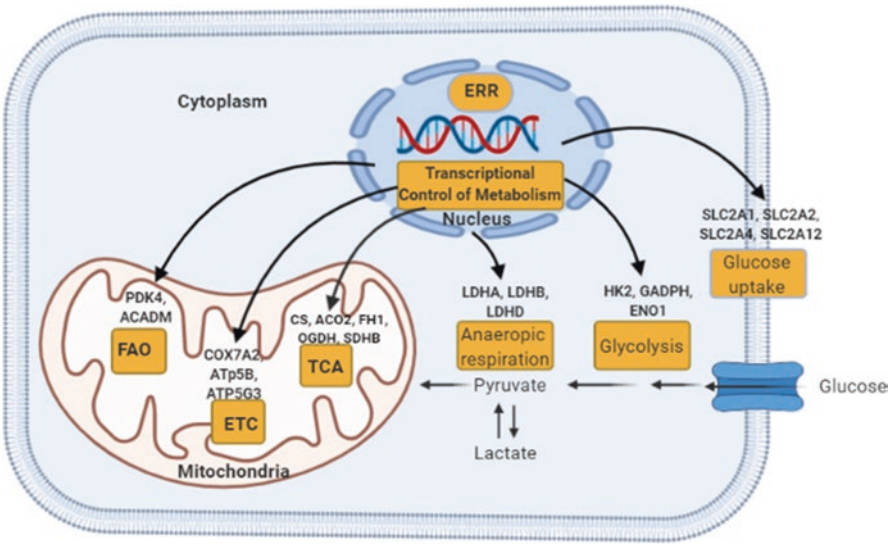


Fig. 5.2 ERRs as regulators of energy metabolism

were found to regulate cellular, liver, and mitochondrial metabolism, mitochondrial activity, biogenesis, and cardiac mitochondrial biogenesis [35], fatty acid oxidation [35], glycolysis, bone morphogenesis by regulating the osteopontin gene [37], bone resorption, and osteoprogenitor cell proliferation and differentiation [11, 38, 39]. Therefore, targeting ERRs therapeutically is of great importance in treatment of diseases such as type 2 diabetes, liver diseases, heart failure, cancers, bone resorption, etc. [39, 40]. For example, modulating ERRs can be of great importance in treating breast cancer where ERRα and ERRγ were found to be the major isoforms expressed in the human breast cancer cell lines [19, 41]. Interestingly, ERRα was found to act as a repressor of ERE-dependent transcription in MCF-7 and Hela cells [9]. Moreover, ERRs can be a good therapeutic target for colon cancer by directly targeting Osteopontin (OPN) [37]. ERRs are promising target in the treatment of diabetes. For example, increasing ERRα expression was found to enhance the mitochondrial function and oxidative capacity and inhibit hepatic gluconeogenesis and lower plasma glucose levels [42]. ERRs directly regulate several genes that are involved in the mitochondrial function, alongside with other factors controlling mitochondrial gene expression, such as NRF1 and NRF2/GABPA [43]. Targeting ERRs can play a role in modulating immunity because ERRα was found to be a selective regulator of effector (Teff) subset of T-cells through regulation of their essential metabolic pathways. Inhibition of ERRα in vivo showed pharmacological effect in autoimmune encephalomyelitis models as it reduced Teff generation and T-cell proliferation [44].

5.2 Structure and Function

Estrogen-related receptors (ERRs) share the common structural features of NRs, and they have three major domains: (i) N-terminal domain (A/B) that contains the transcription activation function (AF-1 domain), which is poorly conserved domain among all NRs; (ii) ligand binding domain (LBD), which sometimes called C-terminal domain that shares 10–60% of homology among the family members; and (iii) a highly conserved DNA-binding domain (DBD) that contains two zinc fingers and share the highest homology (40–90%) among all NRs family. DBD folds to form a single structural domain where it binds to the specific response element called ERRE. ERRs have hinge region described as the D domain [2, 15, 45–47].

Sequence analysis showed that ERRs share a high degree of homology in amino acid sequence with the classical estrogen receptors (ERs) within both DNA binding domains (DBD) and ligand binding domains (LBD). For example, ERRα shares about 68% amino acid sequence identity of its DBD and about 33% of its LBD with ERα (Fig. 5.3a) [48, 49]. Despite this significant homology with ERs, ERRs does not bind to estrogens like estradiol or thyroid hormones and their endogenous ligand is yet to be identified [6, 26, 48–51]. In humans, the major ERRα1 consists of 422 amino acid polypeptide and its coding gene exists on chromosome 11q12–q13 with predicted molecular weight of 46 kDa (NCBI accession NP_004442.3) [52–54]. Another ERRα2 protein that consists of 506 amino acid and a predicted molecular weight of 56 kDa was detected and found to have an additional exon at the 5′-end and an in-frame translational start site [52]. Another processed human ERRα pseudogene was found on chromosome 13q12.1 [53]. In addition, ERRβ consists of 500 amino acids and its coding gene exists on chromosome 14q24.3 with predicted molecular weight of 56 kDa (NCBI accession NB_004443.2) [53, 55]. Human ERRγ has three mRNA species ERRγ1, ERRγ2, and ERRγ3, and they are alternatively spliced at the 5′-end. The major ERRγ1 protein consists of 458 amino acids and encoded by chromosome 1q41 with predicted molecular weight of 51 kDa [5, 55]. ERRγ2 and ERRγ3 consists of 435 amino acid each with a predicted molecular weight of 49 kDa. They are 23 amino acid smaller than ERRγ1 protein, and they

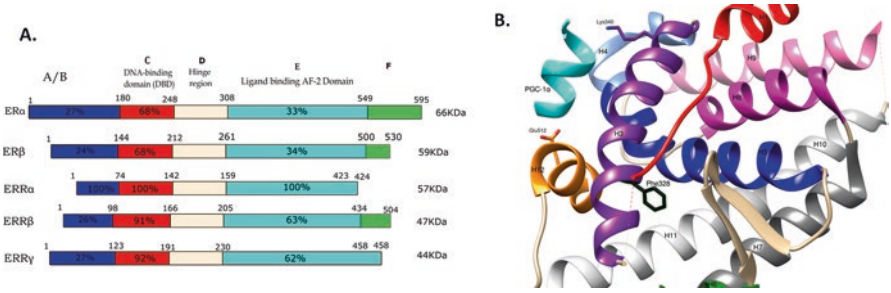


Fig. 5.3 (**a**) Organization of ERRs and sequence identity related to ERRα. (**b**) LBP of ERRα

differ than ERRγ1 in the N-terminal A/B region where they lack the activating function 1 (AF-1) [55–57]. Moreover, ERRs share a significant sequence identity with each other specially within their LBD and DBD. ERRβ and ERRγ shares 63% and 62% of their LBD with ERRα LBD, respectively. They share 91% and 92% DBD identity with ERRα (Fig. 5.3a). ERRβ and ERRγ shares 77% sequence identity within their LBP and 98% within their DBD [3, 47]. ERRα and ERRγ differ in 7 amino acid residues in the LBP; F328/A272 (H3), V366/I310 (H5), F382/Y326 (S1), G402/N346 (H7), L405/I349 (H7), V491/A431 (H11), and L492/V432 (H11) for ERRα/ERRγ, respectively [58].

As we previously mentioned, ERRs regulate the transcription via binding not only as monomers and homodimers but also as a heterodimer of two ERR isoforms to the estrogen response element [21, 22]. The affinity of ERRα for binding to ERRE is affected by its acetylation mediated by the acetyltransferase PCAF and the deacetylases, HDAC8 and SIRT1 on four lysine residues located in the DBD [59]. Transcription activity was also regulated via SUMOylation and phosphorylation of the N-terminal domain (NTD) of ERRα and ERRγ [60]. In ERRs, the less conserved LBD, which contains the activated function-2 (AF-2), adopts a conformation that supports the recruitment of coactivators in absence of any ligand. Hence, they were classified as a constitutively active receptor [15, 28, 61, 62]. X-ray crystallography revealed that ERRs has a small hydrophobic ligand binding pocket (LBP) with few polar residues (e.g., His494, Glu331, and Arg372 in ERRα), which are conserved among all ERRs [58, 63]. This LBP comprises 12 α-helices and small 1β-fold with a canonical α-helical three-layered sandwich structure. The cavity volume in ERRα is about 100 $Å^3$, which can be occupied by a ligand consists of four or five non-hydrogen atoms [58]. This cavity is small compared to that of ERRγ, which is 220 $Å^3$. This small size cavity in ERRα is partially occupied with side chains, particularly the bulky phenylalanine residue Phe328 on H3, which fills the LBP and is essential for the constitutive activity of ERRα (Fig. 5.3b). Consequently, the LBP adopts an agonist conformation that allows it to bind to PGC-1α peptide [58].

In order to reveal molecular basis of small molecule regulations of ERRγ, Notle and his coworkers [51] determined the X-ray crystal structure of ERRγ LBD in three different states: unliganded, inverse agonist bound, and agonist bound. The unliganded structures were solved in the absence and presence of the cofactor peptide RIP140. There were no significant differences between these two unliganded structures. The most important observation was the identification of two distinct LBPs in both structures. The first pocket (pocket 1) (Fig. 5.4) is analogous to the LBP of ERα but much smaller (280 $Å^3$ in ERRγ vs. 480 $Å^3$ in ERα). The second pocket (pocket 2) (Fig. 5.4) was larger in size with a volume of 390 $Å^3$. Phe435 of ERRγ, which corresponds to Leu525 in ERα, blocks common steroidal estrogens from binding to ERRγ. Two more residues, Ala431 and Phe450, define the topology and volume of ERRγ LBP. Ala431 and Phe450 in ERRγ correspond to Gly521 and Leu540 in ERα, respectively. These data provided an explanation for why ERR does not bind to steroids and showed that the LBP is partially filled with bulky residues

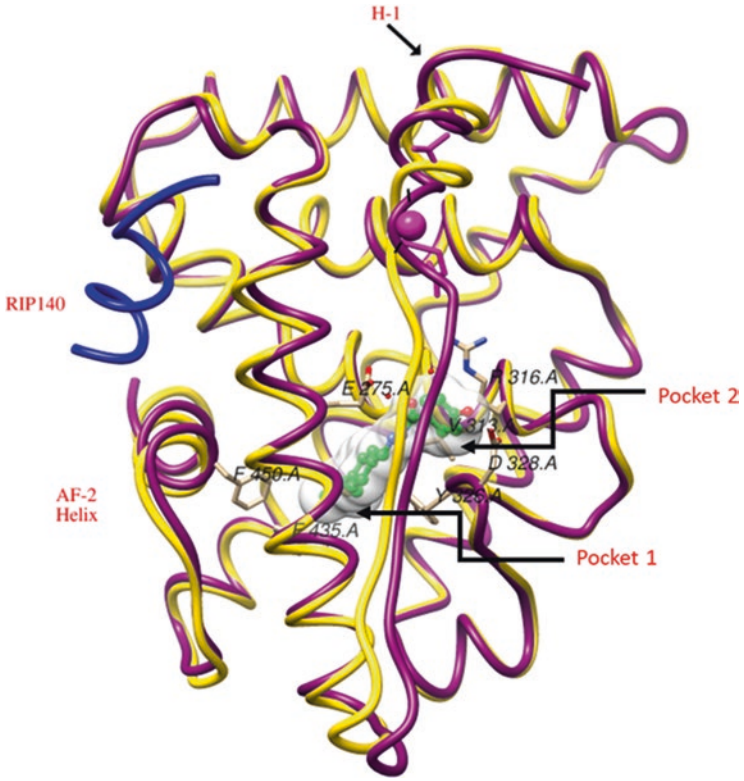


Fig. 5.4 Overlay of unliganded ERRγ (PDB ID: 2GP7) and ERRγ complexed with GSK4716 and RIP140 (PDB ID: 2GPP)

that stabilize the active conformation of the receptor in cells to recruit coactivators [51].

The X-ray structure of inverse agonist 4-OHT complexed with ERRγ showed conformational changes to the end of helix 10 and the AF-2 helix. Phe435 rotated from its original conformation in the apo-ERRγ structure and induced steric clashes with Leu454 and Phe450 on the AF-2 helix (Fig. 5.5). These steric clashes lead to displacement of AF-2 from its original position where it caps the LBP and consequently blocks coactivators from binding to the receptor [51]. When compared to apo structure, no substantial changes in the conformation of helices 1–10 were observed in ERRγ.4-OHT structure. It is worth mentioning that the ERRγ.4-OHT structure is tetrameric and the interaction of one homodimer to another block at least one of coregulatory binding sites. As a result, we do not know if this mode of binding is relevant in vivo or not.

The X-ray structure of agonist **GSK4716** complexed with ERRγ.RIP140 showed AF-2 helix adopting agonist conformation (Fig. 5.6). The overall structure is similar to the apo structure where Phe435 and Phe450 did not change their conformation to

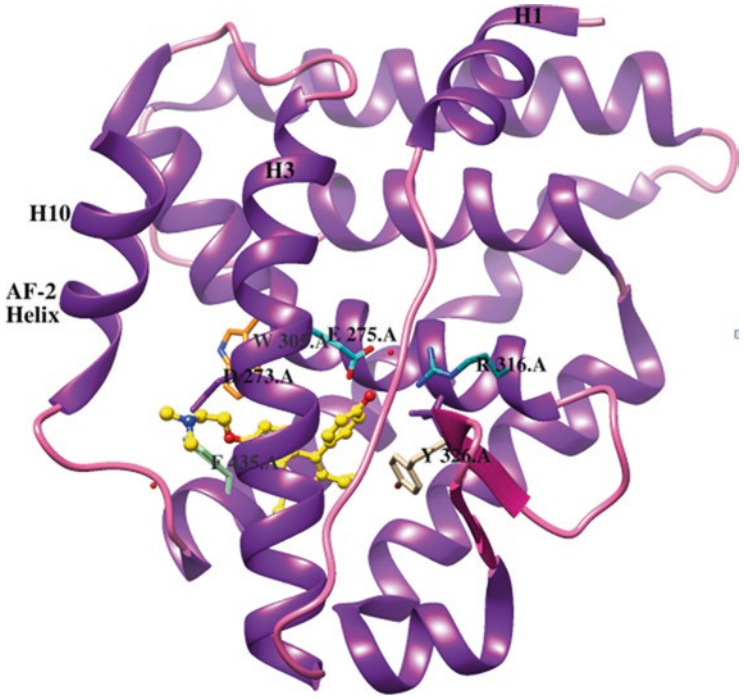


Fig. 5.5 X-ray of ERRγ complexed with 4-OHT (PDB ID: 2GPU)

accommodate **GSK4716**. The major conformational changes were observed in the region between helices 1 and 3 where three residues (i.e., Pro246, Glu247, and Lys248) showed significant movements compared to the apo structure. These movements took place in parallel with the move of the phenolic binding residues Glu275 and Arg316 and allowed **GSK4716** to access the additional pocket away from AF-2 helix and led to formation of a larger pocket with a volume of 610 Å^3. The phenolic hydroxyl of **GSK4716** did not interact with the phenolic binding residues Glu275 and Arg316 but made contacts with Arg328 located near the surface of the receptor. Interestingly, the carbonyl group of **GSK4716** made contacts with Arg316 through a water bridge [51]. This study showed clearly that ERRγ has remarkable plasticity within the LBP and provide explanation for the molecular basis of the receptor regulation via small molecules.

Kallen and his coworkers [64] successfully crystalized ERRα LBD with the inverse agonist cyclohexylmethyl-(1-*p*-tolyl-1*H*-indol-3-ylmethyl)-amine (**6**) and showed that ERRα has significant degree of plasticity similar to ERRγ. The LBP of ERRα underwent dramatic conformational changes to accommodate the ligand. Phe328 (on helix 3) that partially fills the LBP and Phe510 (on helix 12) has to rotate to avoid steric clashes and consequently dislocated helix 12 from its original agonist conformation. Surprisingly, helix 12 binds in the same groove where

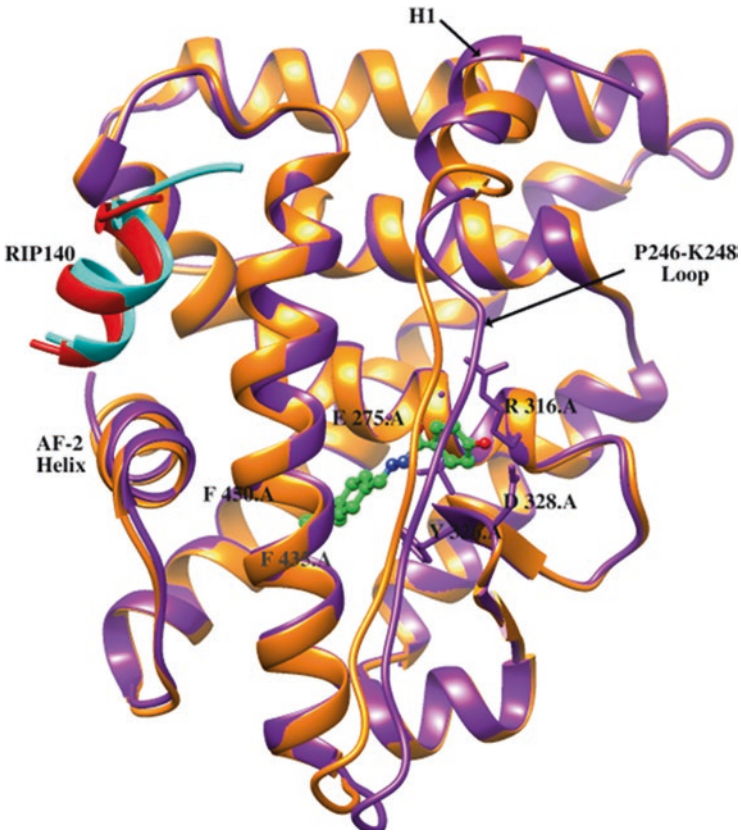


Fig. 5.6 Overlay of unliganded ERRγ.RIP140 (PDB ID:2GPO) and ERRγ with GSK4716-RIP140 peptide (PDB ID:2GPP)

coactivator usually binds and inactivates the receptor (Fig. 5.7). This binding mode is similar to the binding mode observed in ERα.4-OHT complex but different from ERRγ.4-OHT complex where helix 12 was completely dissociated from the LBD.

Exploiting all available structural information is very crucial in the process of structure-based design of novel modulators of ERR receptors.

5.3 Medicinal Chemistry of ERR Modulators

ERRs are orphan nuclear receptors with no endogenous ligand identified, but several synthetic ligands were developed as potent ERR modulators. ERRs are constitutively active with small cavity size that can only accommodate small molecules, which make them a challenging drug target. The majority of identified modulators

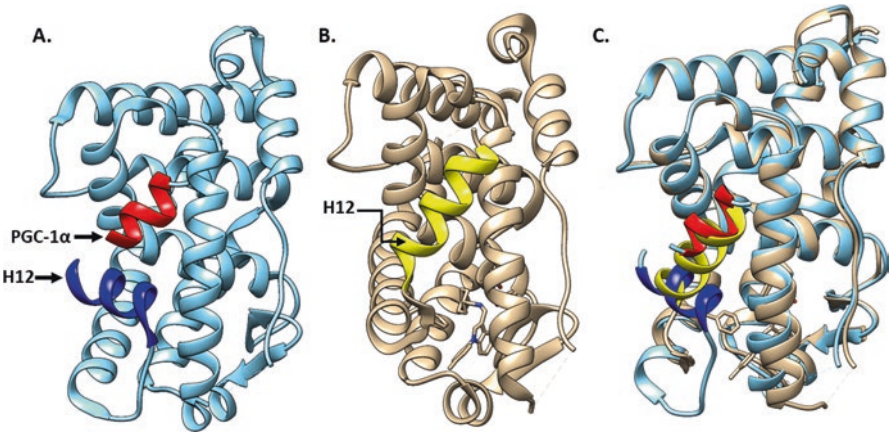


Fig. 5.7 (**a**) The 3D crystal structure of ERRα LBD and PGC-1α peptide complex. (**b**) Co-crystal structure of inverse agonist **6** with ERRα LBD. (**c**) Overlay of (**a**) and (**b**)

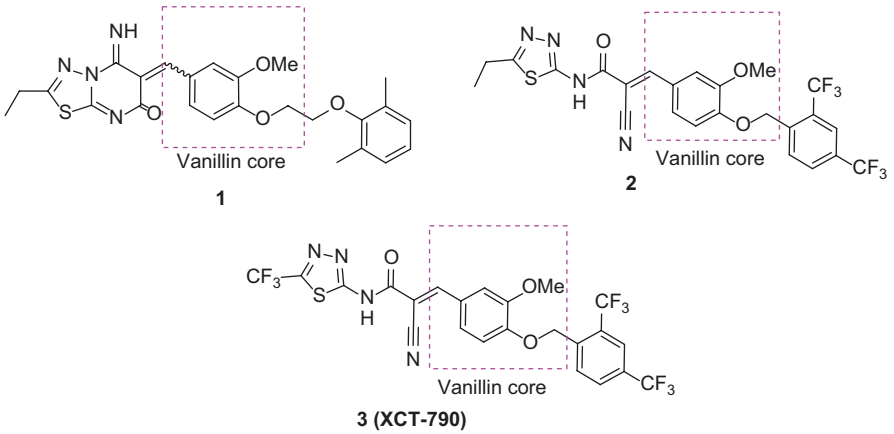


Fig. 5.8 ERRα inverse agonists **1–3**

were inverse agonists (i.e., modulators that suppress ERRs basal activity) for ERRα/γ, and few agonists (i.e., modulators that enhance ERRs basal activity) were also reported.

5.3.1 ERR Inverse Agonists

Inverse agonists **1–3** (Fig. 5.8) were developed from SAR of a hit compound identified through HTS. The thiadiazoleacrylamide **3** (XCT-790) is the first potent and selective ERRα inverse agonist. The activity and potency of **1–3** were measured using fluorescence polarization (FP) assay and were confirmed in a cell-based

co-transfection assay using Gal4-ERR format. XCT-790 (**3**) exhibited the highest potency in the cell-based GAL4-ERRα transfection assay and was selective to ERRα (IC_{50} = 0.37 μM) with no activity observed toward ERRγ, ERα, and ERβ. The thiadiazole analogue **2** (IC_{50} = 0.54 μM) was 4-fold more potent than thiadiazolopyrimidine **1** (IC_{50} = 2 μM).

The three compounds incorporate the vanillin core, which was found to be beneficial to ERRα activity [65]. Suppressing ERRα activity using XCT-790 (**3**) was found to alter the ERRα/PGC-1 signaling pathways, suppress mitochondrial biogenesis, increase the ROS production, activate HIF-1α, and induce glucose transporters expression thus controlling β-oxidation and affect glucose uptake indirectly. This validates the role of ERRα in the regulation of energy metabolism and type II diabetes [65, 66]. Additionally, XCT-790 (**3**) induced apoptosis in HepG2 hepatocarcinoma and its MDR sub-line R-HepG2 and synergized with paclitaxel. This shed light on the importance of ERRα inverse agonists as chemotherapeutic agents [67]. Furthermore, XCT-790 (**3**) was reported to be potent and fast-acting mitochondrial uncoupler that potently activates AMP kinase (AMPK) in a dose-dependent and ERRα-independent manner with concentrations 25-fold more than those used to inhibit ERRα. XCT-790 (**3**) share common structural features with the chemical uncouplers carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), suggesting similar mode of action as a proton ionophore [68]. Moreover, it was demonstrated that XCT-790 (**3**) suppress the proliferation of the triple negative breast cancer cells (TNBC) both in vitro and in vivo by raising the ROS generation, induce the mitochondrial-related apoptosis, and increase the expression of the growth inhibition-related proteins like p53 and p21. XCT-790 (**3**) elevates the proteins related to the endoplasmic reticulum (ER) stress like ATF4/6, XBT-1, and CHOP. Other multiple signaling pathways were found to be involved in the inhibition effects of XCT-790 on TNBC proliferation [69].

Compound **4** (Fig. 5.9) emerged from a high-throughput screening of ERR coactivator HTRF assay of a collection of approximately, 1,256,187 chemical compounds. Its specificity was confirmed by dissociation enhanced lanthanide fluorescence immunoassay (DELFIA) where it showed 100-fold affinity over that of DES with an IC_{50} = 170 nM. Compound **4** did not inhibit the interaction of PGC-1α with ERRβ or ERRγ, but it did with ERRα, which shows excellent selectivity toward ERRα [70, 71]. Further studies revealed that compound **4** inhibits ERRα transcriptional activity in MCF-7 breast cancer cells in luciferase assay but has little to no effect on mRNA levels measured by RT-PCR. It also decreased the level of some ERRα target genes such as pS2 (TFF1), osteopontin (SPP1), and aromatase (CYP19A1) mRNA. Besides, it increased the ERRα protein degradation through ubiquitin proteasome pathway [71].

SR16386 (**5**) is a steroidal antiestrogen that was found to selectively bind to ERRα but do not bind to either ERRβ or γ. It inhibits the transcriptional activity of ERRα and prevents the binding of coactivators to ERRα and also prevents their recruitment to the promoters or enhancers of target genes. SR16386 (**5**) inhibited the proliferation of human prostate tumor xenografts in mice when used alone or

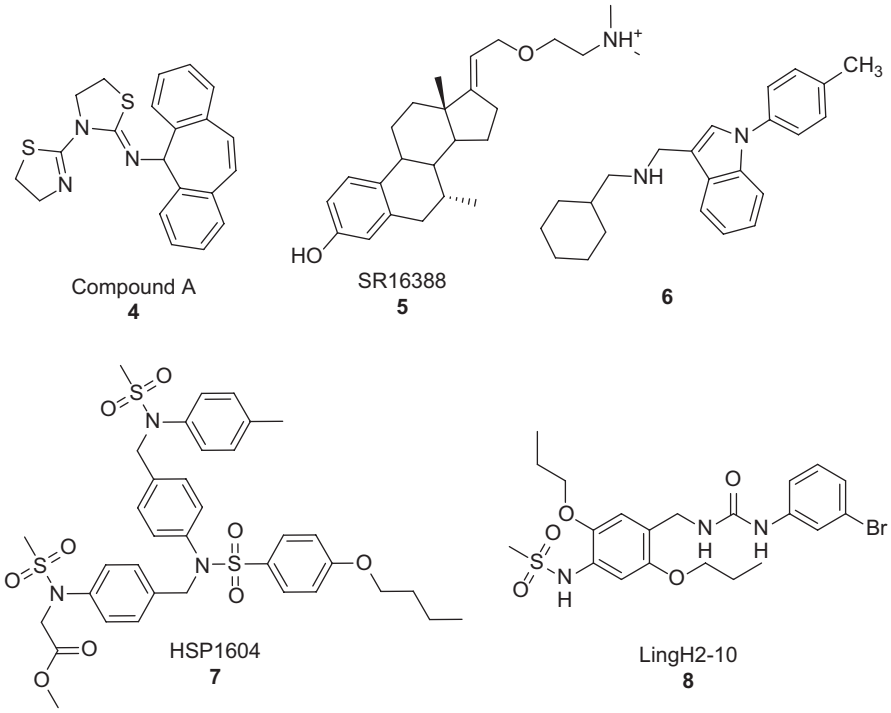


Fig. 5.9 Selective ERRα inverse agonists **4–8**

when combined with paclitaxel by 61% compared to untreated xenograft tumors. It also inhibited the proliferation of broad tumor cell lines after 24 h of treatment by SR16386 (**5**) [72]. Compound **6** is a synthetic inverse agonist that binds to ERRα LBP and induces significant conformational changes. There were unexpected movements in H12 which led to dislocation of H12 to bind in the coactivator binding groove [64]. Zhang and his coworkers identified HSP1604 (**7**) as a novel selective ERRα inverse agonist in a transient transfection luciferase reporter assay and a time-resolved fluorescence resonance energy transfer (TR-FRET) coactivator assay. HSP1604 (**7**) inhibited the ERRα transcription activity with $IC_{50} = 1.477 \pm 0.17$ μM and decreased protein and mRNA levels of downstream target genes such as PDK4, pS2, and SPP1. Additionally, **7** inhibited the proliferation of several human cancer cell lines and inhibited the proliferation of human breast cancer in vivo [73]. Similarly, LingH2–10 (**8**) was found to bind to ERRα and inhibits its transcriptional activity with $IC_{50} = 0.58 \pm 0.09$ μM in cell-based luciferase reporter assay. LingH2–10 (**8**) downregulated several ERRα target genes such as PDK4, SPP1, and pS2. It also inhibited the proliferation in several human cancer cell lines and the triple negative breast human cancer cell lines both in vitro and in vivo [74].

Kaempferol (**9**) (Fig. 5.10) is a dietary flavonoid that has antiproliferative and antiangiogenic activities and also induces cancer cell apoptosis [75]. Kaempferol

(**9**) was found to act as dual ERR inverse agonist that binds to both ERRα and ERRγ LBP blocking the binding site from interacting with the coactivator PGC-1α and suppressing the receptor transcriptional activity. Kaempferol (**9**) suppressed the expression of ERR target genes PDK2 and PDK4 [76]. Troglitazone (**10**) is another dual ERR inverse agonist that was found to interfere with ERRα and ERRγ interactions with PGC-1α. Troglitazone (**10**) suppressed PGC-1α and PGC-1β expression, which are key regulators of mitochondrial functions. Hence, it dwindled the mitochondrial mass and suppressed the expression of superoxide dismutase to increase ROS production, which in turn induce cell cycle inhibitor $p21^{WAF1}$ expression [77]. Recently, endogenous estradienolone-like steroid ED (**11**) was reported as the first endogenous inverse agonist for both ERRα and ERRγ in human as it was found to bind to their LBP and inhibits their transcriptional activity. ED (**11**) exhibited antiproliferative activity against both the negative and positive estrogen receptors breast cancer cell lines (MDA-MB-231 and MCF-7) in a dose-dependent manner. ED (**11**) anti-mitogenic effect in breast cancer cells is ERRα-dependent. Interestingly, it exhibited little to no effect on the normal epithelial breast cells [78]. On the other hand, 4-hydroxy tamoxifen (4-OHT) (**12**) is an ERRβ/γ inverse agonist that binds to their LBP, dissociate the complex between the receptor and coactivator SRC-1, and inhibit their transcriptional activity [79, 80]. 4-OHT (**12**) did not bind to ERRα binding site because of the bulky phenylalanine that corresponds to alanine in ERRγ. [^{3}H]4-OHT bound to recombinant ERRαF232A with affinity similar to ERRγ, which proves the similarity between ERR isoforms LBP [79].

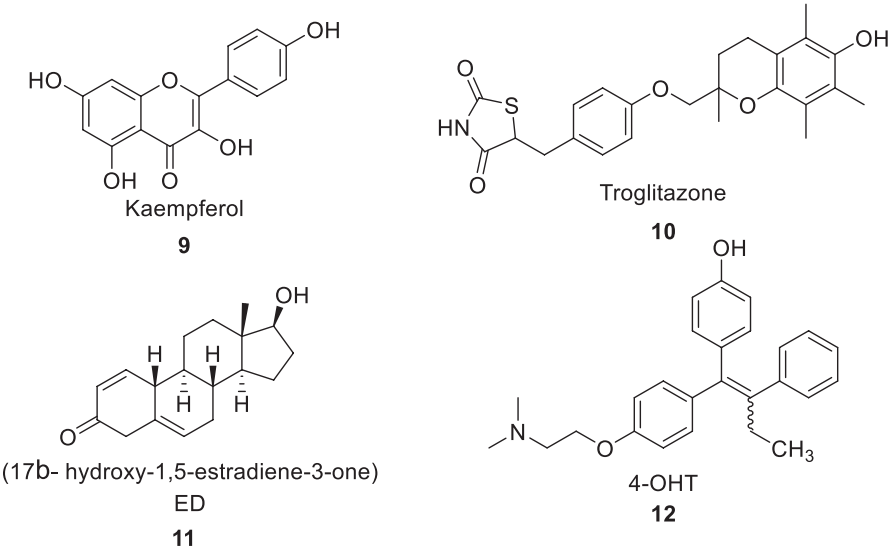
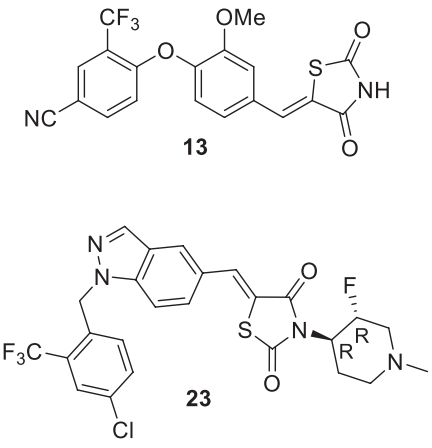
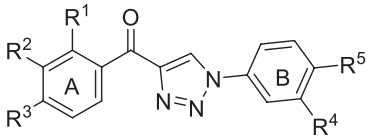


Fig. 5.10 Dual ERR inverse agonists **9–12**

The diaryl ether-based thiazolidinedione **13** (Fig. 5.11) was found to inhibit the peptide coactivators binding to ERRα with IC_{50} = 0.04 μM and 50-fold selectivity over ERRγ (IC_{50} = 2.8 μM). The X-ray crystal structure of ERRα with compound **13** complexed in the LBP revealed a conformational change and side-chain rotation of Phe328 to accommodate the ligand with a reversible covalent interaction between the protein and the ligand. Compound **13** normalized the insulin and circulating TGs level in two mouse models for obesity and type II diabetes with significant improvement of insulin sensitivity and glucose tolerance in vivo [81].

Xu et al. developed and synthesized a series of 1,2,3-triazole derivatives **14–22** (Fig. 5.11) as an orally bioavailable suppressors of ERRα transcriptional activity. Structure-activity relationship (SAR) studies showed that compound **14**, which incorporate OH group in ortho-position of ring A inhibited the transcriptional activity with IC_{50} = 0.39 μM. Replacing the OH group in the *ortho*-position with NH_2 group in **15** increased the inhibition activity 10-fold with IC_{50} = 0.041 μM. On the contrary, repositioning NH_2 group into *meta* (i.e. **16**) or *para* position (i.e. **17**) of ring **A** led to significant decrease in potency compared to **15** with IC_{50} = 0.31 μM and 0.16 μM, respectively. Compound **18** that bears an isopropyl group at *meta* position of ring B exhibited twice the potency of **15** in inhibiting ERRα transcriptional activity. The isopropyl group at the *meta* position of ring B is essential for the high potency of this series, and the existence of smaller or larger hydrophobic group at this position was crucial for suppression of ERRα transcriptional activity. Other compounds in the series with small hydrophobic groups like **20** and **21** were less potent and inhibited ERRα transcriptional activity with IC_{50} = 0.43 μM and 0.20 μM, respectively. The potency was directly proportional to the size of the hydrophobic group. For example, compound **21** with *t*-Bu in the meta position and **22** with phenyl group in the same position showed improved potency with IC_{50} values of 0.094 μM and 0.65 μM,





14: R^1=OH, R^2=R^3=R^4=H, R^5=i-Pr
15: R^1=NH_2, R^2=R^3=R^4=H, R^5=i-Pr
16: R^1=H, R^2=NH_2 ,R^3=R^4=H, R^5=i-Pr
17: R^1=H, R^2=R^4=H, R^3=NH_2, R^5=i-Pr
18: R^1=NH_2, R^2=R^3=R^5=H, R^4=i-Pr
19: R^1=NH_2, R^2=R^3=R^5=H, R^4=Et
20: R^1=NH_2, R^2=R^3=R^4=R^5=H
21: R^1=NH_2, R^2=R^3=R^5=H, R^4=t-Bu
22: R^1=NH_2, R^2=R^3=R^5=H, R^4=Ph

Fig. 5.11 Selective ERRα inverse agonists **13–23**

respectively. The 2-aminophenyl-(1-(3-isopropylphenyl)-1*H*-1,2,3-triazol-4-yl)methanone (**18**) was the most promising compound among the series in inhibiting the ERRα transcriptional activation with IC_{50} = 0.021 μM in a cell-based reporter gene assay. Compound **18** reduced both ERRα mRNA and protein levels. The most potent ERRα inverse agonists **15**, **18**, and **21** exhibited antiproliferative activity against MCF-7 cancer cell line in vitro with an IC_{50} of 6.7, 3.7, and 17.2 μM, respectively. Moreover, they inhibited the growth of MDA-MB-231 breast cancer cells. Compound **18** showed good pharmacokinetics with 71.8% oral bioavailability and 1.5 h in vivo half-life [82].

Patch et al. developed the indazole-based *N*-alkylthiazolidenediones as ERRα selective inverse agonists. Compound **23** was identified as the most potent and selective ERRα repressor among the developed series. It inhibited the recruitment of coactivator peptide by ERRα with IC_{50} value of 0.023 μM, with no activity against other metabolically active nuclear receptors. Besides, **23** enhanced the glucose control and reduced the circulating TGs upon chronic oral administration in two animal models for obesity and diabetes [83].

Du et al. designed novel small molecules inverse agonists for ERRα based on the most potent inverse agonist XCT-790 (**3**). They were able to identify a good hit, 1-(2,5-diethoxy-benzyl)-3-phenyl-urea (**24**) (Fig. 5.12), that resembles XCT-790 (**3**) in possessing 4-atom linker between rings A and B. However, compound **24** lacks an additional ring that exists in XCT-790 (**3**) and interact with ERRα via

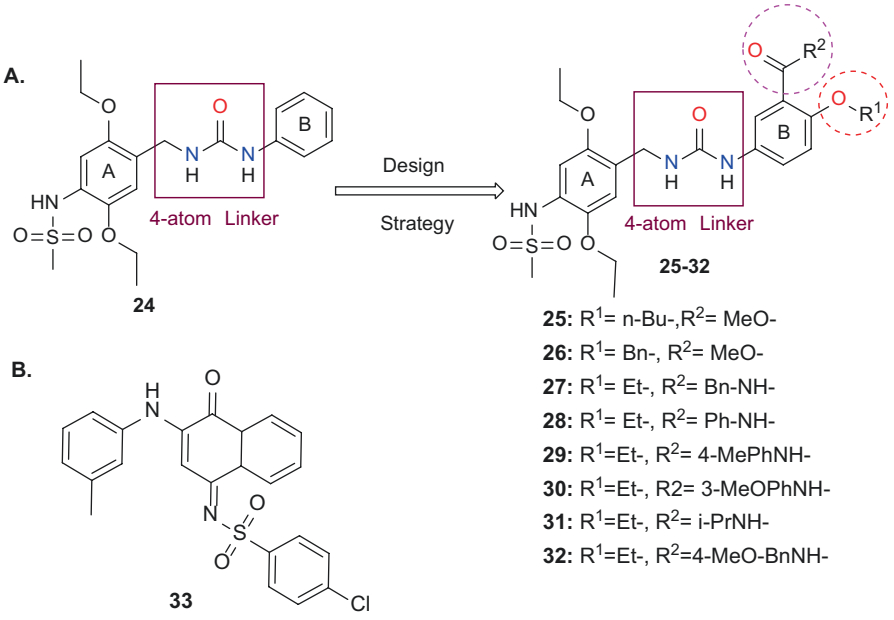


Fig. 5.12 Design strategy for selective: A. ERRα inverse agonist **24–32**. B. ERRα inverse agonist **33**

hydrophobic interactions. Compound **24** was weaker than XCT-790 (**3**), which may be attributed to the lack of ring C. Further chemical modifications led to the identification of compounds **25–32** (Fig. 5.12). Compound **25** which incorporate 3-MeOCO- and 4-BuO-groups in ring B was 6-fold more potent than the starting compound **24** with an IC_{50} value of 3.43 ± 1.17 μM. Compound **26** with benzyl group on ring B has better potency than **24** with IC_{50} = 4.06 ± 0.12 μM. Using a smaller group like ethyl group on ring B alongside with the bulky 3-Bn-NHCO-group in compound **27** resulted in a more potent inverse agonist with IC_{50} = 3.81 ± 0.17 μM. The enhanced activities were correlated to enhancement in the hydrophobic interactions with the LBP of ERRα. Further optimization of compound **27** led to identification of compounds **28–32**, which showed similar potency to compound **27** with IC_{50} < 4.23 μM. Besides, compound **32** exhibited the highest potency in inhibiting the transcriptional activity of ERRα with 2-fold improved potency over **27** with IC_{50} = 1.90 ± 0.39 μM. Further investigation of antiproliferative activity of compound **32** against several human cancer cell lines revealed that it has wide and strong antiproliferative effect on eight different cancer cell lines including ER-positive and ER-negative cancer cell lines. The best antiproliferative effect of **32** was observed against ER-negative MDA-MB-231 cells, where **32** inhibited the growth of this cell line with an IC_{50} value of 1.46 ± 0.12 μM [84].

Zhao and his coworkers used structure- and ligand-based approaches to identify novel ERRα inverse agonists. They succeeded to identify 17 compounds by virtually screening a library of 211,297 compounds. Only compound **33** exhibited superior activity over other compounds when they were tested for their ERRα- PGC-1α inhibition in a TR-FRET assay [85]. Moreover, compound **33** was more potent than XCT-790 (**3**) in inhibiting the ERRα- PGC-1α interaction with IC_{50} value of 0.5 ± 0.04 μM. Compound **33** was found to inhibit proliferation of MCF-7 cells with GI_{50} value of 1.3 ± 0.08 μM. Moreover, **33** inhibited ER-negative MDA-MB-231 breast cells proliferation with GI_{50} value of 2.5 ± 0.4 μM, which further suggest this compound is a good ERRα inhibitor [85]. Further investigation of the effect of compound **33** on the target genes and proteins of ERRα showed that **33** reduced the mRNA level of both PDK4 and PGC-1α in a dose-dependent manner in real-time PCR assay. Additionally, it reduced the levels of ERRα, PDK4, and PGC-1α proteins in western plot analysis. Compound **33** exhibited moderate anti-proliferative activity against both ER-positive and ER-negative breast cancer cell lines.

Molecular docking studies of **33** in the LBD of ERRα (PDB ID: 2JPL) shows that it forms two hydrogen bonds with the side-chain carboxylic acid of Glu331, which is important for the ERRα-PGC-1α inhibitory activity [85].

Lynch and his coworkers [86] identified a set of antineoplastic agents that could inhibit the ERRα signaling using HTS assays. They screened 10,000 compounds library in a HEK293 cells containing the ERRα-reporter or the reporter containing PGC-1 expression. Their study revealed two groups of ERRα antagonists that contains 78 compound that inhibit ERRα with efficacy >50% of the positive control, XCT-790 (**3**). The identified compounds inhibited the PGC/ERRα pathway, and results were confirmed using gene-expression studies. Bortezomib **35** (Fig. 5.13), a tubulin disruptor and topotecan **39**, a topoisomerase inhibitor, was among the most

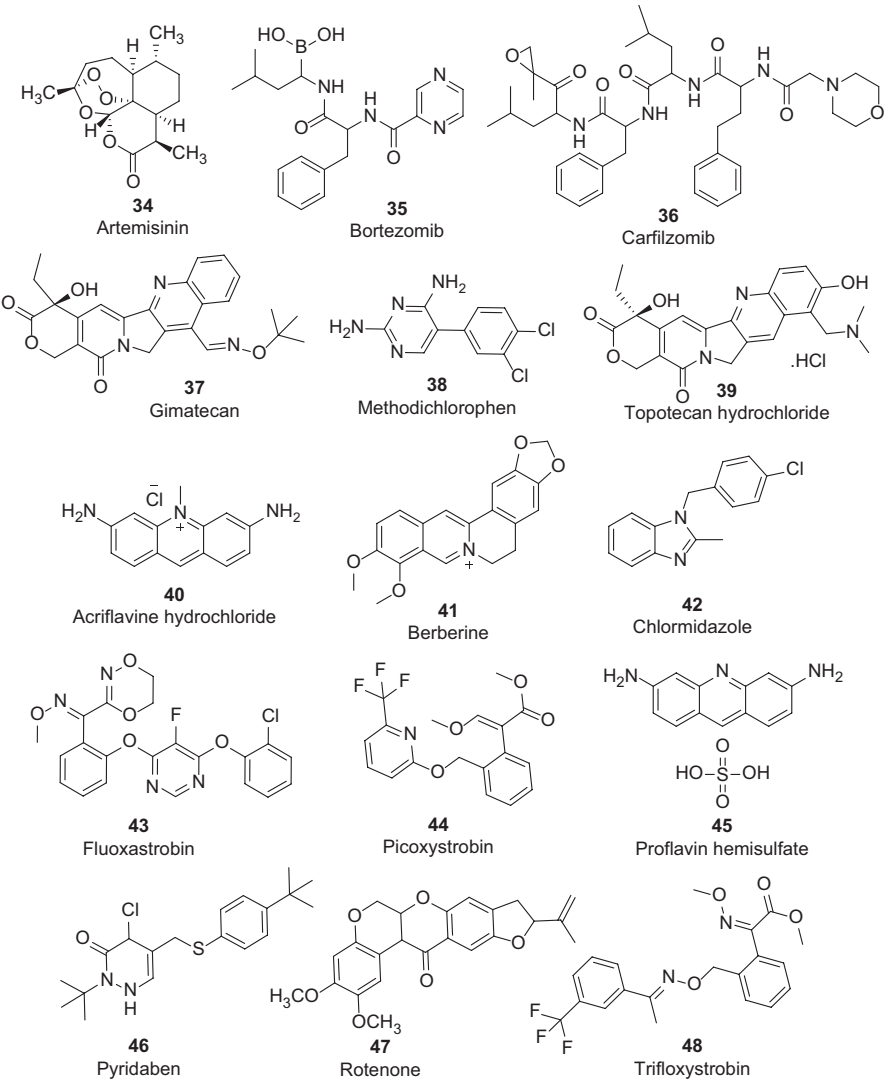


Fig. 5.13 ERRα antagonists **34–48**

potent ERRα antagonists identified from the screening. Five antineoplastic agents (**34–38**) and nine pesticides (**40–48**) (Fig. 5.13) were confirmed as an ERRα antagonists and were able to suppress ERRα target genes in gene expression studies [86].

Selective ERRγ inverse agonists were developed based on 4-OHT using structure-based design. GSK5182 (**49**) (Fig. 5.14) exhibited 25-fold selectivity toward ERRγ over ERα with an IC_{50} value of 0.250 μM. Compound **49** showed a hydrogen bonding interaction with Tyr326 and Asn346 residues in the LBP of ERRγ (PDB ID: 2EWP). It showed a similar binding mode to that of 4-OHT complexed with the

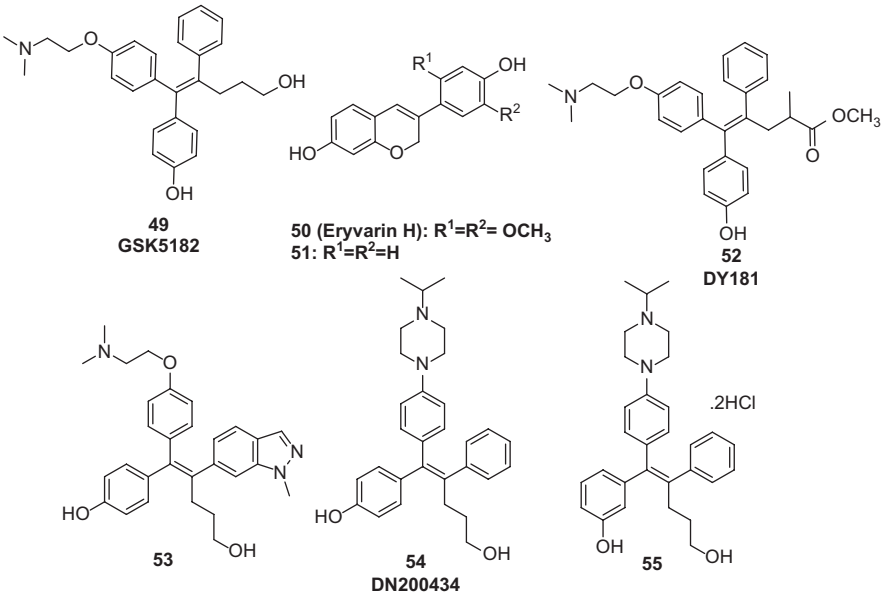


Fig. 5.14 ERRγ inverse agonists **49–55** and ERRβ inverse agonist **52**

LBP of ERRγ. It showed interaction between the phenolic part with the Glu275 and Arg316, while the other part interacts with Asp273 [87].

Koo and his coworkers used structure-based virtual screening of 4000 structurally diverse natural products in the X-ray of ERRγ bound with inverse agonist GSK5182 (**49**) (PDB ID: 2GPU). Eryvarin H (**50**) was identified as an ERRγ inverse agonist. Docking of Eryvarin H (**50**) in ERRγ-LBP revealed both dipole-dipole interactions and hydrophobic interactions. The most important interactions were the hydrogen bonding interaction between the hydroxyl group on Eryvarin H (**50**) and amino acids Asp273, Tyr326, and Asn346 and dipole-dipole interaction between the ether linkage of Eryvarin H (**50**) and Cys269. Eryvarin H (**50**) showed good inverse agonist activity against ERRγ when tested in cell-based reporter gene assay in HEK-293 T cells at 10 μM. Compound **51** (Fig. 5.14), an eryvarin H derivative, exhibited good inverse agonistic activity against ERRγ. The activity of both **50** and **51** was very weak compared to GSK5182 (**49**) but showed selectivity toward ERRγ over ERα [88]. Compound **52** (DY181) (Fig. 5.14) was reported to be the most potent and selective ERRβ to date with IC_{50} value of 0.01 μM. Molecular docking studies revealed that it forms an extensive hydrogen bonding with the ERRβ-LBP residues. In the homology model of the ERRβ protein, **52** formed 3-hydrogen bonds with Asp248, Glu250, and Tyr321 residues in the ERRβ-LBP [89].

Kim and her coworkers developed compound **53** (Fig. 5.14) as an inverse agonist for ERRγ. This compound was identified through structural modification of GSK5182 (**49**). **53** exhibited good binding toward ERRγ over other subtypes (i.e., ERRα, ERRβ, and ERα) with IC_{50} of 0.44 μM, and showed 95% transcription repression at 10 μM while maintaining acceptable AMDET profile [79].

Similarly, Compound **54** (DN200434) was identified based on GSK5182 (**49**) as a novel, highly potent, and selective ERRγ inverse agonist (IC_{50} = 0.006 μmol/L). Compound **54** was validated as lead compound using an array of biochemical and cell-based assays. Binding affinity was measured to assess the subtype selectivity of **54**, and it exhibited good selectivity toward ERRγ (IC_{50} = 0.040 μmol/L) over ERRα, ERRβ, and ERα (IC_{50} > 10, 1.330, 1.240 μmol/L, respectively). X-ray crystal structure of **54** complexed with ERRγ showed a similar binding mode to ERRγ. GSK5182 (**49**). The binding affinity of **54** to ERRγ was higher than GSK5182 (**49**) due to better fitting in the LBP and stronger interactions with Tyr326, Asn346, and Glu275. The OH group of **54** makes two hydrogen bonding interactions with Tyr3266 and Asn346. Phenol makes hydrogen bonding interation with Glu275 while piperazine nitrogen forms a hydrogen bonding interaction with Asp273 (Fig. 5.15). Movement of both His434 and Phe435 led to tighter packing of **54** inside the LBP. Compound **54** retained inhibitory activity of ERRγ upon mutation of key amino acid residues Glu275, Tyr326, and Asn346 into alanine. However, the inhibitory effect of **54** was lost upon mutation of Asp273 into alanine, which suggests that the ionic interaction of **54** basic chain with Asp273 is crucial for **54** to retain activity toward ERRγ.

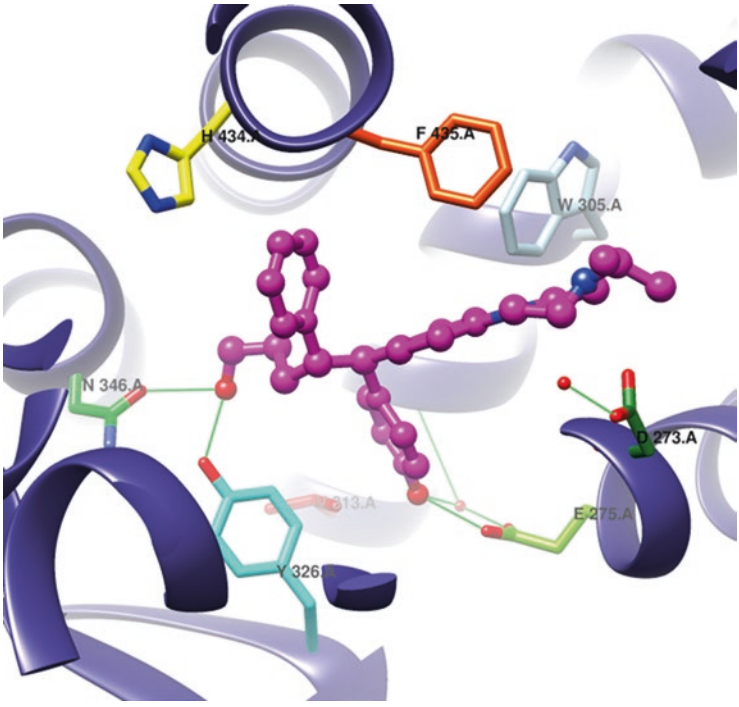


Fig. 5.15 X-ray of compound **54** inside the LBP of ERRγ

From ^{124}I PET/CT imaging analysis, compound **54** enhanced ATC tumor radioiodine avidity in an ATC tumor model via upregulation of iodide-handling genes. This in turn led to successful radioiodine therapy of conventional radioiodine therapy-refractive ATC tumors [90]. These results suggest that **54** and related compounds are potential therapeutic agents toward ERRγ-related cancers [91].

5.3.2 ERRs Agonists

Suetsugi and his coworkers reported flavone **56** and isoflavones **57–60** (Fig. 5.16) as ERRα agonists using a combination of structure-based virtual screening and receptor-binding assays. The virtual screening was performed in a homology model of ERRα. Compounds **56–59** can activate ERRs at 10 μM in both mammalian cell transfection and mammalian two-hybrid experiments [92].

Molecular docking of identified ligands showed that they packed tightly inside the small LBP of ERRα. The ligands' hydroxyl groups form hydrogen bonds with the conserved water molecule and with Glu235, Arg276, and His398 residues. Additional hydrophobic interactions between ligands' aromatic rings and several phenyl alanine residues help in fixing these ligands in favorable binding conformation. The modelling data suggest that planar ligands with aromatic rings will be favorable ERRα agonists [92].

Equol (**60**) (Fig. 5.16), an isoflavone, was found to act as an ERRγ agonist and stimulated its transcriptional activity in transiently transfected PC-3 and U2-OS cells. Moreover, **60** enhanced the interaction between the GRIP1 and ERRγ. **60** induced a conformational change in the ERRγ-LBD and enhanced the ERRγ growth inhibition effect on the prostate cancer PC-3 cell lines [93].

Pyrido[1,2-α]pyrimidine-4-ones (Fig. 5.17) were identified as ERRα agonists, and they were found to enhance the transcriptional activity and to elevate both the

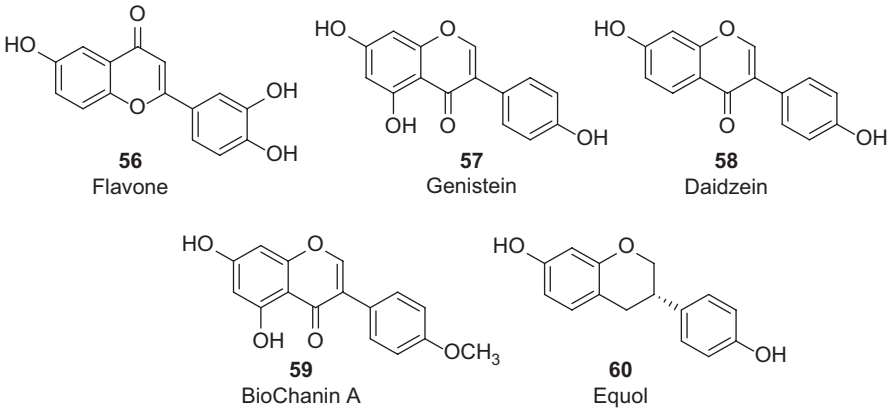


Fig. 5.16 Flavone and isoflavones **56–60** ERRs selective agonists

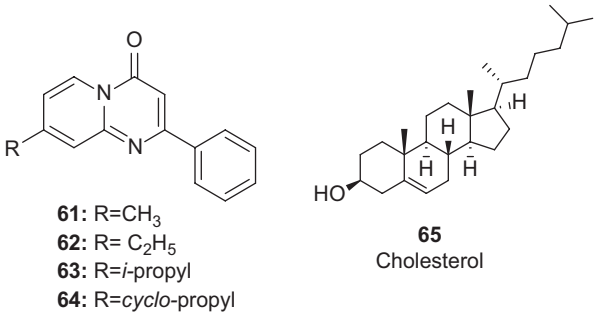


Fig. 5.17 ERRα agonists **61–65**

mRNA levels and the protein levels of ERRα downstream target genes [94]. Compound **61** upregulated the transcriptional functions of ERRα. Replacement of the 8-methyl group of **61** with slightly larger hydrophobic groups like ethyl (*cf*. **62**), isopropyl (*cf*. **63**), and cyclopropyl group (*cf*. **64**) improved ERRα activity 2.7-, 2.5-, and 2.6-fold, respectively at 10 μM. Compound **62** enhanced the transcriptional activity of ERRα in a dose-dependent manner and elevated the ERRα- driven luciferase activity in Kaempferol pretreated 293FT cells where ERRα basal constitutive activity was reduced. Additionally, **62** was found to moderately elevate the transcriptional activity of ERRγ; and improved the glucose and fatty acid uptake in the C2C12 muscle cells [94].

Cholesterol (**65**) (Fig. 5.17) was reported to function as an ERRα agonist and bound to the ligand binding pocket forming a hydrogen bonding interaction between its hydroxyl group and the Glu235 residue in addition to other hydrophobic interactions with Phe232 and Leu228 residues. Upon binding to the LBP of ERRα, **65** induced conformational changes, increased PGC-1 coactivator recruitment, and enhanced the ERRα-mediated transcription [95].

N-Acyl hydrazones were reported as agonists for ERRγ [96, 97]. DY131 (**66**) is a selective ERRγ agonist at lower concentrations. It was reported to effectively enhance the activity of ERRγ by 5-fold at 3 μM and 6.6-fold at 30 μM. **66** was reported to activate ERRβ 3- to 4-fold at 10–30 μM [97]. Compound **66** (GSK9089) has a binding affinity of 0.66 μM in an ERRγ FRET assay with 55% efficacy [96]. GSK4716 (**67**) exhibited remarkable selectivity toward ERRβ and ERRγ over other estrogen receptors. GSK4716 (**67**) was found to activate both ERRβ and ERRγ in comparable way to the protein ligand PGC-1α [96].

Compound **68** (**E6**) (Fig. 5.18) is a synthetic acylhydrazone derivative that was identified as a selective and potent ERRγ agonist derived from **GSK4716** agonist through a combinatorial approach [98]. **68** was identified from a library of 30 compounds that was designed based on analyzing the interactions of agonist **GSK4716** and inverse agonist **4-OHT** in the LBD of ERRγ at the molecular level. Both **GSK4716** and **4-OHT** bind in two distinct pockets in the LBD of ERRγ. The isopropyl group of **GSK4716** extends into the second pocket where the phenolic part of **4-OHT** binds. The new ligands were designed to fit the two pockets and maintain the favorable interactions of **GSK4716**. The designed ligands were screened

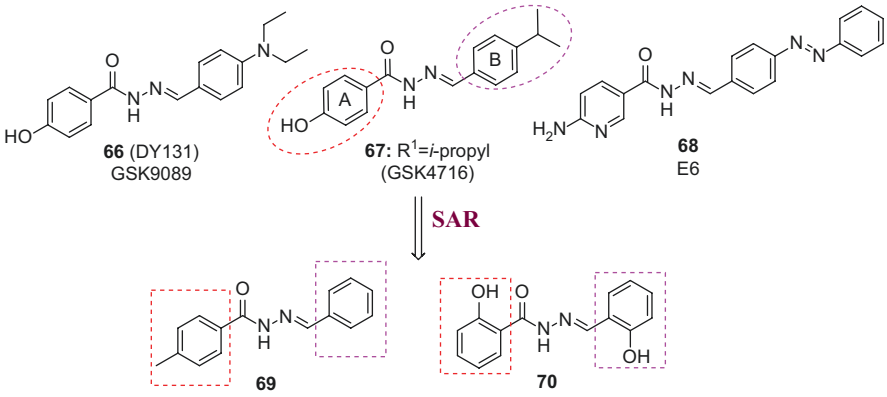


Fig. 5.18 Acylhydrazone agonists based on **GSK4716:** Selective ERRγ Agonists (**66–68**) and ERRα/γ pan agonists (**69–70**)

virtually in the two cavities identified earlier for both **GSK4716** and **4-OHT**. The ligands with highest docking scores and favorable interactions were synthesized using microwave-assisted solution phase parallel synthesis. The synthesized compounds were evaluated in a cell-based reporter-gene assay using **GSK4716** and **4-OHT** as controls. Compound **68** (E6) enhanced the transcriptional activity of ERRγ and did not show agonistic effect toward ERRα and ERRβ. The potency of **68** was comparable to **GSK4716** at both 10 and 1 μM concentrations [98].

Due to the lack of chemical probes to study ERRα, many groups embarked on developing ERRα agonists with limited success so far. Elgendy lab was successful in engineering high affinity ERRα agonism into **GSK4716** (selective ERRβ/γ dual agonist) [101]. They used structure-based design approach to identify the key structural requirement to induce ERRα agonism. Removing hydroxyl group of **GSK4716** that form hydrogen bonding interaction with Glu384 in ERRγ proved to be important to increase affinity toward ERRα. Additionally, removing bulky group at the para position of ring B was important for enhancing ERRα activity since the LBP of ERRα is much smaller in volume. Changing the length of the linker was detrimental to activity toward both isoforms. Compound **69** (Fig. 5.18), which incorporate a *p*-methyl substituent at ring A while no substituent on ring B, enhanced the potency toward ERRα (EC_{50} = 0.209 μM) and maintained good activity toward ERRγ (EC_{50} = 0.194 μM). This compound was equipotent toward both isoforms with EC_{50} α/γ = 1.07. Molecular modeling of compound **69** in the LBD of both ERRα and ERRγ showed that it forms л-л interactions between ring B and Tyr326 in ERRγ and Phe328 on helix 3 in ERRα. The NH of the *N*-acyl hydrazone forms hydrogen bonding interaction with Phe382 in ERRα while it forms the same interaction with Tyr326 in ERRγ. The binding mode of **69** inside the LBP of ERRα showed that it induced conformational changes where Phe328 was displaced to accommodate the ligand inside the cavity.

Compound **70** (Fig. 5.18) with two hydroxyl groups on the ortho-position of both rings was less potent than **69** but was fourfold more selective toward ERRα over ERRγ (EC_{50} = 0.378 μM for ERRα and EC_{50} = 1.645 μM for ERRγ) with EC_{50}

α/γ = 0.23. The binding mode of **70** was similar to **69** with the hydroxyl group on ring A forming a hydrogen bond interaction with Glu331 in the LBP of ERRα.

The best ERR pan agonists identified by Elgendy lab elevated the expression of ERR target genes, PGC-1α, PGC-1β, CPT1α, and PDK4 in C2C12 mouse myoblast cell line. They did not exhibit any signs of toxicity at 1 and 10 μM concentrations [99].

Bisphenol A (**71**) [BPA; 2,2-bis(4-hydroxyphenyl) propane] (Fig. 5.19), an endocrine disruptor and ER ligand, was found to strongly and selectively bind to ERRγ (K_D = 5.5 nM) with high activity in a dose-dependent manner with IC_{50} value of 13.1 nM. It showed 5- to 50-fold higher potency than 4-nonylphenol and DES. The X-ray crystal structure of **71** complexed with the ERRγ-LBD revealed that one of the hydroxyl groups forms a hydrogen bonding with Glu275 and Arg316 residues and the other hydroxyl group with Asn346. Additionally, **71** did not disrupt helix 12 and maintained the active conformation, but it deactivated ERRγ in the reporter gene assay suggesting that it can act as agonist/inverse agonist for ERRγ [100–102]. Furthermore, Okada and his coworkers specified the structural characteristics for a strong binding of Bisphenol A and its derivatives with human ERRγ. They demonstrated that only one hydroxyl group was essential for the full binding and removing one methyl group allowed the maximum activity. The calculated binding affinity constant from the Scatchard plot analysis data exhibited K_D of 5.50 nM, while the receptor density (B_{max}) was 14.4 nmol/mg. 4α-cumylphenol (**72**), a one-hydroxyl group-deficient analogue of BPA, exhibited activity comparable to BPA, which suggests that phenol derivatives are good endocrine disruptor that binds to ERRγ [103]. 4-α-Cumylphenol (**72**) (Fig. 5.19), a derivative of **71** lacking one hydroxyl group, bound tightly to ERRγ- with IC_{50} value of 13.9 nM. This tight binding was attributed to the back and forth rotation of the Leu345-β-isobutyl group in an induced fit manner [104].

Parabens (**73a–e**) (Fig. 5.19), a *p*-hydroxy benzoic acid esters that vary in the ester group, were demonstrated to have an agonistic activity similar to PBA toward ERRγ in a coactivator recruiting assay and in silico molecular docking. All tested parabens **73a–e** with methyl, ethyl, butyl, propyl, and benzyl groups exhibited potent agonistic activity toward ERRγ. Compounds **73a–e** were well-fitted in the ERRγ active site forming a hydrogen bond between parabens hydroxyl group and both Glu275 and Arg316 residues in ERRγ LBD [105].

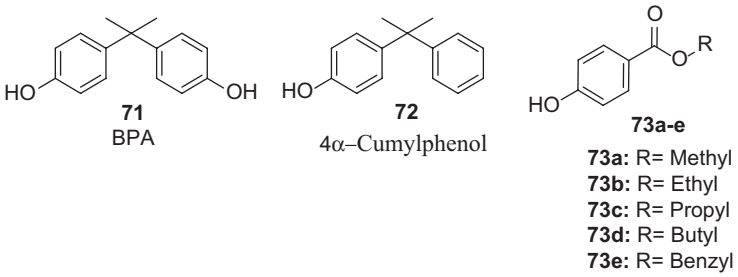


Fig. 5.19 ERRγ agonists **71–73**

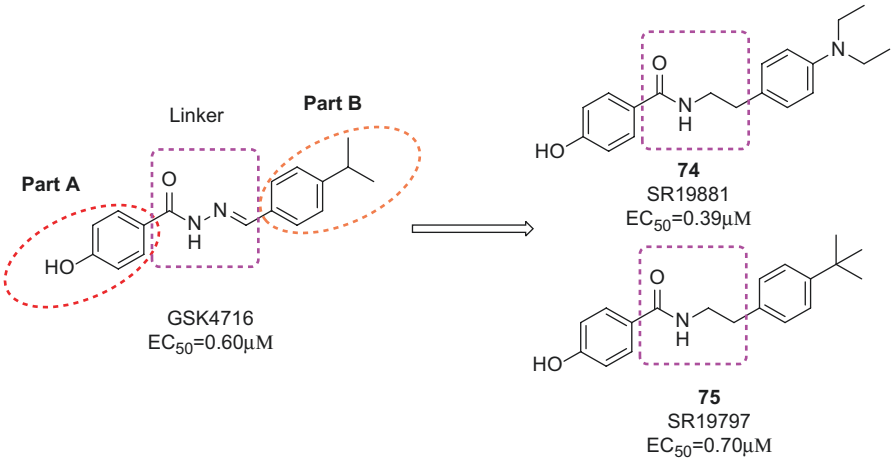


Fig. 5.20 Design strategy for ERRγ amide phenol agonists **74–75**

Recently, Lin and his coworkers designed and synthesized novel ERRγ agonists based on **GSK4716** as lead compound. Their design strategy was based on replacement of the unstable hydrazide moiety, which was most likely responsible for the observed poor metabolic stability of **GSK4716** analogs with more stable amide group. They explored the structure activity relationships of the lead compound where they modified parts A and B, and the hydrazone linker. They explored several bioisosteres of the *N*-acyl hydrazone linker (e.g. ureas, sulfonamides, α,β-unsaturated ketones, … etc) and the amide bioisostere was the only successful replacement. Exploration of parts A and B led to identification of SR19881 (**74**) and SR19797 (**75**) (Fig. 5.20) as the most potent agonists within synthesized analogs. Both compounds are structurally similar to the lead **GSK4716** with the same phenol group in part A and bulky substituent at the para position of part B. SR19881 (**74**) was slightly selective toward ERRγ (EC_{50} = 0.39 μM) over ERRβ (EC_{50} = 0.63 μM) in FRET assay. SR19881 (**74**) exhibited weak agonistic activity in a cell-based assay (EC_{50} = 4.7 μM) [106].

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Chapter 6
PPARα and δ Ligand Design: Honing the Traditional Empirical Method with a More Holistic Overview

Benjamin S. K. Chua and John B. Bruning

Abstract Peroxisome proliferator-activated receptor (PPAR) ligands have been used in clinical therapy to treat metabolic disease since the 1960s. However, these ligands have side effects that restrict their use, thought to be caused, in part, by their broad specificity. Efforts have been made to synthesize new ligands; however, most have failed to pass clinical trials. Here we examine the available crystal structures of PPAR in complex with ligands to identify common ligand design factors for selectivity towards a PPAR subtype. Methods to improve drug-lead identification and optimization and other factors that may contribute to design of a successful PPAR ligand are discussed.

Keywords PPAR · Metabolism · Specificity · Structure guided drug design

6.1 Introduction to the PPAR Protein

The peroxisome proliferator-activated receptors (PPARs) are a group of ligand-activated nuclear receptors that control various cellular pathways, most notably involved in energy metabolism and homeostasis [23, 61]. These receptors belong to the nuclear receptor type II (NR2) subgroup, which dimerizes with the retinoid X receptor Retinoid X Receptor (RXR). There are three subtypes of PPAR in humans, each with a distinct tissue distribution and function [23, 62]. PPARα coordinates the regulation fatty acid metabolism and is predominantly expressed in the liver, heart and intestines [23, 85]. Activation of PPARα improves serum lipid and cholesterol profiles as well as glucose tolerance in type 2 diabetes mellitus (T2D) patients [23,

B. S. K. Chua · J. B. Bruning (✉)
The University of Adelaide, Adelaide, SA, Australia
e-mail: john.bruning@adelaide.edu.au

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116]. PPARγ controls adipogenesis and is expressed mainly in adipose tissue and macrophages [18, 51]. Activation of this receptor induces adipocyte differentiation and improves insulin sensitivity and glucose tolerance [23]. PPARδ is expressed ubiquitously, at lower levels throughout the body [7, 19, 102]. Its exact function has not been determined to date, but PPARδ has been shown to play a role in lipid, glucose, lipoprotein and cholesterol metabolism, cancer, muscular fiber switching and endurance and cell differentiation in the CNS [7, 81, 102, 109, 113, 115]. The PPARs coordinate a wide array of genes that, when disrupted, lead to diseases such as metabolic syndrome, heart and neurological diseases and even cancer [23]. Thus, the PPARs represent a prime target for drug development [23, 82].

In fact, synthetic ligands for the PPARs have already been in development for a few decades. It was observed early on that PPARs were able to bind a wide variety of naturally occurring and synthetic ligands [31, 106]. Fibrates have been in clinical use for dyslipidemia since the 1960s but were only found to act through PPARα later on [80]. The discovery of the PPAR NR subfamily sparked a wave of development of synthetic PPAR ligands, of which two classes are currently licensed for clinical use: Fibrates, which bind PPARα preferentially, are used as hypolipidemic agents and Thiazolidines (TZDs) that bind PPARγ preferentially and are used as hypoglycemic agents and insulin sensitizers [23, 91]. However, these drugs have either limited efficacy or presented side effects that limit their therapeutic utility; these include edema, obesity, osteoporosis, hepatotoxicity, bladder cancer, increased atherogenic risk, and mortality rate [16, 19, 23, 111]. Currently, there are no clinically approved drug classes for PPARδ. In the early 2000s, PPARδ agonist GW501516 was synthesized by GlaxoSmithKline (GSK) and displayed excellent cholesterol-modulating properties. However clinical trials of GW501516 were halted when it was found to be carcinogenic in mice models [28, 64, 65]. Initially, to alleviate the side effects seen from single PPAR subtype activation, the idea of dual or even pan subtype acting agonists was conceived. The Glitazar drug class was aimed at reducing the weight gain from PPARγ activation with the fat-oxidizing or energy-modulating properties of PPARα and δ activation, respectively. However, most have been dropped from clinical trials due to various safety concerns [23, 71, 72]. Later, research moved towards improving subtype selectivity [28, 58, 63–65].

The fear of side effects, widely thought to be caused by the lack of subtype selectivity, limits the exploitation of the enormous therapeutic utility of PPAR activation [16, 23]. Despite this, attempts to identify ligands of PPAR and new therapeutic uses for existing drugs are still underway [23, 28]. To facilitate this effort, this review attempts to provide tools to design a subtype specific ligand. First, we present an introduction to the PPAR protein structure, focusing on its ligand binding domain (LBD), and we review different ligands and examine the structural basis for their selectivity and potency, to provide possible explanations for variations of potency between ligands. We also present common ligand design factors, followed by novel methods and other insights that could streamline the reader's efforts in designing a clinically effective and, more importantly, safe PPAR ligand.

6.1.1 *Overall PPAR LBD Protein Structure*

Like most nuclear receptors, the PPAR protein consists of five domains A–E (Fig. 6.1a). The highly mobile domains A and B contain the activation function 1 (AF1) sequence which is involved in ligand-independent transcriptional activation and nuclear localization and also contain sites of phosphorylation [21, 62, 105]. This is followed by domains C, the DNA binding domain (DBD); D, the hinge region; and E, which contains the LBD and Activation Function 2 (AF2) surface (Fig. 6.1a). Amino acid sequence numbers for the PPAR LBD referred to here were taken from the following UniProt entries: Identifier Q07869-1 for PPARα, P37231-2 for PPARγ and Q03181-1 for PPARδ. The secondary structure elements in the LBD were first numbered based on the RXR crystal, a convention that has been used by most crystallographers since (Fig. 6.1b) [106, 113, 114].

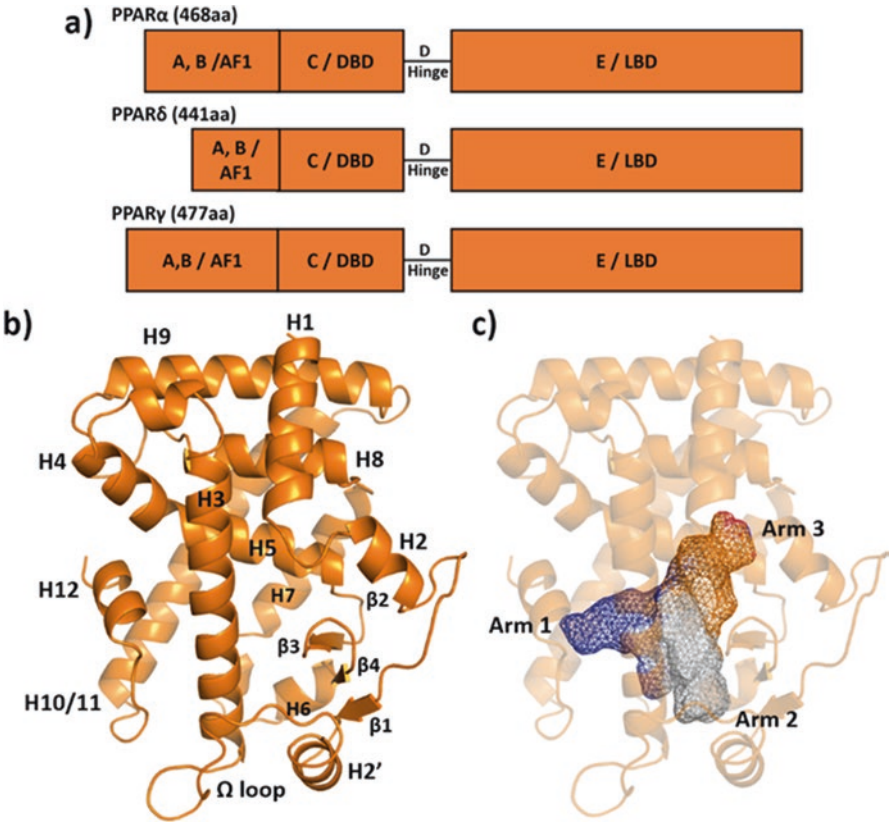

Fig. 6.1 (**a**) The domain architecture of the PPAR protein subtypes. (Adapted from Uppenberg et al. [106]). (**b**) The canonical PPAR LBD with helices labelled according to convention established by Uppenberg et al. [106] (PDB ID: 2GWX). (**c**) The PPARδ Y-shaped LBD cavity highlighted (PDB ID: 2GWX Arms 1: dark blue, 2: light grey, 3: orange)

The LBD (domain E, Fig. 6.1b) consists of 13 α helices and 4 β-stranded sheets. The LBD contains a Y shaped cavity of approximately 1300 Å, bordered by helices H4 and 5 superiorly, H2′ and H6 inferiorly, H3 and Ω loop anteriorly, H7 and H10/11 posteriorly and H12 and β stranded sheets laterally (Fig. 6.1b) [114]. Most ligands of PPAR bind in this cavity, referred to as the ligand binding pocket (LBP). The LBD contains the AF2 surface, which sits in a cleft above H12, formed by H3, H4, H5 and H12. This surface contains the four polar residues that typically form hydrogen bonds with ligands binding in the LBP (Table 6.1). The LBD also contains a dimerization surface made up of H8, H9 and H10 (Fig. 6.2) [42, 115].

The PPAR LBD, compared to other nuclear receptors, contains two unique structural characteristics. Firstly, PPAR contains an extra helix, designated as helix H2′ between the first β sheet strand and helix 3. The extra helix H2′ increases the space in the PPAR LBD (the largest of the NRs) which allows accommodation of a wide variety of ligands [113]. Due to the position of H2′, H2 is arranged differently in the tertiary structure and is hypothesized to allow easier access for ligands [33, 79]. Secondly, the H2′–H3 loop or the 'ω loop' is thermally mobile and typically left unmodelled in most PPAR structures [79, 113]. This loop is the proposed entry site for PPAR ligands and is restricted by a hydrogen bond 'latch', formed by residues α: Y334 and E282, γ: E343 and E291 and δ: N307 and E255 [11, 27, 33]. This flexible H2′–H3 loop is thought to allow PPAR to adapt to chemically diverse ligands [113].

6.1.2 Mechanism of PPAR Gene Transcription

PPAR forms an obligate dimer with RXR, regardless of ligand binding status, via helices 8, 9, 10 and 12 (Fig. 6.2) [7, 21, 42, 121]. The PPARs can dimerize with all three subtypes of RXR, forming different PPAR-RXR subtype complex combinations [108]. These PPAR-RXR complexes then bind to PPAR response elements (PPREs), which are comprised of direct repeat elements of hexanucleotide sequences separated by a single nucleotide spacer (DR1), recruit various cofactors and assemble the gene transcriptional complex to regulate gene transcription [17, 121].

In the apo or antagonist bound state, H12 of PPAR remains flexible and assumes a range of conformations [14, 21, 40, 95, 115, 116, 123]. When H12 is flexible, the larger 3 turn α-helical LXXXIXXXL corepressor motif preferentially occupies the space parallel to H3, bordered by H3, H4, H5 and H12 [52, 116]. This corepressor motif is positioned by interactions between the conserved clamp and motif residues α: K310 and E+2, the carbonyl backbone of α: Y464 and K+7 and α: K292 and carbonyl backbone of L+9 (Fig. 6.2b.). This positions the L+1, I+5 and L+9 residues optimally to interact with the cleft between H3 and H4. The PPAR-corepressor complex then binds to PPREs and causes repression of downstream target genes [88, 102, 121].

Table 6.1 Corresponding residues of the PPAR LBD subtypes
Conserved residues are highlighted in grey, the four polar residues of the AF2 surface that form hydrogen bonds are highlighted in green. Residue numbers used are taken from UniProt ID: P37231-2, Q03181-1, Q07869-1 and aligned using Jalview

Region	PPAR±	PPAR´	PPAR³	Secondary structure
Arm 1	E269	S242	A278	H3
	F273	F246	F282	
	Q277	Q250	Q286	
	S280	T253	S289	
	Y314	H287	H323	H5
	I317	I290	I326	
	F318	F291	Y327	
	F351	F324	F360	H7
	I354	I327	F363	
	H440	H413	H449	H10/11
	V444	M417	L453	
	I447	I420	I456	
	K448	K421	K457	H11-12 loop
	A454	T427	M463	
	A455	S428	S464	
	L456	L429	L465	
	L460	L433	L469	H12
	Y464	Y437	Y473	
Arm 2	I241	I213	I249	β-sheet 4
	M244	I216	M252	H2'
	L247	L219	L255	
	A250	A222	G258	
	E251	E223	E259	
	A268	I241	V277	H3
	R271	H244	R280	
	I272	V245	I281	
	C275	R248	G284	
	C276	C249	C285	
	T279	T252	R288	
	V332	V305	I341	β-sheet 2
	I339	V312	M348	β-sheet 3
	F343	F316	F352	H6
	L344	L317	L353	
	L347	L320	L356	
	M355	I328	M364	H7

Arm 3	N219	N191	P227	H1-2 loop
	M220	M192	L228	
	E282	E255	E291	H3
	T283	T256	A292	
	E286	E259	E295	
	M320	M293	M329	H5
	L321	L294	L330	
	V324	I297	L333	
	M325	V298	M334	β-sheet 1
	M330	L303	V339	β-sheet 2
	L331	L304	L340	
	Y334	N307	E343	β-sheet 2-3 loop
	K358	K331	K367	H7
	F359	F332	F368	

Tab. 6.1 (continued)

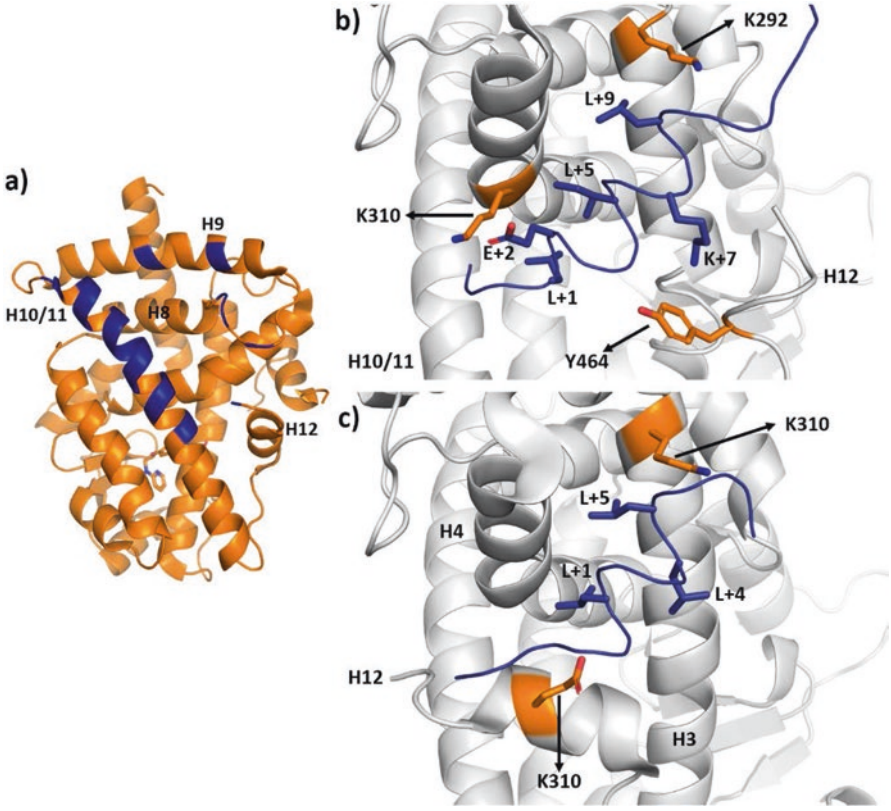

Fig. 6.2 (**a**) PPARδ LBD dimerization surface highlighted (PDB ID: 2GWX), (**b**) PPARα LBD complexed with the corepressor motif (PDB ID: 1KKQ) and (**c**) the coactivator motif (PDB ID: 1K7L)

On agonist binding, H12 is stabilized in the active position through direct or indirect interactions with the ligand [11, 106, 113]. H12 interaction is crucial, but not sufficient for full activation of PPAR [6]. In this active position, the AF2 surface is stabilized – the 'charge clamp' residues α: E462 δ: E436 γ: E472 and α: K292 δ: K265 γ: K301 are optimally positioned to interact with the coactivator motif LXXLL. The charge clamp positions the leucine residues to interact with the hydrophobic pockets created by H3, H4 and H12 and stabilizes the complex. The charge clamp is essential for interaction with the smaller LXXLL motif [116]. The PPAR-RXR-coactivator complex then binds to PPREs, recruits the gene transcriptional machinery and transcribes PPAR controlled genes.

The PPAR complex can also exert functions through non-genomic means, for example, by sequestering coregulators from other metabolic pathways in an apo state or binding to kinases and phosphotases [43, 66, 102].

6.1.3 Differences in LBD Between PPAR Subtypes

Although the overall fold of the three receptors is very similar and about 80% of the LBD residues are conserved, each subtype has a distinct pharmacological or ligand binding profile [41, 101]. The differences in the sequence of the PPAR subtypes lie in regions that are likely to affect ligand binding, such as the Ω loop and, more importantly, the ligand binding pocket [27, 113]. Of note, the Ω loop and H9 regions contain the highest sequence deviation between the PPAR subtypes, which could have implications in interactions with other components of the gene transcriptional complex [21].

Generally, differences in amino acid sequence change the possible interactions that each PPAR subtype can form with the chemical motifs of a ligand; mutation of a single residue was sufficient to change the ligand binding phenotype of one subtype to another [101]. These differences in the ligand binding pocket confer the affinity of each subtype for certain ligands and can be exploited to create subtype specific ligands. In Table 6.1, we list the residues of the ligand binding pocket of the PPAR subtypes [41].

6.2 Catalogue of Known Ligands

Numerous attempts have been made by research groups to exploit the therapeutic effects of PPAR activation. Counting structural-based investigations alone, there are 288 unique entries in the Protein Data Bank (PDB) for PPAR-ligand complexes as of 2020 (rcsb.org) [10]. However, there are currently only 9 PPAR-activating ligands that are approved for clinical use [23]. To attempt to bridge this disparity between effort and successes, we examine the previous attempts at synthesizing PPAR ligands, focusing on ligand design and PPAR-ligand interactions.

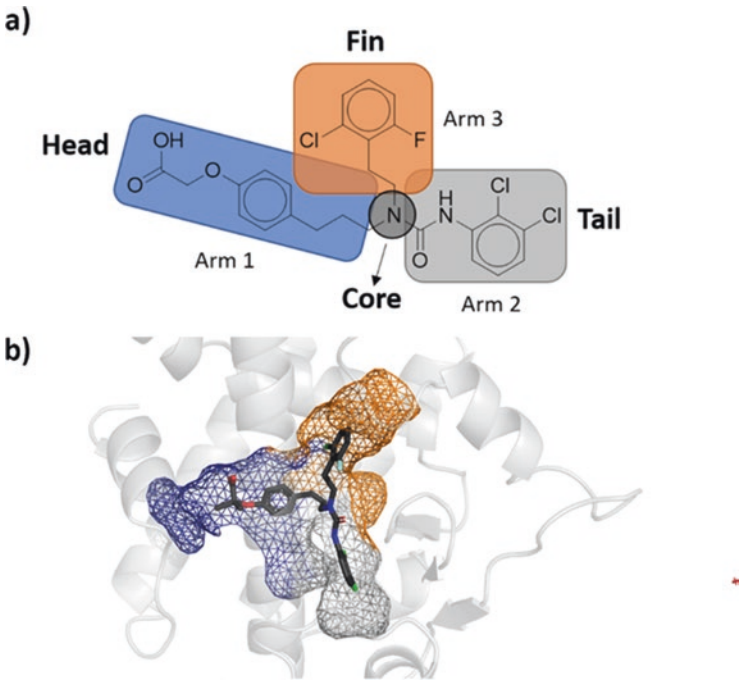

Fig. 6.3 (**a**) The regions of the archetypal Y-shaped PPAR ligand. Arms 1, 2 and 3 are highlighted in blue, orange and grey, respectively (GW2433, PDB ID: 1GWX). (**b**) The PPAR ligand bound to the PPAR LBP. The parts of the ligand are highlighted

Ligands generally consist of a few regions, a 'head' that interacts with residues in Arm 1, a 'tail' that forms hydrophobic interactions with residues in Arm 2 and a 'fin' region that interacts with residues in Arm 3. These three Arms are connected to the ligand 'core, marked by the amino acid residue α: T279, δ: T252, γ: R288' (Fig. 6.3) [113]. PPARγ ligand design has already been discussed in a previous publication [62]. Here we focus on examining attempts at synthesizing chemical tools and therapeutic drugs for PPARα and δ, although we will include PPARγ – activating dual or pan agonist examples. PPAR-ligand interactions were determined using Discovery Studio Visualizer© with the default search parameters [29]. We define the total number of interactions as all unique interactions between receptor and ligand.

6.2.1 PPARα Ligands

In the early 1950s, an analogue of an insecticide was found to have plasma cholesterol-lowering effects [16, 80]. This class of drugs, called fibrates, were later found to mediate their effects through the PPARα receptors [80]. Thus, PPARα

became the first PPAR subtype with a clinically available, subtype specific drug class. Fibrates contain the fibrate head group, a phenoxyisobutyric acid motif in the ligand head region which makes hydrogen bond contacts with the AF2 surface, and are typically full agonists for PPARα [13, 16].

Fenofibrates are one such example in clinical use – they have considerably milder side effects compared to the PPARγ-specific TZDs and have been beneficial in treating cholesterol- or lipid-related conditions [16, 23]. However, PPARα-specific fibrates have low affinity and poor subtype selectivity and may as a result have lower efficacy than statins [16, 23, 76]. As such, research groups have focused on designing more potent full agonists of PPARα, hypothesizing that increasing affinity will increase therapeutic effects [58, 76, 97].

6.2.1.1 PPARα Full Agonists

WY14643 or Pirinixic Acid

WY14643, a potent PPARα agonist, has been used in therapy as an anticholestoremic agent. It was initially used for its peroxisome-proliferating properties in the discovery of PPAR [49, 93] and has since been used as a control for PPARα drug discovery [11]. The WY14643-PPARα complex was first crystallized by Bernardes and group, in their study to understand its binding mode and the role of PPARα in ocular inflammation (Fig. 6.4, Table 6.2) [11]. This is the first crystal structure of a single PPARα LBD monomer binding to two ligand molecules.

WY14643 is smaller than the typical ligand, only containing the head and core regions. Like other PPAR agonists, WY14643 forms hydrogen bonds with 4/4 of the polar residues in Arm 1 and hydrophobic contacts with seven residues from Arm 1, 2 and 3. Selectivity is likely imparted by interactions with the larger side chains of residues α: Y314 and M330 that are unique to PPARα. Surprisingly, it was found that WY14643 also bound to a secondary binding site, around the solvent exposed surface of H3, sandwiched between H2′, H2′–H3 loop and H3. The charge cluster formed between α: D453-H274-K266-WY14643 in the second binding pocket anchors H12 to H3 via the Ω loop, resulting in stabilization of the AF2 surface. The stabilization imparted by the binding of two ligand molecules in the LBD was sufficient to induce gene transactivation despite making substantially fewer contacts in the LBP.

GW590735/Compound 25a

GSK developed the high affinity PPARα agonist GW590735 to treat dyslipidemia and tackle the risk factors of coronary artery disease. GW590735 lowered serum triglycerides (TG), very low density cholesterol (VLDLc) and low density cholesterol (LDLc) and raised high density cholesterol (HDLc) in animal models. It had improved potency and selectivity for PPARα compared to previous Fibrates, which

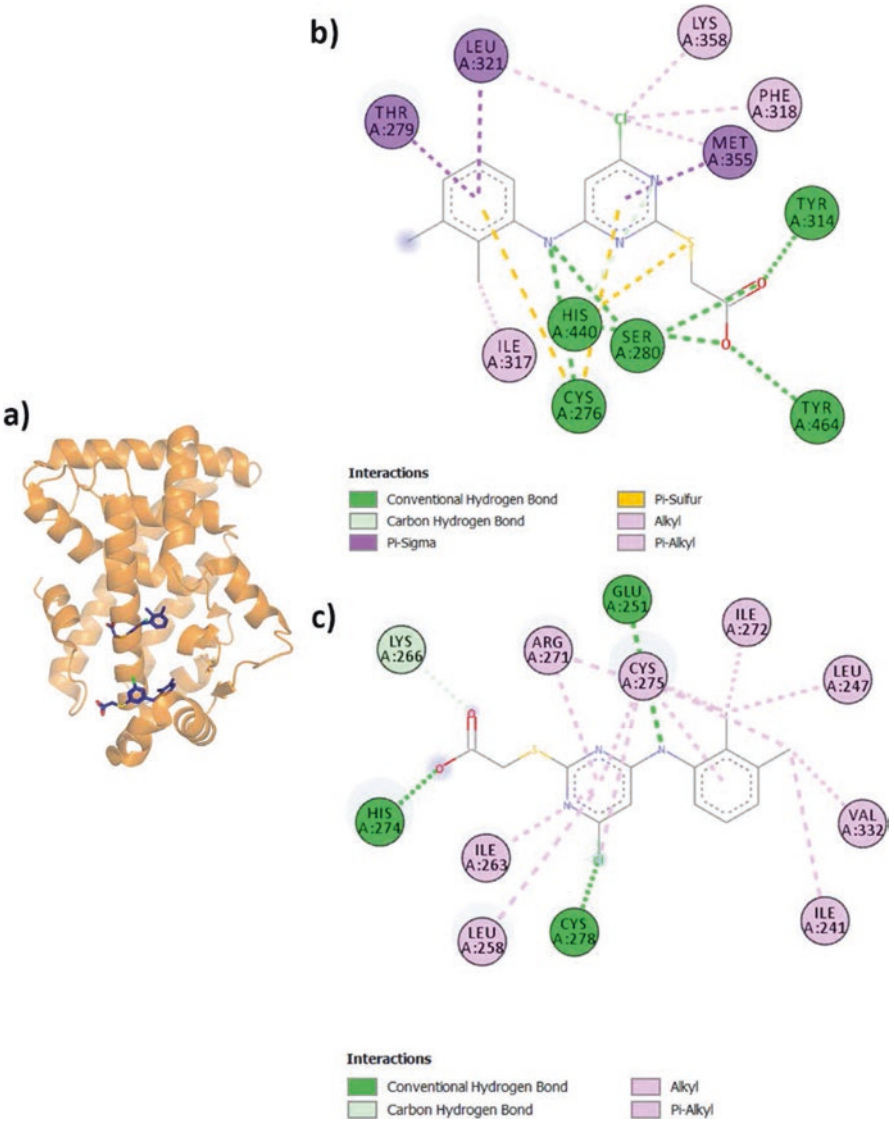

Fig. 6.4 (**a**) PPARα LBD in orange complexed with WY14643 in dark purple (PDB ID: 4BCR). (**b**) Interaction map of WY14643 in the LBP with PPARα. (**c**) Interaction map of WY14643 in the secondary binding site with PPARα

was proposed to increase therapeutic benefits and reduce side effects [58, 97]. The PPARα-GW590735 complex was crystallized by Sierra et al. [97] as part of their SAR study to synthesize a high affinity, PPARα-specific agonist using GW501516 as the lead compound (Fig. 6.5, Table 6.3).

Table 6.2 The activity of WY14643 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
WY14643	5.38	>100	>100

EC_{50} values were determined by dual luciferase assay in HepG2 cells, values were normalized to *Renilla* activity

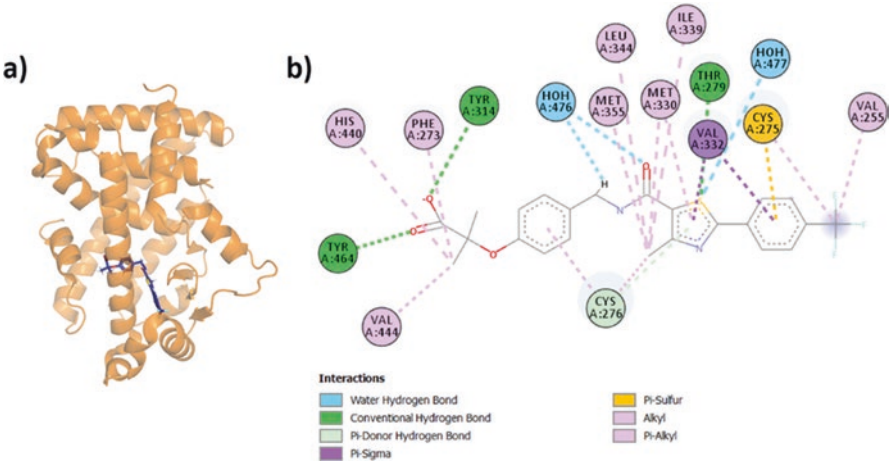

Fig. 6.5 (**a**) PPARα LBD in orange complexed with GW590735 in dark purple (PDB ID: 2P54). (**b**) Interaction map of GW590735 with PPARα

By swapping the oxy-propanoic head with an isobutyric acid head, the L-shaped GW590735 makes hydrogen bond contacts with 4/4 of the polar residues of Arm 1 in PPARα. The gem-dimethyl groups make additional hydrophobic interactions with α: V444 (γ: L453, δ: M417) and F273. The bulkier corresponding residues of α: V444 in the other subtypes may sterically interfere with ligand, accounting for the affinity of the gem-methyl motif for PPARα.

The amide group in the core region confers rigidity to the ligand, which tends to increase the stability of interactions with and affinity for the LBP [113]. The phenoxy motif in the head region as well as the thiazole motif in the tail region interacts with α: C276. The 4-methyl substitution on the thiazole motif makes multiple alkyl interactions with α: M330 and M355 in Arm 3 and I339 on the β3. Corresponding residues of PPARδ and γ in Arm 3 are not bulky enough to form favorable interactions with the 4-methyl group. In addition, corresponding residue of α: I339 in PPARγ (γ: M348) might sterically interfere with this group. The tail region makes hydrophobic and π-donor hydrogen bond interactions with residues in Arm 2 as well as α: V255 in the Ω loop. Only the corresponding residues in PPARδ can make similar interactions with the ligand, which explains why this scaffold was successful in both PPARα and δ but not γ.

Table 6.3 The activity of GW590735 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
GW590735/25a	0.004 ± 0.002 (95%)	2.83 ± 1.18 (82%)	>10

EC_{50} values were determined by dual luminescence assay in CV-1 cells, measuring alkaline phosphatase activity normalized to β-galactosidase activity. Maximal activity (%) was defined as the activity of Compound 25a, Compound 1 and Rosiglitazone for PPARα, δ and γ, respectively [97]

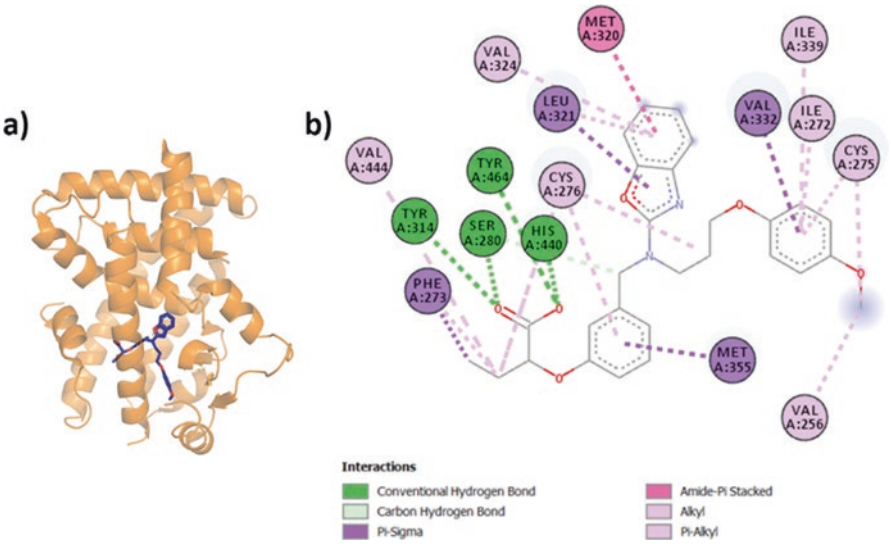

Fig. 6.6 (**a**) PPARα LBD in orange complexed with pemafibrate in dark purple (PDB ID: 6L96). (**b**) Interaction map of pemafibrate with PPARα

Table 6.4 The activity of Permafibrate at each PPAR subtype

Ligand	PPARα	PPARδ	PPARγ
Pemafibrate	IC_{50} (μM)		
	0.13 ± 0.04	–	9.58 ± 1.85
	EC_{50} (μM)		
	0.001	1.58	1.10

IC_{50} values were determined by isothermal titration calorimetry (ITC). EC_{50} values were taken from Yamazaki et al. [119], determined by dual luciferase assay in HepG2 cells, values were normalized to *Renilla* activity

Pemafibrate

Pemafibrate is a selective, high affinity PPARα agonist synthesized by Kowa Pharmaceuticals [119]. Pemafibrate has been in clinical trials for treatment of multiple ailments and is set to replace previous PPARα agonists [23]. The

PPARα-Pemafibrate-SRC1 complex was crystallized by Kawasaki and colleagues (Fig. 6.6, Table 6.4) [58].

Pemafibrate has a Y-shaped design that contains a 2-phenoxybutanoic acid head, instead of a isobutyric acid head as seen in GW590735. This fibrate head forms hydrogen bonds with 4/4 of the polar residues in Arm 1, but sits slightly lower to accommodate the shorter distance to the core, as well as the fin substituent in Arm 3 [119]. The unique α: Y314 residue in PPARα allows the formation of favourable hydrogen bonds with the acid head compared to the histidine residues in PPARδ/γ. The ethyl substituent (versus gem-dimethyl) interacts more closely with α: F273 than V444. α: M355 'stretches out' (compared to GW590735 structure PDB ID: 2P54) to interact with the phenoxy motif in the head region. The head is connected to the fin and tail regions via a nitrogen core.

Pemafibrate is one of the only fibrates that contains a benzoxazole fin substituent that interacts with three residues in Arm 3. The area of the tail region adjacent to the core does not contain substituents. The tail 1-butoxy-4-methoxybenzene motif interacts with residues in Arm 2. The methyl group at the tip of the tail region rotates and interacts with α: V256 or V247. The lack of posterior facing substituents in the core and tail regions might contribute to the decrease of affinity for PPARδ, due to its smaller residues in Arm 2 compared to other subtypes (δ: V245, I328, V312).

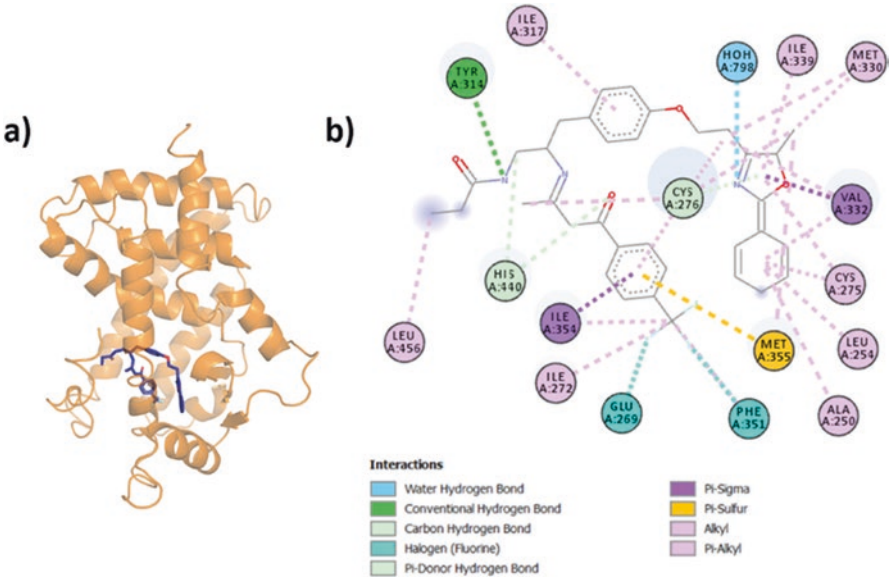

Fig. 6.7 (**a**) PPARα LBD in orange complexed with GW6471 in dark purple (PDB ID: 1KKQ). (**b**) Interaction map of GW6471 with PPARα

Table 6.5 The activity of GW6471 at each PPAR subtype

	IC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
GW6471	0.24	–	–

IC_{50} values were determined by dual luminescence assay in CV-1 cells, measuring alkaline phosphatase activity normalized to β-galactosidase activity, in the presence of 10 nM GW409544

6.2.1.2 PPARα Antagonist

GW6471

GW6471 is an antagonist developed by GSK using the PPARα and γ dual agonist GW409544 as a chemical scaffold. The PPARα-GW6471-SMRT complex was crystallized by Xu and colleagues in 2002 to explore the structural basis for PPAR antagonist binding (Fig. 6.7, Table 6.5) [116]. This was the first PPAR structure crystallized with an antagonist.

The N-shaped GW6471 contains a non-acidic N-phenylpropyl-propanamide head motif. The propylamide group occupies the typical position of H12 residue α: L460, destabilizing H12 and disrupting the AF2 surface. This group also forms interactions with the corepressor. This group sits 5.2 Å further laterally towards H12 than typically seen with the carboxylic acid groups in full PPARα agonists.

GW6471 contains a unique amino-phenylbutanone substituent at the head region that is not normally seen in ligands. This motif occupies the inferior part of Arm 1 and extends into the posterior area of Arm 2, forcing H3 and H7 outward, compared to full agonists. The tail substituent utilizes a similar design to GW590735, having a methyl substituent that projects into the back of the junction/Arm 2 area. This tail substituent makes interactions with Arm 2 and Ω loop residues α: L254 and A250.

6.2.2 PPARδ Ligands

As of 2020, PPARδ remains as the only PPAR subtype without a clinically approved drug. Unlike PPARα and γ, there were no preexisting drug classes that were found to mediate their effects through PPARδ, and early efforts to create such a drug class were plagued by discouraging results [23, 64, 65]. Nevertheless, the clinical success of Bezafibrate shows that PPARδ activation is not inherently toxic [16]. Due to its role in energy and lipid metabolism, and the side effects seen with long term use of PPARα/γ-based therapies, PPARδ makes for a particularly attractive therapeutic target [16, 28, 113]. Most of the work on PPARδ ligands have been directed towards creating full agonists.

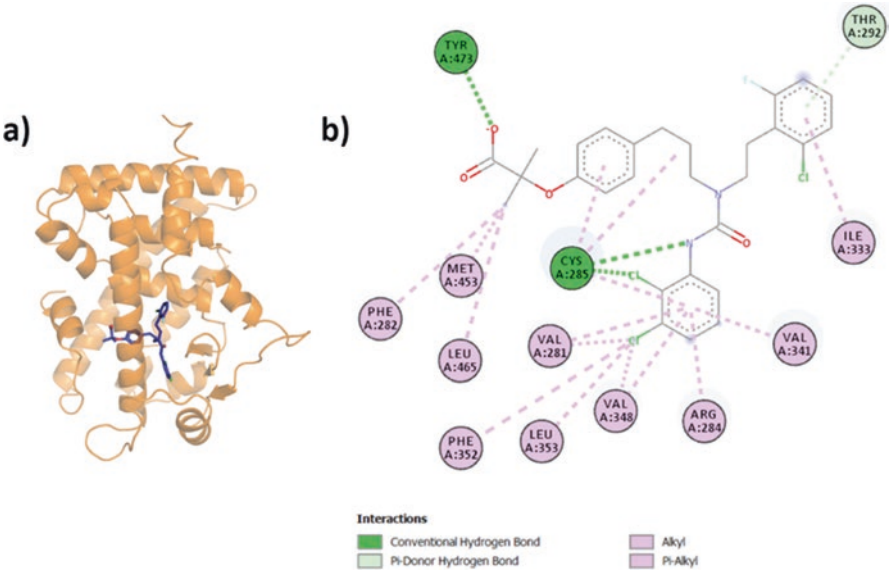

Fig. 6.8 (**a**) PPARδ LBD in orange complexed with GW2433 in dark purple (PDB ID: 1GWX). (**b**) Interaction map of GW2433 with PPARδ

Table 6.6 The activity of GW2433 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
GW2433	0.17	0.19	2.5

EC_{50} values were taken from [112], determined by dual luminescence assay in CV-1 cells, measuring alkaline phosphatase activity normalized to β-galactosidase activity

6.2.2.1 PPARδ Full Agonists

GW2433

GW2433 was the first high affinity PPARδ ligand developed by GSK [13]. GW2433 was developed from a 'biased library' comprised of motifs taken from other successful ligands. The PPARδ-GW2433 complex was crystallized by Xu and colleagues in their study (Fig. 6.8, Table 6.6) [114]. This was the first crystal structure of the human PPARδ LBD. Despite containing a fibrate head, this ligand shows affinity for PPARδ, showing that the fibrate motif is insufficient for PPARα specificity.

GW2433 is a Y-shaped ligand that contains the typical phenoxyisobutyric acid head that forms hydrogen bonds with 3/4 of the polar residues in Arm 1. The gem-dimethyl substituents form hydrophobic interactions with five residues in the inferior region of Arm 1. GW2433 wraps around δ: C249, which interacts with the different regions of the GW2433. The tail phenyl also makes interactions with six

residues in Arm 2. GW2433 contains a chloro-ethylbenzene fin substituent, that interacts with four residues along the Arm 1/Arm 3 region. In PPARγ, residues γ: M348 and R288 likely interferes sterically with the halogen substituents on the ligand in Arms 2 and 3, respectively, selecting against PPARγ affinity. The three ligand regions are connected to a nitrogen core.

GW2331

GW2331 is a fibrate that was developed as a high affinity ligand, to be used as a control for PPAR α and γ in ligand binding assays. In 1997, Kliewer and colleagues designed and used GW2331 in an inhibitory assay to prove that several fatty acids and eicosanoids bind to PPARα directly. GW2331 was shown to activate PPARδ at concentrations of >1 μM). Interestingly, despite being a PPARα/γ dual agonist, GW2331 was only ever crystallized with PPARδ. The PPARδ – GW2331 complex was obtained by Takada and colleagues as part of their study into PPAR subtype selectivity and PPAR phylogenetics (Fig. 6.9, Table 6.7) [101]. Strangely, this compound was successfully crystallized with PPARδ despite having no detectable IC_{50} values.

GW2331 is a Y-shaped ligand that contains a 2-methyl-2-phenoxybutanoic acid head, which makes hydrogen bond interactions with 3/4 of the polar residues in Arm 1. The methyl and ethyl groups in the head region form hydrophobic

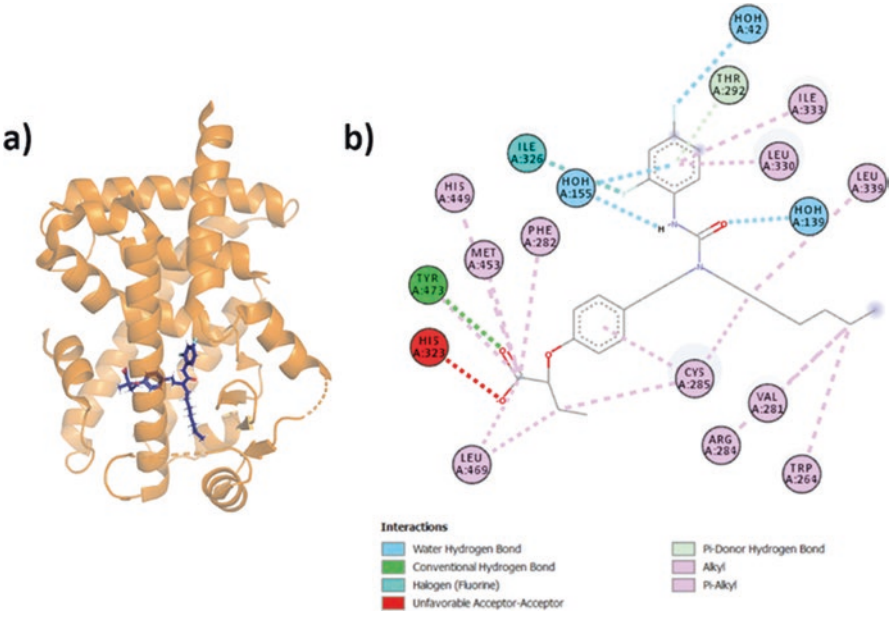

Fig. 6.9 (**a**) PPARδ LBD in orange complexed with GW2331 in dark purple (PDB ID: 1Y0S). (**b**) Interaction map of GW2331 with PPARδ

Table 6.7 The activity of GW2331 at each PPAR subtype

Ligand	PPARα	PPARδ	PPARγ
GW2331	IC_{50} (μM)		
	0.348	ND	0.670
	EC_{50} (μM)		
	0.071 ± 0.035 (79%)	ND	0.249 ± 0.238 (90%)

IC_{50} and EC_{50} values were taken from [32]. IC_{50} values were determined by fluorescence polarization assay. EC_{50} values were determined by luciferase assay in HEK293 cells. Maximal activity (%) was defined as the activity of 1 μM of ligand relative to 1 μM of GW2331 and Rosiglitazone for PPARα and γ, respectively. ND – not determined; compounds had no activity against PPARδ

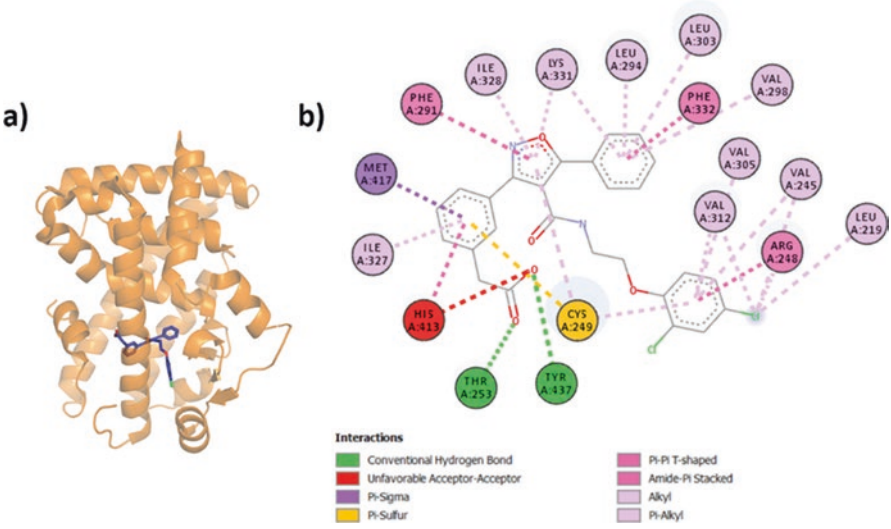

Fig. 6.10 (**a**) PPARδ LBD in orange complexed with LC1765 in dark purple (PDB ID: 2J14). (**b**) Interaction map of LC1765 with PPARδ

interactions with Arms 1 and 2.The larger ethyl group was observed to sit very near to H3 (3.3 Å) possibly causing repulsion. The larger δ: M417 also likely interferes with the accommodation of these alkyl substituents in Arm 1. The heptane chain in the tail region makes interactions with only three residues in Arm 2, 1 in Arm 3 and δ: W228 on the Ω loop. GW2331 contains a N-(1,4-difluorophenyl)amide fin substituent, which makes interactions with four residues in Arms 1 and 3 as well as a bridging interaction to δ: T252 in Arm 2. The three ligand regions are connected to a nitrogen core. The repulsion caused by the ethyl substituent in the head coupled, with the lack of interactions in Arm 2 possibly explains the selectivity of GW2331 against PPARδ.

Table 6.8 The activity of LC1765 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
LC1765	>10	0.07 (83%)	>10

EC_{50} values were determined by cell-based transactivation assay. Maximal activity (%) was defined as relative to GW501516 for PPARδ

LC1765

Epple and colleagues derived PPARδ selective full agonist LC1765, to be used as a tool for investigation of the pharmaceutical benefits of activating PPARδ. The PPARδ-LC1765 complex was crystallized by Epple and colleagues, in their study to improve the pharmacokinetic properties of their 3,4,5-trisubstituted isoxazoles (Fig. 6.10, Table 6.8) [38].

LC1765 had a Y-shaped design, with a phenylacetic acid head region, forming hydrogen bond interactions with 4/4 of the polar residues in Arm 1 and hydrophobic interactions with two and one residues in Arms 1 and 2, respectively. This phenyl ring in the head region is 1,3 substituted as opposed to 1,4 substituted and thus sits lower than other PPARδ ligands to accommodate the core and fin substituents in Arm 3.

The isoxazole core of LC1765 sits further posteriorly than where the typical ligand core sits and interacts with residues in the interface of Arms 1, 2 and 3. Corresponding methionine residues of δ: I328 in PPARα and γ (α: M355 γ: M364) likely clash sterically with the core, selecting for affinity towards PPARδ. The phenyl fin substituent projects backward into H7, making hydrophobic contacts with four residues in the inferior area of Arm 3. The tail region contains a 1-ethoxy-2,4-dichlorobenzene, N-linked via an amide to the isoxazole core. This amide lies in the same plane as the core, pushing the 1-ethoxy-2,4-dichlorobenzene substituent forward anteriorly. The 2,4 substituted chloride groups on the tail substituent points down and backwards, making hydrophobic interactions with six residues in Arm 2.

Compound 48

This PPARδ selective full agonist was developed by GSK, continuing their work on partial agonists [39, 96]. Optimization of a lead from a search of their internal database resulted in Compounds 46 and 47. The Compound 48–PPARδ complex was crystallized to understand the structural basis for the activity of the partial agonist Compounds 46 and 47 in the series (Fig. 6.11, Table 6.9).

This Y-shaped Compound 48 contains a naphthoxyacetic acid head region. It forms the hydrogen bond interactions with 4/4 of the polar residues in Arm 1. The naphthalene interacts with three residues in Arms 1 and 2. The sulfonamide core interacts with δ: F291 and forces a cis conformation between the head and tail regions. The butane fin substituent interacts with five residues in the inferior part of

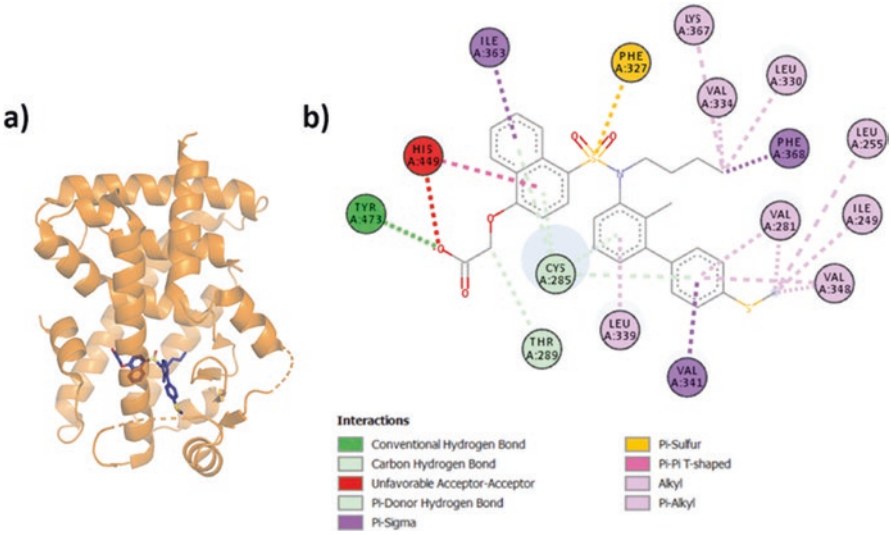

Fig. 6.11 (**a**) PPARδ LBD in orange complexed with Compound 48 in dark purple (PDB ID: 3PEQ). (**b**) Interaction map of Compound 48 with PPARδ

Table 6.9 The activity of Compound 48 at each PPAR subtype

Ligand	PPARα	PPARδ	PPARγ
Compound 48	IC_{50} (μM)		
	–	0.0316	–
	EC_{50} (μM)		
	–	0.0079 (90%)	–

IC_{50} values were determined by scintillation proximity assay (SPA), using [H^3]GW2331, [H^3]GW2433 and [H^3]BRL49653 as radioligands for PPARα, δ and γ, respectively. EC_{50} values were determined by dual luminescence assay in CV-1 cells, measuring alkaline phosphatase activity normalized to β-galactosidase activity. Maximal activity (%) was defined as relative to GW501516 for PPARδ.

Arm 3. The tail biphenyl interacts with four residues in Arms 1 and 2. The methanethiol *para* substituent interacts with five residues in Arm 2 and δ: W228 on the Ω loop, spanning the whole of Arm 2.

Affinity to PPARδ is likely imparted by the close proximity of the core and tail to δ: I328 and V312, respectively, and by the fin interactions with δ: L303 and V298 in Arm 3. The corresponding bulkier methionine residues in PPARα and γ likely clash sterically with compound 48, presumably having a negative impact on PPARα/γ binding.

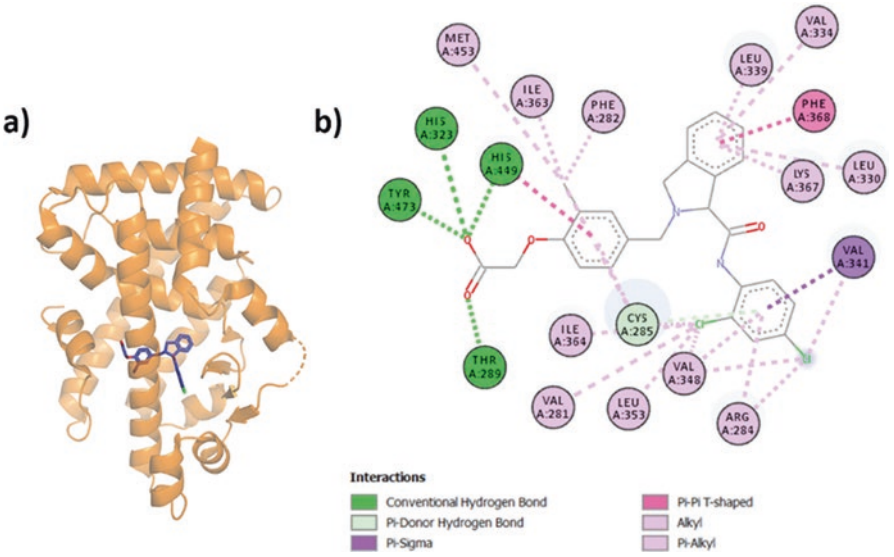

Fig. 6.12 (**a**) PPARδ LBD in orange complexed with Compound 5 in dark purple (PDB ID: 3OZ0). (**b**) Interaction map of Compound 5 with PPARδ

Table 6.10 The activity of isoquinoline compound 5 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
Compound 5	>30	0.025 (110%)	>30

EC_{50} values were determined by luciferase assay in HEK-293 cells. Maximal activity (%) was defined as relative to GW501516

Isoquinoline Compound 5

Compound 5 was part of the optimization process that led to identification of tetrahydroisoquinoline compound 18 by Luckhurst and colleagues using leads from AstraZeneca's hit identification program. The PPARδ-Compound 5 complex was crystallized by Luckhurst and colleagues (Fig. 6.12, Table 6.10) [72] in their study to identify a less lipophilic drug for PPARδ.

In the Y-shaped Compound 5, the (2-methylphenoxy)acetic acid motif makes hydrogen bonds with 4/4 of the polar residues and hydrophobic contacts with five other residues in Arm 1. The tail 2,4-dichlorophenylamide motif also interacts with seven residues in the superior part of Arm 2. The phenyl portion in the core isoquinoline act as a fin substituent, interacting with five residues in Arm 3. However, it does not make any interactions with residues at the bottom of Arm 2 or the Ω loop due to the shorter ligand tail. This double ring core sits further back into H7. Corresponding methionine residues in PPARα and γ (α: M355 γ: M364) likely clash with the core motif, selecting for affinity towards PPARδ. This compound is the

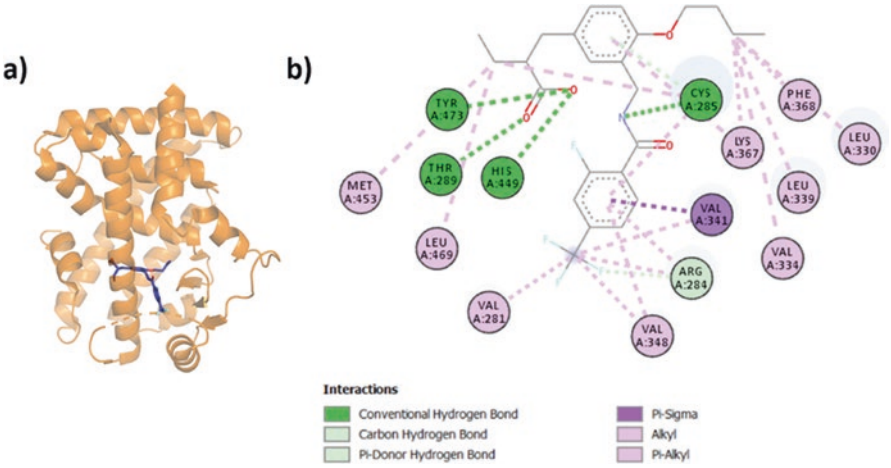

Fig. 6.13 (**a**) PPARδ LBD in orange complexed with TIPP-204 in dark purple (PDB ID: 2ZNP). (**b**) Interaction map of TIPP-204 with PPARδ

Table 6.11 The activity of TIPP-204 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
TIPP-204	0.250	0.00091	1.100

EC_{50} values were determined by GAL4 transactivation activity in HEK-293 cells, relative to GW501516 for PPARδ and rosiglitazone for PPARγ [54]

only reported crystal structure of a full agonist with 110% activity compared to GW501516 in a cell-based assay.

TIPP-204

Kasuga and group synthesized the PPARδ-specific agonist TIPP-204 from the PPARα/γ agonist TIPP-401, which was in turn synthesized from PPARα agonist KCL [54]. The PPARδ-TIPP-204 complex was crystallized by Oyama and colleagues to understand the different binding modes in their series of compounds that includes PPARα and PPARδ-specific agonists and a PPARα/γ dual agonist (Fig. 6.13, Table 6.11) [82]. The TIPP ligands have a reversed amide compared to KCL and differ from each other by an extended fin substituent in TIPP-204 compared to TIPP-401.

TIPP-204 is a Y-shaped ligand. The isobutyric acid head interacts with 3/4 polar of the residues and three other residues (including δ: M417) in Arm 1. The 1,3,4-substituted phenoxy core interacts with two residues in Arm 2 and 3. Its tail substituent, an amino linked 2-fluoro-4-(trifluoromethyl)benzene interacts with

V312, four other residues and δ: W228 on the Ω loop. Its fin butanol substituent interacts with δ: I328 in Arm 2, and four other residues in Arms 3.

Affinity for PPARδ is likely mediated by δ: L303 and V298 in Arm 3. The larger corresponding residues in PPARα/γ (α: M330 and α: M325 γ: M334) sterically clash with the fin. The tail substituent interacts with δ: V312 and potentially clashes with PPARγ (γ: M348). The relatively high affinity for PPARα might be due to the reversed amide, which makes contacts with α: T279 (δ: T252, γ: R288) and the flexibility of α: M355, to accommodate the fin substituent. This might also be the case for the other methionine residues in PPARα (α: M330 and M325). Strangely, increasing the length of the fin also increased the potency towards PPARγ, compared to TIPP-401. This may suggest the ligand adopts an alternative top-down conformation in PPARγ as seen for compounds GL479 and compound 21 and that the fin substituent may be able to make additional contacts in Arm 1 (PDB ID: 4CI5 and 3H0A).

GW0742

PPARδ full agonist GW0742 was synthesized by GSK along with GW501516 and showed superior selectivity against PPARγ compared to GW501516 [100]. Batista and colleagues crystallized the PPARδ-GW0742 complex in their study (Fig. 6.14, Table 6.12) [9]. This compound is still in the preclinical stages as of 2020, although it was found to be associated with side effects [23, 28].

The L-shaped ligand contains a (2-methylphenoxy)acetic acid motif in the head region that interacts with 3/4 of the polar residues and hydrophobic contacts with

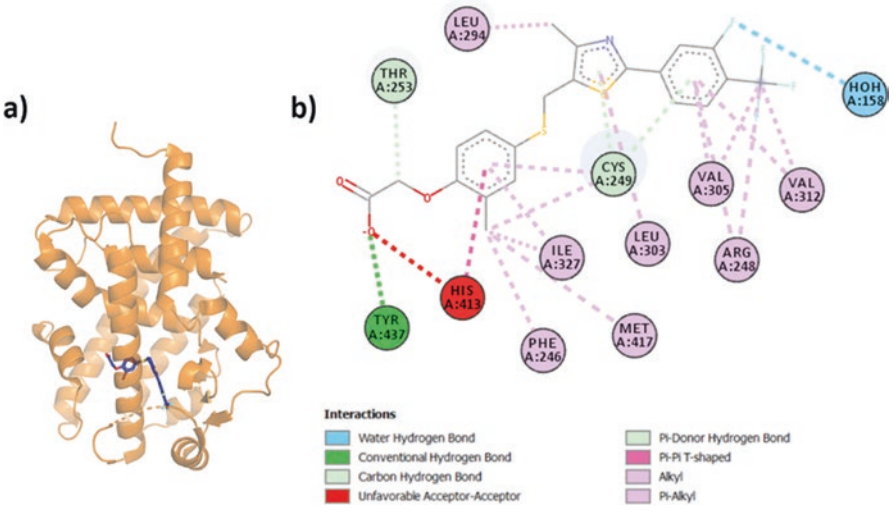

Fig. 6.14 (**a**) PPARδ LBD in orange complexed with GW0742 in dark purple (PDB ID: 3TKM). (**b**) Interaction map of GW0742 with PPARδ

Table 6.12 The activity of GW0742 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
GW0742	ND	0.00325	ND

EC_{50} values were determined by dual luciferase assay in HepG2 cells, values were normalized to *Renilla* activity. ND – not determined; EC_{50} values could not be calculated up to 10 μM of ligand

five residues in Arm 1. The thiazole core interacts with two residues in Arm 3 and one in Arm 2. The 3-fluoro,4-(trifluormethyl)benzene motif in the tail region interacts with three residues in Arm 2. The compound GW0742 does not contain a fin substituent.

The structural selectivity was likely imparted by three residues: δ: I328 α: M355 γ: M364, δ: V312 α: I339 γ: M348 and δ: M417 α: V444 γ: L453. The corresponding methionine residues at position δ: I328 and V312 will clash with PPARα and PPARγ, respectively, increasing selectivity for the PPARδ. This was confirmed by the binding assays of δ: I328M and V312M mutants [9]. δ: M417 in Arm 1 makes closer contacts with the carboxylate head than the corresponding PPARα and γ residues, which might contribute to GW0742's strong selectivity towards PPARδ. The interactions with other residues in Arm 1 likely contribute to the high potency of GW0742.

GW501516, Compounds 1–16

GW501516 was the first highly selective and potent PPARδ to make it to clinical trials when, unfortunately, it was found to be carcinogenic in mice models [64, 65, 81, 100]. Wu and colleagues synthesized Compounds 1–16 to mimic fatty acids and to occupy Arm 3 of the PPARδ LBD, using GW501516 as a benchmark in their SAR study. The PPARδ-GW501516/Compounds 1–16 complexes were crystallized by Wu and colleagues as part of their SAR study to identify new selective and potent compounds for PPARδ (Fig. 6.15, Table 6.13) [113]. Here we discuss GW501516 as well as their representative lead compounds 2 and 4.

The L-shaped GW501516 contains a (2-methylphenoxy)acetic acid head motif. The acetic acid motif forms hydrogen bonds with 4/4 of the polar residues in Arm 1. The 2-meythlphenoxy motif in the head region makes hydrophobic contacts with five other residues in Arms 1 and 2. It is connected to the core tail motif via a methanethiol linker. The tail motif consists of a 3-thiazolidine, which makes contacts with δ: C249 and two other residues, and a (trifluoromethyl)benzene, which makes interactions with five other residues in Arm 2.

In the Y-shaped Compound 2, the phenoxyhexanoic acid head region forms hydrogen bonds with 1/4 polar residues in Arm 1 and forms hydrophobic interactions with five other residues in Arms 1, 2 and 3. The head region is linked via a methyl chain to an amide core. This amide core forms hydrogen bonds with one water molecule and is N-linked to an isopropyl fin substituent. The fin substituent

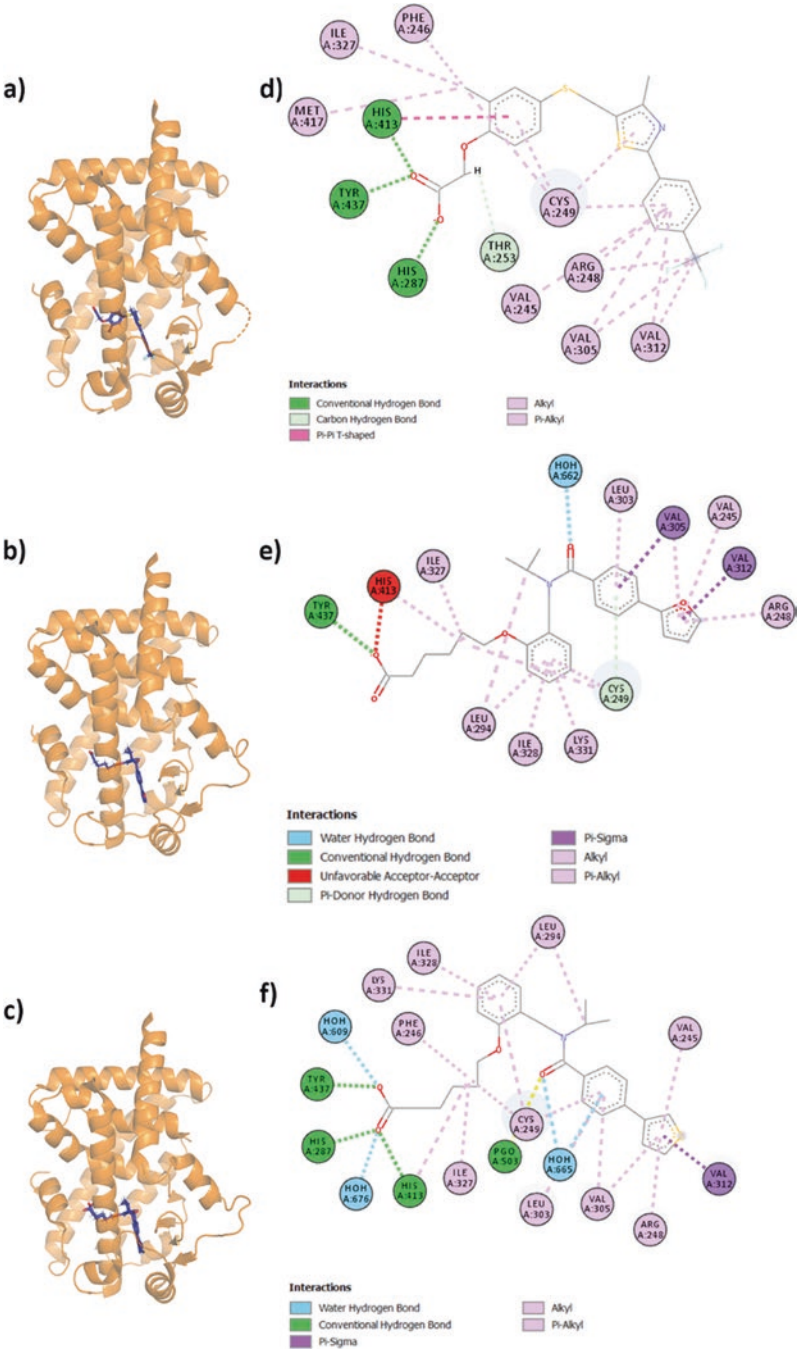

Fig. 6.15 (**a**) PPARδ LBD in orange complexed with GW501516 in dark purple (PDB ID: 5U46). (**b**) PPARδ LBD in orange complexed with Compound 2 in dark purple (PDB ID: 5U3R). (**c**) PPARδ LBD in orange complexed with Compound 4 in dark purple (PDB ID: 5U3T). (**d**) Interaction map of GW501516 with PPARδ. (**e**) Interaction map of Compound 2 with PPARδ. (**f**) Interaction map of Compound 4 with PPARδ

Table 6.13 The activity of lead compounds at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα[a]	PPARδ	PPARγ[a]
GW501516	2.591 ± 0.0012	0.0012 ± 0.0003	2.591 ± 0.0012
Compound 2	>10	0.0090 ± 0.0003	>10
Compound 4	>10	0.0172 ± 0.0007	>10

EC_{50} values were determined by luciferase assay
[a]EC_{50} for PPARα and γ was given as a single value in the study. Luciferase assay methods were not given

forms hydrophobic contacts with one residue in Arm 3. The core is C-linked to the biaryl tail substituent. The biaryl tail substituent consists of a phenyl ring and 2-furyl group, which forms hydrophobic contacts with five residues in Arm 2.

In Compound 4, the phenoxyhexanoic acid head region forms hydrogen bonds with 3/4 polar residues in Arm 1 and forms hydrophobic interactions with six other residues in Arms 1, 2 and 3. The head region is also linked via a methyl chain to an amide core and contains the same N-linked isopropyl fin substituent. The core makes hydrogen bonds with one water molecule and S-1,2-propanediol, an additive used in crystallization. The fin makes hydrophobic interactions with one residue in Arm 3. The core is also C-linked to a biaryl tail substituent, which consists of a phenyl ring and 3-thienyl group, which makes hydrophobic contacts with five residues in Arm 2 and 3.

Affinity of GW501516, Compounds 2 and 4 against PPARα and PPARγ is likely imparted by residue δ: V312. The larger corresponding methionine residues (α: M330 γ: M348) interfere with the tail region of GW501516. Compared to Compounds 2 and 4, GW501516 has lower absolute affinity towards PPARα and γ [100] (EC_{50} α: 1.1 μM and γ: 0.85 μM). This might be due to the lack of posterior facing substituents in the core or tail region of GW501516. Even though Compounds 2 and 4 do not contain such substituents, the core region is positioned slightly lower compared to GW501516 and closer toward H7, causing interference with corresponding residues of δ: I328 and V298 in PPARα and γ (α: M355, M325 γ: M364, M334). In PPARα, interference between the larger α: Y314 (δ: H287 γ: H323) residue on the AF2 surface and the longer hexanoic acid head would possibly force the core and tail substituent of the ligand into an unfavourable position, clashing with corresponding residue of δ: L303 (α: M330). These observations were confirmed by mutagenesis [113].

Compound 18 and Compound 13

Compounds 18 and 13 were synthesized by Astellas pharma in SAR studies which eventually led to MA-0204, a potential therapeutic for Duchenne Muscular Dystrophy (DMD). These compounds were optimized to have better pharmacokinetics and an improved safety profile over GW501516 [63–65]. The

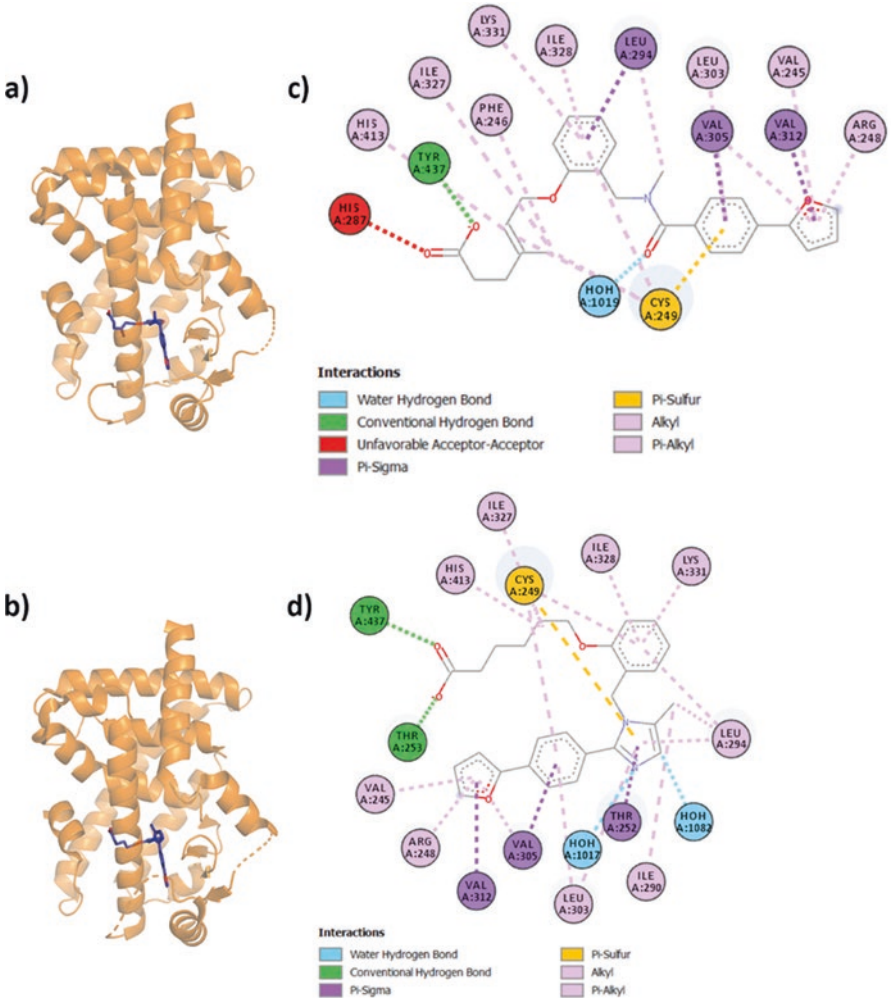

Fig. 6.16 (**a**) PPARδ LBD in orange complexed with Compound 18 in purple (PDB ID: 5XMX). (**b**) PPARδ LBD in orange complexed with Compound 13 in purple (PDB ID: 5ZXI). (**c**) Interaction map of Compound 18 with PPARδ. (**d**) Interaction map of Compound 13 with PPARδ

PPARδ – Compound 18/13 complexes was crystallized by Lagu and colleagues (Fig. 6.16, Table 6.14) [63–65] in their search for a potent, selective and safer PPARδ agonist.

These L-shaped compounds differ slightly in the arrangement of the acidic head and tail region. Compound 18 contains a 4-methyl,4-hexenoic acid head and an amide motif in the tail, and Compound 13 contains a hexanoic acid head and an imidazole motif in the tail. The head region of compounds 18 and 13 makes similar hydrogen-bonding interactions with 4/4 of the polar residues and hydrophobic contacts with two residues in Arm 1 and 2. The extra 4-methyl substituent in compound

Table 6.14 The activity of lead compounds at each PPAR subtype

Ligand	PPARα	PPARδ	PPARγ
	IC_{50} (μM)		
Compound 18	–	0.057 ± 0.001	–
	EC_{50} (μM)		
Compound 18	6.100	0.0370 ± 0.0050	>10.000
Compound 13	6.970	0.0007 ± 0.0002	>10.000

IC_{50} values were determined by surface plasmon resonance (SPR) [63]. EC_{50} values were determined by luciferase assay in CV-1 cells [63]

18 makes an additional contact in Arm 1. The core phenoxy motif in both compounds interacts with four residues in Arms 2 and 3. The 2-phenylfuran tail motif of Compound 18 interacts with 5 residues in Arm 2 and 1 in Arm 3. The larger imidazole motif of Compound 13 makes additional contacts with 3 residues in Arms 1 and 2.

The compound has increased affinity for PPARδ due to having the head and tail connected to the phenyl core at the *ortho* position, pushing the core closer to H7. Corresponding methionine residues of δ: I328 in PPARα and γ (α: M355 γ: M364) would clash sterically with the core. The corresponding residue of δ: V312 in PPARγ (γ: M348) would also interfere with the 2-furyl group in the tail end, selecting for affinity towards PPARδ.

6.2.2.2 PPARδ Partial Agonists

Partial agonism of PPARδ has been studied by several groups, motivated by the increased safety profile seen from partial agonism of PPARγ [14]. Having a suite of chemical tools is beneficial for exploration of PPAR function, but whether partial agonism of PPARδ has any therapeutic benefits over full agonism remains to be seen.

Compound 2

Novo Nordisk synthesized the partial PPARδ agonist Compound 2. The goal of lowered efficacy came from the observations of the reduced side effects seen in partial agonists for PPARγ [14, 84]. This compound was derived from GW501516, to design a rigid PPARδ selective full agonist that could fill the PPAR LBP. The PPARδ-Compound 2 complex was crystallized by Pettersson and colleagues to investigate the structural basis for partial agonism induced by Compound 2 (Fig. 6.17, Table 6.15) [84]. This was the first crystal structure of a partial agonist of PPARδ.

Compound 2 utilizes a Y-shaped design, swapping the methyl substituent in the (2-methylphenoxy)acetic acid in the head region to a cyclopentyl. The head region

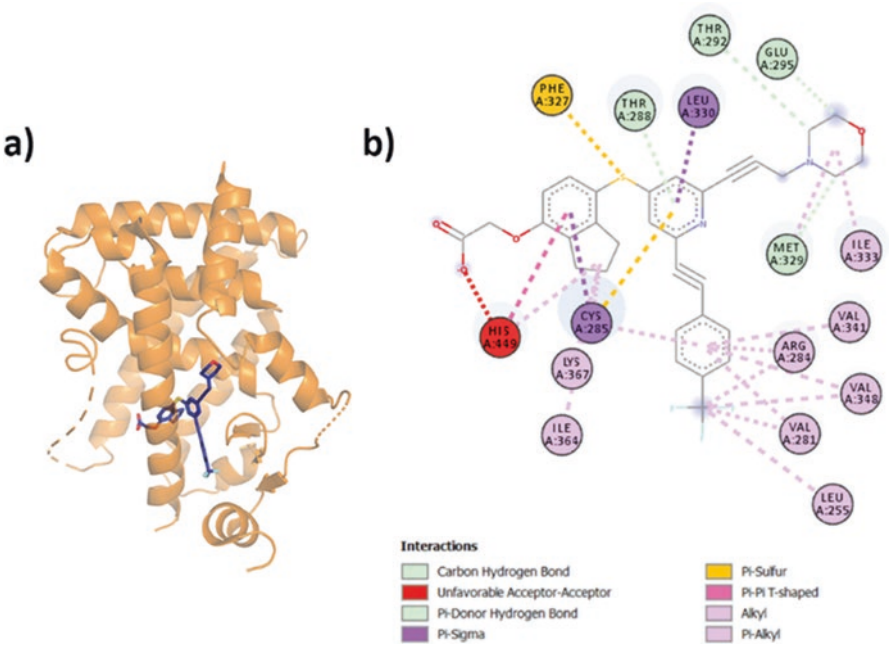

Fig. 6.17 (**a**) PPARδ LBD in orange complexed with Compound 2 in dark purple (PDB ID: 2Q5G). (**b**) Interaction map of Compound 2 with PPARδ

Table 6.15 The activity of Compound 2 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
Compound 2	ND	0.13 (39%)	ND

EC_{50} values were determined by dual luminescence assay in CV-1 cells, measuring alkaline phosphatase activity normalized to β-galactosidase activity. Maximal activity (%) was defined as relative to NNC 61-4655, GW501516 and rosiglitazone for PPARα, PPARδ and PPARγ, respectively. ND – not determined; EC_{50}.was not calculated if activity was lower than 10% at ligand concentration of 30 μM

adopts a lower position compared to full agonists and forms hydrogen bonds with 1/4 of the polar residues in Arm 1 and interacts with five other residues in Arms 1, 2 and 3. The lack of interactions with residues on H12 might have destabilized H12 (unmodelled in structure) and the AF2 surface, contributing to its partial agonistic activity. The core interacts with two residues from Arms 2 and 3. The trifluoromethylbenzene motif in the tail interacts with five residues in Arm 2. The morpholine motif in the fin interacts with four residues in Arm 3, reaching up to H5. The fin is connected to the core via a rigid alkyne carbon linker.

To accommodate the fin, the core sits further anteriorly than usually seen for PPARδ ligands. The cyclopentyl motif in the head is too close to the core due to the short core-head sulphur linker and points posteriorly into δ: I328, selecting against

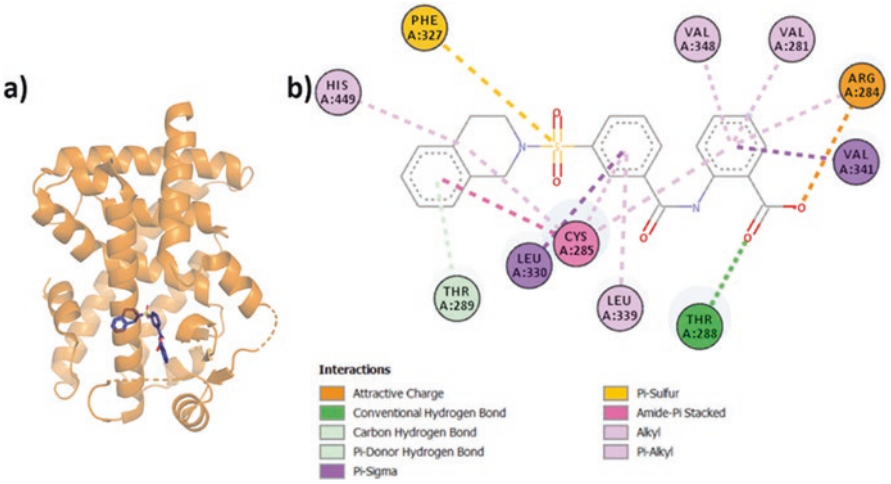

Fig. 6.18 (**a**) PPARδ LBD in orange complexed with GW9371 in dark purple (PDB ID: 3DY6). (**b**) Interaction map of GW9371 with PPARδ

PPARα/γ affinity as previously mentioned and pushing H7 out posteriorly. This causes the carboxylic acid head to sit lower than other PPARδ agonists, preventing interaction with H12. The trifluoromethyl substituent in the tail also interacts with δ: V312, selecting against PPARγ affinity.

GW9371

The PPARδ partial agonist GW9371 was identified by GSK through a high throughput screening (HTPS) of their in-house compound collection. This was part of their efforts to identify a range of chemical tools to investigate biological role of PPARδ. The PPARδ-GW9371 complex was crystallized by Shearer and colleagues in their SAR study that yielded partial agonist compounds GSK1115 and GSK7227 (Fig. 6.18, Table 6.16) [96].

The L-shaped GW9371 contains a tetrahydroisoquinoline interacts with 1/4 of the polar residues in Arm 1 and 2 residues in Arms 2 and 3. This motif does not form hydrogen bonds with H12, likely resulting in its partial agonistic activity. It contains a sulfonyl core which makes a total of four interactions with residues in Arms 1, 2 and 3. A formylanthranilic acid motif comprises the tail region of the ligand and makes polar interactions with δ: T252 and R248 and hydrophobic interactions with four other residues in Arm 2.

Ortho-substituted acids are generally not tolerated in Arm 1 of PPARδ, and thus the compound binds to Arm 2 as predicted by Shearer and colleagues [96]. The proximity of the carboxylic acid and amide substituents likely causes intra-ligand interactions, further reducing the likelihood of accommodation of this motif in Arm 1, which then places the tetrahydroisoquinoline motif in Arm 1. Affinity to PPARδ

Table 6.16 The activity of GW9371 at each PPAR subtype

Ligand	PPARα	PPARδ	PPARγ
GW9371	IC_{50} (μM)		
	>10	0.1258	7.9432
	EC_{50} (μM)		
	–	1.2589 (61%)	–

IC_{50} values were determined by scintillation proximity assay (SPA), using [H^3]GW2331, [H^3]GW2433 and [H^3]BRL49653 as radioligands for PPARα, PPARδ and PPARγ respectively. EC_{50} values were determined by dual luminescence assay in CV-1 cells, measuring alkaline phosphatase activity normalized to β-galactosidase activity. Maximal activity (%) was defined as relative to GW501516 for PPARδ.

is likely due to the proximity of the sulfonyl core to δ: I328 (α: M355 γ: M364), clashing with methionine residues in and selecting against PPARα and γ.

6.2.3 Dual or Pan Agonist Ligands

Dual or pan agonists refer to agonists that bind specifically to at least two or all three of the PPAR subtypes, respectively, and result in transactivation of PPAR genes. Groups that synthesize these agonists attempt to combine the unique pharmacological benefits of activating each PPAR subtype [23]. This was initially done to alleviate the side effects seen in activation of a single subtype, such as weight gain with PPARγ-activating TZDs. Another motive for such therapeutics is that ligands combining the effects of multiple receptors may reduce risks associated with using multiple drugs in combination to treat complex ailments [6]. Some dual agonists combine the effects of different nuclear receptor families or enzymes, opening the possibility of an even wider range of pharmacological outcomes [37, 68]. Examining the structural basis of dual and pan agonists often reveal important insights into selectivity and unexpected binding modes.

6.2.3.1 PPARα/γ Dual Agonists

TZD-based therapies were efficacious in lowering blood glucose levels, decreasing insulin levels and increasing sensitivity to insulin. However, they did not modulate serum lipid levels and often resulted in weight gain. Since fibrates modulate serum lipid levels, the prominent idea during this period was to create ligands with PPAR α and γ affinity. In theory this would combine the insulin sensitization effects of TZDs and the lipid modifying effects of fibrates to reduce the weight gain seen with TZD treatment [34]. Based on this hypothesis, many groups have attempted to synthesize dual α/γ agonists, however many of these drugs have been dropped during various stages of clinical trials due to side effects [23, 110, 123]. Since then, several PPARα/γ dual agonists have been approved for clinical use, such as lobeglitazone.

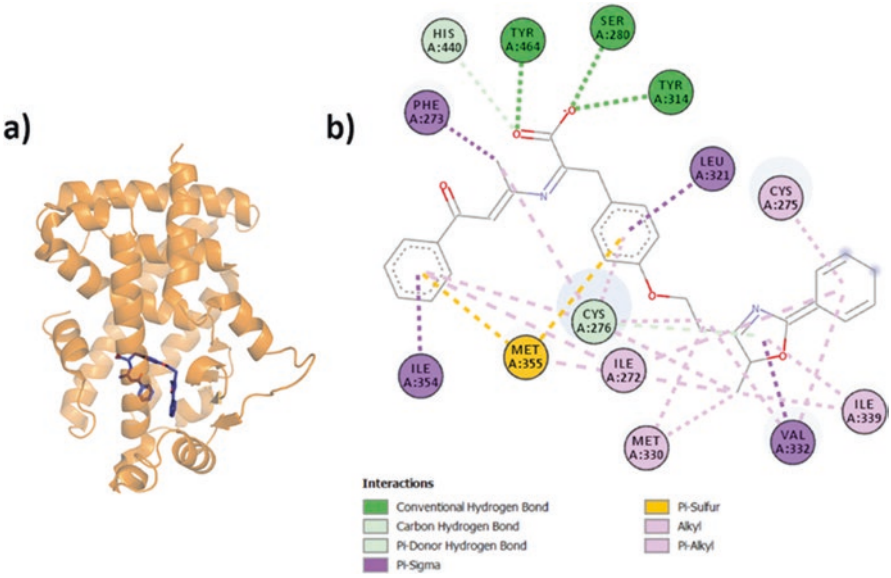

Fig. 6.19 (**a**) PPARα LBD in orange complexed with GW409544 in dark purple (PDB ID: 1K7L). (**b**) Interaction map of GW409544 with PPARα

Table 6.17 The activity of GW409544 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
GW409544	0.0023 ± 0.0005	>10	0.00028 ± 0.00006

EC_{50} values were determined by dual luminescence assay in CV1 cells, measuring luciferase activity normalized to β-galactosidase activity

This suggests that PPARα/γ dual agonism may not be inherently toxic, but further investigation is required to synthesize and determine the safety of new compounds [45, 123].

GW409544

GW409544 is a L-tyrosine analogue, modified from Farglitazar [115]. It was developed to improve on Farglitazar's affinity for PPARα and to balance its affinity for PPARα and γ [115]. The GW409544-PPARα-SRC1 complex was crystallized by Xu and colleagues (Fig. 6.19, Table 6.17) [115], continuing their study into the structural determinants of selectivity between the PPAR subtypes [114, 115]. This is the first PPARα crystal structure published, as well as the first PPARα structure crystallized with a coactivator motif.

The N-shaped GW409544 forms H bonds with 4/4 of the polar residues in Arm 1. It contains an atypical a phenyl-butanone substituent near the acidic head group.

This group points downwards from Arm 1 diagonally towards H7, pushing against F273 and interacting with four other residues in Arms 1 and 2. The phenoxy core wraps around H3 and makes interactions with three different residues in Arms 2 and 3. The tail substituent makes interactions with six residues in Arms 2 and 3.

Selectivity to PPARα and γ is likely imparted by a few features. Firstly, residues α: F273 γ: F282 is flexible and can accommodate the head phenyl-butanone substituent, but in PPARδ, δ: M417 likely prevents flexing of corresponding δ: F246. This substituent is able to occupy the posterior part of Arm 2, a characteristic not normally seen in PPARα agonists due to methionine residues α: M355 and M330. The phenyl ring at the tip of the tail does not contain bulky, polar substituents that interfere with γ: M348. Finally, the tail is connected to the phenoxy core via a flexible alkyl chain linker. The skinny alkyl chain does not interfere with methionine residues in PPARα/γ (α: M355 γ: M364 and α: M330) and allows optimal positioning of the tail substituent to accommodate PPAR γ: M348 in Arm 2. The smaller corresponding residues in PPARδ are unable to form favourable interactions with GW409544.

Azetidinone Compounds 17 and 35

Wang and colleagues from Bristol-Myers Squibb developed the dual α/γ agonist Azetidinone Compounds 17 and 35. These compounds were developed to be structurally distinct from Muraglitazar to avoid the associated side effects seen with the ligand [110]. Only the PPARα-Compound 17 complex was crystallized to confirm the stereochemistry of Compound 17, as part of their SAR study (Fig. 6.20, Table 6.18) [110].

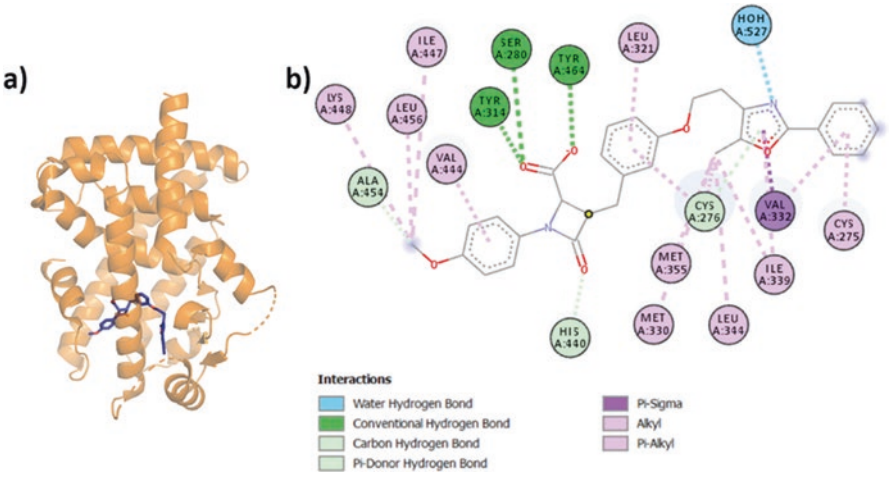

Fig. 6.20 (**a**) PPARα LBD in orange complexed with Compound 17 in dark purple (PDB ID: 2REW). (**b**) Interaction map of Compound 17 with PPARα

Table 6.18 The activity of Azetidinone compound 17 at each PPAR subtype

Ligand	PPARα	PPARδ	PPARγ
Compound 17	IC_{50} (μM) Ki		
	0.360 ± 0.005	–	0.100 ± 0.003
	EC_{50} (μM)		
	0.070 ± 0.005 (>90%)	–	0.090 ± 0.021 (>90%)

IC_{50} values were determined by fluorescence polarization assay (FPA), against probes 7-benzyloxy-4-trifluoromethyl coumarin and 7-benzyloxyresorufin for PPARα and PPARγ, respectively. EC_{50} values were determined by luciferase assay in HEK293 cells. Maximal activity (%) was defined as activity of 1 μM of ligand relative to 1 μM of GW-2331 and rosiglitazone for PPARα and γ, respectively.

Compound 17 is an N-shaped, full PPARα/γ agonist. In the head region, the 2-substituted formic acid group on the azetidinone motif forms hydrogen bonds with 4/4 of the polar residues in Arm 1. It contains an atypical 1-substituted methoxbenzene motif which makes interactions with five residues in the 'Benzophenone pocket', bordered by H3, H11 and the H11–H12 loop. The *para*-substituted phenoxy core interacts with two residues in Arms 2 and 3. This optimally positions the tail substituent, to interact with the bulky methionine residues in the Arm 3 region of PPARα. In the tail region, the aryl rings interact with eight residues in Arm 2 and 3.

Compound 17 selects against PPARδ, similar to GW409544 described above. The core and tail motifs are identical, making contacts with the methionine residues in PPARα and PPARγ. However, α: F273 flexes and points towards H7 allowing the head methoxybenzene substituent to occupy the benzophenone pocket, a conformation previously seen for PPARγ in Montanari et al. [77]. PPARδ cannot accommodate this substituent due to the presence of a bulkier δ: M417 residue in this pocket. This conformation of α: F273 might also push H3 anteriorly (compared to all other PPARαs structures examined) and induce instability to H3, accounting for the variation in affinity between Compound 17 and GW409544.

GL479

In 2012, Giampietro and colleagues synthesized compound GL479, a dual α/γ agonist that had full agonistic activity toward PPARα and partial agonistic activity toward PPARγ. This specific activation profile was selected based on the hypothesis that side effects seen in previous dual α/γ agonists were caused by full agonist activity toward PPARγ [45]. The PPARα/γ – GL479 complexes were crystallized by dos Santos and colleagues in their study (Fig. 6.21, Table 6.19) [33].

The L-shaped GL479 contains a fibrate head which forms hydrogen bonds with ¾ of the polar residues in Arm 1. The gem-dimethyl motif here interacts with four other residues in Arm 1. An ethanol core links the head to the tail region. The tail azobenzene motif interacts with six residues in Arms 2 and 3. Interestingly, in

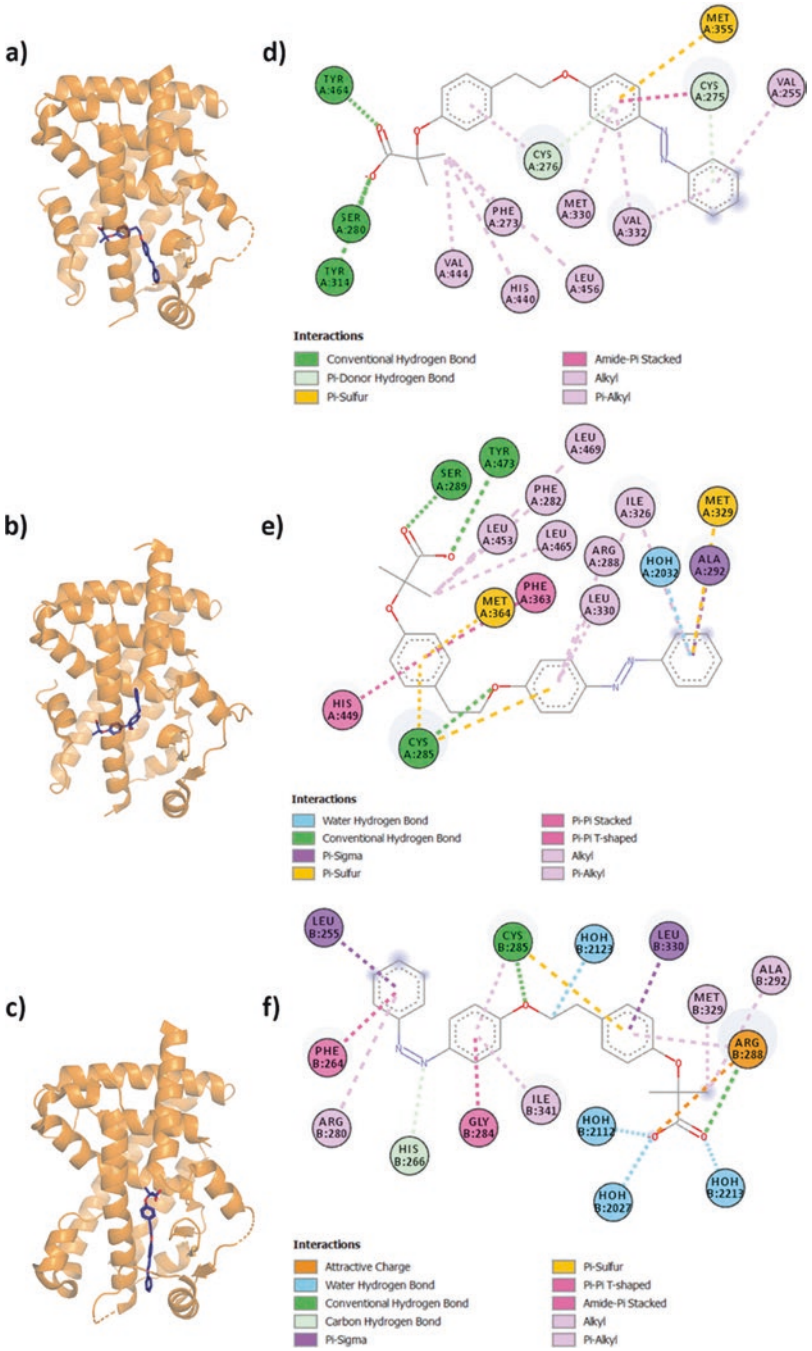

Fig. 6.21 (**a**) PPARα LBD in orange complexed with GL479 in dark purple (PDB ID: 4CI4). (**b**) PPARγ LBD in orange complexed with GL479 in the 'tail-up' conformation in dark purple (PDB ID: 4CI5). (**c**) PPARγ LBD in orange complexed with GL479 in the 'top-down' conformation in dark purple (PDB ID: 4CI5). (**d**) Interaction map of compound GL479 with PPARα. (**e**) Interaction map of GL479 in the 'tail-up' conformation with PPARγ. (**f**) Interaction map of GL479 in the top-down conformation with PPARγ

Table 6.19 The activity of GL479 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
GL479	0.6 ± 0.01 (158%)	–	1.4 ± 0.02 (21%)

EC_{50} values were determined by dual luciferase assay in HEK293 cells, values were normalized to *Renilla* activity. Maximal activity (%) was defined as relative to Clofibric acid Rosiglitazone for PPARα and γ respectively [45].

PPARγ GL479 adopts two distinct conformations. In the first conformation, it adopts a tail-up conformation and interacts with Arm 3 [114]. This can be due to: (1) The smaller γ: H323 (α: Y314) residue forces the ligand to bind closer to H12 in PPARγ. The ligand would be unable to effectively span across H3 into Arm 2, possibly explaining its partial agonism for PPARγ. (2) The tail region likely makes more contacts in the tail up conformation for PPARγ, due to the smaller corresponding residues of α: C275 and M330 in Arms 2 and 3 (γ: G284, V339). It forms interactions with five residues in Arm 3. In the second conformation of PPARγ-GL479 complex, GL479 adopted a top-down conformation spanning Arms 3 and 2. Partial agonists for PPARγ often adopt this top-down conformation [14, 25]. It makes interactions with three residues in Arm 1, six residues in Arm 2 and γ: H266 and F264 on the Ω loop.

The affinity towards PPARα and γ, like GW409544 and Azetidinone compounds 17 comes from the slim core and tail design, allowing accommodation by the methionine residues in PPARα and γ Arms 2 and 3. However, it is the lack of substituents in the tail region that likely accounts for its low potency. GL479 makes 16 interactions with PPARα, which is comparable to other ligands with low affinity like WY14643 or 17-oxoDHA. Although GL479 makes more interactions (20 in both conformations) with PPARγ, it is more potent at PPARα (16 interactions). The top down conformation is possible in PPARγ due to residues γ: G284 and R280.

6.2.3.2 PPARα/δ Dual Agonists

Targeting PPARα and γ has been the focus drug discovery due to availability of selective ligands and thus a better understanding of its biochemistry and therapeutic utility. Tools for PPARδ arrived much later, and consequently the therapeutic benefits of PPARδ were discovered later compared to PPARα and γ [53]. As such, PPARα/δ and δ/γ dual agonists are very rare [23]. A recent example was PPARα/δ Elafibranor which displayed a desirable safety profile but did not perform significantly better than placebo against non-alcoholic steatohepatitis [44, 86]. Whether this problem was specific to elafibranor or due to the inherent biochemistry of PPARα/δ activation remains to be seen.

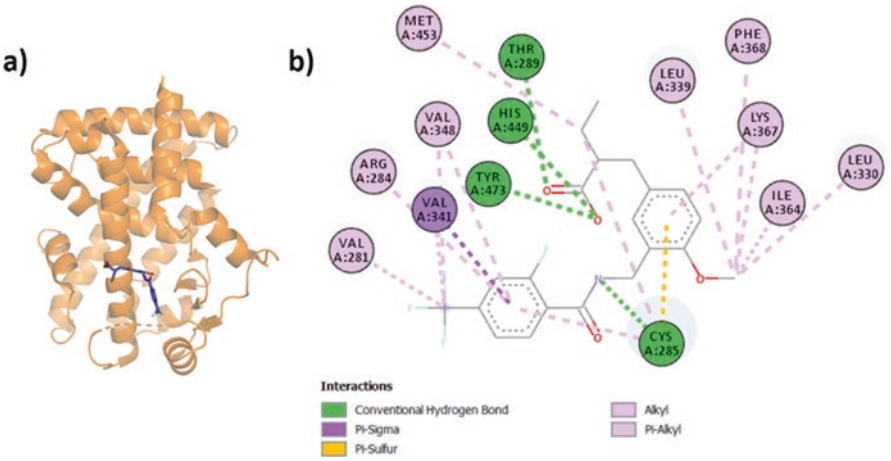

Fig. 6.22 (**a**) PPARδ LBD in orange complexed with TIPP-401 in dark purple (PDB ID: 2ZNQ). (**b**) Interaction map of TIPP-401 with PPARδ

Table 6.20 The activity of TIPP-401 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
TIPP-401	0.010	0.012	4.900

EC_{50} values were determined by luciferase activity in HEK-293 cells. Maximal activity (%) was defined as relative to GW501516 for PPARδ and Rosiglitazone for PPARγ [53]

TIPP-401

Kasuga and colleagues first synthesized TIPP-401 by optimizing the ligand tail and core regions, using their previous PPARα specific compound KCL as a lead [53]. The PPARδ-TIPP-401complex was later crystallized by Oyama and colleagues to explore the structural selectivity between the PPAR subtypes (Fig. 6.22, Table 6.20) [82]. The only difference between the PPARα selective KCL and PPARα/δ dual agonist TIPP-401 is a 2-substituted fluorine atom on the phenyl in the tail motif and a reversed carbon amide core-tail linker.

TIPP-401 is a Y-shaped ligand that interacts with 3/4 of the polar residues and two other residues (including δ: M417) via the isobutyric acid head motif in Arm 1. It contains a 1,3,4-substituted phenyl core that interacts with two residues in Arm 2. The tail amino linker interacts with δ: T288. Its tail substituent, an amino linked 2-fluoro-4-(trifluoromethyl)benzene interacts with δ: V312, five other residues and W228 on the Ω loop, similar to TIPP-204. Its fin methanol substituent interacts with δ: I328 in Arm 2 and four other residues in Arms 3.

Like TIPP-204, affinity for PPARδ of TIPP-401 was likely increased by reversing the amide linker, compared to the PPARα specific KCL, creating interactions

between the carbonyl and residue δ: T288 [54]. Affinity against PPARγ is likely mediated by the close proximity of the tail to δ: V312, corresponding residues in PPARγ would clash sterically (γ: M348). As seen in the PPARα-TIPP-703 complex (PDB ID: 2ZNN), α: M355 flexes to avoid clashing with the fin substituent, explaining the high affinity of TIPP-401 for PPARα.

6.2.3.3 PPARδ/γ Dual Agonists

Phenoxyacetic Acid Compounds 10 and 21

Connors and colleagues identified Compounds 10 and 21 from a SAR study [25]. They attempted to combine the fatty acid-oxidizing properties of PPARδ activation,and the safer, antiadipogenic character of partial agonists of PPARγ. The PPARδ-Compound 10 and PPARγ-Compound 21 complexes were crystallized by Connors and colleagues to understand the structural basis for the binding profile of the compounds (Fig. 6.23, Table 6.21) [25].

Compounds 10 and 21 both uses a Y-shaped design. In the PPARδ – Compound 10 complex, we see that the (2,3-dimethylphenoxy)acetic acid head motif in compound 10 makes hydrogen bonds with 4/4 polar residues in Arm 1 and hydrophobic interactions with five residues in Arms 2 and 3. It contains a phenyl core which interacts with δ: L294 in Arm 3. This core is 1,2,4-substituted with the head, fin and tail regions, respectively. The ethanol fin interacts with four residues in Arm 3. The 4-trifluoromethylphenol interacts with seven residues in Arm 2. In the PPARγ-Compound 21 complex, the ligand adopts a top-down conformation. The head interacts with γ: R288 and three other residues in Arm 3. The core and tail regions interact with five residues in Arm 2 and γ: I262 on the Ω loop. The 2-pentyn-1-ol fin sits between H3 and H7 below Arm 1.

Affinity for PPARδ is likely imparted by the oxygen and sulphur linkers from the head, tail and fin to the core. This 1,2,4-substituted core optimally positions it to interact with δ: I328 and V334. Corresponding methionine residues in PPARα and γ would likely clash with the ligand. This clash likely forces Compound 21 to assume an atypical top-down conformation in PPARγ but not α, which was possible due to residue γ: G284 (α: C275 δ: R248) and R280 (α: R271 δ: H244). These residues create space for the tail substituent to be accommodated in Arm 2, away from γ: M348.

Sulfonylthiadiazole Compounds 6, 11t and 20a

Compounds 6, 11t and 20a are sulfonylthiadiazole analogues synthesized by Sanofi-Aventis from a SAR study of PPAR. The rationale for this series of compounds was similar to the study by Connors et al. [25]. The PPARδ-compound 6, 11t and 20a complexes were crystallized by Keil and colleagues to understand the binding mode of and optimize their compounds (Fig. 6.24, Table 6.22) [59]. The lead compound,

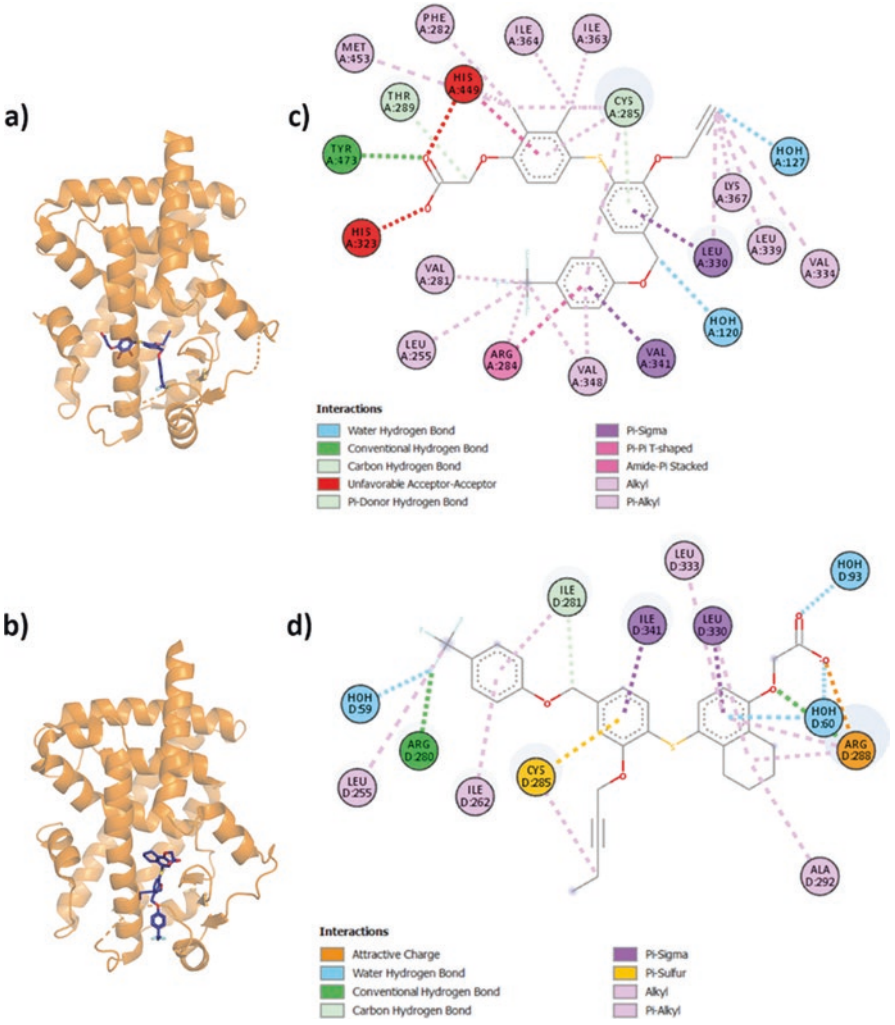

Fig. 6.23 (**a**) PPARδ LBD in orange complexed with Compound 10 in dark purple (PDB ID: 3GZ9). (**b**) PPARγ LBD in orange complexed with Compound 21 in dark purple (PDB ID: 3H0A). (**c**) Interaction map of Compound 10 with PPARδ. (**d**) Interaction map of Compound 21 with PPARγ

Table 6.21 The activity of phenoxyacetic acid compounds at each PPAR subtype

Ligand	PPARα	PPARδ	PPARγ
	IC_{50} (μM)		
Compound 10	0.21	0.004	0.30
Compound 21	0.88	0.005	0.033
	EC_{50} (μM)		
Compound 10	6.1 (48%)	0.054 (86%)	3.0 (40%)
Compound 21	>10	0.044 (94%)	0.16 (34%)

IC_{50} values for PPARα and δ were determined by scintillation proximity assay (SPA), using [^{3}H] GW2433 as a radioligand for PPARα and δ. IC_{50} values for PPARγ were determined by a filtration assay (FA), using [^{3}H] rosiglitazone. EC_{50} values were determined by luciferase assay in CV-1 cells. Maximal activity (%) was defined as relative to GW501516 and rosiglitazone for PPARδ and PPARγ, respectively

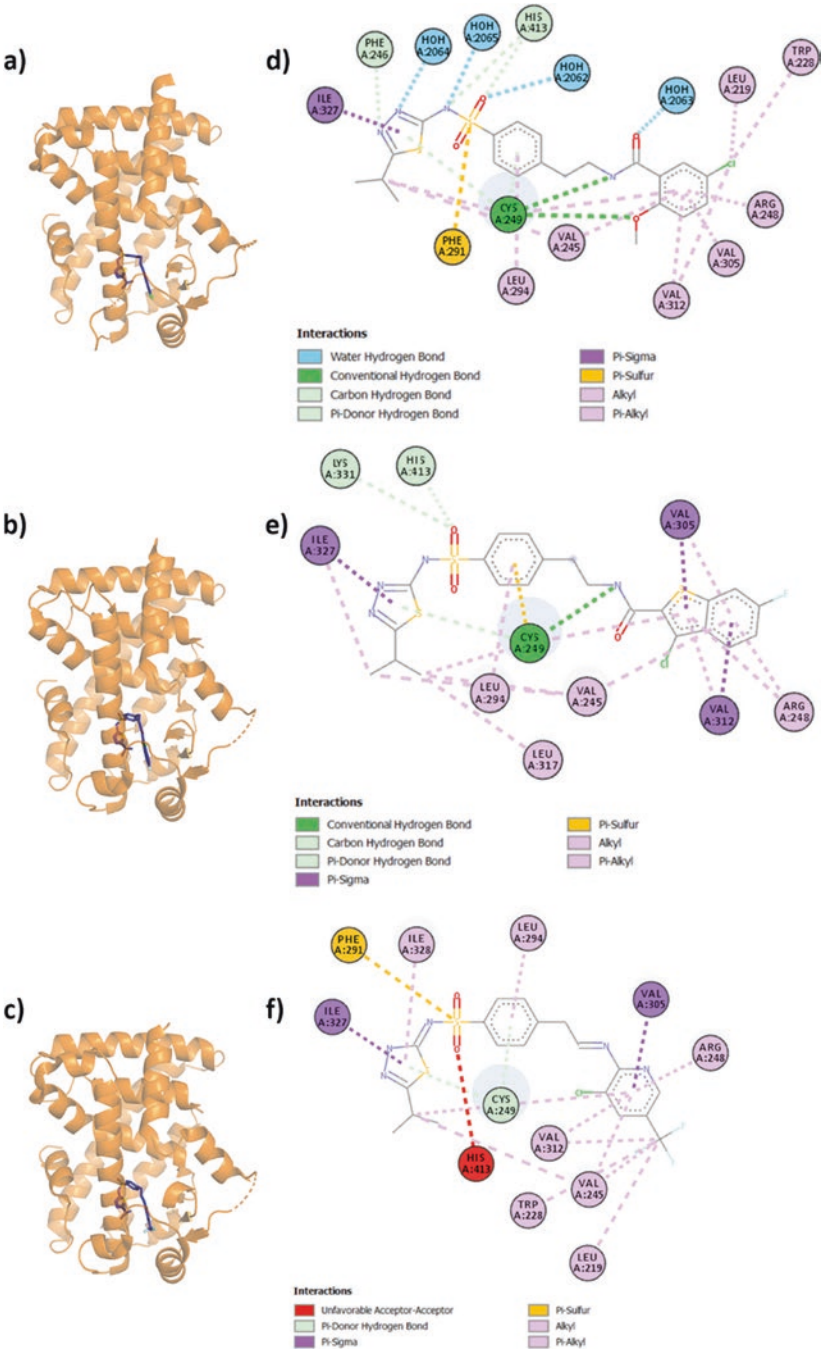

Fig. 6.24 (**a**) PPARδ LBD in orange complexed with Compound 6 in dark purple (PDB ID: 2XYJ). (**b**) PPARδ LBD in orange complexed with Compound 11t in dark purple (PDB ID: 2XYW). (**c**) PPARδ LBD in orange complexed with Compound 20a in dark purple (PDB ID: 2XYX). (**d**) Interaction map of Compound 6 with PPARδ. (**e**) Interaction map of Compound 11t with PPARδ. (**f**) Interaction map of Compound 20a with PPARδ

Table 6.22 The activity of lead sulfonylthiadiazole analogues at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
Compound 6	1.6900 (13%)	0.7380 (30%)	3.2300 (13%)
Compound 11t	ND	0.3180 (40%)	1.1990 (33%)
Compound 20a	ND	0.0016 (26%)	0.3360 (26%)

For PPARα and δ, EC_{50} values were determined by luciferase activity in stably transfected HEK cells. For PPARγ, EC_{50} values were determined by dual luciferase assay in HEK cells, and values were normalized to *Renilla* activity. Maximal activity (%) was defined as relative to fenofibric acid, GW501516 and rosiglitazone for PPARα, PPARδ and PPARγ, respectively. Compounds were tested up to 30 μM. ND – not determined; if compounds exhibited <10% activity, the EC_{50} values would not be calculated

Compound 6, was a low affinity, low efficacy pan agonist compound. The tail region of Compound 6 was optimized to give Compound 11t and Compound 20a with vastly improved affinity, potency and efficacy.

These N-shaped compounds bind to the bottom of Arm 1, between H3 and H7 in the inferior region of LBD and Arm 2. All three compounds occupy similar position and binding mode in PPARδ. These compounds contain a sulfonyl head region with a 2-isopropyl-1, 3, 4-thiadiazole substituent and wrap around H3 at δ: C249.

The sulfonyl head region in Compound 6 forms hydrogen bonds with three water molecules. It makes π-donor hydrogen bonds with two residues in Arm 1 and 2 and interactions with two other residues in the inferior region of Arm 1. The phenyl core interacts with two residues in Arm 1 and 2. The core is linked to the tail substituent via an N-ethylformamide linker, which forms hydrogen bonds with δ: C249 and one water molecule. The 2-methoxy-5-chlorobenzene tail substituent makes hydrophobic interactions with five residues in Arm 2 and δ: W228, and one hydrogen bond with δ: C249 via the methoxy group.

The sulfonyl head region in Compound 11t forms weak hydrogen bonds with three residues in Arms 1, 2 and 3. It makes hydrophobic interactions with four other residues in Arms 1, 2 and 3. The phenyl core makes interactions with two residues in Arm 2. The core is linked to the tail substituent with the same N-ethylformamide linker, which forms hydrogen bonds with δ: C249. The benzothiophene tail substituent makes hydrophobic interactions with five residues in Arm 2.

In Compound 20a, the sulfonyl head region forms a π-donor hydrogen bond with δ: C249 and interacts with four other residues in Arms 1 and 2. The phenyl core interacts with two residues in Arms 2 and 3. The core is connected to the tail substituent via a propanimine linker. The 3-chloro-5-trifluoromethylpyridine tail substituent forms interactions with six residues in Arm 2.

The high potency of this series of compounds can be attributed to the strong interactions between the linker and δ: C249. The rigidity of Compound 20a compared to Compounds 6 and 11t might account for its increased potency. Compared

to GW501516 [97, 113], the trifluouromethyl motif in the tail substituent sits lower, reducing interference with corresponding methionine residue of δ: V312 in PPARγ (γ: M348).

6.2.3.4 Pan Agonists

The rationale behind synthesizing pan agonists follows that for dual agonists – to combine the therapeutic benefits of each PPAR subtype and to reduce the risk of multiple pharmacology [6, 55, 56, 82]. These efforts are spurred on by the clinical success of bezafibrate [16, 55, 56]. Pan agonism presents a challenging design problem, as pan agonists need to interact with areas conserved among all three subtypes – this is difficult as these regions of dissimilarity are spread throughout the LBP (Table 6.1). Here we see two different design approaches: (1) by decreasing the size of the ligand [6, 19] and (2) by using a Y-shaped ligand [82].

Indeglitazar

Artis and group synthesized the pan agonist indeglitazar. The group took an unorthodox approach in the drug screening phase where they selected ligands with weak activity across all three receptors and then optimized for affinity towards all PPAR subtypes, as opposed to selecting ligands with stronger affinity for a specific subtype and then optimizing for selectivity. The rationale was that the side effects of PPAR activation were not only caused by non-specific binding but also by the supraphysiological activation of the PPAR receptor [118]. The PPARδ, PPARα and PPARγ-indeglitazar complexes were crystallized by Artis and colleagues to understand the binding mode of their pan agonist (Fig. 6.25, Table 6.23) [6].

The Y-shaped indeglitazar assumes a nearly identical conformation in all three PPAR subtypes. It binds to the inferior area of Arm 1, in the space between H3 and H7. It has a propionic acid head that interacts with 3/4 of the polar residues in PPARα, δ and γ. In PPARα, α: Y314 pushes this acid motif slightly towards H3 to interact with S280 instead of H440. In PPARγ, the acid motif rotates slightly in comparison, interacting with γ: S289 instead of H449. This acid group is connected to the 5-methoxy-1H-indene group, which makes interactions with six residues in PPARα/γ and seven residues with PPARδ in Arms 1 and 2. The short tail substituent, a 4-methoxy1-sulfonylbenzene, wraps around H3 at the interface of Arms 2 and 3. It makes interactions with five residues in PPARα, with seven residues in PPARδ and γ, in Arms 1, 2 and 3.

The atypical architecture of indeglitazar avoids the methionine residues in Arms 2 and 3 and makes contacts in the inferior region of Arm 1 and posterior region of Arm 2. Although indeglitazar interacts with most polar residues in PPAR, it does not have full agonistic activity, showing that interaction with H12 is not sufficient for full agonism.

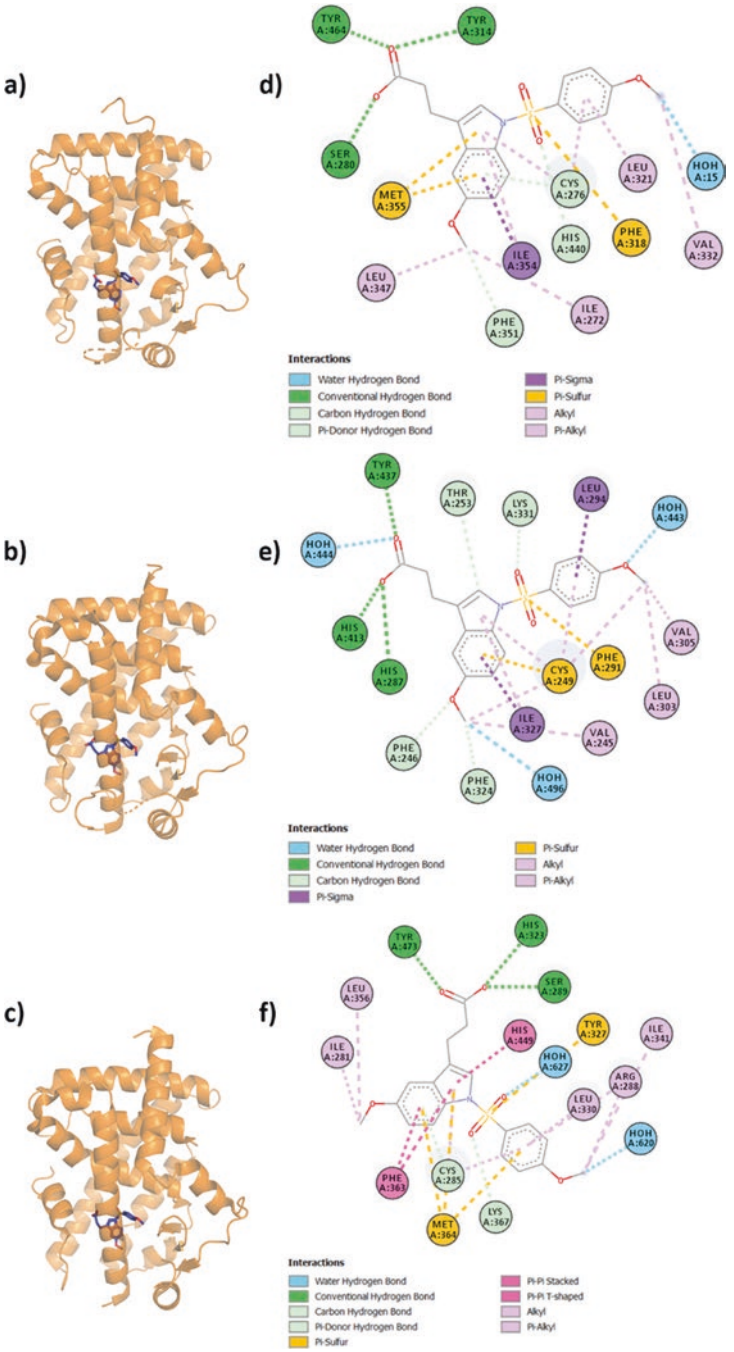

Fig. 6.25 (**a**) PPARα LBD in orange complexed with Indeglitazar in dark purple (PDB ID: 3ET1). (**b**) PPARδ LBD in orange complexed with Indeglitazar in dark purple (PDB ID: 3ET2). (**c**) PPARγ LBD in orange complexed with Indeglitazar in dark purple (PDB ID: 3ET3). (**d**) Interaction map of Indeglitazar with PPARα. (**e**) Interaction map of Indeglitazar with PPARδ. (**f**) Interaction map of Indeglitazar with PPARγ

Table 6.23 The activity of Indeglitazar at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
Compound 1	≈100	>200	≈150
Indeglitazar	0.99	1.3 (67 ± 18%)	0.85 (45 ± 10%)

EC_{50} values were determined by luciferase assay in HEK293T cells. Maximal activity (%) was defined as relative to WY-14643, L-165041 and rosiglitazone for PPARα, PPARδ and PPARγ, respectively

TIPP-703

Kasuga and group synthesized the pan agonist TIPP-703 during their exploration of the effects of the trifluoromethyl tail substituent on structural selectivity [55, 56]. The PPARα/γ-TIPP-703 complexes were later crystallized by Oyama and colleagues to explore the structural selectivity of PPAR (Fig. 6.26, Table 6.24) [82].

TIPP-703 is an L-shaped ligand. In PPARα, the isobutyric acid head motif interacts with 3/4 of the polar residues and three other residues in Arm 1. The 1,3,4-substituted phenyl core interacts with α: C276. The core-tail amide linker interacts with T279. The tail substituent interacts with six residues in Arm 2 and α: L254 V255 on the Ω loop. The fin propanol substituent interacts with four residues in Arms 2 and 3 including α: M330 and M355. In PPARγ, the isobutyric acid head motif forms hydrogen bonds with 2/4 of the polar residues and hydrophobic interactions with four other residues in Arm 1. The 1,3,4-substituted phenyl core interacts with four different residues. The tail phenyl interacts with three residues including γ: G284, while the adamantyl group interacts with γ: R280 and two other residues. In the PPARγ crystal structure the fin propanol substituent is disordered and left unmodelled.

The affinity for all three PPARs is likely explained by the slim core area with the longer tail motif with the bulky adamantyl group. The core avoids the methionine residues in PPAR α and γ. The architecture of the tail substituent avoids clashing with γ: M348 in PPARγ. Additionally the flexible γ: R288 (α: T279, δ: T252) allows the tail motif to move closer to H3 and for the adamantyl group to occupy the space between γ: G284 and R280 in Arm 2 of PPARγ. This adamantyl group is still able to make favourable interactions with Arm 2 of PPARα/δ. The propanol fin substituent makes sufficient contacts with the Arm 3 area which likely increases affinity for PPARδ without interfering with PPARα α: M330 [55, 56].

AL29-26

Capelli et al. [19] synthesized compound AL29-26, selected through a structure-based virtual screening process that considers experimentally determined PPAR-ligand interactions. The PPARα/γ-AL29-26 complexes were crystallized by Capelli

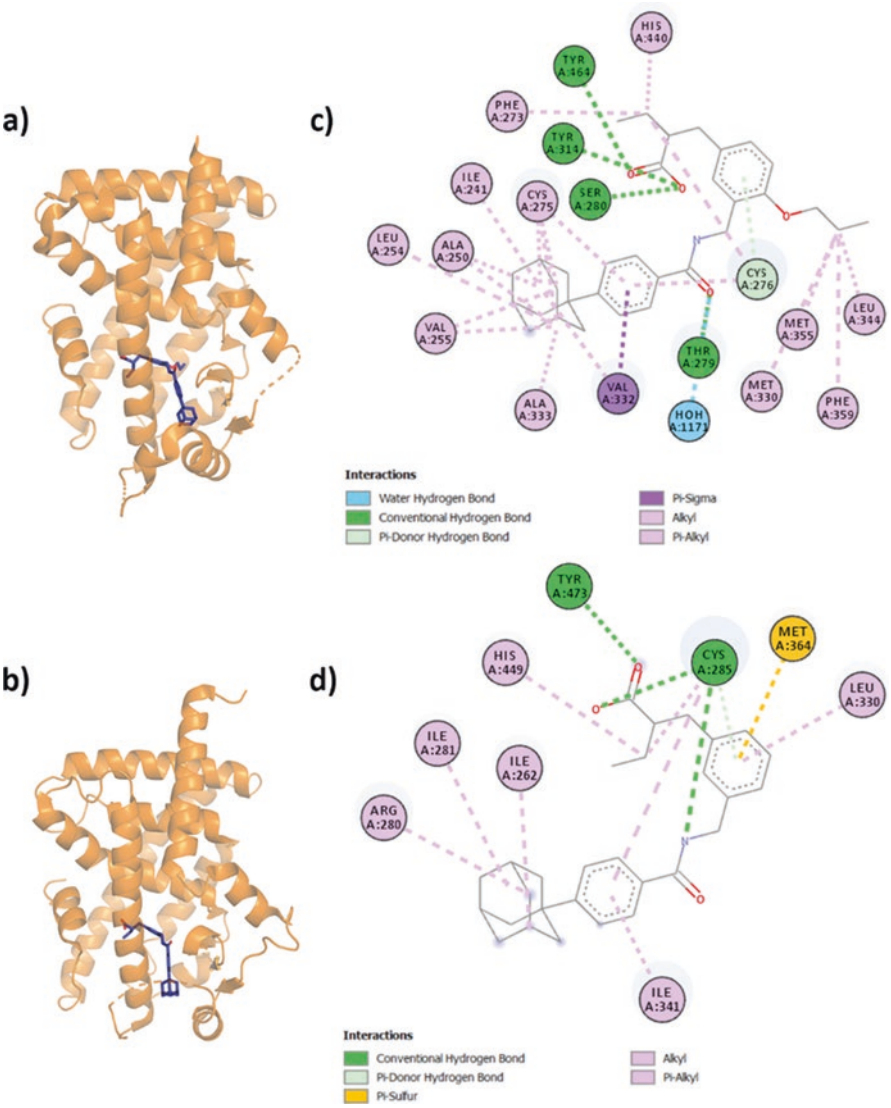

Fig. 6.26 (**a**) PPARα LBD in orange complexed with TIPP-703 in dark purple (PDB ID: 2ZNN). (**b**) PPARγ LBD in orange complexed with TIPP-703 in white (PDB ID: 2ZNO). (**c**) Interaction map of TIPP-703 with PPARα. (**d**) Interaction map of TIPP-703 with PPARγ

and colleagues (Fig. 6.27, Table 6.25) [19] to identify the structural basis for the difference in AL29-26 activity at the three PPAR subtypes.

AL29-26 adopts a similar, L-shaped conformation in both PPARα and γ. In PPARα, the isobutyric acid head forms hydrogen bonds with 4/4 of the polar residues in Arm 1 and hydrophobic interactions with three other residues. The

Table 6.24 The activity of TIPP-703 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
TIPP-703	0.061	0.120	0.043

EC_{50} values were determined by Dual luminescence assay in HEK293 cells, measuring luciferase activity normalized to β-galactosidase activity [53]

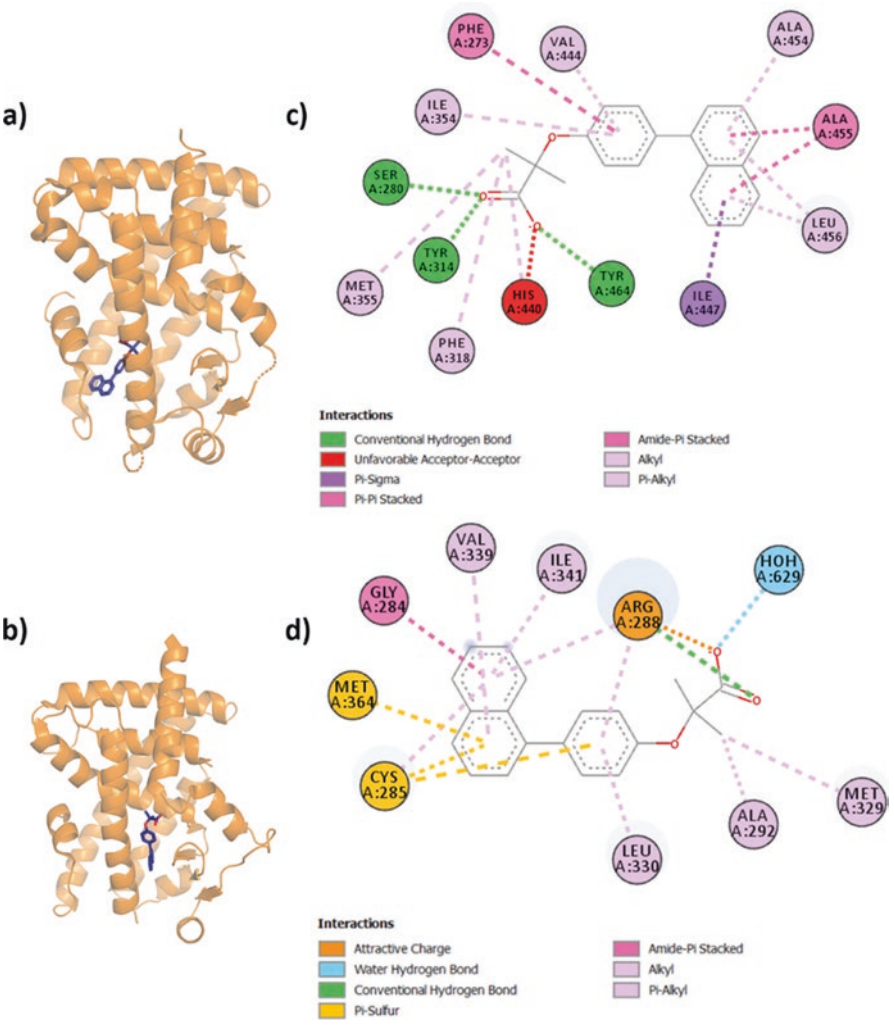

Fig. 6.27 (**a**) PPARα LBD in orange complexed with AL29-26 in dark purple (PDB ID: 5HYK). (**b**) PPARγ LBD in orange complexed with AL29-26 in dark purple (PDB ID: 5HZC). (**c**) Interaction map of AL29-26 with PPARα. (**d**) Interaction map of AL29-26 with PPARγ

Table 6.25 The activity of AL29-26 at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
AL29-26	0.31 ± 0.13 (87 ± 8%)	11.0 ± 2 (54 ± 6%)	5.3 ± 1.6 (27 ± 3%)

EC_{50} values were determined by dual luminescence assay in HepG2 cells, measuring luciferase activity normalized to β-galactosidase activity

phenoxy core interacts with three residues in Arm 1. The diphenyl tail makes interactions with four residues in the 'benzophenone pocket' in Arm 1 [77]. This is made possible by the flexing of α: F273 to accommodate the diphenyl. In PPARγ, the isobutyric acid head forms interactions with γ: R288 and two other residues in Arm 3. The phenoxy core and diphenyl tail interacts with seven residues in Arms 2 and 3.

This ligand possesses a much lower transactivation activity for PPARδ and PPARγ than seen for other ligands. This is likely due to the relatively smaller number of residues it interacts with in the PPAR LBD. Selectivity of this ligand for all subtypes likely arises from the fact that the ligand is small and does not interfere with residues of the different subtypes in PPAR. The fibrate motif in the acidic head may increase affinity towards PPARα, due to the larger α: Y314 residue, positioning it to interact with S280 [19]. AL29-26 resembles another known peroxisome proliferator nafenopin.

6.2.3.5 Endogenous Agonists

The PPAR subtypes have unique and overlapping affinities for different endogenous ligands, usually fatty acids or related metabolites [31, 61]. These fatty acids are usually present at micromolar concentrations in blood in the human body [114]. Various techniques have been used in the discovery of these ligands, but it was Xu and colleagues that first confirmed fatty acids as bona fide endogenous PPAR ligands through protein crystallography [31, 114]. Analysis of the binding mode of endogenous ligands often reveals important insights that can be applied to the design of better or safer synthetic ligands [82, 113].

Eicosapentaenoic Acid (20:5 EPA)

Eicosapentaenoic acid (C20:5) is a polyunsaturated fatty acid (PUFA). These PUFAs are thought to act through the PPAR receptor and has lipid lowering and insulin sensitization effects [114]. The PPARδ-EPA complex was crystallized by Xu and colleagues as part of their study into structural selectivity of PPARδ (Fig. 6.28, Table 6.26) [114]. In this study, EPA was found to bind in two distinct conformations, the 'tail-up' and 'tail-down' modes.

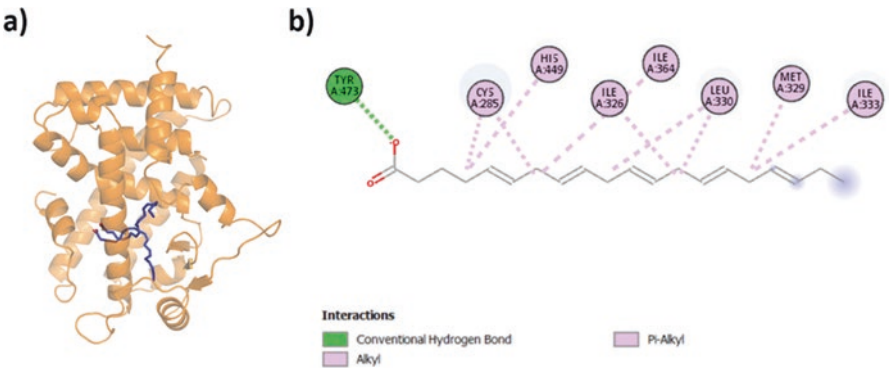

Fig. 6.28 (**a**) PPARδ LBD in orange complexed with EPA in both conformations in dark purple (PDB ID: 3GWX). (**b**) Interaction map of EPA in the tail-down conformation with PPARδ. Interaction map of the tail-up conformation is not available

Table 6.26 The activity of Eicosapentaenoic acid at each PPAR subtype

	IC_{50} (μM)			
Ligand	PPARα	PPARδ	PPARγ	Assay
Eicosapentaenoic acid (C20:5)	1.1 ± 0.23	4.0 ± 0.90	1.6 ± 0.20	SPA

IC_{50} values were determined by scintillation proximity assay (SPA), using [^{3}H] GW2331, [^{3}H] GW2433 and [^{3}H] BRL49653 as radioligands for PPARα, PPARδ and PPARγ respectively [114]

The EPA molecule assumes an L-shaped conformation. The carboxylic acid head forms hydrogen bonds with δ: Y473. Its lipophilic alkyl tail interacts with residues in Arm 2 or Arm 3 of PPARδ in the tail down or tail up conformation respectively. In the tail-up conformation, the lipophilic tail interacts with three residues in Arm 1, two residues in Arm 2, three residues in Arm 3. In the tail-down conformation, the lipophilic tail interacts with δ: H449, Q286 in Arm 1 and I363, I364, L339, C285, V281 and L255 in Arm 2.

In both conformations, all the unsaturated bonds adopt a cis configuration. In the tail-up conformation, the tail curves upwards to δ: M329 in H5 and then back down and out anteriorly towards the plane of H3. In the tail down confirmation, the tail it extends along the lateral edges of Arm 2 interacting with δ: I328, V312, L303, H7 and the β-sheet.

Vaccenic Acid (18:1)

Fyffe and colleagues found that a previously published 'apo' PPARδ protein structure contained significant electron density in the LBP. They found that bacterial fatty acid vaccenic acid binds to PPARδ during the protein purification process,

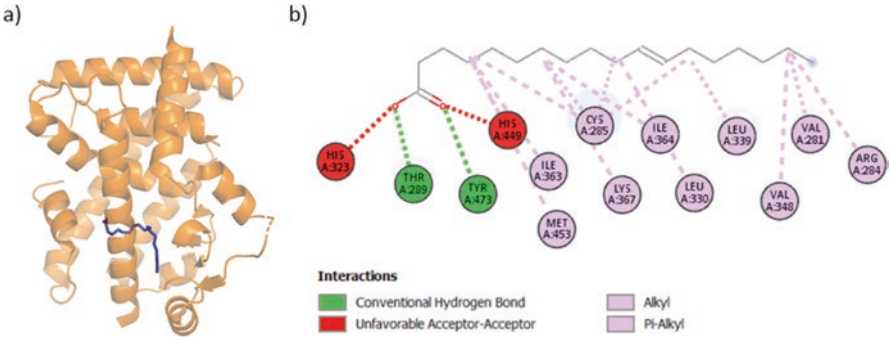

Fig. 6.29 (**a**) PPARδ LBD in orange complexed with vaccenic acid in dark purple (PDB ID: 2AWH). (**b**) Interaction map of Vaccenic acid with PPARδ

causing H12 of PPARδ to be locked in an active conformation. The PPARδ-vaccenic acid complex was crystallized by Fyffe and colleagues to prove that vaccenic acid binds to PPARδ during protein purification (Fig. 6.29) [41].

Vaccenic acid assumes an L-shaped conformation. The acidic head interacts with Arm 1 residues via δ: Y473, H449, H323 and T289. The lipophilic tail interacts with δ: M453, K367, I364, I363, V348, L339, L330, C285, R284 and V281, wrapping around H3 and interacting with Arm 2.

Iloprost

Iloprost is a prostaglandin I_2 (PGI_2) synthetic analogue, developed to study the role of a related but metabolically unstable eicosanoid PGI_3 in cardiovascular homeostasis. It was previously demonstrated that PPAR subtypes are differentially regulated by prostaglandins [122]. Here, the PPARα/δ-Iloprost-coactivator complexes were crystallized by Jin and colleagues to uncover the molecular mechanisms for selectivity of Iloprost towards PPARα and δ (Fig. 6.30 Table 6.27) [52].

The Iloprost assumes an L-shaped conformation. In PPARα, the 5-hexenoic acid head interacts with 3/4 of the polar residues in Arm 1. It forms hydrophobic interactions with five other residues in Arms 1 and 2. The 2-oxy-octahydropentalene core interacts with four residues in Arms 1, 2 and 3. The tail region forms a hydrogen bond with α: T279 in Arm 2 and hydrophobic interactions with two other residues in Arm 2.In PPARδ, the acidic head motif interacts with 2/4 polar residues in Arm 1. It also forms hydrophobic interactions with three other residues in Arm 1 and 2. The 2-oxy-octahydropentalene core interacts with four residues in Arms 2 and 3. The tail region forms a hydrogen bond with δ: T252 in Arm 2 as well as six other residues in Arm 2.

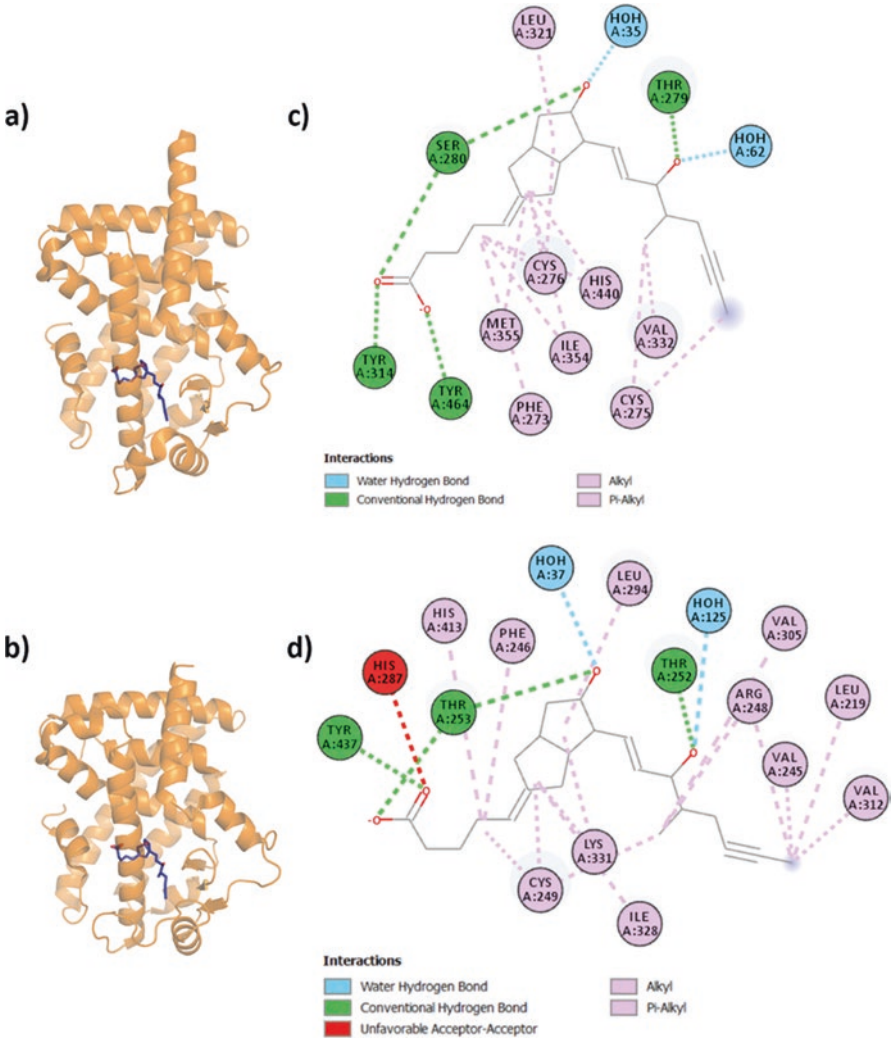

Fig. 6.30 (**a**) PPARα LBD in orange complexed with Iloprost in dark blue (PDB ID: 3SP6). (**b**) PPARδ LBD in orange complexed with Iloprost in dark blue (PDB ID: 3SP9). (**c**) Interaction map of Iloprost with PPARα. (**d**) Interaction map of Iloprost with PPARδ

Table 6.27 The activity of Iloprost at each PPAR subtype

	EC_{50} (μM)		
Ligand	PPARα	PPARδ	PPARγ
Iloprost	≈0.200	≈0.200	>20

The EC_{50} values were determined by dual luciferase assay in Cos-7 cells, and values were normalized to *Renilla* activity

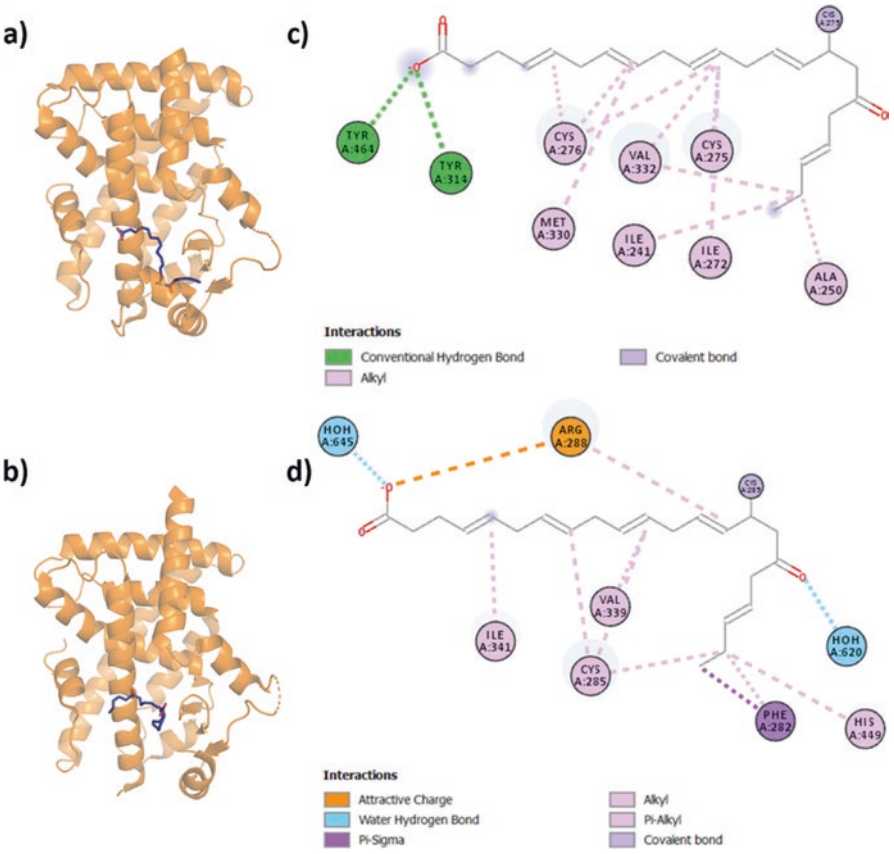

Fig. 6.31 (**a**) PPARα LBD in orange complexed with 17(S)-oxoDHA in dark purple (PDB ID: 5AZT). (**b**) PPARγ LBD in orange complexed with 17(S)-oxoDHA in dark purple (PDB ID: 5AZV). (**c**) Interaction map of 17(S)-oxoDHA with PPARα. (**d**) Interaction map of 17(S)-oxoDHA with PPARγ

Selectivity against PPARγ may be caused by interference with the corresponding residue γ: R288 (α: T279, δ: T252). γ: R288 would also be unable to form hydrogen bonds with the oxygen group in the tail substituent.

17(S)-oxoDHA (22:6)

17(S)-oxodocosahexaenoic acid (17(S)-oxoDHA) is an endogenous ligand of PPAR. The PPARα/γ – 17(S)-oxoDHA complexes have been crystallized by Egawa and colleagues, continuing their previous research into binding mode of endogenous ligands (Fig. 6.31) [35]. This represents the first crystallized example of an endogenous fatty acid bound to PPARα.

17(S)-oxoDHA assumes an L-shaped conformation in PPARα. Here, the acidic head of 17(S)-oxoDHA interacts α: Y464, Y314 and S280. The lipophilic tail interacts with Arm 1, 2 and the Ω loop. It makes a covalent bond with α: C275 and interacts with α: V332, M330, C276, C275, I272, A250, L247 and I241.

In PPARγ 17(S)-oxoDHA adopts a slightly different, folded L-shaped conformation and occupies the Arm 1, 2 area near the β1 sheet. The lipophilic tail interacts with γ: H449, M348, I341, V339, L330, Y327, R288, C285, F282, I281 and forms a covalent bond with γ: C285. The acidic head interacts with R288. This covalent bond is also seen in other PPARγ-endogenous ligand complexes [50; 107].

6.3 Ligand Design Factors

The PPAR ligand binding pocket contains areas of similarity and dissimilarity between the subtypes (Table 6.1). From SAR studies, especially studies regarding dual and pan agonists, interesting trends can be observed – different substituents on the same general ligand scaffold changes affinity of the ligand towards each PPAR subtype [13, 82, 113]. The structural basis for the variations in affinity can be elucidated when structural information is present, and for this reason, drug-discovery studies now typically include some form of structure-related information, protein docking or simulation, NMR, MS or crystallography. Our analysis of the PPAR-ligand interactions in Sect. 6.2 revealed common observations of the binding patterns of ligands, which we describe below.

1. PPARδ has the 'narrowest' Arm 1 and does not appear to accommodate residues in the 'Benzophenone pocket' observed in α or γ [77].

   The size of Arm 1 was noted in [115]. PPARα-specific agonist GW590735 was optimized based on this difference, between PPARδ vs α in Arm 1 by replacing the acid head motif with the 'Fibrate head' motif that tends to be specific to PPARα [97]. Some groups achieve different subtype selectivity by swapping the head region of the ligands. GSK has made multiple ligands with this method: GW590735 from GW501516, GW6471 from GW409544 and compound 48 from GW501516.
2. PPARα contains a unique AF2 hydrogen bonding network α: Y464, H440, Y314 instead of δ: Y437, H413, H287 γ: Y473, H449, H323.

   This difference has been used to optimize for and against PPARα selectivity [115, 119]. α: Y314 in PPARα allows binding of ligands with a shorter head region. This was seen in the optimization of pemafibrate, where it was found that increasing the distance between the core and acid motif decreases specificity for PPARα [119]. A counter example is seen in Sierra et al. [97], where the longer head-core distance allows for better interaction between PPARδ/γ (δ: H287/γ: H323) and the fibrate head.

3. PPARδ has the widest Arm 2, followed by α and γ.

   Ligands selecting against PPARδ tend to contain smaller tail motifs that do not contain substituents that project posteriorly into Arm 2, for example, TIPP-204 and TIPP-401 vs KCL and GW590735 and pemafibrate compared to LC1765 [82]. Kasuga and colleagues also observed that a longer core-tail linker increased affinity for PPARδ [53]. Trifluoride tail designs in this area have been very successful for high affinity activation of PPARs (GW409544, GW0742, TIPP-204, TIPP-401, GW501516, Compound 20a). Adding fluoride atoms to the tail has been used to achieve more favourable pharmacokinetic properties [55, 56]. Wu et al. [113] made a similar observation in their study and confirmed this using mutagenesis of PPARδ residues δ: V298, L303 and V312.
4. Each PPAR has a unique Ω loop forming the anterior wall of Arm 2. PPARδ has the bulkiest Ω loop residues, followed by α and then γ.

   Studies showed that showed that substituents at the tail can interact favourably with the Ω loop. LC1765 and pemafibrate demonstrated the use of halogen substituents in its ligand tail region to make interactions with Arm 2 [58, 97]. In their study, Wu and colleagues demonstrated that their compounds caused a conformational switch in residue δ: W228 [113].
5. PPARδ has the widest Arm 3, followed by γ and then α.

   Kasuga and colleagues observed that a longer fin substituent that extended into the inferior area of Arm 3 increased selectivity for PPARδ [54].
6. PPARα and PPARγ contain a series of methionine residues in Arms 2 and 3 (α: 355 γ: 364, α: 325 γ: 334, α: M330, γ: M348), differing at residue α: M330 and γ: M348. PPARδ contains none of these residues.

   These methionine residues serve as an important selection criteria between the PPARs. The methionine residues in LBD for PPARα and γ seem to have an 'induced fit' effect, stretching or compressing to accommodate chemical groups when needed (PDB ID: 6L69/2ZNN vs 2P54). Many PPARδ specific agonists adopt an ortho substituted cyclic core design which tends to fully occupy the extra space in this region α: M355 δ: I328 γ: M364 [38, 113]. Decreasing the size of the core or ligand substitutions in the tail region just adjacent to the core selects against PPARδ, as less interactions with the PPARδ LBP are formed [53]. These methionine residues are very sensitive to proximity, the same trifluoromethyl substitution affects affinity differently due to slight shifts in position in the LBD (PDB ID: 2XYX vs 5U46)
7. Forming multiple interactions with residue α: C276 δ: C249 γ: C285 on H3 seems to be correlated to high affinity.

   Ligands that form multiple interactions with this cysteine residue on H3 tend to show high affinity [28]. The sulfonylthiadiazole compound series by Keil and colleagues made significant interactions with δ: C249 and displayed high affinity despite having low efficacy [59]. Partial agonists of PPARγ also interact with this residue [14]. In PPARγ, this cysteine residue is able to form a covalent bond with endogenous ligands [35].

6.4 Tools and New Information

In this section, we mention some of the novel methods used by groups in conjunction with protein crystallography during their drug discovery process.

6.4.1 Examples of Computer-Aided Drug Design

Traditionally, new ligands for PPAR were identified and/or optimized from previously synthesized compounds and validated through binding or cell-based assays. These methods are still frequently used [13, 45, 54, 63, 84, 97, 115]. In many SAR studies, researchers identified lead compounds from their own internal databases [13, 39, 59, 72, 96]. As an example, the scaffold for GW501516 was identified from a screen of libraries for compounds with characteristics of lipophilic carboxylic acids, prepared using combinatorial chemistry and structure-based design [100].

These methods have been quickly surpassed by virtual screening methods that are able to sift through massive, open access chemical databases for novel chemical scaffolds [6, 19, 28]. Chemical ligand databases include ENAMINE, CHEMBL, and WOMBAT [28, 104]. Typically, virtual screening methods can be grouped into ligand-based or structure-based [104]. Lu and colleagues adopted a ligand shape-based search method, for ligands that are likely to adopt a specific conformation that would fit the LBP [71]. On top of a multi-step structure-based virtual screening process, Capelli and colleagues utilized a 'structure interaction fingerprints' approach which considers important interactions with the PPAR subtypes, as opposed to the shape of the LBP [19]. Da'adoosh et al. [28] recently identified 13 chemically diverse, novel compounds with nanomolar affinity at the PPARδ receptor by screening a database of 1.56 million molecules, a feat that trumped all previous lead-based compound optimization in terms of number of novel compounds identified in a single study and the affinity of the compounds identified. They made use of a machine learning algorithm to screen for potential PPAR agonists from the ENAMINE database [28]. Ehrt et al. [36] provide an overview on some computer-based methods in rational ligand design, specifically for to distinguishing between different protein subtypes.

Virtual screening methods frequently use docking programs to validate hits in silico or to understand the structural basis for ligand affinity, especially when biophysical or biochemical techniques are inaccessible, impractical or unsuccessful [24]. Research groups have utilized many different programs such as Glide, GOLD, AutoDock Vina, MOE and OEdocking, typically validating docking results with cell-based assays [28, 37, 69, 91, 104]. However, the transferability of in silico docking results to in vitro/in vivo experiments depends greatly on the appropriate validation of the docking program used and scoring criteria. In addition, accurate selection of the ligand binding site, as well as appropriate preparation of both the molecular target and the chemical ligand, such as assigning adding hydrogens,

replacing unmodelled residues and hydrogen atoms and selection of force fields, is also required [28, 99, 104]. Docking tools have been discussed in recent reviews [99, 104].

Currently, it is still a requirement that in silico docking programs and its results are validated by experimental structural information [104]. This is due to inherent limitations in simulation algorithms, computing power or simply due to wrongly processed input data or faulty parameterization [99, 104]. These limitations are exemplified by the fact that crystallization can often reveal unexpected and novel binding modes – although crystallization comes with its own caveats as discussed by Davis and colleagues [30]. In the case of pemafibrate, the docking result predicted a conformation different from that of the crystal structure PDB ID: 6L69 [58, 117]. Uncommon binding modes were also seen in crystal structures of PPARγ antagonist SR10171 and PPARα agonist WY14643, with ligands binding outside of the typically defined ligand binding pocket [11, 40].

6.4.2 Pharmacokinetics and Pharmacodynamics

Early drug discovery is a multistep process that involves the interrogation of the pharmacokinetic and pharmacodynamic properties of a lead compound [48]. These properties can cause discrepancies between their apparent binding affinity in vitro and their potency or efficacy in vivo [74]. Commonly, researchers measure pharmacokinetic properties, such as maximum drug plasma levels (Cmax), ligand half-life and clearance rate and oral bioavailability in mice or rat models [25, 38, 39, 63–65].

One aspect of the pharmacokinetics of a ligand is its absorption, which can be affected by its lipophilicity [48, 74]. Some examples of researchers that optimize against hydrophobicity include Luckhurst et al. [72], who optimized their compounds based on favourable cLogP value, a measure of hydrophobicity, and Evans et al. [39] and Lagu et al. [63] who used various measures to gauge the solubility of their compounds. Other researchers employ cell permeability tests for their compounds. Evans et al. [39] tested permeability of their compounds 46 and 47 through an artificial membrane. Lagu et al. [63] used Caco-2 permeability assays as a measure of cell permeability.

Another aspect is the metabolism of the ligand [48, 74]. The inclusion of certain chemical motifs in the ligand could slow its metabolism, therefore increasing the bioavailability of the ligand [74]. For example, some researchers intentionally adopt fluorine atoms into their ligands, which block metabolically labile sites [55, 56, 74]. Unfavourable rates of metabolism of ligands could also be predicted by its reactivity against cytochromes P450s. Bristol-Myers Squibb, GSK and Astellas Pharma implemented inhibition tests against cytochrome P450 isoforms 3A4, 2C9, 2C19, 2D6 and 1A2 [39, 63, 110]. In their study, AstraZeneca employed a more direct measure of metabolism by checking their compounds for clearance, via incubation

with rat hepatocytes [72]. Other researchers utilized a similar strategy with liver microsomes to check the metabolic stability of their compounds [38, 39]. Astellas Pharma used liquid chromatography–mass spectrometry (LC-MS) to analyse the metabolites formed from incubation with liver microsomes, revealing 'metabolic fault lines' in their compounds [63]. This allowed for optimization of the chemical motifs in the compounds to reduce liver clearance, increasing exposure. However, one should exercise caution when optimizing solely for low clearance. Lagu et al. [64, 65] hypothesize that, at least for PPARδ ligands, maximum exposure achieved with a relatively low dosage and any more might have toxic effects.

Acknowledging the inherent differences between models, some researchers incorporate simultaneous in vitro and in vivo tests with their structural studies [38]. Research groups from GSK, Takara bio, Plexxikon and Astellas Pharma conducted receptor cross-reactivity tests to ensure ligand specificity towards PPAR only [6, 64, 65, 81, 82]. Other cross-reactivity targets include human Ether-a-go-go-Related Gene (hERG). Wang et al. [110] performed a hERG assay to ensure their compounds do not interfere with cardiac repolarization. hERG testing has since become regulation for any compounds to be approved by the FDA and has been performed by other studies [64, 65, 103]. Inherent genetic differences between animal models and humans can also result in discrepancies. One such difference is the absence of a PPRE in the Apo-A1 gene in mice. Some researchers used human Apo-A1 transgenic mice for their in vivo tests to account for this difference [92, 97].

Such in vitro/in vivo tests can be costly and time consuming for smaller research groups or simply impractical during the identification process of new drug leads. Fortunately, there are a range of in silico web tools that can be used to predict pharmacokinetic and even pharmacodynamic properties such as hydrophobicity, CP450 inhibition and hERG inhibition. Online tools such as preADMET can be used to predict pharmacokinetic parameters of any compound. In addition, preADMET can predict the 'drug-likeness' of a compound based on Lipinski's Rule of 5 and other defined drug-like rules [67]. A similar tool, FAF-Drugs3 (now FAF-Drugs4), was used by Capelli et al. [19] as a final screening step in their initial virtual screening process. Ehrt et al. [36] lists binding site comparison tools, which can be invaluable in identifying off-target effects. It is important to note, however, that as of now these web tools are still unable to completely substitute in vitro/in vivo tests [2].

6.4.3 Wider Considerations for PPAR

In this section we examine other factors that might affect the translation of in vitro/in vivo successes into a clinical setting. This could also provide possible explanations as to why some patients respond to currently available drugs, while others do not.

Table 6.28 Non-exhaustive list of PPAR LBD mutations and its associated diseases

PPAR	Mutation	Disease	Reference
α	V227A	Non-alcoholic fatty liver disease	Chen et al. [22]
γ	V290M, P467L	Familial partial lipodystrophy type 3	Barroso et al. [8]
	Q286P, R288H	Colon cancer	Sarraf et al. [90]
	F360L	Familial partial lipodystrophy type 3	Hegele et al. [47]
	R397C	Familial partial lipodystrophy type 3	Agarwal and Garg [1]
	D396N	Familial partial lipodystrophy type 3	Ludtke et al. [73]
	R280P, A233E	Familial partial lipodystrophy type 3	Agostini et al. [3]
	K422Q, L423P	Colon cancer, familial partial lipodystrophy type 3	Broekema et al. [12]
	S221L, M252I, I262M, T447M	Luminal bladder tumor	Rochel et al. [89]

6.4.3.1 LBD Mutations

Mutations in the PPAR protein can result in aberrant gene activation or repression [3, 89]. In fact, these mutations are relatively common – it is estimated that 1 in 500 people have mutations in their PPARγ genes [75]. As previously mentioned in Sect. 6.1.3, in their study, Takada and colleagues demonstrated that even the mutation of one amino acid residue could change the ligand binding phenotype from one subtype to another [101]. The first documented mutation for PPARγ was a dominant-negative mutation of γ: V290M and P467L in the PPARγ LBD [8]. This and other characterized PPARγ mutations have been correlated with familial partial lipodystrophy type 3 and also with various cancers (Table 6.28).

Majithia and colleagues observed that single mutations in the protein sequence altered protein function to a variable degree, resulting in a gradient of phenotypes instead of the traditionally conceptualized 'on/off' effects of mutations [75]. These mutations can have varying effects on the stability and thus on the activity of the LBD, perturbations that can be rescued by synthetic ligands as demonstrated by Agostini and colleagues [3]. These mutations can also potentially alter ligand binding and subtype affinity, causing aberrant cross activation [3, 101]. A patient could even have mutations in other related genes and/or proteins that alter their downstream responses or cross-reactivity to a PPAR ligand [89]. Thus, it is important that mutations in PPAR and other related genes are considered during treatment of patients with drugs [3].

6.4.3.2 PPAR Intact Structure and Implications

While the focus of this review was on the interactions in the LBD, PPAR works in concert with multiple components to exert its gene transcriptional activity [121]. Hence, models of ligand binding and stabilization of the LBD should always be considered in the context of the full intact structure.

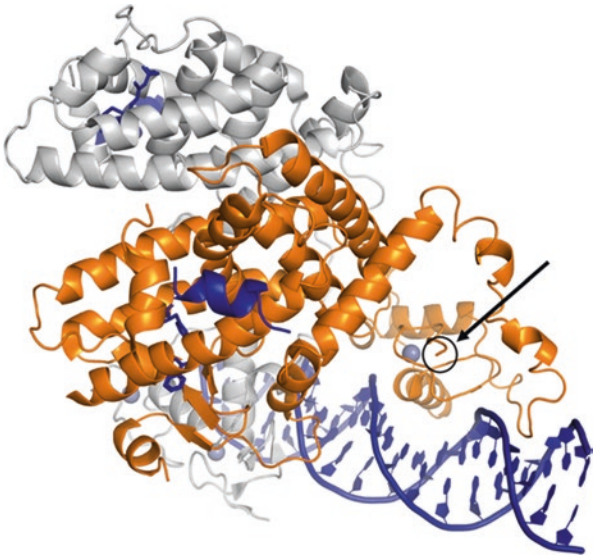

Fig. 6.32 Intact structure of PPARγ LBD-DBD region (orange) complexed with RXRα (light grey) and bound by their respective ligands rosiglitazone and 9-*cis*-retinoic acid (dark blue), coactivator peptides (dark blue) and DNA sequence 5′-GCAAACTAGGTCAAAGGTCAG-3′ and 5′-CTGACCTTTGACCTAGTTTGC-3′ (dark blue). The N terminal end of the modelled protein is circled in black (PDB ID: 3DZY)

Chandra et al. [21] crystallized the ligand-PPAR-RXR-coactivator-DNA complex, providing the first look at a ligand bound DNA/NR complex (Fig. 6.32). Each component of complex assumed conformations similar to previously, individually determined structures [21] (PDB ID: 2ENV). In this complex, the RXRα DBD and PPARγ DBD interact with the β-sheet and H2 region and H7–8 loop and H9–10/11 of the PPARγ LBD, respectively [21, 120] (Fig. 6.32). This suggests that partial agonists, that only stabilize the β-sheet region and/or inhibit phosphorylation of S245 (on H2–H2′ loop), may be able to induce gene transcription through stabilization of the DBD [14, 87].

As mentioned previously in Sect. 6.1.3, the poorly conserved regions Ω loop and H9 in the LBD and the hinge region (domain C) are exposed to the bulk solvent [21, 113]. These regions could possibly form interactions with other components of the gene transcriptional complex [21, 120]. Mutations in H9 and the hinge region could affect assembly of the PPAR gene transcriptional complex. Ligands that aim to bind and stabilize the Ω loop can also have similar effects [57].

The poorly conserved and highly mobile A/B domains were left unmodelled in this structure, and its exact position is still currently unknown even though experiments have been conducted to uncover details of these domains [17, 94]. A recent electron cryo-microscopy (cryoEM) structure of ER suggests that its A/B domains sits lateral to the ER/RXR dimer core, exposed to the solvent [120]. In this PPAR-RXR structure, N terminal end of the modelled PPARγ protein leading to the A/B

domain seems to point towards the anterior face of the PPARγ LBD, suggesting that the PPARγ A/B domains occupy a similar position to that for ER (Fig. 6.32) [21, 120]. If so, mutations or post translational modifications (PTMs) in A/B domains could have a structural effect on the LBD and vice versa [15, 46, 78, 120]. This scenario is likely, as mutations in A/B domains have also been correlated with diseases, and PTMs have been shown to affect receptor activity [4, 7, 17, 89]. It has even been shown that domains A/B are involved in binding of cofactors and increase selectivity of each PPAR subtype [17]. More work needs to be done to understand the structure and implications of domains A and B in PPAR.

Adding on to the puzzle, it was noted that all the intact NR/DNA complexes published so far adopt different quaternary conformations [60]. In the case of PPAR, its length was laterally aligned with the DNA [21]. This observation could possibly challenge the paradigm that ligands in general should aim to stabilize H12 in the active conformation, as this could potentially have varying effects in different NR, assembly of the gene transcriptional complex and transcription of downstream genes.

6.4.3.3 Coregulators and FABPs

Just from the studies examined in Sect. 6.2, we see a wide variety of cell types, assay formats and animal models used. The variety of experimental methods used generally depends on availability or ease of access. However, using different models can become an issue when comparing results between studies, due to inherent differences between models, such as differences in the levels of proteins that could affect potentiation of a ligand's effects [26, 121].

Fatty acid binding proteins (FABPs) bind to and shuttle endogenous and synthetic ligands into the nucleus for interaction with PPARs. Each FABP subtype interact specifically with one PPAR subtype and typically only bind ligands that are specific for its own PPAR subtype (α: FABP1, δ: FABP5 γ: FABP4) [5, 83]. The transactivation assays that are commonly used to test ligand-LBD EC_{50} values should also, in theory, test for ligand-FABP binding. While FABPs can bind ligands specific for another PPAR subtype, it does not potentiate its transcriptional activity. This has potential implications on research conducted with different cellular models as different cells contain different subtypes and levels of FABP [83].

Coregulators play a crucial part in gene regulation. Each coregulator seems to be able to coordinate different and distinct genetic pathways [88, 121]. PPARs are able to bind to a range of coregulators, including NCoR1, SMRT, PGC-1α, CBP and SRC1 [18, 52, 70, 88, 116, 121]. Coregulators play a huge part in potentiation of NR-activated gene transcription [26]. Interestingly, PPAR ligands were able to differentially recruit coregulators. Specifically, this was observed for PPARγ partial agonist PA-082, where PGC-1α seems to be preferentially recruited as opposed to SRC1 when compared to rosiglitazone [18, 70]. In PPARδ, Lagu and colleagues demonstrated that two compounds can activate different subsets of genes, despite having similar affinities and plasma exposure [64, 65]. This difference could possibly be mediated by differential recruitment of cofactors. Considering that different

cells models have different levels of coregulators, the implications on cell-based assays or data could be significant [7].

6.5 Conclusion

The biology of PPARs presents a valuable therapeutic target for the treatment of metabolic diseases, heart and neurological conditions and cancers [23, 82]. However, there are many structural, pharmacological and biochemical challenges to designing a safe but efficacious PPAR drug [6, 64, 65]. There seems to be an interesting irony with PPAR ligand discovery. On one hand, a potential PPAR drug candidate must perform significantly better than currently available drugs on a given set of parameters. However, in the case of PPAR, it seems that most drug candidates that improve on affinity, potency and efficacy often fall short due to unforeseen side effects. A widely held hypothesis suggests that a lack of selectivity for one specific subtype causes side effects [58, 72, 113]. Interestingly, this issue of selectivity is not unique to the PPAR subtypes; two proteins with different sequences and even folds can have similar binding pocket environments, and ligands can adapt to multiple binding pockets [36, 98]. Another recent hypothesis states that PPAR might also have a therapeutic index; if a specific range of ligand concentration in the blood or if a hypothetical 'threshold of activation' is exceeded, side effects will occur [64, 65]. These side effects of PPAR activation may also be due to aspects of cross talk with other nuclear receptors that are still currently unknown [20, 64, 65].

For now, the complexity of the PPAR regulation network will remain an obstacle when scaling from cell and animal-based models to a clinical setting [26]. The differences in cell and animal models used between studies also add to the complexity [7, 102, 109]. An important point not discussed in this review is that different species have different PPAR ligand binding selectivity; the implications of species selectivity becomes significant, considering preclinical safety trials are conducted in animal models [31, 61]. However, advances in global screening methods and single cell quantitative polymerase chain reaction (qPCR) analysis of human tissues may play a key role in unravelling this problem [7, 26]. The research into PPAR ligand discovery thus far has revealed many insights into the activation and function of the PPARs. In this review, we have attempted to tackle the issue of absolute selectivity of PPAR ligands, by examining the available structural information of the PPAR ligand binding domain. From this, we have identified a few observations that can be used to create a subtype-specific PPAR agonist. There still remain a few issues in the field of PPAR structural selectivity: The identification of the full-length structure, including the A/B domains for all the PPAR subtypes, its implications on ligand function and selectivity between subtypes, the elucidation of PPAR gene activation networks and the regulation of those networks in different contexts, such as cell and animal models.

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Chapter 7
Pregnane X Receptor: Understanding Its Function and Activity at the Molecular Level

Sergio C. Chai and Taosheng Chen

Abstract The pregnane X receptor (PXR) is a ligand-activated nuclear receptor recognized as an important player in xenobiotic detoxification because it regulates the expression of drug-metabolizing enzymes and transporters. Because of its notorious role in drug metabolism and disposition, PXR's activity may result in unintended drug-drug interactions, decreasing drug efficacy, inducing resistance, and causing toxicity. As such, PXR has become an attractive target for the development of PXR antagonists that can be used as co-drugs. However, PXR agonists have emerged as potential therapeutics against certain diseases, such as inflammatory bowel disease. This book chapter describes the molecular basis of PXR activity, correlating biochemical and structural elements to describe the promiscuous nature of PXR in recognizing a wide array of chemicals and the challenges in developing PXR modulators.

Keywords PXR · Drug metabolism · Antagonist · Agonist · Inflammatory bowel disease

7.1 Introduction

The pregnane X receptor (PXR, NR1I2) is a ligand-activated transcription factor and a member of the nuclear receptor (NR) superfamily [1]. As part of the xenobiotic detoxification system, PXR recognizes environmental chemicals and modulates the expression of enzymes involved in the biotransformation, metabolism, and elimination of xenobiotics [2, 3]. PXR also plays diverse roles in the biotransformation of endobiotics, being involved in physiologic processes such as gluconeogenesis,

S. C. Chai · T. Chen (✉)
Department of Chemical Biology and Therapeutics, St. Jude Children's Research Hospital, Memphis, TN, USA
e-mail: Taosheng.Chen@STJUDE.ORG

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lipid metabolism, and the homeostasis of bile acids, steroids, and thyroid hormone [4].

In its role as master xenosensor, PXR regulates the concerted transcriptional activation of an array of genes encoding phases I/II drug-metabolizing enzymes (DMEs) and phase III ATP-binding cassette drug transporters [5, 6]. Some of the target genes regulated by PXR encode for oxidative enzymes (e.g., cytochrome p450 or CYP) and conjugative enzymes (e.g., UDP-glycosyltransferases, glutathione S-transferases, and sulfotransferases). Among the transporter proteins regulated by PXR are the multidrug resistance proteins and multidrug resistance-associated proteins [7].

Although the detoxification system is a vital mechanism for organisms to protect themselves against toxic compounds while maintaining appropriate physiologic levels of endobiotics, PXR has become a therapeutic target because of its significant impact on clinical drugs by potentially compromising drug efficacy, causing drug resistance and inducing toxicity [8, 9]. Moreover, PXR has been implicated in physiologic processes related to cancer [10], inflammatory bowel disease (IBD) [11], infectious diseases [12], and energy metabolism [13–15]. However, PXR has proven to be a challenging therapeutic target primarily because of its notoriety for promiscuously binding to structurally diverse chemicals [6].

7.2 The Chemical Landscape of PXR Ligands

As a master xenobiotic sensor, PXR has evolved to recognize a multitude of chemicals that it can bind to. The ligands display extensive diversity in terms of size, lipophilicity, chemical scaffold, and surface area. Figure 7.1 provides a preview of the chemical landscape of reported PXR ligands, reflecting the vast range of physiochemical properties that these ligands encompass and indicating the promiscuity of PXR [16–27]. Principal component analysis (PCA) of common physiochemical properties (molecular weight, partition coefficient, hydrogen bond acceptors, and hydrogen bond donors) provides a simple, yet effective way to visualize the wide spectrum of these ligands (Fig. 7.1a, blue dots). Although the data spread is

Fig 7.1 (continued) tory that are used for high-throughput screening is illustrated alongside (light red dots). The wide spectrum of physicochemical properties of PXR ligands is demonstrated by the distribution in (**b**) molecular weight, (**c**) predicted partition coefficient AlogP, (**d**) predicted molecular surface area, and (**e**) predicted molecular solubility. The two-dimensional PCA sets and physicochemical properties were computed by using the software package Pipeline Pilot (BIOVIA). (**f**) Circular representation of PXR ligands' hierarchical clustering based on chemical structure similarity shown with the respective molecular weight values (blue bars). Reported PXR inhibitors are indicated by a red dot. (**g**) Rectangular tree representation of the hierarchical clustering of PXR ligands, with the distribution of molecular descriptors (JOELib) represented as bars: number of aliphatic hydroxyl groups (purple), number of acidic groups (red), number of basic groups (light blue), number of heterocycles (green), and fraction of rotatable bonds (orange). The clustering trees and JOELib molecular descriptors were generated by using the online tools ChemMine and Interactive Tree of Life

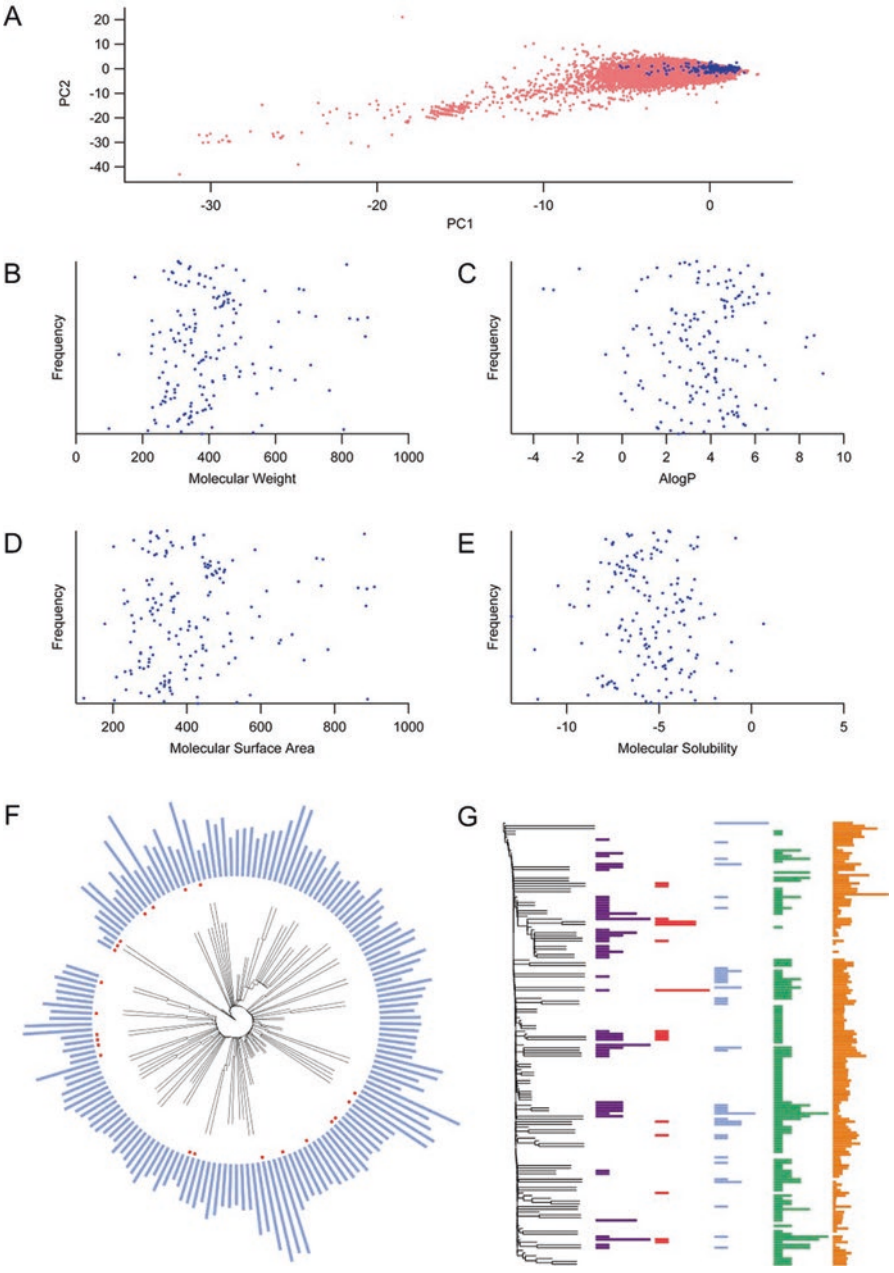

Fig. 7.1 The promiscuity of PXR is reflected in the diversity of PXR ligands (including agonists and inhibitors). (**a**) The chemical space of PXR ligands (blue dots) can be visualized by using principal component analysis (PCA) of several physiochemical properties: molecular weight, partition coefficient (AlogP), hydrogen bond acceptors, and hydrogen bond donors. For comparison, PCA of >1,000,000 compounds from the St. Jude Children's Research Hospital compound reposi

considerable enough to indicate that PXR's activity can be modulated by a variety of chemicals, a boundary for the types of compounds that can be accommodated in PXR's ligand binding pocket likely exists. For comparison, chemicals from a large collection of over a million compounds intended for high-throughput screening (from the St. Jude Children's Research Hospital repository) are plotted alongside the reported PXR ligands (Fig. 7.1a, light red dots). It becomes apparent that not all types of compounds may be able to serve as PXR modulators, as compounds with extreme properties were not identified or reported. Furthermore, the analysis of the individual physicochemical properties cannot provide a narrow range in terms of molecular weight (Fig. 7.1b), predicted partition coefficient AlogP (Fig. 7.1c), predicted molecular surface area (Fig. 7.1d), or predicted molecular solubility (Fig. 7.1e). These observations exemplify the difficulty of predicting PXR modulators without the need to experimentally confirm their effect on PXR's activity. Similarly, the hierarchical clustering of these reported PXR ligands does not show correlations between chemical structure similarity and molecular weight (Fig. 7.1f), nor do chemical structure and reported PXR inhibitors seem to be associated. Likewise, chemical structure and frequency of the different types of chemical groups (aliphatic hydroxyl groups, acidic groups, basic groups, heterocycles) are incongruent (Fig. 7.1g). The unpredictability and the wide distribution of these physicochemical characteristics validate PXR as a master xenobiotic sensor, but they also manifest the challenges in developing PXR antagonists and performing structure-activity relationship studies of PXR modulators.

7.3 The Structural Architecture of PXR

As a master xenobiotic sensor, PXR recognizes a range of chemicals with differing physicochemical properties. The molecular basis of PXR's activity and xenosensing abilities can be elucidated by the results of numerous structural studies conducted on PXR and complemented by findings obtained from other NRs. PXR is a multi-modular protein that displays the prototypical architecture of NRs, consisting of an N-terminal sequence-specific DNA-binding domain (DBD), the multi-functional ligand-binding domain (LBD), and a flexible hinge region that connects the LBD to the DBD [28, 29]. The crystal structure of the full-length peroxisome proliferator-activated receptor gamma (PPARγ) adequately illustrates the organization of these modules (Fig. 7.2). PXR's control of target gene expression is mediated by zinc fingers situated within the DBD, which engage the PXR-responsive element module (PXRRE) in the proximal promoter and distal enhancer regions of target genes [30, 31]. Although PXR exhibits similarities in these domains with other NRs, it lacks the long N-terminal A/B domain and the C-terminal F domain seen in some other NRs [32].

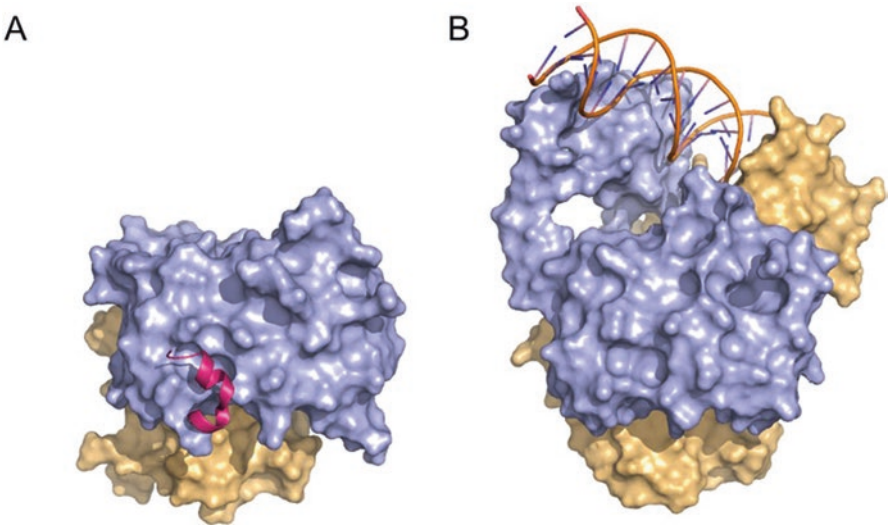

Fig. 7.2 Comparison of the structural architecture of PXR and PPARγ. (**a**) Surface representation of the structures of PXR LBD (light blue) and RXRα LBD (light orange), with the PXR AF-2 helix (cartoon representation) shown in pink (PDB code 4J5X). (**b**) For comparison, the structures of the full-length PPARγ (light blue) and full-length RXRα (light orange) are oriented similarly to those of the PXR-RXRα complex. The DBD of PPARγ and that of RXRα are seen interacting with DNA (PDB code 3DZY)

The LBD is the most distinguishable feature of NRs (Fig. 7.2). It is a multifunctional module that contains the activation function 2 (AF-2) domain at the C-terminus, which engages either coactivator or corepressor proteins depending on the specific positioning of the AF-2 helix. The first reported crystal structure of the LBD of human PXR (hPXR) depicts a three-layered helical sandwich fold comprising the sets H1/H3, H4/H5/H8, and H7/H10 [29]. The PXR LBD consists of 11 α-helices, but in several of the available x-ray structures, H2 and H6 are modeled as an extended flexible loop due to disorder; it is being postulated that the former one is involved in forming a channel for ligand access to the ligand-binding site [33]. In addition, the LBD is composed of a layer of five stranded anti-parallel β-sheets, including the two novel β1 and β1' not observed in other NRs [29].

The B factors (temperature factors) of the available PXR LBD crystal structures uncovered consistent regions of dynamic mobility [34]. The three regions with the highest disorder have been identified, corresponding to the loops between H1 and β1, β1′ and H3, and β4 and H7 [34]. Some of these regions were not modeled because of the high degree of disorder. Additionally, other regions with thermal mobility include the loops between H8 and H9, H9 and H10, and the C-terminal of the AF-2 region.

7.4 Dimerization of PXR

Many of the 3D structures depict PXR as a homodimer, even though it is widely assumed that PXR functions as a monomer when participating in heterodimerization with regulatory proteins. One of the earliest crystallographic reports was focused specifically on proving that homodimerization of PXR is indeed present in solution and is required for activity [35]. The homodimerization interface is formed by the terminal β1′ strands from each monomer, where the corresponding Trp-223 and Tyr-225 residues from each strand create an interlocking system. Analytical ultracentrifugation and size-exclusion studies indicate that Trp-223Ala and Tyr-225Ala double mutants prevent homodimerization. These mutants also affected PXR's transcriptional activity in cell-based assays by reducing CYP3A4 induction.

The LBD also contains a heterodimerization interface enabling association with the coregulatory protein retinoid X receptor α (RXRα), which is a heterodimerization partner for several NRs. The co-crystal structure of PXR LBD with the RXRα LBD reveals that the monomers are attracted by electrostatic and polar interactions. The heterodimerization interface occupies a surface of approximately 1200 $Å^2$, which is formed by H5, H9, and H10 of PXR and H7, H9, and H10 of RXRα [36]. While forming a complex with RXRα, PXR was shown to keep the homodimer conformation through the β1′ interface. Therefore, the crystal structure illustrates a potential heterotetramer complex that can exist in solution. The higher structural order was shown to increase the affinity for steroid receptor coactivator-1 (SRC-1) peptides, which is also enhanced by agonist binding. It appears that the stabilization of the entire complex is a dynamic process in which ligands, partner, and coregulatory proteins work together.

7.5 Coregulatory Recruitment to PXR

Ligand binding to the PXR LBD triggers conformational changes leading to the recruitment or dissociation of coregulatory proteins, depending on whether the ligand is an agonist, antagonist, or inverse agonist. One of the most important structural changes after ligand binding is the positioning of the AF-2 helix in a specific orientation, which directly affects the association or dissociation of coactivators or corepressors to and from PXR. An agonist induces an active conformation in PXR's structure, enhancing the binding of a coactivator protein to PXR's AF-2 region. The NR box motif Leu-Xxx-Xxx-Leu-Leu (where Xxx is any other amino acid residue) within the coactivator is buried in a hydrophobic groove on the AF-2 region, which is formed by helices H3, H4, and the AF-2 helix. Coactivator binding is stabilized by a charge clamp involving PXR's residues Lys-259 of H3 and Glu-427 of the AF-2 helix. Prominent coactivators include the SRC-1 and the transcriptional mediator/intermediary factor 2 (TIF2) [37].

The negative regulation of PXR's transcriptional activity involves the recruitment of corepressor proteins that bind PXR in the AF-2 region through CoRNR boxes with the motif Ile/Leu-Xxx-Xxx-Ile/Val-Ile [4]. Some of the common corepressors are the nuclear receptor corepressor (NCoR) and silencing mediator for retinoid or thyroid hormone receptors (SMRT). No x-ray structures of PXR with a corepressor peptide exist, but crystallographic studies with other NRs indicate that the corepressor peptide resides in the same groove on the surface of the AF-2 domain as the coactivator peptide does in the active PXR conformation [38].

The interplay between ligand binding and coactivator recruitment does not seem to be a unidirectional process. Reciprocally, association of the coactivator may influence the binding of the ligand. The agonist SR12813 is modeled to be in three distinct orientations within the ligand-binding pocket of PXR in the absence of the SRC-1 coactivator peptide [29], but the presence of the coactivator peptide fixes the ligand in a single position [33].

7.6 AF-2 Helix Orientation Dictates PXR's Activation

The AF-2 helix's orientation is the main factor enabling discrimination between coactivator or corepressor recruitment, and ligand binding encourages bias toward an active or inactive form. [39] When PXR is in the active conformation due to agonist binding, the AF-2 helix is positioned against the body of the LBD, trapping the agonist in the ligand-binding site and creating a favorable surface on PXR's LBD for coactivator recruitment (i.e., AF-2 region). This orientation of the AF-2 helix is stabilized by its interactions with residues comprising the ligand-binding site and, in many cases, with the agonist.

There is no crystal structure of PXR in the inactive conformation, where the AF-2 helix is oriented in a position different from that of the active state. However, a few x-ray structures of other NRs provide valuable insights that can be extrapolated to PXR. The 3D structure of RXRα reveals that the AF-2 helix extends away from the LBD [40]. The structure of the apo testicular receptor 4 (TR4) shows that the AF-2 helix is lodged in the coactivator binding site, presumably preventing the binding of a coactivator or corepressor [41]. The autoinhibition by TR4 is a peculiar mechanism that has also been observed in the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) LBD [42]. Surprisingly, all the apo-form structures of PXR and most other NRs show the AF-2 helix in the agonist-induced conformation, probably due to the non-physiologic conditions under which crystals are formed [43]. Therefore, it is argued that crystallography cannot easily illustrate the multiple populations of NRs present in solution, where the AF-2 helix is favored to be pre-locked in the active state by crystallographic conditions [44].

Several lines of evidence indicate that the AF-2 helix is present in several intermediate states instead of either of the two discrete states of active or inactive. Fluorescence anisotropy studies of PPAR concluded that the AF-2 helix is highly mobile and ligand binding diminishes its motion [45]. In addition to lessons taken

from crystallography [46, 47], NMR studies revealed that in the absence of a ligand, an NR can exist in solution in various conformations because of its fluid and flexible nature, displaying unsteadiness in regions including the AF-2 helix [48]. Similar observations have been made using hydrogen-deuterium mass spectrometry (HDX-MS) studies of vitamin D receptor (VDR), where full and partial agonists stabilize the AF-2 helix in a graded manner, with corresponding differential strength for coactivator peptide recruitment [49].

PXR is not considered to have constitutive activity because it is not thought to have basal activity in the unliganded form, unlike the constitutive androstane receptor (CAR). In the absence of agonist, the AF-2 helix of CAR can remain in the active conformation, but the AF-2 of PXR would most likely display high random mobility [50, 51]. A short and rigid AF-2 helix is believed to be a major contributor to CAR's constitutive activity. This rigidity is due to the presence of the short helix H_X between H10 and the AF-2 helix, composed of only four to six residues [39]. CAR's AF-2 helix is further stabilized by charge-charge interactions with H4, and its movement is restricted by the short linker connecting it to H_X [51]. In contrast, these features observed in CAR are not present in PXR: the link connecting H10 to the AF-2 is seen as an extended loop instead. Interestingly, comparative analysis of the relative B factors of the structures of PXR LBD and CAR LBD indicates that the AF-2 region in CAR is more ordered than that in PXR [34].

7.7 Ligand Binding Stabilizes the Structure of PXR

In addition to playing a role in the stability of the AF-2 helix in the active or inactive conformation, ligand binding increases overall structural stability. The unliganded PXR is probably present in solution in a dynamic balance between partially unfolded and fully folded forms. For PXR to associate with coregulatory proteins, a fully folded and stable structure would be needed to enable stability at the protein-protein interaction interface. The crystal structures of PXR show the ligand completely enclosed and buried deep within the core of the LBD, where the AF-2 helix forms a cap that entraps it. In general, there does not appear to be an obvious ligand entry point to the ligand-binding pocket of NRs [52], unlike those of most enzymes. Therefore, LBD may need to partially unfold through structural rearrangements for the ligand to access the ligand-binding site. Several molecular dynamic simulations have been applied to several NRs to identify ligand entry and exit pathways. Flexible regions have been identified close to the ligand "gates" in PPARγ [53]. Multiple pathways of ligand escape from the LBD in thyroid hormone receptors (TRs) have been simulated, identifying several conformational rearrangements involving helices and β-sheets [52]. All these point to the probable partial unfolded state of NRs in the unliganded form.

Solution-based studies indicate that the ligand-binding site and the coregulatory protein interface were identified as fluid and malleable regions in the absence of

ligand, and ligand binding constrains these conformations to fewer and more energetically stable subsets [48]. HDX-MS analysis of TR shows that ligand anchoring in the binding pocket results in a more stable LBD [54]. Protein thermal shift studies investigating direct interactions of ligands with CAR corroborate the partial unstructured state of the protein in the apo-form, which is stabilized in the presence of a ligand [55]. HDX-MS studies of PXR and CAR demonstrate regions of the LBD that are stabilized by agonists and antagonists, with further restraint of structural motion with the addition of coactivator or corepressor peptides [21, 55]. These studies would indicate that any type of ligand (agonist, antagonists, and inverse agonist) would confer the structural integrity needed for coactivator (in the case of agonists) or corepressor recruitment (in the case of antagonists and inverse agonists) by providing internal networks and acting as "glue" [56].

7.8 Ligand-Binding Site of PXR and Ligand Promiscuity

A relatively large number of hPXR structures co-crystallized with various ligands have been reported, providing perspectives into the nature of the ligand-binding site and its relationship with ligand promiscuity. These structures are valuable because of the diversity in scaffold, size, and polarity of PXR ligands that were co-crystallized with PXR (Fig. 7.3). The ligand-binding site is formed by 28 amino acid residues, most of which are hydrophobic [29]. Only eight residues have polar or charged side chains, with salt bridges neutralizing some of these charges (e.g., between Glu-321 and Arg-410 and between Asp-205 and Arg-413). The LBD of PXR is notably large compared to that of other NRs, with a ligand-binding pocket volume of approximately 1150 Å for the apo-form [29]. The fluid character of PXR's LBD can be appreciated in the hPXR co-crystal structure with SR12813, where the ligand is seen to adopt multiple orientations and the ligand-binding pocket volume expands to 1344 $Å^3$ (Fig. 7.3a) [33]. However, the PXR region that interacts with SR12813 shrinks in the presence of a coactivator peptide, resulting in the constraint of SR12813 in a single orientation (Fig. 7.3b). The LBD is very flexible and dynamic, expanding and conforming its shape to accommodate ligands of different sizes and shapes. For instance, in the presence of hyperforin, the volume increases to 1544 $Å^3$ [57]. The induced fit behavior of the LBD occurs in the presence of rifampicin, one of the largest PXR agonists (Fig. 7.3c) [58]. Without the expansion of the LBD, rifampicin would not fit in the ligand-binding site of the apo-form. The dynamic nature of the PXR LBD is also evident by its large variance in B factors, particularly when compared to that of other NRs with similar function [34].

Figure 7.3 compares the binding modes of several PXR ligands in spatial relation to the AF-2 helix (red surface) and Trp-299 (blue surface). Agonists generally stabilize the AF-2 helix in NRs by interacting with some of its residues; however, Trp-299 is a residue residing deep inside the ligand-binding site that interacts, to some degree, with all co-crystallized ligands. In addition to Trp-299, some of the other

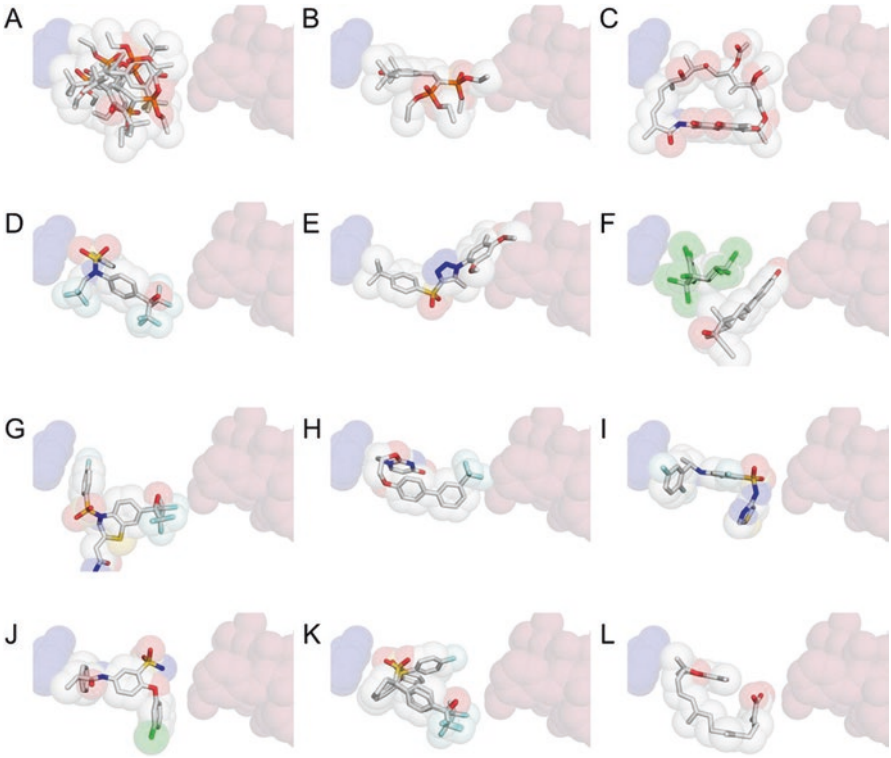

Fig. 7.3 Relevant and recently reported co-crystal structures of hPXR LBD and agonists, with residues from the AF-2 helix shown as raspberry red spheres and Trp-299 shown as purple spheres: (**a**) SR12813 in the absence of SRC-1 peptide, displaying three different conformations of SR12813 (PDB code 1ILH). (**b**) The presence of SRC-1 peptide fixes SR12813 in a single orientation (PDB code 1NRL). (**c**) Rifampicin, one of the largest PXR agonists (PDB 1SKX). (**d**) T0901317 (PDB 2O9I). (**e**) SJB7, an analog of the antagonist SPA70 (PDB 5X0R). (**f**) Binary complex of the two small molecules 17α-ethinylestradiol and the pesticide trans-nonachlor (PDB 4X1G). (**g**) Compound 25a, a retinoic acid-related orphan receptor inverse agonist (PDB code 6BNS). (**h**) Compound 7, a metabotropic glutamate receptor 2 modulator (PDB code 6DUP). (**i**) XPC-7455, a voltage-gated sodium channel inhibitor (PDB code 6S41). (**j**) Compound 25, a P2X4 inhibitor (PDB code 6HTY). (**k**) Compound 3, a retinoic acid-related orphan receptor inverse agonist (PDB code 6NX1). (**l**) Garcinoic acid (PDB code 6P2B). The ligands are represented as sticks, and the transparent spheres illustrate proximity of ligand atoms to the protein residues. Ligand color scheme: carbon, white; nitrogen, blue; oxygen, red; sulfur, yellow; chlorine, green; fluorine, light blue

residues that are commonly seen interacting with ligands include Met-243, Ser-247, Gln-285, His-407, and Phe-420 [59]. Few of these structures exemplify the limited number of hydrogen bonds that can form between the ligand and residues lining the ligand-binding pocket, such as those involving Ser-247, Gln-285, His-407, His-327,

and Arg-410 [56]. Additional attractive forces, such as π-stacking, are provided by Trp-299, Phe-288, and Tyr-306. Given the hydrophobic nature of the ligand-binding pocket, it is evident that the bulk of the interactions occurs through hydrophobic and van der Waals forces.

Crystallography can be a powerful tool in the development of PXR antagonists. Initial efforts to rationally design PXR antagonists relied on the analysis of the PXR-T0901317 structure (Fig. 7.3d) [60]. Attempts were made to generate a PXR antagonist based on the T0901317 scaffold, which failed because of the difficulty in predicting molecules that can antagonize a promiscuous protein such as PXR. More recently, the structure of PXR in complex with the agonist SJB7 provided insights into the molecular basis of PXR antagonism by the analog SPA70 (Fig. 7.3e) [21]. The para-methoxy group in SJB7 stabilizes the AF-2 helix in the active conformation, amenable for coactivator recruitment; the lack of this chemical group in SPA70 would prevent it from doing so. The design of PXR antagonists is largely hampered by the flexibility of PXR's ligand-binding pocket, making it challenging to study structure-activity relationships of closely related compounds. In fact, the binding of ligands to PXR is so unpredictable that a PXR structure with two distinct chemicals was obtained occupying the ligand-binding site (Fig. 7.3f) [61]. The two compounds are the pesticide trans-nonachlor and the contraceptive agent 17α-ethinylestradiol, which were shown to act synergistically in activating PXR. The enhancement in PXR activation as a consequence of the inter-ligand interactions is proposed to result in a "supramolecular ligand," and it opens the possibility that other binary complexes may similarly activate PXR.

There is a great impetus in developing therapeutics that do not activate PXR in order to prevent metabolic liabilities. Structure-based design based on the co-crystallization of these compounds with PXR can lead to chemical modifications that prevent their binding to PXR (Fig. 7.3g–k). Successful examples include derivatives of an inverse agonist of retinoic acid-related orphan receptor (RORγt) (Fig. 7.3g) [27], a metabotropic glutamate receptor 2 (mGluR2) allosteric modulator (Fig. 7.3h) [26], a voltage-gated sodium (NaV) channel inhibitor (Fig. 7.3i) [25], a P2X4 receptor (ligand-gated ion channel) inhibitor (Fig. 7.3j) [24], and a phenyl (1-phenylcyclopentyl)sulfone compound acting as a RORγt inverse agonist (Fig. 7.3k) [23]. These structures reveal features in the agonist that can be substituted to create steric clashes, increase polarity in a hydrophobic pocket, or diminish important contacts to reduce binding affinity to PXR while maintaining potency to their intended target.

Conversely, highly selective and nontoxic PXR agonists that can serve as a potential treatment for inflammatory diseases are being sought. One such example is garcinoic acid (Fig. 7.3l), which was extracted from *Garcinia kola* seeds and determined to be selective against a panel of NRs, with in vivo efficacy [22]. The authors of the study believe, partly based on findings obtained from the crystal structure, that garcinoic acid can serve as a template for medicinal chemistry efforts to improve potency and metabolic stability.

7.9 Species Selectivity in PXR Activation

The amino acid sequences of PXR LBDs differ significantly among species, which account for the species-selective ligand-induced transactivation [62]. The prototypical hPXR ligands rifampicin and SR12813 can markedly activate hPXR while having little effect on the mouse PXR (mPXR) ortholog. Conversely, PCN is a strong mPXR agonist and a poor hPXR activator [3]. An interesting mutagenesis study has been conducted, wherein key LBD amino acids in mPXR were changed to the corresponding hPXR residues on the basis of crystallographic data of hPXR with SR12813. The resulting mPXR mutant could be activated by SR12813 without a response to PCN [29]. These results corroborate that few residues are critically involved in species-based selectivity.

Species-based agonistic effects of PXR can have striking implications in organism-level studies, in which mouse models are often used to evaluate PXR activation by hPXR ligands. To circumvent this issue, several humanized mouse models of PXR have been developed, which have wide-ranging applications in drug development and PXR functional studies. The first such hPXR mouse model was based on the random insertion of the *hPXR* gene into a mouse genome without the *mPXR* gene. Expression of hPXR was under the control of either the liver-specific albumin promoter [63] or the rat fatty acid-binding protein promoter [64]. A second-generation hPXR mouse model was developed by randomly integrating the entire *hPXR* gene and its promoter into a *Pxr*-null mouse genome [65]. A mouse model expressing hPXR and CYP3A4 was generated that displayed robust CYP3A4 induction by rifampicin to levels comparable to those in humans [66]. An alternative model was based on replacing the *mPXR* gene with the *hPXR* gene under the control of the endogenous mouse promoter [67]. Further improvements led to a mouse model expressing a chimeric PXR (mDBD-hLBD) [68], which has the advantage of minimizing the effects of species-based differences in DNA binding.

7.10 PXR Inhibitors

The unintended activation of PXR has been associated with adverse clinical outcomes [16, 20, 69]. The upregulation of PXR-mediated drug-metabolizing enzymes plays major roles in the reduction of drug efficacy, resistance to chemotherapies, and drug-induced liver injury [56, 70]. Consequently, there is great motivation to discover PXR antagonists that can counteract the effects of PXR activation, particularly for clinical applications. However, the development of such PXR antagonists has been hampered primarily because PXR can be activated by different types of chemicals [71], with PXR's promiscuity complicating endeavors to develop highly selective and potent PXR antagonists to prevent off-target mediated side effects [72].

Several natural products inhibit PXR's activity. Ecteinascidin-743 (ET-743), which is derived from the Caribbean marine tunicate *Ecteinascidia turbinata* [73], exemplifies the complex chemical scaffolding seen in many ligands of natural

sources, comparable to that of the classical PXR agonist rifampicin. ET-743 was the first reported inhibitor of agonist-induced PXR transactivation, repressing the expression of CYP3A4 and MDR1 [74]. Its clinical application has been limited due to its potent cytotoxic properties, which are due in large part to its interference with DNA repair pathways by binding to the minor groove of DNA [75]. Conversely, sulforaphane (SFN), a phytochemical in the daily diet, is relatively safe for potential clinical use. It is found in certain cruciferous vegetables and was the first naturally occurring PXR antagonist to be reported [76]. A human clinical study concluded that SFN was not an effective hPXR antagonist in vivo [77]. The relative high concentrations required to abrogate PXR's function in vitro and its off-target effects at lower concentrations are the main concerns used to justify further studies on the direct effects of SFN on PXR antagonism [78]. Among the other reported PXR inhibitors in plant materials are coumestrol (a phytoestrogen from legumes and soy beans) [79], sesamin (a lignan from sesame seeds) [80], and camptothecin (an alkaloid from the plant *Camptotheca acuminata*) [81]. However, these compounds were shown to be weak PXR inhibitors, which could limit their application.

Repurposed compounds have a potential for a more expedited development of drug candidates to be used in clinical settings because of their established safety and desirable PK/PD profiles. Leflunomide is being used in the treatment of rheumatoid arthritis and psoriatic arthritis, and it is considered as the first FDA-approved drug to be repurposed for PXR inhibition [82]. The antifungal compound ketoconazole is among the most extensively studied PXR inhibitors, which include the synthesis of a series of derivatives and their mechanistic investigations [83]. Other notable examples include the antidiabetic agent metformin [84] and the HIV protease inhibitor A-792611 [85].

A handful of PXR inhibitors have been identified, mostly by cell-based assays that monitor reporter gene expression or biological marker. Aside from the many advantages inherent in these types of assays, they cannot conclusively establish if the observed reduction in PXR activity is due to direct interactions of the compound with PXR or by indirectly modulating alternate pathways. Few of them have been characterized at the biochemical level to confirm their binding to PXR. Based on the displacement of radiolabeled ligands for PXR, it is believed that SFN [76], polychlorinated biphenyls [86], and coumestrol [79] interact at the ligand-binding site. More recently, SPA70 was identified from a high-throughput screening campaign and shown to interact in the ligand-binding pocket based on a combination of techniques that include HDX-MS, docking, and crystallographic analysis of its analog SJB7 [21]. Furthermore, SPA70 becomes an agonist for the Trp-299Ala PXR mutant, where Trp-299 is one of the residues lining the ligand-binding site, confirming direct interaction of SPA70 with the ligand-binding site [87]. Other PXR inhibitors are believed to interact directly with PXR but through alternate sites other than the ligand-binding pocket. Among these include ketoconazole [83], SPB03255, and SPB00574 [82], which are thought to reside at the AF-2 region, thus blocking the recruitment of coactivators.

The activity of PXR can be inhibited by several mechanisms. The most attractive approach in repressing PXR's function is by an antagonist, which competes directly with the agonist for occupancy of the ligand-binding site [71]. A PXR inhibitor can

also engage in an allosteric site, either by negatively affecting the recruitment of partner proteins required for transcription (e.g., coactivators or RXRα) or by enhancing the recruitment of corepressors. It is also possible to modulate PXR's activity by interfering with upstream events, such as perturbing posttranslational modifications.

7.11 PXR Agonists as Therapeutics

Although PXR activation can lead to drug-drug interactions and toxicity during drug therapy, evidence suggests that PXR agonists can be used to treat certain diseases, such as inflammatory bowel disease (IBD) [88]. IBD is characterized by inflammation of the gastrointestinal tract, and several observations point to an association of its pathology with PXR. The expression and activity of PXR and its target genes are reduced in the intestines of patients with IBD [89]. Mouse models indicate protection from dextran sulfate sodium (DSS)-induced IBD upon treatment with the mPXR agonist PCN [90]. Similar preventive effects were observed in humanized PXR mouse models after treatment with the hPXR agonist rifaximin. Furthermore, rifaximin effectively alleviated IBD in clinical settings [91]. Mechanistic studies indicate that PXR activation results in the suppression of the NF-κB signaling cascade, with an eventual repression of proinflammatory responses [92].

Considering the encouraging application of PXR agonists in the treatment of IBD, including the approval of rifaximin by the FDA [22], several PXR agonists have been identified and studied as potential IBD therapy. Among these PXR agonists are solomonsterol A (obtained from the marine sponge *Theonella swinhoei*) [93], artemisinin (isolated from the plant *Artemisia annua* and used in the treatment of malaria) [94, 95], chrysin (a plant-derived flavonoid) [96], isorhamnetin (a common flavonoid in food) [97], and garcinoic acid (isolated from *Garcinia kola* seeds) [22]. Unexpectedly, the well-established hPXR agonist rifampicin did not affect IBD [90]. In contrast to rifampicin, rifaximin is concentrated in the intestines and is poorly transported to the liver [98]. The wide distribution of rifampicin in the liver is hypothesized to cause the decrease of hepatic stearoyl-CoA desaturase-1 expression, which results in the reduction of anti-inflammatory unsaturated fatty acids that oppose the beneficial effects of gut-specific PXR agonism [90].

7.12 Conclusion

PXR is an important therapeutic target because of its essential role in drug metabolism and disposition. However, the primary obstacles hindering the development of PXR antagonists are due to its ability to promiscuously recognize a wide range of chemicals, which mostly act as agonists. Ironically, PXR agonists have emerged as potential therapies in treating inflammatory diseases such as IBD. A thorough understanding of the molecular basis of PXR modulation is needed in the pursuit to

successfully develop a PXR antagonist with in vivo efficacy, improved potency, enhanced selectivity, and reduced toxicity.

A simplistic view of the molecular mechanism of PXR transactivation—and probably that of other NRs—can be summarized as a sequential step (Fig. 7.4): (1) Apo-PXR can be present in solution as a mixed population with differing structural

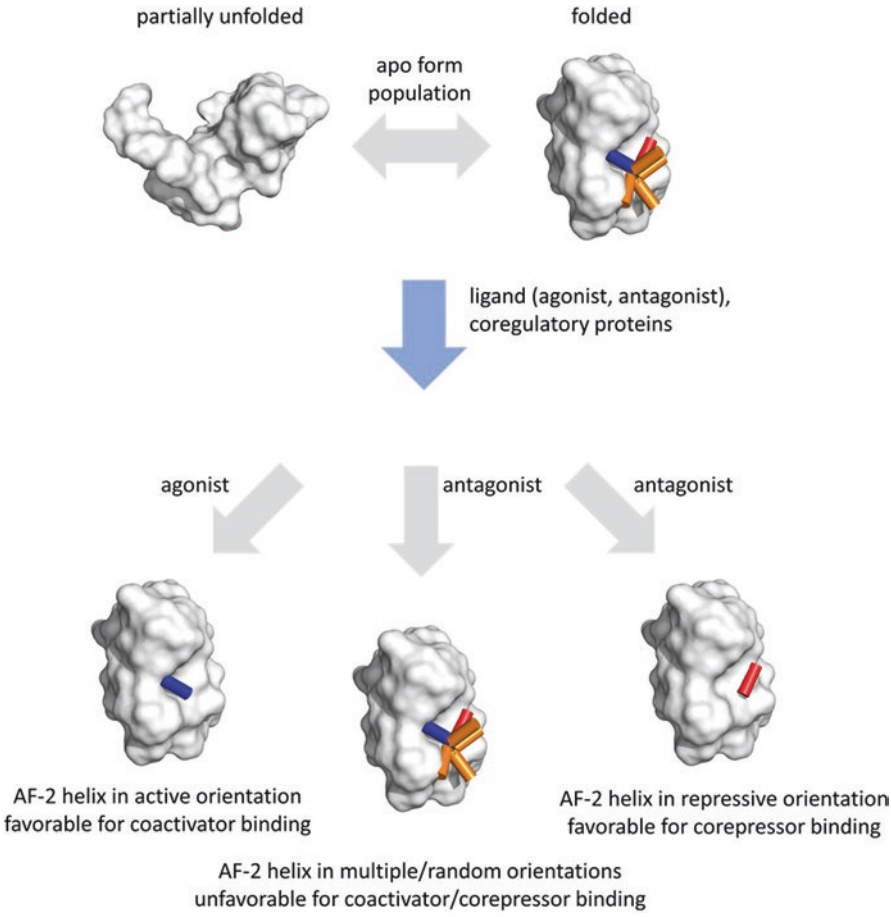

Fig. 7.4 Perspectives on the molecular basis of PXR agonism and antagonism, based on mechanistic investigations of PXR and other NRs. In the unliganded form, PXR is present in a mixture of the population that includes folded and partially unfolded states: in the latter, PXR rearranges its structure to enable a ligand to access the ligand-binding pocket. In the absence of ligand, the AF-2 helix is not fixed in any particular orientation, with a small subpopulation being in a position enabling coactivator or corepressor binding. A ligand (agonist or antagonist) can stabilize the overall structure, forming a more compact and less fluid LBD, which allows for more favorable protein-protein interactions with coregulatory partners. The AF-2 helix is also constrained in either the active (by an agonist) or repressive (by an antagonist or inverse agonist) conformation for coactivator or corepressor recruitment, respectively. However, certain antagonists/inverse agonists could also abrogate PXR's activity by disrupting the positioning of the AF-2 helix from either the active or repressive states, thus inhibiting PXR's activity without forming a complex with a corepressor

conformations due to its flexibility and elasticity, including fully and partially unfolded states. (2) The binding of agonists and some antagonists/inverse agonists shifts the balance toward a fully folded structure by providing an internal network of ligand-to-protein interactions. A compact and properly folded PXR is needed to maintain a stable interface for protein-protein interactions with regulatory and partner proteins. (3) The binding of agonists concomitantly stabilizes the AF-2 helix to provide an accessible surface on the LBD for coactivator association. Certain antagonists/inverse agonists would conversely stabilize the AF-2 helix in a different conformation favorable for corepressor recruitment, while other types of inhibitors fail to fixate the AF-2 helix in any position (sometimes they are referred to as passive antagonists [71]). In such cases, the mobile AF-2 helix would have reduced the ability to recruit the corepressor. (4) Binding of agonists would lead to recruitment of accessory proteins such as RXR along with the coactivator protein.

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Chapter 8
Strategies for the Design of Vitamin D Receptor Ligands

Tania R. Mutchie, Daniel A. Webb, Elliot S. Di Milo, and Leggy A. Arnold

Abstract Structure-activity relationship analysis is a powerful tool to elucidate the structural requirements for high-affinity vitamin D receptor (VDR) ligands. This chapter systematically interrogates the structural features of $1\alpha,25(OH)_2D_3$, the vitamin D metabolite with the highest VDR affinity. It can be concluded that the C1α and C25 hydroxyl groups of $1\alpha,25(OH)_2D_3$ are very important for binding. Optimal spatial arrangement of both hydroxyl groups was achieved with either a hydrophobic semi-flexible secosteroid scaffold or a simplified, flexible carbon chain. Y-shaped ligands with high affinity confirmed a highly inducible VDR ligand-binding pocket, which has been visualized by X-ray crystallography. Substitution of the secosteroid scaffold by other hydrophobic spacers such as carboranes or aromatic ring systems has led to many non-secosteroid VDR ligands. Exploration of ligand substitution has led to the development of antagonists that are accommodated by the inducible VDR ligand-binding pocket but alter the overall conformation of VDR in ways that prevent interactions with coactivator proteins from occurring and ultimately result in reduced gene transcription.

Keywords Vitamin D · Vitamin D receptor · 1,25-Dihydroxyvitamin D_3 · Structure-activity relationship · Agonist · Antagonist · Coactivator

8.1 Introduction

The vitamin D receptor (VDR) takes a special place among nuclear receptors because the biosynthesis of its endogenous ligand, 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), is dependent on sun exposure. Poor living conditions during the industrial revolution, when people were destined to work and live inside with

T. R. Mutchie · D. A. Webb · E. S. Di Milo · L. A. Arnold (✉)
Department of Chemistry and Biochemistry and Milwaukee Institute for Drug Discovery (MIDD), University of Wisconsin, Milwaukee, WI, USA
e-mail: arnold2@uwm.edu

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minimal light exposure, caused bone deformations and skin diseases with symptoms that were alleviated by light therapy. Once irradiated food was found to have the same medicinal effect [1], isolation of vitamin D was rapidly accomplished [2]. Identification of the corresponding receptor turned out to be difficult; however, vitamin D does not bind VDR at physiological concentrations. Once radiolabeled vitamin D was generated [3], $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ were identified [4, 5], which in turn enabled the identification and cloning of VDR [6, 7]. The genomic function of VDR regulates genes involved in calcium homeostasis, cell proliferation, and cell differentiation. The endocrine receptor is expressed in the epithelia of the endocrine organs, digestive tract, kidneys, and thymus [8] but is also found in leukocytes and bone cells. VDR can be found in the cytosol or membrane-bound [9]. In the nucleus, VDR is liganded and binds DNA and the retinoid X receptor (RXR) [10]. VDR-specific gene promoter sequences have been identified [11]. The transcriptional complex includes, among other proteins, nuclear receptor coactivators and corepressors that interact with RNA polymerase II [12], changing chromatin packing and enabling specific gene transcription [13].

This chapter is not a complete review describing more than 3000 VDR ligands that have been reported since 1970. Therefore, I refer the reader to five chapters within the recent edition of *Vitamin D* and cited references, as well as excellent recent reviews [14–20]. This chapter highlights the relationship between molecular ligand structure and VDR affinity. Other downstream biological effects can be found in the references and the recent two volumes of *Vitamin D*. Most VDR ligands have been characterized by their ability to compete with tritium-labeled $1\alpha,25(OH)_2D_3$. Transcription assays have been used to distinguish between agonists and antagonists. VDR coactivator recruitment has been studied with two-hybrid assays or biochemically using homogeneous time-resolved fluorescence (HTRF). Tertiary assays employed for the characterization of VDR ligands included, among others, cell differentiation, cell proliferation, cellular calcium uptake, intestinal calcium transport, and serum calcium changes. Herein, we predominately report VDR affinity independent of cell permeability, metabolic stability, plasma binding (vitamin D-binding protein), and other factors that change with the structure of a small molecule.

8.2 Secosteroid VDR Ligands

Calcitriol, or $1\alpha,25(OH)_2D_3$, has the highest affinity for VDR among all vitamin D metabolites. The competitive VDR binding of $1\alpha,25(OH)_2D_3$ using $[^3H]$-$1\alpha,25(OH)_2D_3$ as a probe has been reported in the range of 0.04–0.16 nM with protein isolated from the tissue and cells or recombinantly expressed VDR as full-length receptor or ligand-binding domain [21, 22]. Other assays such as biochemical coactivator recruitment assays reported EC_{50} of 1.2 nM for $1\alpha,25(OH)_2D_3$ [23]. For most cases, the affinities of new compounds in comparison to $1\alpha,25(OH)_2D_3$ are reported as percent affinity in this chapter.

1α,25(OH)$_2$D$_3$ has six chiral centers and two trisubstituted double bonds that can adopt an E or Z configuration. First, we will compare VDR binding of 1α,25(OH)$_2$D$_3$ epimers and stereoisomers depicted in Figs. 8.1, 8.2, 8.3, and 8.4.

1α,25(OH)$_2$D$_3$ is the metabolic product of vitamin D$_3$, which lacks a hydroxyl group in the C1 and C25 positions (Fig. 8.1). 25-Hydroxyvitamin D-1α hydroxylase, located primarily in the kidneys but also in other tissues, stereospecifically introduces a C1α-hydroxyl group [24–27]. The 3-OH group is present in vitamin D$_3$ and its precursor 7-dehydrocholersterol. The evaluation of A-ring diastereomers of 1α,25(OH)$_2$D$_3$ demonstrated that binding to VDR is more impacted by the stereochemistry of the C1-position than the C3-position [28]. VDR affinity for **2** was 24% in comparison to 1α,25(OH)$_2$D$_3$ but only 0.2% and 0.8% for **3** and **4**, respectively. 1α,25(OH)$_2$-3-Epi-D$_3$ has been identified as a natural metabolite of 1α,25(OH)$_2$D$_3$ [29] and was intensively studied in vivo. 1β,25(OH)$_2$D$_3$ was first synthesized in 1977 [30] and has also recently been identified as a natural metabolite of vitamin D [31]. 1β,25(OH)$_2$D$_3$ has been reported as a non-genomic antagonist of 1α,25(OH)$_2$D$_3$ [28].

Secosteroids in contrast to steroids have a "broken" B-ring resulting in a triene system with 5(Z),7(E) configuration (Fig. 8.2). The formation of secosteroids occurs via a retro Diels-Alder reaction in the presence of light followed by a [1,7] sigmatropic rearrangement. In the skin, this conversion occurs with high stereoselectivity.

Isomer **5** retains a good affinity toward VDR, which is 13% in comparison to 1α,25(OH)$_2$D$_3$ [32]. The E,E stereochemistry can be generated by light in the presence of iodine [33] or by a cheletropic addition-elimination with sulfur dioxide [34]. In contrast, isomers **6** and **7** were not observed for photochemical reactions but synthesized using a chromium(0)-mediated isomerization reaction with a vinylallene precursor [34]. The affinity toward VDR was 0.82% for **6** and 1.6% for **7** in comparison to 1α,25(OH)$_2$D$_3$. Thus, the position of the A-ring with respect to the B- and C-ring is more important for VDR binding than the location of the terminal alkene.

The stereochemistry of the fused B,C-ring system of 1α,25(OH)$_2$D$_3$ originates from 7-dehydrocholesterol. Interestingly, the configuration of the fused system has a direct influence on the equilibrium of the thermal [1,7] sigmatropic rearrangement

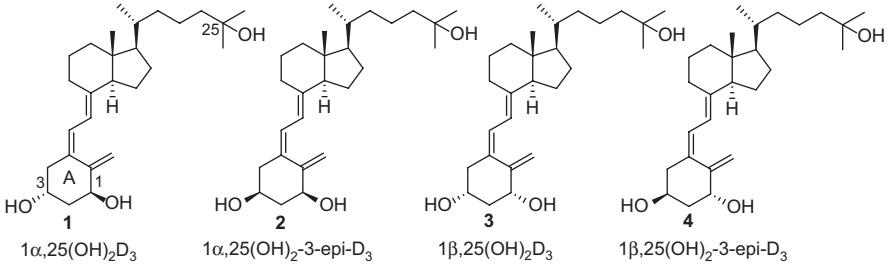


Fig. 8.1 A-ring diastereomers of 1α,25(OH)$_2$D$_3$

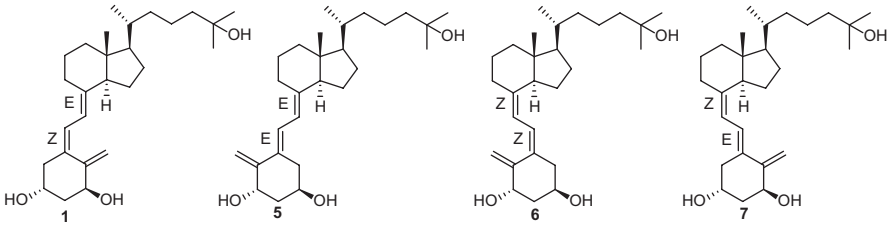


Fig. 8.2 Diene stereochemistry of 1α,25(OH)$_2$D$_3$

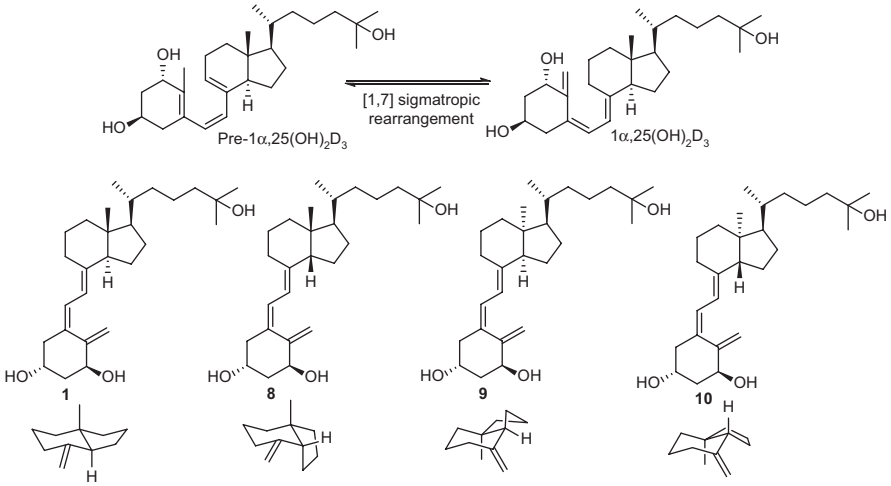
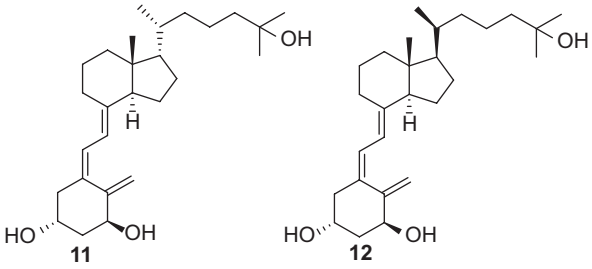


Fig. 8.3 Fused B,C-ring stereochemistry of 1α,25(OH)$_2$D$_3$

Fig. 8.4 C17 and C20 epimers



reaction (Fig. 8.3). When 1α,25(OH)$_2$D$_3$ was heated at 80 °C, only 12% of the pre-1α,25(OH)$_2$D$_3$ was detected [35]. However, when epimer 8 was heated at 80 °C, a 95:5 ratio in favor of the pre-structure was formed.

Pure **8** was synthesized by epimerization of Grundmann's ketone and retained a VDR affinity of 15% in comparison with 1α,25(OH)$_2$D$_3$ [35]. Interestingly, no reports were found for compounds **9** and **10**.

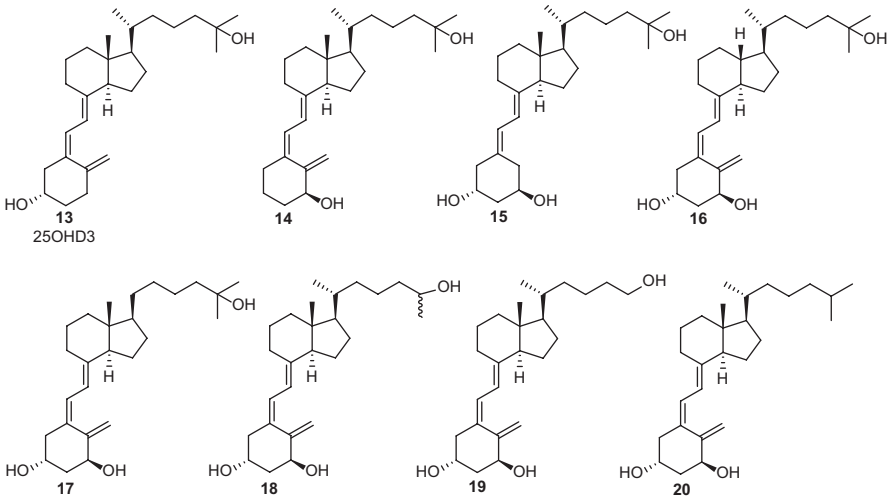


Fig. 8.5 1α,25(OH)$_2$D$_3$ analogs that lack certain structural elements

The synthesis of **11** has been reported [36]. Later, an improved route was developed but VDR binding was not reported [37]. However, inhibition of human breast cancer cell (MCF-7) proliferation was more pronounced in the presence of **11** than 1α,25(OH)$_2$D$_3$. Epimer **12** demonstrated inhibition of T-cell proliferation at picomolar concentrations [38]. The VDR binding was 88% in comparison to 1α,25(OH)$_2$D$_3$ [39].

Next, the importance of functional groups and substituents with respect to VDR binding is discussed. Analogs that lack certain structural elements are compared to 1α,25(OH)$_2$D$_3$ (Fig. 8.5).

25OHD$_3$ is a metabolic product of vitamin D$_3$ and was identified in 1968 [4]. It is abundant in blood and used to determine the vitamin D status in humans [40]. The binding toward VDR is 900-fold less than 1α,25(OH)$_2$D$_3$ [41]. In contrast to **13**, the affinity of **14** was only 1/8 less effective than 1α,25(OH)$_2$D$_3$, making the 1α-OH group significantly more important for VDR binding than the 3-OH group [41]. Compound **15** lacking the methylene group was first synthesized in 1990 and has been shown to induce the differentiation of HL-60 cells at the same concentration as 1α,25(OH)$_2$D$_3$ [42]. The VDR binding was 30% in comparison to 1α,25(OH)$_2$D$_3$ [43]. Compound **16** was reported to be three times more potent than 1α,25(OH)$_2$D$_3$ with respect to porcine VDR binding [44]. Thus the presence of C18 impaired VDR binding, contrasting with compound **17**, in which a lack of the C21 methyl reduced affinity toward chick VDR to 10% that of 1α,25(OH)$_2$D$_3$ [45]. Interestingly, substitution of C20 by oxygen reduced VDR affinity to 0.1% in comparison to 1α,25(OH)$_2$D$_3$ [45], emphasizing the importance of hydrophobicity for good receptor binding. Compound **18** with possible (R) and (S) configurations has not been reported. VDR binding of **19** has not been investigated; however, the ability to

differentiate HL-60 cells compared to 1α,25(OH)$_2$D$_3$ was 1% at the same concentration [46]. For a similar molecule with a terminal alkene in the 2-position, a 1.9% VDR affinity was reported in comparison to the parent compound [47]. Compound **20**, also known as alfacalcidol, was first reported in 1973 [48, 49]. Alfacalcidol is converted to 1α,25(OH)$_2$D$_3$ in vivo and, therefore, exhibits similar activities. The VDR affinity was 900-fold less than 1α,25(OH)$_2$D$_3$ [41]. Thus, it can be concluded that hydroxyl groups in the C1α and C25 positions are the most important substituents to promote VDR binding.

Further investigations into the significance of the bicyclic structure of 1α,25(OH)$_2$D$_3$ with respect to VDR binding is represented by compounds depicted in Fig. 8.6.

Removal of the five-membered ring of 1α,25(OH)$_2$D$_3$ was investigated with **21** and analogs thereof [50]. The relative stereochemistry of C17 marginally influenced VDR binding; however, large differences between these epimers were observed for anti-proliferation and calcium homeostasis. VDR binding of **21** was 60% in comparison to 1α,25(OH)$_2$D$_3$. The VDR affinity of the C20 epimer of **21** was 70%. The same report characterized compounds like **22** with a VDR affinity of 80% in comparison to 1α,25(OH)$_2$D$_3$. The synthesis of **23** was reported but VDR binding was not determined [51]. However, **24** with the terminal alkene moved from position 10 to position 2, exhibited 80 times lower affinity toward VDR than 1α,25(OH)$_2$D$_3$ [21].

Overall, it can be concluded that structural changes to the hydrophobic core of 1α,25(OH)$_2$D$_3$ can still result in high-affinity ligands for VDR. The ligand-binding domain of VDR consists of 12 helices when bound to 1α,25(OH)$_2$D$_3$. The most essential features of 1α,25(OH)$_2$D$_3$ are the C1α and C25 hydroxyl groups, which have been shown to form canonical hydrogen bonds with VDR (Fig. 8.7).

The interaction of 25-OH with His305 (loop H6-H7) and His 397 (H11) is vital to the conformational change VDR undergoes when interacting with coregulator proteins. 1-OH interacts with Ser237 (H3) and Arg274 (H5), anchoring the ligand in the binding pocket. 3-OH interacts with Ser278 (H5) and Tyr143 (loop H1-H2), but compounds like **2** and **14** have shown that these contacts merely provide further stabilization to the complex. 1α,25(OH)$_2$D$_3$ only fills 56% of the VDR-binding pocket, which helps explain the large variety of high-affinity ligands that have been developed for VDR. Important, however, is the spacing and orientation of the

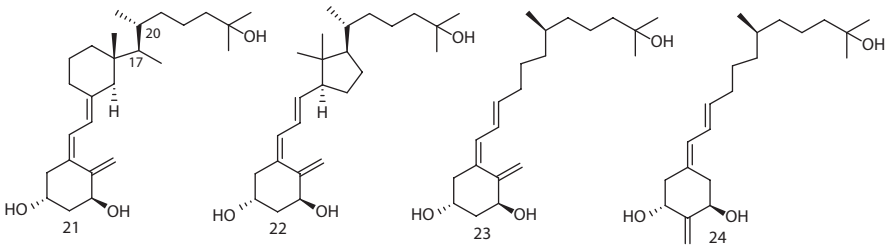


Fig. 8.6 VDR ligands without a fused ring system

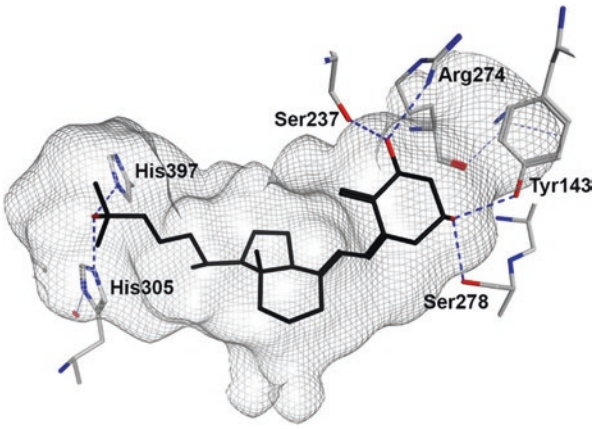


Fig. 8.7 Crystal structure of 1α,25(OH)$_2$D$_3$ bound to human VDR [PDB ID:1DB1] [52]

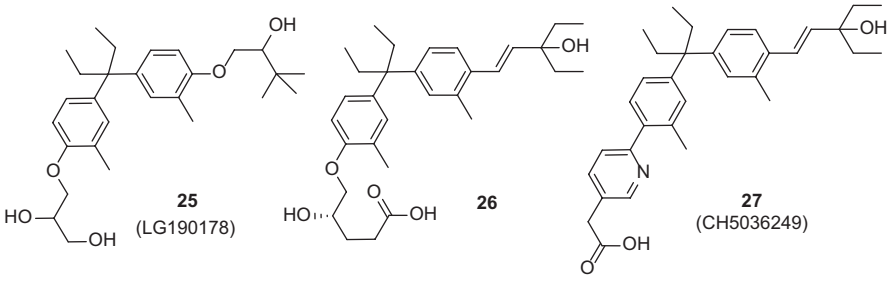


Fig. 8.8 VDR ligands with a diarylmethane moiety

hydroxyl groups, which are supported by ring structures, a diene moiety, and chiral carbon centers. The majority of the central VDR ligand pocket surface is hydrophobic and assembled by leucine, isoleucine, and valine side chains.

8.3 Non-secosteroid VDR Ligands

The first non-secosteroid ligands with a diarylmethane moiety were reported by Ligand Pharmaceuticals (Fig. 8.8). The quaternary carbon center bearing two ethyl substituents was superior to other alkyl substituents, and aligned well with the fused ring system of VDR-bound 1α,25(OH)$_2$D$_3$ [53].

The racemic mixture of LG190178 exhibited a VDR affinity of 0.3% in comparison to 1α,25(OH)$_2$D$_3$ [54]. The synthesis of individual LG190178 stereoisomers identified the (2S, 2'R) isomer as the most active compound with a 28.3% VDR affinity in comparison to 1α,25(OH)$_2$D$_3$ [55]. The systematic development of these ligands resulted in **26**, which was equally as active as 1α,25(OH)$_2$D$_3$ in a cell-based transcription assay [56]. An analog of **26**, which replaced the ethyl groups adjacent

to the tertiary alcohol with trifluoromethyl groups, showed a fivefold improvement in the transcription assay [57]. In recent years, many similar compounds were developed with thiophene, pyrrol [58], and other heterocycles, though compound **27**, which contains a pyridine substituent, exhibited the highest affinity toward VDR at 37% of the VDR-1α,25$(OH)_2D_3$ interaction [59].

Another successful approach for non-secosteroidal VDR ligand design was the incorporation of a dicarba-closo-dodecaborane as a hydrophobic moiety, rather than the fused ring system of natural VDR ligands (Fig. 8.9).

The development of carborane-based VDR ligands with different side chains resulted in **28**, which exhibited an affinity of 640 nM (IC_{50}) [60]. The (R) isomer was one-fifth as active. Subsequent research identified compound **29** being twice as potent as **28** in a HL-60 differentiation assay [61].

Other approaches for VDR ligands included the incorporation of aromatic ring structures, which are absent from natural VDR ligand, 1α,25$(OH)_2D_3$. The earliest examples were identified from a library of bis-aromatic compounds (Fig. 8.10).

CD4528 was characterized with a CYP24A1 reporter assay demonstrating an EC_{50} of 1.7 nM [62]. For the same assay, the EC_{50} of 1α,25$(OH)_2D_3$ was 1.0 nM. Other related VDR ligands exhibited similar low nanomolar activities, for example, CD4849 (0.5 nM) [63]. A recent study employing the A-ring structure of 1α,25$(OH)_2D_3$ resulted in **32**, which displayed a VDR affinity 24% that of 1α,25$(OH)_2D_3$ [64]. Less active was **33**, exhibiting a 0.01% VDR affinity in comparison to 1α,25$(OH)_2D_3$ [65]. Other nutritional ligands with low VDR affinities were reported by Haussler et al. [66].

VDR is highly expressed in the intestine and has been described as a bile acid sensor due to its ability to bind lithocholic acid (Fig. 8.11) [67].

The affinity of VDR for lithocholic acid was less than 0.005% in comparison to 1α,25$(OH)_2D_3$. The corresponding acetate was more potent with a 0.01% VDR affinity in comparison to 1α,25$(OH)_2D_3$ [68]. A later VDR-binding study reported an IC_{50} of 30 μM for both **35** and **36** [69]. Recently, a methylsulfonate analog of lithocholic acid showed an IC_{50} of 1.2 μM [70]. For estrone analog **38**, an EC_{50} of 850 nM was reported in a VDR transactivation assay [71].

The first Y-shaped VDR ligand called Gemini was introduced in 2000 by Norman et al. [72] (Fig. 8.12).

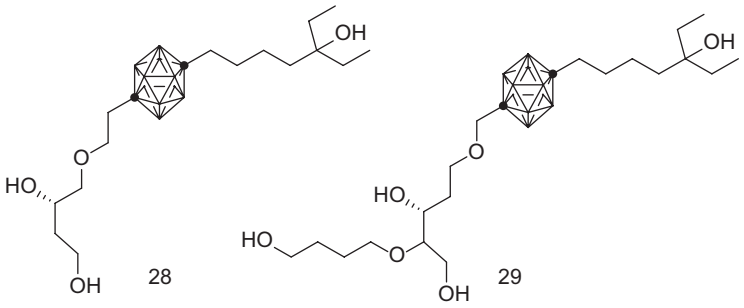


Fig. 8.9 VDR ligands containing a carborane structure

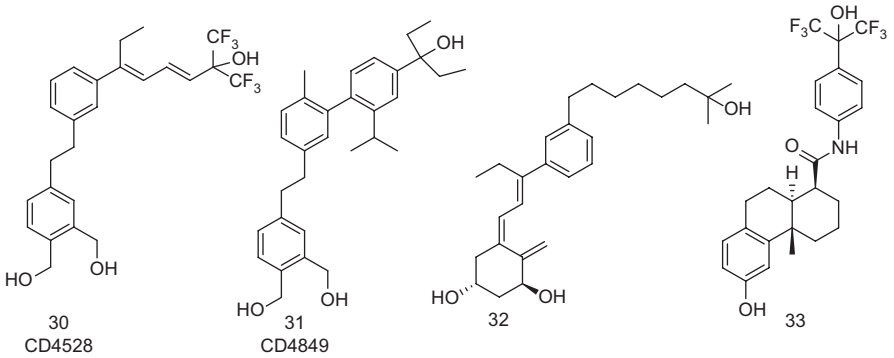


Fig. 8.10 Bis- and tris-aromatic VDR ligands

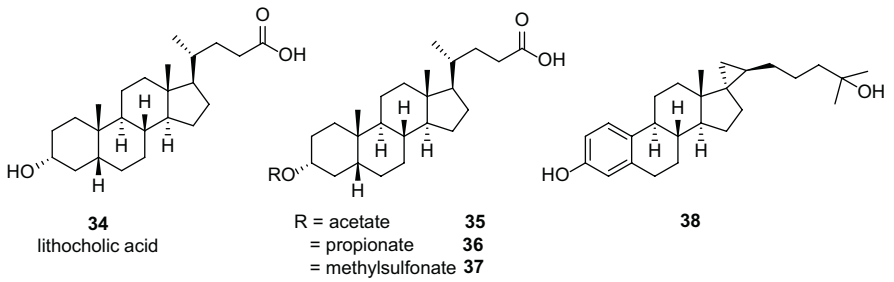


Fig. 8.11 Steroid VDR ligands

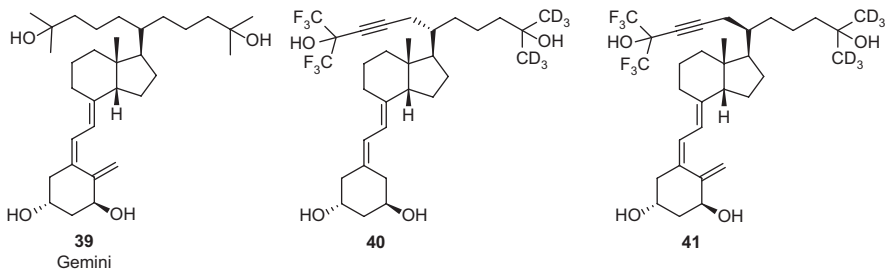


Fig. 8.12 Gemini ligands

Gemini exhibited a VDR affinity of 38% in comparison to 1α,25(OH)$_2$D$_3$. Based on an available crystal structure of VDR bound to 1α,25(OH)$_2$D$_3$, it was hypothesized that VDR might accommodate this second side chain in a so-called A pocket (alternative pocket) [73]. The later reported crystal structure of VDR bound to Gemini confirmed the adaptability of VDR to accommodate Y-shaped ligands [74]. Further developments resulted in **40** and its C20 epimer, **41**, which were 36- and 22-fold more active in a gene reporter assay than 1α,25(OH)$_2$D$_3$ [75].

8.4 VDR Antagonists

Throughout the last two decades, many different antagonists with strong VDR affinities have been reported [76]. The earliest disclosed antagonists were derivatives from the natural-occurring vitamin D metabolite, (23S,25R)-1α,25$(OH)_2D_3$-26,23-lactone [77], such as compound TEI-9647 and its C23 epimer TEI-9648 (Fig. 8.13).

The VDR affinity of TEI-9647 was 10% compared to 1α,25$(OH)_2D_3$. Its C23 epimer TEI-9648 bound VDR with a 8% affinity [78]. Further research confirmed that these unsaturated esters underwent a conjugate addition reaction with cysteine residues in the human VDR ligand pocket [79]. Methyl substitutions at positions 2 and 24 (**43**) significantly increased VDR affinity (63% in comparison to 1α,25$(OH)_2D_3$) [80]. The introduction of a cyclopropyl group on the lactone ring resulted in **44**, which surpassed the VDR affinity of 1α,25$(OH)_2D_3$ (166%) [81].

Based on the structure of calcipotriol (**45**), an approved treatment for psoriasis and a high-affinity VDR ligand, other related ligands were able to be synthesized by Schering (Fig. 8.14).

In contrast to agonist calcipotriol, ZK159222 and ZK168281 were poor inducers of VDR transcription and reduced 1α,25$(OH)_2D_3$-meditated transcription [82]. For ZK191784, a VDR affinity of 33% in comparison to 1,25-$(OH)_2D_3$ was reported. [83] Thus, the VDR affinities of these ligands are strong but induce an antagonistic VDR conformation. Additionally, these antagonists were investigated in vivo and demonstrated promising anti-inflammatory properties [76].

Amide-based VDR antagonists that were inspired by calcitriol lactone were introduced in 2004 (Fig. 8.15).

The corresponding lactam **49** exhibited a VDR affinity of 10.2 nM in comparison to 1α,25$(OH)_2D_3$ with 0.5 nM [84]. The measured VDR binding of **50** was 1.9 nM (IC_{50}). Importantly, **50** inhibited VDR-mediated transcription at nanomolar concentrations without showing any agonist activity in the absence of 1α,25$(OH)_2D_3$ [85].

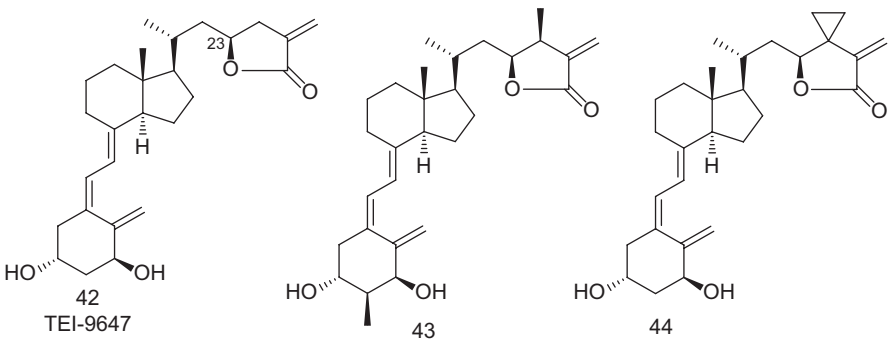


Fig. 8.13 Electrophilic VDR ligands

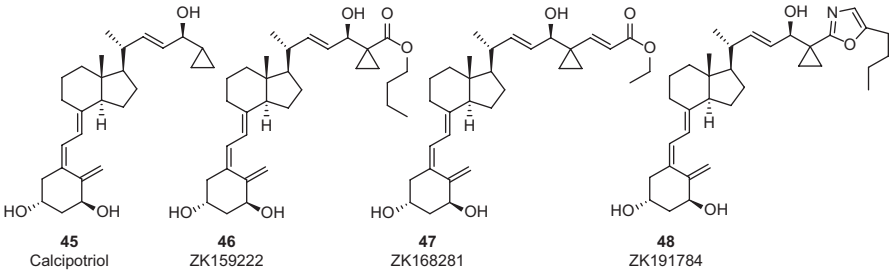


Fig. 8.14 ZK series of VDR antagonists

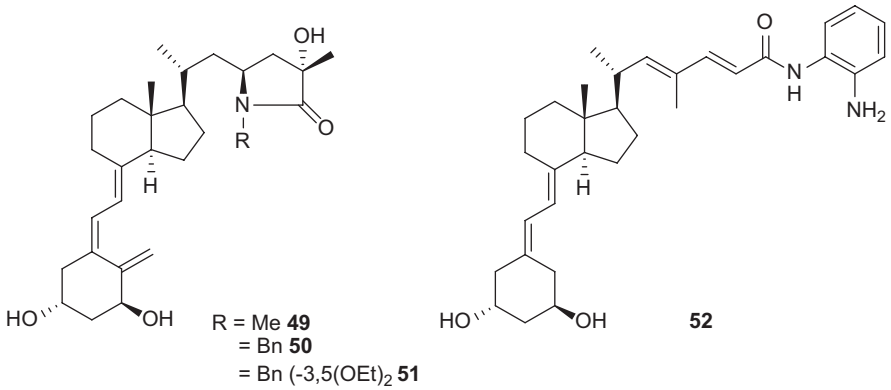


Fig. 8.15 Amide series of VDR antagonists

Further improvement was achieved with **51**, which exhibited 52% VDR-binding affinity compared to 1α,25$(OH)_2D_3$ and inhibited 1α,25$(OH)_2D_3$-mediated transcription with an IC_{50} of 90 nM [86]. For ortho-aniline compound **52**, an IC_{50} of 107 nM was reported [87].

Introduction of a bulky adamantane group was conceived as another approach to change the conformation of VDR (Fig. 8.16).

Compound **53** exhibited 2% VDR affinity compared to 1α,25$(OH)_2D_3$ [88]. In the presence of 1α,25$(OH)_2D_3$, VDR-mediated transcription was inhibited at 100 nM. Among a series of diastereomeric analogs, **54** exhibited the highest affinity toward VDR with 17% affinity in comparison to 1α,25$(OH)_2D_3$ [89]. Further improvements resulted in ligands with an internal alkyne named ADTK1-4 [90]. The compound with the highest VDR affinity among this group was **55** reaching 90% of the VDR-1α,25$(OH)_2D_3$ interaction. This compound behaved as a partial agonist. Finally, a library of VDR ligands with a diyne system were synthesized and evaluated, achieving a 7% VDR affinity in comparison to 1α,25$(OH)_2D_3$ [91].

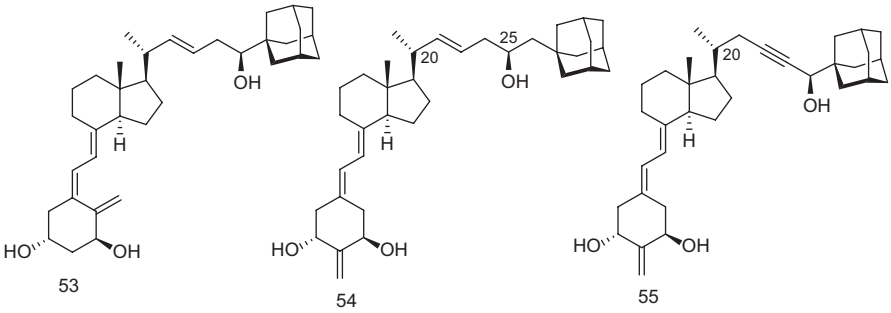


Fig. 8.16 Adamantyl-derived VDR antagonists

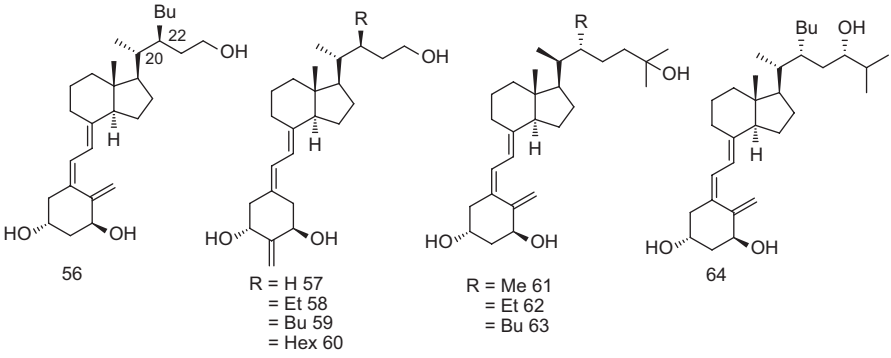


Fig. 8.17 VDR ligands with C22 substitution

A series of Y-shaped VDR ligands that were inspired by Gemini were developed and, dependent upon their substitution pattern, were found to be antagonists, partial agonists, or superagonists (Fig. 8.17).

Among a series of diastereomers with a butyl substituent in the C22 position, **56** was identified as a VDR antagonist [92]. The VDR affinity was 4.1% in comparison to 1α,25-$(OH)_2D_3$ [92]. Interestingly, the C20 epimer of **56** was identified as an agonist with a 2.5% affinity toward VDR in comparison to 1α,25$(OH)_2D_3$. The influence of the alkyl chain length with respect to VDR binding was investigated for very similar compounds containing a methylene group in the C2 position (**57–60**) [47, 93]. The presence of a butyl substituent resulted in antagonist **59**, which had a VDR affinity of 61% in comparison to 1α,25$(OH)_2D_3$. Partial agonists **57**, **58**, and **60** exhibited lower VDR affinity. Further structural changes to compound **59** included the introduction of two methyl substituents in the C24 position or elongation of the hydroxyl-bearing carbon chain by one carbon. Both of these structural changes also resulted in antagonistic ligands; however, implementing the carbon chain inherent to 1α,25$(OH)_2D_3$ resulted in a superagonist with higher VDR affinity than 1α,25$(OH)_2D_3$ [47]. Other superagonists were produced with the introduction of C22 substituents for 20-epi-1α,25-$(OH)_2D_3$ (**12**) [94]. Compound **63** exhibited

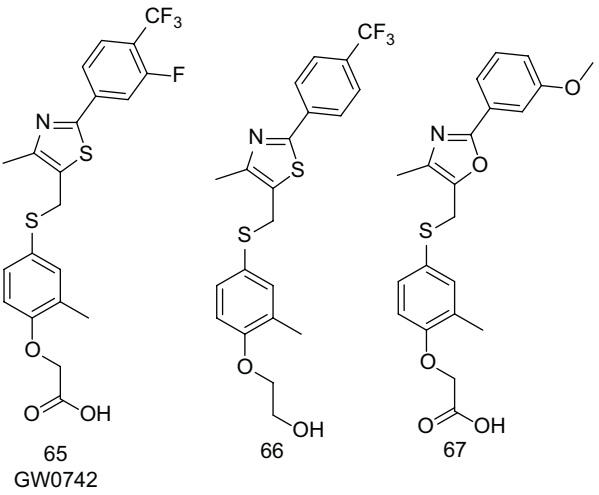


Fig. 8.18 GW0742-based VDR ligands

the highest affinity for VDR (797%) followed by **62** and **61**. Finally, antagonists were produced with an isopropyl group at C24 (**64**) [92]. Among different diastereomers, **64** exhibited the highest VDR affinity of 1.4% in comparison to $1\alpha,25(OH)_2D_3$.

A high-throughput screen of 390,000 compounds identified PPARδ agonist GW0742 as a novel VDR antagonist (Fig. 8.18) [95].

The VDR affinity of **65** was 8.7 μM. The structural change of the acid function into an hydroxyl group resulted in partial VDR agonist **66** with an EC_{50} of 120 nM [96]. The evaluation of a library of compounds related to **65** demonstrated that CF_3 substituents in the meta- and ortho-position reduced the affinity toward PPARδ without influencing the affinity for VDR [97]. Recently, VDR antagonist **67** was reported with activity of 660 nM [98]. This compound did not bind PPARδ. Virtual screening of known nuclear receptor ligands for application in VDR binding identified several compounds as possible candidates. VDR binding was demonstrated for several compounds, including H6036 [99].

8.5 Concluding Remarks and Future Directions

Novel VDR ligand design and synthesis is still a very active research area, with many international research groups working together to develop new drug candidates for disorders caused by vitamin D deficiency, cancer, and inflammatory diseases. Recently discovered VDR ligands are being investigated in clinical trials, reflecting the need for new medications in the respective disease areas. A great number of ligands have been elucidated in complex with VDR using X-ray crystallography. The structural information has guided new ligand design while also

demonstrating that the VDR ligand pocket is amendable to very different ligand shapes. However, as highlighted in this chapter, hydrogen bonding on opposite ends of the ligand pocket is essential for high VDR affinity. Another important feature is distinct spacing of these groups by a flexible hydrophobic spacer. Recently, endogenous ligands for VDR such as lithocholic acid and fatty acids have been identified, although their biological function is still unclear. Furthermore, new vitamin D metabolites have been identified in the last few decades, offering new areas of research in the field of vitamin D.

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Chapter 9
What Makes a Good Antagonist: Lessons Learned from the Estrogen and Aryl Hydrocarbon Receptors

Hollie I. Swanson

Abstract Traditionally, ligands of receptors have been classified as agonists, partial agonists, or antagonists. Study of the estrogen receptor, however, introduced the field of pharmacology to the concept of selective modulators that varied in their ability to either activate or inhibit the receptor. The mechanisms underlying these events were mapped to their unique positions within the ligand-binding cavity of the estrogen receptor and their interactions with key amino acid residues residing within this pocket. Building on these lessons, selective aryl hydrocarbon receptor modulators are currently being developed to finely tune the activities of the aryl hydrocarbon receptor and inhibit disease-modifying processes. These ongoing lessons will challenge modern pharmacologists to develop new tools and approaches for predicting the ultimate pharmacological effects of these emerging therapeutics.

Keywords Agonist · Antagonist · Aryl hydrocarbon receptor · Estrogen receptor · Ligand binding

Abbreviations

6-MCDF	6-methoxy-1,3,8-triCDF
AF1/AF2	Activation function 1/activation function 2
AHR	Aryl hydrocarbon receptor
AHRE/DRE/XRE	Aryl hydrocarbon receptor/dioxin/xenobiotic response element
AHRR	Aryl hydrocarbon receptor repressor
bHLH/PAS	*B*asic *h*elix-*l*oop-*h*elix *P*ER, *A*RNT, *S*IM
CYP1A1/1B1	Cytochrome P450 1A1/1B1

H. I. Swanson (✉)
Department of Pharmacology and Nutritional Sciences, University of Kentucky College of Medicine, Lexington, KY, USA
e-mail: Hollie.swanson@uky.edu

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DBD	DNA-binding domain
DC_{50}	Half-maximal degradation concentration
E2	17β-estradiol
ER	Estrogen receptor
ERE	Estrogen response element
FICZ	6-formylindolo[3,2-*b*]carbazole
HSP90	Heat shock protein of 90 kDa
IC_{50}	Half-maximal inhibition concentration
ITE	2-(1′H-indolo-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester
K_D	Equilibrium dissociation constant
LDB	Ligand-binding domain
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PROTAC	*Pro*teolysis-*ta*rgeting *c*himera
SAHRD	Selective aryl hydrocarbon receptor downregulator
SAHRM	Selective aryl hydrocarbon receptor modulator
SERCA	Selective estrogen receptor covalent antagonist
SERDs	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator
STEAR	Selective tissue estrogenic activity regulator
TCDD	2,3,7,8 tetrachlorodibenzo-p-dioxin
XAP HBV	X-associated protein 2

9.1 Introduction: What Is an Antagonist?

What is an antagonist? This question and its corollary – "why are some drugs agonists and other drugs antagonists?" – are the ones that have bedeviled pharmacologists since the beginning of our discipline's history [32, 64]. As stated in many pharmacology textbooks, "an antagonist is a drug (any substance that brings about a change in biological function via its chemical action) that binds to a receptor and competes with and prevents receptor binding with other molecules." This definition arose from the observations of early pharmacologists who used antagonists to develop several important pharmacological concepts that are core to our understanding of how receptors behave. For example, analyses of competitive antagonism led to the now familiar parallel shift in dose-response curves. An additional pharmacological concept represented by the equilibrium dissociation constant, K_D, used for quantifying the affinity with which a ligand binds to its cognate receptor, also arose from the experimental use of antagonists. Finally, the use of noncompetitive antagonists aided the development of the concept of efficacy. Here, it was found that a relationship existed between a receptor's conformation and the ability of its ligand to incur a biological response. That is, efficacy was a reflection of a ligand's preference for the resting versus active state of a given receptor. In this manner, an agonist with high efficacy would greatly prefer the active conformation of the receptor. An

antagonist, however, would show no preference, be able to bind equally to either resting or active forms, and thus be incapable of producing an effect due to its inability to shift the receptor to its active state. Using these fundamental concepts, early receptor pharmacologists then assigned known ligands as either "agonists" or "antagonists" based on the affinity of the ligand for the receptor and ability of the ligand to induce a measured response that could be quantitatively measured. Several assumptions provided the foundation for their reductionist thinking including theories that (1) the dose-response curve appropriately reflects a receptor's occupancy relationship, (2) the biological response is directly proportional to the receptor's occupancy, and (3) in the absence of agonist, the receptor is silent.

The purpose of this chapter is to reexamine fundamental receptor concepts as they pertain to our understanding of nuclear receptors and contribute to our identification and definition of their antagonists. Using the estrogen and aryl hydrocarbon receptors (ER and AHR, respectively) as specific examples, we will briefly review key events that have led to the development of ligands that selectively activate or inactivate their respective receptors as well as the molecular events that govern these actions. In addition, we will examine current efforts focused on developing novel approaches to be used for blocking the actions of these receptors with high affinity and high specificity.

9.2 Identification and Development of ER Antagonists

9.2.1 A Brief History of the Development of ER Antagonists

The development of ER antagonists began in the early 1960s during efforts to expand the availability of oral contraceptive drug products [31, 62]. Tamoxifen, one of the initial compounds in this drug pipeline, was synthesized by a chemist, Dora Richardson. Known as "compound ICI 46,474," its failure to suppress ovulation would have doomed its further development if not for the tenacity of the team leader, Dr. Arthur Walpole. Dr. Walpole was an astute collaborator who in an effort to revive its patent promise, proposed that it would be useful for treating breast cancer. At that time, cancer was largely treated using either surgical or radiotherapy approaches. Those utilizing chemotherapy were viewed with skepticism as it was considered to be a relatively novel and untested concept. Despite considerable resistance from his company's "suits," Dr. Walpole was able to persevere due in large part to clinical evidence supporting his idea. Thus, tamoxifen was launched into the market both as an agent to be used for infertility and as a breast cancer therapeutic. Nearly two decades later, subsequent clinical trials initiated in the 1980s confirmed that tamoxifen is effective for both treating and preventing breast cancer.

Tamoxifen's dark side, however, was also revealed during these early days of its development and clinical use. Studies performed in mouse models indicated that a correlative relationship between its *anti-estrogenic*/anti-tumor effects and its ability to increase uterine wet weight, a *pro-estrogenic* effect, existed. This proved to be an

early warning sign of a serious side effect of tamoxifen, as it foretold the increased risk of endometrial cancer (by fourfold) in post-menopausal women treated with tamoxifen. It also raised questions as to the true nature of its anti-estrogen actions. Another issue illuminated upon the discovery of the ER and the development of ER binding assays was that tamoxifen exhibited very low affinity for receptor binding. This latter issue was resolved when it was realized that the true nature of tamoxifen's ER antagonist activity lays within the formation of its high-affinity metabolites, in particular, 4-hydroxytamoxifen and endoxifen. That is, the true ER antagonists were 4-hydroxytamoxifen and endoxifen with endoxifen exerting greater efficacy. These findings provided the basis for the development of structure-activity relationships, thereby resulting in the discovery of raloxifene and ultimately ICI 164,384 (fulvestrant) (Fig. 9.1). Additional observations that accelerated the development of ER antagonists were the findings that tamoxifen promoted bone density, a patent-worthy observation. A key characteristic of ICI 164,384 and its descendent, ICI 182,780 (now referred to as fulvestrant), is its long side chain. The importance of long side chains in determining the antagonistic activity of drugs like fulvestrant will be discussed in a latter section.

The idea that the ER could be "selectively modulated" was supported by the clinical observations that tamoxifen, as well as raloxifene, had pro-estrogenic properties (i.e., retarding osteoporosis and atherosclerosis) while also exerting anti-estrogenic, anti-breast cancer activities [41, 56]. Thus, the development of SERMs, selective estrogen receptor modulators, was well on its way. First-generation SERMs are derivatives of triphenylethylene and include tamoxifen as well as toremifene (Fig. 9.1). Second-generation SERMs, which include raloxifene, are benzothiophene derivatives. However, understanding *how* SERMS can selectively activate or inhibit the actions of the ER within tissues of interest requires deeper insights into the structural attributes of the receptor.

9.2.2 Molecular Characterization of ERs

In the late 1970s, the use of radiolabeled binding assays confirmed the existence of a receptor capable of interacting specifically with estrogen [20, 28, 66]. With the advent of molecular biological approaches and the subsequent cloning of the ER during the following decade, the long envisioned molecular structure of the ER (i.e., ERα) became a reality. It is now known that estrogen is capable of binding and activating two forms of the estrogen receptor, ERα and ERβ. In tissues such as the breast and uterus, ERα is thought to be the predominate receptor, whereas in tissues that require estrogen for their structural maintenance, such as the prostate, ovary, vascular endothelium, and immune system, ERβ likely plays a major role in mediating estrogen-induced signaling.

In the absence of ligand, ERα and ERβ are found primarily in the cytosol. In the presence of ligands, like E2 (17β-estradiol), the receptors dimerize, translocate to the nucleus, and interact with specific DNA recognition sites termed estrogen

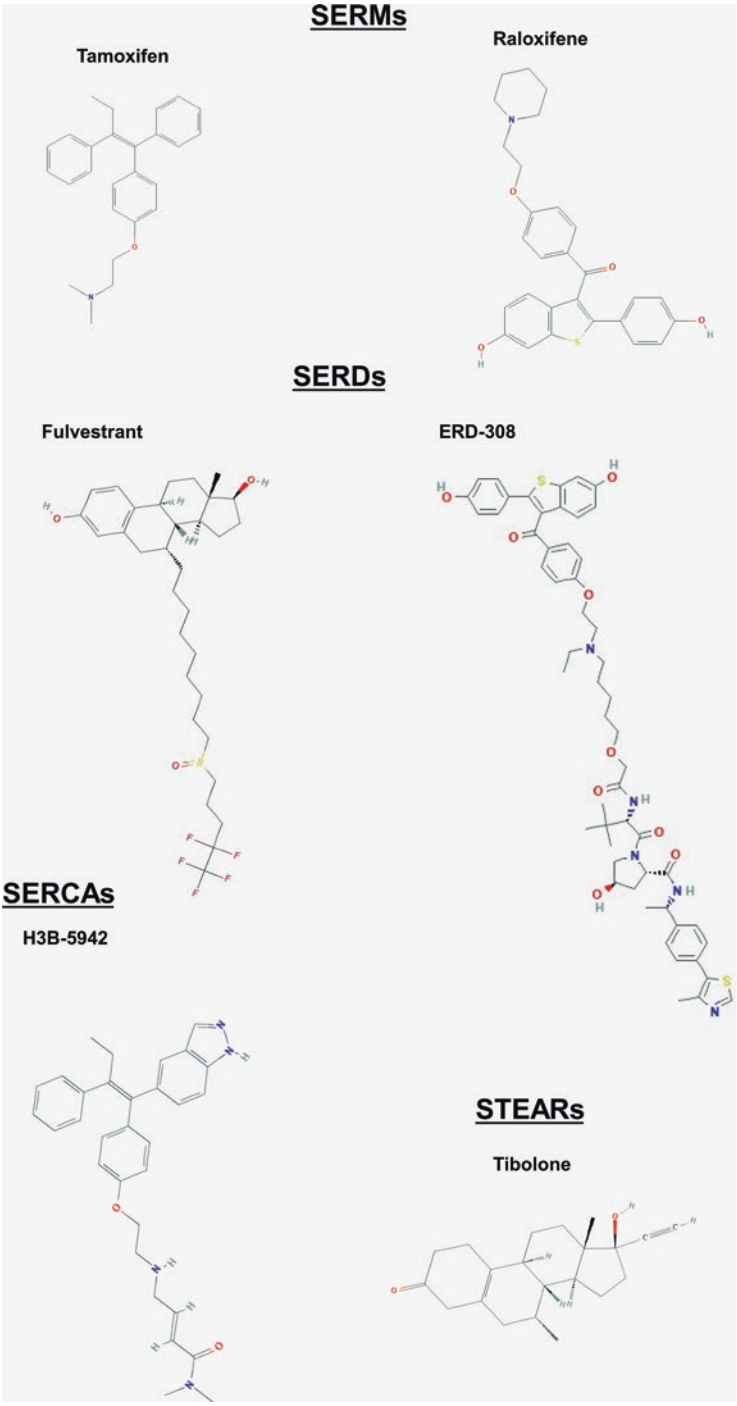


Fig. 9.1 Chemical structures of representative SERMs, SERDs, SERCAs, and STEARS

response elements (EREs). Throughout this series of events, ERα and ERβ interact with a plethora of proteins that facilitate their agonist-induced conformational transition from their "unactivated" to "activated" states, mediate their dimerization and cellular localization, and allow the receptors to either activate (i.e., via interactions with coactivators) or repress (i.e., via interactions with corepressors) gene transcription. Coactivators facilitate gene activation by engaging in activities such as chromatin modification, transcriptional initiation, alterations of RNA processing, and degradation of activated nuclear receptor complexes. Corepressors block transcription by directly interacting with unbound estrogen receptors and/or competitively displacing coactivators from binding to ERα/ERβ.

The ultimate biological effects of ERα/ERβ also impinge on their ability to crosstalk with a number of other transcription factors such as Sp1, AP-1, and the Rel subunit of NF-κB. Many of these protein-protein interactions occur in ligand-specific manners which ultimately determine which genes are regulated and the directionality of their regulation. The transcriptional response to estrogens within a target cell is a combinatorial event involving dynamic populations of ligands, estrogen receptors, estrogen receptor-modifying enzymes, and coregulators. For example, the protein-protein interactions involving the estrogen receptors (i.e., the "interactome") are ligand specific. In addition, the expression levels of the involved proteins are highly variable with estrogen receptor turnover being dependent on the timing of uninterrupted ligand exposure. Further, ERα-/ERβ-interacting proteins are expressed in a cell-type-dependent manner. Given this, it is proposed that assessing the efficacy of estrogen receptor ligands should incorporate temporal measurements of gene transcription within a variety of tissues [63].

The estrogen receptors are composed of five domains; the A/B (N-terminal domain); C, D, E domains; and at the C-terminus, the F domain [20, 28, 66]. A schematic of the key domains of ERα is shown in Fig. 9.2a. The A/B domain mediates transcriptional activation which is facilitated primarily by a region referred to as AF1 (activation function 1). The adjacent C region bears sites involved in receptor dimerization and DNA binding (DNA-binding domain, DBD). The DBD consists of two zinc finger structures that interact specifically with the ERE. The canonical ERE is defined as the palindrome GGTCAnnnTGACC. However, more than 70,000 EREs have been identified in the human genome and vary with respect to their specific sequence compositions and their positions relative to the mRNA transcription start site. While canonical ERE sites were initially identified within gene promoters, more extensive analyses have revealed that the majority of estrogen-induced binding of ERα occurs outside of promoter regions and within introns and intergenic regions [26].

The D domain, also referred to as the "hinge," is involved in nuclear translocation of the receptor [28]. Specific amino acid sequences harbored within this site, nuclear localization sequences, are essential for sequestering the ERs within the cytosol. Ligand binding "unmasks" these sites and allows for the receptors to enter the nucleus. The E domain at the C-terminus harbors the ligand-binding domain (LDB) as well as a second site involved in transcriptional activation, termed the AF2

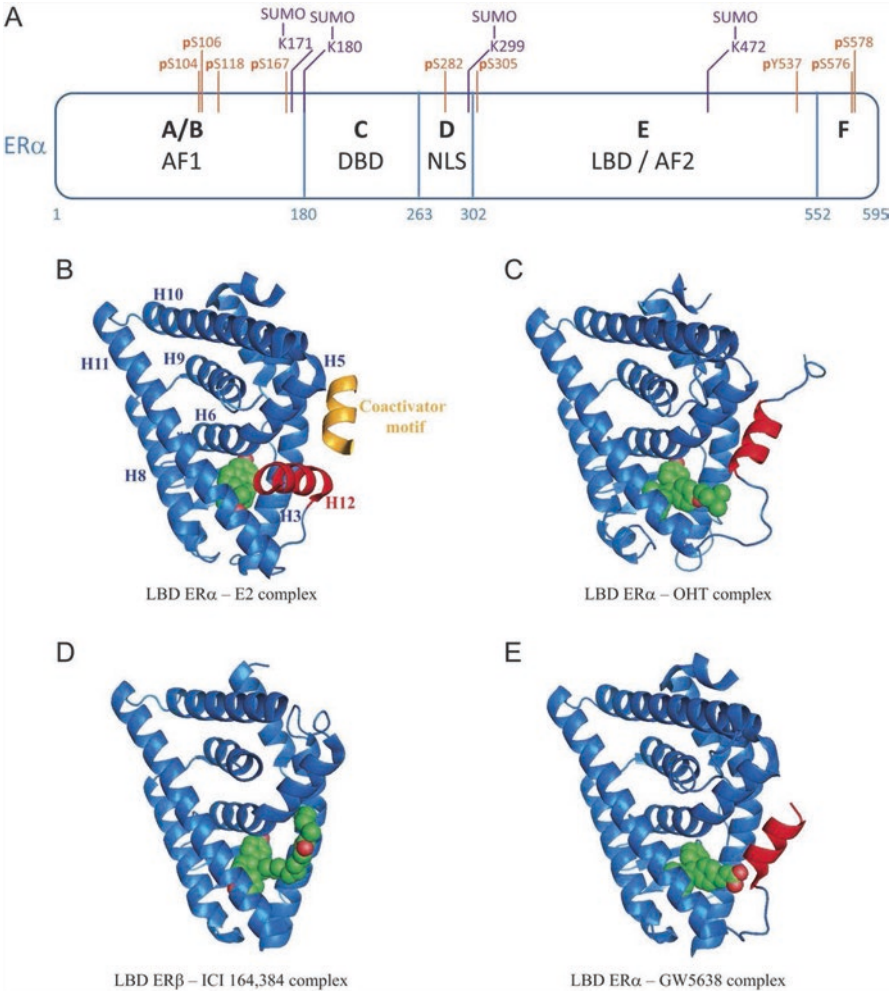


Fig. 9.2 ERα structure, post-translational modifications, and conformational changes induced by different ligands. (**a**) Schematic representation of ERα structure. AF1/AF2, activation function 1/2; DBD, DNA-binding domain; NLS, nuclear localization signal; LBD, ligand-binding domain. SUMOylation sites identified by mass spectrometry in the presence of ICI 182,780 are indicated in purple. Residues phosphorylated in the presence of antiestrogens or implicated in the modulation of sensitivity to antiestrogen treatment are indicated in orange. (**b**) LBD ERα–estradiol (E2)–TIF2 NR box 3 complex [82]. (**c**) LBD ERα–4-hydroxytamoxifen complex (OHT) [70]. (**d**) LBD ERβ–ICI 164,384 complex [58]. (**e**) LBD ERα–GW5638 complex [86]. Representations were generated using PyMOL. Helix 12 is highlighted in red and each ligand is shown in green. The α-helical TIF2 coactivator motif is shown in gold. (Reproduced with permission from Ref. [80])

domain. The hydrophobic nature of the ligand-binding site is determined by the hydrophobic residues that reside within five helices (H3, H6, H8, H11, and H12) as well as the S1/S2 hairpin that lines its cavity [7, 38]. Full transcriptional activation of the ERs requires both AF1 and AF2 domains which function synergistically to recruit coactivators [34]. While both ERα and ERβ harbor fully functional AF2 domains, the AF1 domain of ERβ functions to a lesser extent when compared to that of ERα. Binding of coactivators to only the AF1 domain results in either no or partial transcriptional activation, but this is thought to be promoter and cell type dependent. Studies performed examining the function of the F domain of ERα indicate that at least with respect to activation via 4-hydroxytamoxifen, the F domain governs its species-specific (i.e., human versus murine) transcriptional activation [1].

ERs characteristically bind ligands in a promiscuous manner which is attributed to their large binding cavities and combination of specific polar and nonpolar interactions [53]. Ligand-binding preferences between ERα and ERβ are distinct and thought to be dictated by structural differences within their LBDs. These respective domains are significantly different, sharing only a 59% identity. Interestingly, the amino acids that line their binding cavity are highly conserved differing by only two amino acids with Met-421 of ERα corresponding to Ile-373 of ERβ and Leu-384 of ERα corresponding to Met-336 of ERβ. Given that the subtlety of these amino acid differences contrasts with the wide variety in ligand-binding preferences of ERα versus ERβ, it is highly likely that amino acids positioned beyond the ligand-binding cavity play an important role in determining their ligand specificity.

9.2.3 The Antagonistic Activity of SERMs Involves Repositioning of Helix 12

A consistent theme that has emerged from structural models derived from the analyses of an array of nuclear receptors is the key role enacted by helix 12 within their LBDs that facilitate their ligand-induced conformational changes [80]. Here, agonist (i.e., E2) activation is thought to increase helical integrity, thereby decreasing the mobility of helix 12. Agonist-induced stabilization of helix 12 and its subsequent docking between helix 3 and helix 12 exposes a cleft within the AF2 domain and a site of interaction with the LXXLL motif found within all coactivators (Fig. 9.2b). In the absence of ligand, however, the apo ligand-binding state of the ERα, helix 12 is highly mobile. In this repressed state, ERα interactions with corepressors are favored, while interactions with coactivators are discouraged. Binding of antagonist is thought to incur similar events by preventing helix 12 from assuming its agonist-induced conformation, thereby displacing coactivator binding while providing a surface for interactions with corepressors. The distinct actions of the SERMs described in the previous section are thought to arise from the fact that these ERα ligands have distinct sizes which alter their ability to “fill the space” of the binding cavity. Side chains of SERMs like tamoxifen and raloxifene contain tertiary

amines that engage in steric clashes capable of repositioning helix 12 to the coactivator-binding groove (Fig. 9.2c). It is their "tails" that interact differently with the receptor, thereby differentially altering the ability of helix 12 to establish contact with helix 5. A key event involves Asp351 which resides within helix 3. Here, the tertiary side chain of antagonists like raloxifene forms a salt bridge with Asp351. As a result, helix 12 is forced to reposition over the coactivator-binding groove. SERMs which fail to engage in an interaction with Asp351 fail to achieve "pure" antagonistic activity due to their partial agonist actions.

9.2.4 Additional Classes of ERα Antagonists

SERDs As efforts to develop a "pure" ER antagonist continued, a new class of antagonists arose termed SERDs, selective estrogen receptor downregulators (Fig. 9.1) [56]. These drugs bind ER, induce rapid ER downregulation, and exert no observable ER agonist activity in any tissue. Based on their chemical structures, two groups of SERDs exist, (1) steroidal (e.g., fulvestrant) and (2) nonsteroidal (e.g., GW 5638), which bear structural similarity to tamoxifen. At this time, the only FDA-approved SERD is fulvestrant (also referred to as ICI 182,780). Because of its poor solubility, it is typically administered intramuscularly, and thus its use is limited. The key moieties underlying the pure antagonistic activity of SERDs are their bulky and/or extended side chains which are thought to exert enhanced helix 12 disruption and increase exposure of ERα's hydrophobic surface, thereby facilitating its proteosomal degradation (Fig. 9.2d) [84]. In addition, SERDs such as fulvestrant are efficient at enhancing the ability of ERα to recruit corepressors [83]. Subtle change in the composition of the side chain of SERDs is sufficient for enabling "pure" antagonistic behavior involving disruption of helix 12 and increased conformational helix mobility [18]. For example, side chains of GW 5638, a tamoxifen analog, is capable of forming hydrogen bonds with both Asp351 and the backbone of helix 12 (Fig. 9.2e) [80]. The ultimate consequence is that helix 12 is able to maintain its interaction with the coactivator-binding groove despite the increased exposure of its hydrophobic surface. The role of these structural changes in dictating both the anti-estrogenic action of the antagonist and degradation of the ERα protein is yet to be completely understood. In fact, a recent report has questioned whether the actions of fulvestrant require its ability to degrade the ERα. Instead, it is proposed that the extent to which fulvestrant acts as an anti-estrogen more likely involves its ability to immobilize ERα within the nuclear matrix which subsequently and completely inhibits its ability to transactivate genes [26].

A distinct and emerging class of SERDs are PROTACs (*Pro*teolysis-*ta*rgeting *c*himeras which represent a targeted approach to direct the cell's protein degradation toward a specific protein of interest [67, 74]. Here, bi-functional molecules are used wherein one end is tasked with binding the protein of interest and the other with recruiting proteolytic enzymes. The first of this class linked a peptide derived from

IκBα to the E3 ligase recognition site. Subsequent studies have reported on the use of the PROTAC approach to successfully target dozens of proteins including nuclear steroid receptors, such as the androgen and estrogen receptors as well as the aryl hydrocarbon receptor (AHR). Our efforts at developing PROTACs that targeted ERα included strategies that incorporated a second ligand (E2) resulting in a "two headed" PROTAC which enhanced binding affinity and efficacy as determined by degradation of the ERα protein [15]. An additional improvement was the determination of the optimal distance, a chain length of 16 atoms, between the E3 ligase recognition site and the ligand [14]. The most recently developed ERα-targeting PROTAC, ARV-471, has been developed by Arvmas and is currently being tested in Phase I clinical trials to treat women with locally advanced or metastatic ER+ breast cancer [45]. ARV-471 is orally bioavailable and has been shown in preclinical studies to be more effective than fulvestrant [74]. The efficacy of PROTACs is assessed using DC_{50} values which reflect half-maximal degradation concentrations. While the DC_{50} value of ARV-471 has not been disclosed, that of the androgen-targeting PROTAC, ARV-110 which is also in Phase I clinical trials, has a reported DC_{50} value of 5 nm. Recently, a highly potent ER PROTAC (ERD-308, DC_{50} = 0.17 nM) has been developed (Fig. 9.1). In cultured breast cancer cells, ERD-308 exerts a more complete (i.e., greater than 95%) degradation than that of fulvestrant [29].

STEARs A third class of ER antagonists are termed STEARs, selective tissue estrogenic activity regulators [22]. STEARs are structurally distinct from SERMs and are also capable of impacting the activity of progesterone and androgen receptors as well as altering the metabolism of estrogen. The most commonly used STEAR is tibolone (Fig. 9.1). It is proposed for use as hormone replacement therapy to treat symptoms associated with menopause (vaginal atrophy, vasomotor symptoms, and poor bone density).

SERCAs A major problem that arises in breast cancer patients following their long-term exposure to anti-estrogens is acquired resistance. To circumvent resistance, a new class of ER antagonists, termed SERCAs (selective estrogen receptor covalent antagonists), has been developed (Fig. 9.1) [21, 61]. In a substantial portion of patients who are resistant to anti-estrogens, mutated forms of the ER are enriched within the surviving tumor cells which engage in ligand-independent, ERα-dependent proliferation. Among the mutations involved in conferring constitutive activity are those found within the AF2 helix of ERα. Here, in the absence of ligand, amino acid substitutions (Y537S and D5386) shift the receptor toward its agonist-induced conformation. Targeting a nonconserved cysteine (C530) with a covalently bound pharmacophore (H3B-5942) has been found to be sufficient for shifting the mutated ERα into an antagonist-induced conformation. Further, binding of both wild-type and mutated ERα to H3B-5942 could stimulate formation of a receptor complex that binds DNA but lacks coactivators. Finally, in cultured endometrial cells, H2B-5942 did not impact transcription of the canonical ERα target gene, *PGR*, or impact cell proliferation indicating that its actions may spare ERα-mediated events within the endometrium.

9.3 Development of AHR Antagonists

9.3.1 Early Days of AHR Discovery

The road leading to the discovery of the AHR began with observations that exposures to polyaromatic hydrocarbons increased the protein levels and activity of an enzyme termed "benzopyrene hydroxylase" (subsequently termed "aryl hydrocarbon hydroxylase" and now referred to as CYP1A1 and CYP1A2) in rat liver [2, 52]. The use of inbred mouse strains, C57B6 and C57D2, led to the realization that this response of "polyaromatic hydrocarbon inducibility" localized to a single gene, the Ah locus. Subsequent genetic analyses performed using cultured mouse hepatoma cells ultimately identified three key genes, *Ahr*, *Arnt*, and *Cyp1a1*, that were required for mediating this response. A second line of research utilized a pharmacological approach, i.e., use of radiolabeled ligand-binding assays, to demonstrate that polyaromatic hydrocarbons like 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) bound a cytosolic protein with high specificity and affinity. In addition, it was found that a structure-affinity/structure-activity relationship with respect to ligand-binding affinity and biological response (i.e., induction of enzyme activity) existed. With the advent of molecular biology came the discovery that specific DNA sequences located upstream of the CYP1A1 transcription start site (termed AHREI, aryl hydrocarbon receptor response elements; XREs, xenobiotic response elements, but also referred to as DREs, dioxin response elements) were responsible for the observed increase in CYP1A1 mRNA levels. This observation ultimately led to the definition of a core consensus DNA-binding site that was identified as GCGTG (AHREI), with nucleotides flanking this site playing nonessential but supportive roles [77].

Initial biochemical analyses reported many similarities between the cellular activities of the protein identified as the AHR and that of the steroid receptors, in particular, the glucocorticoid receptor [13]. For example, cellular exposure to agonists of either an AHR or glucocorticoid receptor resulted in relocation of the respective receptor from the cytosol to the nucleus. This event was accompanied by a poorly understood biochemical process wherein the receptors were "transformed" to a form that was capable of binding DNA. While the AHR and steroid receptors share many biochemical features, cloning of the AHR refuted the idea that they were members of a common protein family. The AHR was found to belong to a distinct class of proteins, the basic helix-loop-helix PAS (bHLH/PAS) proteins, that at the time was composed of *P*ER, *A*RNT, and *S*IM [8]. A key, differentiating attribute that distinguishes the AHR from that of the steroid receptors pertains to the manner with which they interact with DNA. While the DNA-binding forms of steroid receptors exist as either homodimers or heterodimers with RXR, that of the AHR exists as a heterodimer with ARNT. Further, the structural motifs of their DNA-binding domains, leucine zippers versus basic regions, are distinct. Nonetheless, as ligand-activated transcription factors, they share key aspects pertaining to their activation and protein-protein interactions that lend insight into how their unique ligands alter physiological homeostasis. These common attributes

provide clues for developing tools to be used for effectively blocking their deleterious or inappropriate actions.

During the past two decades, significant advances have contributed to our understanding of the endogenous function of the AHR and how its activation by a variety of ligands can impact cellular and physiological processes. A model of the events initiated following agonist activation is depicted in Fig. 9.3. In the absence of ligand, the AHR exists as a complex composed of an HSP90 dimer, XAP2, and p23 [50]. In this unliganded form, the AHR engages in dynamic nucleocytoplasmic shuttling but is found primarily within the cytosol. The chaperone proteins, HSP90, XAP2, and p23, play important roles in the ability of the AHR to respond to ligand, its cellular localization, and the extent to which it is subject to degradation. Both HSP90 and XAP2 govern localization of the AHR within the cell. The interaction between the AHR and HSP90 is thought to shield the nuclear localization signal within the AHR from exposure. Upon ligand binding, a conformational change in the AHR ensues that shifts its interaction with HSP90 such that the nuclear localization signal residing within the bHLH domain of the AHR is revealed, thereby allowing the HSP90-bound AHR to enter the nucleus. Within the nucleus, the AHR's dimerization partner, ARNT, displaces HSP90 from the AHR. XAP2 also appears to play a role

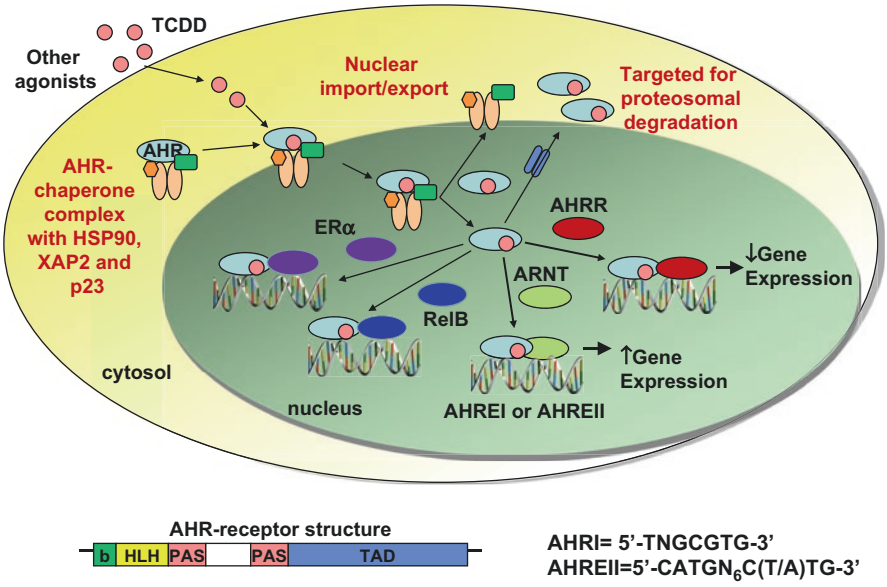


Fig. 9.3 Events initiated upon agonist activation of the AHR. Binding of agonist (e.g., TCDD) induces nuclear translocation of the AHR complex. Within the nucleus, the AHR dimerizes with ARNT or other transcription factors such as ERα or RelB, thereby altering gene transcription. The AHR/ARNT heterodimer complex upregulates genes containing AHREI and AHREII sites. Interaction of the AHR with AHRR results in gene repression and may involve either tethering of the AHR or direct binding of the AHR/AHRR complex. The agonist-bound AHR is also thought to engage in cytoplasmic-nuclear shuttling and serve as a target for proteolytic degradation

in the nuclear translocation of the AHR by inhibiting its ability to interact with the nuclear transport protein importin β. In addition, p23 and XAP2 are involved in maintaining proper protein levels of AHR by modulating its degradation. While AHR degradation occurs following its binding to ligand and subsequent ubiquitination, the degradation pathways incurred by the chaperone proteins (i.e., p23 and XAP2) are thought to be distinct and involve the latent, unliganded receptor [54].

Activation of the AHR pathway via ligand-independent mechanisms has also been reported and is thought to involve tyrosine kinases and cAMP [40]. Evidence of tyrosine kinase-mediated events include the observation that flavonoids, like genistein and daidzein, which harbor tyrosine kinase activity, are able to block omeprazole-induced activation of the AHR. It is proposed that the underlying mechanisms include increased activity of tyrosine kinases as well as enhanced levels of cAMP that trigger protein-protein interactions that promote AHR nuclear translocation.

The ability of the AHR/ARNT dimer to activate gene transcription involves its binding to both the consensus (canonical) AHREI and non-canonical AHREII [75]. Similar to other transcription factors, AHR-/ARNT-induced transcriptional activation requires its recruitment of coactivators, chromatin rearrangement, and enhanced accessibility of the gene promoter. Genes regulated via the consensus AHREI include the prototypical CYP1A1, whereas those regulated by AHREII involve genes encoding transporters and ion channels. The agonist-activated AHR also regulates gene transcription via its interaction with other transcription factors such as ERα, NF-κB (RelA and RelB), and Sp1. The agonist-bound AHR also forms a heterodimer with the AHRR which is capable of repressing gene transcription either via a tethering mechanism or direct interaction with AHREI [87]. Agonist activation of the AHR modulates the expression of genes involved in a myriad of cellular processes including metabolism, proliferation, and the regulation of cell fate decisions such as apoptosis and differentiation. As a consequence key, disease processes, such as inflammatory and immune responses and cancer progression, are impacted. Thus, AHR antagonists, with their propensity for modulating these processes, are attractive, potential anti-inflammatory, and anti-cancer agents and thus are of considerable interest to the scientific and clinical communities.

As we consider the consequences of agonist activation of the AHR, it is important to note that the transcriptional activation of the AHR initiated by its agonists is tightly regulated by a number of negative feedback loops as follows [2]:

1. The prototypical AHR target gene, CYP1A1, often catalyzes the degradation of many AHR agonists, thereby limiting their activities. In the presence of an AHR agonist, CYP1A1 is commonly the most extensively upregulated gene product.
2. The agonist-activated AHR increases the expression levels of a repressor protein, AHRR (aryl hydrocarbon receptor repressor). This bHLH/PAS protein harbors a transcriptional repression domain at its C-termini and competitively displaces ARNT from interacting with the AHR.
3. The cellular levels of the AHR are subject to the above mentioned ligand-dependent and ligand-independent proteolysis of the AHR protein. Thus, the

ultimate effect of an AHR antagonist on this battery of events as well as its ultimate effect is often difficult to predict.

9.3.2 Molecular Structure of the AHR

The 16-member family of bHLH/PAS proteins is widely thought of as "sensor" proteins that allow a host organism to adapt to changes in its environment by transmitting a variety of responses [85]. The bHLH/PAS proteins are classified as either Class I, proteins directly involved in the "sensing" activity, or Class II, which act as common dimerization partners that interact with Class I proteins. Typical of many Class I proteins, the expression of AHR varies in a cell- and tissue-type-dependent manner. In contrast, the protein expression of that of its DNA-binding partner ARNT, a Class II protein, is thought to be constitutively and ubiquitously expressed. As previously mentioned, a third bHLH/PAS protein involved in AHR signaling is the AHRR, AHR repressor protein, which is also classified as a Class I protein.

Like many bHLH/PAS proteins, the basic domain of the AHR lies at its N-termini and is followed by a helix-loop-helix motif and two highly conserved PAS regions (PAS-A and PAS-B) (Fig. 9.3) [2]. The bHLH domain is composed of two α helices that are connected by a short loop. The key DNA-binding interface of the AHR is localized within the first loop of the basic region, wherein 4–6 amino acids, in particular R39, interact with the major groove of DNA [68, 69]. Sites that govern nuclear localization and nuclear export of the AHR have been identified within the HLH domain [25]. However, additional sites identified within the C-terminus using bioinformatics may also be involved. The bHLH and PAS-A domains are essential for mediating dimerization between the AHR and ARNT [2, 52]. Also within the N-terminus (i.e., bHLH/PAS domains) of the AHR lie surfaces that facilitate interactions between the AHR and coactivators/corepressors. The "sensing" activity of the AHR mediated by its ligand-binding cavity, lies within its PAS-B region. While the AHR is as of yet the only identified member of the bHLH/PAS family to bind ligand, based on structural characterizations, it has been hypothesized that all bHLH/PAS may be transcriptionally activated by endogenous ligands.

At the C-terminus lies the TAD, the transcriptional activation domain required for facilitating its interactions with a variety of coactivators involved in transcriptional activation. A common feature of the C-termini of bHLH/PAS proteins is the significant variability in their primary protein structures which is characterized as an "intrinsically disordered region" [35]. It is proposed that the presence of sites for post-translational modification indicates that the C-termini, in addition to contributing to the activity of transcriptional complexes, is involved in regulating the stability/activity of the protein. Further, it is thought that the flexibility and disorder found within this region are relevant to the diverse functions of this protein class. Here, the ultimate structure of AHR's transcriptional activation domain as a component of a given transcriptional complex is likely to be dependent on how it interacts with ARNT as well as coactivator proteins. These protein-protein interactions, in turn,

would be differentially influenced by the bound agonist, thereby eliciting ligand-dependent transcriptional activation.

9.3.3 Agonist-Induced Activation of the AHR

Our understanding of how AHR binding to its agonists promotes changes in its conformation to render it capable of activating gene transcription has been hampered of the lack of crystal structures of the agonist-bound form of the AHR. However, some insights are offered by recent crystal structure analyses of a complex formed by a heterodimer consisting of the bHLH/PAS A regions of both the AHR and ARNT bound to the consensus TTGCGTG sequence [69]. As previously predicted, ARNT was found to interact with the GTG half-site; while the AHR interacted with the 5′ end of the recognition site, GC/CG via H-bonds formed with R39 that resides within its basic region. One of the most striking observations was that the AHR has extensive interdomain interactions (i.e., within its bHLH and PAS-A domains), while within ARNT, these two domains are involved in minimal contact. A second important observation was that the interactions between the AHR and ARNT were found to be highly intertwined consisting of a number of domain-to-domain and cross-domain interactions. These involved interdomain interactions are consistent with allosteric mechanisms that facilitate agonist-induced activation of nuclear steroid receptors [19, 42]. Here, agonist binding that occurs at a distal region of the receptor is capable of "transmitting" this event to promote significant structural alterations in domains engaged in other activities such as interacting with DNA or other proteins. Allosteric interactions occur between the ligand-binding and DNA-binding domains which can reciprocally alter the specificity of interactions occurring at either the ligand- or the DNA-binding site. Further, the identity of the ligand (i.e., agonist, inverse agonist, or antagonist) is thought to induce distinct conformations of the ligand-binding site that are sensed by other regions of the protein, differentially expose the nuclear localization sequences, and differentially determine binding preferences for the LXXLL coregulators, thereby either inducing or repressing distinct gene expression patterns. Thus, the AHR, like many nuclear hormone receptors, is highly attuned to sensing unique ligands and transmitting their distinct signals.

9.3.4 The AHR Is Activated by a Diverse Cadre of Ligands

Study of the interactions between the AHR and a diverse group of ligands over the past four decades has yielded some insights into the rules that govern its agonist activation. The classically defined AHR agonists encompass the high-affinity halogenated aromatic hydrocarbons (i.e., polychlorinated dibenzo-p-dioxins, dibenzofurans, biphenyls, and poly aromatic hydrocarbons) [16]. Many of these interact

with the AHR with high affinity and are poorly metabolized and widely studied because of their toxic, adverse effects. The toxicity of these AHR agonists is thought to arise from their ability to inappropriately and persistently activate the AHR pathway. However, as the number of AHR ligands found in the diet or formed endogenously has expanded, a corresponding transition in our understanding of the AHR-mediated response as a "toxic" to a potentially beneficial response has occurred. Dietary and endogenous AHR ligands, which include indoles, flavones, imidazoles, lipids, and lipid metabolites, are typically less potent than their halogenated aromatic hydrocarbon counterparts and bind the AHR with lower affinity. The variety of structures exemplified by these ligands indicates that the AHR harbors a promiscuous ligand-binding pocket. More importantly, these observations imply that understanding how the AHR ligand-binding domain accommodates this variety in ligand structures is a key step required for improving AHR-based therapeutics.

To better understand the events involved in ligand binding of the AHR, computational molecular docking approaches using homology modeling have been used [23]. Here, a model of the AHR ligand-binding site was created using elements predicted by a closely related protein family member, hypoxia-inducible factor 2, HIF 2α. The model was then tested using site-directed mutagenesis of the AHR LBD followed by ligand-binding analyses. The results from these studies have allowed for an initial grouping of well-characterized AHR agonists based on how they interact with the AHR ligand-binding cavity. Group 1 consisted of prototypical, high-affinity agonists (TCDD, 2,3,7,8 dibenzo-p-furan and benzo[a]pyrene) (Fig. 9.4). Group 2 contained more bulky, polyaromatic hydrocarbons: 3-methylcholanthrene, dibenzo[a,h]anthracene, and 3,3′4,4′5-pentachlorobiphenyl. Group 3 contained flavones and indoles which may be more representative of endogenous ligands: β-naphthoflavone, 6-formylindolo[3,2-*b*] carbazole (FICZ), indirubin and leflunomide. The basis of these three groupings was in large part due to their predicted positions within the binding cavity. Group 1 ligands (containing the high affinity, TCDD) were found to bind deep within the hydrophobic region of the inner cavity (Fig. 9.5). Groups 2 and 3 bind nearer the cavity entrance with Group 3 appearing to be limited in its ability to interact with amino acid resides because of its poorer mobility.

9.3.5 Development of Selective AHR Modulators (SAHRMs)

In addition to observations made by the study of AHR agonists, important advances were also gained while developing AHR antagonists. Early work in this regard was focused on chemically modifying the structures of high-affinity dioxins and furans. For example, the first reported AHR antagonist, 1-amino-3,7,8-trichlorodibenzo-p-dioxin, initially synthesized to aid in detecting TCDD in biological samples, was found to be effective in competitively inhibiting TCDD/AHR binding as well as blocking the ability of TCDD to induce both CYP1A1 enzyme activity and myelotoxicity [39]. The observation that α-naphthoflavone also harbored AHR antagonist

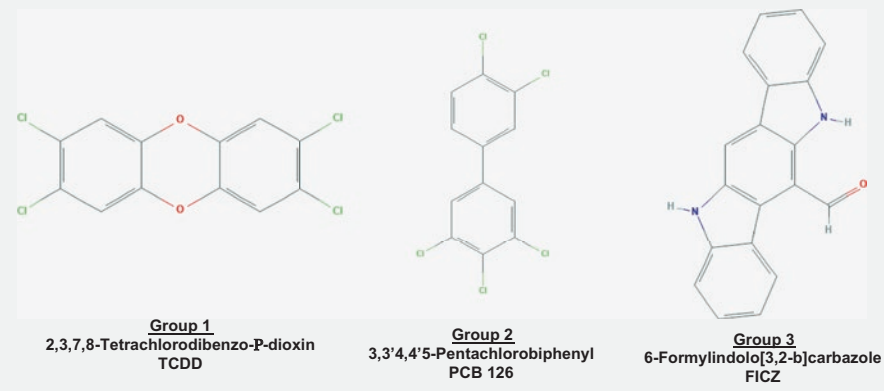


Fig. 9.4 Chemical structures of AHR agonists representing of three different groups

activities led to a second line of investigations that were focused on utilizing flavones as a structural backbone (Fig. 9.6) [5]. This resulted in the development of 3′4′dimethoxyflavone and 3′-methoxy-4′aminoflavone both of which proved to be relatively potent AHR antagonists and, given that they represented a class of compounds that were distinct from the HAH, lessened concerns regarding their potential toxicity [27, 37].

The realization that despite the classification of high-affinity AHR agonists, like TCDD, as "highly toxic," some biological responses could in fact be beneficial initiated efforts to develop selective AHR modulators (SAHRMs). Specifically, could the ability of TCDD to inhibit the estrogen receptor be exploited to develop novel, AHR-based breast cancer therapies? With this in mind, derivatives of 6-MCDF that retained their ability to bind the AHR and exert anti-estrogenic activities but lacked the toxicity typically associated with TCDD were developed [65]. The identification of additional classes of AHR agonists and antagonists was also aided by efforts focused on elucidating the mechanisms by which phytochemicals exerted their chemopreventive actions. This led to the identification of indolo[3,2-b]carbazole that was found to bind the AHR with relatively high affinity but lacked the toxicity associated with prototypical AHR agonists, like TCDD [4]. An additional line of work focused on the study of TCDD-induced immune suppression, a "toxic" effect. Here, it was found that agonist activation of the AHR suppressed the potent pro-inflammatory NF-κB pathway [79] which ultimately led to our current efforts to develop AHR-based therapies for treating immune and inflammatory diseases [51]. The most recent advances in this regard again used α-naphthoflavone as a starting point [46]. The resultant SAHRM was 3,4-dimethoxy-α-naphthoflavone which was capable of suppressing cytokine-mediated gene expression but failed to impact AHR/AHREI-driven events. It is proposed that 3,4-dimethoxy-α-naphthoflavone exerts its anti-inflammatory effects via mechanisms that are ARNT-independent and involve interactions of the AHR with other transcription factors (e.g., Rel B). An agent with similar properties, SGA 360, was also developed via synthesis of

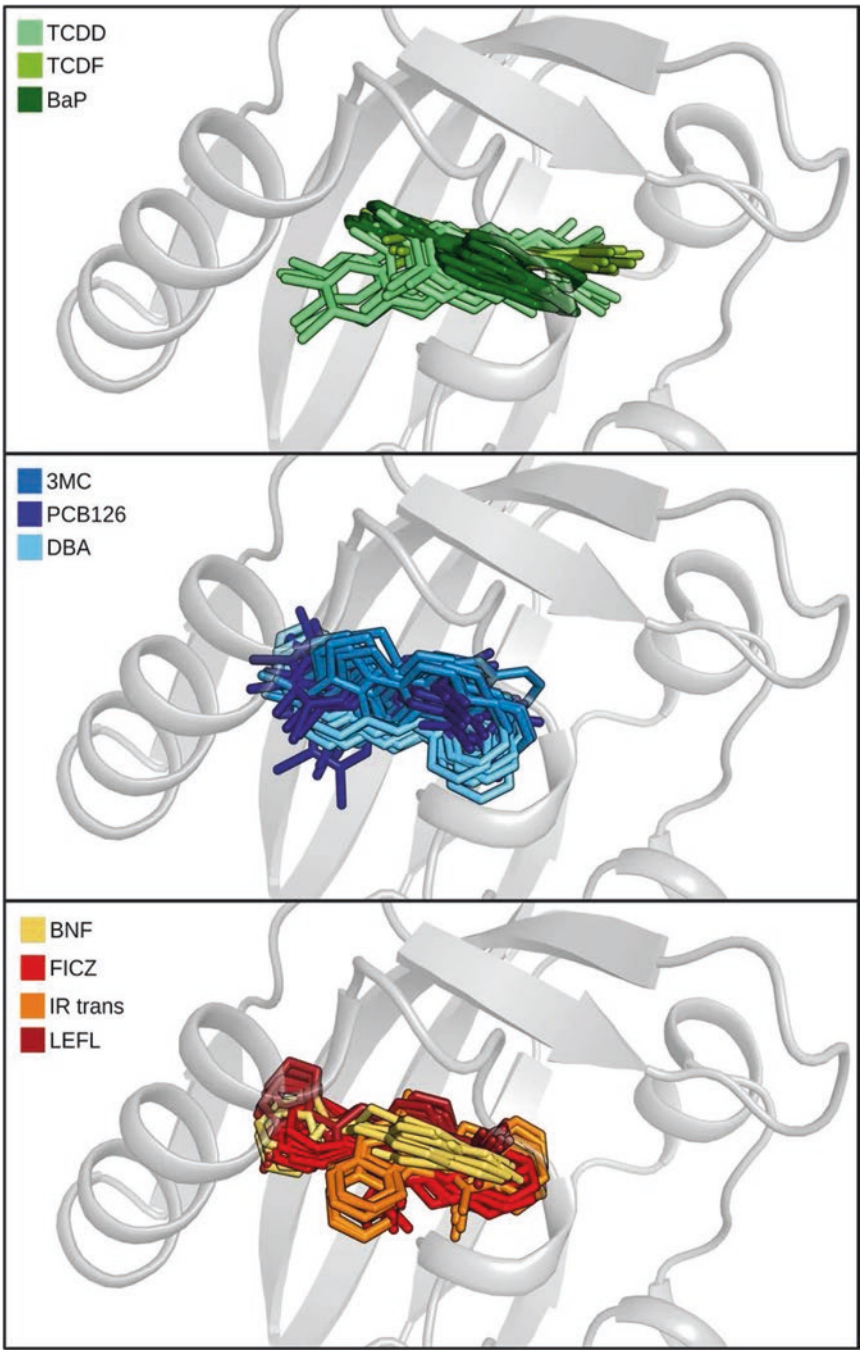


Fig. 9.5 Occupancy of different sites within the AHR cavity by the three groups of AHR ligands as determined by computational molecular docking analyses. Group 1 is depicted in the upper panel, Group 2 in the middle panel, and Group 3 in the lower panel. The ligands are depicted as sticks. (Reproduced with permission from Ref. [23])

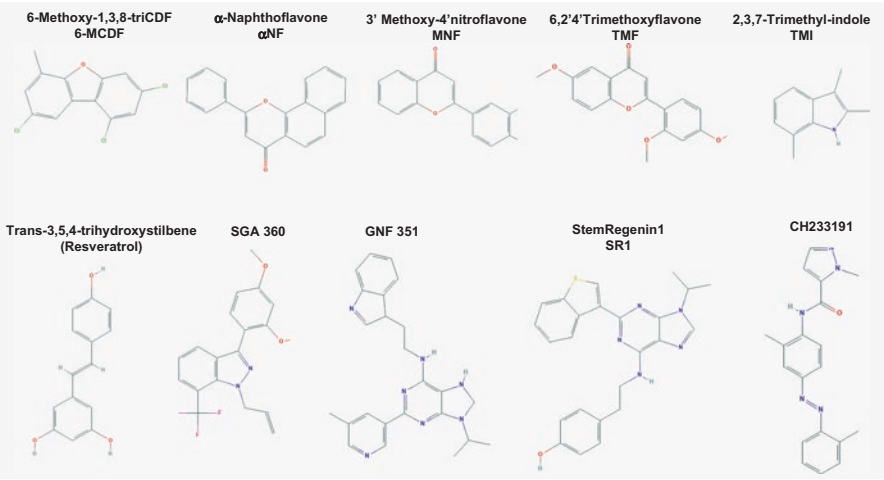


Fig. 9.6 Chemical structures of a variety of SAHRMs

derivatives of WAY-169916, an imidazole with SERM activities (Fig. 9.6) [48]. SGA 360 fails to bind the ER yet exerts anti-inflammatory activities via mechanisms that involve AHR binding, cytosolic retention of the AHR, and inhibition of AHR/NF-κB crosstalk [44]. While the clinical efficacy of these AHR-based anti-inflammatory agents is yet to be determined, they have paved the way for further development of SAHRMs and AHR antagonists.

9.3.6 Toward the Development of "Pure"[1] AHR Antagonists

Problems frequently associated with the use of AHR antagonists that were initially developed included their off-target effects (in particular, inhibition of the catalytic activity of CYP1A1). In addition, they often proved to act as partial agonists exhibiting agonist properties when used in high concentrations. It is important to note that a "pure" AHR antagonist should be able to block all activities of the AHR. These would include genomic events mediated by AHREI and AHREII as well as non-genomic events, such as those involving protein-protein interactions of the AHR with other transcription factors. Efforts to identify "pure" AHR antagonists utilized random screening of a synthetic chemical library and resulted in the discovery of CH223191, containing three connected aromatic rings, which could block the actions of TCDD both in vitro and in vivo (Fig. 9.6) [33]. While CH223191 is capable of blocking the actions of multiple AHR agonists (i.e., TCDD, endogenous

[1] In this context, a "pure" AHR antagonist is capable of blocking all actions of the AHR with high efficacy, exhibits high AHR-binding affinity, and lacks measureable agonist activity.

FICZ, and ITE (1′H-indolo-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester) [11], agonist- and off-target effects have recently been reported [43]. Here, CH223191 was found to exert modest yet significant agonist activities when cells were subjected to highly reduced conditions. Further, CH223191 was shown to inhibit CYP1A1 activity and reduce metabolic clearance of FICZ while also increasing formation of reactive oxygen species in an AHR-independent manner. Hence at this time, it is unclear as to whether the ability of CH223191 to act as an AHR antagonistic lies solely within its occupation of the AHR ligand-binding site or also include its ability to upregulate CYP1A1 and thereby reduce the cellular levels of endogenous AHR agonists.

Structure-activity relationship analyses confirmed that a key aspect pertaining to the potency of CH223191 as an AHR antagonist was the presence of moieties with strong electronegative properties [10]. Interestingly modifications designed to create a form of CH223191 that closely resemble resveratrol (trans-3,5,4-trihydroxystilbene), termed "AL-3," resulted in a compound that was capable of binding the AHR (IC_{50} = 0.76μM) but exerted modest AHR *agonist* activity [11]. However, rather than blocking the actions of AHR agonists (i.e., TCDD, FICZ, and ITE), co-treatment of AL-3 and either of these agonists will result in a substantial and synergistic enhancement of their ability to induce gene transcription. At this time, the mechanisms underlying this type of synergism with respect to AHR agonist activity is undefined but may prove to be invaluable for outlining the rules that govern a ligand's AHR antagonistic activities.

A second "pure" AHR antagonist, StemRegenin-1 (Fig. 9.6), was identified in an unbiased screen of compounds to test their ability to promote expansion of CD34+ hematopoietic stem cells [6]. StemRegenin-1 is a heterocyclic purine derivative that binds the AHR with high affinity (IC_{50} = 40 nM), competitively displaces TCDD, and blocks its ability to induce canonical AHR signaling (i.e., induction of AHRR and CYP1B1 mRNA). Interestingly, StemRegenin-1 displays species selectivity, preferentially inhibiting the actions of the human versus murine AHR. Reports from clinical trials indicate that StemRegenin-1 may be effective for preventing lymphopenia in patients who have undergone hematopoietic stem cell transplants [71].

A third "pure" AHR antagonist that has been described is GNF 351 which is closely related to the analog of StemRegenin-1 [72]. GNF 351 effectively blocks both AHREI-dependent and AHREI-independent activities of AHR agonists. While GNF 351 was shown to be highly potent in vitro, its in vivo properties have been found to be limited by its poor absorption and extensive metabolism [17].

9.3.7 Development of Flavone-Based AHR Antagonists

Flavonoids have intrigued pharmacologists for centuries due to their wide array of purported medicinal properties and extensive use in traditional medicines. With respect to AHR-relevant activities, they are capable of acting as either agonists,

partial agonists, or antagonists [49, 59, 88]. Building on work that had established 3′4′dimethoxyflavone and 3′-methoxy-4′aminoflavone as AHR antagonists [27, 37], a screen of flavonoids identified 6,2,4′trimethoxyflavone as a potent AHR antagonist that lacks partial agonist activity (Fig. 9.6) [47]. A similar luciferase reporter-based screen performed in human hepatoma cells revealed that flavonoids with the most potent, dose-responsive antagonist activities were apigenin, chrysin, and kaempferol [59]. Using competitive ligand-binding assays, kaempferol was shown to interact with the AHR with relatively high affinity (IC_{50}-39.8 nM), inhibited AHR nuclear translocation and DNA binding, and was able to inhibit the ability of cigarette smoke condensate to induce transformation of human lung cells. When examined in human head and neck squamous cell carcinomas from the pharynx (FaDu), oral cavity (PCI-13), and metastatic lymph nodes (PCI-15B), both apigenin and kaempferol reduced cell viability [78]. However, some differences in the in vitro actions of apigenin versus kaempferol were observed. For example, apigenin appeared to be more potent than kaempferol with respect to incurring loss of viability. More importantly, these in vitro results were not consistent with those obtained in vivo using tumor explants. Here, daily administration of apigenin significantly increased growth as indicated by an increase in tumor volume. Similar but less dramatic results were obtained upon administration of kaempferol. These studies illustrate a major problem associated with the use of flavonoids as AHR antagonists – the inability to predict their in vivo actions. A likely explanation is that flavonoids exhibit a plethora of activities which include their activation/inhibition of nuclear receptors, kinases, and transporters, as well as their ability to act as antioxidants [76]. The conditions of in vitro, cell culture models may not appropriately mirror the in vivo tumor environment and thus may not be conducive for measuring this wide range of activities. Whether or not a flavonoid is anti- or pro-tumorigenic may thus depend on the circuitry of these key signaling pathways within either a particular tumor cell or its tumor microenvironment.

A recent examination of the structure-activity relationship of flavones with respect to their AHR agonist versus antagonist activities specifies the importance of three main properties; (1) the number of hydroxyl groups, (2) their relative positions, and (3) the measured biological response [30]. For example, the hydroxyl and carboxyl oxygen residue of apigenin (an AHR antagonist) appears to engage in the formation of three hydrogen bonds as well as hydrophobic and π-π interactions. Quercetin (with AHR agonist properties), like TCDD, appears to interact with similar residues. However, it is proposed that it is the relative strength of these interactions that dictate agonist activity of quercetin versus antagonist activity of apigenin.

9.3.8 Development of Indole-Based AHR Antagonists

As mentioned previously, a number of ligands (i.e., indolo[3,2-b]carbazole, ICZ; 6-formylindolo[3,2-b]carbazole, FICZ; and 2-(1′H-indolo-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester, ITE) that bind the AHR with high affinity contain an

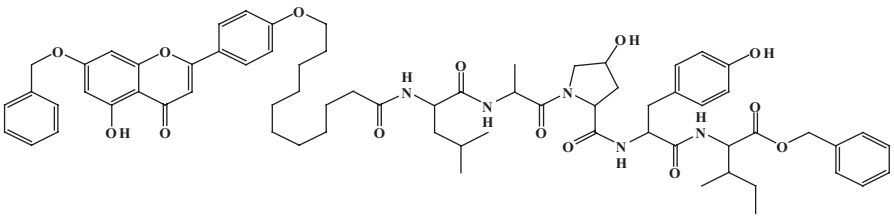
indole moiety. Recently, a recent screen of methylated and methoxylated indoles has offered insights into the rules that may determine how indoles may act as either AHR agonists or antagonists [73]. Here, indoles that exerted high agonist activity were 4-Me-indole and 7-Meo-indole, whereas those with the most potent antagonist activity were 2,3-diMe-indole (IC_{50} = 11μM) and 2,3,7-triMe-indole (IC_{50} = 12μM). Interestingly, 4-methylindole and 7-methoxyindole also exhibited synergistic agonist activity wherein their co-treatment with TCDD significantly enhanced the TCDD-induced response. Molecular docking analyses revealed that key interactions of the agonists involved (1) a hydrogen bond with Thr289, (2) aromatic interactions with Phe324 and His29, and (3) arene-H interactions with Gln383. In addition, a number of hydrophobic and hydrophilic interactions were identified. The synergistic effect of 4-methylindole and 7-methoxyindole was proposed to arise from their ability to simultaneously occupy the AHR ligand-binding pocket. The antagonists, however, lacked many of the conserved interactions favored by agonists and also participated in distinct interactions. For example, 2,3-diMe-indole and 2,3,7-triMe-indole both form an aromatic interaction with Phe 351 that was not observed with those harboring agonist activities.

9.3.9 Development of Stilbene-Based AHR Antagonists

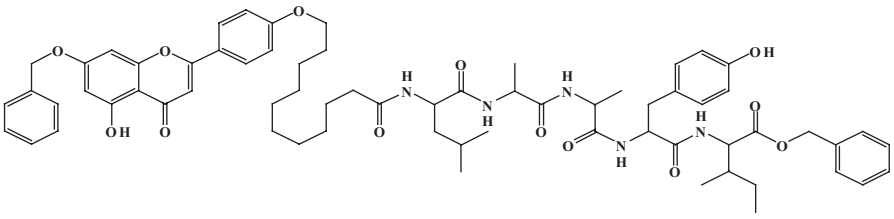
Interest in stilbenes as AHR antagonists was initiated by reports that resveratrol (trans-3,5,4-trihydroxystilbene) (Fig. 9.6) could inhibit the ability of TCDD to activate genes [12] and act as a competitive antagonist [9] that inhibited AHR recruitment at the CYP1A1 promoter [3]. Subsequent efforts that focused on further developing stilbenes as SAHRMS included the synthesis and analyses of derivatives with high hydrophobicity that enhanced their AHR-binding affinity [81]. As of yet, however, the currently reported stilbenes exert dual roles acting as both agonists and antagonists [55]. For example, an analysis of 13 hydroxystilbenes and methoxystilbenes revealed that all exhibited AHR antagonistic activity with IC_{50} values ranging from 1 to 25μM. However, the most potent antagonist (E)-3,4′5-trimethoxystilbene (IC_{50} 1.1μM) retained considerable potency as an agonist (EC_{50} 15.3μM). Thus, efforts to develop stilbene-based AHR antagonists have met with limited success.

9.3.10 Development of AHR-PROTACs (SAHRDs)

The observation that apigenin interacted with the AHR with relatively high affinity (IC_{50} = 0.29μM) and inhibited a number of agonist-induced events in a variety of cultured cells [59] provided support for the idea that it would be a good starting material for developing AHR-PROTAC molecules. We reasoned that the in vivo effects and safety properties of apigenin have been well studied and that the addition of the PROTAC moiety would enhance its ability to block the actions of the



Chemical structure of Apigenin-Protac



Chemical structure of Apigenin-Protac [Ala]

Fig. 9.7 Chemical structures of apigenin-PROTAC and apigenin-PROTAC [Ala]. (Reproduced from Ref. [60] with permission)

AHR. We first determined that modifications of the 4′hydroxyl group of the apigenin molecule did not significantly impact its ability to interact with the AHR [36]. To develop apigenin-PROTAC, we then attached a linker moiety as well as a peptide containing the recognition site of the specific E3-pVHL ubiquitin ligase (Fig. 9.7) [60]. As a negative control, we replaced a key amino acid within the recognition site with alanine (i.e., apigenin-PROTAC [Ala]). In vitro studies demonstrated that apigenin-PROTAC effectively decreased protein levels of the AHR and blocked the ability of TCDD to induce formation of the AHR/ARNT/DNA-binding complex and activate canonical AHR target genes (CYP1A1 and CYP1B1). Apigenin-PROTAC represents the first in class, SAHRD, selective AHR downregulator. The in vivo pharmacological function and efficacy of apigenin-PROTAC is yet to be demonstrated.

9.3.11 Elucidating the Rules That Govern Agonist Versus Antagonist-Induced AHR Activity

A recent structural analysis utilizing a molecular docking approach and "agonist-optimized" homology model has provided some insights into how AHR agonists and antagonists may differ with respect to their interactions with residues of the AHR ligand-binding pocket [57]. The basis of this model was formed from the analyses of 16 known AHR agonists and 26 "inactive" chemicals which assigned

TCDD the top score. This model suggests that hydrogen bonds with His291 and Ser365 are key for determining agonist affinity. Further, it is predicted that agonists must contain two hydrogen bond-accepting groups. Using GNF351 as the AHR antagonist, the model predicts that an antagonist conformation involves more extensive contacts with the amino acids that reside within the region bordered by amino acids 307 and 329 of the AHR. Further, the apo state of the receptor was found to be very dynamic but subsequently stabilized upon binding to either agonist or antagonist. The prevailing hypothesis is that in the apo state, the 307–329 region of the AHR is held in an "open" configuration that is accessible to ligands via its interaction with HSP90. Agonist binding alters the configuration to promote nuclear translocation of the AHR. Antagonist binding, however, favors a distorted configuration which shifts the AHR/HSP90 interaction to a state that prohibits AHR nuclear translocation.

A more recent approach used a combination of cell culture-based and in silico methods to probe a diverse set of AHR antagonists [24]. Here, the characteristics of an AHR antagonist was defined as having (1) a strong hydrophobic character; (2) a connected ring system, in particular aromatic rings with electron-rich and electron-deficient moieties; and (3) an electron acceptor group. These defined characteristics will be useful for future identification of additional AHR antagonists.

9.4 Conclusions and Future Directions

In addressing the question posed many decades ago – "what is an antagonist?", we have learned that holding a perspective of agonists versus antagonists as it pertains to nuclear receptors, like the ER and AHR, presents a false dichotomy. This limited view does not allow for our current understanding of how agonists bind and activate their respective receptors, the finely tuned progression of events that facilitate ligand-induced responses, and the ligand-, context-, and time-dependent nature of their elicited responses. Agonist activation requires pivotal interactions between key moieties of the ligand molecule and specific amino acid residues that are buried deep within the ligand-binding pocket of the receptor protein. These ligand-amino acid interactions initiate events that are propagated throughout multiple protein domains. Ligands vary subtly with respect to characteristic "agonist" interactions and exert activities that may be identified as either selective modulators or "pure" antagonists. The context-dependent responses to this myriad of ligands are multidimensional often involving unique cellular milieus, multiple protein/protein interactions, and a variety of signaling pathways. In addition, ligand-initiated events have proven to be time-dependent with latter events strongly influenced by multiple feedback mechanisms regulating receptor expression and function. Finally, we have learned that antagonists are multifarious in their actions. As described by our early pharmacologists, they may simply block the actions of a given agonist. However, they may also thwart agonist induction of a given receptor by initiating additional events, such as those involving proteolytic degradation of the targeted receptor. Our

challenge then, as modern pharmacologists, is not only to develop antagonists that act with high specificity and efficacy but also to develop innovative tools and approaches to be used for accurately predicting their ultimate pharmacological effects.

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Chapter 10
Design of Novel PPAR Agonist for Neurodegenerative Disease

Ian Steinke and Rajesh Amin

Abstract Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear hormone receptor superfamily comprising three subtypes: PPARα, PPARγ, and PPARβ/δ. The PPAR family of nuclear receptors is centrally involved in regulating whole-body energy homeostasis and metabolic function. Endogenous ligands include free fatty acids, eicosanoids, and leukotrienes. Synthetic ligands developed to serve as full agonists aim at treating diabetes type 2, hyperlipidemia, and other metabolic-related diseases. Further, there has been a developing interest in the role of PPAR agonist's role in neurodegenerative disease. However, many of these clinically practical therapeutics are associated with harmful effects on human health. Therefore, new approaches have led to a new class of selective PPAR modulators (SPARMs), or partial agonists meet this challenge. In addition, these partial agonists have been observed to show a favorable impact on insulin sensitivity, blood glucose levels, and dyslipidemia with significantly reduced side effects on human health. Partial agonists have been found to display differences in transcriptional and cellular outcomes by acting through distinct structural and dynamic mechanisms within the ligand-binding region when compared to full agonists. Recently, a new focus on PPAR agonists' class has intensified for neurodegenerative diseases, as new ligands and novel biological roles have emerged particularly for its therapeutic potential in Alzheimer's disease (AD). The present chapter critically analyzes current PPAR ligands using in silico modeling and the implication of promising new therapeutics in neurodegenerative disorders.

Keywords PPAR · Neurodegeneration · Alzheimer's disease

I. Steinke · R. Amin (✉)
Department of Drug Discovery and Development, Harrison School of Pharmacy, Auburn University, Auburn, AL, USA
e-mail: rha0003@auburn.edu

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10.1 Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily that are ligand-activated transcription factors [1]. These receptors have been linked to many systemic and cellular functions including insulin sensitivity and whole-body energy regulation. PPARs exhibit isotype-specific tissue expression patterns based upon energy demand in the regulation of cellular process. PPARα is abundantly expressed in tissues that utilize fatty acid catabolism such as the heart, liver, brown adipose tissue, and kidney [2, 3]. PPARγ exists in two isoforms, γ1 and γ2, and is principally expressed in white and brown adipose tissues, where they regulate adipocyte differentiation and lipid storage [4, 5]. Although expression of PPARγ2 is mostly observed in adipose tissue, PPARγ1 is ubiquitously expressed in tissues including the gut and immune cells, where they promote anti-inflammatory processes [6]. PPARδ/β has broad expression patterns and prominent roles in the skeletal muscle, adipose tissue, skin, gut, and brain [7, 8].

10.2 Overview of PPARs and Their Structures

PPARs act as transcription factors by binding and functionally responding to endogenous small molecule ligands [9]. These ligands, either endogenous or synthetic, bind to an orthosteric pocket existing in the core of the nuclear receptor ligand-binding domain. PPARs exist as a conserved domain association, including a central DNA-binding domain (DBD) flanked by two regulatory regions, a distinct amine (N)-terminal activation function-1 (AF-1) domain. In addition there is also a carboxyl (C)-terminal ligand-binding domain (LBD) containing the activation function-2 (AF-2) domain that has a coregulator interaction surface [10, 11] (Fig. 10.1). The N-terminal regulatory domain consists of A and B domains and the AF-1 domain, which is involved in ligand-independent coregulator binding [12, 13]. This region is not conserved because it varies greatly between members of this class of nuclear receptors. Two highly conserved zinc fingers are central in the recognition of specific DNA half-sites termed peroxisome proliferator response elements (PPREs) [14]. These sites are represented as either direct or indirect repeats and are separated by a spacer of base pairs. Each zinc finger contains several cysteine residues allowing for the interactions to a zinc ion. These specific zinc finger motifs permit for the discernment of nuclear receptors from other DBDs (Fig. 10.1).

Mechanistically, DNA binding allows for either the activation and recruitment of DNA transcription machinery or the repression of transcription. All members of the PPAR nuclear receptor superfamily bind to DNA as a heterodimer, where the DNA binding is in association with the retinoic acid receptor (RXR) [14]. Each DBD subunit binds to a separate DNA half-site. The most poorly conserved PPAR region is the flexible hinge domain, which allows for rotation between the DBD and the LBD, and contains a nuclear localization signal. The LBD is the largest domain in PPAR molecule and is highly conserved across all PPARs. Ligand binding stabilizes the AF-2 domain and facilitates the interaction with coregulator molecules to

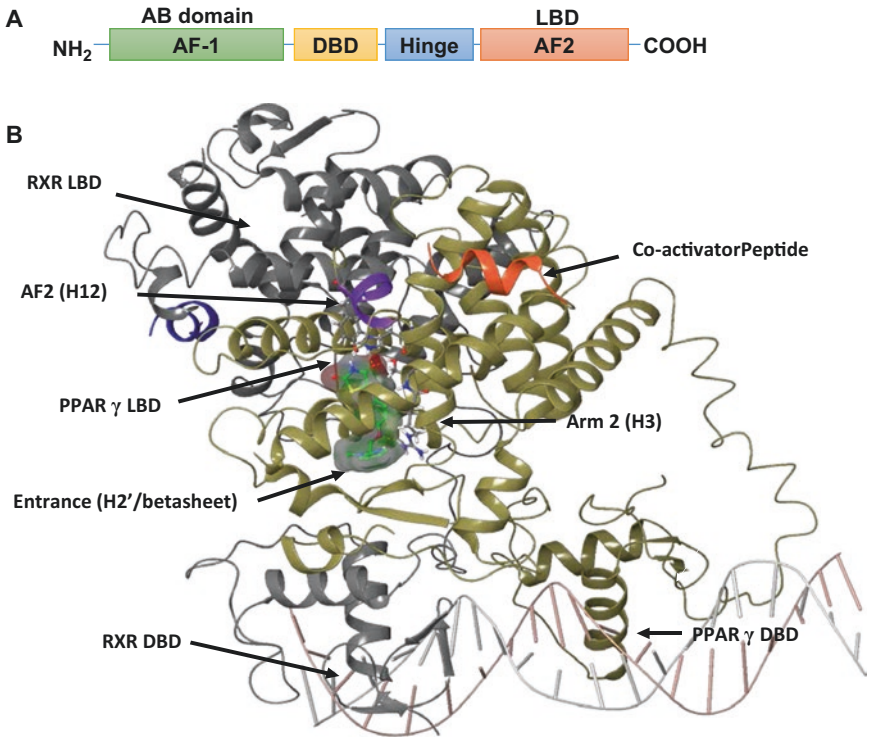


Fig. 10.1 (**a**) Linear illustration of the PPARγ structure, where the ligand binds to the ligand-binding domain (LBD) in the AF-2 domain. (**b**) Full agonist rosiglitazone bound to PPARγ in complex with the heterodimeric partner RXR. PPARγ LBD lies within the RXR-LBD and DNA (DBD) to stabilize interactions with the PPRE. Additional recruitment of coactivator peptides to the PPARγ AF-2 LBD and RXR-LBD allows for additional gene transcription through ligand-stabilized conformations. Ligand stabilization of the β-sheet region and the H2' and H3 helices allow for enhanced stabilization of the RXR-DBD and additional binding of the PPARγ DBD to the PPRE. Specific ligand-induced modifications may allow for enhanced coactivator recruitment and enhanced gene transcription. Crystal structure PDB-(3DZY) was used for constructing the image [10]

remodel chromatin resulting in the induction of gene expression [15]. Although the LBD is highly conserved, specific differences exist in the amino acid make-up of the active site and thus influences the ligand specificity. Ligand binding influences the conformation of the ligand-binding surface of the AF-2 domain, resulting in adapting the binding affinity for chromatin remodeling and transcriptional coregulator proteins. Together these modifications to the ligand-binding domain result in the activation or repression of selective gene transcription [16, 17]. Further knowledge for understanding the conformational changes associated with ligand receptor interactions have been identified by crystal structures that help define the inactive or repressive and active conformations, which result in binding of transcriptional corepressor or coactivator proteins, respectively, by stabilizing specific conformations of the AF-2 region [18].

Recent findings describe how ligands can potentially form interactions with the AF-2 ligand-binding domain via an induced fit or conformational selection mechanism [19]. In the conformational selection scenario, ligands selectively bind to the receptor resulting in selecting a specific conformation that is occupied within the ligand-binding conformational group. In the induced fit model, ligand binding occurs through an encounter complex which results in promoting the ligand-binding conformational group to transform into the final ligand-bound complex. Once this complex becomes stabilized in the active, ligand-bound position, the AF-2 site acts as a binding site for coregulator proteins [19].

10.3 PPAR-Gamma Activation Site

PPARγ relies on cytosolic ligand binding to activate the complex and consequences in the translocation to the nucleus. For gene transcription to occur, PPARγ forms a heterodimeric complex with RXR and binds to the PPAR recognition element (PPRE). Interestingly, the PPARγ LBD intersects the DBD and LBD of RXR, whereby stability of gene transcription is dependent upon the stabilization of this interaction. Stability is greater with the intact nuclear receptor versus the DBD alone. PHE 347 was shown to greatly impact binding to the PPRE indicating that stabilization of this residue is important for gene transcription. Other heterodimeric protein interactions along with coactivator recruitment are also possible leading to increased gene transcription through cooperation [20]. Fatty acids and cyclooxygenase-derived eicosanoids are endogenous activators of PPARγ owing to its specific role in lipid storage, adipogenesis, and glucose metabolism [21, 22]. PPARγ ligands have distinct pharmacophore properties including a carboxylic acid head followed by an aromatic ring with a hydrophobic tail. In a study done with clofibric acid analogs, extension of the hydrophobic tail showed enhanced activity for both the PPARγ and alpha subtypes [23, 24]. This indicates that stabilization of residues outside of the LBD greatly influences the transcriptional potential of novel PPAR ligands. This interaction most likely arises from the ability to influence DBD stabilization to the PPRE outside of initial LBD activation. Specificity for the different PPAR isoforms becomes evident as the length of the hydrophobic tail is increased, highlighting unique gene transcription profiles between them based on the substrate available [25].

10.4 Structure of the Ligand-Binding Domain

The understanding of the PPARγ structure was deciphered by X-ray crystallography and determined that the ligand-binding domain consists of 13 α-helices that are labeled H1–H12 and H2′, as well as one β-sheet region, as shown in Figs. 10.1 and 10.2 [11, 26, 27]. Further, the ligand-binding pocket is located in the core of the

ligand-binding domain [11, 26]. The ligand-binding domain is composed of 270 amino acids and resembles a large Y-shaped cavity and thus three branches, each branch having different properties based upon binding preferences [11, 28]. For example, arm 1, which displays a hydrophobic character, includes H3, H5, H11, and H12 and is the binding site for the acidic head group of ligands such as rosiglitazone [29, 30]. In comparison, arm 2, which is surrounded by helices H2′, H3, H6, and H7, includes the β-sheet region and is hydrophobic in nature, while arm III, which is surrounded by the β-sheet and helices H2, H3, and H5, and has both hydrophobic and hydrophilic regions [29]. The large ligand-binding pocket in PPARγ allows for the promiscuous binding of many ligands (endogenous and synthesized) with lower affinity, thus allowing for targeting of variable ligand interactions. The AF-2 surface includes stands H12, H3, H4, and H5 and forms a hydrophobic binding fork on the surface of PPARγ to which the coactivators bind. Ribbon diagrams of the full-length PPARγ-RXR heterodimer on DNA can be viewed in Fig. 10.1 and partially in Figs. 10.2, 10.3, and 10.4. These structures show that the PPAR/RXR ligand-binding domains dimerize with a view of a PPARγ DNA-binding domain [27]. The hinge region is composed of coils that allow for movement of the ligand-binding domain and DNA-binding domain around each other. Surprisingly, minimal surface contact is observed between the RXR and PPARγ DNA-binding domains. The region of the PPARγ ligand-binding domain that is near the β-sheet, proximal loops, and small helices (H2 and H2′) thus contacts the RXR DNA-binding domain (rather than the ligand-binding domain surface near the AF-2). This interaction allows for understanding as to how signals are conducted from the ligand-binding domain to the DNA-binding domain and vice versa. The LBD of PPARγ consisting of the transcriptional AF-2 motif associated with helix 12 mediates most of the pharmacological actions of PPARγ agonists [31]. The importance of AF-2 domain for regulating PPARγ-targeted gene expression is based upon the mechanism of ligand-induced transcriptional activation by PPARγ [31, 32]. In close inspection (Fig. 10.2), the AF-2 domain exists in an equilibrium state between closed (active) and open (inactive) conformations in the absence of the ligand [31]. Therefore, the binding of a full agonist induces the AF-2 domain conforming into the closed (active) state, thereby allowing the recruitment of coactivators for transcriptional activation [31]. Thus, a rational mechanism for developing novel PPARγ ligands would be to stabilize AF-2 domain in distinct states between closed and open conformations. Several studies have reported that locking the AF-2 domain in its closed conformation is responsible for the anti-diabetic effects as well as unwanted adverse effects from PPARγ agonists like thiazolidinediones [31, 33]. More recently, selective PPAR agonists and dual agonists are proving to be more efficacious in eliciting therapeutic efficacy and avoiding unwanted physiological effects associated with full agonism. More research is needed to understand how specific binding motifs can promote gene transcription to better utilize this drug target and remove the stigma surrounding this class of nuclear receptors. Therefore further evaluation of the stability and conformation and cofactor recruitment if the closed conformation will yield possibilities for the design of better therapeutic agents with increased tolerability.

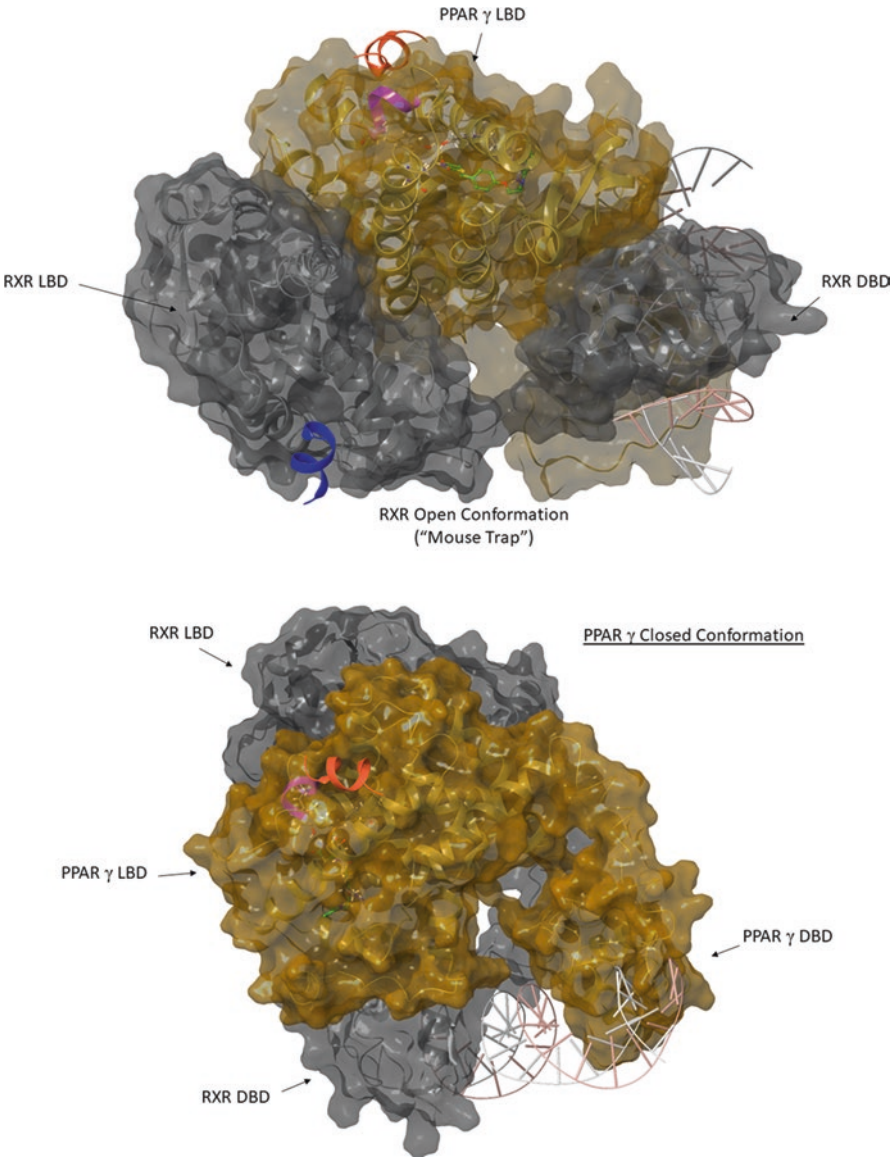


Fig. 10.2 RXR in the (**a**) open conformation illustrating the "mouse trap" model in the heterodimerization with PPARγ. As dimerization occurs, the RXR-LBD and DBD are stabilized through the ligand-induced activation of PPARγ's LBD. PDB(3DZY). (**b**) PPARγ in the closed conformation. As dimerization occurs PPARγ's DBD is able to bind to the major and minor grooves in the DNA allowing for gene transcription to occur. PDB(3DZY) [10]

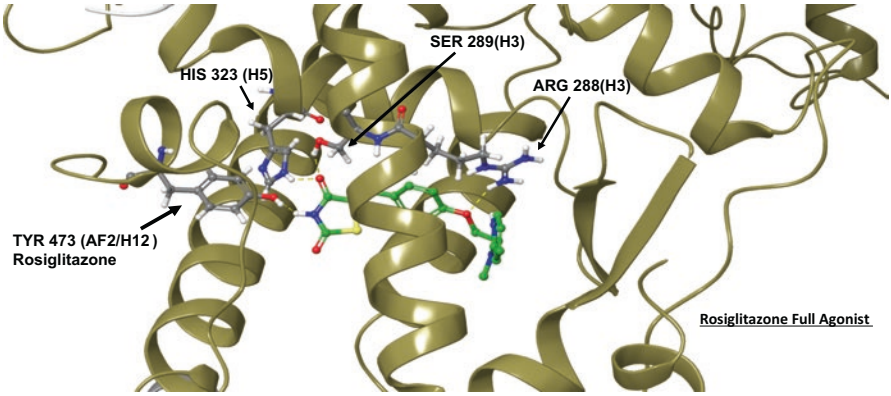


Fig. 10.3 Rosiglitazone bound within the PPARγ LBD active site. Rosiglitazone's polar head forms a tight hydrogen bond network with the AF-2 TYR 473 residue, and additional hydrogen bonds with HIS 323 allows for PPARγ activation and subsequent nuclear translocation. Additional stabilization of the H3 alpha helix in ARG 288 and SER 289 along with hydrophobic interactions to the beta-sheet region provides stabilization upon heterodimerization with RXR allowing for gene transcription to occur. PDB(3DZY)

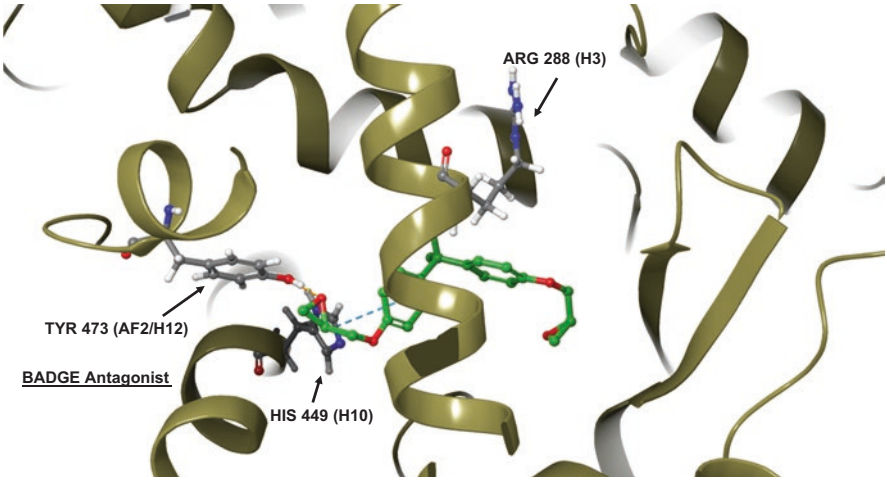


Fig. 10.4 PPARγ antagonist (BADGE) bound within the PPARγ LBD. Strong hydrogen bonding to the AF-2 leads to PPARγ activation. However, conformational changes in the H3-ARG288 result in destabilization of this region opening ARG288 to post-translational modification and gene silencing. PDB(3DZY) [10]

10.5 Structural Dynamics of PPAR Gamma

Initial predictions postulated that mechanism of action of full agonists stabilizes the AF-2 surface through H12, thus allowing less of a physical price for coactivator binding and full transcriptional yield. Likewise, it was thought that partial agonists

can only partially stabilize the AF-2 domain through the H12 helix by generating more of an physical burden for coactivator binding resulting in less transcriptional output. Thus the activating ligands, particularly full agonists, induced the reposition of helix H12 according to the "mousetrap model," and the movement of H12, following ligand binding, traps the ligand within the ligand-binding pocket (Fig. 10.2) [33, 34]. Despite these models involving helix H12, the significance toward stabilization of helix H12 for coregulator binding and transactivation may not be completely understood. In particular, partial agonists are observed to preferentially stabilize other regions of the ligand-binding domain, specifically those associated with the β-sheet region. The connection between the impact of upon stabilization by partial agonists, coactivator recruitment, and PPARγ activity, including insulin-sensitizing effects, is an important question for investigation. The mechanism of action of PPARγ is initiated by ligand binding, resulting in a conformational change of the receptor and the dissociation of any corepressor complexes, including those associated with histone deacetylase activity and the resulting recruitment of coactivators [34]. When the PPAR-RXR receptor heterodimer is unbound to a ligand (natural or synthetic), it becomes associated with corepressor proteins, including NCoR (nuclear receptor corepressor 1) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor). These inactive complex functions to prevent PPAR-activated transcription and keep homeostatic PPAR-mediated transcription minimal. Upon ligand binding (full or partial), the corepressors dissociate from the receptor (PPAR-RXR) complex, permitting for the recruitment of coactivators. These coactivators then implement diverse functions to promote transcription, including altering chromatin structure (acetylation) and recruiting transcriptional machinery to the target gene promoter. Members of the PPAR coactivator family include CBP (CREB-binding protein), MED1 (Mediator 1, also known as PBP/TRAP220/DRIP205), SRC1 (steroid receptor coactivator 1), SRC2, SRC3, and PGC1α (peroxisome proliferator-activated receptor gamma coactivator 1 α) [27].

10.6 Selective PPAR Modulators (SPPARMs)

The popularity of full PPARγ agonist as insulin-sensitizing agents has been constrained because of their association with several adverse effects, including increased plasma volume and edema that is associated with inducing or exacerbating congestive heart failure, osteoporosis, as well as weight gain. Thus, there exists a critical need to develop newer PPARγ-targeted that display robust efficacy with improved tolerability. Consequently we and others have identified and characterized promising selective PPAR modulators (SPPARMs). SPPARMs are PPARγ ligands that serve as partial agonists of the receptor in cell-based transcriptional activity and adipogenesis assays [35, 36]. They have also been shown to generate attenuated and selective gene expression patterns in adipocytes in vitro. The greater therapeutic window of several SPPARγMs has been established in preclinical species. These

findings would include improved insulin-sensitizing activity with attenuated adverse effects on weight gain, adiposity, and cardiac hypertrophy relative to a potent PPARγ full agonist [37–40]. Previous reports indicate that the unique properties of the SPPARMs may be due to their selective physical interaction with the distinct amino acids in the PPARγ receptor, resulting in selective conformational stability of the receptor when compared to full agonists. Findings from X-ray co-crystallographic studies of the PPARγ ligand-binding domain (LBD) associated with full agonist rosiglitazone indicated that the nitrogen of the thiazolidinedione (TZD) ring of rosiglitazone formed hydrogen-bonding interactions with the tyrosine (Y) 473 side-chain hydroxyl group in helix 12 of the human PPARγ LBD [11]. The tyrosine-473 is known to exist deep in the AF-2 ligand-binding domain as demonstrated by in silico analysis [30]. In contrast, X-ray co-crystallography and molecular modeling studies with PPARγ LBD and SPPARγMs indicate that the carboxylic acid moiety of such ligands avoids forming hydrogen-bonding interactions with Tyr473 [41, 42]. The result of these partial interactions demonstrated by biochemical and NMR studies demonstrates that SPPARγMs induce a unique and less stable receptor conformation of the ligand-binding domain than PPARγ full agonists [37]. This instability of the AF-2 ligand-binding domain by SPPARγMs results in the compromised interactions with the transcriptional coactivator-binding pocket of the ligand-binding domain [11, 43] and thus serves as the physical basis for the altered receptor-coactivator interactions, reduced transcriptional activity, and resulting improved tolerability observed in preclinical studies with these ligands [37–40, 44, 45]. In summation, these findings suggest that Tyr473 is a critical site of interaction between the PPARγ LBD and full agonists but not SPPARγMs.

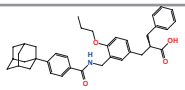
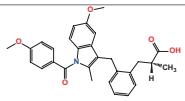
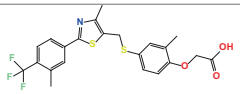
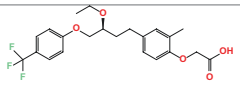
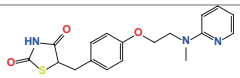
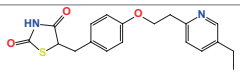
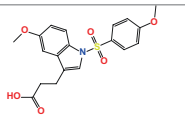
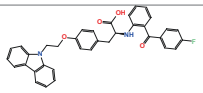
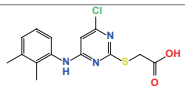
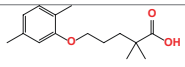
10.7 PPAR Delta Active Site Description

PPARs are approximately 70% conserved in homology, which allows promiscuity of ligand binding between the three PPAR isotypes. Furthermore the key features of the PPARδ active site can be distinguished between PPAR gamma and alpha. Crystallography structures of PPARδ that are bound with the selective agonist GW-0742, a full PPARδ agonist, display a preference for arm 2 occupation with its hydrophobic tail. This occupation of arm 2 of PPAR delta is attributed to the unique interactions involving Valine-312 residue. The VAL312 allows for a slightly larger volume and greater flexibility to accommodate PPAR-delta ligands in the arm 2 occupation, when compared to arm 2 in PPARγ. These observations were verified by mutating the VAL-312 to MET resulting in a 2.5-fold reduction in the EC_{50} of GW-0742 [46]. Crystallographic data showing PPARδ bound with EPA illustrate the dynamic interactions that are crucial for determining ligand-specific binding. EPA's hydrophobic tail can assume a tail that extends up arm 2 or a tail down that extends into the entrance conformation. These interactions induce a conformational stability that can regulate the binding of selective coactivators to PPARδ. Rational drug design for PPAR selectivity must consider not just the AF-2 interaction but

more importantly the stabilizing ability of the hydrophobic tail to increase interaction in PPRE interactions [47].

10.8 PPAR Alpha Active Site Description

In all PPARs, 80% of the active binding site residues are conserved. PPARα is uniquely different when examining the AF-2 region, where a histidine residue is substituted for a tyrosine (TYR) residue on the H5 helix. The larger TYR can explain some of the selectivity of the polar head when designing ligands for PPAR alpha. Although the binding site volume in PPARs is conserved, PPAR alpha is narrower in nature when approaching the AF-2 due to the larger volume of the residues involved in forming the AF-2 stabilizing hydrogen bond network [48].

Compound	Structure	PPARγ	PPARβ/δ	PPARα	References
Alkylphenylpropanoic acid R-7		Partial activity	No apparent activity	No apparent activity	[49]
MRL-20		Partial activity	No apparent activity	No apparent activity	[50]
GW-0742		No apparent activity	Full activity	No apparent activity	[12]
MBX-0825		No apparent activity	Full activity	No apparent activity	[51]
Rosiglitazone		Full activity	No apparent activity	No apparent activity	[52]
Pioglitazone		Full activity	No apparent activity	No apparent activity	[52]
Indeglitazar		Partial activity	Partial activity	Partial activity	[53]
Chiglitazar		Full activity	Partial activity	Partial activity	[54]
WY14643		No apparent activity	No apparent activity	Full activity	[55]
Gemfibrozil		No apparent activity	No apparent activity	Full activity	[56]

10.9 PPARS and Neurodegenerative Diseases

PPARγ agonists have shown efficacy in Alzheimer's disease, Parkinson's disease, brain and spinal injuries, and ALS. Interestingly, in the brain the relative expression of all three isoforms of PPARs are expressed; however PPARδ/β is most abundant in the brain with expression in neurons and microglial cells [57]. PPARγ is observed less in the neuron and microglia, and PPARα is observed mostly in astrocytes [57]. These findings offer PPARs as potential therapeutic targets for mitigating neurodegenerative diseases. Although PPARδ/β is the most abundant PPAR isotype in the brain, PPARγ has been the most extensively studied by using clinical applicable therapeutics in brain injury and degenerative models. The application of PPARγ agonists has been significantly investigated in rodent models of Alzheimer's disease. Findings indicating improvement in amyloid beta burden as well as neurodegeneration have allowed PPARγ agonists to offer promise at the clinical levels. Clinical investigations using PPARγ agonists have revealed a significant reduction in amyloid beta and tau pathology measured in patient samples suffering from AD [58, 59]. More recently, PPARs have been demonstrated to modulate inflammation by inhibiting the production of pro-inflammatory molecules by peripheral immune cells as well as resident inflammatory cells. Furthermore, PPAR agonists have effectively suppressed the development of CNS inflammation in animal models of neuroinflammation and neurodegeneration [60]. In line with this oral administration of the pioglitazone (PPARγ), agonist nullified glial cell activation and the accumulation of Aβ-positive plaques in the hippocampus and cortex [61]. However, the protective signaling mechanisms mediated by central PPARγ activation resulting in improved cognition in AD have not been extensively investigated. Furthermore, PPARγ agonist has been observed to improve cognitive deficits in AD but is limited due to its poor bioavailability in the brain and off-target effects [62, 63].

Furthermore, failures at the clinical level and trials have quenched the clinical applicability of these agonists and have negated volumes of findings verifying these therapeutics for mitigating pathology and neurodegeneration associated with AD. Therefore, there is a perilous need to develop novel PPAR-targeted agents that display improved bioavailability and tolerability. To understand the significance of the chemical interactions on pharmacological consequences will help develop newer PPAR agonists for different cellular targets. Currently, no SPPARγMs have been applied to the clinical level, and mechanistically it remains unclear how to achieve selective PPARγ activation. The current review discusses the role of PPAR in modulating the pathologies of AD followed by SPPARMs under development for treating AD.

10.10 PPARS for Alzheimer's Disease: Overview of AD

The manifestation of clinical symptoms associated with AD is due to years of pathological markers related to AD. For example, the development of AD is thought to be due to the amyloid beta cascade, which involves amyloid beta deposition and damage and leads tau hyperphosphorylation. However, alternative signaling mechanisms associated with the development and progression of pathological mechanisms promote development of clinical AD [64]. Therefore, therapeutics with multi-targets of action against AD pathology may offer potential for treatment of AD (Fig. 10.5).

10.11 PPARs and Neuroinflammation

Neuroinflammation is now considered one of the hallmark's early mediators for developing the pathological process of AD [65]. The inflammatory process is associated with abeta plaque formation as well as microglial activation. Over time, the increase in inflammatory signals associated wit microglia or infiltrating monocytes and macrophages induces cytokine expression and reactive oxygen species in neurons, resulting in spine loss and reduced neural plasticity (Fig. 10.6). The development of memory dysfunction in the later stages of AD is correlated with the levels of synaptic destruction and severity of tau pathology [67]. Therefore, an ideal AD drug would target multiple facets of the disease including Aβ formation and/or clearance, provide anti-inflammatory properties, and reduce tau-related pathologies. However, failures at the clinical level in late-stage AD clinical trials have encouraged researchers to focus on prophylactically administration of PPARγ agonists in the pre-symptomatic phase where Aβ and inflammation play a critical role in the neurodegenerative process and progression. One of the many potential characteristics of PPARγ is to suppress inflammatory signaling pathways in immune cells [68, 69]. For example, PPARγ activation reduces the Aβ burden by inducing microglial phagocytosis of Aβ and consequences in reduced cytokine levels as well [70]. In addition, the activation of PPARγ suppresses transcription factors associated with neuroinflammation including nuclear factor-kB, Stat-1, and transcription factor activator protein-1 [71]. Additionally, PPARγ also downregulates cyclooxygenase-2 (COX-2), metalloproteinase-9 (MMP-9), inducible nitric oxide synthase (iNOS), pro-inflammatory cytokines, chemokines, and interleukins [72, 73]. Similarly, the anti-inflammatory effects of full PPARγ agonists including rosiglitazone and pioglitazone were observed to be efficacious in several rodent models [74]. Specifically, pioglitazone reduced Aβ levels as well as astrocyte and microglial activation in the cortex and hippocampus in APP_{695}SWE mice that are associated with over-expression of Aβ and TGF-β1 [75]. Mechanistically, PPARγ reduces macrophage polarization from M1 to M2, in neurodegenerative diseases. M1

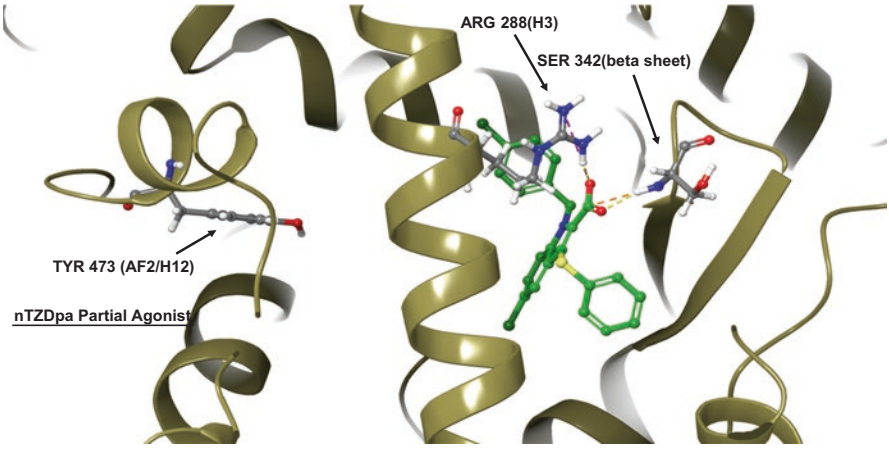


Fig. 10.5 nTZDpa a partial agonist bound within the PPARγ LBD. Partial agonism of the PPARγ LBD progresses in the absence of interactions with the full AF-2 domain. Therefore full stabilization of the AF-2 domain stabilization by the ligand is not required for PPARγ activation. Binding to the ARG288 and SER342 permits stabilization to occur between the RXR-LBD and DBD, and dimerization can proceed. However the recruitment of coactivators may be a factor that arise in regulating gene transcription because of partial agonism PDB(3DZY) [10]

microglia are pro-inflammatory and neurotoxic via secretion of pro-inflammatory cytokines including interleukin IL-1α, IL-1β, tumor necrosis factors (TNF), and nitric oxide (NO) (Fig. 10.6). The alteration of microglia polarization axis from M1 to M2 when treated with PPARγ agonist results in increased anti-inflammatory gene expression profile and increased neurotrophin expression [76, 77]. Surprisingly 12-month-old APP/PS1 mice treated with pioglitazone showed a significant alteration of M1 to M2 microglial in the area surrounding Aβ deposits and a reduction in GFAP-immunopositive astrocytes adjacent to the amyloid plaques [78]. These data validate PPARγ agonist inducing an anti-inflammatory phenotype in microglia and astrocytes and in the process also facilitate the removal of Aβ pathology. However, further research in understanding the mechanisms how TZDs and PPARs confer their anti-inflammatory properties for AD will fill the gaps in knowledge for how newer PPAR agonists can be developed. For example, PPARδ is the most prominent form of PPAR expressed in the brain from the PPAR family. To this, GW501516, a potent PPARδ agonist, demonstrated anti-inflammatory activity [79]. Alternatively, a study by Malm et al. applied a short-term treatment of a PPARd agonist GW0742 to 5XFAD mice and observed a reduction in the parenchymal Aβ load. This was associated with a decrease in overall microglial activation (M1 levels) and reduction in pro-inflammatory cytokines. Instead, microglial immunoreactivity around Aβ deposits was increased [80]. Importantly, the reduction in the pro-inflammatory condition induced by GW0742 resulted in reduction of neuronal loss in the 5XFAD mice.

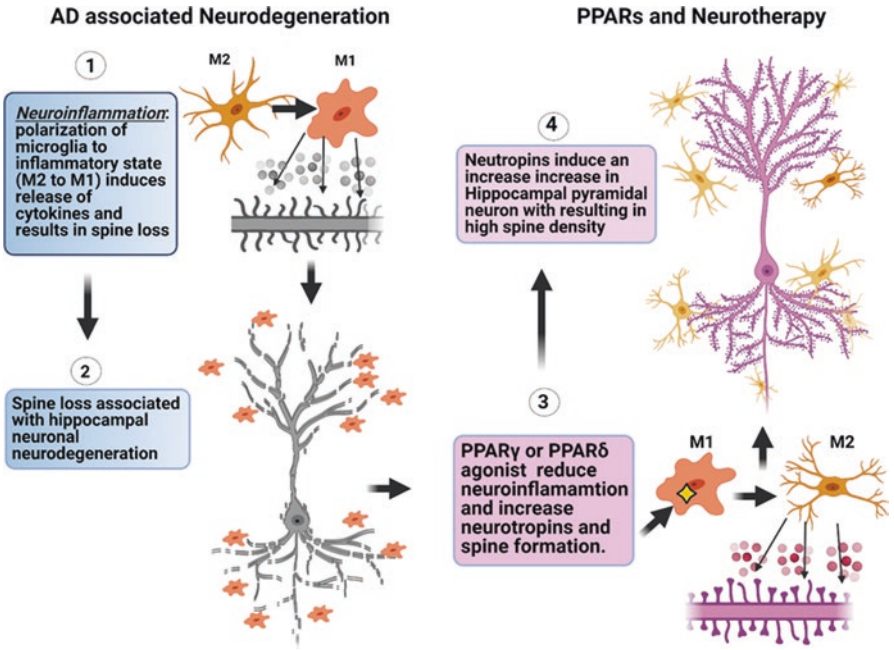


Fig. 10.6 Schematic illustration demonstrates that (1) microglia polarization/activation from M2 to M1 results in secretion of cytokines. Increased cytokines and neuroinflammation are associated with the progression of Alzheimer's disease which is associated with the resulting synaptic deficits and loss in dendritic spines. (2) Further advancement in spine loss potentiates neuronal degeneration. (3) PPARγ/δ agonists are known to reduce neuroinflammation and induce an increase in neurotrophins, including BDNF [66]. (4) The neurotrophins then enhance spine formation resulting in increased spine density

10.12 PPARs and Microglia and Neurotrophins

Microglia are associated with synaptic pruning by regulating spine formation and reduction as a normal process associated with alterations in memory and aging. However advancing AD from moderate to severe stages is highlighted by the development of synaptic deficits and memory impairment. Moreover, neuronal plasticity is associated with synaptic dysfunction, which is due to the loss of dendritic spines. However, neurotrophins mediated by microglia are significantly involved in regulating spine formation and dendritic spine density (Fig. 10.6). Brain-derived neurotrophic factor (BDNF), a major neurotrophin, is known to progressively decrease in expression as AD progresses from moderate to severe stages of AD [81]. Microglia are central mediators associated with inducing neurotrophins including BDNF. Rosiglitazone has been shown to prevent dendritic spine loss and improve synaptic function in hippocampal neurons treated with Aβ oligomers [82]. These findings can be explained mechanistically by the findings that demonstrated ligand activation of PPARγ induced the BDNF promoter in a

dose-dependent manner [66]. Supportive findings from alternative studies in Aβ-injected rats treated with pioglitazone demonstrated reduced levels of active caspase-3 and enhanced BDNF levels yielding improved synaptic plasticity [83]. These observations suggest that PPARγ agonists prevent the development of synaptic deficits by improving BDNF expression and the resulting dendrite spine density.

Alternative protective signaling mechanisms are observed in studies that confirm that the full PPARγ agonist, rosiglitazone, increases the expression of neurotrophic factor-α1 (NF-α1), a neuroprotective protein, which results in the increase of the pro-survival protein BCL 2 expression in the hippocampus [84]. These observations are important because the use of PPARγ agonists improves mitochondrial function and synaptic plasticity and mitigates memory loss. In summation, PPARγ agonism can promote mitochondrial viability while also improving metabolic and energy regulation, modulate neuroinflammation, stimulate spine growth, and clear toxic Aβ from the brain [85]. Findings from our lab has observed that direct PPARg activation induces an increase in BDNF and the ensuing post-synaptic density marker 95 (PSD95), thus representing an increase in spine formation [66].

10.13 PPARS, TREM2, and Amyloid Beta

Dysfunction of microglial appears implicit to the etiology of late-onset Alzheimer's disease (LOAD), as explained in findings from genetic studies that discovered variants in LOAD risk-associated genes that are highly expressed in microglia [86, 87]. One gene in particular from the study, the triggering receptor expressed on myeloid cells 2 (TREM2), a single-pass transmembrane immune receptor was observed to be expressed selectively in microglia within the CNS. TREM2 is a phagocytic receptor and has been demonstrated to be involved in the phagocytosis of apoptotic neurons (Fig. 10.7). However, recent findings demonstrate that TREM2 over-expressing macrophages accumulate on Aβ plaques where they exhibit an inflammatory phenotype yet paradoxically and phagocytically ineffective, as verified by the progressive increase in plaque burden through the course of the disease. Conversely, work by Zhao et al. has observed that TREM2 directly binds to Aβ oligomers with high affinity [88]. Further that TREM2 deficiency results in preventing Aβ degradation in primary microglial culture and in TREM 2 knock-out mice. It is well known that TREM2 suppresses inflammatory gene expression, based on findings from knockdown or genetic knock-out models of TREM2 that show higher levels of pro-inflammatory cytokines and increased levels of Aβ accumulation [89–91]. Thus, neurobiological functions of TREM2 and its pathophysiological ligands remain controversial and need further investigation to understand the role of TREM2 in AD. Clinically, TREM2 levels are regulated by proteolytic cleavage by ADAM10 and ADAM17 at the amino acids His157–Ser158 peptide bonds, resulting in the release of the soluble TREM2 (sTREM2) into cerebrospinal fluid [92, 93]. This soluble form of TREM2 is considered a new biomarker for AD because it is abundantly detected in human cerebrospinal fluid (CSF) and its levels are elevated

in the CSF of patients with sporadic AD [94, 95]. Recent work by Savage et al. [95] provides evidence that nuclear receptors (PPARγ and PPARδ) act to stimulate the Aβ plaques from the brain, by inducing the expression of the phagocytic receptors Axl and MerTK on macrophages. The observation that these cells are from circulating monocytes and express TREM2 suggests that the actions of TREM2 and myeloid cells are involved in ameliorating AD pathogenesis. Additionally, Wang et al. observed that brain-residing TREM2 over-expressing microglia sense lipids that accumulate during Aβ deposition and thus more efficiently clear the Aβ plaques [96]. Further work on understanding the neuroprotective signaling mechanism of TREM 2 is required, including understanding the mechanism and consequence of PPAR-mediated increase in expression of TREM2 on reducing Aβ and tau phosphorylated levels in AD (Fig. 10.8).

PPARs and Astrocytes Astrocytes play a crucial role in brain homeostasis. Among other functions, they provide metabolic support for neurons, uptake neurotransmitters such as glutamate, and blood-brain barrier maintenance [97]. Similar to microglia, astrocytes rapidly react to a wide array of insults or damaging events. Reactive astrocytes, which are characterized by increased expression of glial fibrillary acidic protein (GFAP), a constituent of the intermediate filaments, are typical of most brain pathologies. Thus, astrocytes represent an important target for anti-inflammatory and neuroprotective therapeutic strategies. In addition, rosiglitazone and the non-TZD agonist L-796,449 induced a concentration-dependent increase in glutamate transporter EAAT2/GLT-1 expression and glutamate uptake in primary rat astrocytes, which may help in improving the glutamate dysregulation associated with the progression of AD from mild cognitive impairment to full dementia

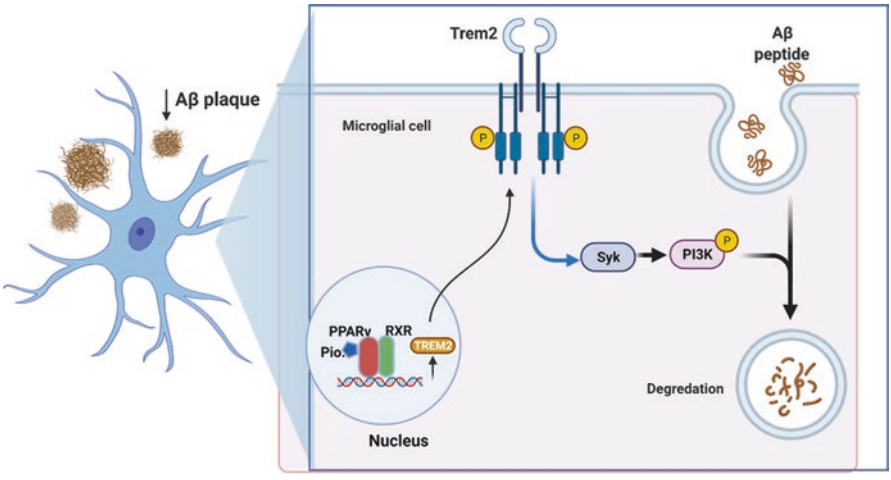


Fig. 10.7 Schematic illustration demonstrating that PPARγ ligands promote amyloid beta clearance by microglia. Mechanistically, PPARγ agonists induce an increase in TREM2 expression by transcriptional regulation. The increase in TREM2 via Syk-PI3K signaling results in diffuse and reduced levels of amyloid beta plaques in the brain by microglia activity

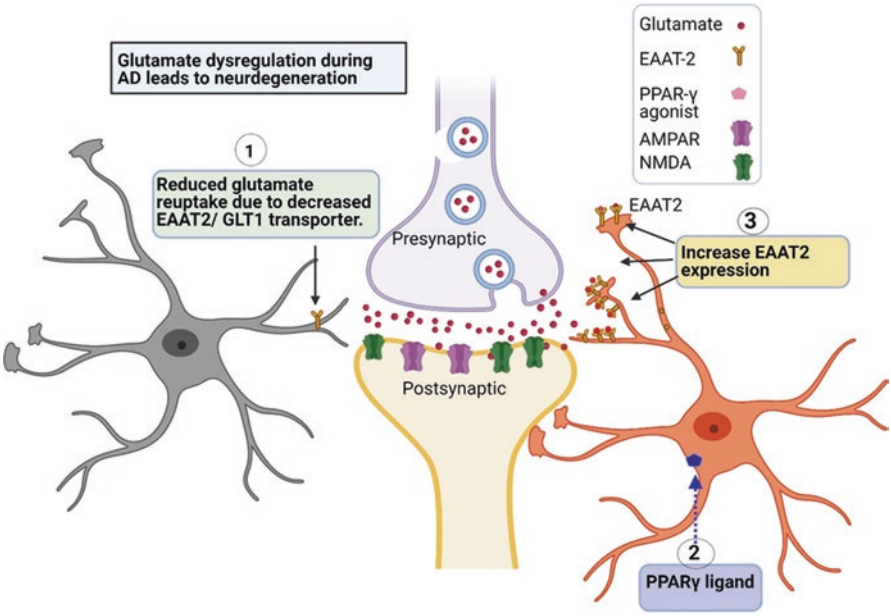


Fig. 10.8 Schematic illustration shows that (1) glutamate regulation is compromised in moderate to late stages of AD. (2) PPARγ ligands can induce an increase in expression of GLT1/EAAT2 glutamate uptake receptor in astrocytes. (3) The increase in surface levels of GLT1/EAAT2 receptors results in improved glutamate handling in the synaptic cleft

(Fig. 10.2). In addition, the authors identified six putative PPREs in the promoter region of GLT1/EAAT2 gene, suggesting GLT1/EAAT2 glutamate transporter is a novel PPARγ target gene [98]. These findings suggest that PPAR ligands can reduce glutamate-mediated neurotoxicity; however critical intervention with the ligands will offer protection against the progression of glutamate-mediated cytotoxicity.

10.14 Conclusion

PPARs offer a unique advantage in treating disease states brought on by chronic inflammation and metabolic dysregulation. Current clinical PPAR therapeutics offer a molecular tool to investigate PPAR roles in neurodegenerative pathologies, yet barriers remain with these compounds as CNS activity is poor at best. High dosages required to achieve CNS activity only compound the unwanted systemic side effects seen at normal therapeutic levels, further complicating in vivo results. Advancements in the design and development of novel selective PPAR agonists may allow researchers clinically relevant compounds that are capable of achieving more efficacious CNS activity without excessive dosing. Achieving selective PPAR agonism may also be beneficial as specific gene transcription can avoid systemic

side effects and elicit cellular-specific gene profiles in targeting various stages of disease pathology. When designing novel selective PPAR agonists, a greater emphasis on understanding the specific roles each arm in the PPAR LBD plays in the ability to recruit coactivators and promotes gene transcription is imperative. PPARs have long been stigmatized as clinically irrelevant therapeutics, but lack of understanding on how to regulate such a powerful molecular tool should not rule out further investigation. Furthermore, understanding the dynamic roles of PPARs in immune cell regulation is crucial to mitigate the pro-inflammatory signaling cascade and improve tissue regeneration. Energy dysregulation and metabolism are at the heart of all disease pathologies, and PPARs offer a unique tool to modulate reorganization of metabolic pathways in various tissue types.

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Chapter 11
Functional Bioassays Lithograph Ligand Reflections in the PPARα Sphere

Pallavi R. Devchand

Abstract The discovery of PPARα led to a global explosion of scientific research in both academia and pharmaceutical companies. Almost 30 years later, this nuclear receptor remains an enigma. Here, I focus on the ligand-binding domain (LBD) of PPARα and chart a trajectory of landmark bioassays for receptor activation and ligand binding. What were the hurdles? And how were they addressed? The LBD facet is just a small part of the PPARα protein, but it plays a pivotal role in the functional machinations. It arguably symbolizes how the interplay of competition and collaboration between research groups is required to fuel discovery toward safe and effective medicines. Perhaps in reflection, we can bring some light to how the LBD of PPARα forms functions to direct dynamic fate.

Keywords PPAR bioassays · Leukotriene B_4 · Glitazone · Drug discovery · Adaptive homeostasis

I am myself: a light.
In me you find your fate.
So be not blind to the truth,
shining from my glow.

—A.E. Drijfhout in Emblamata (1932)

The goal is to take an investigational drug to a medicine. This path of drug development necessitates meandering through animal models before testing an investigational drug in man. But mouse is not always reflective of man. For example, some clinically relevant lipid-lowering drugs can rapidly induce expression of key peroxisomal enzymes and also trigger proliferation of peroxisome organelles in the liver of rodents.

P. R. Devchand (✉)
Department of Physiology and Pharmacology, University of Calgary, Calgary, AB, Canada
e-mail: Pallavi.Devchand@ucalgary.ca

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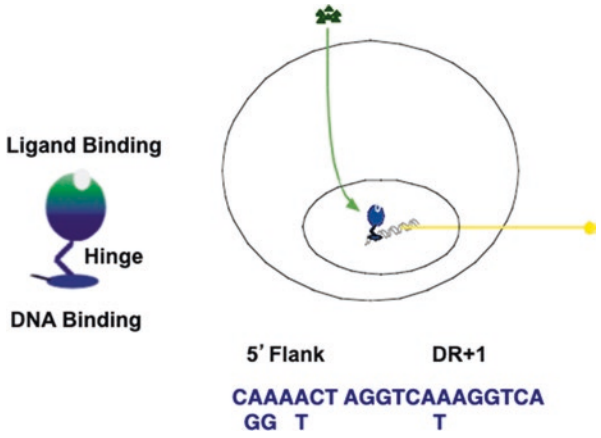

Fig. 11.1 PPARα is a nuclear receptor that conceptually has two major globular domains (DNA-binding and ligand-binding) connected by a hinge. This nuclear receptor can be activated by xenobiotics to rapidly induce transcription from a PPRE, a direct repeat (DR+1) DNA response element

11.1 PPARs Are Nuclear Hormone Receptors

In focused searches, Issemann and Green [10] identified a novel transcription factor as a transducer of the rodent peroxisomal proliferation response to non-genotoxic carcinogens, and named it PPAR (the *p*eroxisome *p*roliferator-*a*ctivated *r*eceptor). The PPAR protein sequence depicted a classic nuclear hormone receptor with distinct DNA-binding and ligand-binding domains connected by a hinge region (Fig. 11.1). Dreyer et al. [6] later cloned three isotypes of PPAR from the frog *Xenopus laevis* and also delineated a DNA response element that mediated rapid induction of transcription by PPARs when activated (Fig. 11.1).

11.2 The RXR Obligation

Further investigation by several research teams indicated that PPARα functioned as a heterodimer with RXR, the retinoid receptor [9, 11, 14]. In the native form, RXRα, β or γ is required for the full-length PPARα activation of transcription from a PPRE. Clearly, activities of both nuclear receptors can be modulated by ligands [14, 17]. The genomic landscape is peppered with PPREs that inherently confer important adaptive and developmental roles of PPARα [2, 3]. The nuances of transcriptional control are in part due to relative affinities of the PPAR-RXR heterodimer for DNA based on the 5′-flanking sequences of the PPRE [12].

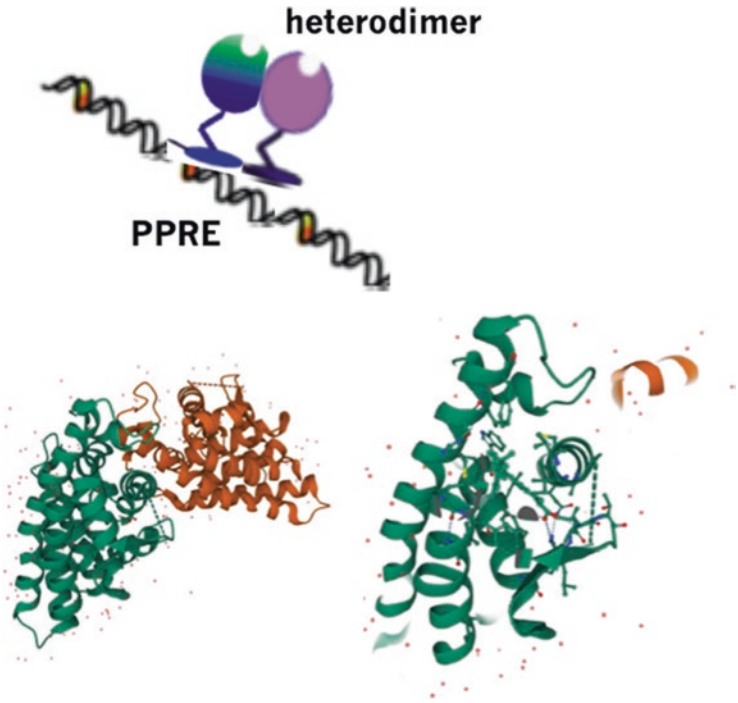

Fig. 11.2 PPAR-RXR heterodimer is the functional unit at a PPRE on a target gene. The crystal structures depict the human RXRα (left) and its interactions with the 9-*cis*-retinoic acid ligand (right). 1FBY deposited by [7] was obtained from RCSB protein database [1]

11.3 Screening for PPAR Activators

Initial mass screenings for activators were performed using the mouse and *Xenopus* PPARs, primarily because these were the tools at hand. Transient transfection experiments in cell culture used two approaches to reporter systems:

1. Use of a native PPRE and optimized combinations thereof
2. Use of an artificial fusion protein that tethers the PPAR(LBD) to an unrelated DNA-binding element

It is worth noting that these systems also differed in basal promoter elements of the reporter construct and cell type. Some examples are presented below.

11.4 Reporter Plasmids of PPAR-RXR Heterodimer Activity

When using the native PPAR-RXR heterodimer as transducer, the primary strategy is to create a reporter plasmid that has a low background and a robust response.

For example, the promoter of cytochrome P450A6 responds robustly to PPARα activators like the xenobiotic Wy 14.643. In this example, eicosanoid modulators are screened in HeLa cells using an efficient reporter system with a palindromic arrangement of two Cyp4A6 PPREs upstream of a thymidine kinase basal promoter that drives CAT expression [4] (Fig. 11.3).

Alternate PPREs used in compound screens for PPARα include those from promoters of adipocyte protein 2, acyl co-enzyme A oxidase and hydratase dehydrogenase [15, 16, 21]. The sensitive reporters have also proved invaluable in pin-pointing differences of species-specific responsiveness [13].

11.5 Power of the Imperfect

An alternate avenue for a screening strategy involved assaying the PPAR-LBD as a fusion with an unrelated protein. This scenario, while pushing aside the RXR obligate and any potential heterodimer to PPRE effects, has been fruitful as a preliminary screen. This approach necessitates characterizing the native PPAR-RXR heterodimer response after identification compounds of interest.

For example, the yeast GAL4 transcription factor has two distinct domains: a DNA-binding domain and an activator domain. GAL4 acts as a homodimer to regulate transcription of galactose-inducible genes via a distinct DNA response element (Fig. 11.4). This system was adopted to define thiazolidinediones as targets of PPARγ [18] and also to follow up in identification of a novel glitazone as a dual PPARα/PPARγ activator [15].

Another successful assay used the bacterial Tet repressor system with a luciferase reporter in U2OS cells [21]. This example indicates differential activation of PPARα by enantiomers of 8-HETE (Fig. 11.5).

In totality, the above screens for activators allowed for two parallel approaches: mass screening of catalogues of compounds – literally! [8, 15, 16] – and also a knowledge-based approach based on the study of published literature [3]. Once an activator of interest is identified, it remains to be determined as to whether the mechanism of activation is indirect or direct.

11.6 Is It a Ligand?

Logically, a compound is a ligand if:

$$\text{free compound} + \text{PPARalpha}\left(\text{LBD}\right) \rightarrow \text{COMPLEX[PPARalpha(LBD) + ligand]}$$

Several methods were developed to measure the outcome, in part due to technical difficulties in expression of mammalian PPARα (LBD) proteins.

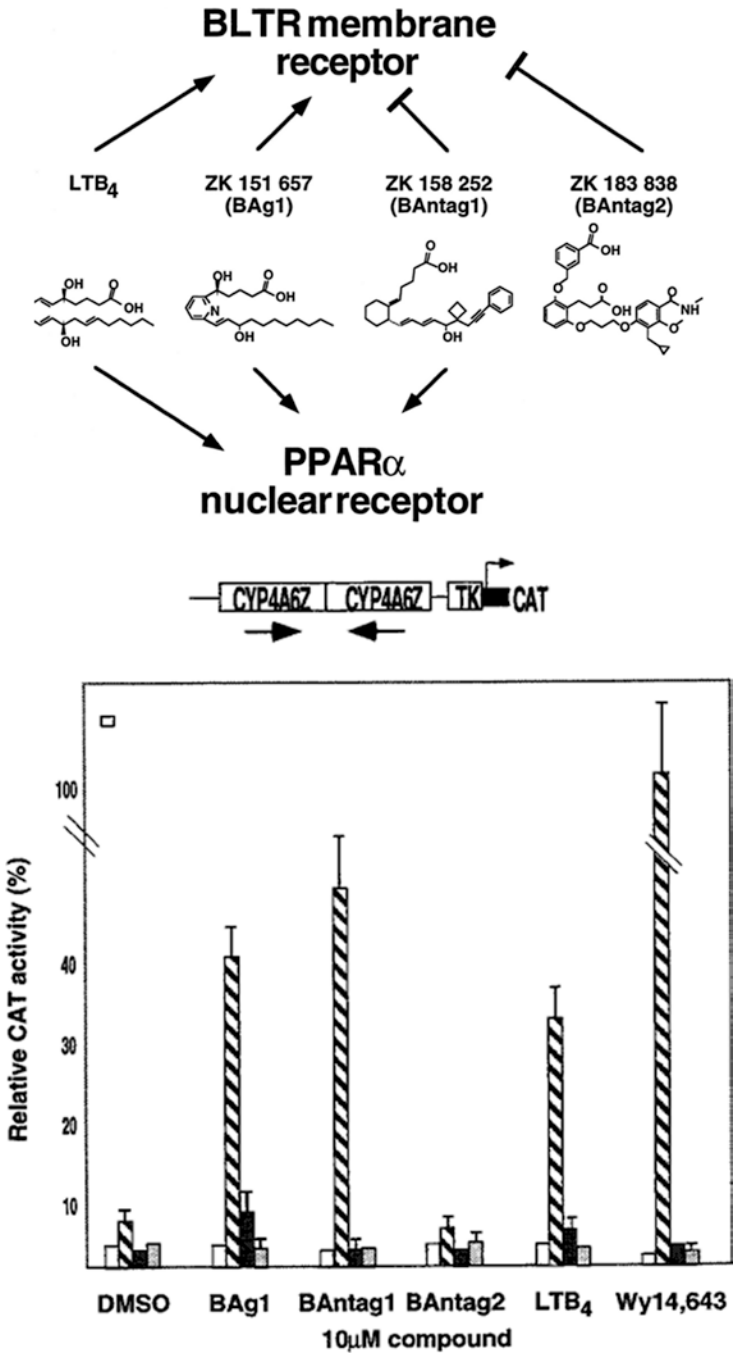

Fig. 11.3 A PPRE reporter generated from the Cyp4A6 promoter sequence used to screen modulators of the BLTR eicosanoid receptor. The Cyp4A6 PPRE is in a palindromic arrangement upstream of a TK basal promoter. Expression of chloramphenicol acetyltransferase activity is then quantified by an enzyme assay. A β-galactosidase construct is used to normalize transfection efficiency between samples. (Figures reproduced from Devchand et al. [4] (Journal of Biological Chemistry))

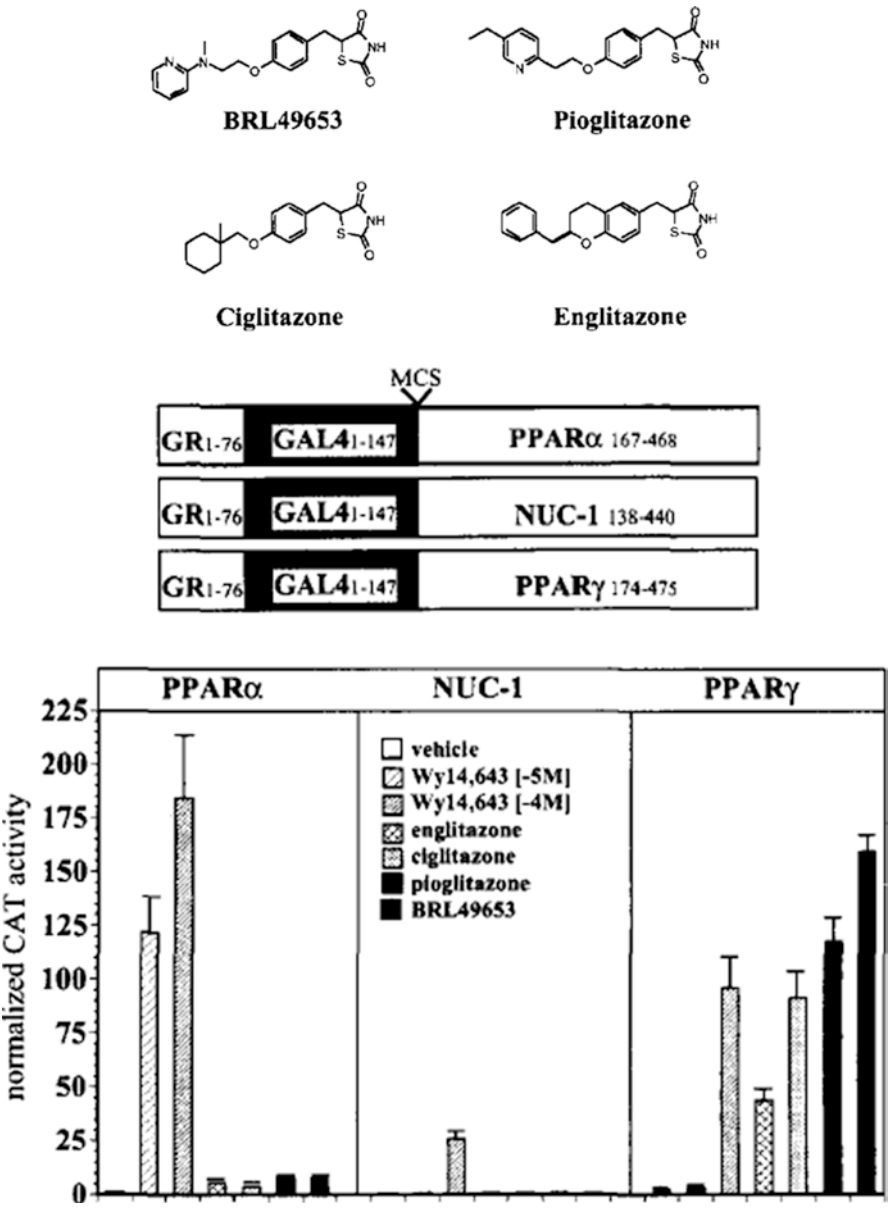

Fig. 11.4 A GAL4-based system. The fusion of PPAR-LBD to a GAL4-DBD, albeit forming homodimers, still proves powerful in measuring PPAR activation via the PPAR ligand-binding domain. (Figures are from [18] (Journal of Biological Chemistry))

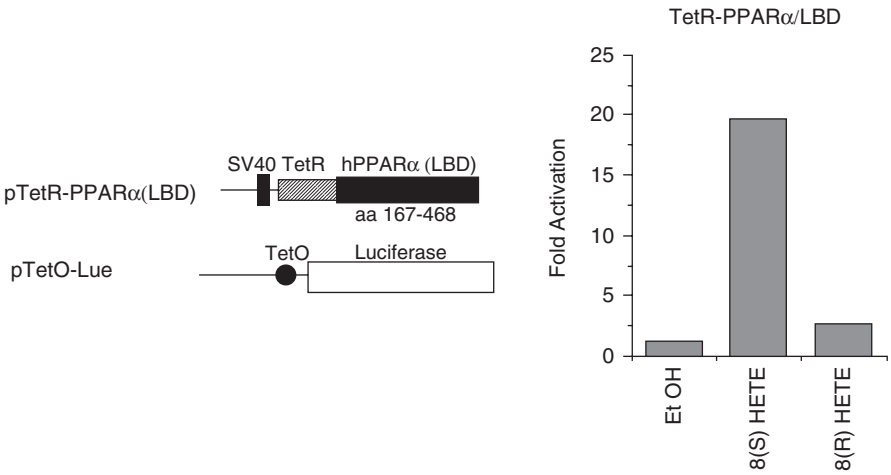

Fig. 11.5 A TetR-based system. The fusion of PPARα LBD to TetR offers a sensitive assay which reports via a Tet operon-driven luciferase reporter. (Figures are from [21] (Journal of Biological Chemistry))

11.7 Saturation and Competition-Binding Analyses

In vitro, the conventional experiment requires a labeled ligand that can be a quantified and also requires soluble, purified PPARα(LBD) protein. Early attempts at producing substantive amounts of GST fusions of human and murine PPARαLBDs proved difficult. Fortunately, the *Xenopus laevis* PPARα (LBD) protein was amenable for direct binding assay studies with radiolabeled eicosanoid LTB_4 and the glitazone GW2331 [3, 13, 15]. Alternately, the use of sensitive fluorescently-labeled *trans*-parinaric acid facilitated calculation of equilibrium constants of binding of ligands with His-tagged mPPARα(LBD) proteins [19].

11.8 Ligand-Dependent Conformational Change

Alternate approaches to determine binding of ligands to the receptor PPARα LBD were based on conformational change of the protein. For example, Dowell et al. [5] developed a differential protease sensitivity assay, radiolabeled the GST–mPPARα (LBD) fusion protein, and resolved fragments generated by specific chymotrypsinogen cleavage at sites that were exposed by conformational change upon ligand binding (Fig. 11.6).

The CARLA assay developed by Krey et al. [16] used the fact that binding of a ligand alters conformation of helix 12 of the PPARα LBD and exposes a surface for binding of the nuclear cofactor SRC-1. This assay was developed using *Xenopus* PPARα LBD and later adapted to the mouse PPARα LBD (Devchand et al., 1997).

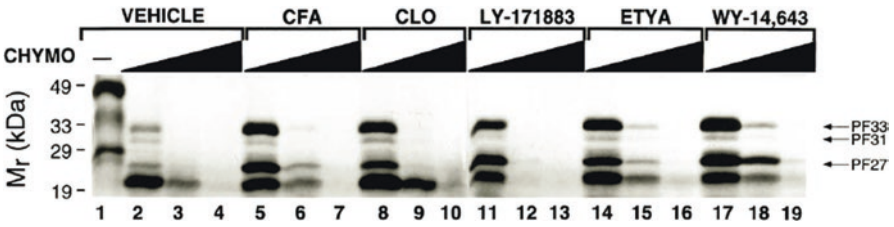

Fig. 11.6 Differential protease sensitivity assay. In this assay, ligand-induced PPAR conformational change is monitored by susceptibility to chymotrypsinogen cleavage. (Figure is from [5] (Journal of Biological Chemistry))

Interestingly, this assay affords generation of dose-response curves that correlate to ED_{50}s in co-transfection assays (Fig. 11.7).

11.9 Altered DNA-Binding Affinity

The PPAR-RXR heterodimer binds to a PPRE DNA response element. This can be measured using a standard electromobility shift assay (EMSA). Interestingly, Forman et al. [8] demonstrated that under very limiting conditions, the binding of the heterodimer to DNA can be ligand-dependent. This ligand-induced complex (LIC) assay is amenable for evaluating PPARα and PPARβ but not PPARγ. Like the CARLA system, this is a semi-functional assay that can provide dose-dependent readouts (Fig. 11.8).

11.10 Crystal Structure

At the finish line, in terms of medication in patients, knowledge on the human system would be the ideal. Xu et al. [20] reported a crystal structure of a His-tagged human PPARα (LBD) in complex with the ligand GW409544 and the cofactor motif of SRC-1 (Fig. 11.9). This is an instructive structure in its detail and no doubt will provide insight into further studies on human PPARα responsiveness to different medications and diets.

11.11 Summary

The narrative of PPARα started with a quest for understanding the species-specific proliferation of peroxisomes in the liver that surfaced in rodents during drug development. After a long journey, the challenge remains to understand how endogenous

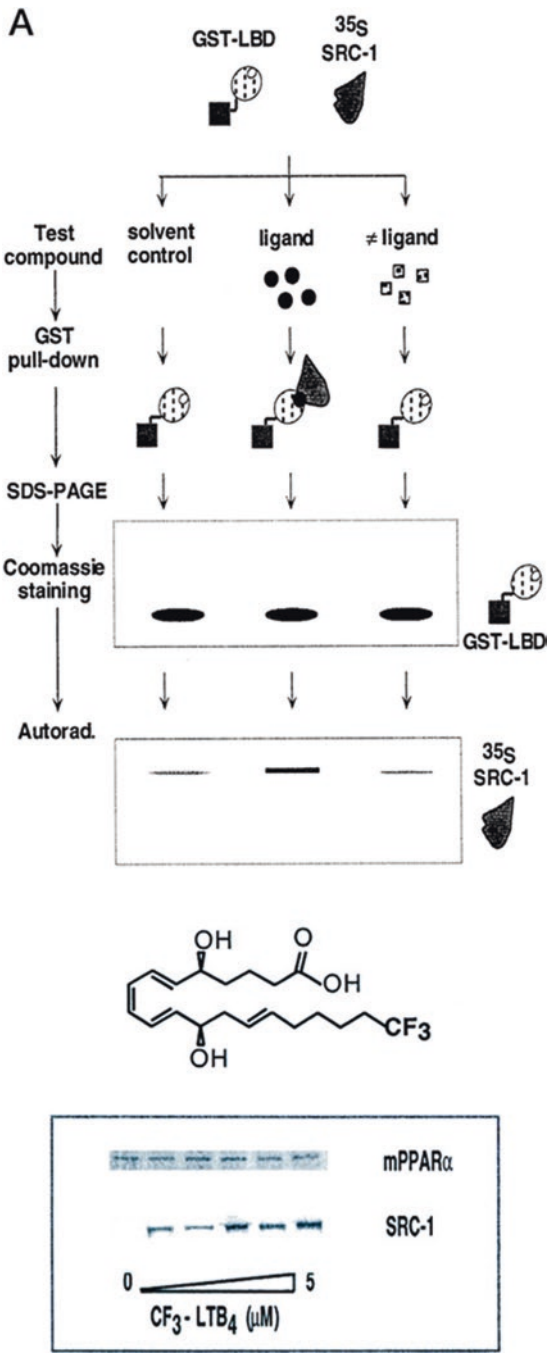

Fig. 11.7 The semi-functional CARLA. This assay correlates the pull-down of radiolabeled SRC-1 cofactor with the binding of GST-PPARα (LBD) to ligand. The dose-dependent nature is reflective of ligand affinity. (Figures are from [4] (Journal of Biological Chemistry))

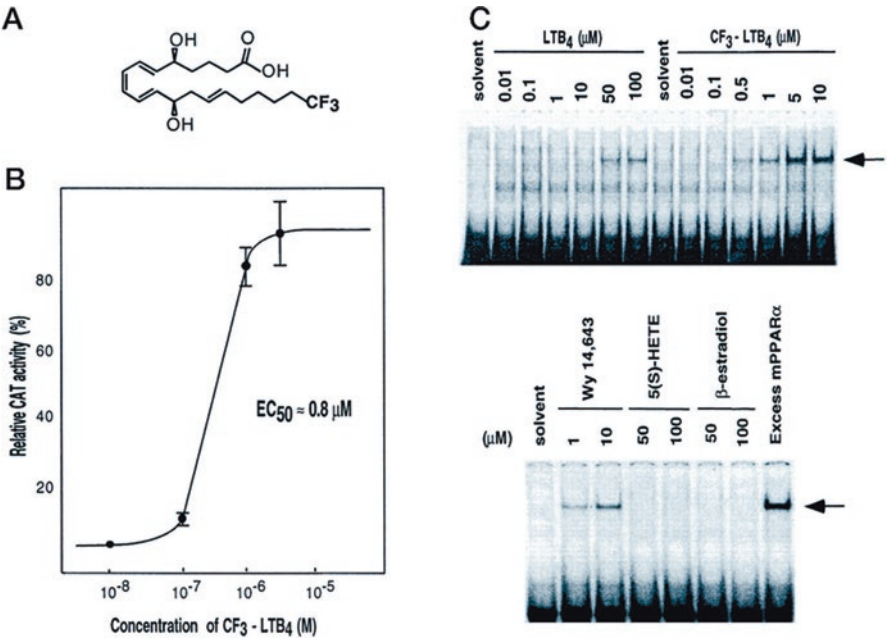

Fig. 11.8 The LIC assay. This figure demonstrates that LTB_4 and its stable analogue CF_3-LTB_4 are ligands of mPPARα. (Figure is from [4]) (Journal of Biological Chemistry))

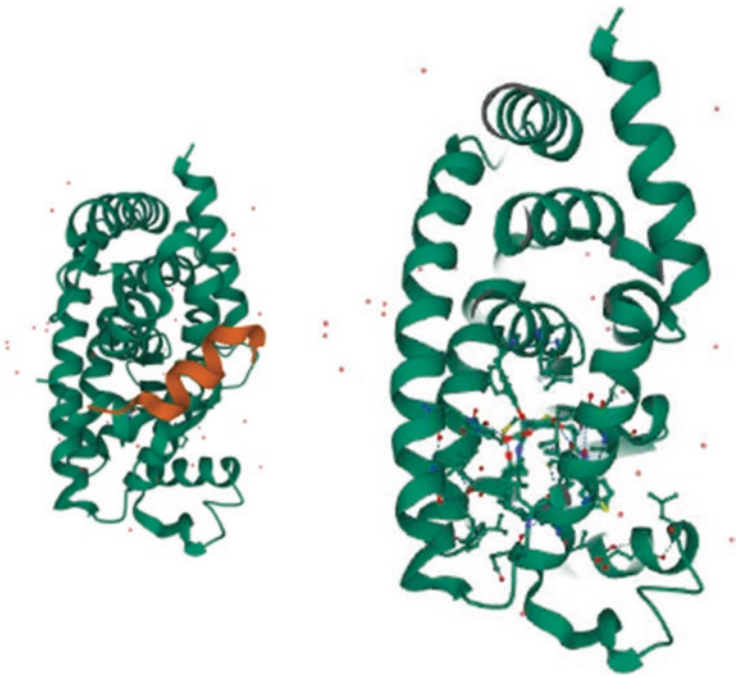

Fig. 11.9 Crystal structure of human PPARα (LBD) complexed with SRC-1 cofactor motif (left) with a weak ligand GW509544 (right). 1K7L deposited by [20] was obtained from RCSB protein database [1]

Fig. 11.10 Photograph by Pallavi R. Devchand (2018) taken in Brooklyn, New York at *Escher: the Exhibition and Experience*

lipid mediators, exogenous natural compounds, and xenobiotics trigger molecular and cellular mechanisms of adaptive homeostasis. A reflection and reevaluation of data from the perspective of the PPARα ligand-binding domain are warranted. We must continue to create lithographs of views from within the PPARα LBD in order to appreciate the dynamics of the bigger picture of health (Fig. 11.10).

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Chapter 12
Computational Applications on Food Contact Chemicals as Nuclear Receptor Binders

Francesca Cavaliere, Giulia Spaggiari, and Pietro Cozzini

Abstract Humans, but also animals, are exposed to chemicals in everyday life. Many of these compounds are present in our food as food contact chemicals (FCCs), naturally occurring (toxins produced by plants), intentionally added (food additives, flavourings) or unintentionally added (pesticides, bisphenols, polychlorinated biphenyls). It is well-known that some of them can act as endocrine-disrupting chemicals (EDCs), which can interfere with the endocrine systems mainly by acting through their interaction with nuclear receptors (NRs). NRs are a superfamily constitute of 48 ligand-regulated transcription factors that are expressed in the animal kingdom and are essential for cell signalling, survival and proliferation. Thus, the alteration of nuclear receptor pathways is correlated to a large number of pathologies. Given the high number of EDCs we are exposed to, it is fundamental to test the endocrine disruptor properties of FCCs with alternative methods to animal testing. In this chapter, we focus our attention on the most common in vitro bioassays and in silico analysis as methods that can consider different endpoints of the NR pathway.

Keywords Endocrine disruptors · Food contact chemicals · Food safety prediction · In silico methodology · In vitro bioassays · Nuclear receptor-associated diseases

12.1 Introduction

Nuclear receptors (NRs) are a superfamily constitute of 48 ligand-regulated transcription factors that are expressed in the animal kingdom. NRs because of the activation of small molecules play diverse roles in cell differentiation/development, proliferation and metabolism. Nuclear receptors share a common structural

F. Cavaliere · G. Spaggiari · P. Cozzini (✉)
Molecular Modelling Lab, Department of Food and Drug, University of Parma, Parma, Italy
e-mail: pietro.cozzini@unipr.it

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organization (Fig. 12.1). The N-terminal region, called the A/B domain, is highly variable and contains the transcriptional activation function (AF-1) and other transactivation domains. The most conserved region is the DNA-binding domain (DBD), or C domain, which contains a P-box and two zinc fingers. The former is responsible for DNA-binding specificity, and it is involved in the dimerization of NRs, while the latter is essential for protein-protein interactions. A D-domain, localized between the DNA-binding and the ligand-binding domains, contains the nuclear localization signal.

The ligand-binding domain (LBD) is the largest, and it is contained in the E-F domain, close to the carboxy terminus. The LBD, contained a conserved core of 12 α-helices (H1–H12) and two short β-sheets, is responsible for ligand recognition but also the coactivator and corepressor binding. These ligands can be classified into two different categories: (i) agonists, which promote the nuclear receptors activity, and (ii) antagonists, which block the effect of agonist through competitive interactions to the same binding site. The activation of LBD is determined by the equilibrium of different α-helix 12 (H12) conformations induced by the ligand. In fact, it rather changes the equilibrium towards more active conformations, characterized by a close H12, in the case of agonists and inactive conformations, characterized by an open H12, in the case of antagonists [8].

Depending on the structure and the ligands, the nuclear receptors could be divided into seven subfamilies (Table 12.1). The first group (*Subfamily 0*) is composed of only two proteins characterized by only a ligand-binding domain. *Subfamilies 1* and *3* are composed of a large variety of receptors (peroxisome proliferation-activated receptors, liver X receptor, progesterone receptor, and many

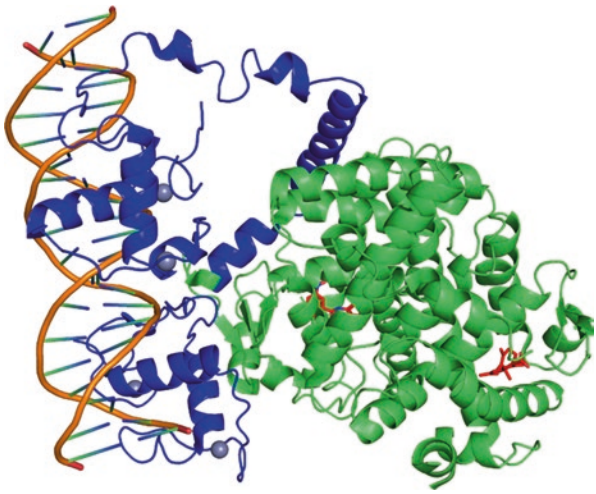

Fig. 12.1 Representative nuclear receptors' structure (PDB ID: 3E00). The ligand-binding site is in green (in this case it is composed of two nuclear receptors, RXRα and PPARγ, and their, respectively, ligands, 9-cis-retinoic acid and 2-chloro-5-nitro-N-phenylbenzamide, in red), while the DNA-binding domain is in blue

others) that can interact with a vastness of ligands. The following receptors' group (*Subfamily 2*) contains orphan receptors, so called because the putative ligand remains to be identified, and the retinoid X receptor (RXR), important receptor because the capability of form heterodimeric complexes with other NRs. Finally, *Subfamilies 4*, *5* and *6* contain orphan receptors important for the development and the metabolism [25].

Along with the endogenous ligands, very different types of molecules are able to bind nuclear receptors. These compounds can be divided into two groups: the first one are molecules that are synthesized to treat NR diseases (drugs) and the other one consists of unintentional binders. In this latter case, they are able to change the important biochemical pathway in which NRs are involved and they are named endocrine disruptor compounds. They include a variety of molecules such as bisphenols (BPs), mycotoxins, food additives, cosmetics, printing ink, plasticizers, etc. From a purely chemical point of view, there are no differences between these different molecules. The common result is that many of them are possible endocrine disruptors. These molecules can be present intentionally or unintentionally in our food, and in this chapter, we define this huge set of molecules simply as food contact chemicals (FCCs).

12.2 Methods to Evaluate Food Contaminants as Nuclear Receptor Modulators

In this chapter, we want to talk about alternative animal tests that can be used to screen food contact chemicals against nuclear receptors to evaluate their endocrine disruptor proprieties. Firstly, we have considered in vitro bioassays that are currently accepted by different agencies involved in this field. Secondly, the in silico methods have been discussed to evaluate the interactions and the mechanism of action of FCCs as possible EDC molecules.

12.3 In Vitro Bioassays to Study the Mechanism of Action (MoA) of Endocrine Disruptor Compounds

Since the 1930s endocrine disruptor compounds have been studied using a range of in vivo models. However, since the use of animals has ethical, economic and scientific limitations, European legislation has prompted the reduction, refinement and replacement (3R) of animal experiments. Considering the higher number of compounds that are synthesized every year, it is not feasible to screen this huge amount using only in vivo study because of their high costs and low throughput. Moreover, it has been shown that a mixture of chemicals can act additively inducing a more potent endocrine-disrupting outcome [15]. Thus, human exposure to different

Table 12.1 List of the 48 nuclear receptors with the respective diseases

No.	Subfamily	Approved name	Gene name	Crystallography PDB structure[a]	NR diseases
1	NR0B1	Dosage-sensitive sex reversal, critical region on the X chromosome	DAX1-AHC	Yes (1)	Adrenal failure; salt-losing crises in the neonatal period; nausea; weight loss; hypotension; hyper-pigmentation; Ewing tumours typical of children, adolescents and young adults; adrenocortical tumours; ovarian, endometrial, prostate, lung and breast cancer; X-linked adrenal hypoplasia congenita (AHC); hypogonadotropic hypogonadism (HHG)
2	NR0B2	Small heterodimer partner	SHP	Yes (6)	Obesity
3	NR1A1	Thyroid hormone receptor alpha	THRA	Yes (8)	Alzheimer's diseases, thyroid neoplasms, osteoporosis, cardiovascular and coronary disorders, osteoarthritis, hypertension
4	NR1A2	Thyroid hormone receptor beta	THRB	Yes (18)	Hyperthyroidism, diabetes, female infertility, end-organ unresponsiveness to thyroid hormone, abnormal growth and bone maturation, and deafness, asthma, abortion, narcolepsy
5	NR1B1	Retinoic acid receptor alpha	RARA	Yes (6)	Cleft lip and palate, diabetes, autistic and bipolar diseases, obesity, myopia, neural tube defects, neoplasms, diabetes, mental disorders, schizophrenia
6	NR1B2	Retinoic acid receptor beta	RARB	Yes (6)	Bipolar and autistic disorders, Creutzfeldt-Jakob syndrome, mental disorders, gout, diabetes, myopia, meningomyelocele, cleft lip and palate
7	NR1B3	Retinoic acid receptor gamma	RARG	Yes (11)	Diabetes, liver cirrhosis, bipolar and autistic disorders, oedema, Alzheimer's disease, neoplasms

(continued)

Table 12.1 (continued)

No.	Subfamily	Approved name	Gene name	Crystallography PDB structure[a]	NR diseases
8	NR1C1	Peroxisome proliferator-activated receptor alpha	PPARA	Yes (18)	Dementia, coronary restenosis and stenosis, carcinoma, brain ischemia, diabetes, oesophageal, lung and liver neoplasms, rhinitis, kidney failure, IGA, ventricular dysfunction, obesity, thrombosis, premature birth, ovarian and prostatic neoplasms, hepatitis C, hyperlipidaemia, hypercholesterolaemia, chorioamnionitis
9	NR1C2	Peroxisome proliferator-activated receptor delta	PPARD	Yes (41)	Bipolar disorder, adenoma, diabetes, adenocarcinoma, colonic and oesophageal neoplasms, oedema, hypertrophy, growth disorders, gout, weight gain and loss, multiple myeloma, personality inventory, obesity, schizophrenia, metabolic syndrome X, peripheral nervous system diseases, coronary and cardiovascular diseases
10	NR1C3	Peroxisome proliferator-activated receptor gamma	PPARG	Yes (178)	Diabetes mellitus, metabolic syndrome X, cardiovascular and coronary artery diseases, colorectal and lung neoplasms, myocardial infarction, weight gain and loss, obesity, rectal and prostatic neoplasms, disease progression, stroke, leiomyoma, atherosclerosis and arteriosclerosis, oedema, pulmonary and metabolic diseases, peptic ulcer, chronic and Hodgkin's diseases, dementia, sleep apnoea, neoplasms, memory and mental disorders, hip fractures, lipid metabolism, and growth disorders

(continued)

Table 12.1 (continued)

No.	Subfamily	Approved name	Gene name	Crystallography PDB structure[a]	NR diseases
11	NR1D1	Rev-ErbA alpha	THRAL/ ErbA	Yes (1)	Hypothyroidism congenital nongoitrous, REM sleep behaviour disorder, major depressive disorder, enhanced s-cone syndrome, delayed sleep phase disorder
12	NR1D2	Rev-Erb beta	Rev-ErbB	Yes (4)	Atrioventricular septal defect (AVSD), metabolic disorders, plasmin system abnormalities, cardiovascular diseases
13	NR1F1	RAR-related orphan receptor A	RORA	Yes (3)	Wet molecular degeneration, mental and macular disorders, oedema, choroidal neovascularization, bipolar and depressive disorders, mood and sleep disorders, vasculitis
14	NR1F2	RAR-related orphan receptor B	RORB	No	Epilepsy, enhanced s-cone syndrome, refractive error
15	NR1F3	RAR-related orphan receptor C	RORG	Yes (81)	Diabetes, celiac diseases, breast neoplasms, carcinoma, lymphedema
16	NR1H2	Liver X receptor B	LXRB	Yes (17)	Calcinosis, dementia, atherosclerosis, encephalitis, oedema, coronary and Crohn's diseases, obesity, diabetes mellitus type 2, metabolic syndrome X, neoplasms, colitis
17	NR1H3	Liver X receptor A	LXRA	Yes (7)	Cardiovascular diseases, diabetes mellitus type 2, metabolic syndrome X, polycystic ovary syndrome, coronary and cerebrovascular diseases, oedema, myocardial ischemia, dyslipidaemias, hypertension, lymphoma, dementia

(continued)

Table 12.1 (continued)

No.	Subfamily	Approved name	Gene name	Crystallography PDB structure[a]	NR diseases
18	NR1H4	Farnesoid X receptor	FXR	Yes (73)	Lung neoplasms, pregnancy complications, inflammatory bowel diseases, insulin resistance, liver cirrhosis, cholestasis, colitis, coronary and Crohn's diseases, calcinosis, diarrhoea, Hepatitis C, dyslipidaemias, liver and cardiovascular diseases, metabolic syndrome X, neoplasms, osteoporosis, overweight, irritable bowel syndrome, urinary bladder neoplasms, Alzheimer's disease
19	NR1I1	Vitamin D receptor	VDR	Yes (45)	Periodontitis, vitamin D deficiency, disease progression, obesity, diabetes, tuberculosis, rickets, melanoma, adenoma, prostatic hyperplasia, psoriasis, lead poisoning, carcinoma, kidney calculi
20	NR1I2	Pregnane X receptor	PXR	Yes (23)	Liver cirrhosis and neoplasms, asthma, diabetes, oedema, lung and liver neoplasms, leukaemia, dementia, head and neck neoplasms, viremia, anaemia, Crohn's and cardiovascular diseases, carcinoma, acquired immunodeficiency syndrome
21	NR1I3	Constitutive androstane receptor	CAR	Yes (2)	Renal carcinoma, neutropenia, prostatic neoplasms, memory and mental disorders, leukopenia, dementia, hypertriglyceridemia
22	NR2A1	Hepatocyte nuclear factor 4 alpha	HNF4A	Yes (5)	Hyperinsulinism, tubulointerstitial kidney disease, diabetes, Fanconi renotubular syndrome 4 with maturity-onset diabetes of the young

(continued)

Table 12.1 (continued)

No.	Subfamily	Approved name	Gene name	Crystallography PDB structure[a]	NR diseases
23	NR2A2	Hepatocyte nuclear factor 4 gamma	HNF4G	Yes (1)	Maturity-onset diabetes of the young, hyperuricemia, chromosome 8q21.11 deletion syndrome, ulcer, diabetes mellitus, colitis, Crohn's diseases, pancreatic neoplasm, carcinoma, inflammatory bowel diseases, dengue fever
24	NR2B1	Retinoic acid receptor alpha	RXRA	Yes (85)	Carcinoma, colonic and colorectal neoplasms, coronary stenosis and diseases, diabetes, autistic and bipolar disorders, keratoconus, microsatellite instability, neoplasms, hypercholesterolemia and hypertriglyceridemia, schizophrenia, pulmonary diseases
25	NR2B2	Retinoic acid receptor beta	RXRB	Yes (6)	Diabetes, gallstones, gallbladder neoplasms, bile duct neoplasms, arthritis, neoplasms, cryptorchidism, pulmonary diseases, psoriasis, hypospadias, lung and prostatic neoplasms, tonsillitis
26	NR2B3	Retinoic acid receptor gamma	RXRG	Yes (1)	Obesity, hypospadias, oesophageal neoplasms, metabolic syndrome X, adenocarcinoma, neoplasms, autistic and bipolar disorders, diabetes, acquired immunodeficiency syndrome, carcinoma, Alzheimer's diseases
27	NR2C1	Testicular receptor 2	TR2	No	Urothelial cancer, infertility
28	NR2C2	Testicular receptor 4	TR4	Yes (1)	Premature aging, lateral myocardial infarction, epilepsy, anterior cerebral artery infarction, teratocarcinoma, cancer

(continued)

Table 12.1 (continued)

No.	Subfamily	Approved name	Gene name	Crystallography PDB structure[a]	NR diseases
29	NR2E1	Tailless homolog	TLX	Yes (1)	Enhanced s-cone syndrome, retinitis pigmentosa, chromosome 17q21.31 duplication syndrome, microphthalmia, autism spectrum disorder, bipolar disorder, neurological diseases
30	NR2E3	Photoreceptor-specific nuclear receptor	PNR	Yes (1)	Enhanced s-cone syndrome, retinitis pigmentosa, colour vision deficiency, cone-rod dystrophy
31	NR2F1	Chicken ovalbumin upstream promoter transcription factor I	COUP-TFI	No	Exotropia, Bosch-Boonstra-Schaaf optic atrophy syndrome, unilateral polymicrogyria, adrenal cortical adenoma, cerebral visual impairment
32	NR2F2	Chicken ovalbumin upstream promoter transcription factor II	COUP-TFII	Yes (1)	Congenital heart defects' multiple types 4 (CHTD4), complete atrioventricular canal-tetralogy of Fallot syndrome, complete atrioventricular canal-left heart obstruction syndrome, complete atrioventricular canal-ventricle hypoplasia syndrome, partial atrioventricular canal
33	NR2F6	V-erbA-related protein 2	EAR-2	No	Patulous Eustachian tube, Eustachian tube disease
34	NR3A1	Oestrogen receptor alpha	ERA	Yes (266)	Osteoporosis, breast and prostatic diseases, cardiovascular diseases, female infertility, hypertension, scoliosis, uterine and colorectal neoplasms, cryptorchidism, polycystic ovary syndrome, inflammation, primary ovarian syndrome, stroke, osteoarthritis, hip fractures, leiomyoma, metabolic syndrome X

(continued)

Table 12.1 (continued)

No.	Subfamily	Approved name	Gene name	Crystallography PDB structure[a]	NR diseases
35	NR3A2	Oestrogen receptor beta	ERB	Yes (32)	Cardiovascular diseases; beast, colorectal and prostatic neoplasms; osteoporosis; endometriosis; male and female infertility, hypertension, oligospermia, obesity, Parkinson's and Alzheimer's diseases, azoospermia, adenocarcinoma, ovarian and testicular neoplasms, colonic and endometrial neoplasms, gallstones, hip fractures, anorexia nervosa, abortion, inflammation
36	NR3B1	Oestrogen-related receptor alpha	ESRRA	Yes (4)	Diabetes mellitus type 2, cardiovascular diseases, oedema, glandular and epithelial neoplasms, obesity, ovarian neoplasm
37	NR3B2	Oestrogen-related receptor beta	ESRRB	No	Deafness autosomal recessive, adrenal hypoplasia, hereditary hearing loss and deafness, autosomal recessive non-syndromic sensorineural deafness-type DFNB
38	NR3B3	Oestrogen-related receptor gamma	ESRRG	Yes (17)	Breast and colorectal neoplasms, diabetes mellitus type 2, hearing, neoplasms, osteoporosis, overweight, stomach neoplasms
39	NR3C1	Glucocorticoid receptor	GR	Yes (43)	Obesity, bipolar disorder, bronchiolitis, cardiovascular and coronary diseases, diabetes mellitus, hypertension, inflammation, fatigue syndrome, metabolic syndrome X, adenoma, premature birth, schizophrenia, mental and psychotic disorders, multiple sclerosis

(continued)

Table 12.1 (continued)

No.	Subfamily	Approved name	Gene name	Crystallography PDB structure[a]	NR diseases
40	NR3C2	Mineralocorticoid receptor	MRL	Yes (25)	Bipolar and attention deficit disorders, myocardial infarction, reward, stress and mental disorders, metabolic syndrome X, hypotension, hyperkalaemia, child behaviour disorders, pseudohypoaldosteronism, pregnancy complications, oedema
41	NR3C3	Progesterone receptor	PGR	Yes (20)	Breast and ovarian neoplasms, premature birth, uterine and prostatic neoplasms, male and female infertility, carcinoma, gallstones, abortion, musculoskeletal diseases, neoplasms, vertigo, thrombophilia, skin and pulmonary diseases, obesity
42	NR3C4	Androgen receptor	AR	Yes (82)	Prostatic and breast neoplasms, infertility (male), polycystic ovary syndrome, alopecia, ovarian neoplasms, oligospermia, prostatic hyperplasia, testicular neoplasms, disease progression, endometrial, neoplasms, carcinoma, insulin resistance, cryptorchidism, neoplasms, hypospadias, hypogonadism, Klinefelter syndrome, diabetes mellitus type 2, adenocarcinoma, acne, androgen insensitivity syndrome, obesity, azoospermia, cardiovascular diseases, Alzheimer's disease, leiomyoma hyperandrogenism, osteoporosis, ovarian failure, gender identity, metabolic syndrome X, abortion, autistic disorder, chromosome aberrations, depressive disorder, endometriosis

(continued)

Table 12.1 (continued)

No.	Subfamily	Approved name	Gene name	Crystallography PDB structure[a]	NR diseases
43	NR4A1	Nerve growth factor IB-like receptor	NGF IB	Yes (15)	Pseudohypoaldosteronism, salivary gland carcinoma, pyomyositis, night blindness congenital stationary type 1, salivary gland disease, metabolic disease, colorectal and pancreatic cancer, lung and breast cancer, inflammatory disease
44	NR4A2	NGFI-B/nur77 beta-type transcription factor homolog	NURR1	Yes (2)	Parkinson's disease, arthritis, rheumatoid arthritis, attention-deficit/hyperactivity disorder, alcohol dependence, colorectal, lung, adrenocortical and cervical cancer
45	NR4A3	Neuron-derived orphan receptor 1	NOR1	No	Chondrosarcoma, epithelial-myoepithelial carcinoma, myxoid and extraosseous chondrosarcoma, Ewing sarcoma (ES)
46	NR5A1	Steroidogenic factor 1	STF1	Yes (4)	46,XY sex reversal 3 (SRXY3), 46,XX sex reversal 4 (SRXX4), premature ovarian failure 7 (POF7), spermatogenic failure 8 (SPGF8), adrenal insufficiency NR5A1-related (AINR), prostate cancer
47	NR5A2	Liver receptor homolog-1	LRH1	Yes (17)	Oedema, diarrhoea, obesity, osteoporosis, irritable bowel syndrome, adenocarcinoma, cardiovascular diseases, diabetes mellitus type 2
48	NR6A1	Germ cell nuclear facto	GCNF	No	Embryonal carcinoma, teratocarcinoma, ureter cancer, retinitis pigmentosa

[a]In brackets are the number of the nuclear receptors' structures present in PDB until 2017

contaminants could also have an additive effect. Although in vivo studies have their advantages, the complexity of the biological system often generates controversial results: for example, where the same experiment was possible between humans and animals, a correlation of 60% has been found [14]. Thus, the results of animal tests cannot be always related to the human outcomes. Keeping this in mind, in silico and in vitro analysis should be preferred over in vivo studies.

This section does not want to be an exhaustive list of in vitro studies to screen endocrine disruptor compounds, but we want to make a brief discussion about the most accepted and used in vitro tests considering the guidelines of agencies that are most active in this field: US Environmental Protection Agency (EPA) (https://www.epa.gov/), European Chemicals Agency (ECHA) (https://echa.europa.eu/it/home), Organisation for Economic Co-operation and Development (OECD) (https://www.oecd.org/) and European Food Safety Authority (EFSA) (https://www.efsa.europa.eu/it). Chemicals intentionally or unintentionally coming in contact with food can adverse human health, in most cases functioning as endocrine disruptor compounds. To evaluate their potential ED proprieties, different in vitro tests are currently used. To understand how they function, a short discussion about how NRs work should be made. Nuclear receptors are composed of two principal domains: a DNA-binding domain and a ligand-binding domain. When an agonist ligand binds to the ligand-binding pocket of LBD, the receptor (as a monomer, homodimer or heterodimer) can migrate inside the nucleus, where the DBD recognized specific DNA sequence named DNA-responsive element located upstream to the gene regulated by the receptor. Once the NR is bound to the DNA, it recruits additional proteins of the transcriptional machinery and activates the transcription and transduction of the gene. Thus, an endocrine disruptor is a compound able to bind the nuclear receptor inducing its activation or deactivation. As a consequence, it determines an upregulation or downregulation of the genes that the receptor modulates. The endocrine-disrupting issue is not a recent discussion. In 1998, EPA convened a committee for developing a tiered approach to evaluate the oestrogen, androgen and thyroid-related effects of a great number of chemical contaminants for a rapid prioritization following by in vivo tests on only relevant compounds. After that, in 2012, the OECD has released a revised guidance document in which test guidelines are exposed for evaluating chemicals for endocrine disruption, and that has been updated in 2018. In vitro assays are part of the Level 2 Framework of OECD, and most of them refer to the oestrogenic and androgenic pathway as well as steroidogenesis (Fig. 12.2).

However, compounds could also interfere with other nuclear receptors/pathways, and thus additional in vitro assays are required to detect all endocrine activity. Operating with the same principle, most of the in vitro tests cited in the OECD document are also available for other NRs.

Since the scope of this section should be to make a brief discussion about in vitro tests that could be useful to validate in silico methodologies and that are used for studying EDCs, we have categorized them according to the biological endpoint under investigation. Accordingly, since an EDC could act at different levels of the biological systems and induce different responses, we discuss bioassays considering the effect resulting from EDC exposure: the chemical interaction with hormone receptors, the induced gene expression by the ligand binding to the receptor, and the cellular responses to EDCs (Table 12.2).

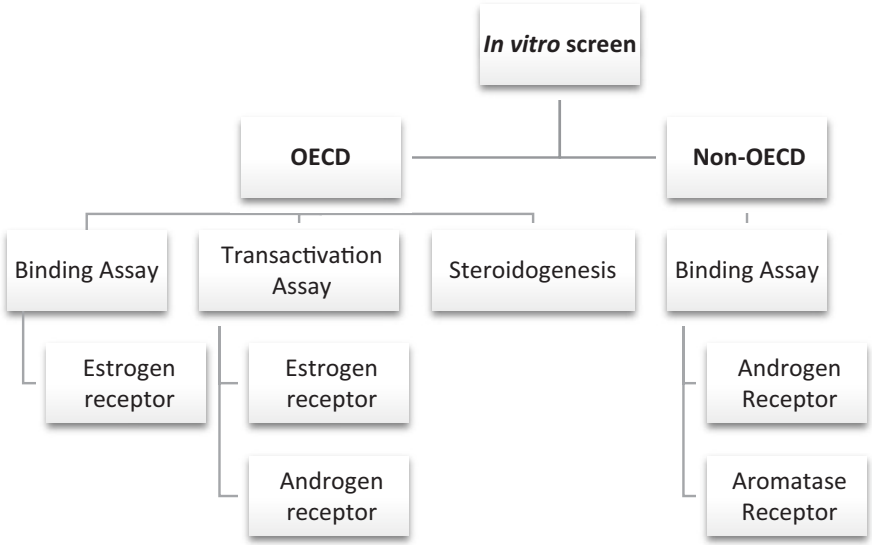

Fig. 12.2 Flowchart of in vitro approaches

Table 12.2 List of the in vitro tests based on the biological endpoint under investigation

Level of response	Type of bioassay	Mechanism	Endpoint
Receptor	Ligand binding assays	Detect the direct ligand binding to nuclear receptors	Receptor binding
	Isothermal titration calorimetry		
	Differential scanning fluorimetry		
Transcription	Reporter gene assays	Detect the agonistic/antagonistic effects	Receptor transactivation
Steroidogenesis	H295R assay	Detect metabolic activation induced by EDCs	Hormone production
	Aromatase assay		

12.3.1 Ligand-Binding Assays

OECD guidelines and EPA documents refer to only the oestrogenic/androgenic binding assay. The first in vitro test to screen the capability of a compound to bind a nuclear receptor is the ligand-binding assay. The older version of the test, applied to the oestrogen receptor, used rat uterine cytosol as a source of the oestrogen receptor protein. Since there are two different isoforms (alpha and beta) of the receptor, the test does not make any distinction between them (although the alpha isoform is the most abundant protein in rat uterine cytosol). Thus, a homogenates tissue extract can be used that must be specific for the receptor under investigation:

that is, it is advisable to use a cell line derived from a specific tissue that expresses the nuclear receptor at higher levels. However, a modern binding assay method uses a recombinant protein of the human receptor produced in and isolated from baculovirus-infected insect cells expressing a full-length human recombinant protein or the human recombinant ligand-binding domain only. Homogenates of cells or tissues with a radiolabelled or fluorescent (fluorescent polarization binding assay) compound are incubated together with different concentrations of tested compounds. Plotting the bound reference ligand against the log concentration of the tested compound gives the possibility to generate the competitive binding curve. The binding activity is quantified as the concentration of the competitor needed to displace half of the reference compound (IC50) or as relative binding affinity calculated from the ratio between the IC50 of the reference compound and the test chemical. Thus, the binding assay cannot determine whether a compound is an agonist or an antagonist since it does not consider the transcriptional activity of the receptor, but it only divides compounds into binders (strong to weak) and non-binders. As an advantage, since the ligand-binding domain of some nuclear receptors is highly conserved across different vertebrate species, the assay results could be referred to many taxa. Among in vitro tests used, this represents the best method for correlating in silico results since it refers to the direct binding of a compound to the receptor. However, the great advantage of in silico methods could be evident: although the rationale of binding assay could be extended to other nuclear receptors, the document of 2018 guidelines is only provided for oestrogen-, androgen- and aromatase-receptor. The great versatility of in silico methods lies in the possibility to consider different NRs all at once to predict the direct binding of chemical contaminants to receptors. This can be done in a very fast and inexpensive way compared to the binding assay. They do not require cell lines; no solutions and no compounds are needed for the experimentation. Moreover, there are fewer interfering factors compared to cell lines, where it has been reported that a certain grade of variability exists in the assay results influenced by protein concentration and/or plate temperature. The phenomenon of partial degradation and/or denaturation of the protein could influence the ligand-receptor interaction inducing a reported decrease of the binding that it is, in reality, a false-positive result. Another important issue can be encountered that when being tested compounds that themselves fluoresce or interfere with light emission report an erroneous interaction.

However, although LBA is the only direct binding assay in the OECD guidelines and the only one accepted by EPA, which refers to OECD documents, in literature some other in vitro methodologies have been used to evaluate the direct endocrine disruptor binding with NRs. Isothermal titration calorimetry (ITC) assay, for example, is often used to study the binding of a small molecule to large macromolecules, such as proteins. It directly measures the heat realized or absorbed along with a bimolecular reaction depending on the type of binding, i.e. whether exothermic or endothermic. The instrument is composed of two different cells that are kept at steady temperature and pressure: (i) the main cell where the NR ligand-binding domain is placed in its buffer solution and (ii) the reference cell which is generally filled with water or with the solvent used for the analysis. During the

experiment, the tested compound is titrated into the receptor solution (main cell). Since the reaction leads to a heat release or consumption, the binding induces a variation in the temperature of the main cell. For maintaining it at the same temperature as the reference cell, the instrument spends energy. The heat change is calculated by integrating the power spent over the time (seconds) that corresponds to the enthalpy of the reaction and, thus, to the fraction of bound ligand. For instance, Zhang and colleagues have used an in silico approach to screen indoor dust contaminants against thyroid hormone receptor β1 (THRβ1) [27]. Of the 31 compounds predicted as potential (THRβ1) binders, 5 have been tested using ITC, and the binding affinity has been calculated. The results showed that four of five molecules were THRβ1 binders. ITC is often useful when the synergic effect of compounds would be studied. Balaguer and colleagues used ITC to study the cocktail effects of two molecules alone and in combination against the peroxisome X receptor (PXR) reporting that the two compounds can interact contemporarily with the nuclear receptor [2]. Thouennon and co-workers used the ITC to characterize the ability of some environmental chemical contaminants to bind oestrogen-related receptor γ (ERRγ) finding that bisphenol E was a more potent binder compared to bisphenol A [22]. An additional in vitro technique that exploits protein thermodynamic characteristic to study ligand-protein binding is the differential scanning fluorimetry (DSF), also known as thermal shift assay (TSA) or thermal denaturation assay (TDA). The methodology is based on the principle that bounded nuclear receptors are more stable than the apo-form and thus are much less prone to denaturation process induced by the heating temperature. DSF uses a real-time PCR instrument to monitor thermally induced denaturation of protein at different ligand concentrations by measuring the fluorescence of a dye that binds preferentially unfolded proteins. Compounds that significantly increase the protein Tm as compared to the vehicle controls are good binders of the nuclear receptor. Since the magnitude of ΔTm is negatively correlated to Kd of the interaction, DSF allows obtaining the binding affinity of different compounds. DeSantis and colleagues have reported the capability of this technique to identify known interactors of ERα contained in a commercially available compound library, showing that the two agonists β-estradiol and estrone and the antagonist tamoxifen citrate can increase significantly the Tm of the receptor compared to the control sample [11].

12.3.2 Gene Reporter Assays

If the experiment's purpose is to distinguish against agonist and antagonist compounds, reporter gene or gene transactivation assay can be applied. Monitoring the transcriptional levels of downstream genes is an efficient in vitro test to screen endocrine-disrupting properties of food contact chemicals. Cell cultures are co-transfected with two plasmids: the first one containing the genomic sequence of a nuclear receptor and the second one reporting the specific DNA-responsive element fused with the genomic sequence of a product that can easily be quantified

(e.g. luciferase, a fluorescent protein or β-galactosidase). Cells are treated with tested compounds, and the agonistic activity could be detected by monitoring the NR-mediated transactivation of the reported gene compared to control cells (normally treated with the vehicle alone). The antagonistic activity of a compound can be instead detected co-treating cells with a chemical and a potent agonist to establish whether it determines a reduction in response and data are compared to cells treated with the potent agonist alone. Finally, if the compound is not able to bind the nuclear receptor and/or induce an agonistic or antagonistic activity, no differences will be reported in the transcription of the reported gene in both experiments. One of the first versions of this assay utilized yeast cell lines carrying the human nuclear receptor together with a vector containing the reported gene, and it is widely used for screening environmental samples. Actually, more specific human mammalian cell lines could be used. They could be properly selected for the type of nuclear receptor under investigation, i.e. cell lines that are well-known to express at high dose the NR. In this latter case, cells are only transfected with report gene construct using selected mammalian cells that naturally express the receptor of interest. Alternative, dual receptor-reporter transfections are also common for mammalian endocrine-screening assays. However, reducing performances could be encountered due to the transcriptional activation of the reporter gene construct induced by non-ER or non-AR-mediated process. To solve this issue, a chimeric construct is utilized in some cases that involve the use of the human ligand binding fused with the DBD of a yeast-specific protein. Importantly, this in vitro test has significant interlaboratory variability, in part influenced by assay parameters such as pH and solvent effect. In silico methods can be, in some circumstances, compared to gene transactivation assay result. Generally speaking, molecular docking allows predicting if a compound is a good, a weak or a bad binder of the receptor since it predicts the binding strength of a protein-ligand interaction without considering the effect of this interaction in terms of agonistic and/or antagonistic activity of a compound. However, for some NRs, such as the oestrogen receptor, two different protein conformations are well-known differing for the helix 12 positions: a close (agonist) conformation where the H12 is located towards the receptor and an open (antagonist) conformation, where H12 is displaced from the receptor. Taking in consideration both the receptor conformations during molecular docking, screening allows to distinguish towards agonist and antagonist compounds: if a compound has a high score in the agonist conformation and not in the antagonist conformation, it can be speculated that it could act as an agonist compound; on the other side, if a molecule has a higher score in the open conformation compared to the agonistic one, it could probably act as an antagonist. Although not included in the OECD guidelines, additional in vitro tests could be performed to analyse the capability of a compound to interfere with the endocrine system. The effects of EDCs on the expression of NR target genes can be also examined using real-time PCR (RT-PCR). Cell lines expressing the nuclear receptor under investigation are treated with different concentrations of a tested compound and incubated for a variable period of time. Total RNA content is then extracted, and the mRNA of specific genes transcribed by the NR is converted into cDNA. Different techniques could be used

in this passage, but generally they allow to detect the mRNA conversion in real time. Since the conversion is a linear reaction, the methodology allows us to quantify the expression levels of mRNA transcribed and thus the ability of a compound to induce the nuclear receptor activation. For example, Dellafiora and colleagues have used quantitative RT-PCR to measure the transcriptional activity of oestrogen receptor-controlled genes (GREB1, growth receptor by oestrogen in breast cancer 1; PR, progesterone receptor) induced by two mycotoxin compounds, the well-known xenoestrogenic zearalenone (ZEN) and zearalenone-14-glucoside (ZEN14Glc), a metabolite produced by plants and is present in food intended for human and animal consumption [10]. They have found that ZEN14Glc can induce a more potent activation of ER target genes and thus supposedly a more potent oestrogenic interference. The same experiment has been used by Yin and co-workers for evaluating different probable EDC compounds for their capability to activate the oestrogenic activity showing that bisphenol A and bisphenol AF consistently can activate endogenous ER target genes [17].

12.3.3 Steroidogenesis Assay

Endocrine disruptor compounds can also affect steroid biosynthesis influencing the NR activity as an indirect effect. A range of in vitro models for steroidogenesis is available, and the H295R assay is the one accepted by OECD (OECD TG 456) and also included in the EPA Endocrine Disruptor Screening Program (EPA 640-C-09-003). The human adenocarcinoma H295R cell line expresses all enzymes needed to convert cholesterol to the key steroids. However, although the interaction of EDCs with steroidogenesis proteins can influence the production of different sex steroids such as oestrogens and androgens as well as progesterone, glucocorticoids and aldosterone, the assay was validated only to detect testosterone and estradiol. In brief, H295R cells are exposed to seven concentrations of the tested compound in at least triplicate for 48–72 h. At the end of the exposure period, the concentration of hormones secreted into the medium can be measured using a variety of methods, such as radioimmunoassay, ELISA (enzyme-linked immunosorbent assay) or chemical analysis. The results are expressed as fold changes in hormone concentration compared with the negative control. Chemicals that may induce steroidogenesis increase the production of estradiol and testosterone; rather, chemicals that inhibit the steroidogenesis decrease the concentration of the two hormones. However, the test does not provide specific information concerning the interaction of the test substance with the endocrine pathway, and thus the results cannot be correlated with in silico studies. Additionally, aromatase assay can be used to identify chemicals that may affect the endocrine system (e.g. steroidogenesis) by inhibiting the catalytic activity of aromatase, the enzyme responsible for the conversion of androgens to oestrogens. It is included in the EPA's EDSP Tier I screening protocol (EPA 740-C-09-004). Human recombinant microsomes are incubated with radiolabelled androstenedione [3H]ASDN, an aromatase substrate

and an essential cofactor (NADPH) for the aromatase activity together with increased concentration of the tested compound. The rate of tritiated water (3H2O) released during the conversion of [3H] ASDN to estrone is quantified, and it is influenced by the activity of aromatase. If a chemical is able to interact and inhibit the enzyme binding to the binding pocket of the androstenedione, a decrease in the tritiated water (3H2O) is reported. Thus, plotting the production of 3H2O as a percent of the solvent control versus the log of the concentration of the test chemical, it is possible to obtain the response curve that allows classifying a compound as an aromatase inhibitor or non-inhibitor.

Although in vitro studies are common usage for screening endocrine disruptor compounds, the huge amount of food contact chemicals highlights the importance of alternative methods (in silico) that can predict EDCs in a faster, safer and better way.

12.4 In Silico Methods for Screening Endocrine Disruptor Compounds

12.4.1 3D Protein Structure: The Starting Point of Computational Methods

Currently, over 700 nuclear receptors' structures have been solved using X-ray crystallography or NMR spectroscopy. When a structure is solved, it is deposited in various structural databases, such as PDB. This database, called Protein Data Bank (PDB), contains the experimental data of the protein structures. In the PDB database, protein 3D structures are represented as a set of coordinate triplets (x, y and z) that define the position of protein atoms. The quality of the PDB structure is defined by two parameters: the resolution (Å) value and the B-factor value. The resolution value is influenced by how well the crystal diffracts and by the amount of time needed to collect resolution data. When a structure has a high resolution, the value is around 1 Å, whereas when it has a lower resolution is around 3 Å and above (Fig. 12.3).

The B-factor monitors the oscillation amplitudes of the protein atoms around their equilibrium positions, or it can be defined as a probability density function for the location of each atom in the protein [7]. The B-factor is defined according to the following equation:

$$B = 8\pi^2 \left(u^2\right)$$

where u is the mean displacement of a scattering centre, measured in Angstroms, and it is an isotropic displacement parameter associated with the reference atom. Usually, an isotropic model is used to model protein motion characterized by a low resolution and a spherical shape, while an anisotropic or ellipsoid model is used to

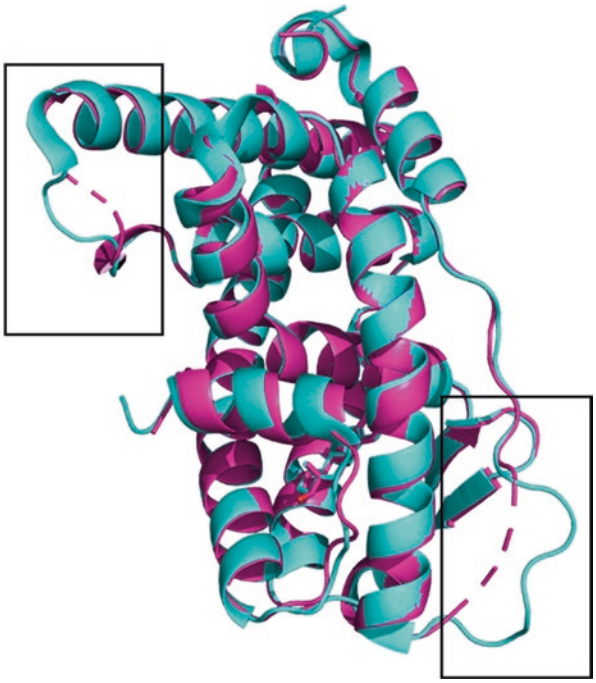

Fig. 12.3 PDB structures of oestrogen receptor alpha with two different resolution values. *In blue* the protein (PDB ID: 2YJA) with a high-resolution value (1.82 Å resolution) and *in magenta* the protein (PDB ID: 1ERE) with a low-resolution value (3.10 Å resolution) are shown. The box highlights the part of the protein resolute in 2YJA

describe the protein motion of small organic crystals (Fig. 12.4) [23]. The latter provides both the magnitudes and the directions of each atom shift, and, thus, it allows a dynamic description of the protein structure.

However, as shown in Table 12.1, not all the nuclear receptors' structures are crystallized. In fact, the major limitation of the X-ray crystallography technique is that the molecules under study must be able to adopt sufficient compact and rigid structures to pack and form a crystal. Instead, nuclear receptors are very complex, both for their flexibility, characterized by an essential biological conformational transition under relatively mild conditions in a wide range of time and space scales, and for the millions mechanism of action given from the relationship between the receptor conformation and the ligand binding. Moreover, some of the structures of the nuclear receptors are unknown, both for the flexibility and the plasticity of the system than for the expenses, labour and time of the procedure. These gaps can be filled in by computational techniques, in particular, due to the use of homology modelling. Homology modelling is the most common and used techniques fundamental to predict the 3D structure of proteins. The basic principle of homology modelling is that proteins with similar sequences may display common structural features. It is for this reason that the accuracy of 3D structures obtained is highly dependent on the sequence identity to the reference structural models.

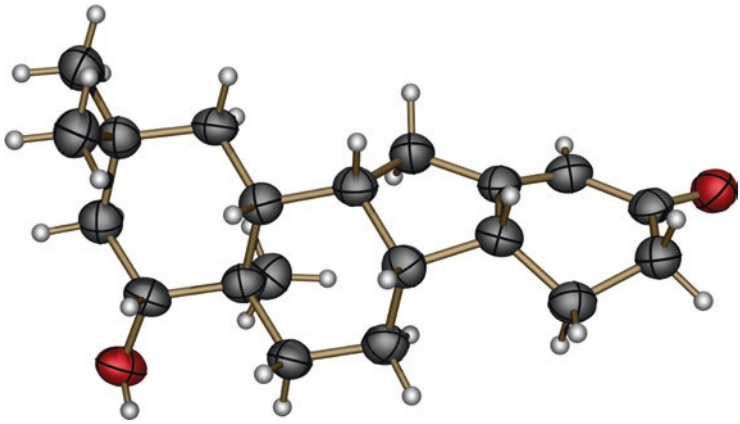

Fig. 12.4 The epitestosterone shown above, made by the program ORTEP [12], illustrates the thermal ellipsoid

12.4.2 Ligand-Based Virtual Screening

In silico methods (Fig. 12.5) are widely used in the fields of computational chemistry, computational biology and material sciences to study molecular systems, ranging from a small system to large biological molecules. Virtual screening is a powerful tool to predict the activity of a huge number of chemicals in a reasonable time. Several databases of molecules are currently available for virtual screening campaigns, such as ZINC, a free database of commercially available compounds; ChEMBL, a database of bioactive molecules; and PubChem (https://pubchem.ncbi.nlm.nih.gov/), a database of chemical information [9, 18, 21]. Virtual screening approaches can be divided into ligand-based when the information of known ligands is used, and structure-based, when the information of the targeted protein-binding site is used.

The increasing number of chemical product synthesized and released in the market every year has necessitated the development of computational approaches to speed up the process of their food safety and security. Although the usage of computational approaches was started from the drug discovery field with the aim to identify new potential drug candidates, in recent years, the usage of virtual screening is becoming more important in the food risk assessment area, too. This is because on the molecular scale, interaction is an interaction, and thus from a chemical point of view, it is not important if a compound is a drug or a food contact chemical (FCC). Thus, in silico methods can be easily moved in the food safety field to screen the capability of FCCs to interact with target proteins interfering with their natural biological activity. In silico screening techniques of a large compound databases are commonly defined as virtual screening (VS), referring to those computing techniques that use a complementary tool to identify potential binder compounds on a pool of chemicals. Like high-throughput screenings (HTS), VS is used as a first step to

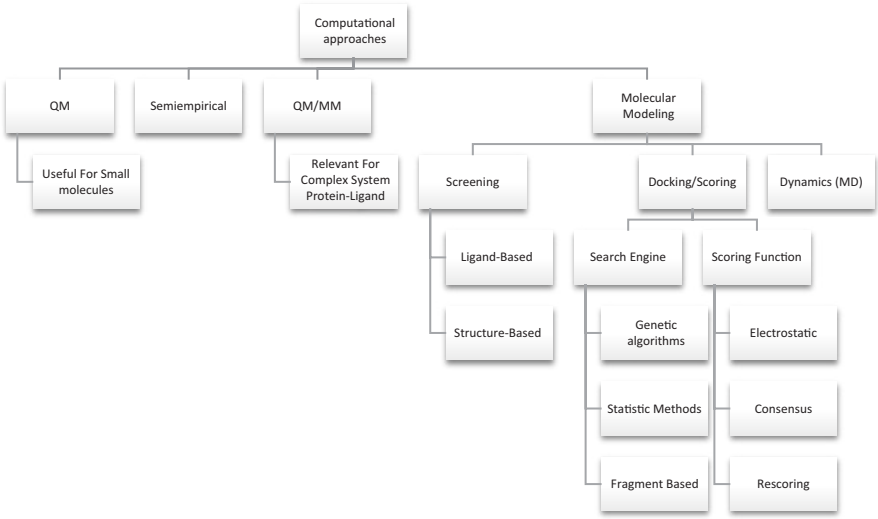

Fig. 12.5 Flowchart of computational (or in silico) approaches

process large libraries of compounds. The main advantage of VS compared to HTS is the rapidity of the screening method and the decreasing costs since it does not require compounds to be synthesized or purchased and tested. Since virtual screening methodologies are knowledge-based approaches, they require structural information about the binding site and/or the nature of ligand that should bind. Thus, based on the available information, virtual screening can be divided into ligand-based virtual screening and structure-based virtual screening. If the three-dimensional structure of target binding site is unknown, ligand-based virtual screening (LBVS) can be used since it faces the problem by the ligand point of view. In fact, based on known active molecules, this methodology searches for similar compounds. Ligand-based methods consider molecule dimensionality, with 1D or 2D methods being considered separately from 3D methods. The former searches for molecules' numerical descriptors that are independent by their molecular structure to attempt to relate them with their known biological activity, and they are mainly described as quantitative structure-activity relationship (QSAR). Instead, three-dimensional (3D) LBVS methods incorporate the molecular conformation and can be mainly divided in subgroups based on the method used for the similarity search: (i) pharmacophore-based, (ii) shape-based, (iii) molecular field-based methods, (iv) fingerprint-based methods and (v) electrostatic potential similarity.

The concept of pharmacophore was introduced by Ehrlich in the nineteenth century based on the idea that specific groups within a molecule are responsible for its biological activity. The pharmacophore concept was developed over time reaching the modern IUPAC (International Union of Pure and Applied Chemistry) definition: "a pharmacophore is the ensemble of steric and electronic feature that is necessary to ensure the optimal supramolecular interactions with a specific biological target

structure and to trigger (or to block) its biological response" [26]. Thus, it describes the essential features that a molecule should have for the binding with the target protein and does not represent a real molecule. The three-dimensional (3D) structure of known active molecules is superimposed considering shared pharmacophore features in order to identify key interaction points for building a skeleton of abstract characteristics that define interaction type, such as hydrophobic and aromatic contacts, hydrogen-bond donors and acceptors and charged interactions.

Shape-based strategies, such as ROCS and shape screening (Schrodinger), are based on the concept that if a molecule has an overall similarity shape with a known binder, then it is likely to fit in the same binding pocket [19, 20]. Thus, they compare atomic radii instead of atom types and do not consider particular properties of the reference ligands. Therefore, shape-based methods are often used in combination with other approaches which consider some chemical properties.

Molecular field-based or grid-based methods, such as CoMFA (comparative molecular-field analysis), CoMSIA (comparative molecular similarity index analysis) and GOLPE based on GRID compared to molecules aligning the dataset compounds using different rules. Steric, electrostatic or hydrophobic potential fields (but also can be included hydrogen-bond donors and acceptor descriptors) are calculated at each grid point using a probe atom for identifying the similarity between the molecules [3].

Fingerprint-based methods are based on the concept to reduce the complexity of the molecular representation considering molecules as a sequence of bits which can then be easily compared. The similarity is then calculated using Euclidean distance or most commonly the Tanimoto coefficient. According to the nature of bits, fingerprints can be classified as sub-structure key-based, topological or path-based, circular and pharmacophore fingerprints [6, 13]. Finally, since electrostatic interactions often play a critical role in ligand binding, another approach of LBVS uses the electrostatic potential of a reference ligand to collect compounds that have similar electrostatic distribution [13].

12.4.3 Molecular Docking

Two molecules can interact in several ways let alone the interaction of a protein and protein/small molecules. Molecular docking is a computational technique that involves finding the most favourable binding mode of a ligand to the target protein. First of all, to have an accurate docking prediction, a high resolution X-ray, NMR or homology-modelled structure is necessary. Molecular docking can be achieved through two steps: (i) the different conformations' prediction of the ligand in the active site of the protein and (ii) the conformations ranked via a scoring function. There are a huge number of binding modes between two molecules. For this reason, various sampling algorithms have been developed in molecular docking software (Table 12.3). These algorithms should be able to reproduce the experimental binding mode between two molecules.

As mentioned before, the nuclear receptors are flexible and plastic systems. The protein may adopt different conformations in the unbound and bound states and may adopt different conformations with different ligands. For these reasons, molecular docking methods can be divided into rigid docking where the bond angles, the bond lengths and the torsion angles of the ligand and the protein are not modified and flexible docking that permits conformation changes. The flexibility could be applied to the ligand and/or to the protein. If the flexibility is imposed on the ligand, it can be able to explore all the conformational space of the protein. The ligand flexibility is commonly considered in docking simulations, while the protein flexibility still remains a challenging goal, mainly because of the dynamic complexity and of the computational time required for running the simulations. A considerable option is to impose the flexibility only to a region of the protein. Then, a limited number of atoms are considered, for example, the pocket side chains.

Obtaining a huge number of ligands binding mode, scoring functions are fundamental to estimate and calculate the ligand-binding affinity between the protein and the ligand, to delineate the correct poses from incorrect poses. Two main aspects characterize a docking simulation and influence its results: (i) a search engine that defines the sampled conformational space and (ii) an empirical scoring function that is used to approximately predict the ligand-protein binding affinity and, in a virtual screening campaign, is used as a measure to rank screened compounds. Scoring functions can be divided into force field-based, empirical and knowledge-based scoring functions. The first estimates the binding energy calculating the sum of the non-bonded interactions. The basis of the second scoring function is that the binding energies of the complex can be approximated by the sum of individual energy components: hydrogen bond, ionic interaction, hydrophobic bond and binding entropy. The knowledge-based scoring function uses statistical analysis of the ligand-protein complex to obtain the interatomic contact frequencies and/or a distance between the two components. As a technique that aims to furnish a quick result for the analysis of a complex biological process, the molecular docking has some limitations: (i) scoring functions are very sensitive to ligand size and are implemented mainly considering electrostatic contributions and underestimating the hydrophobic effect, and (ii) a docking simulation can be performed only between

Table 12.3 The most used molecular docking programs with the respective algorithms

Molecular docking program	Algorithm
DOCK, LibDock	Matching algorithm (MA)
$DOCK_{4.0}$, SLIDE, FlexX	Incremental construction (IC)
AutoDock, $DockVision_{1.0.3}$	Monte Carlo (MC) technique
GOLD, FLIPDock	Genetic algorithm (GA)
GLIDE	Hierarchical method

The MA based on molecular shape maps a ligand into an active site of a protein in terms of shape features and chemical information. The IC fragments the ligand from rotable bonds into various segments. The MC modifies gradually the ligand using bond rotation and translation or rotation of the entire ligand. The GA is similar to the MC method, but it is used to find the global minima. The hierarchical method precomputes and aligns the low energy of ligand

two molecules per time; it cannot predict the effect of water molecules and/or cofactors to the ligand binding. In such case studies, where the role and the position of a water molecule are well established, the water molecule can be explicitly considered even for docking simulations. However, it is challenging to determine the effect of waters in the binding when the experimental structure is not available. In order to deeply rationalize the ligand-protein binding process, molecular dynamics simulations can be used.

12.4.4 Consensus Scoring

A solution to overcome the intrinsic limitations of a specific docking/scoring software is the consensus scoring. Because of any embedded force field used to score the docked solution that is intrinsically linked to the searching engine (the algorithm used to search the possible positions of a ligand within a receptor cavity), a solution for a more reliable result is to use more than one package or more than one evaluation function. This is in order to achieve a "convergence", a "consensus" to the best possible solution. We have three possible approaches: (i) one package with a different internal scoring function, not a great solution because the newest scoring function is, in general, an updated version of the previous one, and then it works better; (ii) two or more packages with the internal scoring function; and (iii) more packages with their internal scoring function plus a rescoring using one or more external independent scoring functions. Compared to a single scoring function, Wang and Wang have reported that using different scoring functions can reduce false positives and improve hit rates [24]. Moreover, Bissantz and co-workers have highlighted that using three different scoring functions allows to reduce the number of false positives and enhance the capability to reach hit rates from 10% up to 65–70% [4].

12.4.5 Molecular Dynamic Simulations

The power of the existing supercomputers allows us to carry out microsecond-scale MD simulations in a few days or a week depending on the architecture of the system. The atoms in a biomolecule are in constant motion, and both the molecular functions and the intermolecular interactions depend on the dynamics of the molecules involved. Molecular dynamic (MD) simulation is a computational technique used for analysing the physical movements of atoms and molecules and for investigating the structure, dynamics and thermodynamics of biological systems with the use of computer. The molecular dynamic simulation is based on Newton's second law or the equation of motion, F = ma (F is the force exerted on the particle, m is the mass and a is the acceleration). From a knowledge of the force on each atom, it is possible to determine the acceleration of each atom in the system. Integration of the equations of motion then yields a trajectory that describes the positions, the velocities and the

accelerations of the particles as they vary with time. From the trajectory, the average values of properties can be determined. MD trajectories provide a view of the motion of a molecular system in a time-space, allowing to consider the macro flexibility and the influence of the solvent. Water molecules solvate the protein but can also enter the cavity-binding site and influence its shape or, more importantly, mediate the ligand-receptor binding. There are different approaches to treat water molecules during the simulations. When water molecules play an important stabilizing effect, explicit water treatment should be used. In the case of HIV1 protease, a water molecule (named W301) functions as a bridge between two lysins in the ligand-binding site and the ligand (i.e. the drug Saquinavir). Without this water, the ligand will not able to interact with the protein.

With the computational cost of MD simulations, it is impossible to screen the huge number of FCCs with this technique. However, if the scope of the analysis is to study the mechanism of action (MoA) of an endocrine disruptor, molecular dynamics can be applied to a limited number of molecules. The analysis can give insight into how an EDC interacts with the NR, i.e. if it induces conformational changes compared to the endogenous ligand, the type of binding interactions inside the binding pocket, the effect of the compound in respect to the coactivator and corepressor binding, etc. Some kinds of parameters can be exploited to analyse the MD simulation results. The most commons are the use of the RMSD (root-main-square deviation) and RMSF (root-main-square fluctuation) values to monitor the stability of the system. Additionally, the hydrogen bond networks between the protein and the ligand and/or the protein and the coactivator/corepressor can be monitored during the simulation time to explore in more detail how ligand interacts with the NR compared to the endogenous ligand.

12.5 Case Studies

In this section, we illustrate some real case studies where in silico methods are applied together with the wet test (in vitro tests). Until a few years ago, the word computational in food science identified statistical applications, QSAR or COMFA applications. Taking into consideration what has been previously done in the medicinal chemistry field, screening, molecular docking and scoring functions can be used to discover new possible endocrine disruptors from a large dataset of food contact chemicals, such as food additives [1]. Starting from a joint FAO-WHO database of 1500 chemicals, Amadasi and colleagues screened 31 compounds predicting 13 of them as potential xenoestrogens towards oestrogen receptor alpha. Four of these compounds have been previously reported as well-known ER endocrine disruptors. Thus, the in silico analysis confirmed the prediction. For the other nine compounds, the binding affinity and oestrogenic effects were determined using in vitro assays. The most interesting result is propyl gallate that is a widely used antioxidant (in particular in the fish industry), and hexylresorcinols (www.fao.org/ag/agn/jecfa-additives) are predicted as oestrogen receptor binders both by in

silico and in vitro analyses. It may be hypothesized that the latter has an indirect effect and facilitates the interaction between unliganded ER and coactivators, inducing the transcription of the reporter.

Recently, EFSA considered "Safety and efficacy of propyl gallate for all animal species" paper important for the panel on additives and products or substances in animal feed [28].

Kenda and co-workers conducted a screening of 1046 US-approved and marketed small-molecule drugs for estimating their endocrine-disrupting properties [16]. Binding affinity to 12 nuclear receptors was assessed with a molecular docking program, Endocrine Disruptome. They identified 130 drugs with a high binding affinity to a nuclear receptor that is not their pharmacological target. Another software, VitualToxLab, has been used to evaluate a subset of molecules, and the results have been compared with in vitro results from the Tox21 database.

Another interesting approach of nonstatistical in silico prediction to screen oestrogenic and androgenic activity and to decipher the mechanism of binding (MOA) of substances of very high concern (SVHC) for the European Union is the case of bisphenols [5].

Bisphenol A (BPA) has been considered at first as toxic for reproduction and subsequently as an endocrine-disrupting chemical that interferes with the endocrine system mimicking the effects of oestrogen. Some European countries banned BPA from industrial production to avoid contact with the food and consequently with the human organism. Instead of BPA, they allowed the use of bisphenol S (BPS) as an alternative less active. The authors analysed a series of BPA alternatives and derivatives with similar physical-chemical properties that have been produced and used by companies for substituting it. They evaluated the oestrogenic and androgenic binding activity of 26 BPs against six different nuclear receptors using literature in vitro data for comparison. In this specific case, they propose a rough classification of the results, high binder, medium binder and low binder compared to bisphenol A as a reference. This rough ranking list could be useful and faster for massive screening instead of complex statistical analysis.

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Chapter 13
Nuclear Hormone Receptors and Host-Virus Interactions

Nadine Ahmed, Noreen Ahmed, Roxana Filip, and John Paul Pezacki

Abstract Viruses are a diverse class of obligate parasites that require a host to propagate. Viruses have evolved to exploit host signaling pathways to promote propagation and facilitation of their life cycle. Host-virus interactions are a complex network that can enable the viral life cycle within the host. These interactions also allow for the host's immune system to overcome the infection. Successful viral infection and the resultant antiviral response are highly dependent on the dynamic molecular interactions between the viral components/factors and the host's antiviral and cellular signaling pathways. These interactions are also modulated by the cellular microenvironment that can be beneficial or detrimental to the viral life cycle. Recently, increasing evidence has emerged highlighting the role of the nuclear hormone receptor superfamily in facilitating host-virus interactions. The nuclear hormone receptor family is a diverse group of transcription factors that share analogous structure and architectures. They can be activated or repressed depending on the upstream signal. This chapter will focus on the diverse roles that nuclear hormone receptors play in modulating host-virus interactions, as well as highlighting the crosstalk between viruses and specific subtypes of nuclear receptors, namely, peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXRs), and retinoid X receptors (FXRs).

Keywords Virus replication · Virus life cycle · Beta-oxidation · Lipid metabolism · Immunometabolism · Antivirals

N. Ahmed · N. Ahmed · R. Filip · J. P. Pezacki (✉)
Department of Chemistry and Biomolecular Sciences, University of Ottawa, Ottawa, ON, Canada
e-mail: john.pezacki@uottawa.ca

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13.1 Introduction

Viruses are obligate parasites that heavily depend on the host's cellular machinery for propagation and production of viral progeny. They lack the molecular machinery required for the replication and spread of their viral genetic material. Viruses have evolved to dysregulate the host's metabolic processes and signaling pathways to favor the efficient progression of the viral life cycle [56, 85, 118, 172]. Viruses have been shown to redirect the cellular energy toward the production and assembly of progeny virions, thus hijacking the translational and metabolic machinery of the host. This digression in the equilibrium results in the dysregulation of the homeostatic balance in cellular signaling pathways that maintain a stable cellular microenvironment. In addition, viruses induce the remodeling of the host structural architecture to promote the replication and the multiplication of the viral progeny [115]. For example, *Flaviviridae* family is found to greatly remodel the host's cytoplasm to promote the replication of the viral RNA. Precisely, the virus associates with endoplasmic reticulum-derived membranes and alters the cellular lipid composition to promotes the formation of virus-induced cellular enclosures called replication organelles (RO) to facilitate its genomic replication [105]. These remodeling events and alteration in signal transduction pathways are tightly regulated by the action of receptors and transcription factors that control cellular and physiological function at the molecular level in order to fine-tune and shift the equilibrium toward a more favorable state for the virus.

The nuclear hormone receptor family is an abundant family of transcription factors that is ubiquitously expressed in a variety of different cell types [175]. This superfamily of transcription factors is composed of key players that modulate a vast majority of biological and physiological processes including metabolism, proliferation, and immune responses [146]. Additionally, this group of proteins has been shown to be involved in the development and the regulation of various pathological conditions, such as cancer, diabetes, and other metabolic conditions [146]. Mechanistically, nuclear hormone receptors regulate gene expression following a response to ligand activation, generally a lipophilic molecule. Commonly, these proteins bind to a specific DNA sequence to regulate the expression of gene downstream. The binding event acts as an on-off switch to control diverse regulatory processes in the cells ranging from development and metabolism to response to infection and immune responses [5, 146].

Historically, nuclear receptors have been generally characterized into three distinct classes. These classes include steroid/thyroid hormone receptors, orphan hormone receptors with unknown ligands, and finally adopted receptors with known and characterized ligands [5]. The adopted family of nuclear hormone receptors includes peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXRs), and retinoid X receptors (FXRs), which will be the focus of this chapter. Over the past few years, nuclear receptors have evolved to have a much-appreciated role in modulating host-pathogen interactions and regulating immune responses against viruses and other pathogens. Therefore, understanding the role of nuclear

receptors in modulating the antiviral responses and viral pathogenesis may create novel therapeutic avenues in the treatment of infectious diseases. In this chapter we will focus on highlighting the roles of PPARs, LXRs, and FXRs in modulating host-virus interactions. How viruses may alter the function, activity, and transcriptional levels of these nuclear hormone receptors to promote their propagation, replication, and dissemination will also be discussed.

13.2 Nuclear Receptor Structure and Function

Structurally, the nuclear hormone receptor family of proteins shares a high degree of similarities among the varying subcategories [61]. To date, roughly around 900 genes known to express nuclear hormone receptor proteins have been identified, making this group of proteins one of the most diverse families of receptors [22, 61]. This group of proteins is described as transcription factors activated by sterols, lipids, fatty acids, and other metabolites [50]. Based on that, they have shown to be involved in a wide variety of cellular processes such as developmental processes, metabolic processes, and cellular proliferation [5, 146]. They have been heavily associated with infection progression, where they contribute to the host-virus interplay [121]. To better understand this connection, it is useful to understand the structural components of these receptors and how these components contribute to viral pathogenesis or host immunity.

Most nuclear hormone receptors share common structural features, which makes them easy to identify and categorize accordingly. Common aspects include an N-terminal region known as the A/B domain, which is variable in length [61]. This domain is characterized by a constitutively active region identified as AF-1 and several autonomous transactivation domains [50]. On the other hand, the C-terminus contains a highly conserved region, which exhibits the DNA-binding functionality. This region is known as the transactivation domain or DNA-binding domain (DBD), which includes the P-box, with binding specificity to the AGGTCA motif [22, 50, 121]. In addition, the C-terminus houses the ligand-binding domain (LBD), which is linked to the DBD via a hinge region known as the C-terminus extension region [61]. The ligand-binding domain is independent from the AF-1 region in the N-terminus of the receptor, which harbors the transactivation function of the protein [22, 61, 121]. The ligand-binding domain, in addition to binding the appropriate ligand, contains an activation function within itself known as AF-2, which helps recruit transcriptional activators [22, 50, 121]. In general, the ligand-binding event regulates the nuclear hormone receptor allosterically, leading to surface regions within the receptor to be exposed for co-regulators and co-activators to bind and interact with hydrophobic regions to be more accessible to lipophilic regulatory molecules. The activation of the nuclear receptor is initiated by the binding of the receptor's ligand. Upon the association of the ligand, the receptor undergoes conformational and structural rearrangements which allow the recruitment of various cofactors that modulate the structure and function of the receptor [22, 50, 121].

13.3 PPAR Signaling and Host-Virus Interactions

Peroxisome proliferator-activated receptors (PPARs) are a part of the nuclear receptor family which have been found to be required for the transcription of genes essential for regulation of cellular processes ranging from lipid and lipoprotein metabolic processes to inflammation and immunity, all of which are indispensable for tissue and cellular function [116]. Imbalance in such PPAR-controlled cellular functions is found to result in the development of a range of metabolic and pathological diseases [176]. There are three primary isotypes within the PPAR subfamily: PPARα, PPARβ/δ, and PPARγ, encoded by NR1C1, NR1C2, and NR1C3, respectively [33, 68, 94]. A study that investigated the differences in the binding of the chimeric PPARα/β/γ proteins highlighted the requirement of AF-1 region for isotype-dependent gene activation and transcription [67]. PPARα/β/γ nuclear receptor subsets, generally, bind DNA as heterodimers with retinoid X receptors (RXR)-α/β/γ [42]. Following association of the dimer, the complex translocates into the nucleus, where it binds to consensus sequence spaced by a single nucleotide, termed peroxisome proliferator-responsive elements (PPREs) [5]. Binding to the PPREs results in the activation of the transcription of target genes required for the modulation of various biological processes depending on the upstream activation signal/ligand**.** Long-chain fatty acids and eicosanoids are examples of endogenous ligands that act on activating PPARα and PPARβ [103, 188]; on the other hand, PPARγ is generally activated by arachidonic acid metabolites such as 5-oxo-15(S)-hydroxyeicosatetraenoic acid and 5-oxo-eicosatetraenoic acid [3, 107]. Although the various PPAR isotypes share a high degree of structural similarities, they display different biological functions within the cells. For example, PPARα primarily regulates fatty acid catabolism, whereas PPARγ regulates lipid storage and adipogenesis [67, 92, 116]. Different functions can be attributed to different tissue distributions of the receptors. Additionally, PPARs are found to control the expression of putative long-chain fatty acid transporters such as CD36 and fatty acid transport protein (FATP) and as a result control the transport of fatty acids into hepatocytes and regulate hepatic lipid homeostasis [99]. As a result, the transported lipids can be stored as triglycerides or can be oxidized via β-oxidation. Both outcomes are controlled by the activity of PPARs [75].

Additionally, PPARs have been found to have a role in the suppression of various inflammatory and innate immunity regulatory transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), signal transducer and activator of transcription (STAT), and activator protein 1 (AP-1) [137, 168]. Given the role that such nuclear receptors play in regulating host cellular signaling pathways that are essential for the viral life cycle, it is not surprising that evidence has been emerging which highlights the role of PPARs in viral pathogenesis. Therefore, the interest in the development of novel drugs that targets PPARs for the treatment or the regulation of viral infection, in addition to other pathologies such as obesity, diabetes, and cardiovascular disease, has been increasing over the past decades.

13.3.1 PPARs and Hepatitis C Virus (HCV)

HCV affects a large percentage of the global population, with a prevalence of roughly 2–3% [119]. The infection results in chronic hepatitis in up to 80% of infected individuals, and it is the major cause of chronic liver disease and liver transplantation worldwide [8]. Additionally, the virus is considered the leading cause of liver steatosis, cirrhosis, and HCV-mediated hepatocellular carcinoma (HCC) [70]. Hepatitis C virus is highly dependent on the host's cellular lipid metabolism, cholesterol, and fatty acid biosynthesis [162]. It has been clear that the virus hijacks host machinery to promote its propagation in the host's cellular environment. Similar to other members of the *flaviviruses* family, HCV greatly alters the host's structural and cellular architecture to enhance its replication and progression [105, 115]. In particular, HCV alters cellular signaling to enhance lipogenesis and modifies the pathways involved in cholesterol/fatty acid biosynthesis in order to promote viral particle formation and facilitate genomic replication [43, 70, 142, 160]. An early study by Su and Pezacki et al. uncovered the critical involvement de novo lipid and cholesterol biogenesis in modulating HCV pathogenesis [160]. Moreover, HCV hijacks the very-low-density lipoprotein (VLDL) and LDL secretion pathways in order to promote viral entry and replication [46]. These events result in alterations in the de novo lipid and fatty acid biosynthetic pathways [46, 142, 160].

During chronic HCV infection conditions, upregulation of triglyceride and fatty acid synthesis in conjunction with a decrease in serum cholesterol levels is usually observed [162]. Pathways controlling these conditions have been found to be tightly regulated by several host factors that orchestrate the interactions between the virus and the host. Interactions between several such factors and viral proteins were proven instrumental for the progression of the viral life cycle in the infected individual. For example, Nasheri et al. confirmed the dysregulation of fatty acid synthase enzyme (FASN) activity and expression in hepatoma cells expressing the subgenomic and genomic HCV replicons. In the same study, the same phenotype was also confirmed in chimeric SCID/Alb-uPA mice infected with HCV genotype 1a [104].

Phosphatidylinositol 4-kinase (PI4K) is another example of a host factor which has been identified to play instrumental roles in promoting HCV replication. PI4K is a lipid kinase which has been found to promote a favorable lipid environment for HCV RNA replication [12]. Thus, modulation of host factors within key lipid regulatory pathways may indirectly suppress the infectivity and spread of HCV. Due to the high degree of dependence of HCV on the host's cellular lipid environment, it is therefore not surprising that the virus would fine-tune the function of regulators of such processes. Nuclear hormone receptors are master regulators of lipid homeostasis, biosynthesis, metabolism, and β-oxidation, all of which are processes that are intrinsically essential for HCV infection and propagation. Dysregulation of such processes has been observed during HCV infection [160]. It is, therefore, essential to understand the intricate crosstalk between HCV and nuclear receptor regulation of host cellular pathways during infection.

PPARs have been found to play crucial roles in the modulation of hepatic metabolic processes including lipid and glucose metabolism and homeostasis [176]. They have been highlighted as targets for the study of HCV-mediated alteration of host's cellular environment given their role in the regulation of various processes hijacked and dysregulated by the virus [36]. One of the most well-studied host factors which has been shown to have roles in modulating HCV pathogenesis is PPARα. PPARα is prevalent in hepatocytes, cardiomyocytes, and brown adipocytes and has been found to regulate the transcription of various genes involved in the fatty acid metabolism and oxidation. Some noteworthy PPARα-regulated gene targets are carnitine palmitoyl acyl-CoA transferase 1A (CPT1A) and fatty acid transporters CD36 and FATP [99, 120]. These PPARα-regulated gene targets have been shown to play instrumental roles in HCV life cycle.

Some studies have provided evidence for dysregulation in the levels and activity of PPARs during HCV infection. For example, in HCV-infected patients, it's been shown that PPARα activity is repressed which results in the suppression of CPT1A transcription, a PPARα target gene. As previously mentioned, CPT1A is a key gene responsible for the transport of long-chain fatty acids across the mitochondrial membrane. Thus, it seems that HCV alters the activity of PPARα to modulate the levels of genes essential for lipid homeostasis in the cell [25]. It is also possible that the altered activity is the result of the host response to the virus. Interestingly, Cheng et al. have highlighted that deficiency in the transcriptional activity and levels of PPARα results in HCV-mediated steatosis. Similarly, another study by Dharancy et al. suggested the same findings, where PPARα expression and activity were impaired in chronic HCV patients, which in turn resulted in lower levels of the PPARα target, CPT1A [32]. In the same study, the group observed an analogous phenotype in HepG2 hepatoma cell lines expressing HCV core protein [32]. Moreover, in a study by De Gottardi et al., the levels of PPARα and PPARγ were significantly lower with genotype 3 HCV infection relative to genotype 1. In the same study, the group demonstrated that HCV-induced steatosis is correlated with a decrease in the levels of PPARα and PPARγ. Thus, from this study, it is clear that both genotype 3 HCV infection and steatosis are associated with a decrease in the levels of PPARs [45]. Altogether, these studies suggest an association of PPARα/γ levels with HCV infection in human subjects.

Consistent with the previous study, Rakic et al. have demonstrated the suppression HCV replication in Huh7 cells through 2-chloro-5-nitro-N-(pyridyl)benzamide (BA)-mediated antagonism of PPARα activity [126]. Interestingly, in the same study, antagonism of PPARγ had no effect on HCV replication, suggesting that only PPARα activity is likely essential for the formation of membranous webs required for HCV replication [126]. Similarly, Lyn et al. [91] highlighted the effects of PPARα antagonism using 2-chloro-5-nitro-N-(pyridyl)benzamide (BA). They observed an induction of hyperlipidemia and increase in triglyceride levels in Huh7 cells. These changes in the cellular lipid microenvironment induced the disruption of HCV replication complexes [91].

A more recent study highlighted the role of calcitriol-mediated activation of vitamin D receptor in Huh7.5 cells in inhibiting HCV infection through the suppression of PPARα/β/γ activity [88]. Conversely, other studies have emerged that highlight the role of PPARα activation in the suppression of HCV infection. For example, bezafibrate, a PPARα agonist, has been found to decrease the levels of HCV titers in the serum of chronic HCV patients [38]. On the other hand, a study by Tanaka et al. has shown that activation of PPARα is essential for the pathogenesis of HCV. Interestingly, chronic treatment of mice with a PPARα agonist resulted in HCV-induced HCC and hepatic steatosis [163]. This may represent more complex dependencies that include the differential sensing and involvement of the innate and adaptive immune responses.

A more recent study by Goldwasser et al. demonstrated that the naringenin-mediated induction of PPARα levels and activity results in a dose-dependent decrease in the production of infectious HCV particle [44]. This may reflect differences in HCV genotype sensitivity as well as differential effects on different steps of the virus life cycle in cell culture models for infection. Figure 13.1 highlights some of the controversial roles of PPARα during HCV infection.

As previously discussed in this chapter, there is crosstalk between the activity of PPARs and the regulation of the inflammatory response during infection. Specifically, PPARα has been shown to control the inflammatory response in hepatocytes during infection by negatively regulating the activity of transcription factors that mediate inflammation, such as NF-κB and AP-1. Consistent with that, HCV core protein has been found to suppress the inhibitory effects of PPARα on NF-κB activity [87].

Connections between PPARγ activity and HCV have also been established. PPARγ activity controls several cellular processes involved in lipogenesis, glucose homeostasis, and inflammation. Studies have suggested that the expression of PPARγ is generally abundant in mature adipocytes and can be found preeminent in fatty livers eventually resulting in the development of liver steatosis [143]. Interestingly, PPARγ is usually referred to as the "master regulator" of lipogenesis [143]. Some of the lipogenic genes regulated by PPARγ are fatty acid synthase (FASN), acyl-CoA carboxylase (ACC), and stearoyl-CoA deyhydrogenase-1 (SCD-1) [41]. Inhibition of any of these genes using pharmacological inhibitors results in the restriction of HCV replication [104, 152, 160]. Given the dependence of HCV on the cellular lipid environment to promote its propagation, it is therefore not surprising that HCV NS5A has been found to induce the transcriptional activity of PPARγ and thus enhancing HCV-induced lipid accumulation in hepatocytes [78]. However in a conflicting study, a decrease in the levels of PPARγ was observed in huh7 cells expressing HCV core protein genotype 3a. Suppression of PPARγ results in a consequential decrease in the levels of suppressor of cytokine signaling 7 (SOCS-7), which has been found to be regulated by PPARγ transcriptional activity [117]. Overall, these findings highlight novel and often complex roles of PPARγ in regulating hepatic metabolism and signaling during HCV infection.

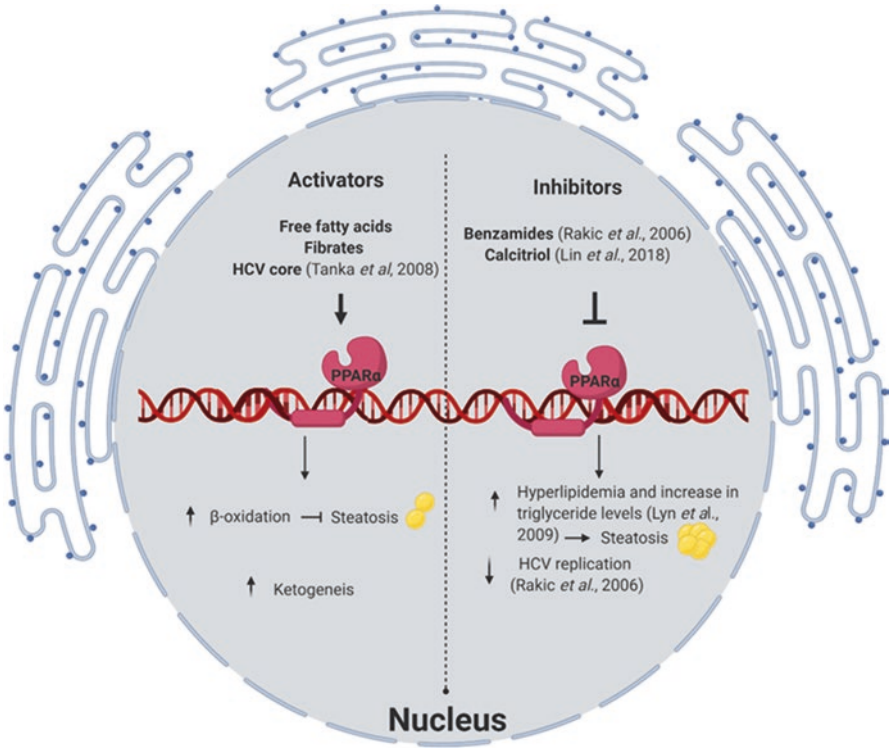

Fig. 13.1 PPARα modulation alters lipid microenvironment and affects HCV pathogenesis. Activation of PPARα chemically or endogenously results in the increase of β-oxidation, ketogenesis, and suppression of steatosis in hepatic cells. HCV core has been shown to activate PPARα-regulated transcription. Antagonism of PPARα results in hyperlipidemia and increase in triglyceride levels, which in turn results in hepatic steatosis. Some studies have demonstrated that inhibition of PPARα activity using benzamide can suppress HCV replication

13.3.2 PPARs and Flaviviruses

Members of the *Flavivirus* genus are arthropod-borne RNA viruses with high dependency on host lipids. The most common examples include the yellow fever virus, the dengue virus, Japanese encephalitis, West Nile virus, and the Zika virus [7]. The last two decades have revealed a complex set of interactions between flaviviruses and cell lipid metabolism. However, PPAR-*Flavivirus* interplay has yet to be defined. Indeed, dengue virus has been shown to increase fatty acid synthesis [56, 167], modulate cholesterol levels [20, 141, 156], and alter membrane compositions [83, 105, 178]. Tongulan et al. showed that genotype 2 dengue infection of HEK293T/17 cells increases PPARα levels for up to 3 days post-infection. However, the implications of this change and the connections between the nuclear receptor and the lipid variations mentioned above have not been further investigated to date.

In addition to its roles in lipid metabolism, PPARα is also known to mediate anti-inflammatory responses in the cell [17]. Another prevalent *Flavivirus*, the Japanese encephalitis virus (JEV) has been shown to cause severe neurological inflammation and lead to encephalopathy [180]. Interestingly, Sehgal et al. have shown that the PPARα agonist fenofibrate protects mice from JEV-induced inflammation and significantly reduces mortality when given prior to infection. Therefore, the prophylactic use of nuclear receptor agonists may be beneficial to combat certain viral infections.

Finally, the Zika virus has shown to be a reoccurring epidemiological threat with the latest outbreak in Central America in 2015–2016. This virus has been associated with microcephaly in newborns and Guillain-Barré syndrome in adults [179]. Recently, de Oliveira et al. used a metabolomics approach to identify biomarkers of the disease in the saliva of infants. From this screen, they identified 15-deoxy-Δ-12,14-prostaglandin J2 as a marker of congenital Zika syndrome. This metabolite is an endogenous ligand of PPARγ, suggesting that the receptor may be activated as response to microglial inflammation [109]. This points to a connection between Zika infection and nuclear receptor function in the central nervous system.

13.3.3 PPARs and Human Immunodeficiency Virus (HIV)

HIV is part of the *Retroviridae* family which has been shown to primarily infect CD4 T lymphocytes; however there are several other examples that highlight the ability of HIV to infect other cell types [73]. It has been estimated that approximately 36.7 million individuals are living with HIV worldwide in 2015 [138]. For cellular entry, it depends on the presence of a CD4 receptor, with which it interacts, as well as the chemokine receptors, CCR5 or CXCR4 [29, 30]. HIV is characterized by the presence of regulatory and accessory genes which modulate viral replication. HIV, similar to other families of viruses, requires specific and often tightly regulated conditions to propagate efficiently. Thus, it is of interest to understand the crosstalk between HIV and the host cell and how the host modulates and alters cellular functions as a response to HIV infection. Among the host factors that were shown to be involved in HIV pathogenesis, the PPAR nuclear hormone receptors are especially noteworthy.

Several early studies examined the effects of PPAR activation in context of HIV infection. A study by Skolnik et al. explored the roles of drugs known to modulate the activity of PPARs, namely, thiazolidinediones (TZDs), which are frequently used to treat type II diabetes, and hyperglycemia, in HIV infection [155]. In this study, Skolnik and colleagues demonstrated the efficiency of TZD-related PPARα and PPARγ agonists in inhibiting HIV replication. Particularly the group investigated the effects of the agonists in peripheral blood mononuclear cells (PBMCs) infected with HIV-1, in chronically infected monoblastoid cell line and in alveolar macrophages from HIV-1-infected and HIV-uninfected individuals [155].

Treatments with PPAR agonist resulted in the suppression HIV-1 levels in all investigated models. Intriguingly, treatment with the drugs also resulted in a decrease in the levels of TNFα, which has previously been shown to increase HIV-1 replication [155]. Similarly, a study by Hayes and colleagues confirmed the suppressive effects of PPARγ agonism on HIV replication [55]. In particular, the group took advantage of cyclopentenone prostaglandins (cyPG), which have been previously shown to act as PPARγ agonists, to investigate the effects of PPARγ agonism on HIV replication. This study further highlighted the suppressive effects of PPARγ activity on HIV-1 gene expression in macrophages [55]. Moreover, a more recent study by Hanley et al. has likewise highlighted the role of PPARγ activation in the repression of HIV infection in macrophages. More specifically, the group highlighted that the inflammatory response mediated by PAM3CSK4, a TLR1/2 agonist, which is required for the activation of HIV replication and propagation in infected cells and macrophages is potently suppressed following PPAR activation [54]. Overall, these studies highlight the major role that PPARs play in modulating HIV infections in various cell types.

The PPAR nuclear receptor subfamily has also been found to be involved in the modulation of host-HIV interactions. A prominent biological function of PPARs is the regulation of oxidative stress and inflammatory responses during infection [31, 40]. Previously, Ramirez et al. have reported that inflammatory responses induced by HIV infection is often subverted by PPAR agonism using synthetic ligands, which include fibrates for PPARα-specific activation and TZDs for PPARγ-specific activation [129]. In particular, the group demonstrated that PPARγ activation in primary human brain endothelial cells (BMVEC) significantly suppresses the adhesion and migration of HIV-1-infected monocytes across primary endothelial cells [129]. Conversely, PPARα activation failed to elicit the same response/phenotype. Functionally, the group demonstrated that PPARγ activation specifically suppressed the activity of Rac1 and RhoA GTPases. Inhibition of their activity prevents the adhesion and migration of HIV-1-infected monocytes across the endothelium [129].

Several more recent studies have suggested that PPARγ activation results in a neuroprotective phenotype in the context of HIV-1 infection [65, 66, 123]. For example, Huang et al. have highlighted the protective role of PPARs against HIV-induced disruptions of tight junctions responsible for the integrity of the blood-brain barrier (BBB) in HIV-infected patients [64]. These HIV-induced modifications are often subverted by the activation of PPARα- and PPARγ-mediated cellular signaling. Functionally, PPARα and PPARγ activation suppresses the function of matrix metalloproteinase (MMP) 2 and 9 and proteasome activities which aids in the maintenance of the BBB integrity [64]. A more recent study by Omeragic et al. highlights the role of rosiglitazone and pioglitazone, PPARγ agonists, in inhibiting the induction of gp120-induced inflammatory response in primary cells of mixed glial cells and in a HIV-1 gp120 rat model [110]. A more recent study by the same group emphasized the role of PPARγ agonist treatment in promoting anti-HIV and anti-inflammatory responses in an EcoHIV in vivo model of HIV infection [111]. Moreover, additional studies showed that the use of rosiglitazone results in the inhibition of HIV infection in other cell lines including Th1Th17 cells and macrophages

[13, 123]. On the other hand, pioglitazone has as well displayed potential in clinical trials for the treatment of HIV-1-associated lipodystrophy syndrome (HALS) [124].

Recent advancement in HIV research, such as development of HIV antiretroviral treatments (HAART), has resulted in the increase and extension of life expectancy and the quality of lives of HIV-infected patients. However, this came with the development of various other chronic conditions because of the toxicity associated with such treatments. Chronic HIV infections are often characterized by lipodystrophy, which has been reported in 50% of HIV patients, and bone abnormalities [11]. Adipocyte and osteoblast development and maintenance is often controlled by the activity of PPARs [11]. In addition, it has been reported that antiretroviral treatments may cause alterations in free fatty acid flux and accumulation of intramyocellular lipids [39, 52]. In addition to these dysregulations, a reduction in the levels of PPARγ has also been observed in subcutaneous adipocytes of HIV patients undergoing antiretroviral treatments [10]. Overall, these findings provide new avenues for the development of effective therapeutic strategies that could acclimatize the levels and activity of PPARs to improve the quality of lives of HIV-infected patients and develop avenues for potential novel treatments and cures.

13.3.4 PPARs and Other Examples of Viruses

13.3.4.1 Hepatitis B Virus

HBV is an enveloped, partially double-stranded DNA virus which belongs to the *Hepadnaviridae* family. Its life cycle includes the repair of its incomplete relaxed circular DNA (rcDNA) to form covalently closed circular DNA (cccDNA). The cccDNA is transcribed into subgenomic RNA (sgRNA) and pregenomic RNA (pgRNA), the latter of which will be reverse transcribed into DNA before being packaged into new virions [47]. Four promoter (core, pre-S1, pre-S2/S, and X) and two enhancer (EN1 and EN2) regions regulate translation [4]. The EN2-core promoter controls the synthesis of the pgRNA, and interestingly it possesses multiple binding sites for host hepatic nuclear receptors, including RXR-PPARα heterodimers [130]. Binding of the RXR-PPAR complex was also shown to occur at the first enhancer [58]. Interestingly, RXR-independent activation of HBV genome transcription by PPARα was also demonstrated in transgenic mice lacking the heterodimer-binding site [131]. Consistent with these observations, Guidotti et al. showed that treatment of HBV transgenic mice with agonists of PPARα significantly increased HBV replication, although they modestly increased genome transcription [48]. Furthermore, Tang and McLachlan demonstrated that expressing RXRα and PPARα in non-hepatic cell lines allows the cells to support HBV replication, suggesting that these nuclear receptors are important in determining viral tropism [166].

In addition to PPARα, there is a growing body of evidence for interactions between HBV and PPARγ. However, the effects of this nuclear receptor on HBV infection have been controversial. Indeed, Choi et al. showed that the HBV X

protein, HBx, binds the receptor and interferes with its nuclear localization. PPARγ activation decreases cell growth and promotes apoptosis, reducing cancer proliferation [139]. It is therefore thought that this HBx-PPARγ interaction plays a role in HBV-induced HCC [27]. On the other hand, Kim et al. showed that HBx overexpression induces the expression of PPARγ and upregulates downstream genes implicated in hepatic steatosis [77]. From a pharmacological perspective, Wakui et al. reported inhibition of HBV replication using the PPARγ agonist rosiglitazone in HepG2 cells. Interestingly, they did not see any effects on the virus with the PPARα agonist bezafibrate [174]. In contrast, Yoon et al. showed that treatments of HBV expressing HepG2 cells with rosiglitazone upregulated viral replication. They also reported that silencing the PPARγ-controlled gene adiponectin reduces HBV infection [186]. From a clinical standpoint, Zhao et al. showed that chronic hepatitis B patients have significantly lower PPARγ expression due to increased methylation of the gene [193]. Interestingly, reduced methylation and recovery of PPARγ expression improved prognosis of patients with acute-on-chronic hepatitis B liver failure [194]. Most recently, Du et al. treated HBV-infected mice with bezafibrate, fenofibrate (a PPARα agonist), and rosiglitazone. In all cases, the viral mRNA, DNA, and serum antigen loads increased, suggesting that agonism of both PPARα and PPARγ increases HBV infection [34]. Based on all these studies, interactions between the hepatitis B virus and PPARs are complex, and the observed effects may be specific to the models and conditions used. Therefore, there is an ever-present need for more research in this area.

13.3.4.2 Influenza A Viruses (IAVs)

IAVs are negative-sense, single-stranded, segmented RNA viruses [9]. Several subtypes have been identified to be the etiological agents of influenza in birds and mammals. Influenza viruses cause heterogeneous respiratory infections which can range from self-limiting symptoms that are mild to more serious non-resolving pathology in the lungs. Worldwide, more than 500,000 lives are claimed yearly by influenza [62]. More recently, immunomodulatory agents have been used as potential treatment approaches to reducing mortality in both seasonal and pandemic influenza [37]. Agonists of PPARα have been shown to affect lipid metabolism but were also demonstrated to have immunomodulatory effects. PPARα and PPARγ agonists (Glitzones and Fibrates) were shown to reduce the mortality in patients with viral pneumonia caused by IAV, as well as in mouse models of IAV [37]. In an early report by Moseley et al., evidence underscoring the success of PPARγ agonists in providing protection in mice infected with highly pathogenic influenza A strains was demonstrated [98]. Pre-treatments with PPAR agonist, rosiglitazone, produced protection against the infection by reducing the morbidity and mortality of infected mice [98]. A later study by Huang et al. further strengthened the association between PPARs and their roles during IAV infection. In this study, PPARγ was shown to be downregulated in alveolar macrophages (AM) following IAV infection [62].

Additionally, myeloid PPARγ deficiency caused by the infection led to a higher rate of host morbidity and increased inflammation [62], thus further cementing the connection between the critical role of PPARγ expression in the lungs and the modulation of the viral response. Furthermore, mice with intrinsic deficiency of myeloid PPARγ showed increased influenza-induced deposition of collagen in the lung tissue, leading to a dysfunctional lung model. Deficiencies in activation of damage repair genes were observed in the cells [63]. It is evident that activation of PPARs, which are critical regulators of inflammation, plays a protective role against IAV infections since they can antagonize inflammatory responses [9].

13.3.4.3 Herpesviruses

Herpesviruses belong to the family of *Herpesviridae*, which is a large family of DNA viruses [165, 172]. Nine herpesviruses are known to infect humans including herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), Epstein-Barr virus (EBV), and human cytomegaloviruses [59, 122, 140]. With respect to host's lipid metabolism, studies have explored the role of these pathways in modulating herpesvirus infections [59, 165]. A unique feature of herpesviruses is that they undergo both a lytic replication cycle and exhibit latency [1]. Drastic differences between both life cycles lead to different requirements for host-derived metabolites. Thus, there is a continuous need for the understanding of the interplay between the viral infections and host lipid metabolism. HCMV is the leading infectious causing agent for congenital defects [165].

HCMV uses a cholesterol-mediated cell entry process [122]. It has been previously shown that treatments with statins, causing inhibition of cholesterol synthesis, led to an antiviral response [122]. Furthermore, HCMV glycoprotein B (gB) was demonstrated to be post-translationally palmitoylated [114]. Due to the reported connections between lipid metabolism and HCMV, it is apparent that nuclear receptors play a part in infection modulation. In an early report by Rauwel et al., it was demonstrated that HCMV infection induces the expression of PPARγ in cells. PPARγ antagonism was shown to impair viral particle production [132]. Additionally, a more recent report by Rolland et al. showed similar effects of HCMV infection in both neural stem cells (NSCs) and brain sections from infected fetuses [140]. HCMV infection was shown to induce the expression of PPARγ. Levels of 9-hydroxyoctadecadienoic acid (9-HODE) were shown to be elevated in infected NSCs. 9-HODE is an endogenous PPARγ ligand [140]. Treatments of uninfected cells with 9-HODE recapitulated the effect of infection on PPAR expression [140]. Overall, these results reveal the role of PPARγ in modulation of HCMV infection. These studies highlight the present link between nuclear receptors and herpesviruses, specifically HCMV.

13.4 Liver X Receptors (LXRs) and Host-Virus Interactions

LXRs are nuclear hormone receptors that act as "sterol sensors," and they respond to the physiological concentrations of sterols in the cells [90]. Their roles in the modulation of host-pathogen interactions have been proven integral for both the host and invading pathogens [81]. Historically, these receptors were identified as orphan receptors with unclear classification. Later, these receptors were classified as nuclear receptors that respond to cholesterol metabolites, oxysterols [69]. Some of the natural ligands for LXRs include derivatives of oxysterols such as 25-hydroxycholesterol (25-HC), 27-hydroxycholesterol (27-HC), 22(R)-hydroxycholesterol, 24(S), 25-epoxycholesterol [24(S), 25-EC], and 5α,6α-epoxycholesterol [5,6-EC] [14, 69, 82]. LXRα (NR1H3) and LRXβ (NR1H2) are two distinct genes that encode two isoforms of the LXR nuclear receptors [69]. While LRXβ is ubiquitously expressed, LXRα expression is restricted to metabolic tissues including the liver, kidney, adipose tissue, and macrophages [136].

Cholesterol regulation has been found to play instrumental roles in modulating the life cycles of various viruses, especially viruses that require enrichment of the lipid and cholesterol microenvironment for their propagation [15, 89, 102, 147, 190]. LXRs play an opposing role to sterol response element-binding proteins (SREBP). Their primary function is to sense oxysterols in the environment and respond by increasing the expression of various genes that regulates cholesterol transport/efflux and the conversion of cholesterol to bile acid and to promote intestinal cholesterol absorption [192]. Some of the genes regulated by LXRs encode phospholipid transport proteins, apolipoproteins and ATP-binding cassette (ABC) transporters, ABCG5 and ABCG8 [135], as well as cholesterol 7α-hydroxylase (CYP7A1), one of the first identified targets of LXRs and an enzyme known to be involved in catalyzing the rate-limiting step of the bile acid biosynthesis pathway [49].

LXRs are as well involved in the regulation of triglyceride and fatty acid synthesis. These functions are mediated through LXR-controlled genes such as fatty acid synthase (FAS), ACC, and SCD1 [71, 134, 149, 187]. The utilization of LXR agonists has been shown to enhance HDL-mediated tissue cholesterol efflux, thus resulting in free-circulating HDL molecules [157]. Moreover, an enhanced incorporation of fatty acids into phospholipids and triglycerides is observed in LXR knockout C57BL/6 mice [79]. Thus, given LXR's role in regulating fatty acid, triglyceride, and cholesterol biosynthesis pathways, it is not surprising that it has been identified as an attractive therapeutic target for various metabolic diseases, such as liver steatosis, and cardiovascular diseases such as atherosclerosis [16, 84].

As previously mentioned, for some viruses such as viruses in the *Flaviviridae* family, fatty acid and cholesterol biosynthetic pathways have been proven vital for viral propagation and dissemination. These processes are generally hijacked by the viruses to facilitate viral replication and the production of viral progeny. Given that LXR nuclear receptors are one of the major regulators of such processes, it is not surprising that they play instrumental roles in modulating host-virus interactions.

Recently, there is an increasing interest in the developments of drugs and small molecules that regulate the activity of LXRs to suppress the life cycle of viruses that depend on fatty acid and cholesterol metabolic processes and thus create an LXR-dependent intrinsic antiviral state.

13.4.1 LXR and HCV

Over the past years, the role of LXR in regulating the viral life cycle during infection has become more apparent. In recent studies, LXR has been reported to control HCV infection and particularly the entry of the virus through modulation of the levels of LDL receptor (LDLR) [189, 190]. In particular, idol, an LXR-inducible E3 ubiquitin ligase, has been found to post-transcriptionally regulate the expression levels of LDLR through the promotion of the ubiquitination of its cytoplasmic domain [189]. Zeng et al. have demonstrated a decrease in the levels of infection of HCV JFH-1 strain in HCV-susceptible Huh7.5.1 hepatoma cell line with the overexpression of LXR-inducible protein, idol [190]. They were also able to demonstrate the inhibitory effects of LXR synthetic ligands (T0–901317 or GW3965) on HCV entry and intracellular levels. Conversely, Nakajima et al. have highlighted the inhibitory effects that a fungus-derived antagonist of LXRs, neoechinulin B (NeoB), exhibits on the HCV life cycle. In particular, the group demonstrated that the treatment of HCV-permissive cells with the compound results in the inhibition of the formation of specialized HCV replication sites and membrane compartments essential for the viral replication cycle [102]. The formation of these sites is controlled by mechanisms modulated through LXR-mediated transcription [102]. Overall, this work opened potential avenues to explore for the impediment of HCV life cycle through LXR-dependent mechanisms.

Another study that highlights the role of LXR-mediated signaling in modulating HCV infection has revealed the potential role of the LXR-regulated gene, ATP-binding cassette transporter A1 (ABCA1), in inhibiting HCV infection [15]. In this study, Bocchetta et al. revealed the importance of cholesterol efflux modulated by ABCA1 function in suppression of HCV infection in hepatoma cells [15]. In this study the group treated hepatocytes with GW3965, an LXR agonist, which resulted in the upregulation of ABCA1 levels and consequentially a decrease in the levels of the virus [15]. ABCA1 is a transmembrane protein that regulates the transfer of cholesterol and phospholipids across the cellular membrane and controls the formation of lipid-free ApoA1, which is crucial for the formation of HDL particles [113, 164]. The function of ABCA1 is essential for the maintenance of cholesterol homeostasis in liver cells. Disruptions in physiological cholesterol levels are generally a characteristic of initial phases of viral infection [173]. Thus, it is not surprising that dysregulations of the levels of ABCA1 levels is generally observed during vial infections.

An earlier study by Moriishi et al. confirmed the role of HCV core protein in the promotion of hepatic steatosis through LXRα-/RXRα-mediated transcription of

SREBP-1c [95]. This activation promotes the initiation of fatty acid biosynthetic pathway due to the activation of sterol regulatory element-binding protein 1 (SREBP-1c) promoter activity. In the same study, the group highlighted the role of PA28γ/REGγ, a proteasome-activating protein, in the degradation of HCV core protein in the nucleus of hepatocytes [95]. They revealed that the HCV core-induced binding of LXRα/RXRα to the LXR response element in the SREBP-1c gene only occurs in the presence of PA28γ [95]. Thus, HCV-induced hepatic steatosis is mediated through the interactions between PA28γ and LXRα/RXRα.

A more recent study by Singaravelu et al. highlighted the role of 25-hydroxycholesterol (25-HC), an LXRα-activating endogenous oxysterol, in inducing the transcription of microRNAs that display potent antiviral activity against HCV infection in Huh7.5 hepatoma cell line [89, 151]. In this study, the group identified, through employing a small molecule-mediated annotation of miRNA targets (SMART) screen, 25-HC-induced miR-185 and miR-130b which displayed antiviral activity against HCV [151]. Overall, these studies highlight the role of LXR-mediated transcription in modulating HCV pathogenesis and hepatic metabolism during infection. Figure 13.2 illustrates some of the effects of LXR modulation on HCV pathogenesis.

13.4.2 HIV-1 and LXR

Sterols and oxysterols have been shown to play crucial roles in HIV life cycle and infectivity [97]. Specifically, HIV-1 requires cholesterol for the assembly and budding of the virions from its target cells [97, 101, 127]. HIV-1 budding occurs at cholesterol-enriched "lipids rafts," and consequently, it has been shown that there is a higher ratio of cholesterol to phospholipids within the viral envelope [18]. It has been reported that cholesterol depletion markedly reduces HIV-1 particle production and release decreasing infectivity of the produced virions. Due to these connections, it is evident that the LXR-regulated signaling can play crucial roles in the modulation of viral progression and production [112, 195]. Both the cholesterol-related and trans-repression activities of LXR contribute to reported suppression of HIV-1 progression and infectivity. Depletion of cholesterol from lipid rafts lead to a decrease in infectivity and virus production [97, 101].

Due to this reported connection, cholesterol-sequestering drugs have been utilized as a mean to inhibit viral infectivity. β-Cyclodextrin, a cholesterol-sequestering drug, has been designed to inhibit viral entry by inhibiting viral attachment to target cells, preventing the membrane fusion step [51]. Morrow et al. have revealed that the use of LXR agonists can affect HIV-1 replication, including T0–901317, a potent stimulator of ATP-binding cassette transporter A1 (ABCA1) expression. T0–901317 restored cholesterol efflux from HIV-1-infected T lymphocytes and macrophages. This ligand has been utilized to suppress HIV-1 replication in these cell models, as well as in ex vivo cultured lymphoid tissue, highlighting the connection between the stimulation of LXR pathways and the induction of ABCA1, which

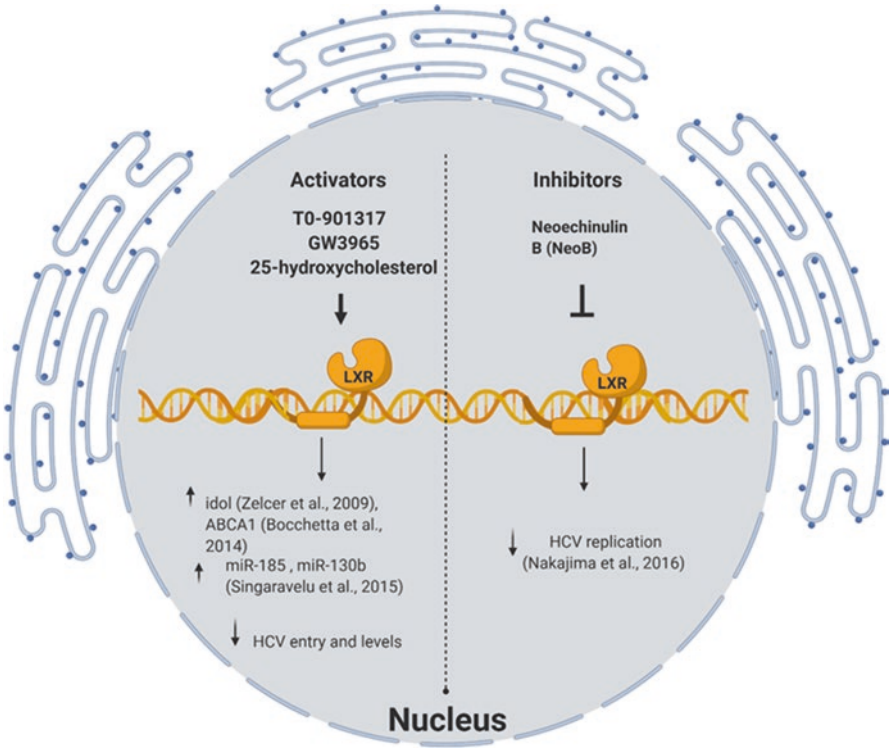

Fig. 13.2 LXR activity modulation alters lipid microenvironment and affects HCV pathogenesis. Activation of LXR results in the increase of idol, an LXR-inducible E3 ubiquitin ligase, which has been demonstrated to suppress the levels of HCV. Treatment of hepatoma cells with LXR agonists (T0–901317 or GW3965) results in the suppression of HCV entry and levels. LXR activation induces ABCA1 transcription, which has been demonstrated to inhibit HCV infection. 25-Hydroxycholesterol (an endogenous LXR agonist) was shown to induce the transcription of miR-185 and miR-130b, which were demonstrated to promote an anti-HCV environment. Conversely, an antagonist of LXR (neoechinulin B) has been displayed to suppress HCV replication

leads to inhibition of the HIV-1 replication cycle [97]. Dubrovsky et al. have demonstrated that treatment with T0–901317 potently reduced viral replication, and they were able to show the prevention of HIV-1-mediated reduction of plasma HDL in humanized NOD.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/SzJ (NSG) mice [35].

Stimulation of LXR has been linked to inhibition of dendritic cell-mediated HIV-1 capture and *trans*-infection [54]. Dendritic cells (DC) contribute to HIV-1 transmission by capturing and transporting infectious particles from the mucosa to the draining lymph nodes, thus transferring the virus to CD4+ T cells. Hanley et al. have shown that LXR prevented DC capture of HIV-1 by decreasing DC-associated cholesterol, due to LXR signaling inducing cholesterol efflux from DC [53]. In addition, LXR stimulation has been linked to ABCA1-mediated cholesterol efflux from DC, which is related to the decreased ability of capturing HIV-1 particles.

LXR ligands have been connected to the repression of DC-mediated trans-infection and DC migration, leading to HIV-1 inhibition [53, 54].

HIV-1-related comorbidities involving diseases such as metabolic and cardiovascular disorders have also been linked to cholesterol metabolism and lipid rafts. More specifically, Nef, a viral protein, has been linked to an increase in viral infectivity by promoting viral budding from lipid rafts [195]. Stimulation of ABCA1 expression via LXR agonists was shown to counteract the effect of Nef [86, 161].

In another experiment by Trabattoni et al., it was demonstrated that thiazolides (TZDs) induce the expression of cholestrol-25-hydroxylase (CH25H), which is an interferon-stimulated gene (ISG) responsible for the generation of 25-HC [170], suggesting that this upregulation could be linked to innate immune responses and production of ISGs involved in cholesterol biogenesis and LXR-regulated pathways, leading to a potent antiviral responses. An 87% inhibition of HIV-1 replication was observed in vitro upon treatment with TZDs, once again linking the potent inhibitory effect on HIV-1 replication to the reduction of intracellular cholesterol levels and systemic administration of LXR agonists or activators of LXR-mediated signaling [170].

Conversely, LXR has been linked to HIV-1 viral pathogenesis. HIV patients have been shown to develop hepatic steatosis and fatty liver disease [2]. A study by Agarwal et al. has reported that transgenic mice-expressing HIV-1 Vpr developed high levels of liver triglyceride leading to hepatic defects such as de novo lipogenesis (DNL). DNL was linked to co-activation of LXR-α by Vpr, leading to increased expression of targets such as SREBP1c, carbohydrate-response element-binding protein (ChREBP), liver pyruvate kinase (LPK), diglyceride acyltransferase (DGAT), FASN and SCD1, and intranuclear SREBP1 and ChREBP [2]. The evidence presented here suggests an association between the LXR response and HIV pathogenesis. Further research needs to be conducted to better understand the molecular mechanism by which this level of regulation is controlled.

13.4.3 LXR and Other Examples of Viruses

13.4.3.1 Influenza A Viruses

Viral pathogens have been shown to interfere with macrophage cholesterol metabolism mainly through inhibition of the LXR signaling pathway [21, 170, 191]. This specific phenomenon has been attributed to the activation of Toll-like receptors (TLR) 3 and 4 by viral pathogen-associated molecular patterns (PAMPs) leading to blocking of LXR target genes including ABCA1 [21]. An early report demonstrated that upon infection of peritoneal macrophages with influenza A in the presence of LXR agonist GW3965, inhibition of LXR target genes was achieved; however the LXR mRNA was not affected [21]. Overall, it is reported that LXR gene expression is strongly compromised in macrophages upon influenza A viral infection, thus linking the innate immunity component to LXR signaling. This was shown to be

achieved through the induction of interferon β (IFN- β) and myxovirus resistence-1 (MX-1) [21].

13.4.3.2 Herpesviruses and LXR

Additionally, a connection has been established between the role LXR and DNA viruses, namely, the herpes simplex virus 1 (HSV-1) [19, 89]. HSV-1 is a member of *Herpesviridae*, which causes viral infections in humans. HSV-1 consists of a double-stranded, linear DNA genome [19]. 25-HC and 27-HC, which were shown to exhibit antiviral effects, have been reported to induce the expression of CH25H in an LXR dependent manner, leading to the conclusion that LXR plays a fundamental role in the antiviral response [19, 89]. Liu et al. have shown that activation of LXR using either natural ligands or synthetic agonists leads to a decrease in HSV-1 infection in HepG2 liver cells or RAW 264.7 macrophages.

Interestingly, it was validated that using genetic deletion of LXRα or LXRβ leads to increased HSV-1 infection and susceptibility. The same phenotype was achieved upon knockout of CH25H, cementing the connection between the role of LXR and the antiviral mechanism against HSV-1 infection [89]. Additionally, LXRs have been shown to modulate gammaherpesviruses. Gammaherpesviruses are viruses that establish persistent lifelong infections [80]. More recently, Lang et al. have demonstrated that deficiency in LXRα/β leads to an increase in cholesterol and fatty acids in the primary macrophages, which in return led to an increase in gammaherpesvirus replication, confirming the antiviral role of LXRs [80]. Upon infection, type I interferon caused an induction of LXRs; however, this was associated with suppression of the target genes' expression, leading to the decrease of fatty acid and cholesterol synthesis and underscoring the intrinsic metabolic mechanism leading to restriction of viral replication in innate immune cells [80]. Furthermore, it was later confirmed that LXRα restricts the reactivation of gammaherpesviruses from latently infected peritoneal cells [81]. LXRα was shown to restrict the reactivation through a mechanism independent of the $CD8^+$ T-cell antiviral response [81].

13.4.3.3 HBV

Furthermore, other viruses that are dependent on components of liver metabolism were shown to be linked to LXR signaling. HBV infection is closely related and associated with liver metabolism, so it is no surprise that the LXR pathway can play a crucial role in this virus' interaction with its host cell [148]. LXR is a crucial regulator of lipid and cholesterol metabolism, which are essential in HBV's life cycle [148]. In a study by Zeng et al., the role of LXR in HBV infection has been studied. HBV-infected primary hepatocytes were shown to potently inhibit the infection if treated with synthetic LXR agonists including T0901317, GW3965, and LXR-623. Reduction of viral RNA was observed, underscoring the role of LXR activation in

eliciting a potent anti-HBV response. This potent inhibition was linked to sustained reduction of covalently closed circular DNA (cccDNA) [191]. Thus, all the previous examples illustrate the interplay between the different viruses and the LXR pathway. Through modulation of the different components of the pathway, it is evident that viruses can promote their own replication or propagation in the host cell; also, the host can affect the virus life cycle by controlling the expression of pathway regulators.

13.5 FXR and Host-Virus Interactions

The Farnesoid X receptor (FXR), also known as bile acid receptor, is nuclear receptor mainly expressed in hepatocytes and enterocytes. Its primary function is to regulate synthesis, transport, and metabolism of bile acids. However, it is also known to regulate aspects of lipid metabolism and lipoprotein homeostasis, making it an important host factor in interactions with hepatotropic lipid-dependent viruses such as hepatitis B and C.

13.5.1 FXR Signaling

Bile acids are synthesized from cholesterol in the liver, stored in the gallbladder, and secreted into the intestine postprandially to facilitate emulsification and absorption of dietary fats. Although 95% of these acids are recovered in the ileum, 5% are lost through excretion and need to be synthesized de novo in the liver each day. In humans, the principal bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA) [26]. In order to prevent toxicity from overaccumulation of these bile salts, they exert a complex feedback mechanism to regulate their own synthesis. In 1999, Makishima et al. identified FXR as a nuclear receptor capable of biding bile acids. Activation of this receptor was shown to downregulate levels of CYP7A1, the first and rate-limiting enzyme in bile acid synthesis [93]. This regulation does not result from direct binding of FXR to the CYP7A1 promoter but rather through FXR-mediated control of the atypical nuclear receptor small heterodimer partner (SHP).

Activation of FXR through binding of bile acids leads to the formation of a FXR-RXR heterodimer which induces expression of SHP. In turn, this partner interacts with the hepatic nuclear factor 4 (HNF4) or the liver receptor homolog-1 (LRH-1) to directly reduce CYP7A1 expression [28]. An alternate mechanism has been identified through which binding of bile acids to FXR in enterocytes leads to expression and secretion of fibroblast growth factor 15 (FGF15). This factor binds FRFR4 receptors on hepatocytes and leads to repression of CYP7A1 expression via activation of the c-Jun N-terminal kinases (JNK) pathway [171].

More recent work has focused on FXR's role in regulating lipid and glucose metabolism. This was based on the early observation that FXR null mice had

elevated levels of not only cholesterol but also triglycerides and lipoproteins in the sera [150]. In contrast, agonism of the receptor decreases plasma triglycerides and VLDL secretion. A study of the mechanisms underlying these changes identified several metabolic genes under the control of the FXR receptor. The previously mentioned effects were found to be mediated via repressed expression of SREBP-1c and microsomal triglyceride transfer protein (MTP), both of these genes being under the indirect control of SHP [57, 177]. Additionally, activation of FXR directly increases expression of apolipoprotein C2 (ApoC-II), a cofactor for lipoprotein lipase, a hydrolase responsible for the breakdown of triglycerides from chylomicrons and VLDL [74]. Other apolipoproteins under the direct or indirect control of FXR include ApoAI, ApoB, ApoCIII, and ApoE [72]. Furthermore, hepatic lipase (HL), an enzyme responsible for remodeling high-density lipoprotein (HDL) particles, has also been shown to be downregulated with FXR ligand treatments [153]. Some effects of FXR activation on triglyceride stores have been linked to induced expression of PPARα [169]. Finally, levels of VLDL receptor (VLDLR) have also been shown to be induced with FXR agonism [154]. Therefore, through modulation of these central factors as well as other target genes, FXR can significantly affect lipid pools in the liver. These changes can be meaningful when considered from the perspective of host-pathogen interactions.

13.5.2 FXR Signaling During HCV Infection

Given the extensive role of the FXR in hepatic metabolism, it is not surprising that its activation could modulate HCV replication. The exact mechanisms through which FXR signaling and the HCV life cycle are linked are still not fully understood, but there is a significant overlap between the metabolic processes controlled by FXR and host factors implicated in HCV pathogenesis. As previously discussed in the chapter, the virus induces hepatic steatosis, decreases serum cholesterol, promotes accumulation of lipid droplets in hepatocytes, and hijacks VLDL assembly [6, 60, 96, 145]. Chronic HCV infection and persistent inflammation often lead to fibrosis, cirrhosis, and hepatocellular carcinoma [24, 158, 185].

Interestingly, two early reports have shown that FXR agonism increases HCV replication, suggesting that elevated levels of bile acids in the liver may worsen viral infection [23, 144]. In both studies, activation of the receptor was achieved by treatment with free bile acids including deoxycholic and chenodeoxycholic acids. These treatments stimulated the levels of HCV 1a and 1b in replicon models, while FXR antagonism by guggulsterone countered this effect [23, 144]. Interestingly, the JFH1-2a genotype was not affected by these treatments [144]. Most recently, Wu et al. showed that the FXR agonist GW4064 inhibits JC1 HCV entry by downregulating protein, but not mRNA, levels of the scavenger receptor class B type I (SR-BI), a receptor required for viral entry. This group also saw a decrease in HCV levels with two other synthetic FXR agonists as well as with CDCA treatment and overexpression of FXR [181]. However, they do not convincingly uncover the

mechanisms behind these observations. Therefore, the effects of the FXR pathway on the life cycle and infectivity of the hepatitis C virus lack consensus and require further investigation. Figure 13.3 illustrates some of the proposed interplay between FXR signaling and HCV pathogenesis.

13.5.3 FXR Signaling During HBV Infection

The connection between the HBV and bile acid signaling is better established than that of its HCV counterpart. Although the interaction between the HBV genome and the HNF4α and PPARα nuclear receptors was demonstrated in the late 1990s, the first evidence of direct interaction with FXR was only presented in 2008 by Ramiere et al. They showed that FXR binds the HBV enhancer II and that maximal transcription can be achieved by co-expressing FXR with RXR in the presence of bile acids [128]. The increase in HBV gene expression mediated by bile acids was later

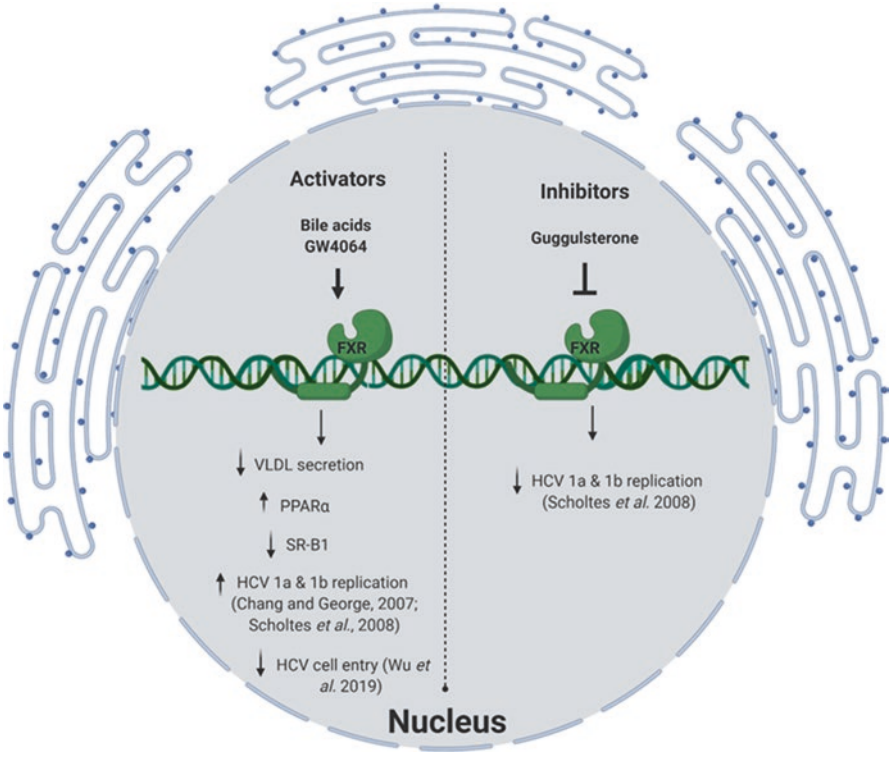

Fig. 13.3 Activators and inhibitors of FXR exert effects on metabolism and HCV replication. Bile acids activate FXR and lead to decreased VLDL secretion and increased PPARα activation which decreases HCV replication [23, 144]. The synthetic agonist GW4064 lowers SR-B1 signaling and reduces HCV cell entry [181]. The FXR antagonist Guggulsterone inhibits HCV replication [144]

confirmed by Kim et al. Interestingly, their study also showed the involvement of the JNK/c-Jun pathway in the CDCA/FXR-mediated activation of HBV transcription and demonstrated that bile acids disrupt the antiviral effects of interferon alpha (IFN-α) [76]. Reese et al. further confirmed that bile acids increase HBV transcription via FXR-RXR without the need for independent RXR activation [133].

An important advancement in our understanding of bile acid-HBV host-virus interactions was the discovery of the sodium taurocholate cotransporting polypeptide (NTCP) as an essential receptor for HBV entry [183]. NTCP is the principal transporter of bile acids into hepatocytes from the portal blood [159]. The pre-S1 domain of the large envelope protein of HBV was shown to interact with NTCP, and interestingly this interaction blocks the normal uptake of bile salts by the transporter. Conversely, occupation of the receptor by bile salts was able to decrease virus entry [184]. This evidence brought forth a new level of complexity to the interactions between the virus and host metabolism. It was later shown that binding of the virus to the NTCP receptor leads to a strong upregulation of CYP7A1 expression. This was coupled with an upregulation of genes involved in cholesterol uptake and biosynthesis [108]. Adding to this complexity, Radreau et al. showed that the FXR agonists GW4064 and 6-ECDCA decrease HBV infection in HepaRG cells and primary human hepatocytes. This was a surprising finding considering the previously demonstrated direct effects of FXR on HBV gene expression. This discrepancy might be explained by differences in the cell models, the use of FXR overexpression vectors, as well as variation in infection incubation times [125]. Since then, the controversial role of FXR in HBV progression has not been defined. Xu et al. showed that the green tea catechin epigallocatechin-3-gallate (EGCG) acts as an antagonist of FXR and inhibits HBV replication [182]. Meanwhile, the HBx was shown to directly interact with FXR as a co-activator capable of increasing its transcriptional activity [106]. Most recently, Mouzannar et al. made the argument that FXR is indeed a pro-viral factor for HBV, as its silencing in infected cells decreased viral DNA pools. However, they showed that agonism of FXR with GW4064 inhibits this pro-viral effect. Additionally, treatment of infected mice with the same agonist had decreased HBV DNA [100]. Therefore, the role of FXR and bile acid metabolism in the progression of HBV infection is of ongoing interest. However, a more in-depth understanding of these host-pathogen interactions is required before this pathway can be considered for therapeutic intervention in HBV infection.

13.6 Conclusions

In this chapter, we have highlighted the critical and diverse roles that nuclear hormone receptors play during viral infection. Associated signaling pathways can be diverted to meet the needs of invading pathogens, and thus the receptors can represent pharmacological targets for antiviral therapies. In addition to their effects on cellular metabolism, diverse roles also include modulation of inflammatory

responses to infection, immunomodulation, and immunometabolism. This is consistent with the rich biological roles of different nuclear receptors. Thus, there is still much work to be done to understand how and why different viruses have evolved to modulate receptor function and exert control over the associated cellular signaling pathways. It is also becoming clear that aspects of the innate immune response restrict viruses in diverse ways that may also include reversing virus-induced changes to metabolic pathways. Ultimately, a greater understanding of this interplay will lead to innovative new strategies to combat viruses that have deleterious effects to human health.

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Chapter 14
Retinoic Acid-Related Orphan Receptor (ROR) Inverse Agonists: Potential Therapeutic Strategies for Multiple Inflammatory Diseases?

Anton M. Jetten, Ju Youn Beak, Andrzej T. Slominski, and Brian Jensen

Abstract Retinoic acid-related orphan receptors (RORs) function as ligand-dependent transcription factors. Several (oxy)sterols have been identified that activate or repress ROR transcriptional activity by functioning as either ROR agonists or inverse agonists. RORs are involved in the control of many biological processes, including the regulation of differentiation and function of neural, immune, and metabolic tissues, bone, and heart. Many of the processes and functions regulated by RORs play a critical role in various pathologies, including autoimmune and other inflammatory diseases, metabolic syndrome and diabetes, neurological and psychiatric disorders, and cardiac injury. Together, these studies raised the possibility that modulation of ROR activity by synthetic ligands might be a useful approach to intervene in these diseases. This led to the identification of many synthetic ROR (inverse) agonists that repress or induce ROR transcriptional activity. Most studies have been focusing on RORγt inverse agonists that repress the generation of interleukin 17 (IL-17)-producing immune cells and the production of pro-inflammatory cytokines, such as IL-17, which play a critical role in various inflammatory diseases. Treatment of autoimmune disease in several experimental rodent models with RORγ inverse agonists was shown to reduce the production of pro-inflammatory cytokines and ameliorate the disease. Thus, ROR (inverse) agonists may potentially provide new therapeutic strategies to treat various pathologies.

A. M. Jetten (✉)
Cell Biology Section, Immunity, Inflammation, and Disease Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Durham, NC, USA
e-mail: jetten@niehs.nih.gov

J. Y. Beak · B. Jensen
McAllister Heart Institute, University of North Carolina School of Medicine, Chapel Hill, NC, USA

A. T. Slominski
Department of Dermatology, Comprehensive Cancer Center, Cancer Chemoprevention Program, University of Alabama at Birmingham, Birmingham, AL, USA

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14.1 Introduction

The retinoic acid-related orphan receptors, RORα, RORβ, and RORγ, constitute a subfamily ligand-dependent transcription factors encoded by the *RORA-C* (or *NR1F1–3*) genes, respectively [23, 28, 54, 64, 65, 79, 80]. By alternative splicing and/or the use of different promoters, *RORA-C* generate several isoforms that exhibit different patterns of cell type-specific expression and control distinct physiological processes and target genes [4, 38, 65, 79, 105, 112]. RORs regulate gene transcription by binding to the promoter-regulatory region of target genes. This binding is mediated by a conserved DNA-binding domain (DBD), containing two C_4-type zinc fingers that recognize ROR response elements (ROREs) consisting of the RGGTCA consensus preceded by an A/T-rich sequence [53, 79, 113]. Whether RORs act as transcriptional activators or repressors is determined by the interaction of their ligand-binding domain (LBD) with, respectively, an agonist or inverse agonist that facilitate the recruitment of coactivators or corepressors, respectively [71, 79, 95, 145, 175]. In addition to (inverse) agonists, several other factors, including various posttranslational modifications and protein interactions, play an important role in modulating ROR transcriptional activity [80].

Various (oxy)sterols, including cholesterol sulfate, 7-dehydrocholesterol, desmosterol, 20α-hydroxycholesterol (20α-OHC), 25-OHC, 7β, 27-OHC, and 27-OHC, have been demonstrated to act as endogenous ligands of RORα and/or RORγ [70, 80, 84, 86–88, 130, 139, 146, 175]. They function as either agonists or inverse agonist of RORs. Moreover, RORs control several biological processes that are critical in the development and progression of a variety of pathologies, including several metabolic, immune, and neurological diseases. Together, these discoveries raised the possibility that synthetic ROR ligands might be potentially useful to intervene in biological processes that underlie these pathologies and that RORs provide potential therapeutic targets to treat a variety of these diseases. This led to an intensive search for synthetic, small molecule ROR (inverse) agonists [44, 49, 80, 86, 144, 153]. In this chapter, we provide a selective overview of RORs and their (inverse) agonists and their role in the development and potential treatment of several inflammatory diseases.

14.2 Functions of RORs in Immune Cells

Using alternative promoters, *RORC* generates two isoforms: RORγ1 and RORγt (RORγ2) [38, 65, 79, 112]. Via a combination of alternative splicing and promoter usage, murine and human *RORA* generate 4 and 2 isoforms, respectively [54]. RORγ1 is expressed in many endocrine and metabolic tissues, whereas RORγt expression is restricted to several immune cell types, including CD4CD8 double-positive thymocytes, T helper 17 (Th17) cells, innate lymphoid 3 (ILC3) cells, lymphoid tissue inducer (LTi) cells, NKp46$^+$, and γδ T cells [28, 38, 39, 75, 76, 79, 96, 114, 133, 154] (see Fig. 14.1).

Both RORα and RORγt have a critical role in thymopoiesis and the development of T lymphocytes [37, 79, 96, 154]. During thymopoiesis, RORγt is selectively expressed in CD4CD8 double-positive (DP) thymocytes. Loss of RORγt function causes accelerated apoptosis in DP cells in part due to the downregulation of the antiapoptotic gene, *Bcl-X$_L$*, resulting in reduced generation of single-positive T lymphocytes [96, 154]. Recent studies have provided additional insights into the role of RORγt in the regulation of positive selection [122, 123]. This study demonstrated that the histone deacetylase HDAC3 represses RORγt transcriptional activity and consequently the expression of the RORγt target gene, the purinergic receptor P2X7, a proapoptotic gene. RORγ-deficient mice have further been reported to exhibit a high probability developing lymphoblastic lymphomas during adulthood [103, 160].

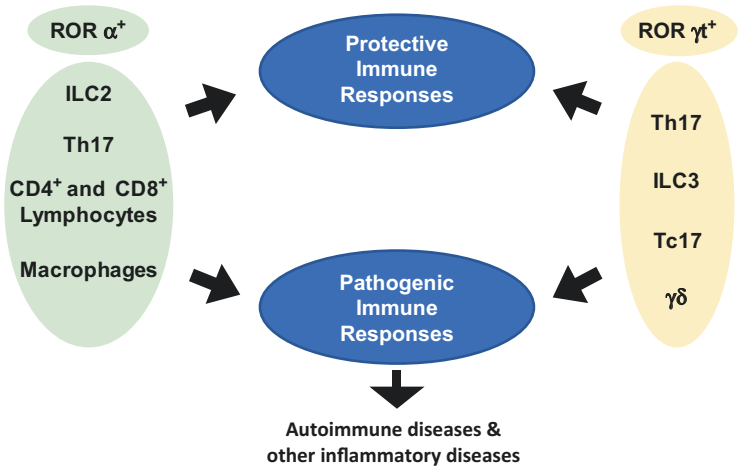

Fig. 14.1 RORα and RORγt are selectively expressed in a variety of immune cells, including ILCs, Th17, macrophages, Tc17, and γδ cells, that have protective as well as pathogenic roles. (Inverse) agonists modulate ROR transcriptional activity and/or regulate the generation and functions of these cells. Subsequently, they can positively or negatively influence inflammatory responses and diseases

RORγt is also expressed in IL-17-producing $CD8^+$ T cells, termed Tc17, that are derived from $ROR\gamma t^+$ CD4CD8 DP thymocytes [109]. These cells can participate in protective, as well as pathogenic, immune responses, including protection against bacterial and viral infections, and contribute to several inflammatory diseases, including psoriasis, multiple sclerosis, and ulcerative colitis.

In addition, RORγt is important in the development of γδ17 T cells during intrathymic differentiation [2]. As in Th17 cells, RORγt regulates the transcription and production of IL-17 in these cells. γδ17 T cells have been implicated in several inflammatory diseases, including psoriasis, multiple sclerosis, and collagen-induced arthritis [2].

In the mouse, group 1–3 innate lymphoid cells (ILC1–3), natural killer (NK) cells, and lymphoid tissue inducer (LTi) cells are derived from common lymphoid progenitors (CLPs) [34, 114]. RORγt is required for the development of ILC3s and their production of IL-17 and IL-22. These cells have a role in the pathogenesis of various autoimmune diseases, host defense responses, and tumor immunity [7]. RORγt is also essential for the development of lymphoid tissue inducer (LTi) cells, which are required for the development of secondary lymphoid organs, including Peyer's patches and intestinal lymphoid follicles ([39] #455; [96, 154]).

Much of the focus of RORγ has been on its role in the differentiation and function of T helper 17 (Th17) cells [28, 36, 75, 76]. Th17 cells produce a series of pro-inflammatory cytokines, including IL17A, IL-17F, and IL-22, that play a critical role in several autoimmune diseases. These cells are also important in protecting the host against bacterial and fungal infections. RORγt is induced during the early stages of differentiation of naïve T cells into Th17 and is required for the generation of Th17 cells and IL-17 synthesis [36, 75, 178]. RORγt directly regulates the transcription of the pro-inflammatory cytokines, including *Il17a*, *Il17f*, *Irf4*, and *Il23r* [173, 178]. RORα4 is also expressed in human Th17 cells, where it also regulates Th17 cell differentiation and cytokine production; however, RORγ plays a more significant role than RORα [24, 178]. The regulation of gene transcription by RORα and RORγ overlaps, and many Th17 genes, including IL-17A, IL-17F, IL-23R, CCL20, and CCR6, are regulated by both RORγt and RORα4.

In addition to the function of RORα4 in Th17 cells, RORα has important roles in several other immune cells (see Fig. 14.1). RORα is important for lymphocyte development, and both T- and B-cell developments are abnormal in RORα-deficient mice [37]. RORα is highly expressed in CD4+ and CD8+ lymphocytes, while DP thymocytes are almost completely absent in the thymus of RORα-deficient mice. RORα is also critical for the development of ILC2s, which produce IL-5, IL-9, and IL-13 [171]. These cells have an important protective role in the innate response to helminth parasites and in the immunopathology of asthma and other allergy-related pathologies. In addition, RORα regulates inflammatory responses in macrophages. Stimulation of RORα-deficient macrophages with lipopolysaccharide (LPS) was reported to induce hyperexpression of several pro-inflammatory cytokines, including IL-1β and TNFα [93]. Subsequent studies reported that RORα promotes M2 polarization of macrophages [61, 172].

14.3 RORs and Autoimmune Disease

Together, these studies revealed that RORα and RORγ have many critical functions in the immune system and control the development and function of various immune cells that play a crucial role in the pathogenesis of autoimmune and several other inflammatory diseases. Autoimmune and allergic inflammatory diseases are complex diseases that involve genetic and environmental factors. In addition, a variety of different immune cells, particularly the IL-17-secreting Th17, ILC3, and γδ T cells, but also ILC2 and Th1/2 responses, have been reported to play a critical pathogenic role in these diseases [85, 110]. The extent to which various immune cells participate in the immunopathology varies among different autoimmune diseases. Studies demonstrating that following: (1) neutralizing anti-IL-17 antibodies ameliorate autoimmune inflammation [11, 101, 111]; (2) RORγt, and to a lesser extent RORα, is critical for the generation of these cells and the production of pro-inflammatory cytokines, including IL-17; (3) RORα- and RORγ-deficient mice show a reduced susceptibility against several inflammatory diseases in several experimental autoimmune and allergic inflammatory disease models [16, 28, 75, 78, 80, 157, 178]; and (4) the discovery that RORs function as ligand-dependent transcription factors [86, 87, 150] suggested that inhibition of ROR activity by ROR (inverse) agonists could potentially provide new strategies in the treatment of these pathologies. Subsequently, these observations induced a strong interest in developing chemical ligands that inhibit ROR activity. Most studies have been focusing on the identification of RORγt inverse agonists that inhibit pro-inflammatory responses by IL-17-producing immune cells and that might be potentially useful in the management of autoimmune disease.

Digoxin and its synthetic derivatives, 20,22-dihydrodigoxin-21,23-diol and digoxin-21-salicylidene, ursolic acid, and SR1001 were among the first small molecules reported to inhibit RORγ activity [71, 145, 175] (see Fig. 14.2a). Digoxin and ursolic acid are RORγ selective, where SR1001 inhibited both RORα and RORγ activity. Subsequently, many additional inverse agonists were identified [13, 43, 44, 48, 50, 63, 80, 83, 94, 134, 148, 153, 165]. Many of these inverse agonists were shown to inhibit Th17 differentiation, repress the transcriptional activation of the *IL17* promoter by RORγ and the production of IL-17, and alleviate inflammatory responses in experimental mouse models of autoimmune disease. Moreover, they inhibited the interaction of RORγ with coactivator peptides and facilitated its interaction with corepressor peptides, suggesting that they function as RORγ inverse agonists. Crystal structure analysis, molecular modeling, and differential hydrogen-deuterium exchange mass spectrometry (HDX-MS) analysis of the RORγ(LBD)-inverse agonist interactions indicated that many of them bound the ligand-binding pocket of the RORγ(LBD) [13, 84, 86, 87, 102, 119, 141, 145, 155, 164]. Some inverse agonists, such as GSK805, do not alter significantly the interaction of RORγ with ROREs in target genes, while others moderately (e.g., TMP778) or significantly (e.g., TMP920) alter the binding to genomic RORγ target sites [173](see Fig. 14.2b). Recently, several other studies reported on alternative modes of action

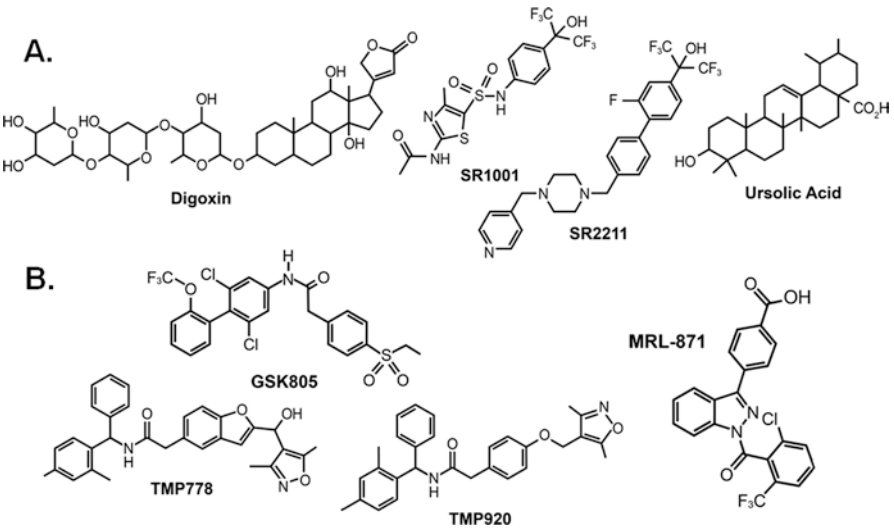

Fig. 14.2 Chemical structures of several RORγ inverse agonists. (**a**) Digoxin [71], SR1001 (RORα and RORγ inverse agonist) and SR2211 [91], and ursolic acid [175]. (**b**) GSK805 [163], TMP778 and TMP920 [173], and MRL-871 [132]

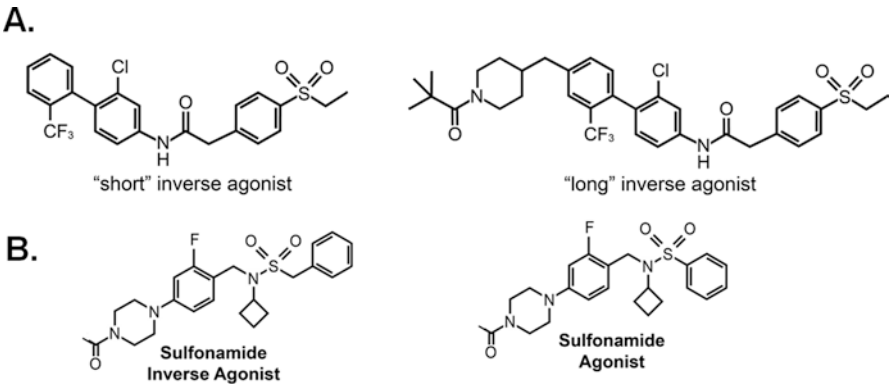

Fig. 14.3 "Short" and "long" biaryl amides [164]. The "short" amide stimulates corepressor peptide binding and blocks coactivator binding, while the "long" amide prevents binding of both. (**b**) A small change in chemical structure of a sulfonamide can determine whether it functions as an agonist or antagonist [125]

of RORγ antagonists. The inverse antagonistic effect of MRL-871 was shown to be mediated through an allosteric interaction with RORγ(LBD) and a different helix 12 conformational change [132](see Fig. 14.2b). Another study identified two additional types of RORγ inverse agonists, referred to as "short" and "long" biaryl amides (see Fig. 14.3a); the "short" amide stimulates corepressor peptide binding and blocks coactivator peptide binding, while the "long" amide prevents binding of

both [164]. Small changes in the chemical structure of oxysterols or synthetic RORγ ligands have been reported to determine whether they act as inverse agonists as shown for two sulfonamides in Fig. 14.3b [125].

14.4 Vitamin D3, VDR, and RORs

Recent studies showed that vitamin D, but particularly iCYP11A1-derived vitamin D3 hydroxyderivatives, can function as weak inverse agonists for both RORα and RORγ [81, 135, 138, 141]. Vitamin D3 (D3) is formed via the photochemical and thermal transformation of 7-dehydrocholesterol (7DHC) by ultraviolet B radiation [67]. Prolonged exposure to UVB leads to production of lumisterol (L3)[162]. D3 is hydroxylated by CYP2R1/CYP27A1 and subsequently by CYP27B1 to generate the biologically active 1,25$(OH)_2$D3, which functions as an agonist of the vitamin D receptor (VDR) (see Fig. 14.4). 1,25(OH)2D3 is inactivated by hydroxylation at C24 by CYP24A1 and further shortening of the side chain [158]. D3 can also be hydroxylated by CYP11A1 generating a series of additional hydroxylated D3 metabolites, including, but not limited to, 20(OH)D3, 22(OH)D3, 20,23(OH)2D3, 20,22$(OH)_2$D3, and 17,20,23(OH)3D3, with a main product and precursor molecule being 20(OH)D3 [140, 158](see Fig. 14.4a, b). These intermediates can also be hydroxylated by other CYPs, including CYP27B1, to produce wide range of vitamin D3 hydroxyderivatives [137, 140, 158]. Lumisterol (L3), a product of photoisomerization of pre-vitamin D3, can also be further hydroxylated by CYP11A1, producing several hydroxylated derivatives, including 20(OH)L3, 22(OH)L3, and 20,22$(OH)_2$L3 [138](see Fig. 14.4a, b). D3 and L3 hydroxyderivatives are able to inhibit RORE-dependent transactivation by RORγ and the recruitment of a coactivator peptide in TR-FRET and reporter assays [138, 141]. In addition, they inhibit ROR-mediated activation of the RORα/γ target gene promoter, *Bmal1,* and the RORγ target gene promoters, *G6pase* and the *Il17* [138, 139, 141]. Molecular modeling of the interaction of the RORα(LBD) and RORγ(LBD) with hydroxylated D3 and L3 derivatives showed good docking scores, suggesting favorable binding [139]. These studies demonstrate that hydroxy D3 and L3 derivatives can function as inverse agonists for RORα and RORγ and suggest that these nuclear receptors provide an alternative mechanism by which vitamin D3 and its derivatives can modulate gene expression and cell functions, and possibly various diseases. The anti-inflammatory action of vitamin D3 and its role in autoimmune disease risk and treatment is well established [17, 136, 142, 177]. Thus, the RORγt antagonistic effects of hydroxylated D3 and L3 metabolites and the effects of 1,25$(OH)_2$D3 on Th17 and T_{reg} lineages might synergistically reduce inflammatory effects in several inflammatory diseases [120, 135, 137](see Fig. 14.4a).

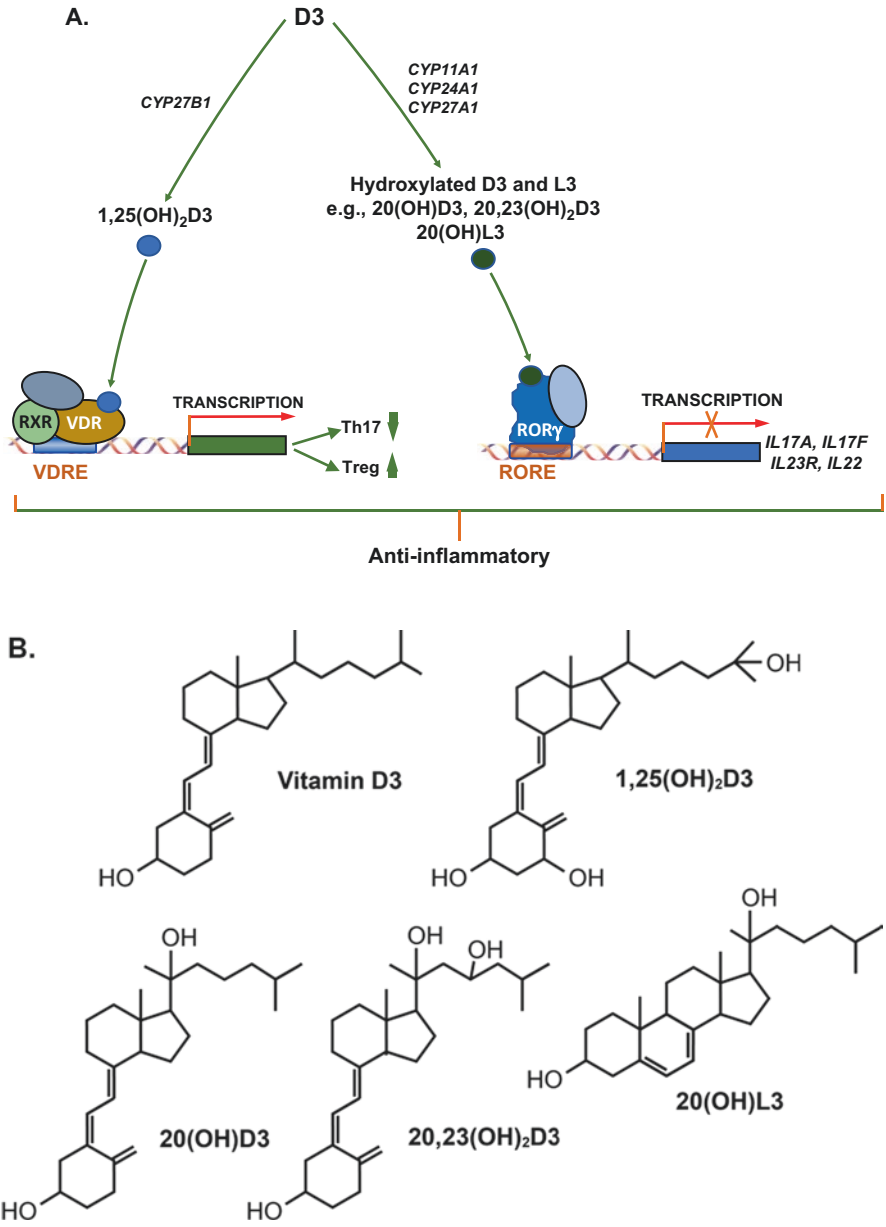

Fig. 14.4 (**a**) Hydroxylated vitamin D3 derivatives through either VDR ($1,25(OH)_2D_3$) or RORγ (e.g., 20(OH)D3, $20,23(OH)_2D3$, 20(OH)L3) act cooperatively, inhibiting inflammation and improving inflammatory disease by reducing Th17 cell generation and function. (**b**) Structures of vitamin D3: $1,25(OH)_2D_3$, 20(OH)D3, $20,23(OH)_2D3$, and 20(OH)L3

14.5 Multiple Sclerosis (MS)

MS is an inflammatory autoimmune disease of the central nervous system (CNS). IL-17-secreting immune cells play a critical role in this disease [110]. Experimental autoimmune encephalomyelitis (EAE) shares multiple features with MS and has served as animal model of MS. RORγ-deficient mice exhibit a significantly reduced susceptibility to EAE, while RORα deficiency was less protective, and RORγ/RORα double knockout mice were completely protected against EAE [75, 178]. Digoxin and several of its derivatives, ursolic acid, and SR1001 were among the first RORγt inverse agonists shown to effectively suppress the clinical severity of EAE in mice [47, 71, 145, 166, 175]. Subsequent studies in administration of other RORγ inverse agonists, including GSK805, demonstrated that they could efficiently ameliorate the severity of EAE [173]. This protection is likely due to the inhibition of pro-inflammatory cytokine synthesis by IL-17-producing cells.

14.6 Psoriasis

Psoriasis is an autoimmune disease that affects the skin and is often associated with psoriatic arthritis affecting the joints. Psoriasis vulgaris is the most common form of this disease that is characterized by inflammation, hyperproliferation, and aberrant differentiation of epidermal keratinocytes, resulting in thickening of the epidermis [56]. Keratinocytes and several different immune cells, each producing various cytokines, are all involved in the development and progression of psoriasis. Psoriatic lesions are characteristically associated with infiltration of lymphocytes, macrophages, and neutrophils into the skin. RORγt$^+$ and IL17-producing cells, including Th17, ILC3, Tc17, and γδ T cells, play a critical role in this disease. Cytokines, such as IL-17 and IL-22 produced by IL-17-producing cells, stimulate epidermal keratinocyte proliferation, while production of IL-23 further stimulates Th17 cells and maintains chronic inflammation. Gene expression profiling analysis showed that IL-17A induces many changes in gene expression in epidermal keratinocytes, including increased cholesterol biosynthetic genes [161]. This increase in cholesterol biosynthesis promotes RORγt activation and NFκB-mediated activation of several chemokines in keratinocytes and stimulates the recruitment of neutrophils. The critical role of IL-17 and IL-23 in psoriasis is supported by clinical data showing that monoclonal antibodies against IL-17A, IL23, and the IL17 receptor (e.g., secukinumab, brodalumab, and guselkumab) alleviate the severity of psoriasis and psoriatic arthritis [11, 101, 111].

RORγ-deficient mice were shown to be resistant to IL-23-induced skin inflammation, suggesting that inhibition of RORγ activity might do the same [156]. Several RORγ-selective inverse agonists (e.g., GSK2981278, A213, JNJ-54271074, S18-000003, BIO-0554019, Cpd A, Cpd 1, AZD-0284, VTP-938)(see Fig. 14.5) have been reported to inhibit the expression of IL-17A/F and IL22 in CD4$^+$ T cells

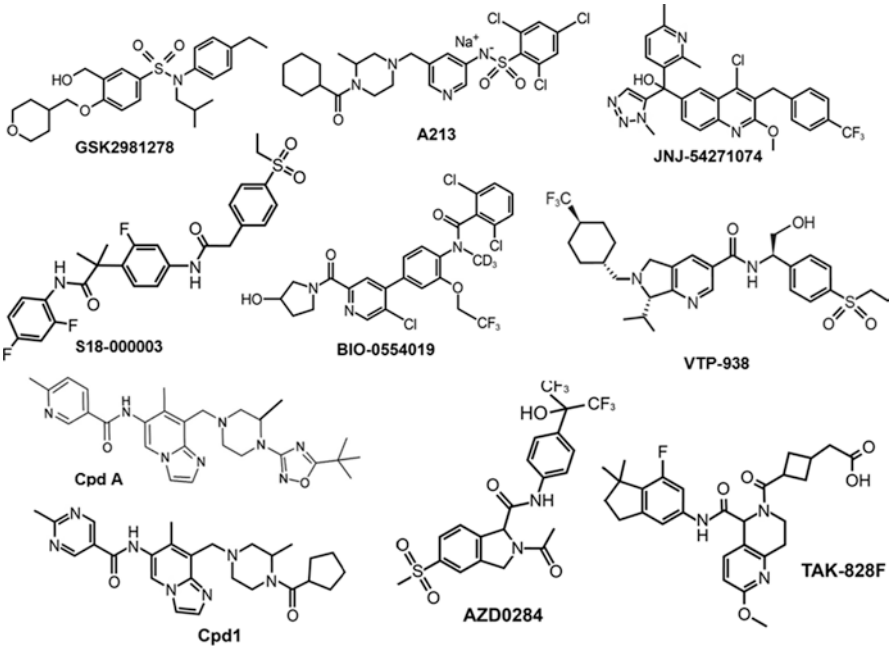

Fig. 14.5 Chemical structures of several RORγ inverse agonists reported to alleviate autoimmune disease in animal models. GSK2981278 [143], A213 [156], JNJ-54271074 [176], S18-000003 [74], BIO-0554019 [12], VTP-938 [168], Cpd A [40], Cpd 1 [58], AZD-0284 [8], and TAK-828F [72]

as well as [40, 58, 74, 143, 156, 168, 176]. Cpd A was shown to inhibit the induction of IL-17-dependent genes, such as defensin 2, in cultured keratinocytes and human skin biopsies [40]. Topical application of GSK2981278 imiquimod-induced mouse model of psoriasis inhibits the expression of pro-inflammatory cytokines in skin explants obtained from biopsies of psoriatic patients [143]. However, a phase 1 trial showed that topical treatment did not result in any improvement of psoriatic lesions [89]. Oral treatment with JNJ-54271074 significantly diminished skin inflammation in an IL-23 psoriasis-like mouse model [176]. In a separate study, oral administration of the RORγ antagonist A213 or topical treatment with S18-000003 attenuated skin inflammation in two mouse models of psoriasis: K5.Stat3C mice and mice injected with IL-23 [74, 156]. This was accompanied with a reduction in IL-17-producing Th17, Tc17, and γδ T cells. Similarly, oral treatment with the RORγ inverse agonist, BMS-986251, greatly reduced skin thickness and the production of IL-17 in IL-23- and imiquimod-induced skin inflammation in mice [27]. Intraperitoneal injection of BIO-0554019 reduced blood IL-17 levels in mouse after IL-1β/IL-23 challenge and attenuated imiquimod-induced skin inflammation [12], while the RORα/γ inverse agonist SR1001 suppressed inflammation in mouse models of atopic dermatitis, likely by targeting cells important in this disease,

particularly Th2 and ILC2 cells as well as Th17 cells [30, 68]. Another study reported that Cpd 1 delayed methylated bovine serum albumin (BSA)-induced hypersensitivity (DTH) responses in female Lewis rats [58]. However, prolonged treatment with Cpd 1 caused accelerated apoptosis in thymocytes and preneoplastic thymic hyperplasia.

Together, these studies suggested that RORα/γ inverse agonist might be a useful approach in treating skin inflammatory diseases. A number of additional RORγ inverse agonists, generated by several pharmaceutical companies, have been or are being tested for their clinical efficacy in treating psoriasis and potential side effects (for details see [20, 22, 49, 153]). Although several clinical trials with RORγ inverse agonists have been abandoned due to toxic side effects, several new inverse agonists show promise [8, 50, 148, 153]. For example, a recent phase I clinical trial reported that inverse agonist AZD-0284 (see Fig. 14.5) reduced IL-17A and was well-tolerated [8]. RORγ-deficient mice and treatment with several RORγ inverse agonists have been shown to increase the risk of lymphoma development [58, 103, 160]; however, recently, RORγ inverse agonists, including S18-000003, have been identified that do not show dysregulation of CD4CD8 double-positive thymocytes and not to increase cancer risk in topically treated mice, even at a high dose [74]. Currently, several RORγ inverse agonists are at different stages in clinical trials (for review see [153]).

14.7 Role of RORα in Cardiac Injury and Heart Failure

Little is known about the roles of RORβ and RORγ in the heart; however, recent studies have indicated that RORα has an important cardioprotective function. RORα-deficient mice developed exaggerated pathological ventricular remodeling and heart failure after chronic infusion of angiotensin II (Ang II), a commonly used model of neurohormonal induction of heart injury [16]. Compared to WT littermates, the hearts of Ang II-infused RORα-deficient mice exhibited more pathological cardiomyocyte hypertrophy, fibrosis, energy depletion, and inflammation. Another study reported that RORα protected against heart injury in myocardial ischemia/reperfusion and diabetic cardiomyopathy models [62, 174]. The absence of functional RORα also facilitated activation of critical pro-inflammatory IL-6 and NFκB pathways. RORα protein expression is significantly decreased in failing heart tissue [16, 174]. Together, these studies suggest that RORα has a protective role in the development of heart failure.

The heart consumes more ATP than any other organ by virtue of the requirement for constant cardiomyocyte contraction and relaxation. Much of this ATP is produced by oxidative respiration in the mitochondrial electron transport chain, using fatty acids as substrate, though cardiomyocytes are capable of adaptive switching in substrate utilization to favor glucose or ketones [31]. The metabolic profile of the failing heart is characterized by progressive decreases in myocardial fatty acid

oxidation and mitochondrial respiration, resulting in energy deprivation and oxidative stress [116, 182]. As such, maintenance of a large and optimally functional pool of mitochondria through careful orchestration of mitochondrial biogenesis and the clearance of dysfunctional mitochondria is critical [124]. RORα-deficient hearts contain less ATP and more reactive oxygen species than WT littermates, and in vitro knockdown of RORα with lentiviral shRNA in primary neonatal rat ventricular myocytes (NRVMs) recapitulates these in vivo findings [16]. Furthermore, lentiviral knockdown of RORα in NRVMs resulted in decreased citrate synthase activity and a marked reduction in mitochondrial membrane potential, indicating a reduction in viable mitochondria [15]. Basal increase in oxidative stress is exacerbated in the setting of in vivo heart injury induced by either diabetes or ischemia-reperfusion and is accompanied by activation of mitochondria-mediated apoptosis [92, 180]. Although the mechanisms underlying the protective function of RORα in heart failure still need to be determined, regulation of mitochondrial quality via mitophagy appears to be part of this process [16, 62, 181].

In addition to mitochondrial dysfunction, myocardial inflammation also plays a critical role in heart failure [46, 60]. This includes production of pro-inflammatory mediators by cardiomyocytes and activation of various pro-inflammatory immune cells, including RORα$^+$ macrophages and RORα$^+$RORγ$^+$ Th17 cells [28, 172]. Thus, suppression of cardiac inflammatory responses might play an important role in the cardiac protection by RORα (see Fig. 14.6). Data showing NFκB activation and markedly elevated IL-6 and STAT3 levels in the hearts of RORα-deficient mice exposed to chronic angiotensin II are consistent with this hypothesis [16]. In vitro studies using NRVMs revealed that RORα knockdown led to greater IL-6 release whereas RORα overexpression suppressed IL-6 release. The expression of *Il6* was shown to be under direct transcriptional control of RORα. Collectively these findings showed that cardiomyocyte RORα suppresses pro-inflammatory responses after cardiac injury and is consistent with a report demonstrating that RORα-deficient mice exhibit increased susceptibility to lipopolysaccharide-induced lung inflammation [147]. In addition to the critical role of RORα in regulating myocardial inflammation, RORγ might also have a role in myocardial Inflammation by regulating Th17 cell differentiation and IL-17 production, which contribute to pathological remodeling and heart failure in several contexts. Interestingly, digoxin, which is used to treat heart failure but also functions as a RORγ inverse agonist [71], is potentially supporting a role for RORγ in the regulation of Th17-mediated cardiac inflammation [55].

The protective effect of RORα in the development of heart failure suggested that RORα agonists might have a beneficial effect in injured and failing cardiomyocytes. This possibility was supported by data demonstrating that the RORα agonist, SR1078, promoted autophagy whereas the RORα inverse agonist, SR3335, suppressed autophagy in cardiomyocytes [15]. The expression of classical autophagy mediators was increased by SR1078 and decreased by SR3335. Together, these findings support a cardioprotective role for RORα (see Fig. 14.6) and suggest that RORα agonists might offer a novel strategy for treating heart failure.

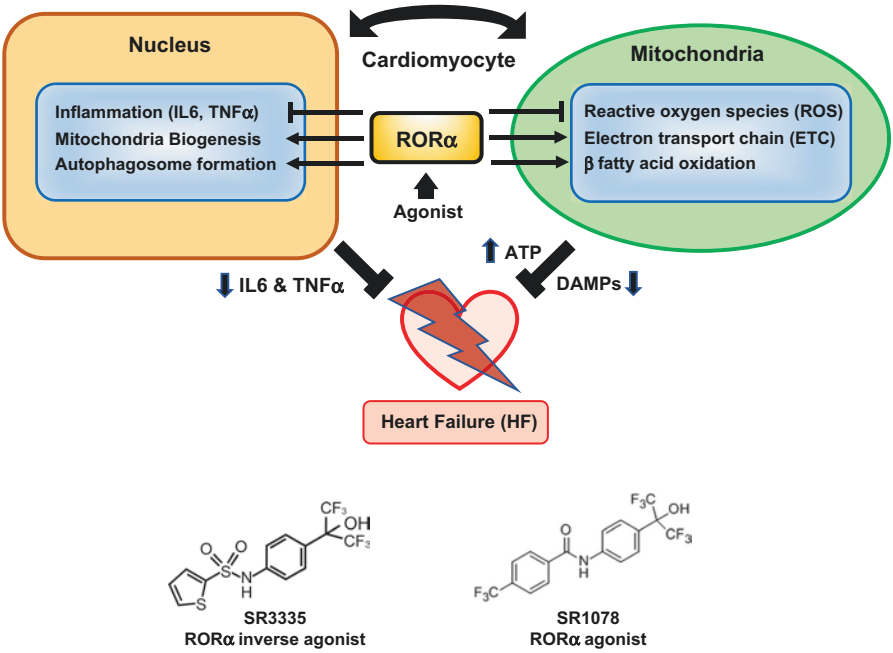

Fig. 14.6 RORα has a protective role in heart failure. RORα inhibits the expression of pro-inflammatory genes (e.g., *Il6*, *Tnfa*) and upregulates the expression of genes that play a role in mitochondria biogenesis and autophagosome formation. RORα inhibits the production of reactive oxygen species (ROS) and maintains the electron transport chain (ETC) function and β fatty acid oxidation in mitochondria. RORα agonist, SR1078, stimulates and RORα inverse agonist, SR3335, inhibits these functions. RORα appears to protect against heart failure by inhibiting the production of pro-inflammatory cytokines and damage-associated molecular patterns (DAMPs)

14.8 RORs in Asthma and Acute Respiratory Distress Syndrome (ARDS)

Asthma is a complex and heterogeneous allergic respiratory disease that involves cooperation of environmental and genetic factors and involves innate and adaptive immune responses and various immune cells, including several IL-17-producing cells, ILC2, Th2, and neutrophils [66, 85]. Asthma has been divided into several subtypes that includes steroid-sensitive and steroid-insensitive allergic lung inflammation. A recent study indicated that RORγt may be an important factor in determining the subtype of asthmatic airway inflammation [5]. This report revealed that inflammation in OVA-sensitized/challenged RORγt-overexpressing mice is a steroid-insensitive form of neutrophilic inflammation associated with Th17 cells and IL-17. The latter was supported by data showing that anti-IL17 antibodies alleviated allergic lung inflammation in these mice, suggesting that targeting the Th17

pathway might be useful in the treatment of this pathology. Oral administration of the RORγ-selective inverse agonist VTP-938 (see Fig. 14.5) attenuated Th17 development in lung draining lymph nodes and production of IL-17 in the airways in a house dust extract model of asthma [168]. This was accompanied with a reduction in neutrophilic inflammation and airway hyperresponsiveness. These observations suggest that RORγ inverse agonists may provide a novel strategy in the management of (steroid-insensitive) asthma.

ARDS is a major cause of acute respiratory failure. The expressions of RORγt and its coactivator, histone acetyltransferase p300, were found to be elevated in peripheral blood mononuclear cells (PBMCs) from patients with ARDS [26]. Inhibition of p300 reduced RORγ expression and IL-17 production. Lipopolysaccharide-induced acute lung injury in mice significantly increased p300 and RORγt mRNA expression. Treatment with RORγ inverse agonists might be useful in reducing lung tissue inflammation and lung injury.

RORα also plays a role in lung inflammatory disease. Recent studies reported an association between single-nucleotide polymorphisms in *RORA* and increased asthma risk [1, 104]. *RORA* variants have also been found to be associated with chronic obstructive pulmonary disease [179]. RORα-deficient mice were shown to exhibit an attenuated allergic airway response, as indicated by reduced induction of several inflammatory chemo- and cytokines, suggesting a pro-inflammatory role of RORα [78]. This was supported by a study indicating that ILC2-deficient mice, obtained by transplantation of RORα-deficient bone marrow, failed to develop lung inflammation in response to protease allergens [60]. In contrast, RORα-deficient mice were shown to be more susceptible to lipopolysaccharide (LPS)-induced airway inflammation, suggesting that RORα has a protective role under these conditions [147]. These studies suggest that RORα (inverse) agonists by targeting RORα$^+$ Th2 and ILC2 cells might be useful in the management of inflammatory lung disease [18, 60, 85, 171].

14.9 Rheumatoid Arthritis

Rheumatoid arthritis is an autoimmune disorder that involves chronic inflammation of the joints. The balance between Th17 and T_{reg} cells plays a critical role in this pathology. Intraperitoneal injection of the RORγ inverse agonist, SR2111 (see Fig. 14.1), diminishes joint swelling in a collagen-induced arthritis mouse model [25]. Similarly, oral administration of the inverse agonist, JNJ-54271074 (see Fig. 14.5, was shown to decrease arthritic scores in a mouse collagen-induced arthritis model and inhibit IL-17A synthesis in PBMCs from rheumatoid arthritis patients [176]. The RORγ inhibitors, MRL-248 and MRL-367, were also reported to suppress secretion of IL-17A in PBMCs from patients with rheumatoid arthritis [32]. Oral treatment with the RORγt inverse agonist, Cpd 1, reduced joint swelling in antigen-induced arthritis in rats and caused a decrease in the number of

IL-17-producing cells [57]. Oral administration of N-(5-(arylcarbonyl)thiazol-2-yl) amide RORγt antagonists were described to diminish collagen-induced arthritis in mice [165].

14.10 Sjögren's Syndrome (SS)

Sjögren's syndrome is a chronic autoimmune disease characterized by IL-17 and infiltration of Th17 lymphocytes into the salivary and lacrimal glands and increased levels of pro-inflammatory cytokines, including IL-17, IL-23, and IL-6. T-cell-specific RORγt-transgenic mice under human CD2 promoter (RORγt-Tg mice) developed severe spontaneous inflammation of the salivary glands (SS-like sialadenitis) [73]. Oral administration of the RORγ inverse agonist A213 to RORγt-Tg mice significantly improved SS-like sialadenitis [118]. In addition, the infiltration of inflammatory cells and level of pro-inflammatory cytokines were reduced, while salivary volume was increased. A recent study provided evidence that RORα may also have a role in SS [167]. These findings suggest that RORγ, and possible RORα, inverse agonists might be a useful therapeutic approach for SS.

14.11 Inflammatory Bowel Disease (IBD)

IBD comprises two chronic inflammatory conditions of the gastrointestinal tract: Crohn's disease and colitis. Chronic enteric bacterial infection causes severe intestinal inflammation and fibrosis and serves as a mouse model for Crohn's disease. Mice transplanted with bone marrow derived from RORα-deficient mice are protected against *Salmonella*-induced fibrosis [108]. Although RORα is expressed in ILC2s, this pathology appears to be driven by IL-17 and IL-22 produced by ILC3s. These pro-inflammatory cytokines are reduced in the RORα-deficient mice. The authors concluded that RORα preserves ILC3 fate under inflammatory conditions [107]. Th17 and IL-17 are critical in IBD pathogenesis. This was supported by a report showing that, in contrast to wild-type T cells, transfer of RORγ-deficient T cells into RAG1-null mice failed to increase mucosal IL-17 cytokine levels and did not induce colitis [99]. Moreover, temporal deletion of RORγt in Th17 cells reduced intestinal inflammation in two mouse IBD models [170]. These studies suggested that inverse agonists of RORα and/or RORγ might be effective in the treatment of IBD. This was supported by studies showing that treatment with RORγ inverse agonists, BMS336, GSK805, Bi119, VPR-254, and TAK-828F (see Figs. 14.2b and 14.5), diminishes the severity of IBD in several mouse models of IBD as indicated by improved histopathology and reduced colonic pro-inflammatory cytokine levels [14, 45, 72, 152, 170]. Moreover, RORγ inverse agonists decreased the expression of Th17-related genes, such as *Il17* and *Il22*, in microbial-stimulated peripheral blood mononuclear cells (PBMCs) and in cultured colon biopsies obtained from IBD patients.

14.12 RORβ

RORB generates two isoforms, RORβ1 and RORβ2, that are under the control of two different promoters [23, 105, 115, 131]. RORβ1 is expressed in many tissues, including several regions of the brain, retina, and testis. Expression of RORβ2 is more restricted and primarily expressed in the pineal gland and retina. In the brain, RORβ is expressed in several regions that process sensory and circadian information, including the suprachiasmatic nuclei (SCN) and pineal gland, both of which play a central role in coordinating the regulation of the circadian rhythmicity of physiological processes in many tissues.

Study of RORβ-deficient mice revealed that RORβ plays an important role in the regulation of many biological processes, including vision, locomotion, osteogenesis, male fertility, and circadian behavior [3, 9, 105, 127, 131] . RORβ-deficient mice exhibit loss of visual function due to retinal degeneration [3]. It is expressed in several retinal subpopulations and plays an essential role in the development of amacrine and horizontal cells, the interneurons that integrate the visual information transmitted from the photoreceptors to the optic nerve [19, 82, 105, 106].

RORβ regulates cytoarchitectural patterning, the neuronal positioning in several regions of the brain, and RORβ-deficient mice exhibit a duck-like gait that has been recently linked to dysfunction of dorsal spinal cord inhibitory neurons, which are needed to ensure a smooth walking gait. RORβ is expressed in inhibitory neurons and selective RORβ knockout in inhibitory $PAX2^{+}$ and spinal inhibitory neurons results in a strong duck gait phenotype. These studies indicated that the *RORβ* mutant locomotor gait deficit is due to reduced presynaptic inhibition and changes to sensory-mediated reflexes [3, 33, 90, 169]. RORβ is also expressed in the sensory areas of the cerebral cortex (in certain thalamic nuclei and neurons of layer IV), important in perception, cognition, and other processes, and in the superior colliculus in the midbrain that is important in orienting behaviors and defense responses [21, 77, 105, 117].

RORB mutations and variants have been implicated in a number of neurological and psychiatric disorders, including bipolar disorder, epilepsy, autism, and schizophrenia [10, 19, 29, 41, 52, 59, 97, 98, 128, 129, 151, 159]. In addition, variants in *RORA* and *RORB* have been associated with sleep disorders that may involve alterations in circadian behavior. Abnormal sleep cycle has been linked to increased risk of several diseases, including obesity, cancer, and several psychiatric disorders [69, 100].

RORβ also plays a critical role in the regulation of osteoblast differentiation [6, 42, 126, 127]. RORβ is highly expressed in osteoblast precursors and becomes downregulated during osteoblast differentiation. The expression of RORβ during osteogenesis was found to be controlled by miR-219a-5p [6]. RORβ-deficiency promotes osteogenesis and preserves bone mass during aging and is shown to involve β-catenin-dependent activation of the Wnt signaling pathway [42]. In contrast, RORβ overexpression in MC3T3-ME cells and increased RORβ expression in the bone during aging were associated with reduced osteogenesis [6, 42, 127]. Therefore,

the observed bone loss in postmenopausal women might be in part due to the increased RORβ expression. Together, these studies indicate that RORβ functions as a repressor of osteogenesis and suggest that inhibition of RORβ by inverse agonists might potentially provide a therapeutic target to treat age-associated bone loss.

RORβ-deficient mice have further been reported to exhibit a transient male infertility. RORβ was shown to be expressed in Leydig cells, where it might be involved in the circadian regulation of gene expression [9]. This may be related to the transient infertility.

14.13 Therapeutic Potential of RORβ Ligands

X-ray crystallography analyzing the structure of RORβ(LBD) complexed with a coactivator peptide provided the first indication that RORβ also functions as a ligand-dependent transcription factor. This study found stearate bound to the RORβ ligand-binding pocket [149]. A subsequent study identified all-trans-retinoic acid and the synthetic analog ALRT 1550 as low-affinity functional inverse agonists [150] (Fig. 14.7a). Several N-(5-(arylcarbonyl)thiazol-2-yl)amides that act as potent RORγ inverse agonists, were found to exhibit also a high affinity for RORβ, but not RORα, suggesting that they function as dual RORβ/RORγ inverse agonists [51,

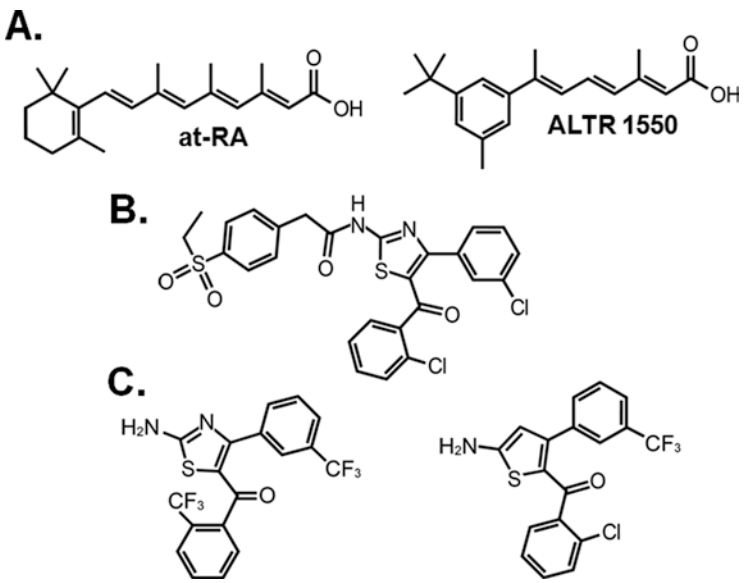

Fig. 14.7 Structures of RORβ inverse agonists. (**a**) All-trans-retinoic acid (at-RA) and RA derivative ALTR 1550 [150]. (**b**) Dual RORβ/RORγ inverse agonist [51]. (**c**) RORβ-selective inverse agonists [35, 121]

121] (see Fig. 14.7b). Subsequently, several aminothiazoles and aminothiophenes were identified as effective RORβ-selective inverse agonists [35, 121] (Fig. 14.7c). These studies are opening the door for the discovery of additional RORβ-selective (inverse) agonists that might be potentially useful in the management of osteoporosis and psychiatric disorders. For example, treatment with RORβ inverse agonists might prevent or reverse age-induced osteoporosis. In addition, RORβ-selective ligands might potentially provide new strategies in the management of several neurological and psychiatric disorders, including bipolar disorder, epilepsy, autism, and schizophrenia.

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Chapter 15
Therapeutic Strategies to Target Activating Estrogen Receptor α Mutations

Kristine Donahue and Wei Xu

Abstract Breast cancer is a hormone-dependent disease. Despite great strides, estrogen receptor α (ER)-positive breast cancer remains a challenging disease. About 60–70% of breast cancers are ER positive, which is predictive of response to endocrine therapies, including selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs). However, many patients will still develop therapeutic resistance. One mechanism of resistance is the development of gain-of-function mutations in the ligand-binding domain (LBD) of ER. Mutant ER exhibits ligand-independent, pro-metastatic activity, and higher concentrations of antiestrogens are required to inhibit its activity. Fulvestrant, currently the only FDA-approved SERD, possesses dose-limiting pharmacological properties and promotes only partial degradation of ER. New orally bioavailable SERMs and SERDs are being developed to overcome the shortcomings of current mainstay treatments but are challenging classes of drug to develop. Taking a ligand-binding domain-independent approach by modulating molecular chaperones and E3 ligases that control ER stability could be an alternative approach to circumvent endocrine resistance and could also be used to target additional oncogenic drivers in mutant ER tumors.

Keywords Estrogen receptor · Selective ER degrader · Breast cancer · Endocrine therapy resistance · Estrogen receptor mutations

K. Donahue
Cancer Biology Graduate Program, University of Wisconsin-Madison, Madison, WI, USA

McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI, USA

W. Xu (✉)
McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI, USA
e-mail: wxu@oncology.wisc.edu

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15.1 Introduction

In 2019, breast cancer was the most commonly diagnosed cancer in the USA [1]. It is a heterogeneous disease and can be subclassified based on expression, or lack of expression, of different classes of receptors. About 60–70% of breast cancers are luminal tumors, which arise from the luminal cells of the mammary duct. They express estrogen receptor α (ERα or ER) and can be subgrouped into luminal A and luminal B tumors. Luminal B tumors express high levels of Ki67 and can also express human epidermal growth factor receptor 2 (HER2). In luminal A tumors, tumor growth is driven primarily by estrogen and ER. Even before the discovery of estrogenic hormones produced in the ovary (1923) [2] and ER (1958) [3, 4], as well as the cloning of ER (1986) [5, 6], it was recognized that endocrine ablation through oophorectomy, first performed in 1895, could lead to regression of some breast tumors [7]. The ER is a nuclear receptor and ligand-activated transcription factor encoded by *ESR1* and is responsible for sensing and mediating the effects of its ligand, estrogens. In humans, estrogens are produced throughout life, with 17β-estradiol (E2) being the most potent and predominant circulating estrogen, particularly during the reproductive years. Estrogen signaling and ER are important regulators of diverse functions, including the normal development of mammary glands and reproductive tissues as well as inflammation, bone density, cognitive function, and cholesterol homeostasis. As a result, dysregulation can lead to a variety of disease states.

15.2 ER Structure

Full-length ER α protein is ~66KDa and has several domains. The intrinsically disordered N-terminal activation functional domain 1 (AF-1) is involved in ligand-independent activation (Fig. 15.1) [8, 9]. The structure of AF-1 remained elusive until recently due to its flexible and intrinsically disordered nature [10]. Phosphorylation in the AF-1 domain represents a major mechanism of ER activation [11]. Most notably, S118 can be phosphorylated [11] by cyclin-dependent kinase 7 (CDK7) in response to E2 stimulation [12] and by mitogen-activated protein kinase (MAPK) in response to the activation of growth factors [13–15].

The DNA-binding domain (DBD) contains zinc finger motifs, allowing ER to bind to estrogen response elements (EREs) within the genes it regulates [16, 17]. The hinge region of ER connects the DBD and ligand-binding domain (LBD) and contains a nuclear localization signal (NLS) [18] responsible for nuclear translocation. This region is subjected to a variety of posttranslational modifications. For example, lysine residues in the hinge domain can be acetylated, which fine-tunes and regulates the hormone and ligand responsiveness [18, 19], as well as sumoylated [20]. In addition, K302 and K303 can be polyubiquitinated, which is important for regulating receptor stability [21]. The LBD, which resides in activation functional

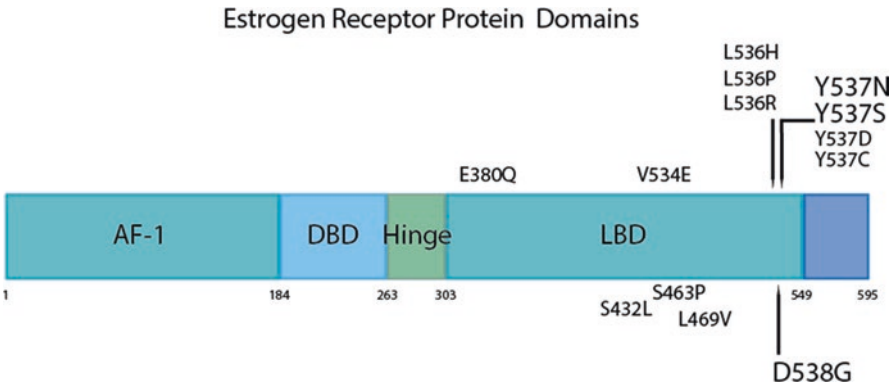

Fig. 15.1 Estrogen receptor protein domains and *ESR1* mutations. Schematic of ER's protein domains as well as the relative position of selected *ESR1* point mutations found in clinical samples. The majority of *ESR1* point mutations occur within the ligand-binding domain

domain 2 (AF-2), is required for the interaction with endogenous estrogens, synthetic ligands, and coactivators [22, 23].

15.3 ER Signaling Pathways

15.3.1 Ligand-Dependent Genomic Functions of ER

The canonical or genomic action of ER as a transcription factor involves the binding of endogenous ligand, such as E2, or synthetic ligands, to the LBD. This induces the C-terminal helix 12 to change to the agonistic conformation [22], leading to dissociation of ER from the HSP90 chaperone complex [24], dimerization [25], and association with DNA motifs, such as EREs found in the promoter, enhancer, and intergenic regions of target genes [26]. Though the ERE consensus sequence is 5′-GGTCAnnnTGACC-3′ [27], the actual sequence of many EREs can differ, resulting in changes to the binding affinity of the receptor to an ERE [28]. Some genes regulated by ER do not contain an ERE at all.

Once bound to an ERE, the complex interacts with coregulators, including corepressors and coactivators which interact via their LXXLL motif [29], and epigenetic enzymes, to regulate the transcription of estrogen-responsive genes, some of which are important in tumor growth and survival. Posttranslational regulation promotes the dissociation of the complex, and finally, ubiquitylation of ER induces its degradation by the 26S proteasome and results in either further transcriptional activation or silencing [30–32]. ER-coregulatory complexes can also be recruited by other transcription factors, such as AP-1 [33], SP-1 [34], and NF-kB [35, 36], to regulate transcription.

15.3.2 Ligand-Independent Genomic Functions of ER

Ligand-independent activation of ER is also well-documented. One mechanism is through receptor tyrosine kinases. Membrane receptor tyrosine kinases, such as EGFR, ERBB2 (HER2), and IGF1R, can activate kinase cascades that phosphorylate serine and tyrosine residues in the AF-1 domain of ER. For example, ERBB2 can trigger activation of MAP kinase ERK, which can phosphorylate ER at S118, resulting in ER transcriptional activation [37, 38].

15.3.3 Non-genomic Functions of ER

Many effects of estrogen that take place within seconds to minutes are too rapid to be explained by transcriptional and translational mechanisms, which can take hours to days. This rapid signaling can be mediated by membrane-associated ERs, which can account for ~5–10% of the total cellular ER [39], depending on the cell type. Palmitoylation of serine 522 and cysteine 447 on ER allows for association with caveolin 1 and for ER to be transported to the plasma membrane, where ER can modulate signaling cascades, including PI3K/AKT and MAPK/ERK [39].

15.4 A Brief History of Breast Cancer Treatment and the Discovery of Estrogen Receptor

In 1882, William Halstead began pioneering the radical mastectomy for breast cancer patients, which involved removal of cancerous breast tissue as well as the surrounding tissue where cancer may have spread, including the pectoralis major and minor and axillary lymph nodes (Fig. 15.2) [40]. This certainly significantly improved local recurrence, but was a horribly disfiguring and brutal procedure, and ignored estrogen produced by the ovaries, a major contributor to breast cancer growth. Endocrine ablation through oophorectomy, first performed by George Beatson in 1895, had a significant advantage over radical mastectomy alone as it could lead to regression of some breast tumors, even those that were metastatic [7]. This is considered one of the earliest forms of endocrine therapy. However, Stanley Boyd observed that only one-third of patients responded to ovarian ablation [41], indicating that not all breast tumors were equally dependent on an unknown factor secreted from ovaries that drive tumor growth. This factor was later identified as estrogen [2].

Despite variable responses, oophorectomy was part of the standard of care until advances in surgery, radiotherapy, and chemotherapy. It was unclear how estrogens functioned and what mediated their effects until Elwood Jensen's laboratory discovered estrogen receptor in 1958. His lab demonstrated that tritiated estrogen was

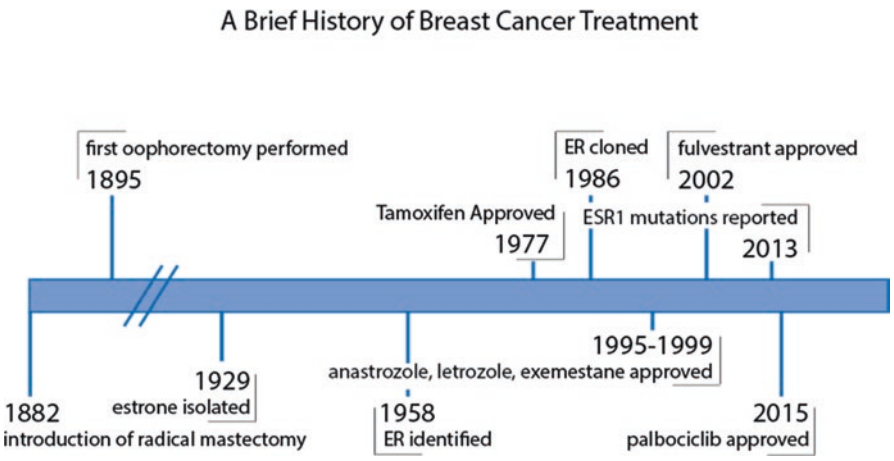

Fig. 15.2 Timeline of the History of Breast Cancer Treatment. Selected major milestones in the history of the treatment of breast cancer

bound and retained in estrogen-sensitive tissues such as the uterus and vagina in immature rats [3, 4]. Along with Elwood Jensen's lab, Jack Gorski's lab was also working to address this question, and was able to isolate and characterize estrogen receptor from rat uterine tissue [42]. These discoveries were particularly notable, given that the prevailing hypothesis at the time was that estrogens participated in enzymatic reactions to exert their effects [3, 4]. Unlike an enzyme, which fundamentally changes its substrates, steroid hormone receptors leave their ligands unaltered. It would take another 20 years before ER would be cloned and sequenced by Chambon and colleagues [5], Shine and colleagues [43], as well as Waterfield and colleagues [6]. Collectively, these discoveries laid the foundation for the idea that if some tumors are indeed dependent on estrogen and ER is necessary for estrogen stimulated growth, then identification of ER may be predictive of clinical outcome. This idea would revolutionize breast cancer treatment.

Jensen and colleagues showed in 1971 that breast cancers expressing high levels of ER were more likely to respond to endocrine ablation than those expressing low levels of ER [44]. Breast cancer task force data would corroborate this observation, showing 60% of ER-positive patients responded to endocrine ablation, whereas only 8% of ER-negative patients responded to the same treatment [3]. These findings established ER as a predictive biomarker for response to endocrine therapy. However, even then, it was recognized that ER+ breast tumors were very heterogenous, with some cells expressing ER while others did not, as seen by immunocytochemistry [45, 46]. Around this time, Harper and Walpole at Imperial Chemical Industries (ICI) were investigating antiestrogens, not as anticancer agents, but rather as modulators of the reproductive system [47]. ICI 46,474, now known as tamoxifen, was one of the more notable molecules developed because of its tolerability and potent antifertility properties in rats [3, 47]. Walpole encouraged V. Craig Jordan to investigate tamoxifen's anticancer properties because tamoxifen had been shown to

inhibit binding to tritiated estradiol of mouse and rat estrogen-sensitive tissues [3, 48]. Jordan and colleagues showed that tamoxifen could not only inhibit the binding of tritiated estradiol to rat and mouse tumors [48, 49], and human tumors [50], but also inhibit tumor growth, and initiation of DMBA-induced rat mammary carcinogenesis, establishing tamoxifen not only as an antineoplastic agent, but also as a chemopreventative for ER+ breast cancer [51]. This discovery laid the foundation for the approval of tamoxifen as a chemopreventative drug for breast cancer by the FDA.

15.5 ER-Based Therapy

Because luminal A tumors are highly dependent on the ER for growth, methods that either inhibit production of its ligand, such as aromatase inhibitors (AI), or antagonize the receptor directly, such as selective ER modulators (SERMs), are highly effective in the adjuvant setting (Fig. 15.3). E2, a potent ER agonist, binds ER via the LBD. SERMs like tamoxifen often compete with E2 for binding to the LBD, forcing ER into an antagonistic conformation and blocking ER-mediated transcription [22, 32]. Tamoxifen, the first clinically approved SERM used in the adjuvant setting, is now primarily used to treat premenopausal breast cancer patients at low risk for recurrence with, or without, interventions to achieve ovarian suppression. Though tamoxifen competes with E2 and inhibits LBD-mediated coactivator recruitment, it can also promote activation of the AF-1 domain [52–54]. This results in weak agonist activity in some tissues (e.g., uterus), increasing the risk for other kinds of cancer, and incomplete ablation of ER transcriptional activity. Fulvestrant (Faslodex), on the other hand, a selective ER degrader (SERD) and pure antiestrogen, also directly antagonizes the ER and can promote degradation of ER through the 26S proteasome [55]. Fulvestrant is used as a second-line therapy in the recurrent and metastatic setting. Aromatase inhibitors, such as exemestane, anastrozole, and letrozole, stop estrogen biosynthesis, thereby preventing ER-mediated transcription. AIs in the adjuvant setting are used as the frontline endocrine therapy in postmenopausal patients, or in high-risk premenopausal patients when combined with ovarian suppression. Recently, cyclin-dependent kinase (CDK) 4/6 inhibitors, such as palbociclib (Ibrance), have been approved for treating metastatic ER+ BC in combination with letrozole for advanced or metastatic breast cancer or in combination with fulvestrant in those previously treated with endocrine therapy [56].

15.6 ESR1 Mutations

ER+ tumors are typically associated with the most favorable prognosis, and the expression of ER indicates a more differentiated and luminal state. In addition, the expression of ER predicts response to endocrine therapies. However, approximately

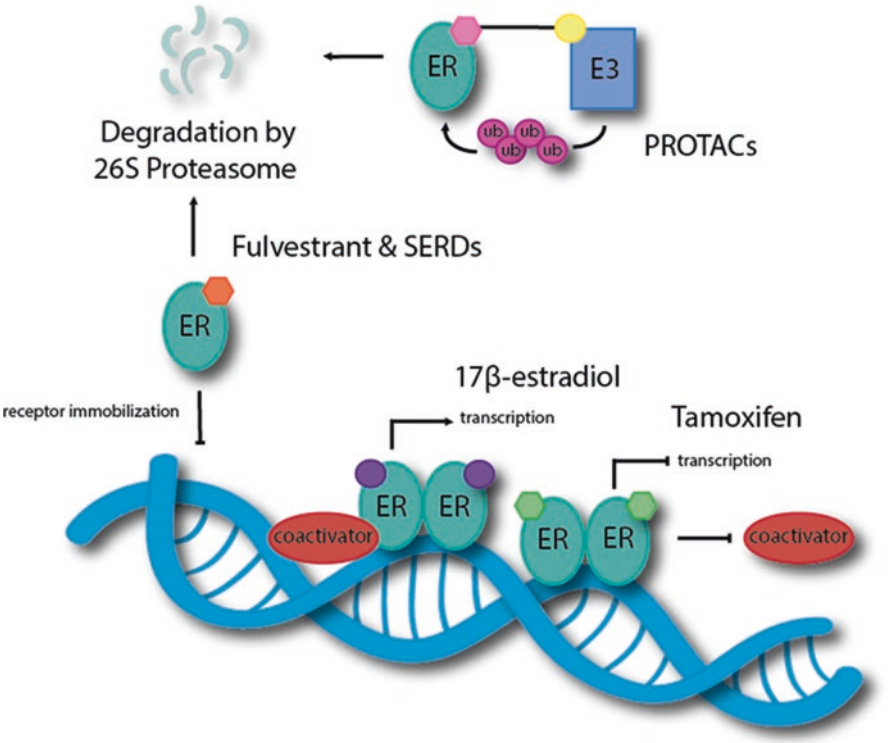

Fig. 15.3 Summary of the mechanism of action of various ER ligands. Binding of estrogen to the ER's ligand-binding domain releases the ER from its chaperone protein. The ER can then homo- or heterodimerize and bind to EREs and recruit coregulators to regulate the transcription of ER target genes. Tamoxifen also binds to the ER via the ligand-binding domain. Tamoxifen transcriptionally represses the ER and prevents the recruitment of coactivators. Fulvestrant and other SERDs also bind to the ligand-binding domain, but slow receptor mobilization, and the ER is eventually degraded by the 26S proteasome. PROTACs link a moiety to recruit an E3 ligases as well as an ER ligand. When the ER ligand on the PROTAC binds to the ER, it brings the ER into close proximity of the recruited E3 ligase. The ER can then be polyubiquitinated and targeted for degradation by the 26S proteasome

25% of patients with primary disease, and almost all patients with metastatic disease, will eventually develop resistance to these therapies [57].

Several mechanisms of resistance have been reported, including loss of ER, increases in ER expression, gene fusions, bidirectional cross talk between ER and growth factor receptors, as well as aberrant activation of growth factor receptors and their downstream signaling cascades [58, 59]. This review will focus on one recently established mechanism of resistance in breast cancer patients treated previously with endocrine therapies is the development of hotspot missense mutations in the LBD of *ESR1*. These mutations were originally identified in the late 90's, but because they were rarely found in primary tumors, they were largely ignored, until recently, when ER mutations were identified by deep sequencing of metastatic

tumors [60–64]. These experiments identified "hotspot" *ESR1* mutations in the of LBD in ~20–50% of metastatic breast cancers following endocrine therapy, depending on the study, cohort, and sequencing technology used [60–64]. Some hotspot mutations found in metastatic sites include the Y537S, Y537N, and D538G mutations [60–64]. For a more detailed breakdown of the frequency of each mutation, and the specific methodologies used for each of the cohorts, the author directs readers to other excellent reviews on the topic, Jeselsohn et al. 2015 [57] and Dustin et al. 2019 [65].

The D538G mutation has been shown to induce an increased migratory capacity in MCF7 cell models in 2D cell culture [60], and the D538G and Y537S mutations are associated with increased metastatic potential *in vivo* [66]. Moreover, the *ESR1* mutations are prognostic of poor outcomes in patients with metastatic disease [67, 68]. *ESR1* LBD mutations result in a constitutively active receptor. In addition, they have reduced ligand binding, including to E2 and fulvestrant [69–71]. Therefore, higher concentrations of antiestrogens are required to inhibit its activity [5, 7, 8, 69–71]. For more detailed information on the structural underpinnings, as well as a detailed table of K_d measurements for ER and mutant ER to various ligands, see Fanning et al. 2016 [69] and Katzenellenbogen et al. 2018 [71]. Interestingly, not all LBD mutations are involved in hormone insensitivity. K303R (though technically at the interface of the LBD and hinge region) and E380Q result in estrogen hypersensitivity [72, 73], and S432L and V534E are neutral mutations [72]. However, there are still many LBD missense mutations that have yet to be functionally characterized. Though ER mutations are not the primary drivers of carcinogenesis, under selective pressure, such as long-term antiestrogen therapy in a post-menopausal breast cancer patient, clonal expansion of rare mutants, or acquisition of de novo mutations, can lead to resistance [57].

Phenotypically, the wild type (WT) and mutant ER are distinct, and their structures help to explain their respective phenotypes. In WT ER-expressing cells, binding of agonists, such as E2, to ER changes the C-terminal helix 12 to the agonistic conformation [36, 74]. However, in ER LBD mutants, the helix 12 is maintained in the agonistic conformation, mimicking ER bound to estrogen [63, 69], even in the absence of ligand (Fig. 15.4). This may explain the hormone-independent activity of mutated receptors, and reduced efficacy of antiestrogens and ER antagonists.

Because of mutant ER's ligand independent activity, many groups have found that coactivator recruitment is constitutive. Gates et al. found coactivators such as SRC1, SRC3, AIB1, p300, RNA polymerase II, KMT2C, and KMT2D were recruited by mutant ER, even in the absence of hormones [75]. Fanning et al. found that SRC3 did not bind to the WT ER α LBD in the absence of hormone [69]. However, the ER Y537S and ER D538G bound SRC3 in the absence of E2, albeit with reduced affinity as compared to E2-bound WT receptor [69]. Toy et al. found that compared to WT ER, the D538G mutant co-immunoprecipitated with a much higher amount of AIB1 [61], indicating that, in addition to constitutive coactivator recruitment, coactivator recruitment to mutant ER is enhanced compared to WT ER.

Despite structural similarities, the mutant ER does not simply behave like constitutively active ligand-bound WT ER, but instead has its own unique phenotype.

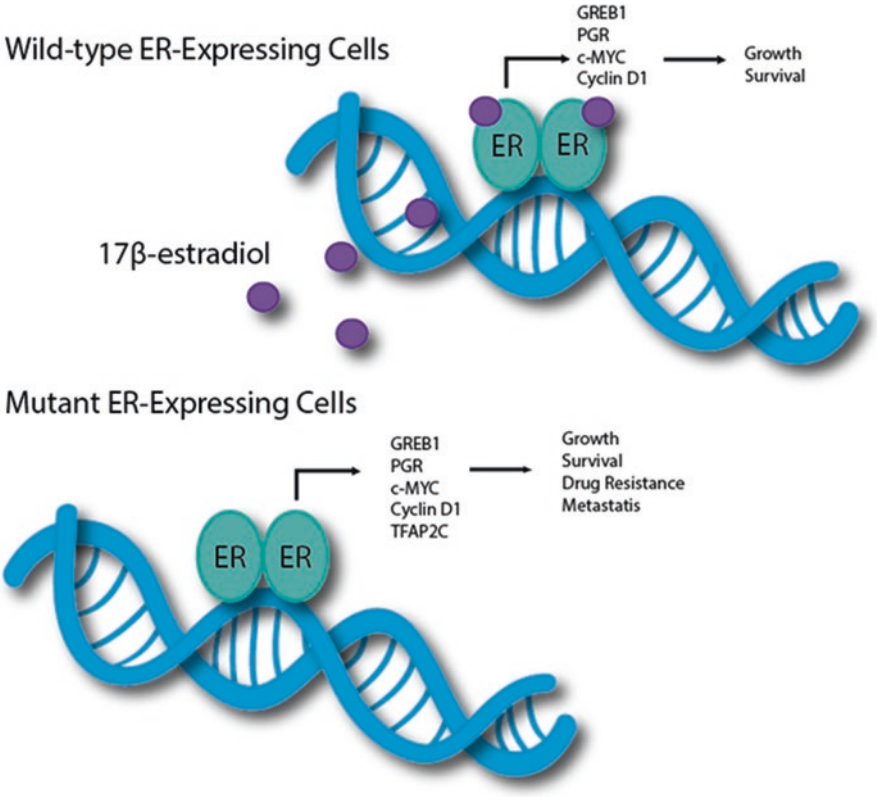

Fig. 15.4 Comparison of wild-type ER and mutant ER transcription. In the presence of E2, the ER can homodimerize and bind to chromatin to regulate the expression of estrogen-regulated genes, including GREB1, PGR, and MYC, some of which are important for normal development, as well as tumor growth and survival. However mutant ER can initiate transcription of estrogen-regulated genes, even in the absence of ligand, as well as ER mutant-specific regulated genes, which results in drug resistance and metastasis

Further, not all mutations are equivalent, and each mutation is distinct, and displays varying degrees of antiestrogen resistance. Several reports have shown that ER residue Y537 (S and N) mutations are more resistant to antiestrogens than mutations at D538 [66, 70] and S463 [72]. This is also evidenced by coregulators with mutant selectivity for the Y537S mutant over the D538G mutant, and vice versa [75]. Jeselsohn et al. compared the transcriptomes of dox-inducible ER mutants Y537S, Y537N, and D538G in the absence of E2 with WT ER cells stimulated with E2 using RNA-seq [66]. Based on their structural similarities, one would hypothesize that there would be large overlaps in their respective transcriptomes. However, only 18% of the Y537S-induced genes, and 33% of the D538G-induced genes overlapped with the E2-induced genes in WT ER cells [66], indicating that many mutant ER-regulated genes in hormone-deprived conditions were unique. In addition, each mutant exhibits differing degrees of E2 independence. When examining the

E2-regulated genes in the ER mutant cells, only 12 genes were upregulated by E2 in the Y537S mutant cells. In the D538G mutant cells, 416 genes were upregulated in response to E2, and 64% of these genes overlapped with the E2-induced genes in WT ER-expressing cells [66]. Analysis of patient derived xenograft (PDX) RNA-seq data and patient RNA-seq data confirmed the relevance of the cell line data, and demonstrated high correlation [66]. This indicates several points. The transcriptional activity of these mutants is more E2-independent compared to the WT ER. Further, each ER mutant drives a unique transcriptional program, and even ER Y537S and ER D538G elicit distinct transcriptional differences [66].

Jeselsohn et al. also found that the mutant cistrome is indeed E2-independent using ChIP-seq, with the number of binding sites in the Y537S, Y537N, and D538G mutants correlating to the known resistance phenotype of each [66]. The Y537S mutant cells had the greatest number of binding sites, and D538G mutant cells had the fewest [66]. In addition, the ER binding sites gained in the presence of the mutations occurred at transcriptionally active regions, and >30% of the super enhancers detected in the Y537S mutant cells, marked by acetylation of histone 3 lysine 27 (H3K27ac), overlapped with the mutant gained binding sites [66]. However, no specific pioneer factors co-occupied these sites [66]. Motif analysis showed that the ERE motif was the most significantly enriched of all of the binding sites, indicative of direct ER binding among all receptors and treatment conditions [66]. There were many enriched motifs common to WT and mutant ER, including FOXA1, AP1, and GRHL2 [66]. FOXA1 was the second most enriched motif in the WT ER-selective binding sites, whereas ERE motifs were enriched in the Y537S and D538G selective motif sites [66]. The FOXA1 motif was not significantly enriched in the mutant-selective binding sites, suggesting that FOXA1 may be less essential for mutant-specific ER DNA binding [66]. Knockdown of FOXA1 did not significantly affect growth of mutant ER-expressing cells in hormone-depleted conditions [66]. Fu et al. showed that FOXA1 overexpression in ER^+ breast cancer cells drives genome-wide enhancer reprogramming to activate pro-metastatic transcriptional programs, and, using clinical $ER^+/HER2^-$ metastatic breast cancer datasets, that the aberrant FOXA1/HIF-2α transcriptional axis is largely nonconcurrent with the *ESR1* mutations [76], implying different mechanisms are employed to drive endocrine resistance.

Using a CRISPR knock-in model, results from Harrod et al. confirm that there was a greater magnitude of ER binding in the absence of estrogen in MCF7 Y537S cells compared to WT cells [77]. In the mutant ER transcriptome, estrogen-regulated gene expression was still a dominant feature. GSEA hallmark gene sets, such as estrogen response early and estrogen response late, were among the most upregulated gene sets compared to MCF7 vehicle treatment [77]. However, in the unstimulated Y537S knock-in model, where only one ER allele was mutated, most of the peaks found were shared with estrogen-treated Y537S cells, as well as MCF7 WT cells [77], which does not agree with Jeselsohn et al. [66] In addition, motif analysis showed that the Y537S mutation does not cause ER binding to new unique sites [77]. In all conditions, ERE, FOXA1, AP-1, and GATA3 were the most enriched binding motifs [77]. To complicate things further, Martin et al. performed rapid

immunoprecipitation with tandem mass spectrometry of endogenous proteins to delineate the WT and mutant ER interactomes [78]. These analyses demonstrated that, though many of the proteins bound by mutant ER were also bound by WT ER, there were increased interactions between ER and transcriptional regulators, like GREB1 and FOXA1 [78]. ChIP-Seq analyses also demonstrated a ligand-independent enrichment of FOXA1 motifs in mutant ER-expressing cells [78]. Targeted knockdown of *FOXA1* in WT and mutant cells resulted in a greater growth inhibition in mutant ER-expressing cells compared with WT ER-expressing cells, suggesting a role for FOXA1 in mutant-specific biology [78], which directly contradicts the results from Jeselsohn et al. [66]

One explanation for the discrepancy between these studies is the differences in models used, with one study using an MCF7-inducible overexpression approach [66], one using an MCF7 knock-in approach [77], and the last using long-term estrogen-deprived SUM44 cells with a naturally occurring *ESR1* mutation [78]. Indeed, Andreano et al. showed that response to ligands was not dictated simply by the presence of a mutant allele but rather by the relative WT ER levels coexpressed in cells [79]. Specifically, dysregulated response to antiestrogens was only evident in cells in which the mutants were overexpressed relative to the ligand-activated WT ER [79]. This underlines the importance of using multiple models as well as paying specific attention to "allelism."

Phosphorylation may also contribute to the constitutive activity of mutant ER. Serine 118 (S118) is a major phosphorylation site within the AF-1 domain. S118 is phosphorylated in response to many stimuli but most notably by E2 [11, 80]. S118 phosphorylation (pS118) is important for receptor stability and is required for proteasome-dependent degradation of ER, which is often coupled to transactivation [81]. Mutations in this site were shown to impair ER transactivation [11]. Helzer et al. performed ChIP-seq to define the pS118-ER and ER cistromes in MCF-7 cells treated with estrogen and found that pS118 promotes direct DNA binding at active enhancers, which is associated with increased transcriptional activity [80]. In addition, pS118-ER sites were enriched in GRHL2 DNA-binding motifs [80]. E2 treatment enhanced GRHL2 recruitment to pS118-ER-occupied sites [80]. Interestingly, mutant ER is constitutively phosphorylated at S118 [82]. CDK7 functions as a CDK-activating kinase (CAK) for CDK1, CDK2, CDK4, and CDK6 but has also been shown to modulate ER activity through S118 [12]. CRISPR-Cas9 gRNA-mediated silencing of CDK7 resulted in suppressed proliferation in both WT ER cells in full medium and mutant ER cells in estrogen-deprived conditions [66], demonstrating CDK7's importance in regulating WT ER and mutant transcription. Normally, WT ER is rapidly phosphorylated within a matter of minutes in response to E2 treatment [80]. However, even in cells expressing HA-tagged ER D538G, the endogenous WT ER could be phosphorylated in hormone-starved conditions, whereas this effect was not seen in the Y537S mutant-expressing cells, perhaps suggesting that the D538G mutant has a greater propensity to heterodimerize with WT ER [66]. This may also explain to some of the phenotypic differences observed between the two mutants. In addition to phosphorylation of S118, phosphorylation on residue Y537 by SRC kinase increases E6AP recruitment and is involved in ER

proteolysis and transcriptional activation [83]. When this residue is mutated to a residue that cannot be phosphorylated (e.g., Y537F), it fails to undergo ligand-dependent proteolysis, stabilizing the receptor [83]. Indeed, Martin et al. discovered the naturally occurring *ESR1* mutations in SUM44 cells, following long-term estrogen deprivation, and showed that *ESR1* mutations are sufficient for driving acquired resistance [78]. The expression of these mutations has also been shown to be sufficient to drive metastasis. Jeselsohn et al. also demonstrated that the Y537S mutation drives metastasis using in vivo orthotopic xenografts with dox-inducible ER Y537S and ER D538G cells in ovariectomized mice with no E2 supplementation. These metastases were dependent on the expression of mutant ER, as the removal of the dox-diet resulted in the regression of the Y537S tumors [66]. Using CRISPR-Cas9-engineered homozygous knock-in ER Y537S xenograft models, Fuqua et al. also reported that the *ESR1* Y537S mutation drives spontaneous distant metastasis in vivo [84].

15.7 Clinical Significance of Mutant ER

Despite many preclinical studies demonstrating that mutant ER-expressing cells are less responsive to fulvestrant treatment, ER-positive metastatic breast cancer patients are not currently stratified based on *ESR1* mutation status. A recent retrospective analysis of the PALOMA-2 clinical trial published by O'Leary et al. showed that patients continued to acquire the Y537S *ESR1* mutation during fulvestrant monotherapy, or fulvestrant and palbociclib treatment [85]. Analysis of the BOLERO-2 clinical trial suggested that patients with the Y537S mutant allele may have worse outcomes compared with patients whose tumors harbor the D538G mutation [67]. Mutant alleles are also associated with shorter progression-free survival [67, 86, 87]. Even in early-stage local recurrence and metastatic lesions, mutant alleles are associated with worse prognosis [88]. *ESR1* mutations occur at a higher incidence rate in patients previously treated with AI compared to those whose treatments did not include AI [88]. Though there is evidence from several studies that *ESR1* mutations are associated with worse progression-free and overall survival [67, 86, 87, 89], the authors of one particular meta-analysis observed that when inspecting specific mutations, the D538G, but not the Y537S mutation, was associated with a worse prognosis and shorter progression-free survival (PFS), regardless of what treatment was administered [87, 89]. Additionally, *ESR1* mutations were predictive of aromatase inhibitors resistance, but were not predictive of resistance to other endocrine therapies [87, 89], which disagrees with the conclusions and observations of many in vitro and in vivo studies. However, additional studies with larger cohorts harboring these ER mutations will be needed to conclusively determine the clinical differences among the various mutant alleles, including responses to specific endocrine treatments, and prognosis.

PET imaging with 16α-^{18}F-fluoro-17β-estradiol (^{18}F-FES) is a common imaging modality that can be used to measure ER in metastatic sites, optimize endocrine

therapies doses, and predict therapeutic response in breast cancer patients [90]. Despite reduced binding and responses to endocrine treatments and E2, as well as the structural similarities between E2 and ^{18}F-FES, surprisingly, Kumar et al. found that tumoral uptake of ^{18}F-FES in MDA-MB-231 cells stably expressing either WT ER, ER Y537S, or ER Y537C was not found to be significantly impacted by the Y537S or Y537C mutations [91]. This indicates that ^{18}F-FES PET imaging may be used for breast cancer patients, regardless of ER mutation status [91].

Frequent metastatic sites for ER+ breast cancer include the bone, liver, lymph nodes, and brain. Toy et al. found that *ESR1* mutations were most frequently detected in liver and bone biopsies, and did not find any *ESR1* mutations in brain biopsies [72]. Zundelevich et al., however, were able to detect one brain metastasis in their cohort. Jeselsohn et al. [66] were able to confirm their findings from Merenbakh-Lamin et al. [60] that the D538G mutant allele has a liver organotropism.

15.8 Methods to Target Mutant ER

Because the ER is constitutively active in these tumors, it remains an important therapeutic target. Several approaches are being pursued to target the mutant ER. One notable class of drugs being used to directly antagonize mutant ER is selective estrogen receptor degraders, or SERDs. Fulvestrant (also called faslodex and ICI 182,780) is currently the only FDA-approved SERD and was shown to reduce ER Y537S and ER D538G expression to basal levels at very high doses [61, 63]. Though fulvestrant has been shown to be effective in the metastatic setting, fulvestrant possesses dose-limiting pharmacological properties, such as low bioavailability [124–127]. Further, fulvestrant is administered intramuscularly, and it is unclear whether fulvestrant occupies receptor at saturating levels at the current clinical dosages [124–127]. Therefore, there is a strong rationale for developing more potent, orally bioavailable pure antiestrogens that are insensitive to *ESR1* mutations.

The SERM, lasofoxifene, which was originally developed for the treatment of vulvovaginal atrophy and osteoporosis, has been shown to not be impacted by mutant *ESR1* status [79]. In addition, it has been shown to be efficacious in mammary intraductal mouse models, where MCF7 or MCF7 expressing the Y537S or D538G mutations was introduced [92]. It was more effective than fulvestrant (250 mg/kg 1/week SQ) at inhibiting primary tumor growth (5 and 10 mg/kg SQ) [92]. Lasofoxifene also inhibited both mutants from metastasizing to the lung and liver, whereas fulvestrant only inhibited the D538G mutant [92]. It is now in phase 3 clinical trials for osteoporosis and is being clinically evaluated as a treatment for patients with advanced ER-positive breast cancer whose tumors harbor *ESR1* mutations (Table 15.1).

Bazedoxifene is a rather unique antiestrogen that possesses both SERM and SERD properties. It has been studied extensively and clinically and is approved for

Table 15.1 Summary of therapies targeting mutant ER and their related publications and clinical trials, if applicable

Class	Therapy Name	Citation	Clinical trial
SERM	Lasofoxifene	Andreano et al. 2020 [79], Laine et al. 2019 [92]	NCT03781063
SERM/ SERD	Bazedoxifene	Fanning et al. 2018 [93], Wardell et al. 2013 [94], Wardell et al. 2015 [95]	NCT02448771
SERD	AZD9496	Toy et al. 2017 [72], Nardone A et al. 2019 [96], Weir et al. 2016 [97]	NCT02248090, NCT03236874
SERD	AZD9833	Hamilton et al. 2020 [98]	NCT03616587, NCT04214288
SERD	B-SERDs	Lu et al. 2019 [99]	
SERD	D-0502	Zhang et al. 2019 [100]	NCT03471663
SERD	G1T48	Andreano et al. 2020 [101]	NCT03455270
SERD	GDC-9545	Metcalfe et al. 2019 [102]	NCT03332797, NCT03916744
SERD	GDC-0810/ brilacestrant	Guan et al. 2019 [103], Joseph et al. 2016 [104], Lai et al. 2015 [105]	NCT01823835
SERD	GDC-0927	Guan et al. 2019 [103]	NCT02316509
SERD	LSZ102	Tria GS et al. 2018 [106]	NCT202734615
SERD	LY3484356		NCT04188548
SERD	RAD1901/ elacestrant	Bihani et al. 2017 [107], Patel et al. 2019 [108], Garner et al.2015 [109], Wardell et al. 2015 [110]	NCT02338349, NCT03778931
SERD	SAR439859	Campone et al. 2020 [111], Shomali et al. 2017 [112]	NCT03284957
SERD	SHR9549	Bardia et al. 2019 [113]	NCT03596658
SERD	ZN-c5		NCT03560531
SERCA	H3B-5942	Puyang et al. 2018 [114]	
SERCA	H3B-6545	Hamilton et al. 2019 [115], Rioux et al. 2018 [116]	NCT03250676, NCT04288089
Novel	K-07	Zhao et al. [70]	
PROTAC	AM-A3	Roberts et al. [117]	
PROTAC	ARV-471	Flanagan et al. 2019 [118]	NCT04072952
PROTAC	ERD-148	Gonzalez et al.2020 [119]	
Novel	Diptoindonesin G	Zhao et al. 2015 [120], Liu et al. 2016 [121], Gao et al. 2017 [122], Fan et al. 2020 [123]	

use in combination with conjugated equine estrogens for hormone replacement therapy in postmenopausal women as well as a single agent for the prevention of osteoporosis [94, 128]. It has a strong antagonist and SERD profiles in breast and agonist properties in bone and, unlike many SERMs and SERDs, did not stimulate endometrial tissue in preclinical studies [94, 129, 130]. In addition, bazedoxifene showed good oral bioavailability and improved pharmacokinetics compared with fulvestrant [94]. Preclinical studies found that bazedoxifene possesses antitumor

activity in several models of endocrine resistance, including AI, SERM-resistant tumors [94], as well as ER mutants, though the Y537S mutant was found to be relatively resistant to degradation [93]. Bazedoxifene was also found to have antitumor activity in multiple ER+ PDX models, including those expressing WT ER, the ER Y537S mutation, as well as PI3K mutations [93]. However, some data suggest that at low concentrations, bazedoxifene may behave more like a SERM than a SERD, with mixed agonist/antagonist activity [93].

New orally bioavailable nonsteroidal SERDs, such as AZD9496, GDC-0810 (also known as brilacestrant), and GDC-0927, have been evaluated in preclinical models and were found to be effective in models of endocrine resistance and mutant ER [72, 96, 97, 103–105]. In addition, they all have improved pharmacokinetics, are orally bioavailable, and do not exhibit the dose limitations of fulvestrant [72, 96, 97, 103–105]. Although exhibiting desirable mechanistic features over fulvestrant and GDC-0810, GDC-0927 still suffers from suboptimal drug-like properties [103]. In addition, both GDC-0810 and GDC-9027 are not pure antiestrogens and showed some weak agonistic activity [103]. GDC-0810 and AZD9496, in endometrial cells and rat models, both drugs exhibited uterotrophic effects in endometrial cells and rat models, raising the concern that these drugs may have agonistic activity in reproductive tissues [97, 104]. Though Genentech is no longer actively investigating GDC-0810 and GDC-0927, both have proven to be useful tools in SERD development and for understanding ER biology.

GDC-9545 is the replacement molecule for GDC-0927. Though not much has been reported on this molecule thus far, an abstract from the SABCS touts major improvements over both GDC-0927 and fulvestrant [102]. GDC-9545 has high binding potency and an improved DMPK profile when compared to GDC-0927 and fulvestrant [102]. GDC-9545's increased oral bioavailability and reduced metabolism improved oral exposure in multiple species means that the same degree of antitumor activity can be achieved but at 100-fold lower doses in the HCI-013 patient-derived xenograft (PDX) model compared to GDC-0927 [102]. In addition, GDC-9545 can achieve full suppression of ER signaling, resulting in robust antiproliferative activity, which may indicate lack of detectable agonist activity, as was seen in GDC-0810 and GDC-0927 [102, 103]. A study of GDC-9545 alone or in combination with palbociclib and/or luteinizing hormone-releasing hormone (LHRH) agonist in locally advanced or metastatic ER+ breast cancer is currently recruiting patients for a phase I clinical trial (Table 15.1).

Elacestrant (RAD1901), developed by Radius, is currently the only next-generation nonsteroidal orally bioavailable SERD in phase III clinical trials [131], underlining the difficulty of developing this class of drug. Elacestrant has demonstrated evidence of single-agent activity, with confirmed partial responses in heavily pretreated patients with advanced ER+ breast cancer, including those with *ESR1* mutations [132]. It displayed potent antitumor activity in multiple ER-expressing tumor models, including PDX models originating from patients who previously received multiple lines of endocrine therapy, those harboring *ESR1* mutations, and those with de novo or acquired resistance to CDK4/6 inhibitors [107, 108, 110]. Elacestrant is also unique in that it can pass the blood-brain barrier [110]. If clinical

studies demonstrate significant efficacy, elacestrant would be one of the first clinically approved oral SERDs that may also be useful for patients with *ESR1* mutations.

Many new SERDs have improved ER-targeting and drug-like properties compared to fulvestrant but lack the ability to penetrate the blood-brain barrier [99]. One recently developed class of SERDs, benzothiophene SERDs, contains a basic amino side arm (B-SERDs) [99]. Though its efficacy is comparable to that of fulvestrant in models of endocrine resistance, including *ESR1* LBD mutants, in vitro and in vivo, B-SERDs possess both oral and brain bioavailability, an advantage over acidic SERDs and fulvestrant [99]. Currently, there are other numerous new ER modulators under evaluation in the clinic listed in Table 15.1, though relatively little preclinical data was available when this manuscript was being prepared.

One consideration for identifying and characterizing SERDs in vitro moving forward suggested by Guan et al. is that ER degradation alone is not sufficient for choosing lead molecules that consistently and fully antagonize ER and its transcriptional activity, particularly with a heavy reliance on MCF7 cells [103]. Many ER+ models should be used when evaluating new SERDS, as they showed that ER+ breast cancer cell lines had variable responses to GDC-0810 and GDC-0927, even though GDC-0810 and GDC-0927 demonstrated potent ER degradation in MCF7 [103]. Sreekumar et al. showed that though in invasive ductal carcinoma (IDC) AZD9496 and fulvestrant behaved equivalently in terms of ER turnover and cell growth, in invasive lobular carcinoma (ILC) cell lines, AZD9496 behaved as a partial agonist and was not as potent of an ER degrader [133]. In addition, ER mobility should be considered, rather than potency of degradation alone [133]. Fulvestrant and other SERDs do not act simply by depleting the receptor, but rather through slowing and immobilizing the receptor. Indeed, in 2006, Long et al. recognized this and showed that fulvestrant immobilizes ER at the nuclear matrix, followed by receptor degradation through the ubiquitin proteasome system (UPS) [55]. This immobilization is mediated by cytokeratins 8 and 18, and helix 12 of ER is essential for association with cytokeratins 8 and 18 [55]. These cytokeratins are essential for fulvestrant's mechanism of action, as siRNAs targeting cytokeratins 8 and 18 partially blocked fulvestrant's effects [55]. This suggests that fulvestrant induces ER to interact with CK8 and CK18, drawing the receptor into close proximity to nuclear matrix-associated proteasomes that facilitate ER turnover [55]. Receptor turnover is therefore a result of receptor immobilization, which distinguishes full antagonists from partial agonists [55]. While the capacity to degrade ER may be a key feature of SERDs, it is not sufficient. This agrees with the previous work by Wardell et al. that demonstrated that fulvestrant-mediated degradation of ER indeed varies between cell models [134], as found by Guan et al. [103] The efficacy of fulvestrant as an inhibitor of ER transcriptional activity under saturating conditions and was neither influenced by the extent of ER degradation, nor by the expression levels of ER [134]. Although fulvestrant binding partially denatures ER, it can be reactivated by competing off bound drug with estradiol [134]. Collectively, competitive inhibition of ER, and not degradation, is a more important consideration [134]. One way to measure receptor mobility is live-cell imaging, which was performed in Guan et al. [103] Live-cell imaging can capture highly dynamic and transient nature

of transcription factors and transcription complexes, and could be incorporated into drug characterization pipelines to measure receptor mobility and receptor antagonism [103], in addition to the typical measurements of ER target gene expression, proliferation, tumor growth, as well as ER degradation.

Another consideration for developing and evaluating SERDs in vivo is the dose of fulvestrant used as a benchmark. Wardell et al. demonstrated that 25 mg/kg dose of fulvestrant is a more accurate and clinically relevant dose of fulvestrant for a mouse model [124]. A dose of 25 mg/kg of fulvestrant exhibited antitumor efficacy comparable to the historically used 200 mg/kg dose, but at this lower dose, it did not result in robust ER downregulation [124]. The antitumor efficacy of the lower dose of fulvestrant was comparable to that observed for other oral SERDs currently in development [124]. Using clinically unachievable doses of fulvestrant as a benchmark may negatively affect SERD development [124]. These studies suggest that receptor immobilization and antagonist efficacy, as opposed to ER degrading activity, are likely to be the primary drivers of clinical response. In the future, using these parameters could improve the selection of lead ER antagonists.

In addition to SERDs, other approaches being used to directly antagonize the ER include selective estrogen receptor covalent antagonists (SERCAs), specifically H3B-5942, that covalently binds the C530 residue of both WT and mutant ER, enforcing an irreversible antagonist conformation [114]. This residue is not conserved among other steroid hormone receptors, rendering SERCAs very specific to ER [114]. H3B-5942 demonstrated better antagonistic properties in mutant overexpressing models and growth inhibition properties than fulvestrant in vivo using cell line-derived xenografts and a PDX model expressing the Y537S mutation [114]. Like some SERMs and SERDs, H3B-5942 exhibits uterotrophic activity in immature rat models [114]. In addition, because of its high dependence on covalent engagement specific to residue C530, it is not beyond the realm of possibility that a mutation at C530 could be one mechanism of resistance [114]. A related compound, H3B-6545 [115, 116], is now in phase I/II clinical trials.

Finally, Zhao et al. developed a series of structurally novel antiestrogens [70]. They had demonstrated efficacy in vitro and in vivo against ER D538G and Y537S mutant-expressing cells, as well as their respective cell-line derived xenografts, with compound K-07 being the most effective against WT, Y537S, and D538G mutant tumor growth, and having the best pharmacokinetic profile [70]. K-07 is also orally bioavailable [70].

15.9 Additional Targets and Combination Treatments

Though *ESR1* mutations are sufficient for driving acquired resistance, other drivers can coexist in many of these tumors and justify combination treatment. Toy et al. observed that the CTC-174 PDX model that expresses the D538G mutation used in their studies was only partially inhibited by AZD9496 treatment, indicating that this mutant tumor model is not exclusively dependent on ER signaling for its growth

[72]. When this model was sequenced, an activating PIK3CA^{N345K} and two inactivating ARID1A truncation mutations E1776* and S705fs were found [72].

Tumor genotyping of *ESR1* mutant breast cancers also revealed recurrent alterations in the PI3K/AKT pathway, cyclin D1, and FGFR [61]. These alterations likely reduce tumor dependence on ER signaling and may benefit from combinations of antiestrogens with inhibitors of these pathways [72]. Despite decreased dependence on ER signaling, tumors under a single selection agent can restore ER dependence, and combinatory antiestrogen treatment still remains important as, interestingly, inhibition of growth signals such as PI3K/AKT restored ER signaling activation and dependence [135].

Furthermore, a more recent retrospective correlative analysis of the PALMOA-3 trial evaluated whether early changes in *ESR1* or *PIK3CA* mutations measured using ddPCR of ctDNA were predictive of response to therapy [85]. Although total *ESR1* mutant abundance was shown to decrease in both treatment arms, these changes were not predictive of response to fulvestrant [85]. In contrast, *PIK3CA* mutation frequency was lower in the fulvestrant- and palbociclib-treated group and was significantly predictive of PFS [85]. This study suggests that truncal mutations, such as *PIK3CA*, may be more useful to predict treatment responses than *ESR1* mutation status [85]. Differences in the predictive value of these two genetic biomarkers may be due to the clinical resistance of selected *ESR1* mutant cells to fulvestrant as well as the truncational nature of PIK3CA mutations that are shared by all subclones in the metastatic tumor [85]. O'Leary et al. also showed that other driver mutations in RB1, growth factor receptors, TP53, and PIK3CA were acquired over the course of treatment [85]. The acquisition of these mutations was associated with a longer time of treatment, and acquired mutations at the end of treatment correlated with shorter PFS [85]. These data support the conclusion that driver mutations may be acquired later in therapy as a consequence of therapeutic pressures but perhaps not always in the early treatment setting. These studies also suggest that there may be limited clinical utility to stratify patients to treatment based on *ESR1* mutation status alone and that concurrent acquisition of other driver mutations should be taken into account when designing therapeutic regimens to overcome resistance.

Jeselsohn et al. performed a genome-wide CRISPR-cas9 KO screening to determine what genes were essential for E2-independent growth of ER mutant-expressing breast cancer cells [66]. As these ER mutants are constitutively active, it is not surprising that many of the negatively selected genes, or essential genes, identified in the screen are known drivers of ER$^+$ breast cancers, such as *GATA3, TFAP2C*, *MTOR*, *MYC*, and *ESR1* itself, as well as ER coregulators, such as NCOA3, EP300, MED1, and MEN1 [66]. *CCND1* and *CDK4* remained essential genes in the mutant cells also [66], which is consistent with a retrospective clinical study in which patients with ER mutations remained sensitive to CDK4/6 inhibitors [136], and Jeselsohn et al. confirmed that mutant ER-expressing cells retain sensitivity to palbociclib, a CDK4/6 inhibitor.

Wardell et al. tested the activity of the cyclin-dependent kinase 4/6 (CDK 4/6) inhibitor palbociclib administered as both monotherapy and in combination with the

SERM bazedoxifene, in PDX models derived from patients with ER+ endocrine-resistant BC [95]. Palbociclib monotherapy was effective in a PDX expressing WT *ESR1*, and in a PDX with amplification of *ESR1*, but was ineffective in a PDX with *ESR1* D538G mutation [95]. However, this observation may be explained by the concurrent loss of RB expression in this model, a well-described mechanism of resistance to CDK4/6 inhibitors [95]. Conversely, in an ER Y537S mutant PDX model, palbociclib alone or in combination with bazedoxifene similarly inhibited tumor growth, but the combination proved more effective in decreasing Ki67 expression than either agent alone [95]. This further demonstrates that *ESR1* status, including the specific mutation, is important but is not sufficient for stratifying and predicting responses to therapy, and other mutations and alterations should be taken into consideration.

As mentioned previously, CDK7 has shown to modulate ER activity through S118 phosphorylation [12] as well as contribute to WT and mutant ER-expressing cell growth [66]. Therapeutic inhibition of CDK7 using THZ1 resulted in a dose- and time-dependent inhibition of Y537S and D538G S118 phosphorylation in vitro [66]. THZ treatment of ER Y537S cells resulted in the downregulation of pathways enriched in ER mutant-expressing cells related to ERBB2, PI3K, and MTOR, implying that THZ may be targeting ER mutant transcription as well as other components of mutant ER's transcriptional network [66]. The combination of THZ1 with fulvestrant showed significant synergism in MCF7 and T47D DOX-inducible cell lines expressing WT ER and ER Y537S, as well as the ER Y537S mutant knock-in cell line. Orthotopic xenografts of MCF7 cells expressing the Y537S mutant demonstrated that the combination of THZ1 with fulvestrant had improved efficacy in inhibiting tumor growth compared with either single agent [66]. These results support the potential of this combination as a therapeutic strategy to overcome endocrine resistance caused by the ER mutants. Harrod et al. also confirmed this in vitro, showing that THZ itself can inhibit MCF7 and MCF7 Y537S cell growth and co-treatment of THZ with fulvestrant significantly augmented the growth inhibition of MCF7 Y537S cells compared to either agent alone [77]. However, the difference in sensitivity to THZ in mutant ER- and WT ER-expressing cells was not statistically significant [77].

An additional target shown to be involved in tamoxifen resistance in *ESR1* mutant models by Gelsomino et al. is insulin growth factor 1 receptor (IGF1R) signaling [137]. IGF1R and mutant ER showed enhanced cross talk and co-localization, as shown by ER immunoprecipitation and proximity ligation assays [137]. Treatment with IGF1R pathway inhibitors sensitized mutant ER-expressing cells to tamoxifen [137]. Using similar mutant models, Li et al. also demonstrated that the IGF1R pathway contributes to endocrine resistance [138]. The mutant ER-expressing models had an enhanced IGF gene signature compared to the WT ER-expressing models, based on RNA-seq analyses [138]. In addition to an enhanced IGF gene signature, mutant ER-expressing cells, tamoxifen resistant cells, and long-term estrogen-deprived cells all showed enhanced growth in response to IGF1 stimulation [138], indicating that IGF1R-mediated endocrine resistance may be shared among many models of endocrine resistance and is not specific only to mutant

ER-expressing models. Using inhibitors or small interfering RNA knockdown to target the IGF1R pathway sensitized mutant ER-expressing cells to endocrine therapy [138], confirming the results from Gelsomino et al. [137] Despite promising results from these preclinical studies, IGF1R inhibitors do not yet have demonstrated efficacy in the clinic in the context of metastatic breast cancer.

Though they play a large role in endocrine resistance as a whole, other growth factor receptors, including the HER1–3 family members and FGFR, need to be evaluated as potential mechanisms of endocrine therapy resistance specifically in *ESR1* mutant models. It appears that at least at a mutational level, *ESR1* mutations and growth factor receptor mutations, including those in HER2, FGFR1, FGFR2, and FGF3, are mutually exclusive [139, 140].

15.10 Modulating Molecular Chaperones, and E3 Ligases, and the Ubiquitin-Proteasome System: New Ways to Target Mutant ER

One of the primary strategies for targeting the ER in breast cancer is small-molecule-mediated receptor degradation. However, as mentioned previously, degradation can be coupled to both transcriptional activation and transcriptional repression and can be explained by the ER being regulated by two different ubiquitin proteasome pathways, depending on the ligand [141]. As a result, it is important to understand the mechanisms that regulate ER stability in order to develop effective ER-targeting therapies. ER stability is known to be affected by multiple factors. HSP90 is a molecular chaperone that is responsible for the folding, maturation, and activation but also stabilization of its over 200 clients [142]. It accounts for 2% of cytosolic protein [143]. Many of these clients include steroid hormone receptors [144, 145]. It maintains the ER in a ligand-binding conformation and also protects it from proteasomal degradation [146].

HSP90 in cancer behaves very differently from HSP90 in normal cells and protects overexpressed and mutated oncoproteins, mediating oncoprotein addiction [142]. Interestingly, HSP90 inhibitors in general have a higher affinity for HSP90 in tumors than in normal cells, and selectively accumulate in tumors [147]. One explanation for this phenomenon is that soluble HSP90 in tumor cells is present in assembled multi-chaperone complexes that are more active than the HSP90 in normal cells, which is in an uncomplexed inactive form [147].

HSP90 inhibitors that belong to the benzoquinone antibiotic family, such as geldanamycin and its analogs 17AAG and 17-DMAG, bind to the N-terminal ATP binding pocket, inhibiting its ATPase activity essential for performing its chaperone functions [148, 149]. ATP binding and hydrolysis are coupled to the "opening" and "closing" of HSP90 protomers [144, 150, 151], and these structural rearrangements regulate the interactions with co-chaperones and client proteins [152]. Inhibition of HSP90 results in the inhibition of this chaperoning cycle [144, 150, 151], the

recruitment of E3 ligases, and the degradation of its client proteins by the 26S proteasome [31, 153].

The UPS is another primary regulator of ER stability and is critical for maintaining protein homeostasis and unfolded protein turnover [80, 154]. There are many players involved in the UPS. Three enzymes, ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3), catalyze the covalent binding of the protein ubiquitin to lysine residues [80, 154]. In humans, though there is only one major E1, E3 ligases help impart substrate specificity to E1 and E2, and there are estimated to be between 500 and 1000 E3 ligases [155]. Some of the most well-known E3 ligases that regulate ER stability include MDM2 [156], C-terminus of HSC70-interacting protein (CHIP) [31], BARD1 [157]/ BRCA1 [158], SKP2 [159], and E6AP [83]. Residues K302 and K303 are essential for ER ubiquitination and subsequent degradation by the 26S proteasome [21]. For a more comprehensive review of ubiquitylation of nuclear receptors, the author directs readers to Helzer et al. 2015.

Geldanamycin was shown to enhance the CHIP-ER interaction, resulting in increased ER degradation [31]. This effect was abrogated by CHIP knockdown using siRNAs, indicating that CHIP is required for geldanamycin-mediated ER degradation [31]. Though wild-type ER is known to be chaperoned by HSP90 until bound to E2, it is currently unknown if mutant ER depends on molecular chaperone proteins. Structural studies indicate that mutant ER is stabilized in the ligand-bound conformation [69, 71]. As a result, it is possible that mutant ER does not associate with HSP90, because it is already in a ligand-bound conformation. Toy et al. treated MCF7 cells transfected with vector expressing HA-tagged wild-type ERα or Y537S and D538G mutant ER with SNX2112, an N-terminal ATP binding site-targeting drug, at various doses for 3 hours. They found that though levels of WT ER decreased in a dose-dependent manner, the Y537S and D538G mutants were unaffected by HSP90 inhibition, indicating that HSP90 does not regulate mutant ER stability [61]. On the contrary, Yu et al. found that ganetespib (STA9090) had cytotoxic effects not only in ex vivo cultured circulating tumor cells (CTCs) expressing *ESR1* mutations as a single agent, but also in combination with raloxifene and fulvestrant [160]. They also found that sensitivity to HSP90 inhibition was associated with mutant *ESR1* allele frequency [160]. Because the conclusions of these two studies contradict one another, further mechanistic studies are required to determine conclusively whether HSP90 associates with mutant ER, and whether this phenomenon holds true for other classes of N-terminal as well as C-terminal-targeting HSP90 inhibitors. Establishing whether HSP90 can interact with *ESR1* LBD mutants could open up a new class of drug for the treatment of mutant ER-expressing tumors, and help better understand the biology of *ESR1* ligand binding domain mutants.

One way to circumvent HSP90 dependence is to directly modulate E3 ligases to target proteins for degradation. There are several molecules that have been reported as E3 ligase modulators. The most notorious small-molecule E3 modulator is thalidomide. Despite its teratogenicity, it has since been shown to bind directly to cereblon and to be an effective treatment in the refractory multiple myeloma setting [161, 162]. Once bound to thalidomide or related compounds, such as lenalidomide

and pomalidomide, cereblon can promote degradation of the IKAROS family of transcription factors, which results in the downregulation of downstream targets that regulate cell death [163]. One study reported the use of novel cereblon modulator CC-92480 to treat multiple myeloma in the relapse, refractory, and lenalidomide-resistant setting by targeting Aiolos and Ikaros for degradation by the 26S proteasome [164]. CC-92480 had a superior degradation efficiency and kinetics compared to lenalidomide and pomalidomide. CC-92480 is reported to act as a molecular glue, allowing cereblon to interact with proteins it would not normally [164]. Further experiments would be needed to know if CC-92480 would be useful in the context of mutant ER in breast cancer.

Diptoindonesin G (DipG) is another example of a small molecule reported as a modulator of the E3 ligase CHIP, and has been studied in the context of breast cancer [120, 121], as well as AML [122]. Zhao et al. showed that DipG could not only promote degradation of ER α but also reciprocally stabilize ER β, implicating a commonly shared E3 ligase, CHIP [120]. When CHIP was knocked down using shRNA, DipG-mediated ER degradation was abrogated, indicating that CHIP is essential for DipG's mechanism of action [120]. In addition, the 26S proteasome was also essential for DipG-mediated ER degradation, and treatment with MG132 resulted in stabilization of ER, even in the presence of DipG [120]. However, it remains unclear whether CHIP is truly the direct target of DipG or whether DipG perhaps modulates another component of the HSP90-ER-CHIP ternary complex, or acts as a molecular glue. Regardless, because of this ligand-binding domain-independent mechanism of ER degradation, our group has demonstrated that DipG is indeed unaffected by ER mutant status, and can indeed promote degradation of WT and mutant ER (unpublished observations), and may be a promising molecule to overcome endocrine resistance.

A new emerging class of drug, proteolysis targeting chimera technology (PROTAC), can link ER ligands to a small molecule that binds an E3 ligase, leading to ubiquitination and proteasomal degradation of ER (Fig. 15.3). Arvinas' PROTAC was effective against mutant ER and thus far has shown no agonist activity [118]. Preclinical studies showed that the ER PROTAC ARV-471 promoted potent degradation of ER in multiple ER-positive cell line models [118]. Furthermore, ARV-471 showed robust growth inhibition of WT and mutant ER xenograft models [118]. Clinical development of ARV-471 is ongoing and, if successful, will represent a novel class of ER protein degraders that can also be used to target other proteins in breast and other cancers. Gonzales et al. developed a series of ER-targeting degraders based on PROTAC, including ERD-148 [119]. Likely because of its ligand-dependent mechanism, ER mutants exhibit resistance to ERD-148 to the same degree as fulvestrant as measured by cell proliferation and downregulation of GREB1 [119]. Though its biological activity in vitro is comparable to that of fulvestrant, it has the advantage of potentially being orally bioavailable [119]. Roberts et al. reported a two-stage strategy to develop PROTACs against ER [117]. A promising molecule generated, AM-A3, elicits potent ER degradation activity and decreased the proliferation of MCF7 [117]. This approach can significantly simplify as well as increase the throughput of PROTAC development and could theoretically

be expanded to other targets of interest [117]. However, PROTAC is still based on a ligand-dependent mechanism, and its large bulky size (~700–1000 Da) may restrict cell permeability. In addition, it is unknown how PROTACs may affect the endogenous substrates of the E3 ligases they modulate.

15.11 Conclusions

Despite great strides in not only understanding estrogen signaling and the biology of breast cancer but also developing breast cancer treatments just the last century, ER-positive breast cancer remains a challenging disease. The majority of mortality is related to metastatic disease and the development of resistance to mainstay therapies. Endocrine resistance caused by *ESR1 hot spot mutations* is still an active area of research, and good drug candidates specifically for mutant ER-expressing breast cancers remain at large. *ESR1* mutations have a unique biology and do not simply recapitulate the phenotype of WT ER in the agonist conformation, nor are *ESR1* mutations at different residues equivalent. Rather, the *ESR1* mutants have their own distinct cistromes and transcriptomes and resistance phenotypes. New orally bioavailable SERDs are being developed to overcome the shortcomings of aromatase inhibitors, fulvestrant, and tamoxifen. However, SERDs have been a challenging class of drug to develop. New SERDs and SERMs still rely on the ligand-binding domain for their mechanism of actions; many of them also exhibit tissue-specific mixed agonist-antagonist activity. This may explain why many SERDs have failed in clinical trials. One way to improve lead molecule selection in the future is considering receptor mobility in addition to the potency of degradation, downregulation of ER target genes, and in vivo efficacy. Currently, elacestrant is the only SERD in phase 3 clinical trials. Taking a ligand-binding domain-independent approach by modulating molecular chaperones and E3 ligases that control ER stability could be a promising approach to circumvent endocrine resistance, and could also be used to target additional drivers in mutant ER tumors. However, the efficacy of HSP90 inhibitors and E3 ligase modulators requires an additional investigation.FundingThis work was supported by NIH R01s CA213292 and CA236356, DOD BC190650 to W.X. and NIH T32 CA009135 to K. D.

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Chapter 16
Androgen Receptors in the Pathology of Disease

Jacky K. Leung, Amy H. Tien, and Marianne D. Sadar

Abstract Androgen receptor (AR) belongs to the steroid hormone receptor group of ligand-activated transcription factors in the nuclear receptor superfamily. AR mediates the action of physiological and exogenous androgens to regulate the expression of a network of genes in target tissues that are essential for the development and maintenance of the male phenotype and reproductive function as well as the function of numerous other tissues in both males and females. AR is ubiquitously expressed throughout the body. AR is a modular protein that comprises an N-terminal domain (NTD) that contains all of its transcriptional activity, a DNA-binding domain, a flexible hinge region, and a C-terminal ligand-binding domain (LBD). All clinically approved hormonal therapies target the AR LBD, either directly with antiandrogens and selective AR modulators or indirectly by reducing levels of androgens. Pathological conditions related to AR dysfunction involve altered levels of androgens and structural alterations in the AR. These include mutations, polymorphisms in the polyglutamine tract of the NTD, and alternative splicing of AR to yield constitutively active receptors. From the extensive list of AR-related diseases, herein we describe prostate cancer, androgen-insensitivity syndrome, polycystic ovary syndrome, breast cancer, and a few more pathological conditions in more detail.

Keywords Androgen receptor · Androgen receptor mutation · Prostate cancer · Breast cancer · Androgen insensitivity syndrome · Polycystic ovary syndrome

Abbreviations

AF-1	activation function 1
AF-2	activation function 2
AR	androgen receptor

J. K. Leung · A. H. Tien · M. D. Sadar (✉)
Canada's Michael Smith Genome Sciences Centre at BC Cancer, Vancouver, BC, Canada
e-mail: msadar@bcgsc.ca

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ARKO	*AR* knockout
AR-Vs	androgen receptor splice variants
CAIS	complete androgen insensitivity syndrome
CRPC	castration-resistant prostate cancer
CTCs	circulating tumor cells
CTE	C-terminal extension
DBD	DNA-binding domain
DHEA	dehydroepiandrosterone
DHT	5α-dihydrotestosterone
E2	17β-estradiol
EMS	external masculinization score
ER	estrogen receptor
fl-AR	full-length AR
GR	glucocorticoid receptor
HSP	heat-shock protein
KLK3/PSA	prostate-specific antigen
LBD	ligand-binding domain
LH	luteinizing hormone
LH-RH	luteinizing hormone-releasing hormone
MAIS	mild androgen insensitivity syndrome
NTD	N-terminal domain
PAIS	partial androgen insensitivity syndrome
PCOS	polycystic ovary syndrome
PR	progesterone receptor
SARM	selective androgen receptor modulator
SBMA	spinal-bulbar muscular atrophy
SHBG	sex-hormone-binding globulin
TAU	transactivation unit
TNBC	triple-negative breast cancer

16.1 Androgens

Historically, androgens have been referred to as male sex hormones due to their importance in the control of normal development and reproductive function in males. The most abundant endogenous androgens are testosterone and its more active metabolite 5α-dihydrotestosterone (DHT). For the average adult male, 3–10 mg of testosterone is produced per day, and approximately 4% of it is converted to DHT by 5α-reductase and 0.2% to 17β-estradiol (E2) by aromatase. The Leydig cells in the testis synthesize >95% of the testosterone in circulation from cholesterol, through a pathway of enzymes in response to luteinizing hormone (LH) signaling. Peripheral tissues including the adrenal glands as well as the ovaries are also sources of weaker androgens, which include androstenedione and dehydroepiandrosterone (DHEA). The normal physiological range of testosterone in healthy

men is between 350 and 600 ng/dL (>12 nM), and levels below 300 ng/dL are considered low testosterone [1]. The upper range of testosterone levels in women is between 12 and 58 ng/dL (0.4–2 nM). Chronically elevated levels of testosterone in women can be associated with polycystic ovary syndrome (PCOS, 0.34–5.5 nM) or congenital adrenal hyperplasia (1.32–5.62 nM). Virilization is observed in women with three times above the normal concentrations of testosterone. In the circulation, only 2% of testosterone is free, whereas 50% is bound to albumin with low-to-moderate affinity, 44% tightly-bound to sex-hormone-binding globulin (SHBG), and 4% loosely-bound to corticotropin-binding globulin [2]. Testosterone bound to SHBG is not bioavailable, since it restricts cell permeability, and thereby SHBG is involved in regulating biological responses to androgens. SHBG levels are down-regulated by androgens and are decreased in pathological conditions, such as diabetes, obesity, hypothyroidism, and aging. Estrogens, hyperthyroidism, cirrhosis, and tamoxifen increase the levels of SHBG [3]. Tissue concentrations of androgens may therefore not reflect changes in the concentrations of circulating androgens [4]. The biological effects of androgens are mediated by the androgen receptor (AR). Pathologies associated with the androgen axis are carried out by AR and may involve altered levels of androgens and/or changes in the structure or function of AR.

16.2 Androgen Receptor Structure and Function

16.2.1 Expression of AR

AR is ubiquitously expressed throughout the body, with the possible exception of the spleen [5]. The AR has important roles in the reproductive tissues of men and women, and it influences cognition, hematopoiesis, coagulation, skin, hair, bone, muscle, and some brain malignancies [6–8] (Fig. 16.1). Tissue-specific expression of AR cofactors mediates the differential effects measured between different tissues [9]. The AR is encoded by a gene (AR; NR3C4) located on the X chromosome (locus: Xq11-Xq12). Males carry a single copy of the *AR* gene, whereas females have one functional copy due to X-chromosome inactivation (also known as Lyonization) [10]. The regulatory regions of the *AR* gene lack TATA and CCAAT elements and have binding sites for SP1, NF-kB, and c-MYC (for reviews, see [11, 12]). Androgen autoregulates AR expression to increase as well as decrease levels of AR mRNA (for a review, see [12]).

16.2.2 AR Structure

Full-length AR (fl-AR) is a 98.8 kDa protein encoded from eight canonical exons in the *AR* gene and at least seven other cryptic exons (Fig. 16.2). Generally, the wild-type full-length protein is described to be 910 to 919 amino acid residues, with

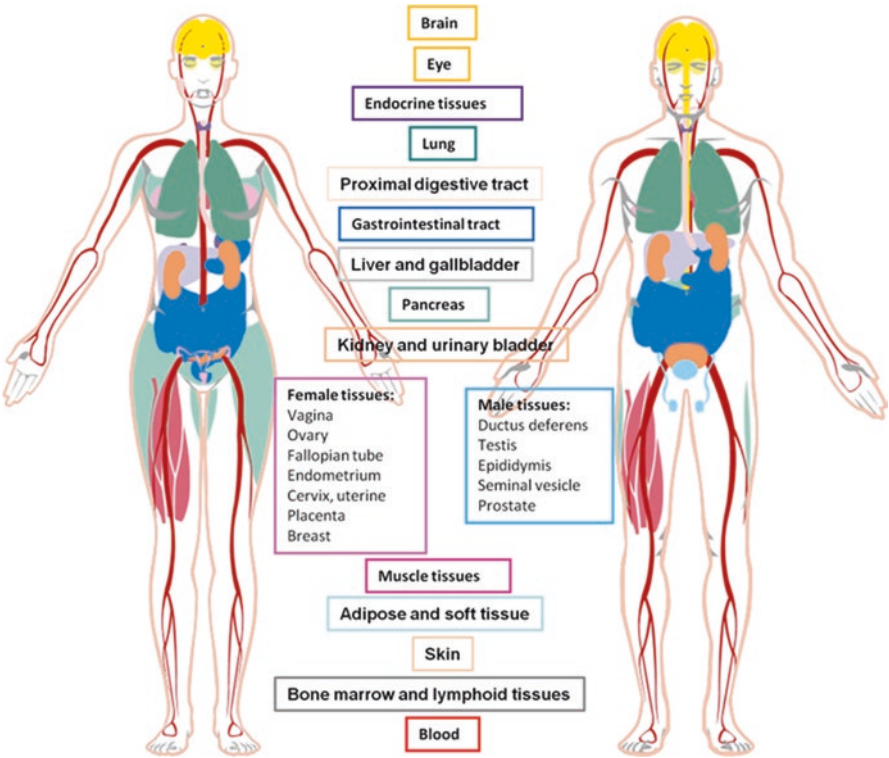

Fig. 16.1 AR expression in the human body. AR expression is detected in various organs in both males and females. Diagram of the human body showing the expression of AR in different organs was retrieved from the RNA and Protein Expression Summary in Human Protein Atlas (https://www.proteinatlas.org/ENSG00000169083-AR/tissue) [7]

deviations predominantly due to polymorphisms in the polyglutamine (CAG) and polyglycine (GGC) repeats in the amino-terminal domain (NTD). Posttranslational modifications of AR include phosphorylation, SUMOylation, methylation, and ubiquitination and can impact AR structure, protein-protein interactions, transcriptional activity, cellular localization, and stability. The amino acid sequence similarity between human AR and related steroid hormone receptors is crucial for understanding its specificity for ligands, DNA-binding sites to regulate gene expression, and drug development. For examples, the AR C-terminal ligand-binding domain (LBD) shares 54% sequence similarity with the LBD of progesterone receptor (PR), and antiandrogens can inhibit the transcriptional activity of PR [13, 14]; the AR DNA-binding domain (DBD) is 76% identical to that of the glucocorticoid receptor (GR), and they share common regulatory sequences within the same loci of chromatin [15, 16]. The specificity of steroid hormone receptors is generally believed to be achieved through receptor-specific residues in their ligand-binding pockets and tissue-specific expression (for a review, see [17]). Using the prostate as an example, benign prostate epithelial cells express AR but do not express GR,

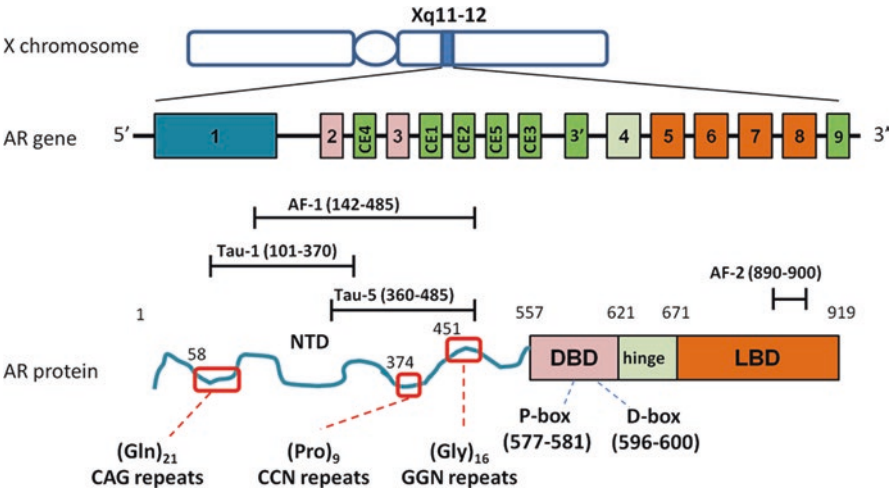

Fig. 16.2 Domains and functional regions of AR. AR gene is located on X chromosome and contains 8 exons that encode for full-length AR. Domains of AR are shown in the same color as respective exons. AF-1 is within NTD whereas AF-2 is in LBD. Tau-1 and tau-5 are located in AF-1. Locations of polyglutamine (CAG repeats), polyproline (CCN repeats), and polyglycine (GGN repeats) on AR NTD are indicated. P-box and D-box are located in the two zinc fingers within DBD. CE, cryptic exon

whereas in advanced prostate cancer, both AR and GR are coexpressed following castration [18]. Based upon these observations, the GR has been suggested as a mechanism of resistance to hormonal therapies for advanced prostate cancer [19].

16.2.3 AR Domains

AR is a modular protein with an intrinsically disordered polymorphic NTD (polymorphic, 547 to 556 amino acid residues), a folded DBD (65 amino acid residues), a flexible hinge region (49 amino acid residues), and a structured LBD (249 amino acid residues).

16.2.3.1 AR NTD

The AR NTD is essential for its transcriptional activity and acts as a hub for interactions with many other proteins. No crystal structure for the AR NTD has been resolved due to its limited stable secondary structure. The AR NTD contains all of its transcriptional activity with activation function-1 (AF-1) instead of AF-2 in the LBD like estrogen receptor (ER). At 547 to 556 amino acid residues, the AR NTD is approximately three times longer than the NTDs of ERα and ERβ. AR AF-1 has

approximately 13% helical secondary structure which is increased with binding to interacting proteins [20, 21]. There are two transactivation units (tau) within AF-1, tau-1 (amino acid residues 101–370) and tau-5 (amino acid residues 360–485), that interact with basal transcriptional machinery to mediate the transcriptional activity of the AR.

The polymorphic AR NTD contains multiple repeat regions that vary in length that include the polyproline tract (average 9 repeats), polyglycine tract (average 16 repeats), and polyglutamine tract (average 21 CAG repeats) (Fig. 16.2). Variable lengths of the polyglutamine tract are the most studied due to its association with diseases such as infertility [22], male pattern baldness [23], symptomatic benign prostatic hyperplasia [24], spinal-bulbar muscular atrophy (SBMA), PCOS, prostate cancer, breast cancer, and ovarian cancers [25–29]. The length of the polyglutamine tract impacts AR solubility and its transcriptional activity. A tract of 9 to 39 is considered in the "normal" range [30]. Short polyglutamine tracts have increased AR transcriptional activity, whereas a longer tract has less activity. Tracts longer than 37 CAG residues can form cytotoxic fibrillar aggregates that are associated with SBMA. The propensity for aggregate formation is increased with androgens due to the release of heat-shock protein (Hsp) 40 and Hsp70 chaperone proteins from the $_{23}$FQNLF$_{27}$ motif in the AR NTD. Shedding of Hsps allows AR NTD interaction between the $_{23}$FQNLF$_{27}$ and the AR C-terminal LBD (called N/C interaction) that is required to mediate transcriptional activity in response to androgen [31]. N/C interactions delay dissociation of androgen from the ligand-binding pocket, stabilize the AR protein, and most importantly provide the main site for binding of coregulators to mediate transcriptional activity through AF-1 rather than AF-2 unlike ER [31–33]. Low-resolution cryoelectron microscopy (cryo-EM) has revealed the structure of transcriptionally active fl-AR to be unique from ERα [34] (Fig. 16.3). Dimerization of AR is in a head-to-head and tail-to-tail manner which allows direct interactions at different sites in the AR NTD with a single molecule of the cofactors SRC-3 and p300 [34]. These data revealed that the AR dimer consists of two different conformations of NTD. One conformation directly interacts with SRC-3 close to its $_{23}$FQNLF$_{27}$ motif [34] that is consistent with coimmunoprecipitation studies from two decades ago that showed SRC interacts within 1–233 amino acid residues of the AR NTD [35]. The p300 molecule interacts with both conformations of NTD [34]. Presumably, interactions with CREB-binding protein (CBP), which is highly related to p300, may also behave similarly to p300 in its mechanism of interaction with the AR NTD. Such direct interactions and stoichiometry for SRC-3 and p300 are unique to AR compared to ERα, which has a strong AF-2 function and weak AF-1 function. The AR NTD is also highly modified by phosphorylation and SUMOylation and contains multiple sites for the peptidyl-prolyl *cis/trans* isomerase Pin1 [36–38]. These modifications can impact the conformation of a protein to potentially alter protein-protein interactions.

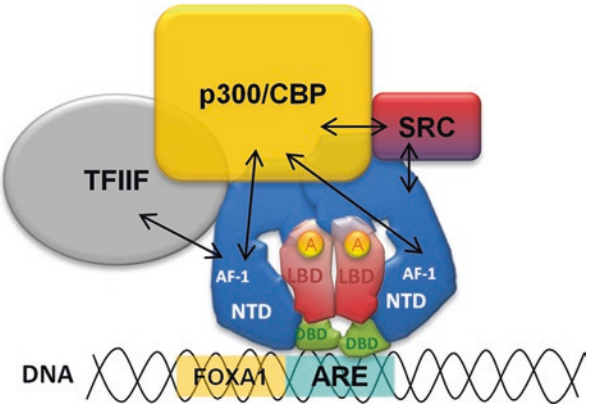

Fig. 16.3 Structure of transcriptionally active AR. The AR dimer forms when androgen binds to LBD. DNA-bound AR dimer interacts with one molecule of SRC-3 and p300 (CBP) through NTD. SRC-3 interacts with a region close to the $_{23}$FQNLF$_{27}$ motif on AR1-233 of one AR monomer. p300 interacts with AF-1 on two AR monomers [34, 35]. CBP is presumed to be similar to p300 in its interaction due to their structural similarities. CBP and the RAP74 subunit of TFIIF interacts with AR 423–448 [306]. Arrows indicate interactions between molecules. FOXA1 binding site is shown on DNA. A, androgen; ARE, androgen response element

16.2.3.2 AR DBD and Hinge Region

The AR interacts with DNA through its structured three-dimensional DBD that has a resolved crystal structure [39]. The AR DBD has three helices consisting of two zinc fingers with four cysteine residues that bind a zinc ion plus a C-terminal extension (CTE). Within AR DBD are the P-box and D-box that are essential for AR transcriptional activity. The first zinc finger is the recognition helix that binds AREs through the P-box [40]. The second zinc finger contains the D-box required for dimerization between monomers of AR [41]. The CTE provides specificity for AR to recognize AREs [42]. These AREs are found in enhancers and less so in promoter regions of target genes and are arranged as repeats of a hexamer separated by a spacer of three base pairs (for a review, see [17]). The hinge region is unstructured and links the AR DBD to its LBD. Nuclear translocation is a major function of the hinge region, but it has other functions and is regulated by acetylation, methylation, phosphorylation, and ubiquitination [43].

16.2.3.3 AR LBD

The effects of androgen are mediated through binding the folded C-terminus LBD. To date, there are only crystal structures resolved for the agonist conformation of AR, which reveals two antiparallel β-sheets and 11 α-helices that encompass a ligand-binding pocket [14]. The AR is missing helix 2, and this lack of helix 2 is seen in PR, GR, and mineralocorticoid receptor, but not in ER [44]. Androgens

cause a shift in conformation to reposition helix 12 over the ligand-binding pocket to create the AF-2 surface for N/C interactions [45]. The ligand-binding pocket consists of hydrophobic residues that interact with lipophilic testosterone and DHT. The AR LBD is the direct or indirect target for all currently FDA-approved drugs against the androgen axis. These drugs include those that reduce the levels of androgen that bind to the AR LBD such as luteinizing hormone-releasing hormone (LH-RH) analogs and CYP17 inhibitors that block steroidogenesis, selective androgen modulators (SARMs), as well steroidal and nonsteroidal antiandrogens. Antiandrogens compete with androgens for the AR LBD. Therefore, since DHT has a binding affinity in the low nM range for AR LBD, an effective antiandrogen must have a very strong affinity to be able to compete with DHT for the ligand-binding pocket in the AR LBD. Structural alterations in the AR LBD involved in disease include deletion or truncation of LBD that results in constitutively active AR that is independent of androgens [46]. Expressions of these constitutively active AR splice variants (AR-Vs) lacking the AR LBD have been detected in numerous tissues [47] and are a major mechanism of resistance to hormonal therapies for the treatment of prostate cancer [48]. Gain-of-function mutations in the AR LBD are also a major mechanism for the failure of current hormone therapies [49].

16.2.3.4 Transactivation of AR

Androgens enter into cells from the circulation by passive diffusion. Within the cell, testosterone can be converted by 5α-reductase to the more active androgen, DHT. Both testosterone and DHT bind with strong affinity within the ligand-binding pocket in the LBD of the cytosolic AR. DHT has approximately ten times improved affinity for the AR compared to testosterone predominantly due to the small difference in its chemical structure that impacts its interaction within the ligand-binding pocket of the LBD to result in a slower dissociation rate compared to testosterone [50]. Here, we focus on genomic signaling of AR and direct readers to a recent review on non-genomic signaling of the cytosolic AR [51]. Genomic signaling of AR is initiated upon androgen binding to the AR LBD to induce a conformational change that decreases AR interactions with chaperones which results in the reduction of its solubility that enhances its affinity for DNA. The nuclear localization signal in the hinge region becomes unmasked, thereby allowing the AR to form intramolecular N/C interactions and translocate into the nucleus. Within the nucleus, the AR forms an intermediate AR homodimer through intermolecular N/C interactions through their D-boxes [52]. Upon binding to androgen-response elements (AREs) within the regulatory regions of androgen-responsive genes, N/C interactions are lost to allow interaction with coactivators and recruitment of the basal transcriptional machinery. Over 300 coregulators have been described for nuclear receptors that function to stimulate or repress transcription without binding directly to DNA. These include proteins that regulate the structure of chromatin and bridge components of the basal transcriptional machinery to the site of transcription. Coregulators include ATPases and histone modifiers (for reviews, see [53, 54]). The

p160 steroid receptor coactivator-1 (SRC-1) and SRC-3 are examples of coactivators of AR that have histone acetyltransferase (HAT) activity. The bHLH/PAS, S/T, and HAT domains of SRC-3 directly interact with region 1–233 amino acid residues of the AR NTD [34, 35]. The AR is unique from other steroid hormone receptors in that p300, and presumably also CBP, directly interacts with the AR NTD rather than indirectly through recruitment to SRC [34]. The AR NTD is the site for interaction with the basal transcriptional machinery including recruitment of RNA polymerase II which is necessary for transcriptional activity. Other important coactivators of AR include the methyltransferases CARM1 and PRMT1 [55, 56]. The requirement of tissue-specific pioneer factors that co-localize with the AR on DNA-binding sites include FOXA1 with fl-AR as well as HOXB13 with AR-V7 in the prostate [57–60]. In response to androgens, the AR both induces and represses the expression of genes that are involved in development, metabolism, differentiation, proliferation, and DNA damage repair [61–65]. Thus, altered transcriptional activity of AR due to structural changes and/or variation in the levels of available androgens has a profound impact on human physiology and disease. An important recent discovery is the role for AR in modulating the expression of androgen-regulated genes such as *TMPRSS2* and *ACE2* that are required for the entry of the SARS-CoV-2 virus into cells to mediate COVID-19 disease [66, 67]. Due to space constraints, in the following sections, the roles of AR will be discussed in only a handful of these diseases, such as prostate cancer, androgen insensitivity syndrome (AIS), PCOS, breast cancer, and a few other AR-associated diseases.

16.3 Prostate Cancer

The prostate is part of the male reproductive system and is an androgen-dependent tissue that relies on functional androgen signaling for growth and survival. Castration leads to involution of the prostate in the mature male with apoptosis of prostate luminal epithelial cells. The androgen dependency of the prostate provided the rationale for Dr. Charles Huggins to test if a reduction of circulating levels of testosterone could induce tumor regression in prostate cancer patients [68, 69]. The success of those studies paved the way for the development of numerous approaches to block the androgen axis for the treatment of prostate cancer and other diseases driven by the AR. Today, androgen ablation therapy remains the standard of care for various stages of prostate cancer and can be combined with antiandrogens that target the AR LBD or other treatment modalities such as radiation therapy [70, 71]. Unfortunately, remissions to first-line androgen ablation for advanced prostate cancers are not durable and within 2–3 years the disease returns. These patients' disease will progress to lethal metastatic castration-resistant prostate cancer (CRPC). The transition to CRPC is characterized by a gradual rise in serum levels of prostate-specific antigen (PSA), the AR-regulated gene *KLK3*, which signifies a resurgence of transcriptionally active AR and biochemical recurrence. Mechanisms of resistance to androgen ablation therapies and antiandrogens implicated in the

progression to CRPC include synthesis of intratumoral androgens, amplification or overexpression of the AR gene, gain-of-function mutations in AR protein, ligand-independent activation by alternate signaling pathways, and expression of constitutively active truncated AR variants [72]. Regardless of androgen deprivation therapy and current AR-targeted therapies, genomic profiling shows a disproportional alteration of the AR signaling pathway compared to other pathways, which suggests that AR remains a key regulator of prostate cancer and an essential therapeutic target [73, 74]. Neuroendocrine prostate cancer is also considered to be a type of CRPC and represents about 20% of CRPC cases, but it does not rely on AR for growth and survival.

16.3.1 AR Mutations and Alterations in the Progression of Prostate Cancer

Amplification of the *AR* gene is the most common gene alteration and occurs in ~28% of CRPC tumors and 50% of CRPC metastases compared to less than 1% for primary prostate cancer tumors [73, 75, 76]. These frequencies support the notion that amplification of the *AR* gene is an adaptive response to androgen deprivation and that CRPC cells remain reliant on AR signaling. Increased sensitivity to a lower threshold of androgen is proposed to be a response to the elevated expression of AR. Castrate levels of androgen where serum testosterone is less than 50 ng/dL may be sufficient to transactivate the AR. Extragonadal sources of androgen including steroidogenesis from the tumor [77] or residual androgen biosynthesis from the adrenal glands [78] also contribute to AR signaling in spite of castrate serum levels of testosterone. Due to these discoveries of androgen still driving the disease, the nomenclature of this stage of the disease was changed from “hormone-refractory” or “androgen-independent” to “CRPC” [79].

AR mutations have been long suspected to drive the etiology and progression of prostate cancer and include the following: (i) point mutations that result in an amino acid substitution or premature stop codon, (ii) nucleotide insertions and deletions that cause a frameshift, (iii) complete or partial deletion of the *AR* gene, and (iv) intronic mutations that interrupt the processing of *AR* transcripts. Currently, there are more than 150 mutations reported in the Androgen Receptor Gene Mutations Database (http://androgendb.mcgill.ca) at the Lady Davis Institute for Medical Research [80]. These mutations predominantly occur in the LBD (48%) or NTD (39%) and are less commonly found in the DBD (7%) (Fig. 16.4a). Intronic mutations and large deletions that span multiple exons are considered to be rare and represent 3% and 2% of all detected mutations, respectively.

Missense mutations in the coding region of exon 8 that encodes the AR LBD are the most frequent and can confer ligand promiscuity and activation by antiandrogens or alternative steroids (Fig. 16.4b and Table 16.1). These AR LBD mutations primarily occur in “hot spots” that impact the structure of the ligand-binding pocket

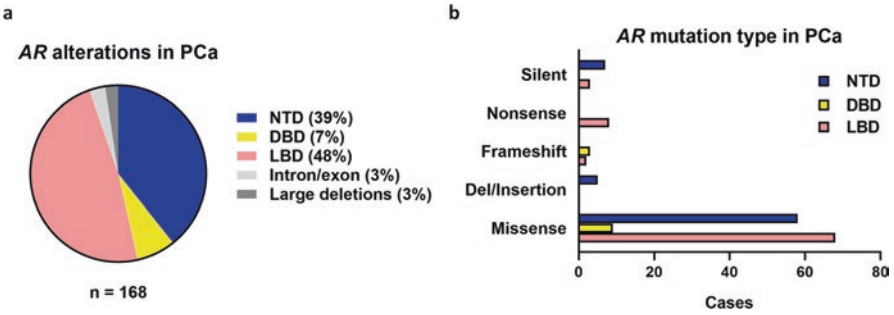

Fig. 16.4 Summary of *AR* gene alterations reported in prostate cancer patients. (**a**) Relative distribution of gene alterations in the AR N-terminal domain (NTD), DNA-binding domain (DBD), or ligand-binding domain (LBD), and intronic mutations or large deletions spanning multiple exons. (**b**) The number of cases reported for alterations occurring in the AR NTD, DBD, or LDB is shown, based on the type of mutation. Data shown were retrieved from the AR Gene Mutations Database (http://androgendb.mcgill.ca)

and are associated with therapies blocking the AR signaling axis, such as antiandrogens or CYP17 inhibitor abiraterone acetate. AR T877A was the first mutant identified to confer agonist activity to the antiandrogen flutamide. T877A reduces the specificity of the LBD for androgen such that the mutant AR can be activated by progesterone, E2, and various antiandrogens (hydroxyflutamide and bicalutamide) [81–83]. This mutation is present in the LNCaP human prostate cancer cell line, a widely used androgen-sensitive model of prostate cancer. Mutations associated with bicalutamide gain-of-function include W741C and W741L [84]. Another noteworthy mutation, AR F876L, was discovered in CRPC patients and confers agonist activity to second-generation antiandrogens enzalutamide and apalutamide [85, 86]. This AR F876L mutation remains sensitive to inhibition by bicalutamide, thereby indicating a difference in mechanism between these highly related compounds [87]. AR L701H was found in CRPC and is less sensitive to androgen but highly responsive to the glucocorticoids cortisol and cortisone [88]. Mutant AR harboring an L701H/T877A double mutation can be found in MDA-PCa cell lines, which were originally derived from a prostate cancer bone lesion.

Polymorphisms in the length of the AR NTD may influence the risk for men to develop prostate cancer. Most men have on average 21 repeats. Fewer CAG repeats, and therefore a shorter NTD, increases AR transcriptional activity in vitro, whereas increasing the length of the CAG region reduces transactivation [89, 90]. Several studies have shown an increased risk of developing prostate cancer for men with shorter (<21) CAG repeats [29, 30], but others have found no association between CAG repeat length and prostate cancer risk [91]. Thus, whether CAG repeat length of the AR NTD predisposes men to prostate cancer remains somewhat controversial.

Table 16.1 Recurring AR alterations from human prostate cancer

Domain	Exon	Mutation[a]	Findings	References
NTD	1	E43G/V	No endocrine treatment	Steinkamp et al. (2009) [281]
NTD	1	Q58L	Treated and untreated	Robins (2012) [282]
NTD	1	Q84del	Treated and untreated	Steinkamp et al. (2009)
NTD	1	S119S	Synonymous mutation, bicalutamide- and flutamide-treated	Steinkamp et al. (2009)
NTD	1	L192Q	Bicalutamide- and flutamide-treated	Steinkamp et al. (2009)
NTD	1	E211E	Synonymous mutation; bicalutamide- and flutamide-treated	Steinkamp et al. (2009)
NTD	1	T227A	Treated and untreated patients	Steinkamp et al. (2009)
NTD	1	T227C	Bicalutamide- and flutamide-treated	Robins (2012)
NTD	1	E250V	Adjacent to conserved CHIP E3 ligase interaction site, bicalutamide- and flutamide-treated	Steinkamp et al. (2009)
NTD	1	A251V	Bicalutamide- and flutamide-treated	Robins (2012)
NTD	1	E253K	Adjacent to conserved CHIP E3 ligase interaction site, prolonged protein half-life and nuclear localization without hormone, bicalutamide- and flutamide-treated	Steinkamp et al. (2009)
NTD	1	A356V/T	Flutamide-treated	Steinkamp et al. (2009)
NTD	1	R360H	Treated and untreated	Robins (2012)
NTD	1	G414S/D	Treated and untreated	Steinkamp et al. (2009)
NTD	1	W433C	Treated and untreated	Steinkamp et al. (2009)
NTD	1	W433L	Impact on WxxLF motif, increased transactivation function and N/C interaction, bicalutamide- and flutamide-treated	Steinkamp et al. (2009)
NTD	1	T438P	Bicalutamide- and flutamide-treated	Robins (2012)
NTD	1	G454S	Bicalutamide- and flutamide-treated	Steinkamp et al. (2009)
NTD	1	G455D	Bicalutamide-treated	Robins (2012)
NTD	1	R484C	Treated and untreated	Robins (2012)
NTD	1	T497I	Treated and untreated	Robins (2012)
NTD	1	V508L	Bicalutamide-treated	Robins (2012)
NTD	1	V508L/G	Bicalutamide- and flutamide-treated	Steinkamp et al. (2009)
DBD	3	C619Y	Cannot bind DNA and is transcriptionally inactive	Nazareth et al. (1999) [283], Marcelli et al. (2000) [284]
H	4	E665D	Bicalutamide- and flutamide-treated	Robins (2012)
LBD	4	L701H	Less responsive to androgens, responsive to glucocorticoids, partial agonist activity with flutamide and bicalutamide	van de Wijngaart et al. (2010) [88], Lallous et al. (2016) [285]
LBD	4	V715M	Responsive to progesterone, partial agonist activity with flutamide and bicalutamide	Culig et al. (1993) [286], Lallous et al. (2016)

(continued)

Table 16.1 (continued)

Domain	Exon	Mutation[a]	Findings	References
LBD	5	R726L	Activated by estradiol, germline mutation	Elo et al. (1995) [287], Mononen et al. (2000) [288]
LBD	5	V730M	Partial agonist activity with flutamide and bicalutamide	Lallous et al. (2016)
LBD	5	W741L	Confers agonist activity to bicalutamide	Bohl et al. (2005) [289]
LBD	5	W741C	Confers agonist activity to bicalutamide	Yoshida et al. (2005) [82]
LBD	5	R752Q	Reduced ligand-binding and N/C interaction, differential gene expression, reported as a germline mutation in some cases of AIS	Robins (2012)
LBD	5	R760R	Bicalutamide- and flutamide-treated	Steinkamp et al. (2009)
LBD	5	R760K	Bicalutamide-treated	Robins (2012)
LBD	6	T786*	Treated and untreated	Steinkamp et al. (2009)
LBD	6	L797P	Flutamide-treated	Robins (2012)
LBD	7	Q867*	Treated and untreated	Steinkamp et al. (2009)
LBD	8	L873P	Flutamide-treated	Robins (2012)
LBD	8	H874Y	Responsive to progesterone and estrogen; partial agonist activity with bicalutamide, enzalutamide, and apalutamide	Taplin et al. (1995) [81], Lallous et al. (2016)
LBD	8	H874Q	Partial agonist activity with flutamide and bicalutamide	Lallous et al. (2016)
LBD	8	F876L	Partial agonist activity with flutamide, enzalutamide, and apalutamide	Korpal et al. (2013) [86], Joseph et al. (2013) [85]
LBD	8	T877A	Responsive to progesterone and estrogen, confers agonist activity to flutamide and bicalutamide, present in LNCaP cells	Wilding et al. (1989) [290], Veldscholte et al. (1992) [291]
LBD	8	T877S	Responsive to progesterone and estrogen, confers agonist activity to bicalutamide	Taplin et al. (1995), Lallous et al. (2016)
LBD	8	D879E	Partial agonist with flutamide and bicalutamide	Lallous et al. (2016)
LBD	8	L881I	Partial agonist with flutamide and bicalutamide	Lallous et al. (2016)
LBD	8	S888G	Responsive to progesterone and estrogen, confers agonist activity to flutamide and bicalutamide	Lallous et al. (2016)
LBD	8	D890H	Confers agonist activity to flutamide and bicalutamide	Lallous et al. (2016)
LBD	8	E893K	Partial agonist with flutamide and bicalutamide	Lallous et al. (2016)
LBD	8	M895V	Confers agonist activity to bicalutamide, partial agonist activity with flutamide	Lallous et al. (2016)

(continued)

Table 16.1 (continued)

Domain	Exon	Mutation[a]	Findings	References
LBD	8	M895T	Confers agonist activity to bicalutamide, partial agonist activity with flutamide	Lallous et al. (2016)
LBD	8	E897G	Partial agonist activity with flutamide	Lallous et al. (2016)
LBD	8	T918S	Partial agonist activity with flutamide and bicalutamide	Lallous et al. (2016)

Note. [a]del, deletion; *, stop codon

16.3.2 Roles of AR Splice Variants in Prostate Cancer

The expression of some truncated AR splice variants that lack the LBD is now established as a major resistance mechanism for CRPC. To date, 22 AR splice variants have been reported in the literature with available transcript sequences [92]. AR-V7 (also known as AR3) is the most extensively studied and the most common splice variant expressed in CRPC. AR-V7 is comprised of the NTD, DBD, and a unique C-terminus with 16 amino acids encoded by cryptic exon 3 [93, 94]. AR-V7 is constitutively active and does not encode the LBD, which is the therapeutic target all of currently approved therapies for CRPC that target AR. Thus, AR-V7 is considered a major resistance mechanism for all therapeutic approaches that target the AR LBD, including next-generation antiandrogens (enzalutamide, apalutamide, and darolutamide) and abiraterone acetate. Clinical evidence for the importance of AR-V7 in CRPC has been drawn from studies showing AR-V7 expression is associated with shorter survival and limited responses to approved AR-targeted therapies for CRPC patients [95–97]. Approximately 10%–30% of patients with metastatic CRPC have detectable AR-V7 expression, based on clinically validated assays that detect nuclear protein or mRNA in circulating tumor cells [98]. Alternative splicing of AR-V7 transcripts is induced by androgen deprivation and antiandrogens. Both the rate of *AR* gene transcription and recruitment of RNA splicing factors and enhancers (U2AF65 and ASF/SF2) that generate the AR-V7 transcript are upregulated when fl-AR transcriptional activity is suppressed [99, 100]. AR-V7 is almost always coexpressed with fl-AR, but V7 transcript levels are usually lower (5%–30%) than fl-AR. AR-V7 is commonly detected in samples that also have *AR* gene amplification.

Proliferation of prostate cancer cells that express mixed populations of fl-AR and AR-Vs tends to be androgen-independent and resistant to antiandrogens. This is observed in clinical findings, where AR-V7-positive CRPC patients treated with enzalutamide or abiraterone had poor responses and lower overall survival than patients without detectable AR-V7 [96]. AR-V7-positive patients are associated with better PSA responses with taxane chemotherapy compared to treatment with enzalutamide or abiraterone, whereas for AR-V7-negative patients, there were no obvious differences in efficacy between taxanes and these hormonal therapies [101]. Serial analysis of AR-V7 expression in CRPC patients further revealed that

inhibition of AR signaling by androgen-deprivation, enzalutamide, or abiraterone may exert a selective pressure for promoting the expression AR-V7 [102], confirming in vitro observations [96, 103]. Reversion to AR-V7-negative status is observed in some taxane-treated AR-V7-positive patients; however, this did not occur with all taxane-treated patients, and thereby further investigation is warranted to understand the mechanism of this phenomenon.

Protein-protein interactions between fl-AR and AR-Vs remain an important area of investigation. A study by Xu et al. in 2015 [104] using bimolecular fluorescence fusion constructs reported that truncated splice variants, AR-V7 and AR^{v567es}, can interact with fl-AR by N/C interactions mediated by AF-2 of fl-AR or by DBD-DBD interactions mediated by the D-box motif. These data suggest that constitutively active AR-Vs may promote transactivation of fl-AR in the absence of androgen or transactivate target genes without fl-AR by using their D-box to form variant homodimers or heterodimers [104]. Analysis of an AR-V gene expression signature in CRPC cell lines suggested that AR-V7 and AR^{v567es} can activate some canonical fl-AR target genes, in addition to a subset of variant-specific genes that include *AKT1* and cell cycle genes, such as *UBE2C*, *CDC25B*, and *CCNA2* [93, 105]. Ectopic expression of AR-V7 can increase the expression of *ETS2* and *EDN2,* which are otherwise co-repressed by fl-AR and the pioneer factor FOXA1 [106]. Cofactors and interacting proteins that uniquely interact with AR-Vs but not fl-AR have also been reported. An analysis of AR-V7 cistromes in CRPC cell lines and patient specimens suggested that homeobox protein HOXB13 may interact with AR-V7 as an essential coactivator and pioneer factor to open the chromatin for access to DNA-binding sites [107]. Genomic profiling of AR-V7 and fl-AR binding sites in 22Rv1 human prostate cancer cells showed a proportion of sites (2221 out of 17,409) were specific to AR-V7 binding [108]. In contrast to the binding sites shared by fl-AR and AR-V7, which were enriched in ARE and FOXA1 motifs at enhancer regions, these AR-V7-specific binding sites were associated with zinc finger X-chromosomal protein (ZFX) and located primarily at promoter regions of MYC-bound genes or genes regulating cell cycle progression (*SKP2*), autophagy (*ZNF32*), and WNT signaling (*FZD6*) [108]. ChIP-sequencing analysis supports the notion that fl-AR and AR-V7 can heterodimerize to mostly the same genomic foci, but AR-V7 preferentially interacted with transcriptional corepressors (NCOR1 and NCOR2), whereas fl-AR was associated with both coactivators and corepressors [109]. These findings suggest that AR-V7 may have a significant repressor function in CRPC, which may contribute to prostate cancer progression by preventing the expression of tumor suppressor genes [109].

16.3.3 Treatments Targeting AR

The two main therapeutic approaches to inhibit AR signaling are surgical or pharmaceutical reduction of androgens and the direct inhibition of binding of androgen to the AR LBD with competitive antagonists called antiandrogens. Castration by

orchiectomy or analogs of LH-RH quickly reduce circulating levels of androgen by >90%. Abiraterone acetate is a CYP17 inhibitor that blocks steroid synthesis to reduce de novo androgen synthesis. The development of antiandrogens as antagonists of the AR commenced approximately 60 years ago first with the development of steroidal progestogens such as cyproterone acetate and then later with the development of flutamide as a first-in-class nonsteroidal pure antagonist that lacked partial agonist activity (for a review, see [110–112]). Steroidal antiandrogens are used today for numerous indications mediated by AR, including prostate cancer, PCOS, congenital adrenal hyperplasia, benign prostatic hyperplasia, acne, hirsutism, and androgenic alopecia. All nonsteroidal antiandrogens, including flutamide, nilutamide, bicalutamide, enzalutamide, apalutamide, and darolutamide, are competitive AR LBD inhibitors with chemical structures based upon flutamide and bicalutamide with the exception of darolutamide. The crystal structure of the folded AR LBD has only been resolved for the agonist conformation bound to ligand with no antagonist conformation reported. The mechanism of how antiandrogens antagonize AR involves blocking N/C interactions required for agonist activity and preventing essential protein-protein interactions with AF-2 in the AR LBD. Differences in AR-binding affinity to the chromatin and reduction of AR nuclear localization have also been reported for the various nonsteroidal antiandrogens [100, 113].

Enzalutamide is a second-generation antiandrogen developed for CRPC using LNCaP human prostate cancer cells engineered to express elevated levels of wild-type AR in the background of the LNCaP AR mutation T877A [114]. Enzalutamide binds to the AR LBD, with about an eightfold improved affinity compared to bicalutamide, and impairs AR nuclear translocation and chromatin binding [114]. Enzalutamide was FDA-approved in 2012 as second-line therapy for metastatic CRPC following results of the AFFIRM trial that showed an improvement for overall survival by 4.8 months [115]. Enzalutamide was subsequently approved for first-line therapy for metastatic CRPC following the PREVAIL study [116] and was later approved for nonmetastatic CRPC after results from the PROSPER trial showed a 71% reduction for the risk of progression for nonmetastatic CRPC patients on androgen deprivation therapy [117].

Apalutamide is a second-generation antiandrogen with high chemical similarity to enzalutamide. It was discovered using the same screen as used for enzalutamide [118]. Apalutamide is the first drug to be approved for the treatment of nonmetastatic CRPC. The SPARTAN trial for nonmetastatic CRPC patients reported a significant improvement to metastasis-free survival by 23.3 months with apalutamide compared to a placebo [119]. Apalutamide has comparable properties to enzalutamide including its binding affinity for the AR LBD and reducing AR nuclear translocation or DNA binding [118]. Preclinical evaluation of apalutamide demonstrated that it has a greater in vivo efficacy on human CRPC xenografts compared to enzalutamide, such that 30 mg/kg/d of apalutamide had a maximum response that was equivalent to 100 mg/kg/d of enzalutamide [118].

Darolutamide is the most recent FDA-approved second-generation antiandrogen for nonmetastatic CRPC. Darolutamide and its active metabolite have an eight to tenfold improved the binding affinity for AR compared to enzalutamide and

apalutamide in ligand competition assays as well as having activity against the AR F876L mutant, which is resistant to enzalutamide and apalutamide [120]. Darolutamide has also been shown to inhibit other clinically relevant AR LBD point mutations, including F876L, H874Y/T877A, F876L/T877A, and T877G [121]. In contrast to enzalutamide and apalutamide, darolutamide does not share structural similarity to first-generation antiandrogens and has negligible brain penetrance [120, 122]. The ARAMIS trial of darolutamide for men with nonmetastatic CRPC reported a metastasis-free survival of 40.4 months compared to 18.4 months for the placebo group [123]. These results are consistent with the PROSPER and SPARTAN studies, where metastasis-free survival was 36.6 and 40.5 months for enzalutamide and apalutamide, respectively [117, 119]. Since these second-generation antiandrogens all target the AR LBD and appear to provide a similar clinical benefit, their differences in cost and improvement on the quality of life are important factors to consider.

Abiraterone acetate is a selective inhibitor of CYP17A1 that blocks androgen biosynthesis from steroid precursors in the testes, adrenal glands, or any sources from the tumor itself [124]. Cytochrome P450 enzymes (CYP11A1 and CYP17A1) synthesize the weak adrenal androgens DHEA and androstenedione, which can be converted by some prostate cancer cells to testosterone and DHT. Castrate levels of serum testosterone following surgical or chemical castration are typically within the 20–50 ng/dL range. The addition of abiraterone can further reduce serum testosterone to a "super-castrate" level of 1–2 ng/dL [125]. Tumor biopsies from CRPC patients following abiraterone therapy showed an upregulation of CYP17A1 expression, which suggests that CRPC cells may remain steroid-dependent [126].

All current FDA-approved hormonal therapies for CRPC target the AR LBD and will inevitably fail from de novo or acquired resistance. Targeting solely the AR LBD is inadequate to completely block all AR signaling. The AR NTD contains the AF-1 region which is required for transcriptional activity, including the activity of truncated AR-Vs lacking the LBD. Thus, targeting the AR NTD would potentially inhibit fl-AR and all transcriptionally active AR-Vs. Ralaniten acetate is a prodrug of ralaniten, which is a first-in-class AR NTD antagonist that specifically binds to AF-1. Ralaniten inhibits the growth of prostate cancer in vitro and in vivo and maintains AR inhibition despite overexpression of AR coactivators, gain-of-function mutations in the AR LBD, or expression of AR-V7 [127–129]. Nuclear magnetic resonance studies revealed that ralaniten and its stereoisomers bind to a pocket formed by amino acids of 345–448 of tau-5 in AF-1 [130]. Proof of concept for the chemical scaffold and efficacy of ralaniten was provided in a phase I clinical trial with heavily pretreated CRPC patients who had previously failed enzalutamide or abiraterone [131]. Due to the rapid metabolism of ralaniten acetate, there was an excessive pill burden that stopped the trial. A second-generation analog, EPI-7386, is more potent with an improved pharmacokinetic profile compared to ralaniten, and it commenced clinical trials in mid-2020 (NCT04421222).

On-target complications associated with blocking the AR axis are associated with anemia, bone and muscle loss, gynecomastia, cognitive impairment, depression, diabetes, coronary heart disease, and cardiovascular disease [132–134].

Cycling of androgen levels by application of intermittent androgen suppression has been proposed as an approach to reduce the incidence of adverse side effects from decreased levels of androgen (NCCN Guideline 2020). High levels of androgen may also be beneficial in blocking the progression of some prostate cancers [135]. Phase 2 clinical trials of bipolar androgen therapy that cycles high and low levels of androgen were well-tolerated but did not improve the outcomes for AR-V7-positive disease [136]. Thus, stimulating AR activity such as with SARMs or exogenous androgen may have beneficial effects for some prostate cancers in addition to other diseases, such as for some breast cancer, sarcopenia or cachexia, osteoporosis, hypogonadism, Duchenne muscular dystrophy, and AIS [137].

16.4 Androgen Insensitivity Syndrome

Testosterone and DHT both play roles in virilization during embryogenesis, with testosterone for the Wolffian structures and DHT for the virilization of the Anlagen, which forms the prostate and external genitals (for a review, see [138]). In the absence of androgens or functional AR, male sexual differentiation fails to occur. Inactivating mutations of the *AR* gene that cause a partial or complete inability of androgen-sensitive cells to respond to androgen is associated with AIS, which is a disorder of sex development [139]. The first detailed report of AIS (formerly known as testicular feminization) was described by John Morris in 1953, who recognized it to be an inherited disorder affecting male sexual differentiation. In general, individuals affected by complete AIS (CAIS) have developed testes and physiological production of testosterone plus its conversion to DHT, but they appear phenotypically female [140, 141]. Over the decades with the identification of the *AR* gene and an increased understanding of the structure and function of AR and the androgen axis, it is now appreciated that this overall feminizing effect arises predominantly from the lack of androgen action and an abundance of E2 resulting from the aromatization of testosterone.

Insensitivity to androgen during development of the male fetus prevents the masculinization of external genitalia. Instead, partial female external genitals are formed from the urogenital sinus, which in most cases results in a blind-ended vagina. Phenotypic variation of the external genitalia in AIS is directly attributed to the binding affinity of androgen to a mutant AR and its residual function. Table 16.2 provides a list of AR mutations. Most individuals impacted by AIS have undescended testes that can be located anywhere along the path of embryonic testicular descent, for instance, in the abdomen, inguinal canal, or labia, since androgen signaling regulates testicular descent to the scrotum [142]. Secondary sexual characteristics that are regulated by androgen actions include the development of axillary and pubic hair, and deepening of the voice at puberty, which can be absent or minimal in AIS. Breast development occurs at the onset of puberty, which is supported by the aromatization of testosterone to E2. AIS is the most common disorder of sex development reported in genetic (46,XY) males. The prevalence of CAIS is estimated to

Table 16.2 Select AR alterations identified from individuals with AIS

Pathology	Domain	Exon	Mutation[a]	Sex of rearing	External genitalia	Findings	References
CAIS	NTD	1	V30M	F	Normal		Topcu et al. (2015) [155]
CAIS/ PAIS	NTD	1	E211E	M or F	Normal to ambiguous	Synonymous mutation at the end of 3′ acceptor splice site	Topcu et al. (2015), Gottlieb et al. (1999) [292]
PAIS/ MAIS	NTD	1	P390S	M	Ambiguous to normal		Hiort et al. (2000) [170], Audi et al. (2010) [169]
MAIS	NTD	1	R405S	M	Ambiguous	Creates a phosphorylation site that inhibits interaction with MAGE-11 and p300	Lagarde et al. (2012) [171]
MAIS	NTD	1	A474V	M	Normal	Male infertility	Zuccarello et al. (2008) [168]
MAIS	NTD	1	L547F	M	Normal	Male infertility, azoospermia	Zuccarello et al. (2008)
CAIS	DBD	3	R607*	F	Normal		Cheikhelard et al. (2008) [293], Boehmer et al. (2001) [143], Giwercman et al. (2000) [294]
PAIS	DBD	3	R607Q	M or F	Ambiguous	Partial AR transactivation	Deeb et al. (2005) [161]
CAIS	DBD	3	R615C	F	Normal		Zhou and Wang (2013) [159], Hughes et al. (2012) [141]
CAIS	DBD	3	R615H	F	Normal	Absence of epididymis and vas deferens	Boehmer et al. (2001), Hiort et al. (1998) [151]
CAIS	DBD	3	R615P	F	Normal		Ahmed et al. (2000) [146], Marcelli et al. (1991) [156]
CAIS	DBD	3	R615S	F	Normal		Sharma et al. (2011) [158]
CAIS	LBD	4	N705S	F	Normal		Hughes et al. (2012), Audi et al. (2010), Pinksy et al. (1992) [295]
PAIS	LBD	4	Q711E	F	Ambiguous		Méndez et al. (2001) [296], Topcu et al. (2015)

(continued)

Table 16.2 (continued)

PAIS	LBD	4	A645D	M	Ambiguous	2 cases with long polyQ (30/28) and short polyG (10)	Hughes et al. (2012), Hiort et al. (1996) [297]
CAIS	LDB	5	D732Y/N	F	Normal		Hannema et al. (2004) [298], Pinksy et al. (1992)
PAIS	LBD	5	M742V/I	F or M	Ambiguous		Audi et al. (2010), Boehmer et al. (2001)
CAIS	LBD	5	M749V	F	Normal		Bouvattier et al. (2002) [299]
CAIS	LBD	5	P752*	F	Normal		Ledig et al. (2005) [300], Hughes et al. (2012), Pinksy et al. (1992)
CAIS	LBD	5	P752Q	F	Normal		Hughes et al. (2012)
PAIS	LBD	5	Y763C	M	Ambiguous		Ahmed et al. (2000)
CAIS	LBD	5	A765T	F	Normal		Ledig et al. (2005), Hannema et al. (2004), Hiort et al. (2000)
CAIS	LBD	5	P767fs	F	Normal	1 nucleotide frameshift and stop codon at 807	Hannema et al. (2004)
CAIS	LBD	6	R774C	F	Normal		Hiort et al. (2000)
CAIS	LBD	6	R774H	F	Normal		Ledig et al. (2005), Ahmed et al. (2000)
CAIS	LBD	6	R779W	F	Normal		Ledig et al. (2005), Ahmed et al. (2000)
PAIS	LBD	6	M780I	F	Ambiguous		Boehmer et al. (2001)
PAIS/ MAIS	LBD	6	Q798E	F or M	Ambiguous to normal	Male infertility and azoospermia, defective AR transactivation	Hiort et al. (2000), Wang et al. (1998) [167], Bevan et al. (1996) [301]
PAIS	LBD	7	I816I	M	Ambiguous	Synonymous mutation, additional E211E synonymous mutation	Topcu et al. (2015)
PAIS	LBD	7	Q824L	M	Ambiguous	EMS = 12	Hellmann et al. (2012) [302]
CAIS	LBD	7	R831*	F	Normal		Giwercman et al. (2000)
CAIS	LBD	7	R831Q	F	Normal		Audi et al. (2010), Ahmed et al. (2000)

PAIS	LBD	7	R840C	M or F	Ambiguous	AR is transcriptionally active only at high concentrations of androgen (EC50 ~300x Wt)	Georget et al. (1998) [303], Bevan et al. (1996)
PAIS	LBD	7	R840H	F	Ambiguous		Beitel et al. (1994) [304]
CAIS	LBD	7	R855C	F	Normal		Ledig et al. (2005), Ahmed et al. (2000)
PAIS	LBD	7	R855H	M or F	Ambiguous		Audi et al. (2010), Deeb et al. (2005)
PAIS	LBD	7	V866L/M	M or F	Ambiguous		Ledig et al. (2005)
CAIS	LBD	8	H874R	F	Normal		Cheikhelard et al. (2008)
CAIS	LBD	8	V889M	F	Normal		Audi et al. (2010), Ledig et al. (2005)

Note. Notable mutations and alterations reported five or more times for CAIS and PAIS, or three or more times for MAIS were retrieved from the Androgen Receptor Gene Mutation Database [80]. The numbering of residues is based on the 919 amino acid reference sequences for the human androgen receptor, NCBI Accession No. AAA51729.1 [305]

[a]*, stop codon; fs, frameshift

vary between 1:20,000 and 1:99,000 in 46,XY live births [80, 143, 144] and is identified in 0.8% to 2.4% of phenotypic females with inguinal hernia [145].

16.4.1 Clinical Presentation of AIS

Androgen resistance in AIS may be suspected when serum androgen levels are physiological or elevated but clinical effect is lacking or suboptimal. Individuals affected by AIS are classified by their clinical phenotype as either CAIS, partial (PAIS), or mild (MAIS) [141]. The external masculinization score (EMS) was devised as a tool for the initial assessment of ambiguous genitalia in infants (ranging from 0 to 12); however, it should be noted that gender assignment does not necessarily depend on the appearance of the external genitalia and gender identity may change during or after puberty [146, 147]. For CAIS, there are no clinical indications of androgen action, and individuals are born with female-appearing external genitalia, but structures, such as the clitoris, labia minora, and labia majors, are typically underdeveloped. CAIS individuals are almost always raised as females, and the condition is rarely diagnosed in childhood unless a family history is known. CAIS can be suspected prenatally when the karyotype (46,XY) of the fetus is not consistent with the developing female phenotype [139]. It is also not uncommon for CAIS to be diagnosed during puberty when breast development occurs but pubic and axillary hair is lacking and menarche does not occur.

PAIS includes a broad range of external genitalia phenotypes, which may vary from female-like to male-appearing depending on the level of residual AR function. The management of PAIS is highly complex, since sexual identity and gender assignment may be unclear at birth. In milder presentations of PAIS, the external genitalia appear morphologically male, but there may be an underdeveloped penis, severe hypospadias, and bifid scrotum with or without undescended testes [145]. PAIS is thought to be as commonly occurring as CAIS. In the case of MAIS, individuals have unambiguous male external genitalia, but there may be evidence of mild impairment of masculinization, such as decreased terminal body hair or perhaps isolated micropenis. Impotence is commonly reported as a concern in MAIS, and spermatogenesis may be impaired but may be sufficient to preserve fertility [148]. MAIS is the least understood type of AIS, since it has the mildest phenotype and may not be actively investigated unless there are issues regarding fertility. In many cases, male infertility is the only reported symptom in patients with MAIS [148]. Other phenotypic characteristics of MAIS include minor gynecomastia, sparse terminal body hair, and lack of vocal deepening at puberty [149]. The prevalence of MAIS is not known, but it is reported at a lower frequency than CAIS and PAIS [141]. Expansion of the AR polyglutamine tract to more than 38 CAG repeats is related to a progressive onset of MAIS in the form of gynecomastia and reduced fertility in adulthood [150].

16.4.2 AR Mutations in Patients with AIS

More than 500 unique mutations in the *AR* gene have been identified from over 900 AIS patients [80] (Fig. 16.5a). The majority of mutations that cause AIS are inherited with about 30% identified as de novo mutations [151]. Defects in the *AR* gene that result in a loss of AR function are sufficient to be a single causative factor for AIS; however, polymorphisms in AR coactivator genes or genes related to steroid biosynthesis and metabolism are also important factors that may contribute to the AIS phenotype [143, 152]. Mutations in the *AR* gene are detected in the majority (90%–95%) of CAIS cases [153]. According to the AR mutation database (http://androgendb.mcgill.ca), the majority of *AR* mutations from CAIS patients affect the AR LBD (66%) and are predominantly missense single base-pair substitutions, resulting in an amino acid change (Fig. 16.5b–d). AR LBD mutations that cause CAIS are clustered in the amino acid regions 688–712, 738–784, and 827–870 [80, 141]. These mutations are predicted to primarily alter the AR LBD protein structure and disrupt the ligand-binding pocket and ligand specificity or render the mutated AR to be functionally inactive [14, 141]. Mutations associated with AIS have also been found in the AR LBD dimerization interface mediating AR LBD-LBD interactions and may disrupt allosteric regulation and impair AR transcriptional activity [154]. Mutations of the AR NTD and DBD have also been reported for CAIS but are less common than the LBD, representing 17% and 13% of detected mutations, respectively (Fig. 16.5e). Notably, a missense mutation resulting in a substitution of valine to methionine (V30M) in the AR NTD was identified from a patient with CAIS [155]. Mutations of arginine 615 (R615C, R615H, R615P, R615S) of the second zinc finger of AR DBD has been documented in several CAIS cases [156–159]. Other *AR* gene alterations identified in CAIS include mutations that impact the intron or exon splice sites and large deletions spanning multiple exons, which are less frequent and cover less than 5% of all detected *AR* mutations (Fig. 16.5e).

In contrast to CAIS, *AR* gene mutations are only identified in 20%–40% of PAIS patients [80, 160]. Mutations associated with PAIS are more frequently detected in the AR LBD (71%) than in the DBD (19%), NTD (8%), or at intron-exon junctions (2%) (Fig. 16.5f). Whether a PAIS patient carries an *AR* gene mutation is phenotypically indistinguishable from the external genitalia and the EMS criteria; however, birth weight was reported to be significantly lower for the gestational age of PAIS infants without an *AR* mutation [160]. It is noteworthy to mention that identical mutations have been associated with different conditions of PAIS [161, 162], such that related affected individuals with the same *AR* mutation may have a different phenotype and sex assignment [143, 163]. These cases imply that additional factors are accountable for the extent of virilization in PAIS. Other identified genetic causes that may promote PAIS in the form of underdeveloped male external genitalia include defects in LH receptor and deficiencies in androgen biosynthesis enzymes (i.e., 17,20-lyase, P450 oxidoreductase, 17β-hydroxysteroid dehydrogenase, and 5α-reductase) [164, 165]. Fewer AR mutations are reported in patients with MAIS, with 40% identified in the NTD and 47% in the LBD (Fig. 16.5g).

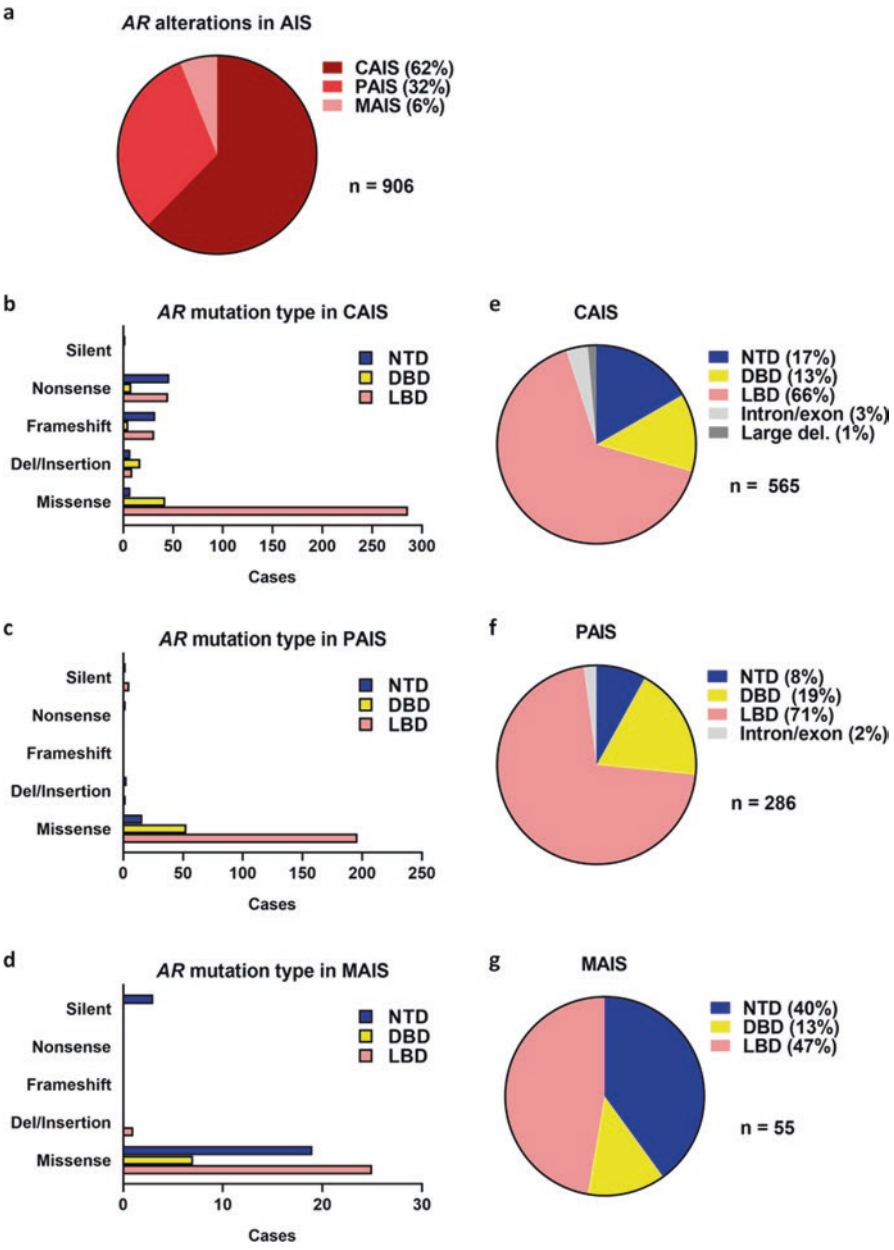

Fig. 16.5 Summary of *AR* gene alterations reported from patients with androgen insensitivity syndrome. (**a**) Relative distribution of AIS phenotypes (complete, partial, or mild) associated with a mutation in the *AR* gene. (**b-d**) The number of cases with a mutation impacting the AR NTD, DBD, or LBD is shown, based on the type of mutation. (**e-g**) Relative distribution of the gene alterations occurring in the AR NTD, DBD, or LBD, or introducing a large deletion or intronic mutation

The AR NTD has a flexible disordered structure; therefore, mutations in this domain have a milder effect on protein structure and are less likely to be detrimental to AR function. The pathogenicity of AR NTD mutations may also be difficult to prove since transactivation of AR in vitro can vary depending on the promoter of the reporter gene or cell line used. Such studies suggest that mutations of the AR NTD associated with a MAIS phenotype might impact coactivator binding or impair the structural flexibility of the AR NTD rather than cause a significant structural change as those observed with mutations in the folded AR LBD [166]. Missense mutations located in the AR NTD have been documented in mild to partial states of AIS (Table 16.2). G216R is associated with reduced AR transactivation and has been reported in multiple patients with PAIS [161, 167]. A474V has been detected in several cases of infertile men with MAIS [168] and P390S in mild to partial AIS cases [169, 170], but neither the A474V and P390S mutations are associated with a significant difference in transcriptional activity in vitro compared to the wild-type AR. Interestingly, an R405S mutation in AR from a PAIS patient creates a phosphorylation site that inhibits interaction with essential transcriptional coactivators such as p300 [171]. Although hyperexpansion of the AR polyglutamine tract is associated with SBMA and progressive onset of androgen deficiency in the form of MAIS, CAG polymorphisms within the normal range of CAG repeats are not single causative factors for AIS [170, 172–174].

16.4.3 Clinical Management of AIS

There is currently no standardized treatment for patients impacted by AIS. Individualized care with a multidisciplinary approach is strongly recommended for the management of a disorder of sex development, such as AIS, from pursuing a diagnosis and providing information about the condition appropriately to monitoring puberty and considering the need and optimal timing for gonadectomy [139]. Gonadectomy is recommended for CAIS due to the increased risk of testicular malignancy that increases with age which is estimated to be about 3.6% at 25 years and 33% at 50 years [175]. Continued support for the adult patient is especially important to promote adequate sexual function and quality of life.

The management of PAIS is far more complex than for CAIS since it encompasses a range of ambiguous phenotypes and patient sexual identity may not be clear. Gender assignment for PAIS should not only consider the external genitalia but also the virilization potential, complexity of genioplasty, likelihood of gaining fertility, and projected gender identity in a case-by-case manner. The majority of PAIS infants are raised as males and would require surgery to repair hypospadias, orchiopexy for the undescended testes, and corrective mammoplasty after puberty. Several studies have demonstrated that some PAIS patients respond to high pharmacologic doses of androgens to improve virilization and masculine self-identity [149, 162, 176, 177]. Patients with shorter CAG repeats appear more likely to respond to testosterone supplementation, but further investigation is required to completely

assess the value of CAG length as a selection marker [178]. PAIS assigned as females would require gonadectomy to prevent further virilization and the risk of developing gonadal tumors later in life, and they may elect for vaginal reconstruction procedures to improve sexual function. Most patients affected by AIS are infertile, but since AIS is an X-linked recessive heritable disorder with significant consequences, genetic counseling is recommended to affected families.

16.5 Polycystic Ovary Syndrome

An excess of androgen in women can be associated with a hormone disorder known as PCOS. The first description of PCOS was reported by Stein and Leventhal in 1935 [179] from a series of women with enlarged bilateral polycystic ovaries, hirsutism, and infrequent or absence menstrual periods. PCOS is a heterogeneous disorder that affects 6% to 20% of women of reproductive age [180]. It is the most common endocrine condition for childbearing age. According to the Rotterdam criteria, a patient diagnosed with PCOS will have two of the following features: clinical or biochemical androgen excess, infrequent or lack of ovulation, and a characteristic polycystic ovarian morphology as observed by ultrasound [181, 182]. Approximately 60% of PCOS patients have high levels of circulating androgen (hyperandrogenism) in the form of testosterone, androstenedione, and DHEA, and possibly also elevated levels of 3β-hydroxysteroid dehydrogenase [183, 184]. In PCOS patients, a greater number of follicles are recruited to the preantral and antral stage; however, the follicles fail to progress to ovulation. This leads to follicular atresia, giving rise to ovaries with the characteristic polycystic appearance. Moreover, increased LH pulse frequency by the anterior pituitary stimulates testosterone production by theca cells of the follicle to further exacerbate the hyperandrogenic state and PCOS condition. There is a wide variety of comorbidities with PCOS comprising of endocrine, reproductive, and metabolic symptoms [185]. The primary endocrine and reproductive features of PCOS include LH excess and hyperandrogenism, ovulatory perturbations, aberrant follicle development, and reduced fertility. Women with PCOS who achieve pregnancy also have an increased risk of miscarriage and for developing complications including gestational diabetes, hypertensive disorders, and premature delivery [186]. A metabolic component of PCOS is associated with hyperinsulinemia and insulin resistance, increased intra-abdominal fat, fatty liver disease, and dyslipidemia, all of which amplify the risk of cardiovascular disease and type 2 diabetes [187].

16.5.1 AR-Mediated Actions in the Ovary and Brain

The phenotype of *AR* knockout (ARKO) mice has been critical for our understanding of androgen action and how AR maintains ovarian function, primarily in regulating the early stages of folliculogenesis. Although AR is not essential for the survival and reproduction of female mice, ARKO females have reduced fertility and show a progressive decline in reproductive potential with age. ARKO female mice produce fewer offspring and smaller numbers of litters, where fecundity is reduced by about 70% compared to wild-type littermates [188, 189]. Ovaries of ARKO female mice appear relatively normal at 4 weeks of age, but by 8 weeks, there are fewer corpora lutea and more atretic follicles compared to wild-type littermates, and the follicles are completed depleted by 40 weeks [189]. Analysis of ARKO mouse ovaries suggests that several genes that are involved in folliculogenesis are regulated by AR signaling, including *Kitl*, *Bmp15*, *Gdf9*, and *Hgf* [189]. Chronic exposure to exogenous androgens is sufficient to induce PCOS-like traits in rodents, including disruption of the estrous cycle, the appearance of polycystic atretic follicles, and metabolic symptoms such as increased body fat and glucose intolerance [190, 191]. Global ARKO female mice supplemented with excess androgen do not develop PCOS, which supports the hypothesis that functional AR is required for the development of PCOS phenotypes [192]. Neuron-specific ARKO prevented the development of most reproductive and metabolic symptoms induced by androgen excess but still had cycle irregularity and partial polycystic ovary morphology [193]. A more recent mouse model with double ARKO in the brain and adipocytes showed further protection against developing irregular cycles, polycystic ovary morphology, and hepatic steatosis in response to androgen excess [194]. Collectively, these findings support that AR-driven neuroendocrine actions from the brain are major drivers to the onset of reproductive and metabolic PCOS traits induced by hyperandrogenism. Other potential tissue targets include adipocytes, liver, and muscle cells, which are believed to be involved in the pathogenesis of PCOS and could also involve AR.

16.5.2 Regulation of AR Signaling in PCOS

Ovulatory women with an AR that has more than 23 CAG repeats in AR NTD are associated with higher aromatase levels and lower intrafollicular testosterone than in patients with fewer than 20 CAG repeats [195]. This suggests that CAG length may influence hormone levels in the follicular milieu. Studies examining the association between CAG length and hyperandrogenism in PCOS have yielded conflicting results. In cohorts of Australian and Chinese women, longer CAG repeats were more frequent in PCOS women [196, 197]. CAG length in a Croatian population was reported to be associated with total testosterone in PCOS women, but it was not a significant predictor of PCOS or PCOS traits like hirsutism or acne [198]. Although

polymorphism in CAG length of AR does not appear to be a major determinant of PCOS, there may be an association between CAG length and the variations in androgen levels among women with PCOS.

The presence of alternatively spliced *AR* transcripts has been identified in granulosa cells of some patients with PCOS. A study by Wang et al. in 2015 identified two AR-Vs, which were found in the granulosa cells of most (62%, 42/68) women with PCOS but not from non-PCOS control subjects [199]. One of these variants has a 69-base-pair insertion in intron 2 in the *AR* gene, whereas the other has a deletion skipping exon 3 [199]. Both of the splice variants are in-frame alterations that only affect the second zinc finger of the AR DBD. The expression of either of these AR-Vs was more common in PCOS women that had severe hyperandrogenism [199]. Notably, these AR-Vs were shown to attenuate AR nuclear translocation in response to androgen and reduce the overall number of DNA sites of AR in ChIP-sequencing analyses [199, 200]. Since these AR-Vs appear to primarily suppress the transcriptional activity of AR, these findings imply that nongenomic AR functions could be involved in hyperandrogenism and PCOS at the ovarian cell level. Furthermore, an analysis of AR phosphorylation from marmoset ovaries by immunostaining showed that phosphorylation of AR can occur at serine resides 81, 309, and 650 in granulosa and theca cells [201]. Phosphorylation of these serine residues was not impacted by hormone manipulation with testosterone or LH-RH antagonist. The biological significance of AR phosphorylation in ovarian cells remains to be fully elucidated, but posttranslational regulation of AR could potentially have a distinct function in ovarian cells.

16.5.3 Targeting AR for the Treatment of PCOS

Currently, there is no cure for PCOS, and the management of PCOS relies primarily on alleviating symptoms to improve quality of life. There are no therapies approved for PCOS specifically, and the majority of treatments for PCOS are used in an off-label fashion. Thus, there is a significant need for continuing research to improve our understanding of the etiology of PCOS and to develop mechanism-specific drugs that are more effective. Therapies targeting the AR signaling axis, including antiandrogens (spironolactone, cyproterone acetate, and flutamide) or the 5α-reductase inhibitor finasteride, have been able to provide some clinical benefit in alleviating PCOS symptoms for women [202–207].

16.6 Breast Cancer

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer mortality among women worldwide [208]. Its incidence is approximately 100 times more common in women than in men [209]. Breast cancer is highly

heterogeneous and may be categorized into four major molecular subtypes based on the expression of ER, PR, and human epidermal growth factor receptor 2 (HER2). The major subtypes include luminal A (ER^+, PR^+, and $HER2^-$), luminal B (ER^+, PR^+, and $HER2^+$), HER2-expressing (ER^- and $HER2^+$), and basal-like, which are mostly triple-negative (ER^-, PR^- and $HER2^-$). Approximately, 75% to 80% of the basal-like subgroup is triple-negative breast cancer (TNBC) [210, 211]. TNBC is an aggressive disease that is usually associated with higher grade, poor prognosis, and an increased rate of mortality [212, 213]. Although TNBC patients respond to chemotherapy, they commonly develop distant recurrence and metastasis [214]. Lack of molecular targets for therapies is a challenge for the treatment of TNBC. In recent years, targeting AR for the treatment of TNBC has been a growing interest in translational research and clinical trials. In addition to TNBC patients, other patients with AR-expressing breast cancers may also benefit from AR-targeted therapies. AR expression is detected in all stages of breast cancer: ductal carcinoma in situ, primary breast cancer, and metastatic disease [215]. From different studies employing various methodologies that vary in their sensitivity of detection and antibody of choice, AR can be detected in 70% to 90% of all breast cancers and 20% to 40% of TNBC patients [213, 216, 217].

16.6.1 AR Roles in Different Types of Breast Cancer

AR signaling plays a role in regulating normal breast development as demonstrated by ARKO mice [218]. Although female ARKO mice appear healthy in general, they display abnormal phenotypes that include decreased ductal branching during prepuberty and decreased lobuloalveolar development with fewer milk-producing alveoli in the mammary gland in adulthood. Most research has been focused on ERα due to its proliferative effects on breast cancer cells; however, AR is more abundantly expressed than ERα and PR in mammary epithelial cells [219]. The main active androgen in females is testosterone, which is produced by the ovaries. In mammary tissues, testosterone and DHT can transactivate the AR, and testosterone can be converted to E2 [213, 220]. The levels of circulating androgens are not consistently correlated with the risk of developing breast cancer [216, 221]. Some studies have shown an increased risk with elevated circulating androgens; however, others have shown that increased levels of circulating estrogens, but not androgens, are linked to the increased risk. Since testosterone can be converted to E2 by aromatase, both androgen and estrogen might be indirectly associated with breast cancer risk [221]. Indeed, both AR and ERα signaling pathways appear involved in the development and progression of breast cancer.

AR is expressed in approximately 75% of ER-positive breast cancers [222]. Depending on the disease stage and ER expression level, AR signaling may have a proliferative or antiproliferative effect, depending on the subtype of the breast cancer cells. In ARKO mice, abnormal development of the mammary gland is associated with impaired ERα and MAPK signaling [218]. There are similarities between

the AR and ERα in regard to their genomic and non-genomic actions. Studies demonstrating cross talk between AR and ERα signaling have been discussed in many reviews [209, 213, 215, 219, 221]. In clinical studies, higher levels of AR are generally associated with improved outcomes and better survival in patients with ERα-positive breast cancer [223, 224]. In these patients, AR behaves as an antiproliferative factor to mitigate estrogen-driven proliferation; therefore, AR expression may have a prognostic value for predicting patient outcomes [213, 215]. In addition to using AR as an independent prognostic biomarker, the ratio of AR to ERα is used as an indicator to predict treatment outcomes, although controversial results have been reported in different studies [225–227]. These discrepancies may be explained by the subtype of breast cancer and the threshold criteria for ratio cutoffs. A more standardized evaluation of these receptors will be required to have a reliable and consistent outcome prediction. The AR has also been reported to support ER signaling in breast cancer growth. D'Amato and colleagues demonstrated that inhibition of AR nuclear translocation with enzalutamide could reduce estrogen-mediated growth driven by ER in breast cancer cell lines and patient-derived xenografts [228]. Furthermore, gene expression analysis of AR-positive circulating tumor cells (CTCs) isolated from patients with metastatic breast cancer identified 18 genes associated with AR. Six of these 18 genes – *XBP1*, *ERBB2*, *CELSR2*, *ESR1*, *TFF1,* and *CA12* – are also regulated by ERα, which further supports the notion that the ERα and AR signaling pathways are connected for certain breast cancers [229]. Interestingly, a correlation between the duration of treatment with aromatase inhibitor and AR expression was determined from CTCs derived from breast cancer patients with bone metastases [230]. These findings support that increased AR expression might enhance tumor cell survival in response to long-term endocrine treatment in some breast cancers.

The AR is expressed in approximately 50% of ERα-negative breast cancers and may replace ERα as an oncogenic driver [215]. Cells that express the AR but not ERα tend to differentiate into apocrine-like cells (molecular apocrine cells). In clinical samples of ERα-negative breast cancer, there is a correlation between AR and HER2 expression [209, 213, 215]. Cross talk between AR and HER2 regulates cell proliferation and apoptosis in molecular apocrine cell lines. Activation of HER2 leads to increased AR binding to target genes (such as *FOXA1*, *XBP1*, *TFF3,* and *KLK3*), and AR reciprocally upregulates the expression of the HER2 gene (*ERBB2*) [231, 232]. Despite cross-regulation between AR and HER2 signaling pathways, the AR in breast cancer with amplified HER2 has no clear association with overall survival [209, 213]. Among ERα-negative breast cancer patients, TNBC patients have the worst prognosis. TNBC can be further stratified into molecular subtypes based on gene expression profiling: basal-like 1, basal-like 2, mesenchymal, and luminal AR (LAR) [233]. Approximately 22% of TNBC is the LAR subtype [234], which is associated with a worse clinical outcome [235]. Clinical LAR tumors can express high levels of AR and coactivators or downstream targets, such as *FKBP5*, *APOD*, *PIP*, *DHCR24*, *ALCAM*, *FASN*, *SPDEF,* and *CLDN8* [214]. The AR has proliferative effects in TNBC based upon studies showing that modulation of AR

can reduce the growth of some subtypes of TNBC. EGFR and PI3K signaling pathways were shown to be involved in AR-mediated proliferation [236–238].

Since AR signaling is involved in breast cancer progression, there has been a considerable effort to investigate alterations in AR structure and function. One such alteration is the length of the polyglutamine tract in the AR-NTD. Some reports reveal no association for patients younger than 40 years of age or in patients that are carriers for BRCA1 and BRCA2 mutations [239, 240]. However, an earlier study reported that a short polyglutamine track with less than 20 CAG repeats may protect against breast cancer [241]. This is contrary to a meta-analysis that revealed a longer polyglutamine track of more than 22 CAG repeats might be protective [242]. Another important AR alteration to consider in breast cancer patients is the expression of AR-Vs. Transcripts and protein of AR-Vs are detectable in breast cancer cell lines as well as in some primary breast cancer specimens from patients without prior antiandrogen treatments [243, 244]. High levels of AR-V7 protein were detected in a subset of ERα-negative/HER2-enriched breast cancer cells, which were likely to be molecular apocrine cells [243]. Moreover, the expression of AR-V7 was detected in CTCs from patients with metastatic breast cancer and was associated with bone metastasis [230] similar to findings from patients with metastatic CRPC [95]. AR-V7 was upregulated in ex vivo primary breast cancer cells treated with enzalutamide [243]. In advanced prostate cancer, AR-V7 upregulates the expression of *UBE2C*, which is involved in cell cycle progression and enhances malignancy [105]. AR-V7-regulated genes in the breast cancer cell line MDA-MB-453 were found to be involved in immune function and cell movement [243]. The exact roles of AR-Vs in breast cancer continues to be an active area of investigation.

16.6.2 Treatments Targeting AR

Historically, androgens were used systemically to treat breast cancer patients and provided tumor regression in 15%–30% of patients. Following the advent of antiestrogen therapies, systemic androgen treatment is no longer used due to the undesirable side effects of virilization and the conversion of testosterone to E2 [209, 215, 220]. With more research on the roles of AR in breast cancer, specific therapeutic strategies for targeting AR are actively being tested and developed. The finding of LAR subtype in TNBC [214] was embraced with numerous preclinical studies and clinical trials to test if existing therapies that target the AR signaling axis by antagonizing the AR LBD or by inhibiting steroidogenesis would be effective. Antiandrogens (bicalutamide and enzalutamide) and ablation of androgen biosynthesis with abiraterone acetate are being evaluated in clinical trials for breast cancer patients. Preclinical studies have shown that bicalutamide could inhibit androgen-induced tumor growth in vivo in mice bearing MDA-MB-453 human breast cancer xenografts [245]. Moreover, bicalutamide has also been shown to inhibit the growth of TNBC xenografts with different subtypes and variable sensitivities [214]. Phase 2 clinical trial was conducted to evaluate the safety and efficacy of enzalutamide in

patients with ≥10% nuclear AR in locally advanced or metastatic TNBC [246]. Patients were dosed daily with 160 mg of enzalutamide until disease progression. Results from the trial indicated that enzalutamide was well-tolerated and led to a median overall survival of 17.6 months for the evaluable subgroup who met the criteria (78 patients) compared to 12.7 months for all 118 enrolled patients [246].

Clinical trials evaluating antiandrogens or an androgen biosynthesis inhibitor have shown promising results for AR-positive TNBC patients especially [246–249], but these therapeutics rely on targeting the AR LBD and would be expected to have limited to no response on the transcriptional activity of truncated AR-Vs. AR-Vs are expressed in breast cancer [243, 244]. Clinical resistance to drugs that only target the fl-AR may also develop from these agents as already seen for prostate cancer. Targeting both fl-AR and AR-Vs by an AR NTD inhibitor, such as ralaniten, may yield therapeutic responses in some breast cancer patients. Next-generation and more potent ralaniten analogs have been developed and undergone preclinical testing for their activity against AR-Vs [61, 250]. A more potent and metabolically stable second-generation ralaniten analog EPI-7386 is in clinical trials for metastatic CRPC patients (NCT04421222).

16.7 AR in Other Diseases

Discoveries from global ARKO murine models and cell-type-specific and tissue-specific ARKO models have vastly expanded our understanding of the pathophysiological roles of AR that were not previously possible by castration and AIS experiments in mice [251]. The unique roles for AR in the function of immune cells, bone mineralization, muscle, brain, liver, wound healing, metabolism, regulating insulin sensitivity, and glucose homeostasis have been described from such murine models [251]. In the following, we highlight some key findings on the role of AR in hypertension and atherosclerosis in humans as well as some other malignancies.

16.7.1 Role of AR in the Progression of Hypertension and Atherosclerosis

The AR is involved in cardiovascular diseases, where its role in hypertension and atherosclerosis is the most established. Men with cardiovascular diseases are observed to have lower levels of serum testosterone [252, 253]. Notably, men with total testosterone levels lower than 241 ng/dL were 40% more likely to die from cardiovascular disease compared to those with higher testosterone levels [254]. Androgen deprivation therapy for prostate cancer patients is also associated with an increased risk for peripheral artery disease [255]. In general, men have a higher blood pressure than women, where the difference is gradually diminished after

women have gone through menopause and men have decreased testosterone levels from the age of 70 years old. Thus, androgen appears to be involved in modulating blood pressure. In preclinical models, castration or flutamide could reduce mean arterial pressure in spontaneously hypertensive male rats to levels that were comparable females [256]. Interestingly, blocking the conversion of testosterone to DHT with a 5α-reductase inhibitor was not able to decrease blood pressure. It is noteworthy to mention that having low endogenous testosterone levels is also associated with higher blood pressure in male populations [257, 258]. Overall, androgens and AR signaling have a role in modulating arterial pressure and may exacerbate the progression of hypertension.

Atherosclerosis is a cardiovascular disease that is associated with the chronic expansion of arterial intima by a gradual accumulation of lipids, cells, and extracellular matrix, which may lead to occlusion and thrombosis, myocardial infarction, sudden cardiac death, or stroke. Males in general have a thicker intima-media during early carotid atherosclerosis relative to females. Total testosterone and SHBG levels are inversely correlated with atherosclerosis [259], where low androgen levels are strongly linked to the production of triglycerides, total cholesterol, and low-density lipoprotein cholesterol [260]. Androgen deprivation therapy for prostate cancer can increase the metabolic burden, which may accelerate the progression of atherosclerosis [261]. In castrated rabbits, DHT supplement was sufficient to inhibit the accumulation of foam cells from oxidized low-density lipoprotein [262]. These findings suggest that physiological levels of testosterone could help to prevent the formation of atherosclerosis.

16.7.2 AR in Other Types of Cancers

AR signaling is also implicated in the development of other cancer types and may be a therapeutic target to influence patient survival such as in salivary duct carcinoma (reviewed in [263]), glioblastomas [8], bladder (reviewed in [264]), kidney [265], endometrial [266], pancreatic [267], and liver cancer [267, 268]. While the bladder is not generally considered to be an androgen-responsive organ, AR expression has been described in the urothelium, submucosa, smooth muscle cells, and neurons of the bladder in primates and humans [264]. Males innately have a three to four times increase in the risk of developing urinary bladder cancer than females, even after accounting for lifestyle and environmental factors that include cigarette smoking and occupational exposure to carcinogens [269]. Notably, the oncogenic action of *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine, a known carcinogen for bladder cancer, was identified to act through AR signaling [270]. Miyamoto et al. identified that the incidence of urothelial carcinoma was much greater in male mice treated with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine compared to female mice (92% vs. 42%, respectively), where tumors did not develop in ARKO mice. In rodents, androgen deficiency induces a decrease in bladder capacity, smooth muscle bladder mass, and autonomic nerve function, whereas testosterone supplementation can reverse

these effects [271, 272]. AR signaling may also promote migration, invasion, and metastasis of bladder cancer cells by interaction with β-catenin/Wnt signaling and its downstream targets c-myc and cyclin D1 [273–275].

In renal cell carcinoma, elevated *AR* expression is generally associated with better patient outcomes [276]. AR expression is negatively correlated with tumor stage, tumor grade, and tumor status [277, 278]. In the case of hepatocellular carcinoma, the AR was reported to be overexpressed in the nuclei of hepatocellular carcinoma cells in approximately one-third of tumors and was associated with advanced disease and poor survival [279]. It was proposed that co-targeting the AR and the mTOR pathway may be a necessary therapeutic approach for hepatocellular carcinoma, since feedback activation of AKT-mTOR from inhibiting the AR could promote AR expression and nuclear localization [279]. Others have also demonstrated that the AR may have a protective role in suppressing hepatocellular carcinoma metastasis, supporting cell adhesion, and increasing tumor cell death by anoikis mechanisms [280]. Thus, the AR may have distinct and opposing roles in hepatocellular carcinoma cells, by promoting tumor initiation and inhibiting metastasis.

16.8 Summary

Some common trends in the AR-associated diseases discussed in this chapter include mutations in AR, polymorphic variants of AR, and the expression of AR-Vs. Although most diseases caused by an imbalance of androgen, deviation from the physiological levels of androgen, or alteration of AR transcriptional activity are clinically manageable, most are not curable and have severe consequences on quality of life and may lead to mortality. These tend to be genetic diseases, and therefore examining the genetic alterations in AR from patients may be beneficial for selecting optimal and effective therapeutic options for personalized medicine. Furthermore, standardizing methodologies to detect and to define AR positivity and status, either at a genetic or protein level, will be required to identify patients who might benefit from AR-targeted therapies. Continued research remains paramount to facilitate drug discovery and to develop more specific, efficacious, and cost-effective therapeutic strategies to target AR and the androgen axis.

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Chapter 17
Bilirubin: A Ligand of the PPARα Nuclear Receptor

Stephen Hong, Darren Gordon, David E. Stec, and Terry D. Hinds Jr

Abstract Bilirubin is the product from red blood cell lysis, which releases heme that is reduced to biliverdin by heme oxygenases (HO). Later, the biliverdin is converted to bilirubin by the biliverdin reductase (BVR) enzyme. Studies have revealed that bilirubin is significantly lower in obese patients with nonalcoholic fatty liver disease (NAFLD). While the mechanisms that reduce plasma bilirubin are unknown, it has been shown that increasing plasma bilirubin lowers body fat percentage and liver fat content in obese animal models. The bilirubin actions have been attributed to a newly revealed function that it is a hormone, which binds directly to the PPARα nuclear receptor transcription factor. PPARα regulates fatty acid oxidation (FAO) and peroxisomal function to maintain cellular homeostasis and catabolism of fatty acids. Here, we discuss the partnership of bilirubin-PPARα, along with the two other PPAR isoforms PPARβ/δ and PPARγ, and how they function to control peroxisomes and mitochondria that mediates fatty acid β-oxidation and adiposity. There may be clinical interest in bilirubin-PPARα functionality to rectify NAFLD and insulin resistance in the obese.

Keywords Mitochondria · Peroxisome · Fatty acid oxidation · Obesity · Fatty liver disease · NAFLD · Biliverdin reductase · BVR · BVRA · Heme oxygenase · HO-1 · HO-2 · Bilirubin

S. Hong · D. Gordon
Department of Neurosciences, University of Toledo College of Medicine and Life Sciences, Toledo, OH, USA

D. E. Stec
Center for Excellence in Cardiovascular-Renal Research, Department of Physiology and Biophysics, University of Mississippi Medical Center, Jackson, MS, USA

T. D. Hinds Jr (✉)
Department of Pharmacology and Nutritional Sciences, University of Kentucky, Lexington, KY, USA
e-mail: Terry.Hinds@uky.edu

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17.1 Introduction

With the growing obesity epidemic, multiple diseases have manifested that affects tens of millions of people. Consequentially, nonalcoholic fatty liver disease (NAFLD) is on the rise, which plays a role in developing insulin-resistant diabetes and cardiovascular diseases. NAFLD progression without corrective measures can worsen to nonalcoholic steatohepatitis (NASH), a fatty degeneration that can culminate into cirrhosis or hepatocellular carcinoma [1]. When examining the pathogenesis of NAFLD, the "two-hit" model focuses on the steatosis of the liver (first "hit") and then inflammation that follows (second "hit") [2]. The liver's fatty buildup can be initiated by hepatic insulin resistance, which increases circulating insulin inducing peripheral tissue to develop glucose intolerance [3]. The circulating glucose is collected by the liver to be converted into fat for de novo lipogenesis, leading to fatty liver development [4]. This process is exacerbated in patients with hyperinsulinemia, as there is an even higher amount of glucose deposition into the liver [4]. Consuming high caloric intake causes hepatic fat accumulation by two primary methods: (1) hepatic de novo lipid synthesis or (2) peripheral fat content in the body is redirected to the liver [5]. Sustained elevated levels of fat in the liver can result in pathological sequelae, such as inflammation and oxidative stress, which are natural responders against the adverse environment. NAFLD treatments are currently limited to lifestyle changes and diet modifications, as weight loss is the most effective method to reverse NAFLD [6]. However, drugs are being developed to target the fat accumulation pathways and inflammation, but these are yet to be approved for clinical use.

To reduce fat, mitochondrial or peroxisomal oxidation can play a vital role and serve as primary therapeutic targets for NAFLD. Although most oxidation is mediated through mitochondrial β-oxidation, peroxisomes assist mitochondria in oxidizing fat [7]. Peroxisomes have the ability to begin the oxidative process of very-long-chain fatty acids (VLCFAs) that cannot be oxidized by mitochondria [8]. Increasing this activity could be crucial in correcting NAFLD, which can be enhanced by activating the peroxisome proliferator-activating receptors (PPARα) nuclear receptor transcription factors. The PPARs were initially discovered by ligands that were thought to increase cellular peroxisomes directly, hence, peroxisome proliferation [9]. However, peroxisomal proliferation is based on the cellular response of PPARα activation. PPARβ/δ and PPARγ are two isoforms that were classified as PPARs based on their homology to the PPARα gene; however, neither induces peroxisome proliferation [10]. PPARα has been mostly studied for its role in lipid-lowering effect and, more recently, anti-inflammatory role [11, 12]. All of the PPAR isoforms reduce inflammation, which can provide additional protection from NAFLD [13]. Recently, bilirubin was shown to be a ligand for PPARα [14–16], which also significantly reduces hepatic fat accumulation [13, 17, 18]. In patients, bilirubin levels are inversely correlated with body weight [19–22]. These finds suggest that antioxidants, at least in the case of bilirubin, may reduce adiposity and NAFLD via PPARs. Here, we aim to elucidate the protective pathway that

PPARs and peroxisomes play against NAFLD with a discussion of the role of bilirubin.

17.2 PPARα Protein Structure

The three PPAR isoforms, α, β/δ, and γ, have different tissue expression [9]. They all work similarly in that ligand activation induces binding to DNA and heterodimerization with the retinoid X receptor (RXR) on PPAR response elements (PPREs), eliciting a gene response (Fig. 17.1) [23]. PPARα is a nuclear protein produced in response to reduced serum nutrients, such as in the case of fasting. It should be noted that after discovering that PPARs bind fatty acids, the mainstream thinking was that increased fatty acids during fasting must be inducing PPARα in the liver during this time. However, this is no longer the mainstream thinking as there was a published paper showing that PPARβ/δ is increased in the liver by free fatty acids

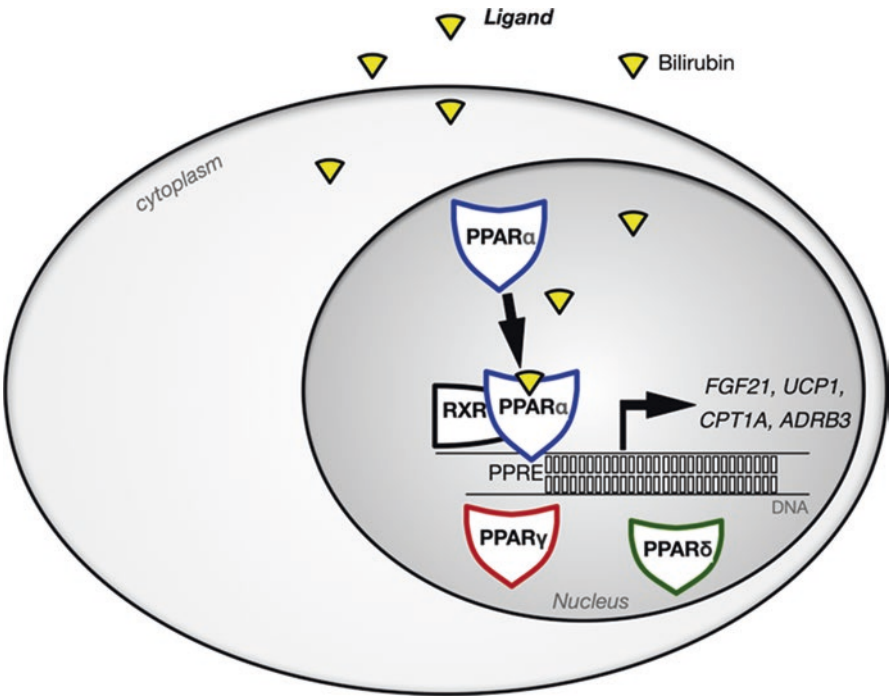

Fig. 17.1 PPAR Transcriptional Signaling Pathway. The PPARs are a superfamily of nuclear receptors, and three isoforms exist: α, β/δ, and γ. All isoforms perform through ligand activation that induces binding to DNA on PPAR response elements (PPREs) and heterodimerization with the retinoid X receptor (RXR), which controls gene transcription. PPARα binding with its ligands (e.g., bilirubin) upregulates transcription of genes that increase metabolism, such as *FGF21, UCP1, CPT1A*, and *ADRB3*

and that it serves as the "plasma free fatty acid sensor in liver" [24]. Since this paper, others have shown data supporting these findings and have rerouted the thinking of free fatty acids for increasing PPARα in the liver during fasting. PPARα being increased during fasting most likely occurs by lower circulating insulin levels, but this is still inclusive.

The PPARα gene is located on Chromosome 22 in humans and 15 in mice and is studied primarily for its role in lipid and glucose regulation in the liver, adipose, and other tissues [25, 26]. The PPAR proteins are arranged in similar domains: the N-terminal domain that includes the amino-terminal transactivation region (AF-1), DNA-binding domain (DBD), the hinge region, and the ligand-binding domain or LBD (also known as activation factor-2, AF2) [27]. Investigating the role of these domains can enhance our understanding of their significance in cellular metabolic pathways and potential impact on metabolic disease. While there are structural similarities, the PPARs have different LBDs that lead to diverse functionalities when bound to their cognate ligand. PPARα activation can lead to specific coregulator (coactivators and corepressors) recruitment that might affect the other PPAR isoforms differently. The AF-1 region is critical in recruiting coregulators that lead to the control of gene activity by the PPARs [28]. Our lab has previously identified an inhibitory PPARα phosphorylation amino acid at serine 73, which is in the AF-1 region. Serine 73 phosphorylation is lower in mice with elevated bilirubin levels [13]. The DBD plays an essential role in the heterodimerization of PPARα with RXR, enhancing PPARα functionality [29], and this cooperativity enhances the expression of metabolic genes [30, 31]. The DBD also contains phosphorylation sites, including the threonine 129 site, which is based on PKC activation and enhances PPARα activity [32]. The hinge region of PPARα serves several roles, which include coregulator binding [33] and is a target of both phosphorylation (serine 179) as well as SUMOylation (lysine 185) [32, 34]. Lastly, while there are some pan-PPAR agonists [31, 35, 36], structural differences in the LBD/AF2 cause preferential activation of specific PPAR isoforms to regulate gene-specific responses [15, 37]. Understanding the structural layout of PPARα is crucial as we explore the effects of several ligands and their ability to alter the coregulator recruitment to PPARα, which controls gene-pathway specific actions. We have previously shown by in silico analysis that bilirubin docks in the LBD of PPARα [15], which we later showed by competitive ligand-binding assays that bilirubin and fenofibrate compete for the same binding regions in the LBD [16]. We also showed that bilirubin induced a specific set of coregulators to PPARα protein (mouse and human) [16]. These indicate that bilirubin has a hormonal function by activating PPARα by direct binding that induces a physiological change.

17.3 Bilirubin Generation and Excretion

One enzyme that may have a key role in attenuating the detrimental impacts of liver pathology is biliverdin reductase (BVR) [13, 38–40]. During red blood cell destruction, hemoglobin is released into the plasma and is converted into biliverdin via heme oxygenase (HO) [41]. Then, through the conversion of a double bond to a single bond by BVR, biliverdin is converted to bilirubin [42–45]. The excretory process for bilirubin is mediated by the UDP-glucuronosyltransferase 1-1 (UGT1A1) enzyme that conjugates bilirubin [46], allowing for excretion into the biliary canaliculi and deposition in the intestine [5, 47]. The gut microbiome transforms conjugated bilirubin into other forms, such as urobilinogen and stercobilin, which are mostly excreted, although some can be reabsorbed [5]. The role of bilirubin metabolism and hepatic function has yet to be elucidated. However, recently, bilirubin nanoparticles were shown to improve fatty liver and reduce hepatic biomarkers AST and ALT enzymes [17].

The two known isozymes of BVR (BVRA and BVRB) have different structural and functional properties, which is further implicated as the genes are located on two different chromosomes [20]. BVRB compared to BVRA in zebra fish larvae has increased expression in states of oxidative stress [48], and global BVRA knockout mouse was subjected to greater oxidative stress [49]. Hepatic BVRA has also been implicated in metabolism as it was shown to protect PPARα for GSK3β inhibition by phosphorylated PPARα at serine 73 to increase turnover and decrease transactivity [13, 40]. BVRA may protect against Alzheimer's disease, which is known to be associated with insulin resistance [50–56]. There remain many scientific questions on BVRA and BVRB and their involvement with the PPAR isoforms, such as regulating all PPAR isoforms, or do they play an integral role in the use of bilirubin by PPARα. Future work on the BVR isozymes and how they signal to the PPAR isoforms is needed.

17.4 Bilirubin as a PPARα Ligand

The beneficial effects of elevated bilirubin levels have been observed in humans with the Gilbert's polymorphism. They contain a polymorphism in the *UGT1A1* promoter that lowers its expression, increasing plasma bilirubin. People with Gilbert's have lower rates of ischemic heart disease and higher rates of high-density lipoproteins (HDL), also known as "good cholesterol" [57]. A humanized Gilbert's syndrome mouse model has been generated using the human UGT1A1*28 polymorphism and has been found to have significantly less fat mass, body fat percentage, cholesterol in the liver, liver stenosis, fasted blood glucose levels, and plasma insulin levels on a high-fat diet (HFD) compared to control on HFD [58]. In addition, there was a significant increase in the CYP4A subfamily of enzymes that are also activated by PPARα [59]. This same model was later shown to

hyperphosphorylate PPARα in white adipose tissue (WAT) and enhanced coactivator recruitment [16].

Previous research has shown that PPARα ligands, such as WY 14,643 and fenofibrate, act as selective PPAR modulators (SPPARM) based on different binding affinity or the recruitment of different coregulators [60–62]. Ultimately, the transcriptional differences provide different protective factors as fibrates are better for anti-inflammatory processes, while WY 14,643 is more suited for lowering blood glucose levels [63]. Based on nonpathogenic increased levels of bilirubin, it may influence the positive effects of both fibrates and WY 14,643.

These beneficial outcomes can be seen in experiments with the direct application of bilirubin to diseased tissues. Colitis-induced rats were given intragastric gavages of unconjugated bilirubin and were shown to have decreased inflammation and a faster recovery rate than controls [64]. These anti-inflammatory effects were further supported using intravenous administration of polyethylene glycol (PEG)-bound bilirubin (bilirubin nanoparticles) to colitis-induced rats. Bilirubin nanoparticles preferentially localized to areas of inflamed colon and significantly halted the process of inflammation in these areas [65]. However, neither of these studies could elucidate the mechanism of how bilirubin was able to reduce inflammation.

To further stress the point of bilirubin activation of PPARα, bilirubin has been shown to attenuate inflammatory processes similarly to PPARα [66, 67]. Two different studies examined the effect of fenofibrate and bilirubin on the proliferation of Th17 differentiation, a T-helper cell associated with autoimmune diseases [68]. Chang et al. found that the activation of PPARα via fenofibrate decreased Th17 cell differentiation by inhibiting the IL-6/STAT3/RORγt pathway [69]. Congruently, Longhi et al. showed that the introduction of bilirubin to mononuclear cells downregulated Th17 cells and IFNγ production via ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1 or CD39) [70]. CD39 is essential for autoimmunity as it hydrolyzes extracellular ATP down to AMP that is converted to immunosuppressive adenosine [71]. In both cases, the downstream effect leads to decreased IL-17, a pro-inflammatory cytokine of Th17 cells, and increased Foxp3 expression, the master regulator of Treg cells. In addition, multiple sources have found that PPARα can upregulate the activity of CD39, showing a more significant correlation between bilirubin and its ability to activate PPARα pathways [72, 73]. The similarities of these results continue to support the interaction between bilirubin and PPARα.

Recent studies have shown that bilirubin activates the PPARα pathway by directly activating the receptor [13, 15]. It had been previously established that activation of PPARα and its other two isoforms leads to increased heme oxygenase-1 (HO-1), the rate-limiting enzyme responsible for synthesizing the bilirubin precursor biliverdin [74–80]. This indicates that there is a positive feedback mechanism between PPARα and bilirubin. There are also limits on this positive feedback mechanism as PPARα has been shown to activate UDP-glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), the enzyme that conjugates bilirubin, its primary clearance mechanism [79, 81]. This correlation between bilirubin and PPARα may be

able to explain bilirubin's protective ability against NAFLD by a peroxisomal mechanism.

By establishing the relationship between bilirubin and PPARα, it is possible to formulate a mechanism the body naturally forms to protect against the increased inflammation and hepatic damage in NAFLD. The process begins with an increase in bilirubin levels, a general indicator of liver damage or disease. The activation of PPARα ensues, which activates multiple pathways, including the positive and negative feedback loops, indicated previously. The major pathways important for NAFLD protection are the increase in fatty acid oxidation (FAO) to lower the overall fat content within the body and the attenuation of inflammation. Although PPARs were discovered to increase peroxisome levels [9], it is essential where these cells are activated as it can be assumed that more FAO indicates greater protection from NAFLD. Fenofibrate increases PPARα transcription and induces general downstream gene expression of enzymes in skeletal muscle [82], including FAO gene production in lean and obese patients [83]. This is highly warranted as the sheer amount of muscle mass outweighs any other organ that contains high levels of PPARα. It can be assumed that bilirubin can have a similar effect in skeletal muscle as it can be transported ubiquitously in the body. In addition to just increasing peroxisomal content, there is evidence that bilirubin through PPARα activation can bolster peroxisomal and mitochondrial FAO through other supportive mechanisms.

17.5 Peroxisomal Protection against NAFLD

Peroxisomes are single-membrane organelles that contain matrix proteins used for fatty acid metabolism, sequestration of reactive oxygen species (ROS), and biosynthesis of phospholipids [84]. Peroxisomes have often been characterized as the sidekick to mitochondria for their ability to break down VLCFAs and eliminate ROS generated by mitochondria (Fig. 17.2) [85]. Thus, a known partnership exists among the two, which is supported by both organelles' FAO, following the same process of dehydrogenation, hydration, dehydration, and thiolytic cleavage, albeit with different enzymes [86]. However, peroxisomal FAO is not limited to very-long-chain fatty acids. Recent research found that in mitochondrial fatty acid transport dysfunction or overload, peroxisomes can oxidize medium- and long-chain fatty acids [87], making them relevant in reducing NAFLD. The closeness in the relationship between peroxisomes and mitochondria is shown in the coordination of peroxisome-mitochondria FAO using shared enzymes and the mirroring between transport enzymes such as the peroxisome's ATP-binding cassette subfamily D (ABCD) transporters and the mitochondria's carnitine palmitoyl-transferases (CPT) [88, 89]. Furthermore, the main regulator of mitochondrial biosynthesis, peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α), utilizes the PPAR pathway to increase the expression of peroxisomal enzymes required for FAO [11, 90].

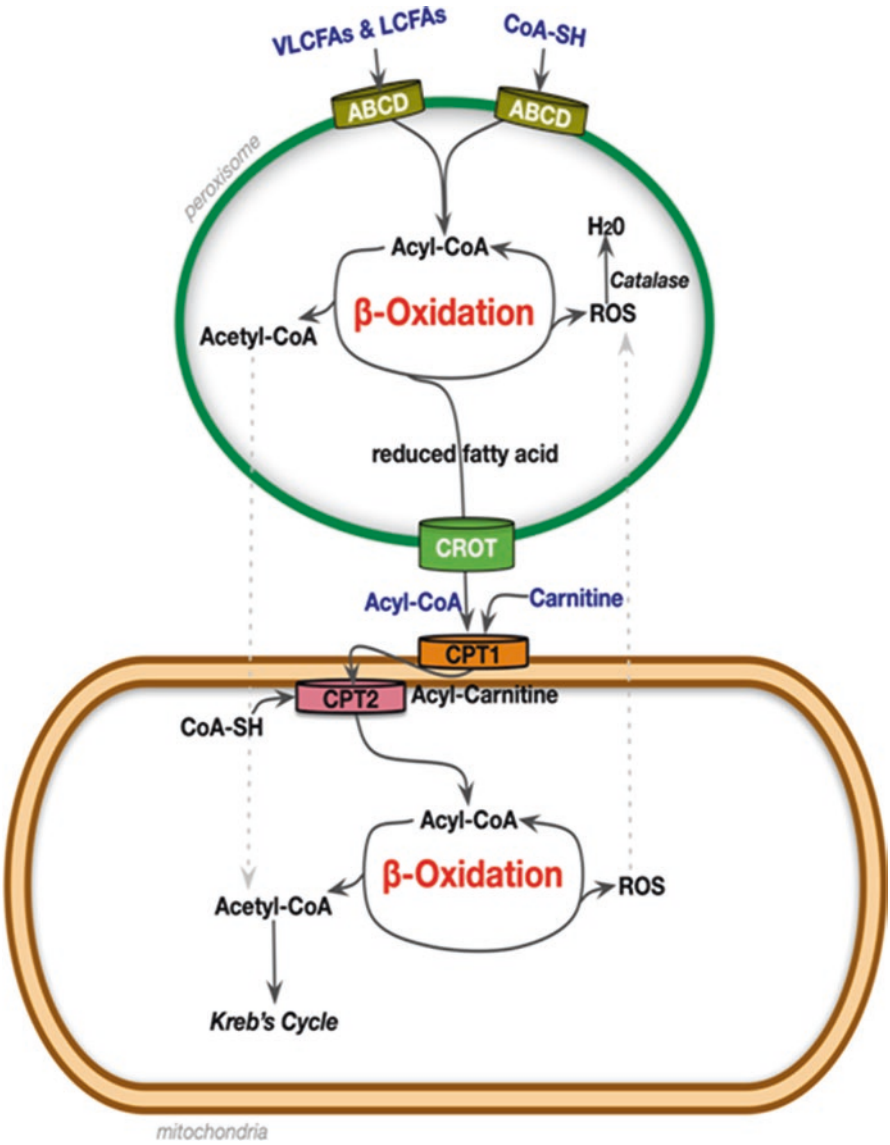

Fig. 17.2 Cross talk of mitochondrial and peroxisomal pathways. Peroxisomes communicate with mitochondria by preparing very-long-chain fatty acids (VLCFAs) into long- and medium-chain fatty acids (LCFAs and MCFAs) for β-oxidation. The VLCFAs and LCFAs enter the peroxisome via ABCD transporters, and, after catabolism, shorter-chain fatty acids are exported via CROT, a peroxisomal carnitine O-octanoyltransferase. The oxidizing of reactive oxygen species (ROS) created from mitochondria and peroxisomes protects the cell. The mitochondria import medium-chain fatty acids via the carnitine palmitoyl-transferase (CPT1) and communicate to CPT2 inside the mitochondria to signal for β-oxidation. Upregulation of the peroxisome allows for improved metabolic activity

In addition to FAO, peroxisomes contain both catalase (CAT) and superoxide dismutase 1 (SOD1), which are vital in the reduction of ROS generated through FAO and other cellular processes [91]. These enzymes represent the main mechanism of ROS removal by converting it into water. It is vital to eliminate these ROS as they damage DNA, including mitochondrial DNA [92]. This becomes a central issue in NAFLD as Nassir et al. have established the link between mitochondrial dysfunction and NAFLD [93], indicating that higher oxidative stress and ROS damage are the root cause of this dysfunction. Peroxisomes act as a significant defensive line against ROS-mediated mitochondrial DNA damage to maintain proper transcription and FAO. With the peroxisome's FAO and ROS sequestration abilities, these intracellular organelles' proliferation and activation may be a key in preventing and correcting NAFLD.

The biogenesis of peroxisomes is regulated by the transcription of peroxin (PEX) genes, which produces a family of proteins necessary for the formation and activity of peroxisomes [94]. Knockouts of essential PEX genes, such as Pex11a, showed significant increases in fat mass, body weight, blood glucose, hemoglobin A1C, insulin, hepatic triacylglycerol (TG), and many other factors [95–99]. This is mirrored in PPARα knockout mice, as similar results were found [11, 100, 101]. Removal of these integral proteins cause increased blood glucose and lipid deposition into the liver, which exacerbate NAFLD. Although it is unclear if most peroxisomes are formed de novo from the endoplasmic reticulum or the fission of preexisting peroxisomes [102, 103], the activation of PPARs directly expands the number of peroxisomes in cells [9].

17.6 Peroxisomes, Oxidative Stress, and Antioxidants

Peroxisomes can be both sources and traps for reactive oxygen species (ROS) due to the oxidative metabolism of fatty acids and the degradation of H_2O_2 by catalases. Peroxisomes contain a subset of enzymes, including the flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)-dependent oxidase, which generate H_2O_2 [104]. Peroxisomes also contain several H_2O_2-eliminating enzyme systems, including catalase and peroxiredoxins (PRDXs). Catalase is abundant in peroxisomes; however, during increased ROS production conditions, the importation of catalase into peroxisomes can be reduced to increase the amount of catalase in the cytosol to protect against H_2O_2 damage [105]. PRDX5 reduces peroxynitrite ($ONOO^-$) and a variety of lipid peroxides (LOOH) via NADPH-dependent thioredoxin (TXN)/TXN reductase (TXNTR) system. They can also transfer oxidizing equivalents from H_sO_s to target proteins through thiol-disulfide reshuffling [106]. PRDX5 and CAT play nonoverlapping roles in H2O2 clearance, supported by the distinct kinetic characteristics of both antioxidant enzymes. Catalase scavenges H_2O_2 in the low millimolar range, while PRDXs work in the low micromolar range [107]. Glutathione S-transferases and epoxide hydrolases can also contribute to ROS balance in peroxisomes [108].

Peroxisomes play an essential role in a healthy redox balance demonstrated in conditions that interfere with normal peroxisomal function and biogenesis. Long-term exposure to peroxisomal proliferators, such as dibutyl phthalate and gemfibrozil, in rodents has been reported to cause oxidative liver damage, and ROS imbalances the induction of ROS-generating peroxisomal enzymes [109]. However, this effect of long-term exposure of peroxisomal proliferators on liver function may be species dependent. Humans appear to be relatively insensitive or nonresponsive at dose levels that produce a marked response in rodents.

17.7 Peroxisomes and Inflammation in NAFLD

The ABCD family of genes, found on the peroxisomal surface, helps import fatty acids or fatty acyl-CoAs [110]. Activation of PPARα significantly increases the transcription of both *ABCD2* and *ABCD3* transporters, increasing peroxisomal FAO [111–114]. In support of this greater import of fat into peroxisomes, bilirubin decreases the levels of the ATP-binding cassette subfamily A member 1 (ABCA1) transporters on macrophages [115]. This may seem counterintuitive as ABCA1, also known as cholesterol efflux regulatory protein (CERP), pumps cholesterol and phospholipids to an extracellular acceptor, apolipoprotein A1, a vital process in the formation of HDL [116]. However, this decrease in the ABCA1 export of lipids out of macrophages may reduce fat in the body. One of HDL's major functions is to return peripheral fat in the body to the liver to be excreted as bile [117], which is unwarranted in NAFLD. To correlate this decreased ABCA1 expression, HO-1 and PPARα agonists have been shown to promote M2 macrophage over M1 macrophage polarization {Stec, 2019 #25713} [118, 119]. M2 macrophages have anti-inflammatory properties and are considered the "repair" macrophages, following injury by promoting growth factors such as PDGF and VEGF [120, 121]. Unlike its M1 counterpart that uses glycolysis, these macrophages are powered by FAO through AMP-activated protein kinase (AMPK). Therefore, it is favorable that macrophages would want to lower the efflux of cholesterol and other fatty acids as they are actively degrading them for the activation of PPARα.

To highlight the importance of peroxisomes in glucose regulation, mice that lacked the *Cpt1b* gene, a key enzyme in mitochondrial FAO, exhibited enhanced glucose regulation by increasing peroxisomal activity [122]. Furthermore, mice with double knockouts of muscle-specific *Cpt1b* and *Pex5* exhibited impaired glucose tolerance due to the lack of peroxisomal compensatory activity [123]. To support this relationship in an endogenous system, our lab has shown that mice with the human *UGT1A1* locus with the Gilbert's polymorphism, a mutation in the gene that reduces bilirubin clearance increasing plasma levels, were protected against hepatic steatosis alongside improved glucose regulation as compared to control [58]. We revealed this is due to a decrease in PPARα phosphorylation in the S73 site, which downregulates PPARα activity [58], resulting in an enhanced gene activity.

Activation of PPARα by WY 14,643 and fenofibrate, both known PPARα ligands, increases catalase and SOD1 activity within cells [124, 125]. Whether this is due to increasing peroxisomal number or individual peroxisomal ROS removal efficiency is unknown. Regardless, this shows that PPARα activation protects the cell from ROS damage at least partially by peroxisomal activity [11]. In addition, upregulation of HO-1 and therefore bilirubin's peroxisomal activation via PPARα have been shown to increase catalase and SOD activity [126], which are further supplemented with bilirubin acting directly as an antioxidant by reducing ROS [127]. In relation to obesity and insulin resistance, why adiposity lowers bilirubin levels and, as a result, progresses to NAFLD will benefit millions of patients.

17.8 Extra-Peroxisomal PPARα Pathways Against NAFLD

PPARα produces several other responses that combat NAFLD through extra-peroxisomal mechanisms via the upregulation of fatty acid transport proteins (FATPs) and solute carrier family 27 members 1, 2, and 4 (Slc27a1, Slc27a2, and Slc27a4) [128–130]. Complementary to this, PPARα has also been shown to increase adipose differentiation-related protein (ADRP), which stimulates fatty acid storage in cytosolic lipid droplets rather than the formation of very-low-density lipoproteins (VLDLs) that are released into the bloodstream {Stec, 2019 #25713} [131]. The increases in fatty acid transport into the cell and the decrease in VLDLs lower plasma TG levels [132]. PPARα is also a mediator for the activation of cytochrome P450 enzymes of 4A subfamily (CYP4A) [11, 133], which is a class of enzymes capable of hydroxylating the terminal ω-carbon of saturated and unsaturated fatty acids [134]. Not surprisingly, mice with hyperbilirubinemia have higher CYP4A expression [58]. This provides an additional possible mechanism of how PPARα lowers the levels of fatty acids.

NAFLD progression increases with inflammation, and PPARα and bilirubin have been shown to function in an anti-inflammatory capacity. PPARα activation reduced the initiation and progression of several inflammatory diseases, such as Parkinson's disease and autoimmune disorders [69, 135]. The protection against autoimmune disorders may be based on the reduced amount of reactive immune cells needed to handle infections. Peroxisomes are required for proper phagocytosis and clearance of bacteria through oxidative burst [136]. The activation of PPARα increases peroxisome concentration in immune cells and their ability to kill bacteria, indicating a need for less immune cells for infection clearance and a lowered chance for autoimmune disorders to develop. However, this hypothesis has yet to be proven. Nevertheless, the activation of PPARα is paramount in attenuating NAFLD as it reduces fat content and inflammation seen in the disease progression.

17.9 PPARγ and PPARβ/δ Effects on Peroxisomes and NAFLD

In the case of the other two isoforms of PPARs, not all things were created equally in protecting the body against NAFLD. Bilirubin was not shown to interact or activate the other PPAR isoforms PPARγ or PPARβ/δ [16]. In particular, PPARγ is critical in lipogenesis and adipocyte differentiation [137–140]. It is important to note that peroxisomes are also vital in producing phospholipids and other phospholipid derivatives critical for cellular functions, such as neuronal myelin sheaths and the formation of the pro-inflammatory precursor, arachidonic acid [89]. This lipid synthesis is typically associated more with PPARγ activation rather than the two other isoforms. Thiazolidinediones (TZDs), a well-known PPARγ activator, are used as an antidiabetic medication to increase insulin sensitivity without increasing hepatic glucose production [140, 141]. This may seem a practical pathway to nullify lipid accumulation as insulin sensitivity allows for greater efficiency of carbohydrate uptake and less lipid production. However, TZDs have been implicated in weight gain as a common side effect, significantly increasing subcutaneous fat compared to visceral fat due to a higher concentration of PPARγ receptors in this tissue [142].

PPARβ/δ is the least studied isoform of the three. This isoform may play a role similar to PPARα's protective nature as it has been shown to work with AMP-activated protein kinase (AMPK) [143, 144]. AMPK is a master regulator of energy metabolism and homeostasis at the cellular and full-body levels by controlling food intake [145, 146]. PPARβ/δ activation via exercise with AMPK synergy has been shown to increase β-oxidation in skeletal muscle cells [147]. However, with PPARs generally associated with anti-inflammatory properties, PPARβ/δ's role is complicated by conflicting reports [148]. One study has found that PPARβ/δ knockouts could not access the anti-inflammatory properties of exercise on vascular inflammation [149]. Counter to this, patients with psoriasis, an autoimmune condition of the skin, had higher PPARβ/δ, and activation of these receptors sustained inflammation [150]. Further studies will be required to understand a possible selective transcription and modulation of PPARβ/δ.

17.10 Conclusion

The recent discovery that bilirubin is a hormone that interacts with PPARα potentially explains the bilirubin-mediated improvement of several metabolic diseases, including obesity and diabetes. Potential mechanisms of how bilirubin is reduced during metabolic disease have implications for improving therapeutics. The bilirubin-PPARα axis appears to be essential in regulating peroxisomes and mitochondria that control fat-burning mechanisms to improve adiposity. Mounting experimental evidence has demonstrated a vital role for peroxisomes in protecting hepatic lipid accumulation and inflammation. Future studies are needed to precisely

determine if bilirubin acting through PPARα can directly stimulate peroxisomal fatty acid metabolism as well as peroxisomal proliferation. Further investigation into the role of PPARα in peroxisomes is needed to completely understand how peroxisomes contribute to the regulation of hepatic function so that novel therapies could be developed in the future to treat conditions such as NAFLD.

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Chapter 18
Nuclear Receptor Ligands in Flavivirus Infection Control

Salma El Adl and Alaa Badawi

Abstract Flavivirus infectious diseases, such as dengue fever, chikungunya, and West Nile and Zika virus, represent a public health concern worldwide. The role of nuclear receptor ligands in the prevention and control of the severity of such vector-borne diseases is yet to be fully elucidated. This chapter provides systematically reviewed information on the role of nuclear receptor ligands in the control of flavivirus infections. Furthermore, we aim to assess the effect of modulating the function of these receptors in influencing disease severity. We conducted a comprehensive search in PubMed, Ovid Medline, Embase, and Embase Classic to extract human studies discussing the role of nuclear receptors and their ligands in flavivirus infections. Seventeen studies were extracted and included in this systematic review. Human studies pertaining to peroxisome proliferator-activated receptors (PPARs) suggest that antagonizing these receptors increases the susceptibility to viral infection. On the other hand, liver X receptors (LXRs) that are involved in cholesterol homeostasis may play a role in flavivirus infection control. LXR agonists have shown to inhibit viral replication via activating cholesterol export from infected cells. Studies on vitamin D receptor (VDR) ligands suggest that VDR polymorphism is associated with infection severity, e.g., in dengue fever. Vitamin D supplementation was shown to inhibit viral replication. One study focused on the reverse strand of erythroblastic leukemia viral oncogene homologue (REV-ERB) receptor and concluded that receptor agonists can markedly reduce both viral load and replication. In conclusion, modulating nuclear receptors can play a role in the control and prevention of flavivirus infectious diseases by either influencing the susceptibility to viral infection, inhibiting its replication, or affecting the disease severity.

S. El Adl
Human Biology Program, Faculty of Arts and Science, University of Toronto, Toronto, ON, Canada

A. Badawi (✉)
Public Health Risk Sciences Division, Public Health Agency of Canada, Toronto, ON, Canada

Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto, ON, Canada
e-mail: alaa.badawi@canada.ca

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18.1 Introduction

Flaviviridae is a group of enveloped, positive sense single-strand RNA viruses, consisting of four different genera: *Flavivirus, Pegivirus, Pestivirus, and Hepacivirus* [1]. Genus *Flavivirus* in particular consists of more than 70 species that include the West Nile virus (WNV), dengue virus (DENV), tick-borne encephalitis virus (TBE), yellow fever virus (YV), Zika virus (ZIKV), and several other viruses, which may cause encephalitis, as well as insect-specific flaviviruses (ISFs), such as cell-fusing agent virus (CFAV), Palm Creek virus (PCV), and Parramatta River virus (PaRV) [1, 2]. Flaviviruses are transmitted to humans through an arthropod vector or a mosquito vector from animal reservoirs [2]. Human to human transmission can also occur through transfusion of infected blood or infected tissue transplants [3]. Climate change, the population dynamics of the intermediate host (i.e., mosquitos), population growth, and urbanization can all play a role in the reemergence and wide spread of flavivirus infectious diseases, rendering them into a significant public health concern [4, 5].

Flavivirus infections can be presented without any clinical symptoms. Some individuals, however, can develop severe clinical symptoms that can be varied from fever to encephalitis and hemorrhage [6]. Although factors responsible for the manifestation of infection are yet to be fully characterized, there are known interindividual variations that can determine disease [7]. For example, existing chronic conditions, such as cardiovascular disease, diabetes, and renal disorders, can contribute to the increasing severity of flavivirus infections [8–10]. With DENV specifically, the virus undergoes an incubation period of 3–14 days before any clinical symptoms start to manifest [11]. These symptoms range from fever, headache, and nausea to joint pain and typically last for 3–7 days before recovery [12]. However, in some individuals, these symptoms may further progress and develop into vascular leakage and pleural effusion [12]. At that stage, patients develop dengue hemorrhagic fever (DHF) [12]. Dengue shock syndrome (DSS), on the other hand, can develop once hypotension starts to emerge, leading to fatal outcome [12]. Therefore, it is important to monitor patients during this phase in order to prevent the deterioration of the vital organs. Similarly, WNV is mainly asymptomatic; however, the disease may progress in some individuals to neurological conditions, including encephalitis, meningitis, and paralysis [10, 13]. Furthermore, ZIKV also has an incubation period of 3–14 days before clinical symptoms start to arise [14] and manifest to fever, headaches, and edema [15]. More severe outcomes include organ failure, thrombocytopenia, meningitis, Guillain-Barré syndrome, and other neurological disorders [16, 17]. Chikungunya (CHIKV) infection is usually characterized by a range of clinical symptoms that vary from fever and maculopapular rash

[18–20] to neurological and rheumatologic conditions in severe cases that can persist for a long period of time [15, 21–29]. This apparent interindividual variation in response to flavivirus infections is related to a number of host-related factors, including the presence of coexisting medical conditions, the competency of the immune system, host genetics, and the function of a wide array of cell receptors (see below).

Flaviviruses are known to target immune-related cells, including macrophages, dendritic cells, and monocytes, by attaching to their surface and entering the cell through receptor-mediated endocytosis [30–32]. The viral particle fuses with the cell membrane, releasing the viral RNA into the cell. The viral RNA is then able to replicate in the endoplasmic reticulum and is transported into the cytoplasm to infect neighboring cells [33]. Nuclear receptors play a key role in viral infection and consequent inflammatory responses and immune-related homeostasis [34]. These functions are mediated by a number of nuclear receptors, such as liver X receptor (LXR), peroxisome proliferator-activated receptor (PPAR), vitamin D receptor (VDR), and reverse strand of erythroblastic leukemia viral oncogene homologue receptor (REV-ERB). LXR plays a role in pro-inflammatory responses, and thus, LXR receptor agonists can play a role in controlling viral infection [34]. Furthermore, LXR is responsible for the regulation of cholesterol homeostasis through the formation of heterodimers with retinoid X receptor (RXR). This heterodimerization was shown to affect the susceptibility to infection with DENV [35]. PPARs, commonly expressed in macrophages [36–38], can negatively regulate macrophage activation and reduce oxidative stress and, thereby, were shown to be effective in the therapy of viral infection. VDR is another nuclear receptor known to be expressed in macrophages. Genetic variation, i.e., single nucleotide polymorphisms (SNPs), in the VDR gene has been increasingly linked to controlling a number of infectious diseases via mediating the host immunity and affecting disease severity [39–41]. REV-ERB is a member of the Rev-ErbA family of nuclear receptors and is a transcriptional repressor. Agonists of REV-ERB were shown, over the past few years, to be effective in the treatment of a number of bacterial and viral infections [42–46].

This chapter provides a systematic review of published literature to describe the role of a range of nuclear receptor ligands in the control and prevention of flavivirus infectious diseases. This will permit exploring the possible contribution of these factors in facilitating the development of public health measures and actions to curb the increased incidence of flavivirus diseases.

18.2 Methods

18.2.1 Literature Search

The systematic review presented in this chapter was conducted according with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA; see Supplementary Table 1). A search was conducted using PubMed, Ovid Medline,

and Embase databases using the following search terms (MeSH): "*Flaviviridae*," "*Flavivirus*," "Zika," "West Nile virus," "dengue," or "chikungunya," and "LXR," "VDR," "PPAR," or "REV-ERB." The time period of the search was from the inception of the databases to the end of February 2020. Only English-language articles on human subjects were included. Letters to the editor, vaccine trials, case reports, animal studies, conference abstracts, and duplicated studies were excluded. Studies were included when containing data on the role of nuclear receptors and their ligands on the control of flavivirus infections or with information on the association between a specific ligand and its effect on disease progression. The reference lists of the eligible studies were also reviewed for relevant studies for additional inclusion. Seventeen reports were selected to be systematically reviewed in the present chapter (see Table 18.1).

The studies included here were chosen using the population, intervention, comparison, outcome, study design, and time (PICOST; see Supplementary Table 2) table. We included studies examining human subjects (of any age) who were diagnosed with a flavivirus infections (Supplementary Table 3). Infected individuals or cells were compared to uninfected controls for the activity of the nuclear receptor and the effect of its ligands.

18.2.2 Inter-reviewer Agreement

Two reviewers (SEA and AB) independently reviewed the abstracts yielded from the search to determine those eligible for full-article review and inclusion. Disagreements regarding study inclusion were resolved by discussion. Percentage agreement and Cohen's Kappa (κ) statistic and 95% confidence interval (95% CI) were calculated as previously described [7]. Agreement between reviewers was based on the Landis and Koch's kappa (κ) statistic divisions as poor (<0), slight (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), and perfect (0.81–1.0) [47]. The agreement for inclusion between the two reviewers was 94.7% with a substantial weighted κ of 0.65 (95% CI: 0.36–0.94).

18.2.3 Data Extraction

The data included from the selected studies included the author's name, year of publication, nuclear receptor, ligand, model, objective, and the overall findings related to the role of the nuclear receptor ligands in the control of flavivirus infection control. Upon completion of data extraction, the studies were grouped into those reporting a particular nuclear receptor (see below).

Table 18.1 Characteristics of the selected studies

Study ID [Ref.]	Year	Nuclear receptor	Model	Overall objective
Hwang et al. [60]	2019	LXR	Human foreskin fibroblasts (HFFs)	To determine whether LXR-623 (LXR agonist) is capable of controlling CHIKV infection in human fibroblasts
Sierra et al. [35]	2017	LXR	Human subjects	To conduct a genome-wide association studies (GWAS) in South American and African populations to determine which gene influences individuals' risk to DENV
Audouze et al. [57]	2018	PPAR	Human subjects	To determine the pathways related to microcephaly related to ZIKV infection
Fusco et al. [58]	2017	PPAR	Human HeLa cells	To identify the human gene(s) that suppresses DENV through interferon effector genes (IEGs)
Devignot et al. [59]	2010	PPAR	Human blood cells	To conduct a GWAS for 48 affected Cambodian children to characterize genes influencing *dengue* shock syndrome (DSS)
Zhuang et al. [61]	2019	REV-ERB	Huh-7 cells (human liver cell line)	To investigate the role of REV-ERB in the replication of DENV and ZIKV
Pereira et al. [4]	2017	VDR	Human blood cells	To analyze the genotypic and allelic frequencies of polymorphisms (SNPs) related to DENV
Laplana et al. [48]	2018	VDR	Human subjects	To conduct a systematic review to examine the role of VDR polymorphisms on infection susceptibility to enveloped viruses, specifically DENV
Giraldo et al. [54]	2017	VDR	Human peripheral blood cells	To determine whether vitamin D supplementation has an effect on DENV susceptibility and pro-inflammatory cytokine production in macrophages
Dettogni et al. [50]	2015	VDR	Human blood cells	To evaluate the relationship between SNPs in VDR genes with symptom persistence in DENV
Harapan et al. [51]	2012	VDR	Human subjects	To evaluate non-HLA gene polymorphisms and their relationship with the susceptibility and severity of DENV infection
Alagarasu et al. [49]	2012	VDR	Human blood cells	To investigate whether 3'UTR gene variants are associated with DENV in infected patients from India
Loke et al. [52]	2002	VDR	Human blood cells	To assess candidate genes related to DENV susceptibility and disease severity
Coffey et al. [53]	2009	VDR	Human subjects	To evaluate genetic variants related to DENV susceptibility and factors affecting DENV infection outcome

(continued)

Table 18.1 (continued)

Study ID [Ref.]	Year	Nuclear receptor	Model	Overall objective
Puerta-Guardo et al. [3]	2012	VDR	Human Huh-7 and U937 cells	To evaluate the effect of vitamin D in DENV therapy
Beard et al. [55]	2011	VDR	Human subjects	To investigate the antiviral effect of vitamin D against enveloped viruses
Alagarasu et al. [56]	2012	VDR	Human blood cells	To investigate the effect of vitamin D levels on DENV infection control

18.3 Results and Discussion

18.3.1 Search Results

From the initial database search, 32 articles met the search criteria. After removing ten duplicate reports, the 22 remaining studies were screened through abstract review. Of the latter, nine studies were excluded during the abstract review process as shown in Fig. 18.1 (one conference abstract, six review studies, and two primary studies). Full-text screening was conducted on the remaining 13 studies that were selected for inclusion in addition to four studies that were identified through bibliography search (Table 18.1).

18.3.2 Vitamin D Receptor (VDR)

Of the selected studies, 11 reports examined the role of VDR ligands on the susceptibility and control of DENV infections (Table 18.2). These studies evaluated factors which varied from the effect of the VDR SNPs on the ligand binding to subsequently the disease outcome, the effect of the ligands in immune cells, and the role of the ligand supplementation on disease risk. For example, significant differences in susceptibility to DENV infections were noted to be associated with SNPs in the VDR gene [4]. FokI polymorphisms of VDR in African, European, Asian, and Central and South American populations was linked to varying risk of susceptibility to bacterial and viral infections [48], particularly enveloped viruses such as DENV. The FokI polymorphism seems to alter the transcriptional activity and subsequently the ligand (vitamin D) binding to VDR, influencing the effect of the ligand on the susceptibility to viral infection. Furthermore, studies focused on Taq1 polymorphisms in VDR were primarily linked to the extent of DENV severity [49, 50]. TaqI C allele, as compared to the T allele, was shown to offer more protection against the severe outcome of DENV infection [49, 50]. This observation was further confirmed in cases of sever DHF [51, 52], where VDR polymorphisms were shown to regulate the activity of monocytes, B cells, and T cells at the sites of infection [51, 52]. This was suggested to be due to the role of VDR in controlling the

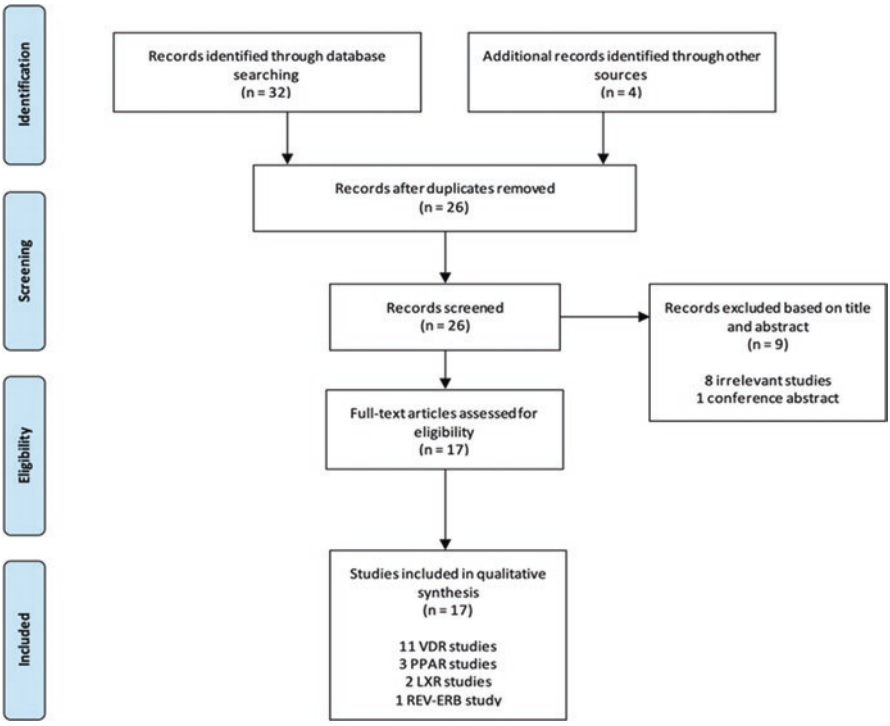

Fig. 18.1 PRISMA flowchart

downstream immune response by activating monocytes and inhibiting lymphocyte proliferation [53].

Apparently, the level of supplementation with VDR ligand seems to also affect the susceptibility to DENV infections [54]. Vitamin D was shown to mediate the expression of LL-37, an antimicrobial peptide, which then inhibits the viral replication at the cell entry phase. Lower levels of vitamin D were shown to be associated with decreased LL-37 expression and increased susceptibility to infection [54]. In this respect, individuals who receive vitamin D supplementation at 4000 IU/day were found to be more resistant to DENV infections and have lower levels of pro-inflammatory cytokines [54]. In support, when myelomonocytic cells (U937) and human hepatocytes (Huh-7) were infected with DENV and subsequently treated with vitamin D, both cell lines exhibited inhibition of DENV replication [3]. This substantiates the inhibitory effort of vitamin D in viral infection and proliferation. Indeed, when Vietnamese subjects with DENV infection were orally supplemented with vitamin D, they exhibited lower disease severity and shorter length of DENV symptoms [55].

In contrast to these findings, one study demonstrated that vitamin D serum levels are higher in patients diagnosed with DENV compared to healthy individuals [56]. This discrepancy was however proposed to be either due to the increased expression of Fcγ receptors on monocytes from the DENV patients, which may increase the

Table 18.2 Role of vitamin D receptor (VDR) and VDR ligands (vitamin D) in dengue fever

Study ID	Study population and design	Findings
Pereira et al. [4]	1560 individuals from Brazil	The genotypic frequencies for all VDR SNPs did not differ between the asymptomatic DENV-positive cases and DENV-negative cases
Laplana et al. [48]	Systematic review and meta-analysis on VDR FokI T-allele frequency on DENV infection	FokI polymorphism is associated with an increased susceptibility to DENV infection
Giraldo et al. [54]	Macrophages from 20 healthy individuals randomly divided into two groups	Healthy donors received high doses of vitamin D. Macrophages were more resistant to DENV, leading to a decreased number of viral RNAs and DENV-infected macrophages Donors who received low doses of vitamin D had a significant increase in the number of DENV-infected macrophages and viral RNA copies Vitamin D supplementation inhibits DENV infection and viral replication in a dose-dependent manner Vitamin D binding to VDR increases the expression of LL-37 that inhibits DENV replication at the level of viral entry into the cell
Dettogni et al. [50]	315 adult patients from emergency care units in Brazil	VDR influences the immunoregulatory function of vitamin D (increased differentiation, functions of T cells and monocytes, and suppression of IgG and B cells) VDR Taq1 T allele increases the susceptibility to DENV VDR Taq1 C allele is associated with protection against severe DENV
Harapan et al. [51]	Review findings from countries in Southeast Asia and South America	There is an association between VDR polymorphism and DENV severity VDR agonists activate B- and T-lymphocyte function
Alagarasu et al. [49]	DENV patients (n = 112) and controls (n = 106) from India	VDR polymorphism affects the ligand's bindings and the outcome of DENV infection
Loke et al. [52]	DENV patients (n = 315 grade III and 37 grade IV) and healthy controls (n = 251) from Vietnam	The less frequent T allele of a dimorphism at position 352 of the VDR gene is associated with DENV severity and DHF
Coffey et al. [53]	Literature survey	Clinical presentation of DENV is affected by SNPs in VDR
Puerta-Guardo et al. [3]	The effect of vitamin D on DENV infection in human hepatic cells and on viral infection	VDR ligand reduced the number of Huh-7 and U937 cells infected with DENV in a dose-dependent manner
Beard et al. [55]	Literature survey	The antiviral effect of VDR activation may be linked to the ability of vitamin D to upregulate the antimicrobial peptides LL-37 and human beta-defensin-2
Alagarasu et al. [56]	Case-control study (48 DF cases, 45 DHF cases, and 20 healthy controls) from India	Higher concentrations of vitamin D might be associated with secondary DHF

viral load, or due to the elevated levels of the anti-inflammatory interleukin (IL)-10, which potentiates the DENV pathogenesis [56]. Overall, the effect of VDR ligands on flavivirus infection, e.g., DENV, is suggested to be regulated by the polymorphisms in the VDR gene to ultimately influence the cell's ability to control the viral entry and replication and eventually the individual's overall response to disease manifestation, i.e., symptom relief and severity of infection.

18.3.3 Peroxisome Proliferator-Activated Receptors (PPARs)

Only three studies were identified evaluating the effect of PPAR ligands on the control of flavivirus infectious diseases such as DENV and ZIKV (Table 18.3). *Exposure to pyriproxyfen (PPF) during ZIKV infection was suggested to activate and* differentially modulate *PPARs to subsequently lead to elevated levels of* maternal inflammation [57]. The latter was found to be associated with the increased risk of congenital disorders in the infants from ZIKV-infected mothers [57]. In support, the PPAR signaling pathway is known to be expressed in placental development and is involved in brain functions and neurodegenerative diseases. Differential modulation of this pathway may, therefore, play a role in fetal neurotoxicity and microcephaly during ZIKV infection.

Infection with DENV downregulates host production of the antivirus cytokine interferon (IFN)-α at early steps of this signal transduction pathway. This downregulation allows uncontrolled viral replication, which subsequently triggers the synthesis of IFN-α which, again, is downregulated [58]. IFN-α stimulates HELZ2 (helicase with zinc finger 2) gene. The protein encoded by HELZ2 gene is a transcriptional coactivator for PPARα. In an effort to determine which nuclear receptor is the mediator of HELZ2-IFN-α antiviral effects during DENV infection, Fusco et al. [58] knocked down PPARα in Huh-7.5.1 hepatoma cells and in HeLa cells to

Table 18.3 Role of peroxisome proliferated-activated receptors (PPAR) in flavivirus diseases

Study ID	Ligand	Infection	Findings
Audouze et al. [57]	Pyriproxyfen	ZIKV	*Exposure to pyriproxyfen during Zika infection modulates PPARs* Modulation of PPAR signaling may play a role in fetal neurotoxicity and microcephaly
Fusco et al. [58]	Fenofibrate	DENV	PPARα knockdown rescued DENV from the effect of IFN in Huh-7.5.1 cells Treatment with fenofibrate upregulated suppressed DENV infection in HeLa cells but with cytotoxic effect
Devignot et al. [59]	Atherosclerosis drugs (proposed)	DENV	PPARα and PPARγ genes and their transcripts to be present in decreased abundance, particularly, in DSS patients Activation of PPARγ is proposed in the systemic vascular dysfunction leading to DSS

evaluate the effect of this PPARα downregulation on DENV infection. PPARα knockdown did rescue DENV from the effect of IFN-α in Huh-7.5.1 cells. In support, treatment with the PPARα ligand fenofibrate upregulated the mRNA expression of PPARα in the Huh-7.5.1 cells and suppressed DENV infection in HeLa cells but with cytotoxic effect. Although these findings did not support a clear role for PPARα as the major nuclear receptor in the HELZ2-IFN-α interaction, it provides evidence for the role of PPARα ligands in the protection against DENV infection outcome.

In a study aiming to provide an overview of the molecular mechanisms altered during dengue shock syndrome (DSS) and how they may interact to lead to final vascular homeostasis breakdown, Devignot et al. [59] analyzed the genome-wide expression profiles of whole blood cells from 48 matched Cambodian children: 19 progressed to DSS, while 16 and 13 presented, respectively, classical dengue fever (DF) or dengue hemorrhagic fever grades I/II (DHF). The study identified PPARα and PPARγ genes and their transcripts to be present in decreased abundance, particularly, in DSS patients. However, a particular function for the activation of PPARγ nuclear lipid receptor was proposed in the systemic vascular dysfunction leading to DSS. The authors proposed that drugs used to treat metabolic disorders, such as atherosclerosis (i.e., PPAR agonists), should deserve further attention for their role in controlling the pro-inflammatory processes in DENV-infected patients. Taken together, since PPAR expression was reduced in DENV infection [59] and upregulating this expression was noted in human cells to suppress the infection in human cells [58], it can be argued that PPAR ligands may play a critical role in the control and treatments of DENV infection. However, further studies are warranted to substantiate this effect in human population and to employ these molecular targets in curbing the increased incidence of DENV and other flavivirus-related diseases.

18.3.4 Liver X Receptor (LXR)

LXR is a member of the nuclear receptor family of transcription factors, is closely related to nuclear receptors such as the PPARs, and is an important regulator of cholesterol, fatty acid, and glucose homeostasis. Two human studies were focusing on the role of LXR ligands in the control of flavivirus infections with CHIKV and DENV (Table 18.4). The findings from the two studies suggest that downregulating LXRs, either by agonists or modulating their downstream signaling, can protect against the viral cell entry as well as its replication, thereby providing a protection against the flavivirus infectious diseases.

In a recent study examining whether the LXR agonist LXR-623 is capable of modulating CHIKV infection, Hwang et al. [60] have concluded that LXR is a host factor that plays a critical role in reducing CHIKV replication, e.g., in physiologically relevant human fibroblasts. This study also demonstrated that endogenous and synthetic activation of INF-stimulated genes synergizes with LXR to protect the host cells against the viral effect. However, LXR was found to also possess anti-inflammatory effects that may be relevant during CHIKV infection. In this regard,

Table 18.4 Role of liver X receptors (LXRs) in flavivirus diseases

Study ID	Ligand	Infection	Findings
Hwang et al. [60]	LXR-623	CHIKV	LXR agonist (LXR-623) inhibits CHIKV replication by upregulating the cholesterol exporter ABCA1 Activation of IFN signaling pathway partners with LXR antagonism to have an antiviral effect
Sierra et al. [35]	OSBPL10 knockdown	DENV	OSBPL10 interacts with the LXR/RXR activation pathway that integrates lipid metabolism and immune functions OSBPL10 is a key player in DENV entry into cells and its replication Lower OSBPL10 expression profile plays a role against DENV disease Knockdown of *OSBPL10* expression led to a significant reduction in DENV replication

inflammatory monocytes, known to be present in joints during the chronic phase of CHIKV pathogenesis, were found to be also modulated by LXR agonism. The study also presented evidence to suggest that LXR agonist induces the expression of ATP-binding cassette transporter 1 (ABCA1), a cholesterol efflux regulatory protein (CERP), a major regulator of cellular cholesterol and phospholipid homeostasis. Upregulating ABCA1 was the primary pathway by which LXR-623 inhibits CHIKV replication in primary human fibroblasts. The study concluded that endogenous and pharmacological activation of inflammatory signaling pathway (particularly the innate immunity related) will synergistically interact with LXR agonism to generate antiviral effect.

The oxysterol-binding protein-related protein 10 (*OSBPL10*) is known to interact with the LXR/RXR activation pathway that integrates lipid metabolism and immune functions [35]. OSBPL10 was shown to be a key player in DENV entry into cells and its replication. Knockdown of *OSBPL10* expression in THP-1 cells followed by DENV2 infection led to a significant reduction in DENV replication, being a direct functional proof that lower OSBPL10 expression profile plays a role in the control of DENV disease. Given the interaction between LXR signaling and OSBPL10, the findings of this study provide evidence demonstrating that LXR pathway has a central role in DENV protection and support pursuing therapeutic techniques involving synthetic ligands of LXR, particularly those that interact with proteins involved in lipid metabolism.

18.3.5 Reverse Strand of Erythroblastic Leukemia Viral Oncogene Homologue Receptor (REV-ERB)

The cell-autonomous circadian clock directs a network of physiological processes that delineate the daily pace of cell proliferation, metabolism, and inflammation. The nuclear hormone receptors REV-ERB are regulators of this clock components

and control the expression of metabolic genes in a circadian- and tissue-dependent manner [61]. Our search resulted in a single study examining the effect of modulating REV-ERB on flavivirus diseases [61]. This study demonstrated that overexpression or activation of REV-ERB with synthetic agonists inhibits the replication of flaviviruses DENV and ZIKV via perturbation of lipid signaling pathways. The study highlighted a role of REV-ERB in restricting RNA virus replication. When treating human liver cell lines (Huh-7) with the REV-ERB agonist SR9009, the ZIKV and DENV infections were significantly reduced. This pathway involves inhibiting Bmal1, which, in turn, reduces viral replication in a dose-dependent manner with a minimal (if any) cytotoxic effects. Overall, that study characterized new pathways for the circadian network to impact multiple stages of the DENV and ZIKV replication processes, particularly when the viruses rely on host metabolic activities to replicate.

18.4 Conclusion

The introduction of the flavivirus infection competent vectors (i.e., mosquitoes) into natural environments and urban areas and the changing societal factors (see below) all have facilitated the geographical expansion of these diseases into different regions of the world [7]. Such incursion of the disease vector into new world regions was proposed as a major factor influencing the increased incidence of flavivirus diseases and their severity [62, 63]. Indeed, the decreases in mosquito control efforts, rapid changes in climate and vector's demography, dense urbanization, population growth, and globalization with increased transportation and trade activities all have contributed to the elevated global burden on flavivirus diseases [64]. Additionally, identifying and characterizing host-related factors that play a role in the severity of flavivirus infections and defining approaches to modulate the effect of such factors can be significant in designing measures that aim to prevent the severe outcomes of infection.

Nuclear receptors have been identified to play a key role in flavivirus infection and diseases by influencing the viral entry into the host's cell [54], viral replication [3, 61], and the individual's response to infection [3, 56]. Modulation of nuclear receptors either due to gene polymorphism [49, 50] or by treatment with the corresponding ligands [54] was shown to offer protection against the severe outcome of flavivirus infection, e.g., with VDR [49, 50]. *Furthermore, PPAR ligands were suggested to activate and* differentially modulate *the receptor-mediated downstream signaling pathways to subsequently lead to elevated levels of* inflammation and suppression of flavivirus infection, e.g., DENV [57]. Additionally, activation of REV-ERB inhibited the replication of flaviviruses DENV and ZIKV via perturbation of lipid signaling pathways [61]. On the other hand, downregulating LXRs, either by agonists or modulating their downstream signaling, were suggested to protect against the viral cell entry as well as its replication to provide a protection against diseases such as DENV and CHIKV [35, 60]. Overall, it appears that the effect of nuclear receptor ligands on the control of flavivirus infection is receptor specific,

where ligands (e.g., of VDR, PPAR, and REV-ERB) or agonists (e.g., of LXR) can have significant protection against viral entry, replication, and disease outcome.

Infection with flaviviruses downregulates the host production of a number of innate immunity-related antivirus cytokines [30–32]. Modulation of nuclear receptors can similarly affect the inflammatory responses and immune-related homeostasis [34]. Agonizing (VDR, PPAR, and REV-ERB) or antagonizing (LXR) these receptors can, therefore, be employed in rectifying the inflammatory signaling pathways disrupted by viral infection. In this respect, overproduction of pro-inflammatory cytokines, such as ILs, TNF-α, IFN-γ, and TGF-β, is known to occur in severe DENV [65], WNV [66], and yellow fever [67], leading to cytokine storm and vasculopathy, hemorrhage, tissue damage, and septic shock characteristic of severe flavivirus infections. Cytokine synthesis shift to Th1 (microbicidal action of pro-inflammatory IFN-γ) from Th2 (anti-inflammatory IL-4, IL-10, and IL-13) in severe infection can lead to endothelial dysfunction and a subsequent range of complications, including allergy, vascular leakage, ascites, and pericardial effusion, as observed in DENV [68–70]. Such an impairment of the immune system increases the level and duration of viremia and facilitates the passage of, for example, neurotropic flavivirus across the blood-brain barrier to predispose patients to severe disease outcome, e.g., neurological complications [71, 72]. Impairment of the innate immune system, which mediates the host defense to infection, renders individuals more susceptible to a range of infectious diseases and severe illnesses [73]. This may subsequently mediate anti-inflammatory responses and generate a pro-inflammatory state, to exacerbate the infectious disorders [73]. Given the effect of nuclear receptors (e.g., VDR, LXR, PPAR, and REV-ERB) on inflammation and its related processes (see above), evaluating the modulatory effects of the receptor ligands on flavivirus infection can be essential to controlling the infection-induced pro-inflammation. This can consequently play a role in reducing the burden of the disease via guiding approaches for improved patient outcome or differential case management.

This chapter provided evidence for a possible role of nuclear receptor ligands in flavivirus infection control. The findings presented here, however, are still fragmentary and do not implicate causality between ligand treatment and infection control. It simply demonstrates the possibility that this approach can be further investigated in disease therapy or intervention. Indeed, the data presented in this chapter simply warrants further assessments to identify the nature and extent of effect that can be attained by introducing nuclear receptor ligands into flavivirus infection control. However, even in the absence of causal inference between ligand treatment and disease outcome, it may be justified that ligand treatment may retain the innate immunity homeostasis disrupted during early stages of infection.

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Author Contribution SEA screened and selected the studies and wrote the first draft of the paper. AB conceived the study idea and design, assisted in the data interpretation, and wrote the final manuscript. Both authors critically reviewed the manuscript, approved the final draft, and agreed to be accountable for all aspects of the work.

Supplementary Materials

Supplementary Table 1 PRISMA checklist

Section/topic	#	Checklist item	Page #
Title			
Title	1	Identify the report as a systematic review, meta-analysis, or both	1
Abstract			
Structured summary	2	Provide a structured summary including the following, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number	1
Introduction			
Rationale	3	Describe the rationale for the review in the context of what is already known	1–3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS)	Supplementary Table 2
Methods			
Protocol and registration	5	Indicate if a review protocol exists and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number	None
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale	3
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched	Table 1.1
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated	3
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis)	Figure 1.1
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators	4
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made	Supplementary Tables
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level) and how this information is to be used in data synthesis	None

(continued)

Supplementary Table 1 (continued)

Section/topic	#	Checklist item	Page #
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means)	Table 1.1
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis	None
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies)	None
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analysis, meta-regression), if done, indicating which were prespecified	None
Results			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusion at each stage, ideally with a flow diagram	Figure 1.1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations	Table 1.1, Figure 1.2
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see Item 12)	None
Results of individual studies	20	For all outcomes considered (benefits or harms), present the following for each study: (a) simple summary data for each intervention group and (b) effect estimates and confidence intervals, ideally with forest plot	Tables 1.2–1.5
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency	None
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15)	None
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16])	None
Discussion			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., health-care providers, users, and policy makers)	16
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias) and at review level (e.g., incomplete retrieval of identified research, reporting bias)	16
Conclusions	26	Provide a general interpretation of the results in the context of other evidence and implications for future research	16
Funding			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data) and the role of funders for the systematic review	16

Supplementary Table 2 PICOST table

Component	Criteria
Population	Human individuals of any age with a flavivirus infection (Zika virus, dengue virus, chikungunya virus, West Nile virus, yellow fever) or human viral infected cell lines
Intervention	No intervention criteria
Comparison	Compared flavivirus-infected individuals to healthy controls via nuclear receptor activity
Outcome	Association of specific ligands with flavivirus infection progression
Study design	English-language studies Human subjects or human cells
Time	No time restriction

Supplementary Table 3 Articles obtained for each search term

Search terms	Number of articles generated	Number of articles included
"*Flaviviridae,*" "*Flavivirus,*" "Zika," "West Nile virus," "dengue," or "chikungunya" and "LXR"	3	2
"*Flaviviridae,*" "*Flavivirus,*" "Zika," "West Nile virus," "dengue," or "chikungunya" and "peroxisome proliferator-activated receptor"	9	3
"*Flaviviridae,*" "*Flavivirus,*" "Zika," "West Nile virus," "dengue," or "chikungunya" and "REV-ERB"	1	1
"*Flaviviridae,*" "*Flavivirus,*" "Zika," "West Nile virus," "dengue," or "chikungunya" and "VDR"	9	7

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Chapter 19
Use of Nanotechnology to Improve 15d-PGJ$_2$ Immunomodulatory Activities

Marcelo H. Napimoga, Henrique B. Abdalla, and Juliana T. Clemente-Napimoga

Abstract A growing body of evidence demonstrates that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$) which is an endogenous ligand of peroxisome proliferator-activated receptor gamma (PPAR-γ) has multiple physiological properties. It has been demonstrated the efficacy at low doses even lower when combined with nanotechnology, as a promising therapeutic approach as immunoresolvents and some of them present long-lasting anti-inflammatory effects. In this chapter, we focus on how 15d-PGJ$_2$ is involved in the resolution of inflammatory responses and as potential analgesic molecule. Importantly, we will present evidences that nanotechnology (nanocarriers) is a helpful tool to improve its action and bioavailability.

Keywords 15d-PGJ2 · Inflammation · Nanotechnology · PPAR-gamma · PPAR · Prostaglandins

19.1 Introduction

Nuclear receptors are ligand-dependent transcription factors that regulate several genes related to the control of cell growth, cell differentiation, and homeostasis [67, 68]. In particular, peroxisome proliferator-activated receptors (PPARs) are a type of nuclear receptor, which regulates the transcriptional response [82]. When inactivated, PPARs are constitutively associated with the 9-cis retinoic acid (RXR) receptor as a heterodimer [50]. However, upon activated by natural or synthetic ligands, a conformational change occurs in PPARs structure, allowing PPAR complex binds

M. H. Napimoga (✉) · H. B. Abdalla · J. T. Clemente-Napimoga
Faculdade São Leopoldo Mandic, Instituto São Leopoldo Mandic,
Laboratory of Neuro-Immune Interface of Pain Research, Campinas, SP, Brazil
e-mail: marcelo.napimoga@slmandic.edu.br; juliana.napimoga@slmandic.edu.br

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to the peroxisome proliferator response element (PPRE) in the promoter regions of target genes [50]. Subsequently, the recruitment of transcriptional coactivators leads to an increase of transcriptional responses [77].

The PPAR-gamma (PPAR-γ) was the first transcription factor identified in 1990 [34]. Since then, surveys on PPARs have increased dramatically, revealing more and more intriguing functions in human biology. Three different isoforms of PPARs exist—PPAR-α, PPAR-β/δ, and PPAR-γ, which demonstrate diverse functional capabilities, including, but not restricted to, the control of inflammatory gene expression, lipogenesis, and cell cycle [28]. Nuclear receptors can bind small lyophilic ligands that induce their transcriptional activities. However, the identity of their endogenous ligands remains largely elusive. Despite that, due to several studies focused on identifying PPAR-γ ligands, several candidates were found, including unsaturated fatty acids and arachidonic acid metabolites [37, 52, 76].

Prostaglandins (PGs) are a family of biologically active lipid compounds derived from arachidonic acid with a broad spectrum of functions in our body. PGs have a diverse range of actions depending on the PG type and cell target; however, most of their activity is specially related to inflammation outcomes. The A and J PGs series contain a cyclopentenone ring structure, which is characterized by a chemically reactive α,β-unsaturated carbonyl [74], in which includes 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). 15d-PGJ_2 is one of the final products of the arachidonic acid cascade. This cascade is a fundamental metabolic pathway in the human body that synthesizes a wide range of chemical mediators. Its synthesis begins by several stimuli (whether physiological, pharmacological, or even pathological), resulting in the activation of A_2 phospholipase (PLA_2) enzyme. Therefore, PLA_2 hydrolyzes the phospholipid membrane, releasing the arachidonic acid as a substrate for different metabolic pathways. The two main enzymatic pathways are cyclooxygenase (COX) and lipoxygenase (LOX). Particularly, the arachidonic acid is converted into prostaglandin G_2, through COX-pathway, and subsequently into prostaglandin D_2, which, through a dehydration process, becomes 15d-PGJ_2 [76]. The 15d-PGJ_2 is a cyclopentenone prostaglandin that is formed by dehydration and isomerization of PGJ_2 (a metabolite product of PGD_2), that unlike the other PGs exerts antitumor, anti-inflammatory, analgesic, and anti-fibrotic effects in a vast range of cellular systems [42, 49, 75]. Although 15d-PGJ_2 is a putative ligand for PPAR-γ, the production of 15d-PGJ_2 in a pathophysiological setting for biological effects in mammalian systems was a doubt [8]. To clarify this issue, it was demonstrated that 15d-PGJ_2, synthesized in vivo, controls the balance of cytokines and chemokines that regulate leukocyte trafficking during acute inflammation as well as the efflux of macrophage to draining lymphatics leading to its resolution [66]. Therefore, this chapter will address the findings regarding the immunomodulatory effects of 15d-PGJ_2, in a range of pathological conditions.

19.2 Analgesic Properties

PGs, in general, induce pain through an indirect mechanism of tough sensitization of primary afferent nociceptors by activating prostaglandin ($EP_{1–4}$) and prostacyclin (IP) receptors [78, 85]. In contrast, cyclopentenone prostaglandins, including 15d-PGJ_2, lead their biologicals actions not mediated by classical EP receptors but rather over interacting with other targets, especially PPAR-γ receptors [76]. Napimoga and colleagues [58] were the first to describe the antinociceptive properties of 15d-PGJ_2. It was demonstrated that 15d-PGJ_2 induces peripheral antinociceptive effect, and interestingly direct administration of 15d-PGJ_2 into the dorsal root ganglion (DRG) was ineffective in blocking PGE_2-induced hypernociception. These findings support the concept that 15d-PGJ_2 cannot directly block nociceptor sensitization but rather prevent the nociceptor sensitization. However, and intriguingly, the blockage of opioids receptors abrogated the analgesic effect induced by 15d-PGJ_2. Considering that intraganglionar injection of 15d-PGJ_2 is ineffective to block pain, it was demonstrated that endogenous opioids release was involved in this effect released by macrophages [58]. In addition, it was demonstrated that the antinociceptive effect of intra-articular injection of 15d-PGJ_2 is mediated by activation of PPAR-γ with κ- and δ- opioid receptors in primary sensory neurons, that stimulate the intracellular activation of L-arginine/NO/cGMP/PKG/K^{ATP} channel antinociceptive pathway resulting in membrane hyperpolarization [45, 61]. Interestingly, PPAR-γ activated by 15d-PGJ_2 are located in leukocytes subpopulations of opioid-peptide-containing cells, such as macrophages cells [45]. The mechanisms of 15d-PGJ_2-induced antinociception is summarized in Fig. 19.1.

Considering these evidences, researchers focused on the resident macrophage cells as a target of therapeutic effect of 15d-PGJ_2 in the peripheral tissues. Macrophages highly express PPAR-γ receptor [12] and could be the source for the endogenous opioids released [46, 54]. As imagined, 15d-PGJ_2 augmented the release of endogenous opioid peptide β-endorphin and dynorphin, the ligands for κ- and δ- opioid receptors, respectively [45]. Further it has been raised the hypothesis that, after PPAR-γ activation by 15d-PGJ_2, macrophages drove to a phenotypic shift from M1-like (macrophages that increases pro-nociceptive mediators) toward M2-like (macrophages that have homeostatic and resolutive functions), inducing transcriptional response and augmenting the genes related to endogenous opioids [31, 32, 45]. Although it is already known that PPAR-γ activation by synthetics [31, 32, 81] or natural ligands [62, 71] induce macrophage phenotypic shift, it remains unknown whether macrophage polarization induced by 15d-PGJ_2 is necessary for the analgesic effect induced. In this scenario recently, it was demonstrated that 15d-PGJ_2 counteracts human soluble epoxide hydrolase enzyme (sEH), which is responsible for block bioactive epoxy fatty acids [4]. Resolvins and maresins are also important epoxy fatty acids with a crucial role in macrophage's resolutions actions, such as analgesia [22, 26, 63]. Besides the PPAR-γ axis, 15d-PGJ_2 showed to regulate bioactive lipids compounds, leading to analgesic effects.

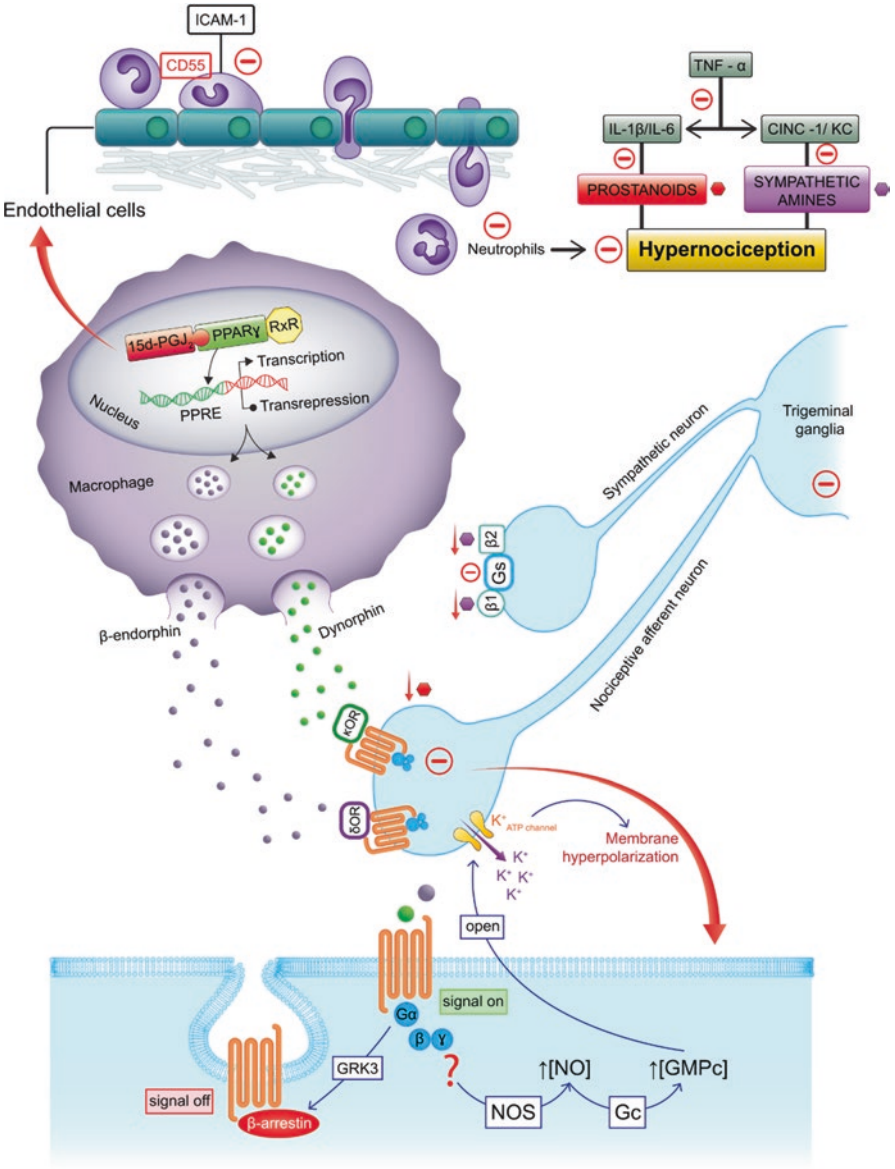

Fig. 19.1 Mechanisms of 15d-PGJ_2-induced anti-nociception in the TMJ. The 15d-PGJ_2 activates PPAR-γ expressed in the resident macrophage of TMJ tissues. Once activated by 15d-PGJ_2, PPAR-γ forms a heterodimer with the retinoid acid receptor (RXR) that in turn allows the release of endogenous opioid peptides activating κ- and δ-opioid receptors (KOR and DOR) in primary sensory neurons. Opioid receptors activated by their ligands induced conformational changes allowing intracellular coupling of heterotrimeric Gi-protein. The subunits of G-protein (β/γ) activated the L-arginine/NO/cGMP/K^{+ATP} channel antinociceptive pathway resulting in membrane hyperpolarization. Opioid receptors are desensitized following phosphorylation by G-protein receptor kinase type 3 (GRK3) and subsequent β-arrestin binding. As a result of opioid receptors activation, the membrane hyperpolarization could inhibit signal of inflammatory chemotaxis. Otherwise, PPAR-γ activated represses the expression of several pro-inflammatory response genes in activated macrophages, including TNF-α – a central mediator of hypernociceptive cascade

The transient receptor potential A1 (TRPA1) ion channel is involved in painful states and has been described as a possible target for the 15d-PGJ$_2$ analgesic effect in DRG [5, 83]. The intracerebroventricular injection of 15d-PGJ$_2$ mitigates peripheral inflammatory inputs and reduces carrageenan-induced inflammatory pain and paw edema [51]. In the neuropathic pain model, 15d-PGJ$_2$ intrathecal administration reduced mechanical and cold hypersensitivity [15]. Lastly, 15d-PGJ$_2$ loaded into nanocapsules demonstrated robust pain killer effects in an arthritic model of pain [70]. However, it remains uncertain if 15d-PGJ$_2$ has a direct or indirect action on sensory neurons. It is important to highlight that the importance of neuroimmune interactome to pain induction and maintenance is a fact [6, 35]. In addition to the neuronal events, the literature has been discussing the two networking between neuronal cells and immune system cells present in the peripheral and central nervous system, such as glial cells, endothelial cells, T cells, and resident tissues macrophages [9, 29, 40, 73]. Immune system cells have been shown to play a role in modulating neural transmission involving painful conditions, a process referred to as the neuroimmune interface [9, 29]. Thus, it is possible to suggest that the principal target of therapeutic effects of 15d-PGJ$_2$ is immune system cells and 15d-PGJ$_2$ may have an indirect action on sensory neurons.

19.3 Anti-inflammatory Properties

The 15d-PGJ$_2$ is well-recognized as a potent anti-inflammatory agent. Considering PPAR-γ is highly expressed in endothelial cells, vascular smooth muscle cells, and monocytes/macrophages, surveys focus on the impact of 15d-PGJ$_2$ on those targets [42]. However, 15d-PGJ$_2$ also demonstrated to regulate lymphocytes (T cells) and osteoclast. Thus, in this section, we will address the anti-inflammatory properties of 15d-PGJ$_2$ in several models.

The first reports on the potential 15d-PGJ$_2$ anti-inflammatory effects were associated with its activities in adipocyte cells, as well as its relationship with the PPAR-γ receptor [23, 37, 38, 41]. Further, it was observed that 15d-PGJ$_2$ was able to upregulate PPAR-γ in macrophages, leading to inhibitory on the signaling of activator protein 1 (AP-1), signal transducer and activator of transcription (STATs), and factor nuclear kappa B (NF-κB) [68]. These findings aroused great interest in 15d-PGJ$_2$ as a potential therapeutic target in inflammatory conditions, and since then, research exploring the molecular mechanisms has not stopped until today.

In addition to the NF-κB signaling pathway, 15d-PGJ$_2$ shown the ability to abolish the inflammasome activation. The inflammasome is a multiprotein complex responsible for the inflammatory response in immune cells [84]. Inflammasome cascade initiates by the reaction of the cytosolic pattern recognition receptors (PRRs), including the NLRs (nucleotide-binding oligomerization domain and leucine-rich repeat-containing receptors) [84]. Subsequently, recruitment of procaspase-1 through ASC (the adaptor molecule apoptosis-associated speck-like protein containing a CARD) triggers the maturation and release of inflammatory

cytokines, such as IL-1β and IL-18 [84]. It has been demonstrated that 15d-PGJ_2 inhibits NLRP1 and NLRP3 inflammasome activation in murine anthrax infection model and mouse peritonitis model of gout, respectively. Consequently, IL-1β release was also inhibited [47]. Also, it has been evidenced that 15d-PGJ_2 blocked neutrophil migration in an NLRP3-dependent manner in the gout model [47]. At the same model of gout, it has been shown that 15d-PGJ_2 decreases the expression of NLRP3, ASC, pro-caspase1, and also inflammatory cytokines, such as IL-1β, TNF-α, IL-6, IL-17, and IL-33 [70]. Moreover, it demonstrated an augment of Nrf2/HO-1 signaling and thereby antioxidant effect [53, 70].

In addition, in ovalbumin (OVA)-induced model of lung inflammation, 15d-PGJ_2 reduced TNF-α, IL-5, IL-13, and IL-17 release, as well as NF-kB phosphorylation. The authors also demonstrated a protective role of 15d-PGJ_2 by blocking neutrophils and eosinophils accumulation, subepithelial fibrosis, and mucus exacerbation [17].

Endothelial cells have high expression of PPAR-γ receptor, and it was demonstrated that 15d-PGJ_2 regulates proteasome in human endothelial cells, by inhibiting NF-kB signaling [48]. The systemic administration of 15d-PGJ_2 prevents neutrophil adhesion and rolling to the inflamed milieu, in a nitric oxide-dependent manner. The authors also elucidated a blockage in ICAM-1 (intercellular adhesion molecule 1) expression on the microcapillary vessels in mesenteric tissues; however, higher levels of inflammatory cytokines were still founded [59]. Similarly, in a lung injury model, 15d-PGJ_2 reduced levels of TNF-α and ICAM-1 through NF- B inhibition [43]. On the other hand, when 15d-PGJ_2 was locally administered (intra-articular injection into temporomandibular joint), ICAM-1 levels were not altered. Instead, CD55 (complement decay-accelerating factor) was augmented, and consequently, lower neutrophil migration, plasma extravasation, and inflammatory cytokines released were observed in the joint [65].

The 15d-PGJ_2 has been implicated as therapeutic strategies in the osteoimmunology field, particularly in illness with a robust implication of the immune system, such as arthritis. It was reported that 15d-PGJ_2 induces synoviocyte apoptosis, and intraperitoneal treatment suppresses pannus formations and mononuclear cell infiltration in an adjuvant-induced model of arthritis [36]. In addition, the inhibition of cyclooxygenase (COX-2) and cytosolic phospholipase A2 (cPLA2) by 15d-PGJ_2 mitigated IL-1β synthesis in human rheumatoid synovial fibroblasts [79]. At the same line of thought, it was demonstrated in human synovial fibroblasts collected from synovial membranes of arthritics patients that the inhibitory effects of 15d-PGJ_2 in COX-2/IL-1β released partially explained the reduced histone H3 acetylation at the COX-2 promoter [21]. These effects on the H3 histone is associated with an inhibitory mechanism in the recruitment of acetyltransferase (HAT) p300 [21].

In albumin-induced arthritis model in TMJ of rats, intra-articular injection of 15d-PGJ_2 prevents inflammatory hypernociception, by avoiding TNF-α, IL-1β, and keratinocyte chemoattractant (KC). Inhibitory effects were also described in the expression of the protein kinase (PK) A and PKCε, preventing the lowering of the nociceptor threshold and thereby inflammatory pain [64]. The pannus formation in the TMJ, as well as the leukocyte migration, was also prevented by 15d-PGJ_2

treatment. In addition, 15d-PGJ$_2$ reduced the release of IL-6, CINC-1, IL-12, and L-18 while increasing the release of IL-10, an important anti-inflammatory cytokine capable of driving to resolution process, leading to homeostasis in arthritics conditions [65]. These higher levels of IL-10 were recently attributed to a macrophage polarization induced by 15d-PGJ$_2$ from macrophages M1-like towards M2-like, where the balance favoring M2-like is one of the keys to the anti-inflammatory features of 15d-PGJ$_2$ [2].

In the collagen-induced arthritis (CIA) model, 15d-PGJ$_2$ improves clinical scores, such as pain and edema. These effects were associated with the regulatory actions on T cells profile, dampening the differentiation of Th17 cells, while inducing Tregs and thereby protecting the joint against arthritics damage [10]. Moreover, 15d-PGJ$_2$ also exerts an immune-modulatory effect on dendritic cells by promoting a rearrangement of membrane-bound costimulatory molecules consisting in a reduction in the expression of costimulatory surface molecules (MHC-II, CD80, and CD86) and in the secretion of pro-inflammatory cytokines by this cell. Interestingly, the glitazone PPAR-γ agonist rosiglitazone showed a lesser modulatory effect [20, 39]. Dendritic cells are regarded as professional antigen presenting cells and provide an important link between the innate and the adaptive immune responses and play a critical role not only in the host defense against pathogens and cancer but also in the tolerance and prevention against autoimmunity. Also, human synovial fibroblasts from patients previously diagnosed osteoarthritis (OA) were treated with 15d-PGJ$_2$ in combination with prednisolone and demonstrated to prevent pro-fibrotic pathways partially by the inhibition of ALK5/Smad2 signaling and β-catenin accumulation [80]. Lastly, in a K/BxN serum transfer arthritis model, the authors through a lipidomics liquid chromatography analyzed the bioactive lipid profile in the spinal cord. Interestingly, levels of 15d-PGJ$_2$ were reduced in the spinal cord compared to control. Moreover, intrathecal injection of 15d-PGJ$_2$ prevents mechanical hypersensitivity in the arthritic animals [14].

Overall, 15d-PGJ$_2$ has stood out as a promising therapeutic option to control inflammatory sickness, in a vast range of models.

19.4 Nanomedicine and Nanotecnology to Improve the Therapeutic Potential of 15d-PGJ$_2$

The nanomaterials could provide a revolution in technology that will soon impact the diseases treatment methods through new nanoparticles delivery systems. Nanotechnology is shown to bridge the barrier of biological and physical sciences by applying nanostructures and nanophases at various fields of science. The use of ideal nano-drug delivery system is decided primarily based on the biophysical and biochemical properties of the targeted drugs being selected for the treatment [60]. It has been shown that around 50% of the 15d-PGJ$_2$ administered exogenously to a biological system binds to albumin [66]. Thus, improved bioavailability and

efficiency of such compound has been achieved from different strategies to couple the 15d-PGJ_2 molecule to carrier systems.

Nanoencapsulation has gained great interest in the pharmaceutical field due to the ability to modify drug release profile, increasing their bioavailability compared to the free drug [69]. Polymeric nanoparticles are defined as solid colloidal particles, ranging from 5 to 1000 nm, and can be classified as nanospheres (NS) or nanocapsules (NC), depending on the technique and materials used [19]. Polymeric NC can be prepared using biodegradable components, such as the polyesters poly-ε-caprolactone (PCL), poly(lactide) (PLA), or poly(lactide-co-glycolide) (PLGA). PLGA is a copolymer well-worn to synthesize nanocapsules able to be loaded with drugs and, as an advantage feature, exhibit low or zero toxicity [19].

Several studies have been performed using nanocarriers to encapsulate 15d-PGJ_2 to enhance bioavailability and extend the effect without toxicity and high dosages [3]. Nanoencapsulation with poly(D, L-lactide-co-glycolide) nanocapsules (NC) improves 15d-PGJ_2 efficiency against inflammation in the peritoneum induced by endotoxin (LPS), carrageenan (Cg), or mBSA (immune response). 15d-PGJ_2-NC loaded reduces neutrophils migration into the peritoneal cavity, while free 15d-PGJ_2 does not, and sustained 15d-PGJ_2 levels in the serum for up to 24 hours [11]. At the same polymer nanostructure system, however, in an inflammatory model in the TMJ, 15d-PGJ_2-NC loaded in nanocapsule impressively reverse hypernociception in picogram levels, while intra-TMJ injection of free 15d-PGJ_2 showed no effects [16].

Also, in a periodontal disease model, 15d-PGJ_2-NC prevents bone loss and inflammatory markers for bone osteolysis. The animals treated with 15d-PGJ_2-NC showed reduced CD4+ T cell infiltration, which may also have contributed to the lower RANKL expression, and consequently decreased bone resorption, supporting the contribution of inflammatory cells in regulating osteoclastogenesis-related factors and bone loss. T-regulatory cells (CD4+CD25+FOXP3+) were elevated in the infected animals and diminished significantly when treated with 15d-PGJ_2-NC. The elevated number of Treg in periodontitis is in accordance with previous reports suggesting that Treg infiltration could reflect an attempt to control tissue destruction promoted by the chronic inflammatory [57]. Complementary low doses of 15d-PGJ_2 improve the osteoblast activity in a PPAR-γ-independent manner due to, at least in part, the elevated expression of Histone Deacetylase 9c [56], which plays a crucial role in the acceleration of mesenchymal stem cells osteogenesis and attenuation of mesenchymal stem cells adipogenesis through interaction with PPARγ-2, which interrupts PPARγ-2 transcriptional activity resulting in attenuation of adipogenesis and acceleration of osteogenesis [13].

The subcutaneous injection of 15d-PGJ_2-NC increased the levels of 15d-PGJ_2 in gingival tissue [57], as well as reduced pain and inflammation in an arthritis gout model in mice, by modulation of the expression of inflammasome markers [70]. Solid lipid nanoparticles (SLN), submicron lipid carriers sized between 50 and 1000 nm, are composed of biocompatible materials able to incorporate mainly lipophilic drugs since they are constituted by an external phase (an emulsifier and water) and an inner layer composed of lipid matrix, where the drug is dispersed. Such

nanocarriers feature low toxicity and cause no irritation to tissues, hence the growing interest in their use in the treatment of inflammatory diseases [7]. As demonstrated, 15d-PGJ$_2$ encapsulation into SLN was highly efficient (>95%), confirming the affinity of the drug to the lipid phase of the SLN. It has previously been reported that the encapsulation efficiency of PLGA nanocapsules was approximately 77% for 15d-PGJ2 [11]. Besides, the SLN system was able to modify drug release profile and to maintain cell viability compared to the free drug. 15d-PGJ2 encapsulation reduced neutrophil migration in three different inflammatory models due in part to a decrease in IL-1β and IL-17 levels as well as an increase in IL-10 [18].

Also, it has been proposed the association between 15d-PGJ$_2$-NC in PLGA polymer with human decellularized amniotic membrane scaffold (AHAS) in the post-infarct model [25]. The AHAS presents the advantages of low immunogenicity, many growth factors, and easy technique. The major point of scaffold implantation in the infarcted myocardium is a prompt settlement of the fibrotic sites with new contractile cells to regain the ventricle function [24, 30]. The authors demonstrated that the association between AHAS and 15d-PGJ$_2$-NC yields cardioprotection by reducing cardiac dysfunction. Specifically, the AHAS +15d-PGJ$_2$-NC group increased satisfactorily the ejection fraction when compared with the negative control. Also, increased collagen type I and cardiomyocytes, along with new blood vessels in the myocardial fibrosis sites were observed in the AHAS +15d-PGJ$_2$-NC treated group. In agreement, AHAS +15d-PGJ$_2$-NC mitigated cardiac infarcted sites. Collectively, authors indicate that acellular amniotic membrane scaffold combined with 15d-PGJ$_2$ loaded prevents myocardial dysfunction [25].

Another approach is when topically applied in combination with a poloxamer 407 hydrogel, considering that micellar dimensions were reduced at physiological temperature, micelles can remain at the site of administration for long periods of time and be small enough (<100 nm) to avoid uptake by the reticuloendothelial system, favoring the therapeutic efficacy of the drug carrier 15d-PGJ$_2$ ameliorates atopic dermatitis by abolishing the expression of ROR-γt and TNF-α, suppressing the excessive immune response. Thus, emerging possible new treatment for atopic dermatitis could be as effective as the current available with potentially fewer side effects and a wider spectrum of action in the mechanism of atopic inflammation [55]. Recently, a binary micellar system composed of poloxamer (PL) 407-(PL) 188 was explored as a carrier system for 15d-PGJ$_2$. The system morphology was a spherical and compact shape, with a diameter range of 120–150 nm, indicating micelles formation. This PL-15d-PGJ$_2$ system, when intra-articularly injected, sustained the antinociceptive effect for 14 days, in a nanogram scale [3]. Curiously, the contralateral treatment with 3 ng of 15d-PGL$_2$ associated with the micellar system blocked nociception induced by formalin. This result reflects the ability of the micellar system to improve viability, effectiveness, and longevity. Also, plasma extravasation, leukocyte migration, and inflammatory cytokines were abrogated in the PL-15d-PGJ$_2$-treated group [3].

Over the past decades, transdermal routes have been attractive to researchers in the drug delivery field, due to their simple form of use, mostly painless, and possible self-applicability. Notably, microneedles (MNs) raise as a promising option to

deliver drugs in transdermal settings. The MNs patch consists of micron-scale needles designed to rupture the stratum corneum layer of the skin, creating micropores over the skin, where the contact of the drug with the interstitial fluid results in drug absorption [1, 27, 33]. Particularly with 15d-PGJ_2, microneedles were applied over the skin as a pretreatment before a 15d-PGJ_2 cream was applied [44]. The cream alone did not evoke antinociceptive effects. On the other hand, the association with microneedles improves efficiency and longevity effect for up to 8 hours, while directly intra-TMJ injection only evokes antinociception for 2 hours. Also, TNF-α and IL-1β, two of the main inflammatory cytokines, were abrogated into baseline levels for 8 hours, supporting long-lasting anti-inflammatory activities [44].

Collectively, 15d-PGJ_2 proved to be a polyvalent bioactive lipid, with excellent ability to solve inflammatory conditions in a vast range of models, in different delivery systems and routes of application. Nanoparticles and poloxamer hydrogel have proven their ability to improve 15d-PGJ_2 bioavailability in lower dosages. Moreover, effectivity and long-lasting effects were also highlighted in these drug delivery systems. Therefore, the proven effect of 15d-PGJ_2 allied with an adequate delivery system paves the way for further improvements in the pharmaceutics field to treat inflammatory sickness and others.

19.5 Conclusions and Perspectives

In the past, inflammation was thought to be resolved after a simple dilution of inflammatory mediators leading to the reestablishment of tissue function. Nowadays, it was recognized that endogenous specialized lipid mediators are produced to block leukocytes recruitment and resolve inflammation [72]. The discovery of 15d-PGJ_2 changed the concept of how inflammation ends and opened new perspectives for the treatment of inflammatory diseases. Understanding the mechanism by which 15d-PGJ_2 and its precursor cyclopentenone PGD_2 regulate inflammatory responses shed light on improved strategies for treatment of inflammatory painful conditions and other inflammatory conditions. The 15d-PGJ_2 is an immunoresolvent molecule (i.e., do not present immunosuppressive effects, one of the undesirable side effects of corticosteroids, immunobiological agents, and opioids) and, therefore, harnessing the pharmacology of resolution, could provide the basis for reprogramming immune cell and neuronal and host response at very low doses, even lower when associated with nanotechnology. Therefore, 15d-PGJ_2 represents a new class of non-immunosuppressive and non-opioid analgesic drugs.

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Chapter 20
Ligand-Independent Coactivation of Peroxisome Proliferator-Activated Receptor Gamma

Robert G. Bennett

Abstract The peroxisome proliferator-activated receptors (PPARs) are related transcription factors in the nuclear hormone receptor family. The regulation of PPAR activation by ligand-dependent and ligand-independent mechanisms has been intensely studied in efforts to develop new approaches to the treatment of pathological states. In particular, PPARγ has been a focus of study due to its important roles in insulin sensitivity and adipogenesis. Since its discovery, many regulatory coactivator complexes were shown to regulate PPARγ, many of them in the absence of ligand binding. This chapter will focus on those coactivator complexes shown to activate PPARγ transcriptional activity in a ligand-independent manner.

Keywords Peroxisome proliferator-activated receptor gamma · PPARγ · Ligand-independent activation · Mediator complex · p300/CREB binding protein · CITED2 · SWI/SNF complex · PGC1α

20.1 Introduction

The peroxisome proliferator-activated receptors (PPARs) are a family of transcription factors in the nuclear hormone family [48]. They are active as heterodimers with the retinoid-X receptor (RXR) at specific target sequences in gene promoters, known as PPAR response elements (PPREs). Three distinct genes have been identified, including *PPARA, PPARD*, and *PPARG*, encoding the protein products *PPARα, PPARδ* (also known as PPARβ/δ), and PPARγ, respectively [48]. There are two

R. G. Bennett (✉)
Research Service, VA Nebraska-Western Iowa Health Care System, Omaha, NE, USA

Departments of Internal Medicine and Biochemistry & Molecular Biology, University of Nebraska Medical Center, Omaha, NE, USA
e-mail: rgbennet@unmc.edu

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protein products from the *PPARγ* gene, resulting from alternative promoter sites, with a shorter, widely expressed form (PPARγ1) and a longer protein with an N-terminal extension (PPARγ2), largely restricted to adipocytes, where it plays a critical role in adipogenesis [43, 78].

All PPARs contain an N-terminal domain (known as the A/B domain), a DNA-binding domain (the C domain), a hinge region (D domain), and a C-terminal ligand binding region, known as the E/F domain [23] (Fig. 20.1). The PPARs are regulated by ligand binding at the E/F domain, also known as the activation function-2, or AF-2 region [55, 84]. In addition, the activity and specificity of the PPARs are regulated by coactivator or cosuppressor protein binding, DNA modifying enzyme complexes, and phosphorylation [78]. Many of these regulatory processes are dependent on ligand binding to the AF-2 region of PPARs, but their actual interaction could also include the other domains. There is also evidence that some of these coactivators interact with the A/B region, also known the AF-1 region [83]. While in most cases, these interactions are dependent on ligand-binding to alter PPAR activity, coactivator association can regulate activity in the absence of ligand binding. This chapter will focus on the evidence for ligand-independent activation of PPARγ by coactivator proteins.

20.2 The Mediator Complex

The Mediator complex is a large multiprotein assembly that can enhance both ligand-dependent and ligand-independent transcriptional activities of several nuclear receptors [5, 49]. This effect generally involves interaction between Mediator and RNA pol II, facilitated by additional transcriptional regulators, particularly TFIIB [4]. The complex was first identified in yeast as an activator of RNA pol II [40, 75]. The mammalian form was identified as binding to thyroid hormone receptor and was named the thyroid receptor-associated protein (TRAP) complex [24]. Later, the complex was found to be associated with other transcription factors, such as SREBP (ARC complex), vitamin D receptor (DRIP complex), and several others [15]. Mediator complexes are present in all eukaryotes and are now known to affect transcriptional activity of many nuclear hormone receptors, both as a coactivator and as a cosuppressor [68, 71].

The specificity and function of Mediator complex is controlled by regulatory subunit proteins. One such subunit was identified by yeast two-hybrid screening as a PPARγ binding protein and was named PPAR binding protein (PBP) [93]. It was later found to be the same protein as identified in other studies of Mediator complex as binding to thyroid hormone receptors (TRAP220) and vitamin D receptor (DRIP205) and is now known as MED1 [15]. The importance of MED1 in the PPARγ-regulated gene expression was revealed using *Med1*-deficient mice in a model of atherosclerosis [6]. The loss of *Med1* was associated with a shift in polarization of macrophages toward the inflammatory or M1 phenotype. Furthermore, overexpression of *Med1* led to promotion of the M2 anti-inflammatory phenotype.

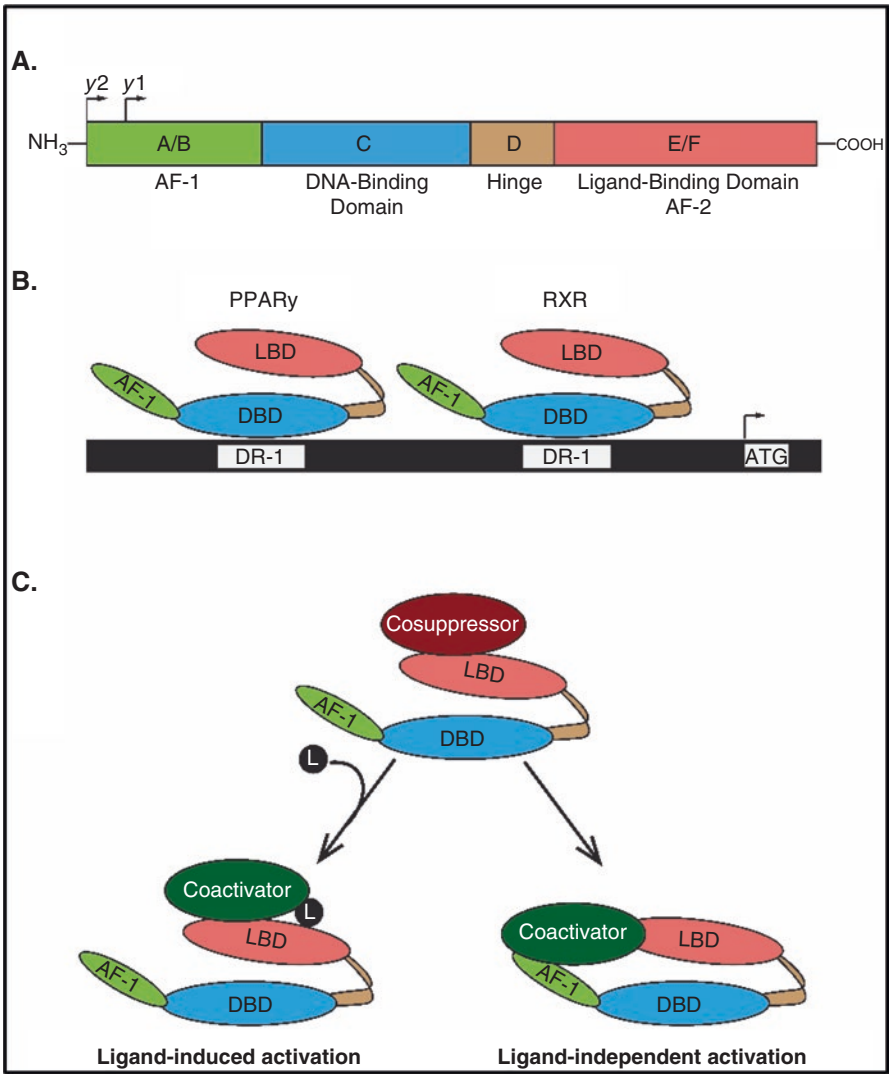

Fig. 20.1 (**a**) Overall structure of PPARγ, showing the four major domains. The alternative transcriptional start sites of PPARγ1 and PPARγ2 are indicated near the N-terminus. (**b**). The PPARγ/RXR heterodimer relationship at the transcriptional regulatory region of a PPARγ target gene. The binding of the DBD to the PPRE region is represented by the DR-1 domains, and the transcriptional start site is labeled by the ATG start codon. (**c**) Ligand-dependent and ligand-independent activation of PPARγ. Binding of corepressor proteins maintain PPARγ in an inactive state. Ligand binding induces exchange of corepressor for coactivator proteins at the LBD. Alternatively, in the absence of ligand, some exchange of coactivator proteins can occur to induce ligand-independent activation. This coactivator binding can occur at either the AF-1 of LBD domains

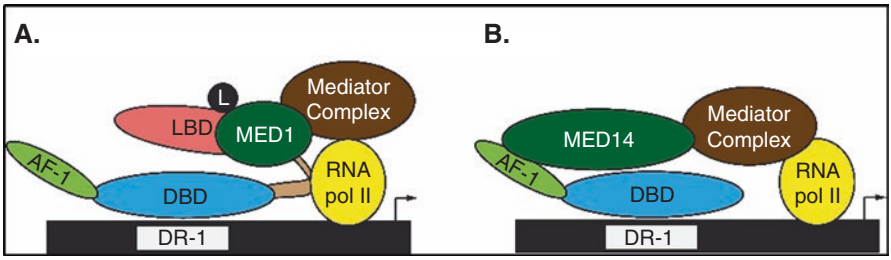

Fig. 20.2 (**a**) MED1 links ligand-dependent binding of Mediator complex to RNA polymerase II. (**b**) MED14 binds to the AF-1 domain in the absence of ligand to link Mediator complex to RNA pol II

The association of PPARγ with M2 gene promoters was reduced in the *Med1*-deficient mice, which was reversed upon restoration of *Med1* expression, and the binding of both PPARγ and MED1 to target gene promoters was increased by interleukin-4.

The binding of MED1 to PPARγ is facilitated by two LXXLL domains, upon which the activity of PPARγ is increased (Fig. 20.2). This interaction takes place in the hinge or c-terminal regions of PPARγ, as an A/B region deletion mutant of PPARγ failed to impair MED1 binding, but a C-terminal mutant abolished binding [11, 93]. The binding of MED1 to PPARγ and other nuclear hormone receptors is ligand-dependent [53, 88]. However, mutant MED1 lacking both LXXLL domains, but incorporated into the complex, still results in association of Mediator with PPARγ, suggesting that additional factors may contribute to Mediator-PPARγ interactions. Indeed, the Mediator complex subunit MED14 was found to be such a factor. MED14 is a central protein in the Mediator complex, linking the head, middle, and tail Mediator modules [64]. Grøntved et al. demonstrated ligand-independent binding of MED14 to the A/B domain of PPARγ [29]. This binding occurred independently of MED1, since pull-down experiments revealed equivalent binding in murine embryonic fibroblasts from wild-type and *Med1* knockout mice after expression of *Med14* and that unlike MED1, MED14 binds to the PPARγ A/B domain. This was accompanied by increased basal and ligand-induced activation of a subset PPARγ target genes, particularly those regulating lipid storage, including *Fabp4, Cd36, Acqp7,* and *Cidec*, and this effect was reversed with siRNA-mediated knockdown of *Med14* [29]. Finally, MED14 was found to be critical for PPARγ-induced differentiation of a preadipocyte cell line. In summary, MED14 is a critical hub linking the Mediator complex to PPARγ target gene expression and is necessary for full basal and ligand-induced of a subset of adipogenic genes.

20.3 NCoA6/PRIP

A PPAR regulatory protein was identified using PPARγ as bait in a yeast two-hybrid system and was named PPAR Interacting Protein (PRIP) [92]. It was found that PRIP was identical to proteins identified in other studies as receptor-activating protein 250 (RAP250), thyroid receptor binding protein (TRBP), activating signal cointegrator 2 (ASC2), and nuclear receptor coactivator (NRC) and is now known as nuclear receptor coactivator 6 (NCoA6). NCoA6 had both ligand-dependent and independent effects on PPARγ activation [92]. Adipogenic regulation by PPAR was dependent on NCoA6 [63]. The mechanism for NCoA6 activation of PPARγ is through interaction with its associated protein NCoA6 interacting protein (NCoA6IP), a methyltransferase. Together, NCoA6 and NCoA6IP act as bridging proteins for Mediator and p300/CBP to affect transcriptional activity [63].

20.4 p300/CBP

The family of lysine histone acetyltransferases (KATs) functions as histone-modifying enzymes that relax chromatin structure to facilitate gene transcription. Two members of the KAT3 family, p300 and CBP (cAMP response element binding protein (CREB) binding protein), have extensive homology and largely overlapping physiological functions. They are considered highly promiscuous acetyltransferases, with hundreds of identified substrates, representing both histone and non-histone proteins. In addition, they function as scaffold proteins that participate in protein-protein interactions transcriptional complexes.

The first report of an interaction of p300/CBP with PPARγ was a yeast two-hybrid study which showed interaction and transcriptional activation of the ligand-binding PPARγ domain with CBP [50]. A later study provided evidence of a complex interaction between both p300 and CBP and PPARγ [27]. In this study, p300/CBP bound to the AF1 domain in a ligand-independent manner and to the AF2 domain in a ligand-dependent manner (Fig. 20.3). Importantly, the p300/CBP binding to the AF1, but not AF2 domain, enhanced the PPARγ basal transcriptional activation.

The role of p300/CBP in regulating PPARγ target gene expression was elegantly explored in a study by Bugge et al. [11]. A truncated PPARγ construct with a deletion of the A/B domains was compared with full-length PPARγ2. Using electrophoretic mobility shift assays, they observed no differences in PPARγ DNA-binding activity between the two constructs. However, when cells expressing the constructs were analyzed by microarray for changes in gene expression in response to rosiglitazone, of the 257 genes found to be differentially expressed in response to rosiglitazone with the full-length PPARγ2, a subgroup of 25 had significantly impaired

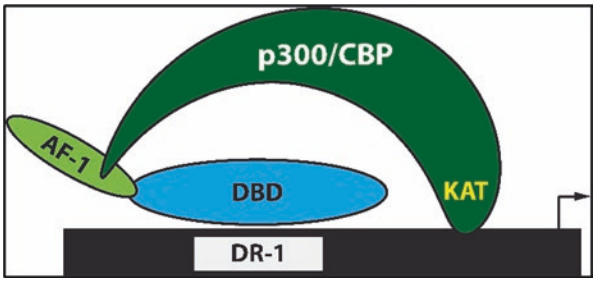

Fig. 20.3 Ligand-independent activation of PPARγ by p300/CBP. The association of p300/CBP with the AF-1 domain of PPARγ allows the lysine acetyltransferase (KAT) to modify histones and regulate transcription

rosiglitazone response with the A/B domain deletion construct. Most of these genes involved lipid storage, such as *Cd36, Fabp4*, and *Plin*. This effect appeared to require the PPARγ A/B domain, as substitution of the PPARα A/B domain did not rescue the rosiglitazone response. The basal activity was also reduced in the A/B--deleted mutant construct, and there was no effect of GW9662 on activity of either construct in the absence of rosiglitazone, consistent with a modest ligand-independent activation dependent on the A/B domain. To determine the transcriptional regulators associated with the PPARγ constructs at target genes, chromatin immunoprecipitation assays were performed. These revealed that while PPARγ-DNA binding was unaffected, recruitment of RNA polymerase II was impaired in cells expressing the A/B mutant at genes with reduced rosiglitazone response. This was related to reduced binding of CBP and p300 to the complex at the A/B--dependent target genes.

A ligand-independent relationship between p300 and PPARγ has also been reported macrophages. In response to various stimuli, macrophages undergo a polarization response to adopt a largely inflammatory (M1) phenotype or an anti-inflammatory (M2) phenotype. The transition to the M2 phenotype, in response to signals such as interleukin-4 (IL-4), requires PPARγ [57]. However, evidence suggested that ligand activation of PPARγ did not result in a full macrophage polarization transcriptional program. For example, while IL-4 potently activated PPARγ at the *Arg1* promoter, rosiglitazone alone failed to stimulate activity [57]. These findings were later supported in another study, showing that rosiglitazone was not sufficient to fully activate M2-related genes [73]. Daniel et al. [21] further explored the mechanism behind IL-4 activation of PPARγ in macrophages. In response to interleukin-4, activation of some PPARγ target genes (*Arg1, Fabp4, Tgm2, Hbegf)* was stimulated by IL-4, but not inhibited by GW9662. The ligand-independent response to IL-4 involved recruitment of p300, as well as the additional transcriptional regulators STAT6 and RAD21, facilitate chromatin opening and transcriptional activation. Importantly, these effects were not observed in rosiglitazone-sensitive genes, further suggesting separate ligand-dependent and ligand-independent mechanisms of PPARγ activation. In addition to the initial response to IL-4, with repeated or

prolonged exposure to IL-4, p300 and RAD21 remained bound to PPARγ to regulate transcriptional memory, by bookmarking the chromatin resulting in a stronger response with repeated exposure to IL-4. These findings represent strong evidence for a physiological role for ligand-independent activation of PPARγ in macrophage polarization.

20.5 CITED2

A cytokine-inducible transcriptional coactivator protein was cloned from human melanocytes and mouse T-helper cells and named Mrc1 (melanocyte-specific gene-1-related gene 1) [67, 72]. Shortly afterward, Bhattacharya et al. identified p35srj, a p300/CBP binding protein that regulated the activity of hypoxia-induced factor-1α (HIF1α), as an alternatively spliced form of Mrc1 [10, 44]. Now known as CITED2 (CBP/p300 interacting transactivator with Glu/Asp-rich C-terminal domain 2), it plays a role in the response to hypoxia via HIF1α [10], as well as critical roles in development [7, 8, 87, 90] and cancer [36, 42, 66]. The first study to examine the interaction between CITED2 and PPARs was by Tien et al. who showed that CITED2 associated with and activated PPARα in a ligand-dependent manner [76]. In contrast, when CITED2 and PPARγ were co-expressed in HepG2 cells, the basal PPARγ activity was robustly increased, with little additional activation after treatment with the PPARγ ligand PGJ2. However, this effect was not seen using a Gal4-PPARγ-LBD reporter system in COS-1 cells, suggesting the effect may be cell-type specific. Another study showed that in cortical neurons, CITED2 was found to associate with PPARγ in neurons subjected to DNA damage and increased its basal transcriptional activity in the absence of exogenous PPARγ ligand [28]. Other studies found no ligand-independent activation of PPARγ by CITED Hep3B hepatoma cells or in RAW-264.7 macrophages, further underscoring the apparent cell-type specificity of this interaction [17, 39].

20.6 SWI/SNF Complex

A complex of proteins with ATP-dependent chromatin-modifying activity was originally identified in yeast and named SWI/SNF (faulty mating-type switching/sucrose non-fermenting) based on the phenotypes screened [54, 58]. The *Drosophila* homologue of SWI/SNF was identified as the product of the brahma gene (*brm*) [74]. Later, two mammalian proteins were identified as homologues of the SWI/SNF ATP-dependent catalytic subunits, Brahma (Brm), also known as SMARCA2, and *brahma*/SWI2-related gene-1 (Brg1), also known as SMARCA4 [18, 38, 52]. These proteins use ATP hydrolysis to drive chromatin remodeling, including histone dimer ejection, nucleosome ejection, or sliding [19]. In addition to these catalytic subunits, SWI/SNF activity is regulated by a family of regulatory/binding subunits

collectively known as Brg1/Brm-associated factors (BAFs). There are currently over 20 distinct BAF proteins that have been identified, and the various combinations of Brm or Brg1 with associated BAFs determine their chromatin remodeling properties, as well as their specificity [14, 82]. The SWI/SNF complexes can act as either gene activators or repressors, depending on their action on chromatin or on the binding of specific transcription factors.

The SWI/SNF complex can interact with PPARγ in a ligand-independent manner and increase its transcriptional activity [22]. In this study, a yeast two-hybrid system using the PPARγ N-terminal activation and ligand-independent activation domains was used as bait for screening a human cDNA library. The SWI/SNF subunit BAF60c (also known as SMARCD3) was found to bind to the PPARγ construct in a ligand-independent manner and that the associated SWI/SNF complex contained Brg1 (Fig. 20.4). Using a series of deletion mutants of PPARγ, it was confirmed that that the N-terminal region strongly interacted with BAF60c, and that in HeLa cells, the overexpression of either BAF60c1 or BAF60c2 isoforms enhanced the ligand-independent and rosiglitazone-stimulated activities of PPARγ. However, neither depletion of BAF60c by siRNA, nor overexpression, affected differentiation of 3T3-L1 cells to adipocytes. Therefore, the functional relevance of the basal elevation of PPARγ activity is unknown.

In a more recent study using brown adipocytes, SWI/SNF binding to PPARγ was regulated by Jumonji domain containing 1A (JMJD1A), a histone H3K9 demethylase which acted as a scaffold protein linking SWI/SNF-PPARγ to RNA polymerase II [2]. This interaction was upregulated by β-adrenergic stimulation and involved BAF60b, as well as an additional SWI/SNF subunit, ARID1A. Interestingly, the transcriptional activation of PPARγ target genes did not depend on the demethylase activity of JMJD1A but rather on long-range enhancer interactions [2]. Later it was found that chronic exposure to cold stress caused a different interaction at PPARγ target gene promoters, which involved a complex between JMJD1A, PGC1α, and PRDM16, and required JMJD1A histone demethylase activity [1]. Adding to the complexity, there is evidence that a third protein, BAF60a, can also interact with PPARγ, albeit weakly, in a ligand-independent manner [35]. Another study found interaction between PPARγ and BAF60a in cold-induced beiging of white adipose

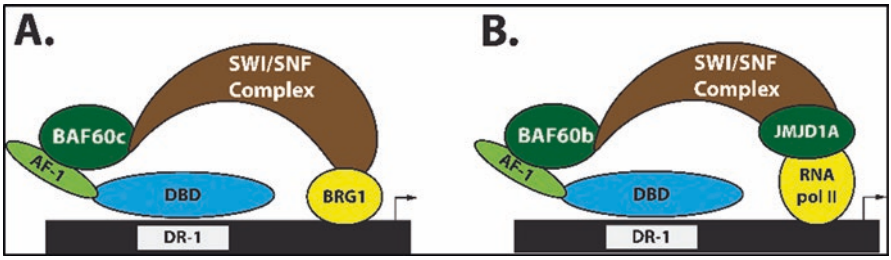

Fig. 20.4 (**a**) SWI/SNF is recruited to PPARγ by BAF60c, promoting histone modification via the ATPase Brg1. (**b**) BAF60b links SWI/SNF to RNA polymerase II through the accessory protein JMJD1A

cells [47]. However, in each of these cases, the transcriptional activation was not determined in the presence of PPARγ ligand-binding inhibitors, and therefore it is unknown whether any of these interactions result in ligand-independent activation of PPARγ target genes.

Interestingly, the SWI/SNF complex is also required for activation of the *PPARG2* promoter during adipogenesis of human fibroblast cell lines and murine 3T3-L1 cells [65]. In this case, SWI/SNF promoted the function of preinitiation complex. Furthermore, PPARγ can activate *PPARG2* transcription at its promoter during adipogenesis, but this relationship is not ligand-independent, since it was inhibited by the PPARγ ligand-binding site inhibitor T0070907 [81].

20.7 PGC1α

A yeast two-hybrid screen of brown adipose tissue extract revealed a PPARγ-binding protein that was named the PPARγ coactivator-1α (PGC1α, gene name *PPARGC1A*) [62]. PGC1α interacts with PPARγ through one of its two LXXLL domains, which increase both its basal and ligand-induced activation [46]. In the case of ligand-independent activation, the binding is to the hinge region of PPARγ, whereas in the ligand-stimulated state, the binding is to the AF2 region in the c-terminus [61, 86]. PGC1α functions at promoter sites by recruiting proteins with HAT activity, such as p300/CBP or p160/SRC [59]. The interaction between PGC1α has been tied to a number of critical cellular processes, including the cold-induced thermogenic response and mitochondrial activity in brown adipose and skeletal muscle tissue [60, 62]. The expression of PGC1α in liver and muscle tissue is regulated by the p38-MAPK pathway, as well as in response to cAMP production and activation of protein kinase A (PKA) and subsequent phosphorylation of CREB [12, 20, 30, 33, 34, 85]. Studies in human 293T fibroblasts overexpressing the relaxin receptor RXFP1 revealed that the hormone relaxin increased both basal and ligand-induced PPARγ activity, but that the basal activation was not inhibited by the ligand-binding inhibitor GW9226, suggesting a ligand-independent mechanism [69]. This activation was associated with increased expression of the PPARγ target genes *CD36* and *NR1H3* (LXRα) in 293T cells overexpressing the RXFP1, or in the human monocyte cell line THP1, which endogenously expresses RXFP1. It was later discovered that the mechanism for this effect was via activation of p38 MAPK and PKA, leading to CREB phosphorylation and subsequent PGC1α expression [70].

Cannabinoids can act through activation of PPARγ, by activation of their receptors CB1 and CB2, or by direct binding and activation of PPARγ [56]. In most of these cases, the effects were blocked by PPARγ antagonists, indicating a ligand-mediated mechanism. Using a diet-induced model of obesity in rats, Youssef et al. provided evidence that the dietary cannabinoid β-caryophyllene, though activation of CB2, resulted in reduced fasting insulin and glucose levels, lower weight gain, and improved behavioral parameters [91]. Expression of both PPARγ and PGC1α

was reduced in prefrontal cortex tissue on the obese rats, which was reversed by β-caryophyllene. Furthermore, these effects of β-caryophyllene, presumed to be mediated through PPARγ, appeared to be ligand-independent, as they were not blocked by co-administration of BADGE. However, it must be noted that PPARγ transcriptional activity was not measured in this study, only PPARγ gene expression, and therefore it is not possible to assess how thoroughly BADGE suppressed PPARγ ligand-activated activity in the prefrontal cortex, or if other pathways are responsible for PPARγ and PGC1α expression. Furthermore, in rodent models of colitis and Alzheimer's disease, β-caryophyllene activation of CB2 also resulted in increased PPARγ expression, but this effect was blocked by GW9662, suggesting a ligand-mediated effect [9, 16]. Therefore, it is currently unclear whether the failure of BADGE to inhibit the CB2 pathway is a tissue-specific effect in the prefrontal cortex.

20.8 SDP1/PGC2

A PGC1α-related protein was discovered by using PPARγ as bait in a two-hybrid screen of a mouse gene library and was named PGC2 [13]. Similar to PGC1α, PGC2 expression increased the transcriptional activity of PPARγ, especially in the presence of ligand. Furthermore, forced expression of PGC2 increased adipogenic differentiation of preadipocytes. The human orthologue of PGC2 was later found to be SCAN-domain containing protein-1 (SDP1), encoded by the *SCAND1* gene [3]. Both PGC2 and SDP1 interact with the A/B domain of PPARγ [3, 13]. Interestingly, while SDP1 increased both ligand-dependent and ligand-independent activation of PPARγ, the latter effect was dependent on the cell type, suggesting additional factors regulating this interaction [3]. To date, little else is known about PGC2/SDP1.

20.9 Tip60/KAT5

Pull-down experiments from osteosarcoma cells overexpressing Tip60 and PPARγ2 revealed association of the proteins in the absence of PPARγ ligand (rosiglitazone), with no increase in binding in the presence of rosiglitazone [79]. Using PPRE reporter vectors, expression of Tip60 increased both the basal and rosiglitazone-induced transcriptional activity. However, when using a reporter vector driven by the *Fabp4* promoter, the ligand-independent activation was modest. Mutation studies revealed that the Tip60 interaction occurred at the A/B domain of PPARγ2 and was not mediated by the Tip60 LXXLL motif. Both ligand-independent and ligand-dependent activation required the lysine acetyltransferase activity of Tip60. This PPARγ-Tip60 interaction had functional relevance, as the interaction of Tip60 with PPARγ increased during preadipocyte differentiation to mature adipocytes, and adipogenesis was blocked by Tip60 knockdown by siRNA. Interestingly, this increase

in Tip60 was not through altered gene expression but by stabilization of Tip60 protein. This was later found to be due to deubiquitylation of Tip60 in the early adipogenic program [26].

20.10 NCoA4/NRC4/ARA70

A nuclear receptor coactivator protein was first identified from a two-hybrid screen that increased the ligand-induced activation of androgen receptor and was named androgen receptor-associated protein 70, or ARA70 [89]. It was later found that the gene encoding ARA70, now known as *NCOA4*, is a coactivator associated with other steroid receptors, as well as the thyroid hormone and vitamin D receptors [25, 41, 51, 77]. A mammalian 2-hybrid reporter system in human prostate (DU145) or mouse adrenal tumor (Y-1) cell lines was used to determine the relationship between NCoA4 and PPARγ [31, 32]. The increased expression of NCoA4 resulted in a robust increase in basal (ligand-independent) activation of PPARγ transcriptional activity. This activity was associated with a direct interaction of NCoA4 with PPARγ, which was eliminated after mutation of the LXXLL motif of NCoA4 [32]. Interestingly, this interaction was recently detected in an in vivo model of *Clostridium difficile* infection in mice, in which infection resulted in a loss of NCoA4 levels in the spleen and lamina propria, resulting in impaired PPARγ activation, and enhanced Th17 immune response [80].

20.11 Ajuba

The LIM domain protein Ajuba functions as a scaffold protein acting as a modulator of a variety of signal transduction pathways, including nuclear receptors [37]. Ajuba was identified as a binding protein interacting with the ligand-binding domain of PPARγ [45]. Interestingly, this is the same PPARγ domain responsible for binding of PGC1α. Knockdown of Ajuba expression reduced both the basal and rosiglitazone-induced expression of PPARγ target genes in 3T3-L1 adipocytes, such as *Fabp4*, *Plin1*, and *Cd36*, but had no effect on *Pparg*. Conversely, overexpression of Ajuba in the same cells resulted in increased expression of the target genes, even in the absence of exogenous PPARγ ligand. These findings were supported at the functional level, as Ajuba knockdown impaired adipocyte differentiation, while overexpression increased adipogenesis, although it should be noted that rosiglitazone was included in the differentiation medium for these experiments. The authors also provide evidence that Ajuba, through its LIM moiety, binds to the coactivator p300/CBP to synergistically stimulate PPARγ transcriptional activity. In summary, Ajuba was identified as a novel PPARγ binding protein that stimulates transcriptional activity in the absence of exogenously applied ligand and enhances the association of the coactivator p300/CBP. One caveat associated with this study is that no PPARγ

antagonists, such as GW9662, were used to rule out involvement of endogenous PPARγ ligands in Ajuba-induced transcriptional activity. Such studies are necessary to prove that Ajuba induces ligand-independent activation of PPARγ activity.

20.12 Summary

The factors associated with the control of PPARγ target gene expression are extraordinarily complex and involve ligand binding, DNA modification, histone modifications, and coactivator/corepressor protein association. The activity of the unliganded or basal state of PPARγ can be changed by the combination of proteins and protein complexes associated with it. While the majority of current studies are focused on the development of selective or partial PPARγ agonists, or dual- or pan-PPAR activators, few studies have sought to target coactivator complexes to alter the basal activity of PPARγ in recent years. It is possible that this may be an alternative or complementary approach to the next-generation PPARγ agonists.

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Chapter 21
PPAR Modulation Through Posttranslational Modification Control

Natália B. Videira, Marieli M. G. Dias, Maiara F. Terra, Vinícius M. de Oliveira, Marta García-Arévalo, Thayná M. Avelino, Felipe R. Torres, Fernanda A. H. Batista, and Ana Carolina M. Figueira

Abstract The peroxisome proliferator-activated receptors (PPAR) are transcription factors modulated by ligands and members of the nuclear receptor superfamily. There are three different human PPAR isotypes: PPARα, PPARδ/β, and PPARγ, which regulate the transcription of their target genes involved with energy metabolism, inflammatory process, and cellular differentiation in different human tissues. Because of these activities, PPARs are considered important targets for drugs to treat metabolic diseases, including diabetes, dyslipidemia, and obesity. Besides ligand modulation, PPARs activities can be modulated by posttranslational modifications (PTM), such as phosphorylation, SUMOylation, ubiquitination, acetylation, and O-GlcNAcylation. The understanding of PTMs modulation of PPARs function could contribute for the development of metabolic diseases treatment with more specificity and fewer side effects. Therefore, in this chapter, we present an overview

N. B. Videira · V. M. de Oliveira · M. García-Arévalo · F. R. Torres · F. A. H. Batista · A. C. M. Figueira (✉)
Brazilian Biosciences National Laboratory (LNBio), Brazilian Center for Research in Energy and Materials (CNPEM), Campinas, Sao Paulo, Brazil
e-mail: ana.figueira@lnbio.cnpem.br

M. M. G. Dias · M. F. Terra
Brazilian Biosciences National Laboratory (LNBio), Brazilian Center for Research in Energy and Materials (CNPEM), Campinas, Sao Paulo, Brazil

Post-Graduation Program of Functional and Molecular Biology, Institute of Biology, State University of Campinas (Unicamp), Campinas, Sao Paulo, Brazil

T. M. Avelino
Brazilian Biosciences National Laboratory (LNBio), Brazilian Center for Research in Energy and Materials (CNPEM), Campinas, Sao Paulo, Brazil

Post-Graduation Program in Pharmaceutical Sciences, State University of Campinas (Unicamp), Campinas, Sao Paulo, Brazil

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of PTMs that modulate the activity of each PPAR isotype and strategies to modulate these PTMs and thus regulate PPARs action.

Keywords Post translational modification · PPAR modulation · PPAR Phosphorylation · PPAR Acetylation · PPAR Sumoylation · PPAR Ubiquitination

21.1 Introduction

Peroxisome proliferator-activated receptor (PPAR) is a transcription factor included in the nuclear receptor (NR) superfamily, within are included the receptors for steroid hormones, thyroid hormone, lipophilic vitamins, and cholesterol metabolites [10, 179]. All of them have central roles as regulators of energy metabolism, tissue development, and cell differentiation, and most of them binds ligands and modulates gene expression in response to them.

The PPAR structure is highly conserved, a characteristic shared with the other members of the NR superfamily [274], and is composed of six functional regions, named from A to F (Fig. 21.1a). In the N-terminal portion, the A/B region is responsible for transcriptional activity and harbors the activation function 1 (AF-1), a

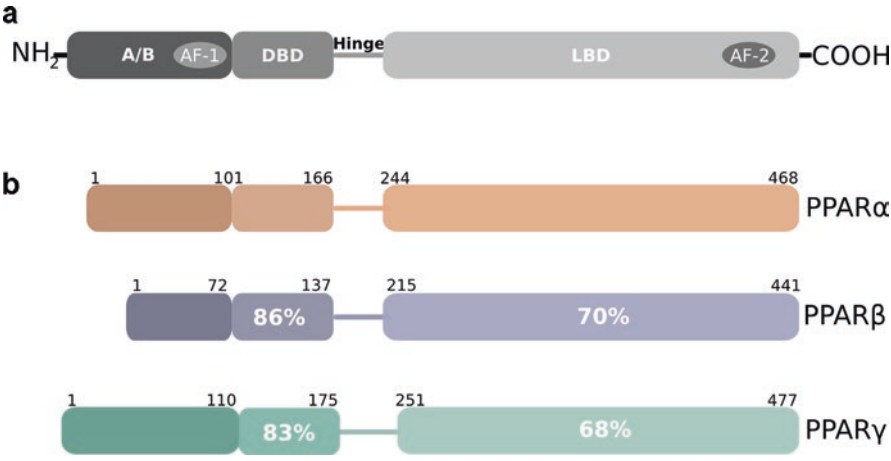


Fig. 21.1 PPARs primary structure. (**a**) The general domain structure of nuclear receptors encompasses four domains. The A/B domain at N-terminal, which contains the ligand-independent activation function 1 (AF-1); the DNA-binding domain (DBD); the hinge region; and the ligand-binding domain (LBD), that contains the ligand-dependent activation function 2 (AF-2). (**b**) The three isoforms of PPARs present different domain lengths. PPARα in orange, PPARβ in purple, and PPARγ in green. The numbers inside each domain correspond to the amino acid sequence identity of human PPARβ and PPARγ relative to PPARα

constitutive activation function independent of ligand binding, which is modulated by PTMs. The C region, also called the DNA-binding domain (DBD), is the most conserved among NRs and consists of two zinc-finger motifs involved in DNA recognition and protein-protein interaction [10, 179]. The DBD recognizes the promotor region of target the genes for peroxisome proliferator response elements (PPREs), formed by six nucleotide sequences with one nucleotide spacer (Direct Repeat 1, AGGTCAnAGGTCA) [179, 274]. The D region is the hinge region, a flexible structure that connects DBD with the ligand-binding domain (LBD), region E/F, in the C-terminal portion of PPAR. This last domain is an essential region responsible for dimerization, where the ligand binding pocket (LBP) and the activation function 2 (AF-2) are present [10, 248].

Although less conserved than the DBD, LBD structure is well conserved compared to all the NR members and is composed of 12 α-helices and 1 β-sheet harboring the LBP [18, 333]. The variation in the LBP residues contributes to the PPARs distinct physiological roles and ligand selectivity among PPAR subtypes [177, 248, 333].

21.1.1 PPAR Isotypes

PPARs are found in three subtypes: PPARα (nuclear receptor subfamily 1, group C, member 1, NR1C1, encoded by the PPARA gene), PPARδ/β (NR1C2, encoded by the PPARD gene), and PPARγ (NR1C3, encoded by the PPARG gene) (Fig. 21.1b) [3, 248]. These three different isotypes mediate the physiological actions of a large variety of fatty acids (FAs) and FA-derived molecules. Despite overlapping roles, each subtype has a distinct role and owns their expression profiles in different tissues, sensitivities to agonists, and regulation of target genes. They play essential roles in energy metabolism; however, they differ in a spectrum of their activity [48, 220].

PPARα The primary function of PPARα is to regulate the expression of genes related to FA oxidation, an activity that is linked to its presence in different tissues [153, 197]. PPARα is highly expressed in high energy requiring tissues, like kidney, liver, brown adipose tissue (BAT), heart, and skeletal muscle, tissues with high levels of mitochondrial and peroxisomal FA catabolism [153, 197, 370]. This isotype is implicated in the lipid regulation through the lipid metabolism control, and its activity is connected to the nutritional (fed and fasted) states [56, 152]. Moreover, this receptor activity is related to inflammation, mainly by limiting inflammatory responses by inhibiting transcription of vascular cell adhesion molecule-1 (VCAM-1), which is essential for leukocyte adhesion and entry into the vessel wall. PPARα also inhibits the secretion of pro-inflammatory cytokines and the nuclear factor kappa B (NF-κβ) signaling pathway [170, 360]. It is important to highlight the liver's PPARα role, where a selective deletion of the receptor was sufficient to promote hepatic steatosis, impairing whole-body FA homeostasis [223].

PPARδ/β This isotype has a broader expression pattern, being found in high levels in tissues related to FA metabolism as the skeletal muscle, adipose tissue, heart, and in gastrointestinal tract, kidney, and skin [14, 220, 232, 310]. Besides roles on FA metabolism, PPARδ/β is involved in suppressing macrophage-derived inflammation, reducing the expression of inflammatory mediators and adhesion molecules [54, 196]. Many studies have already revealed the important role of this receptor on the transcriptional regulation of mitochondrial biogenesis in skeletal muscle, mainly due to the regulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) expression [131, 282]. In addition, it was reported that PPARδ/β is also a key regulator of antioxidant defense and mitochondrial biogenesis in adult heart [337]. In summary, PPARδ/β not only regulates plasma lipid levels through FA oxidation (FAO) in several tissues but also modulates glucose handling in muscle and liver and mitochondrial biogenesis in skeletal muscle and heart [319].

PPARγ The third isotype, PPARγ, is found in two different protein isoforms, PPARγ1 and PPARγ2, which differ from each other by amino acid extension: PPARγ1 lacks the first 30 amino acids due to alternative splicing in mouse (28 amino acids in human) [48, 319]. PPARγ1 is expressed in a wide variety of cells, including the gut, adipose tissues, immune, and brain cells, while PPARγ2 is highly expressed in white adipose tissues (WAT) and BAT [48, 319]. PPARγ is considered a master regulator of adipogenesis and lipid storage, controlling FA uptake and lipogenesis, especially in WAT and BAT [48, 202, 325]. This NR also has an indispensable role in insulin sensitivity and lipid metabolism by forming different transcription complexes with distinct cofactors depending on the physiological condition to regulate a specific set of genes. PPARγ anti-diabetic effects are significantly linked with its anti-inflammatory ones, acting as a suppressor of cytokine release by macrophages and monocytes [209], also inhibiting endothelial cell migration and controlling immune cells differentiation and function [149]. Besides, this NR also acts in controlling the balance between browning of white fat and bone marrow adipogenesis and bone formation mainly by posttranslational modifications, which guides its transcriptional activity to osteogenesis or adipogenesis [202].

21.1.2 Classic Modulation and Activation

To start its activity, all PPAR isotypes form obligate heterodimers with the Retinoic X Receptor (RXR), and a ligand binding induces a conformational change in the receptor, promoting the closure of the LBP entrance by helix 12 repositioning (Fig. 21.2a) [179, 370]. Such change leads to the dissociation of corepressors complexes and the recruitment of coactivators, as CREB binding protein (CBP), steroid receptor coactivator (SRC-1), and PGC1α, promoting the transcription of target genes by binding to the specific PPRE in each promoter region [172] (Fig. 21.2b).

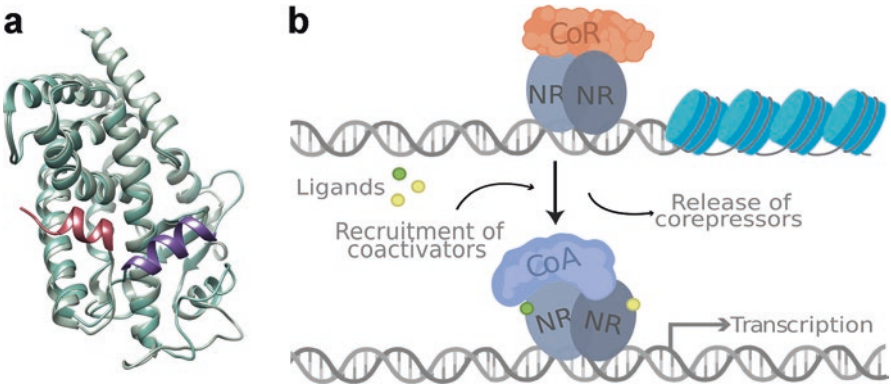


Fig. 21.2 Classical mechanism of action of nuclear receptors. (**a**) Aligned structures of PPARγ-LBD in its agonist (PDB ID: 2prg) and antagonist (PDB ID: 6c5t) conformation; the H12 is highlighted in red for agonist and in purple for the antagonist structure. (**b**) In absence of ligands, the receptors are coupled to corepressor proteins (CoR) that repress transcription. In the presence of ligands, the receptor undergoes a conformational change, main in H12, that leads to the release of corepressors and the recruitment of coactivators (CoA) that activates the transcription of the target gene. (Created with BioRender.com)

In this scenario, coactivators interact with the PPAR-LBD through the LXXLL motif (L, leucine; X, any amino acid), recruiting chromatin modifiers, which act acetylating the nucleosome histones and improving the access of the polymerase machinery to transcript genes [179, 211] (Fig. 21.2b). In contrast, in the absence of a ligand, PPAR-RXR forms a complex with corepressors, as nuclear receptor corepressor 1 (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), blocking transcription of the target genes and keeping it in basal levels [172] (Fig. 21.2b). This mechanism occurs as the corepressors interact with PPAR through the LXXXIXXXL/I motif and are capable of maintaining the chromatin closed by deacetylating it, inhibiting the transcription of the PPAR gene targets [136, 169].

Beyond the mechanism described above, i.e., the classic activation of PPAR/RXR, other action mechanisms have been described for NRs. One example is the formation of atypical heterodimers, which are still not well characterized for PPARs and are limited to certain types of cells or strict physiological conditions, but which can have substantial effects on gene expression [63]. The formation of atypical heterodimers is an example of crosstalk and can be formed either by direct or indirect interaction. Regarding the direct interaction, it involves physical contact between each NR, and one, both, or none of them bind the DNA, involving the participation of other transcription factors [63]. In the case of PPARα, a report of direct

interaction with liver X receptor alpha (LXRα) was shown, in which the atypical heterodimer binds to two directly adjacent hexameric sequences in overlapping PPARα and LXRα response elements, resulting in antagonizing the interaction of PPARα:RXRα or RXRα:LXRα with the murine cytochrome P450 family 7 subfamily A member 1 (*Cyp7a1*) gene promoter [94]. PPARα was also reported to directly interact with glucocorticoid receptor alpha (GRα) by cellular immunoprecipitation and in vitro assays, interfering in GRα gene regulation [30, 269].

In indirect crosstalk, the NR pair has no physical interaction and can affect each other's activity on chromatin by competing for overlapping DNA binding sites, by redistributing common protein partners of the transcriptional machinery, by up- or downregulation of shared coregulators, or by acting as a pioneering factor, facilitating chromatin loosening, and allowing binding of another nuclear receptor [63]. One example of indirect crosstalk is the interaction between PPARα and ERR subfamily members, in which they regulate overlapping pathways [5, 139, 246], and there are some reports of ERRα upregulating PPARα [5, 58, 139, 246]. The relationship of PPARα and GRα can also be described in some cases as indirect interaction, in which reports of sharing control in various steps of the intermediate metabolism and inflammatory pathways signal transduction cascades [184, 264, 302] and of GRα regulating PPARα expression [184, 302].

With all this information in mind, it is clear that the knowledge about modulation of NR is increasing, which is extremely positive on the development of new ligands, which may have a versatile approach by targeting dual receptors and various disorders at the same time [63].

21.1.3 Posttranslational Modulation

Another mechanism of PPAR regulation is mediated by posttranslational modifications (PTMs). The PTM is a covalent attachment of chemical groups to certain amino acids side chains that can lead to a broad spectrum of consequences on the properties of target proteins by modulating their functions [116, 322]. Therefore, PTMs are important regulators of practically every aspect of protein biology, including protein stability, cellular localization, enzyme function, and cofactor interaction [116, 322]. Some examples of PTMs that modulates PPAR are phosphorylation, SUMOylation, ubiquitination, acetylation, and O-GlcNAcylation [8, 34, 332]. In this chapter, we aim to give an overview of research on PTMs present in the PPAR isotypes (PPARα, PPARδ/β, and PPARγ) and their functional roles (Fig. 21.3a, b). Moreover, here we present several mechanisms of how to modulate PTMs and thus regulate PPAR action.

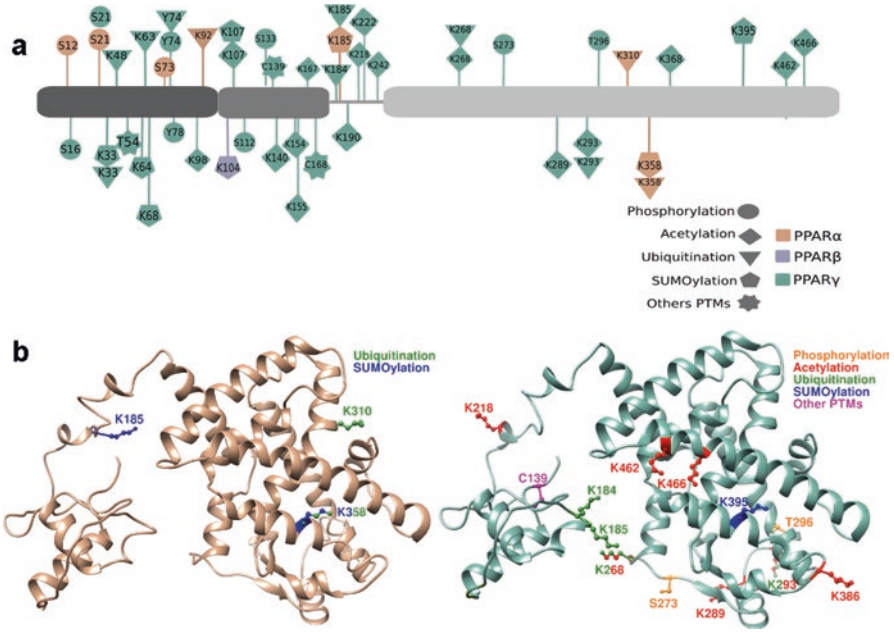
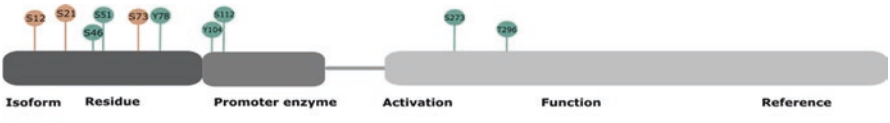


Fig. 21.3 Representation of reported PPARs PTMs. (**a**) Primary structure of PPARs with the identified PTMS in three PPAR isoforms. PTMs without residue specification, such as polyubiquitination, are not showed. (**b**) Structures of PPARα in orange (modeled from PDB ID: 3e00) and PPARγ (PDB ID: 3e00) in green. Residues involved in PTMs had their side chains highlighted in orange for phosphorylation, red for acetylation, green for ubiquitination, blue for SUMOylation, and pink for other PTMs. The structure of the A/B domain at N-terminal is not presented here because this region is intrinsically disordered

21.2 PPARs and Their Posttranslational Modifications (PTM)

21.2.1 Phosphorylation

Protein phosphorylation is the most frequent PTM, a reversible mechanism that occurs through the action of protein kinases, such as cAMP-dependent protein kinase (PKA) and cyclin-dependent kinase 5 (CDK5), which add a phosphate group (PO_4) to the polar group of serine, threonine, and tyrosine. Phosphorylation adds a negative charge to the residue, increasing its size, causing conformational changes that may affect the protein functions [11]. In the case of PPARs α and γ, phosphorylation alters the mechanisms of ligand, DNA, and cofactors binding, affecting their action on insulin sensitivity, inflammation, cancer, and osteogenesis, among others [35, 265, 300, 350] (Fig. 21.4). Up to now, no phosphorylation sites were identified at PPARδ/β.



Isoform	Residue	Promoter enzyme	Activation	Function	Reference
PPARα	S12 and S21	p38 MAPKs or CDKs	↑	Increases coactivation by PGC1 Insulin-induced transactivation Dissociation of corepressors	Barger et al. 2001 Juge-Aubry et al. 1999 Molzer et al. 2016
	S73	GSK3β	↓	Induces hepatic steatosis Increases plasma glucose Increases insulin levels Decreases glycogen storage	Hinds et al. 2016; 2017
PPARγ	S46 and S51*	Casein-kinase II	↓	Cytoplasmatic localization	von Knethen et al. 2010
	Y104*	EGFR kinase	–	Increases cell proliferation and survival Degradation signaling	Xu et al. 2016 Shi et al. 2016
	Y78	c-Src	↑	Anti-inflammatory Insulin sensitivity	Choi et al. 2015
	S112	MAPK, CDK7, CDK9 and ERK	↓	Decreases adiponectin levels Increases FFA levels Insulin resistance Decreases osteoblastic effects Decreases adipocyte differentiation	Hu et al. 1996 Adams et al. 1997 Camp and Tafuri 1997 Shao et al. 1998 Rangwala et ak. 2013 Compe et al. 2005 Iankova et al. 2006 helenius et al. 2009 Pang et al. 2014 Shu et al. 2016 Ge et al. 2018
	S133	ERK or MERK	–	–	Banks et al. 2014
	S273	CDk5 and ERK	↑	Insulin resistance Decreases osteoblastic effects Decreses adipocyte differentiation Glucose intolerance High fasting glucose and insulin levels Decreases diponectin	Choi et al. 2010 Stechschulte et al. 2016 Banks et al. 2014
	T296	CDK5	–	–	Banks et al. 2014

Fig. 21.4 PPARs phosphorylations. (Top) Primary structure with representative phosphorylation in PPARs. (Bottom) Table summarizing identified PPARs phosphorylation sites and their effects. Residues in PPARγ are numbered after γ2 isoform. There is an asterisk for PTM only described for PPARγ1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression

PPARα Three phosphorylation sites were identified in AF-1 of PPARα: S12/S21[17, 147] and S73 [127].

Phosphorylation of S12/S21 in PPARα is targeted by mitogen-activated protein kinases (MAPKs) [17, 147] or by cyclin-dependent kinase 7 (CDK7) of transcription factor II H (DNA-binding domain IIH) complex [55]. These phosphorylations could be stimulated by insulin treatment in human hepatocytes [147]. Functionally, S12/S21 phosphorylations correlate to increased PPARα basal activation, independent of ligand, in rat cardiac myocytes and in human hepatocytes in the presence of insulin, possibly due to decreased corepressor interaction with NCoR or increased interaction with the coactivator PGC1α [17, 147]. In the presence of PPARα ligand, oleic acid, the phosphorylation-promoted activation is even further increased [17, 147]. Phosphorylation-defective mutants (S12A/S21A) are not responsive to p38 MAPK in vitro, in the presence or absence of ligands, confirming that these are the phosphorylation sites for this enzyme [17].

Inhibition of phosphorylation by phosphorylation-defective mutant (S12A and S21A) or by MAPK inhibitor PD98059 decreased the ligand-dependent or insulin-dependent PPARα activation [147], corroborating the hypothesis that the

phosphorylation state modulates PPARα activity. Trichothiodystrophy (TTD) mice, a mouse model, carrying a mutation in the CDK7-containing TFIIH complex, showed lower levels of S12/S21 phosphorylation [55]. This decreased phosphorylation resulted in lower PPARα ligand-induced activity in TTD fibroblast cells, with downregulation of cytochrome P450 4A1 (*Cyp4a1*) and peroxisomal acyl-CoAthioesterase (*Pacoth*) expression, and decreased PPARα recruitment on the CYP4A1 promoter in TTD liver cells [55]. Phosphomimetics (S12E/S21E) showed increased ligand-dependent activation in both native and TDD fibroblasts [55].

Another phosphorylation that regulates PPARα function is at S73 [127], which seems to have an opposite effect of S12/S21 phosphorylations. This PTM is mediated by Glycogen synthase kinase 3β (GSK3β) [127] and not p38 MAPK [17]. In Cos7 green kidney monkey cells, the PPARα activator WY-14643 increased GSK3β concentrations, decreasing PPARα activation, indicating that this phosphorylation decreases the receptor activity [127]. Moreover, phosphorylation-defective mutant (S73A) increased basal activation, and phosphorylation mimetic mutation (S73D) decreased activation in the presence and absence of ligand, corroborating the initial observations [127]. Co-expression of PPARα and GSK3 dramatically increased the ubiquitination of PPARα in Cos7 cells. This ubiquitination was smaller with the phosphorylation-defective mutant, indicating that the receptor's reduced activity after phosphorylation might be due to increased ubiquitination and protein degradation [127].

Liver-specific Biliverdin reductase A (BVRA) knockout (KO) mice, which does not reduce Biliverdin to Bilirubin, had shown increased GSK3β activity and S73 phosphorylation of PPARα, leading to hepatic steatosis, increased plasma glucose and insulin levels, and decreased glycogen storage [127]. In LBVRA-KO, it was also observed a reduction in PPARα activity indicated by a decrease in the expression of several of its target genes in the liver (fibroblast growth factor 21 - *Fgf21*, carnitine palmitoyltransferase I - *Cpt1a*, and fatty acid translocase - *Cd36*). In contrast, a mouse model of human Gilbert's syndrome, a genetic condition that results in moderate hyperbilirubinemia, showed lower S273 phosphorylation levels, along with higher levels of PPARα protein [128]. These animals also showed an improved glucose tolerance, a protective effect against hepatic steatosis, and against insulin resistance, alongside with increased expression of PPARα target genes and increased resistance to metabolic effects of a high-fat diet (HFD) [128]. These two animal models contribute to the hypothesis that phosphorylation modulates PPARα activity in the liver. Bilirubin was reported to also act as a PPARα agonist, increasing receptor activity in high concentration (>50 μM) and upregulating the *Cd36, Cpt1a,* and *Fgf21* in adipocytes and pyruvate dehydrogenase kinase 4 (*Pdk4),* angiopoietin like 4 *(Angptl4)*, and *Fgf21* in liver cells [300]. Furthermore, bilirubin's effect on lowering glucose and reducing body fat percentage was absent in PPARα KO mice [300]. Treatment with bilirubin seems to agree with the hyperbilirubinemia mouse model, where bilirubin's presence favors PPARα activation. More experiments are necessary to confirm if bilirubin is involved in increasing or allowing S73 phosphorylation.

PPARγ Phosphorylations at PPARγ have been reported since 1996, stimulated by insulin and 12–0-tetradecanoylphorbol-13-acetate (TPA) treatment [135, 361] . The first phosphorylation site identified in PPARγ was S112 (or S84 in PPARγ1) [135] which is located at AF-1 domain and is modulated by MAPKs and CDK7 action [122, 135], decreasing PPARγ activity. Specifically, PPARγ1 S84 is phosphorylated in vitro by the MAPKs extracellular signal-regulated kinases 2 (ERK2) and Jun NH2-terminal kinase (JNK), also decreasing the receptor activity [2, 37], without altering its DNA binding activity [37].

Experiments with CDK7 knockdown and PPARγ2 phosphorylation-defective mutants S112A showed that inhibition of phosphorylation leads to increased receptor activity in the presence and absence of rosiglitazone, a PPARγ strong agonist and member of thiazolidinediones (TZDs) family, increasing adipocyte differentiation in vitro [122, 135, 287]. Phosphomimetic mutant S112D showed decreased activity in rosiglitazone's presence, reduced interaction with the coactivator SRC-1, and increased proteolysis [287]. Inhibition of MAP kinase and ERK kinase/ERK (MEK/ERK), reducing S112 phosphorylation, also decreased PPARγ degradation, indicating that phosphorylation at this residue may favor protein stability [83]. Interestingly, CDK9 was also reported to phosphorylate residue S112; however, CDK9-mediated phosphorylation increased PPARγ activity in the presence or absence of rosiglitazone [141]. Pharmaceutical inhibition of CDK9, with DRB, impaired adipocyte differentiation, indicating that CDK9 activity by phosphorylating PPARγ has an opposite effect as MAPK and CDK7-mediate phosphorylation [141].

Concerning the S112 phosphorylation effects (same as PPARγ1 S84), several studies using genetic S112A mutant mice aimed to elucidate this phosphorylation's roles in vivo. Blockage of this phosphorylation with S112A mouse preserved insulin sensitivity on HFD-induced obesity, retrieving smaller fat cells, increased serum adiponectin, and reduced free fatty acid (FFA) levels without increasing body weight [265]. The S112A mice also showed a reduction in bone formation, with decreased osteoblastic activity and increased expression of adipocyte markers: CCAAT/enhancer-binding protein alpha (*Cebpa*), fatty acid-binding protein 4 (*Fabp4*, also called aP2), *Pparg*, and adiponectin (*Adipoq*), revealing its importance on controlling bone mass and marrow adiposity, also affecting energy metabolism [95]. Phosphorylated S112 PPARγ directly interacts with a circadian clock protein, called period circadian protein homolog 2 (PER2), which represses the NR transcriptional activity by blocking its recruiting to target promoters [105]. On the other hand, phosphorylation-defective mutant S112A reduced PER2 binding to PPARγ, and Per2−/− mice cells showed in vitro increased activation of adipogenic genes and brown adipogenic markers [105].

The PPARγ1 S84 phosphorylation was upregulated in a diethylnitrosamine (DEN) mouse model of hepatocellular carcinoma (HCC) and in human liver tumors, respectively. Inhibition of this phosphorylation through phosphorylation-defective mutant S84A or kinase pharmaceutical inhibition (by MEK inhibitor PD0325901) decreased proliferation of human tumoral and normal liver cells [293]. The presence

of this mutation also downregulated genes related to glycolysis and pro-proliferation genes, indicating that S84 phosphorylation may have a role in promoting glycolysis and cell proliferation in hepatocellular carcinoma [293]. Moreover, S84 phosphorylation was reported in another tumor cell line to increase cell proliferation of human fibrosarcoma cells [243]. The PPARγ1/2 (S84 and S112), as well as PPARα (S12/S21), is significantly less phosphorylated in the adipose tissues and liver from the TTD mice model, carrying a mutation in the CDK7-TFIIH complex [55]. Contrary to previous S84 phosphorylation and S112 phosphorylation studies, this decreased phosphorylation in TTD mice was accompanied by decreased PPARγ1/2 ligand-induced activity in TTD fibroblast cells, whereas phosphomimetic mutants (S84E and S112E) showed increased ligand-dependent activation in both native and TDD fibroblasts [55].

The importance of S112 phosphorylation blockage was observed in W1P1 deficient mice [187]. W1P1 is a serine/threonine phosphatase belonging to the protein phosphatase Mg2+/Mn2+ (PPM) family, which plays a critical role in adipogenesis and fat accumulation. W1P1-deficient mice showed impaired body weight growth, decreased fat mass, triglycerides, and leptin levels on circulation. These phosphatase's pro-adipogenic roles were shown to be due to its interaction with PPARγ and dephosphorylation of S112, in vitro and in vivo [187].

Another phosphorylation site, the S273 (or S245 in PPARγ1), was first reported in 2010, and it is one of the most studied posttranslational modifications of PPARγ [45]. This residue is located at the LBD domain of PPARγ and is preferentially phosphorylated by the activated form of CDK5 [45]. However, the MEK/ERK signaling pathway can also be involved in this modification, in which ERK kinase promotes S273 phosphorylation [16]. S273 phosphorylation did not change the basal activity of PPARγ, but its inhibition, for the mutant phosphorylation-defective S273A, increased basal and ligand-dependent activity of this receptor [66]. Moreover, this phosphorylation does not affect DNA binding [45], being the reduced activity explained by increased corepressor recruitment, as shown by the phosphorylation-defective mutant S273A, which presented decreased affinity for the corepressors SMRT and NCoR [66]. In fact, NCoR seems to have a role as an adaptor protein that enhances the ability of CDK5 to associate with and phosphorylate PPARγ.

The phosphorylation of S273 is increased in obesity and has been associated with insulin resistance, occurring mainly in adipose tissues [45]. Studies in vitro confirmed that S273 phosphorylation by CDK5 is related to a scenario of obesity-induced by the tumor necrosis factor alpha (TNFα), mainly due to CDK5 activation through released pro-inflammatory cytokines [45]. It was also shown that reduction of S273 phosphorylation is correlated with pro-osteoclastic activity in vitro, increasing bone turnover through Wnt/β-catenin signaling pathway [301].

In vivo, mice on HFD showed an increased level of S273 phosphorylation, accompanied by insulin resistance and glucose intolerance [45]. S273 phosphorylation was also reported to deregulate genes involved with insulin resistance in vivo, such as *Adipoq*, leptin, and complement factor D (*Cfd*, Adipsin), among others [16, 45].

Phospho-defective (S273A) homozygous PPARγ$^{A/A}$ mice showed no differences in body weight compared to wild type in chow and HFD [108]. Regarding glucose metabolism, on HFD PPARγ$^{A/A}$, mice were as glucose intolerant as wild type; however, they were less insulin resistant. Hyperinsulinemic-euglycemic clamp experiments confirmed an improvement on insulin sensitivity due to an increase in glucose uptake. RNA-seq in epididymal WAT (eWAT) of PPARγA/A mice revealed a downregulation of growth differentiation factor 3 (*Gdf3*), a secreted protein member of transforming growth factor β (TGFβ) family, which was found upregulated in wild-type mice under HFD in both eWAT and inguinal WAT (iWAT), as well as skeletal muscle. Overexpression of GFD3 in vitro decreased glucose uptake in the presence of insulin and in vivo impaired glucose and insulin tolerance tests [108], suggesting the importance of S273 phosphorylation for insulin resistance by the influence of GDF3 factor.

In another mice model, NCoR-KO mice, it was reported a decreased S273 phosphorylation, and PPARγ was found in a constitutive active state, with upregulation of its target genes (*Fabp4*, *Cd36*, solute carrier family 2 member 4 (*Slc2a4*, former *Glut4*, Periplin, long-chain acyl-CoA synthetase 1 - *Acsl1*) in the adipose tissue [189]. These NCoR-KO mice showed enhanced insulin sensitivity, indicating that modulation of S273 phosphorylation has an essential role in insulin resistance and that PPARγ activation, independent of the phosphorylation state, has an adipogenic role [189]. NCoR importance on CDK5-mediated phosphorylation may be revealed during ligand binding to PPARγ. The association of an agonist or non-agonist at the NR LBD may induce conformational changes that dismiss NCoR from the transcriptional complex, decreasing S273 phosphorylation [189]. In addition, it was verified that some PPARγ ligands could block S273 phosphorylation in vitro, promoting an improvement in glucose tolerance and improving insulin sensitivity, as it will be further discussed later in this chapter.

Moreover, in 2020 it was found a phosphatase of protein phosphatase Mg2+- or Mn2+-dependent (PPM) family, called protein phosphatase 1A (PPM1A), that is capable of dephosphorylating S273, restoring the expression of most genes dysregulated by S273 phosphorylation, as adiponectin and CFD [156]. This activity occurs due to the physical interaction of this protein with PPARγ in a phosphorylation-independent manner. PPM1A is positively associated with insulin sensitivity since in vitro assays showed that its expression is decreased when adipocytes are treated with TNFα. In agreement, it was shown that HFD-fed animals have lower expression of this phosphatase, indicating its negative association with S273 phosphorylation and its potential role as a target for obesity and metabolic disorders [156].

PPARγ1 Y74 is another site already described for phosphorylation by epidermal growth factor receptor (EGFR) kinase. This PTM is related to inhibition of the receptor, since Y74 phosphorylation leads to PPARγ1 degradation by murine double minute 2 (MDM2), a ubiquitin ligase system, which recognizes the phosphorylation, destabilizes the receptor, and signalizes for ubiquitin complex PPARγ degradation pathway [350]. This modification occurs more frequently in colonic cancer tissues, being related to its progression and metastasis since the inhibition of

this phosphorylation using a Y74A mutant decreased tumor-associated gene expression (c-MYC proto-oncogene, Ciclo-oxygenase-2 - *COX2*, and interleukin-6 - *IL-6*) and inhibited cell proliferation, colony formation, and antiapoptotic gene expression in human cell culture. These results revealed the importance of Y74 phosphorylation on cell survival and proliferation due to the activation of the EGFR/NF-κβ signaling pathway [350]. Moreover, another study showed that pioglitazone, a known TZD, can block Y74 phosphorylation and consequently inhibit cancer cell chemoresistance by increasing PPARγ protein stability [289].

PPARγ Y78 (Y48 on PPARγ1) was reported as other PPARγ phosphorylation site, being phosphorylated by the proto-oncogene tyrosine-protein kinase (c-SRC) and dephosphorylated by protein tyrosine phosphatase (PTP-1B) [49]. Tyrosine kinase Abelson murine leukemia viral oncogene (c-ABL) was also reported to promote Y78 phosphorylation, once its physical association with PPARγ2 resulted in the receptor phosphorylation on two tyrosine residues (Y78 and Y102) [154]. This phosphorylation promotes the activation of PPARγ, increasing its transcriptional activity, being involved with the suppression of pro-inflammatory cytokines and chemokines expression in adipocytes, also reducing macrophage migration [49]. Pharmacological inhibition of c-SRC kinase raised insulin resistance on obese mice, increasing fasting insulin levels without altering body weight, suggesting that Y78 phosphorylation might have positive effects on controlling insulin and obesity. PPARγ phosphorylation-defective mutants (Y78F) resulted in an increased expression of chemokines and cytokines involved in inflammation in vitro [49].

The other two PPARγ1 phosphorylations, on S16 and S21, are located at AF-1 and are related to the ligand-independent transcriptional activity being the target of casein-kinase II (CK-II) activity under control conditions and promoting a decrease on PPARγ activity. These effects were confirmed using phosphomimetics (S16E/S21E) and phosphorylation-defective mutants (S14A/S21A), demonstrating that CK-II-dependent phosphorylation of PPARγ1 at S16 and S21 provokes its cytosolic localization, impairing this receptor shuttle for the nucleus of the cells, reducing its transcriptional activity in vitro. However, the physiological relevance of these modifications remains unclear [331]. Finally, two other PPARγ sites were identified as targets for phosphorylation: T296 by CDK5 and S133 by MEK/ERK, but their physiological effects are still unknown [16].

21.2.2 Acetylation

Protein acetylation encompasses a transfer of an acetyl group (CH3CO) onto protein lysine residues. However, acetylations on serine, threonine, and histidine residues were also reported, and the acetyl addition can change the protein hydrophobicity, solubility, and surface properties, leading to alterations in the protein physiological effects [50]. Regarding PPARs, this PTM occurs only in PPARγ (Fig. 21.5) and was firstly identified in 2010, being more frequent on lysine residues and promoted by the action of histone acetyltransferases, as CBP and p300 [110,

Isoform	Residue	Promoter enzyme	Function	Reference
PPARγ	K98	CBP	No study about its effects	Qiang et al. 2012
	K107	CBP	No study about its effects	Qiang et al. 2012
	K170*	–	No study about its effects	Tian et al. 2014
	K184 and 185*	–	Lipogenic diferrentiation	Tian et al. 2014
	K197*	–	No study about its effects	Tian et al. 2014
	K218	CBP	No study about its effects	Qiang et al. 2012
	K220*	–	No study about its effects	Tian et al. 2014
	K252*	–	No study about its effects	Tian et al. 2014
	K272*	–	No study about its effects	Tian et al. 2014
	K268 and K293	CBP	Favors lipid storage Insulin resistance	Qiang et al. 2012
	K289	–	No study about its effects	Qiang et al. 2012
	K386	–	No study about its effects	Tian et al. 2014
	K462	–	No study about its effects	Jianget al. 2014
	K466	–	No study about its effects	Jiang et al. 2014
	K184 and 185*	SIRT1	Decrease lipogenic activity	Tian et al. 2014
	K268 and 293	SIRT1	Insulin sesivitivity Increase "browning" gene expression Protec from obesity	Qiang et al. 2012

Fig. 21.5 PPARs acetylations. (Top) primary structure with representative acetylations in PPARs. (Bottom) Table summarizing identified PPARs acetylation sites and their effects. Residues are numbered after PPARγ2 isoform. There is an asterisk for PTM only described for y1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression. In black is described acetylation studies and in red the deacetylation studies

260]. Moreover, this PTM importance was revealed by studies focusing on PPARγ deacetylation, reporting beneficial metabolic effects, as browning and insulin sensitization [34].

PPARγ This PPAR isotype was reported to suffer acetylation by acetyltransferase CBP [260] and p300 [110] and to be deacetylated by NAD-dependent deacetylase sirtuin-1 (SIRT1) [110, 260] and histone deacetylase 3 (HDAC3) [146]. PPARγ acetylation levels were increased in differentiated adipocytes, and the acetylated state was shown to promote activation of the receptor. Inhibition of deacetylation by knockdown or pharmaceutical inhibition of the deacetylase HDAC3 leads to an increased expression of the target genes *Fabp4* and *Adipoq*, increased adipocyte differentiation, and insulin-induced glucose uptake in 3T3 cells [146]. In vivo, HDAC3 inhibitor significantly reduced glucose levels and enhanced insulin sensitivity [146]

In 2004, a report of SIRT1 repressing PPARγ activity described that the deacetylase promoted fat mobilization in white adipocytes by repressing PPARγ, reducing its activity, and reducing fat and triglycerides content during 3T3-L1 differentiation [250]. Despite of this clues, the confirmation that SIRT1 deacetylates PPARγ emerged later [110, 260]. Deletion of Sirt1 from adipocytes led mice to exacerbated insulin resistance, glucose intolerance, and inflammation on short-term HFD feeding. However, these mice fed chronic HFD showed reduced inflammation, improved glucose tolerance, and enhanced insulin sensitivity, relative to wild-type mice [210]. PPARγ acetylation levels increased through HFD in both groups, indicating that this PTM has a role in the metabolic syndrome phenotype [210].

Nine acetylation residues were identified on PPARγ2 by mass spectrometry: lysines 98, 107, 218, 268, 289, 293, 386, 462, and 466 [146, 260, 314]. Native acetylation levels of PPARγ are very low (1%) [314], and the residues were only identified by mass spectroscopy after CBP treatment for acetylation enrichment [260] or PPARγ overexpression in 293 cells [146]. It was the case of K268 and K293 residues, in the helix 2-helix 2′ region of the ligand-binding pocket, which are highly acetylated in obese tissue and were identified after acetylation enrichment with CBP in 293 cells [260]. PPARγ acetylated in both residues interacts with corepressor NCoR in human cells, favors cell proliferation in 3 T3 fibroblasts, and favors lipid storage in adipocytes in vitro and in vivo [260].

K268 and K293 were reported to be deacetylated by SIRT1 [260]. Treatment with rosiglitazone or resveratrol (RSV, SIRT1 activator) also promoted the deacetylation of K268 and K293 by SIRT1 [260]. Deacetylations mimetics promoted expression of "browning" genes (*Ucp1, Cidea, Elovl3, Cox7a1, Pgc1a*) and increased mitochondrial activity in adipocytes under differentiation, whereas acetylation mimetic (K293Q) delayed adipocyte differentiation, failed to induce "brown" genes, and favored expression of "white" genes in adipocytes [260].

In rosiglitazone's presence, the browning effect of deacetylated PPARγ could be explained by its interaction with the brown adipogenic activator PR domain containing 16 (PRDM16). This interaction with the PRDM16 occurs mainly by deacetylated K293 [260]. PPARγ overexpression and Sirt1 gene deletion in mice liver upregulated lipid metabolism pathways as biosynthesis of unsaturated FA, FA metabolism, and FAO in a micro-array screening [314]. On the other hand, SIRT1 gain-of-function in mice promotes "browning" of WAT by deacetylating PPARγ at K268 and K293 [260]. Corroborating the previous findings, another report showed that mice with constitutive deacetylation mutation (K268R/K293R, 2KR) are protected from obesity and its associated comorbidities, through increased energy expenditure and augmented brown remodeling of WAT [171]. These results combined indicate that control of the PPARγ acetylation state could serve as a metabolic switch to regulate lipid metabolism and thermogenesis, where the acetylated receptor increased lipogenesis and the deacetylated receptor favors "browning" of WAT and thermogenesis. With this in mind, selective modulation of PPARγ K268/K293 could have therapeutic importance in obesity and type II diabetes (T2D).

Among the nine acetylation residues identified by mass spectroscopy analysis, K107, a strongly acetylated residue, did not have its acetylation affected by rosiglitazone, indicating that SIRT1 does not deacetylate this residue [260]. This residue was also reported to suffer SUMOylation [84], which indicates potential crosstalk among these two PTM, as will be discussed later in the chapter. Regarding K98, K107, K218, K289, K386, K462, and K466, we did not find reports better characterizing these acetylations in PPARγ2.

Nine other lysine residues were later identified as targets of acetylation in PPARγ1: K140, K154, K167, K188, K190, K222, K238, and K242 in human HEK293 cells [314], of which K188 and K238 correspond to the same site observed in PPARγ2 (respectively, K218 and K268 sites) [260]. K154, one of the lysyl targets identified by mass spectroscopy, is, together with K155, part of a conserved lysine motif (RIHKK) present in PPARγ1 [314]. This motif is present in other evolutionarily related NRs, and it is located just carboxyl-terminal to the zinc finger DBD [314]. Lysines present in this motif were reported to suffer acetylation in estrogen receptor alpha (ERα), androgen receptor (AR), progesterone receptor (PR), and glucocorticoid receptor (GR) [62, 88, 163, 336], strongly suggesting that PPARγ1 K155 could also suffer acetylation. An enzymatic deacetylation assay confirmed K155 deacetylation by SIRT1, and K154/K155 acetylations were confirmed by labeling assay on human HEK293 cells [314].

PPARγ1 acetylation mimetic mutant (K154R/K155R) was very similar to native PPARγ on lipogenic differentiation verified by Oil Red Staining and mRNA expression of multiple lipogenic genes in microarray analysis in ERbB2-positive breast cancer cells [314]. Moreover, acetylation-defective mutants of PPARγ1 showed decreased lipogenic differentiation, protein expression of FAPB4, and mRNA expression of lipogenic genes in a human lineage of breast cancer cells [314]. Besides this, both residues are deacetylated by SIRT1 through enzymatic assay, being the deacetylation inhibited in the presence of nicotinamide (NAM, a SIRT1 inhibitor) [314].

21.2.3 SUMOylation

SUMO (small ubiquitin-related modifier) proteins are <10-kD polypeptides that are bound covalently to the ε-amino group of lysine residues. This process involves a cascade of enzymatic steps that requires an E1 activating enzyme, an E2 conjugating enzyme, and an E3 SUMO ligase [82, 96]. SUMOylation can affect molecular interactions by adding or disguise of surface interactions. In consequence, it can alter the activity, localization, and stability of target proteins. SUMOylation of transcription factors such as NRs frequently is related to inhibition of transcription [96]. On PPARs, SUMOylation predominantly induces negative regulation of target genes (Fig. 21.6).

Isoform	Residue	SUMO	Promoter enzyme	Activation	Function	Reference
PPARα	K185	SUMO-1	PIAS1	↓	Increases NCoR interaction Decreases L-fabp and pkd4 expression	Pourcet et al. 2010
	K358	SUMO-1	–	↓	Increases corepressor interaction	Leuenberger, Pradervand and Wahli 2009
PPARβ	K104	–	SENP2	↑	Increases FAO gene expression	Koo et al. 2015
PPARγ	–	–	PIAS1	–	Decreases chemokines expression	Lu et al. 2013
	K63*	SUMO-2	–	↓	–	Diezko and Suske 2013
	K94*	SUMO-2	–	↓	–	Diezko and Suske 2013
	K98*	SUMO-2	–	↓	–	Diezko and Suske 2013
	K107	SUMO-1/2	PIASxβ	↓	Increases CoR interaction Anti-inflamatory Insulin resistance Adipogenesis	Chung et al. 2011
	K395*	–	PIAS1	↓	Increases CoR interaction Anti-inflamatory	Pascual et al. 2005
	K107	–	SENP2	↑	Increases DNA binding Increses cd36 and pabp3 expression	Chung et al. 2011

Fig. 21.6 PPARs SUMOylations. (Top) Primary structure with representative SUMOylation in PPARs. (Bottom) Table summarizing identified PPARs SUMOylation sites and their effects. Residues are numbered after PPARγ2 isoform. There is an asterisk for PTM only described for y1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression. In black is described SUMOylation studies and in red the deSUMOylation studies

PPARα SUMOylation on PPARα is linked to increased repressive activity by improving corepressor recruitment. Two lysine residues have been reported undergoing this modification: K185 and K358 [185, 257]. In this way, K185 SUMOylation, on the hinge region, downregulates PPARα activity favoring the selective recruitment of the corepressor NCoR [257]. Studies with both COS-7 and HuH-7 cell lines reveals that the presence of proteins related to SUMO enzymatic cascades, such as SUMO-1, SUMO E3, and protein inhibitor of activated STAT1 (PIAS), decreases the transcriptional activity of PPARα and expression of its specific target genes [185]. Cellular assays showed that PPARα ligand GW-7647 blocks this SUMOylation, suggesting that although it does not occur in the receptor's LBD region, it may be ligand-regulated.

The second identified SUMOylation, at K358, leads to a sex-specific and ligand-dependent PPARα repression [185]. K358 SUMOylation in female mice livers

enables PPARα to interact with GA-binding protein (GABP) on the cytochrome P450 family 7 subfamily B member 1 (CYP7B1) promoter via the NR-interacting motif, LKKLL, also recruiting NCoR, HDACs, DNA, and histone methylases, resulting in *Cyp7b1* downregulation [185]. Physiologically, CYP7B1 repression indirectly results in increased testosterone levels and ER activity reduction, which would confer to female mice protection against estrogen-induced toxicity [185]. This effect was reproduced in the male liver using PPARα ligand WY-14643, indicating that the ligand-induced repression was SUMOylation dependent and an agonist-mediated conformational change of the LBD may be necessary for K358 SUMOylation [185].

PPARδ/β This PPAR isotype is the least studied PPAR family member, and the only SUMOylation reported for this isotype is a constitutive one, at K104, which is removed by the SUMO-specific protease 2 (SENP2) [168]. This same protease also acts deSUMOylating PPARγ, and together, these modifications promote the expression of genes involved in FAO, such as carnitinepalmitoyl transferase-1 (*CPT1b*) and *ACSL1* in muscle cells [168].

PPARγ PPARγ conjugation with SUMO proteins commonly results in the negative regulation of its transcriptional activity, either by enhanced transrepression [98, 245] or decreased activation [84, 237, 353]. SUMO-1/2 modification on K107 residue of PPARγ2 (K77 on PPARγ1) is the most studied PPARγ SUMOylation. Through mutational analysis, it was found that inhibited PPARγ K107 SUMOylation can increase the transcriptional activity of the target genes [67, 84, 150, 237, 291, 353]. One possible mechanism that explains this repression is the enhancement of corepressor recruitment by providing a novel interaction site to PPARγ sumoylated [84, 143, 150, 237, 353]. Another possible explanation is that this modification affects PPARγ stability and transcriptional activity, but not its nuclear localization [84].

This repressive state related to corepressor recruitment was found to be important for the anti-inflammatory response. On macrophages, where PPARγ1 acts in the repression of inflammatory responses, K77 (K107 on PPARγ2) SUMOylation triggered by apoptotic cells leads to stabilization of the corepressor NCoR, thereby blocking activation of NF-κβ [143]. In human renal cells, PPARγ ligand-dependent SUMOylation by the PIAS1 inhibits NCoR degradation and NF-κβ activation in lipopolysaccharide (LPS)-stimulated HK-2 cells, also presenting downregulation of chemokines expression [199]. Another SUMOylation site of PIAS1 was identified at residue K365 (K395 on PPARγ2) [245]. In macrophages, this ligand-mediated modification results in repression of the inflammatory response by recruiting PPARγ monomers to NF-κβ and AP1 DNA-binding sites, promoting increased interaction of PPARγ with NCoR and HDAC3, and preventing LPS-induced NCoR degradation [245]. PPARγ agonists also block the activity of the proinflammatory NF-κβ, inhibiting the inflammatory response in macrophages [245].

PPARγ K107 SUMOylation by SUMO-1 can regulate insulin resistance [150], body weight, and adipogenesis [216, 353]. Studies with SUMO-1-null mice demonstrated reduced adipogenesis, resistance to rosiglitazone treatment, decreased weight gain on HFD, and deregulation of PPARγ signaling pathways in adipose tissue [216]. However, SUMOylation-defective (K107R) mutants were able to recover the insulin-sensitizing actions of rosiglitazone without increasing body weight or adiposity [150], presenting increased transactivation [353].

Several studies have reported that the SUMOylation at K107 is regulated by phosphorylation at S112 of PPARγ2. Thus, the lack of phosphorylation at this site promotes K107 SUMOylation, increasing the potency of the SUMOylation repressive effects [291, 353]; however, this correlation is still not clear. Fibroblast growth factor (FGF21), which is a key mediator of the physiologic and pharmacologic actions of PPARγ, was reported to inhibiting the NR SUMOylation at K107 in WAT [71]. The FGF21-KO mice had an increase in K107 SUMOylation, but not in S112 phosphorylation [71]. Additionally, growth differentiation factor 11 (GDF11) can promote the SUMOylation of PPARγ, decreasing its transcription activity in mesenchymal stem cell (MSCs), and in that, this modification occurs without changes in S112 [364]. Interestingly, GDF11 was also reported to induce osteoblastogenesis and to inhibit adipogenesis of MSCs, and these events were supposed to occur via PPARγ modulation through SUMOylation [364].

Moreover, the deSUMOylating at K107 and K104 of PPARγ and β, respectively, were reported to enhance the recruitment of both receptors to the promoter region of their target genes. The SENP2 acts on skeletal muscle, where it selectively increases the expression of some PPARγ target genes (as fatty-acid-binding protein 3 – *Fabp3, Cd36, Cpt1b* and *Acsl1*) [168]. Another SUMO-related mechanism that increases the PPARγ activity is regulated by the E3 ligase PIASxβ/PIAS1 and the SUMO-conjugating enzyme UBC9, which are inhibitors of activated signal transducer and activator of transcription (STAT), leading to the enhancement of the transcriptional activity of PPARγ independent of PPARγ SUMOylation [237].

In addition to the K107, other modification sites can be target by SUMO1 and SUMO2 in PPARγ1: residues K33, K64, and K68 (respectively, K63, K94, and K98 in PPARγ2), and all of them were reported to repress basal and ligand induced PPARγ transactivation when SUMOylated [67]. Besides the K365 (K395 in PPARγ2) SUMOylation in macrophages [245], in adipocytes, this PTM has a role on isoform-specific regulation between PPARγ1 and y2 [12].

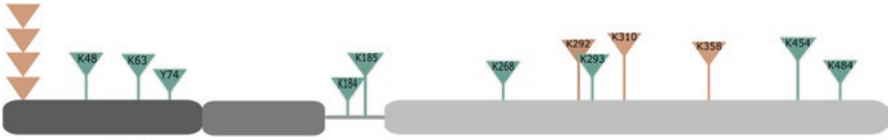
21.2.4 Ubiquitination

Ubiquitination is the covalent coupling of ubiquitin-protein, a 76-amino-acid peptide, to lysine residues in the substrate protein [124, 252]. Through a series of enzymatic processes, ubiquitin can be attached to their substrate proteins as a single

molecule or as polymeric chains in which successive ubiquitin molecules are connected through specific peptide bonds [167, 251].

The ubiquitin-proteasome system (UPS) is an intracellular protein degradation system that regulates the transcriptional activity in different levels [100]; its action goes beyond of the proteolytic role, controlling diverse activities as receptor internalization [312] and ribosome function [297]. The proteasomal degradation of transcription factors is a fundamental step in the fine-tuning regulation of its target genes because this process enables the sequential arrangement of protein complexes at the gene promoter [228].

PPARs ubiquitination regulates the protein content in cells (Fig. 21.7). In most cases, ubiquitination of all isotypes targets them for protein degradation, decreasing receptor activity [97, 102, 157, 158]. Otherwise, treatment with their agonists increases protein stability by inhibiting proteolysis, thereby increasing the receptor



Isoform	Residue	Promoter enzyme	Activation	Function	Reference
PPARα	poliubiquitination	26S proteasome	↓	Degradation	Blanquart et al. 2002
	poliubiquitination A/B domain	MDM21	↓	Degradation	Leuenberger, Pradervand and Wahli 2009
	K292, K310, and K358	MuRF1	–	Inhibits FAO gene expression signalizes cytoplasmatic export	Rodriguez et al. 2015
PPARβ	poliubiquitination	26 S proteasome	–	Degradation	Genini and Catapano 2007
	poliubiquitination	–	–	Degradation	Rieck et al. 2007
PPARγ	poliubiquitination	–	↓	Degradation enhanced by ligand	Hauser et al. 2000
	poliubiquitination	SIAH2	↓	Degradation Adipogenesis	Kilroy et al. 2012
	AF-1 and LBD	–	↑	Degradation	Kilroy et al. 2009
	K48 and K63	NEDD4	–	Decreases stability Promotes adipogenesis	Li et al. 2016
	K63*	Smurf-1	↓	Decreases lipid metabolism gene expression	Zhu et al. 2018
	Y74ph	MDM2	↓	Decreases stability Degradation	Xu et al. 2016
	K184 and K185	MKNR1	↓	Degradation Decreses adipogenesis	Kim et al. 2013
	K268 and K293	CRL4B	–	Decreases stability Decreses adipogenesis	Qiang et al. 2012 Dou et al. 2019
	K454 and K484	pVHL	↓	Degradation Downregulation of ACLY	Noh et al. 2020

Fig. 21.7 PPARs ubiquitination. (Top) Primary structure with representative ubiquitinations in PPARs. (Bottom) Table summarizing identified PPARs ubiquitinations and their effects. When ubiquitination residues were identified, they are numbered after PPARγ2 isoform. There is an asterisk for PTM only described for y1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression

activation [27, 97]. However, some ubiquitin ligases can also increase PPARγ half-life through non-proteolytic ubiquitination, promoting adipogenesis [188].

PPARα Ubiquitination in this isotype was firstly observed in HepG2 cells, in which it was shown that PPARα is degraded by the ubiquitin-proteasome system in a ligand-dependent manner, since the WY-14643 (a PPARα selective agonist) increases the half-life of PPARα, thus protecting the receptor against the ubiquitination [27]. In addition, treatment with MG132, a selective proteasome inhibitor, increases the level of ubiquitinated PPARα and inhibits its degradation [27]. This mechanism allows rapid responses in tightly regulated processes, such PPARα expression in circadian rhythm [183] and acute-phase inflammatory response [20].

MDM2, a ubiquitin ligase, promotes polyubiquitination at the A/B domain of PPARα, regulating its transcriptional activity and promoting its degradation [102]. Furthermore, this process is ligand-dependent, as the increasing concentrations of MDM2, in the presence of WY-14643, leads to decreased PPARα activity.

There is also a report of a mono-ubiquitination of PPARα by the ubiquitin ligase Muscle ring finger-1 (MURF1) in rat cardiomyocytes in vitro [278]. This modification inhibits FAO by inhibiting this isotype activity in cardiomyocytes in a proteasome independent manner, as this single ubiquitination targets PPARα export from the nucleus to the cytoplasm. Residues K292, K310, and K358, located around a newly identified nuclear export signal in the LBD (aa300–308), were identified as putative sites for the mono-ubiquitination [278].

PPARδ/β This isotype can undergo a constitutive polyubiquitination and degradation by 26S proteasome to keep low levels of the receptor in the absence of ligands, despite DNA binding [97]. The presence of PPARδ/β-specific agonists such as L-165041, GW-501516, and prostaglandin (PGI2) completely inhibits PPARδ/β proteolysis, increasing the half-life of the DNA-bound receptor, thus allowing the time for transactivation of target genes. This increase in PPARδ/β half-life can also be achieved in the presence of proteasome inhibitor, such as PS341 [97].

Another level of modulation was revealed by a study that show, in mouse fibroblasts, that the ligand-dependent ubiquitination of PPARδ/β and its subsequent degradation are also influenced by PPARδ/β protein levels [275]. At high PPARδ/β expression levels, the agonist GW-501516 strongly inhibits the receptor ubiquitination and degradation processes, which was not observed at low PPARδ/β levels.

PPARγ The ubiquitination of PPARγ has a role on its stability, as this NR has a short half-life ($t_{1/2}$ = 2 h) [334] and is regulated by ubiquitin proteasome system. The polyubiquitination that marks for degradation usually occurs on AF-2 region [157, 158]. However, the PPARγ activation by ligands, as TZDs, was demonstrated to accelerate the process of ubiquitination and degradation [83, 119]. 3 T3-F442A cells treated with pioglitazone presented increased ubiquitination

levels in a dose-dependent manner and a subsequent decreased in PPARγ2 protein expression [119].

PPARγ degradation by polyubiquitination plays diverse roles in different cell types. In adipocytes, the E3 ligases makorin RING finger protein 1 (MKRN1) [160] and seven in absentia homolog 2 (SIAH2) [157] target PPARγ for proteasomal degradation, determining its physiological effects on adipogenesis. While SIAH2 is required on this process [157], overexpression of MKRN1 inhibits adipocyte differentiation targeting K184 and K185 [160]. It was observed that PPARγ polyubiquitination and degradation by EGFR/MDM2 regulate cancer progression by accumulation of NF-κβ/p65 protein levels and increasing NF-κβ activation [350].

PPARγ polyubiquitination by the ligase complex Von Hippel-Lindau tumor suppressor (pVHL) also leads to the NR proteasomal degradation, being K404 and K434 the potential major ubiquitin acceptor residues in this case [234]. PPARγ degradation via pVHL resulted in the downregulation of ATP citrate lyase protein (ACLY), which is involved on tumor progression and is related to de novo synthesis of lipids, promoting cholesterol synthesis [234].

Despite the proteolytic function of ubiquitination, some ubiquitin ligases can play a role in prolonging PPARγ half-life [188]. In this case, the ubiquitin ligase neural precursor cell-expressed developmentally downregulated 4 (NEDD4) lengthen PPARγ half-life, adding ubiquitin in the hinge (K48 PPARγ2) and in the LBD, stabilizing PPARγ, and promoting adipogenesis in 3 T3-L1 cells, without changing the receptors activity [188]. Another ubiquitin E3 ligase, the tripartite motif protein 23 (TRIM23), has a critical role in the switching from early to late adipogenic function, stabilizing PPARγ protein by atypical polyubiquitin conjugation, that leads to reduced proteasomal degradation [342]. In the liver, the smad ubiquitin regulatory factor 1 (SMURF1) regulates the lipogenic activity of PPARγ attenuating its activity by K63 linked non-proteolytic ubiquitination, leading to hepatocytes protection against nonalcoholic fatty liver disease (NAFLD) [369].

Sites for other covalent modifications were also reported to be sites for ubiquitination. The EGFR-mediated PPARγ Y74 phosphorylation leads to PPARγ ubiquitination and degradation by MDM2 ubiquitin ligase in HEK293 and SW480 [350]. In addition, the targets for acetylation K184/K185 and K268/K293 were reported to suffer ubiquitination, making the protein prone to subsequent proteasomal degradation [69, 160, 260], indicating a possible crosstalk between these PTMs. For example, K184 and K185 are targets for MKRN1 ubiquitin addition, decreasing basal and ligand-dependent activation and targeting PPARγ for protein degradation [160], and K268/K293, for CUL4B-RING E3 ubiquitin ligase (CRL4B), leading to reduced PPARγ stability, as well as adipocyte differentiation (Dou 2019). In this last case, there is one report of aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, acting as the substrate receptor in CRL4B complex [69]. It was observed that after AhR overexpression, PPARγ stability was reduced, as well as adipocyte differentiation. On the other hand, AhR stimulated adipocyte differentiation in 3 T3-L1 cells. These results indicate that AhR could mediate PPARγ activity through posttranslational modifications [69].

21.2.5 Other PTMs in PPARs

21.2.5.1 PPARγ S-Nitrosylation and Nitration

Nitrosative stress occurs with an increase in reactive nitrogen species (RNS) and reactive oxygen species (ROS) formed from oxidative stress. Proteins can suffer two kinds of posttranslational modifications after nitrosative stress: reversible S-nitrosylation of thiol groups and irreversible protein tyrosine nitration [286, 306]. In the case of PPAR, only the isoform γ was shown to be modified by S-nitrosylation and tyrosine nitration (Fig. 21.8).

S-nitrosylation is the reaction of thiols at cysteine residues in the substrate proteins with NO or NO-derived species, resulting in an S-nitrosothiol derivative (RSNO), through a -SNO group formation [125, 306]. This reversible modification is mediated by nitric oxide synthases (NOS), affecting protein activity, protein-protein interactions, and protein location [286, 306]. There are two physiologically relevant denitrosylases to remove NO group from S-nitrosylated Cys thiol side chains: glutathione/S-nitrosoglutathione reductase (GSH/GSNOR) and the thioredoxin/thioredoxin reductase (Trx/TrxR) [125]. PPARγ was first identified to suffer S-nitrosylation in 2003, among S-nitrosylated proteins of activated murine mesangial cells treated with NO donors or appropriate controls [175].

PPARγ Pro-inflammatory macrophage negatively regulated the transcriptional activity of PPARγ in adipocytes by S-nitrosylation of PPARγ1 at the C168 (C198 in isoform γ2), promoted by the release of pro-inflammatory factors like nitric oxide (NO) [358]. This PTM reduced PPARγ ligand-dependent activation in HeLa cells, downregulated PPARγ target genes (*ADIPOQ, FABP4*, and periplin) in 3 T3-L1 adipocytes, and blocked adipogenic differentiation in Rat epididymal preadipocytes and mice 3 T3-L1. This downregulation of PPARγ is due to a decreased binding to the promoters of its target gene, possibly by protein degradation, as 3 T3-L1 cells treated with a NO donor (S-nitrosoglutathione - GSNO) had a decreased level of PPARγ protein. Pretreatment with the proteasome inhibitor MG132 partially prevented the decrease of PPARγ levels, suggesting that the proteasome-dependent degradation might account for the impaired PPARγ stability [358]. These in vitro results agreed with in vivo results showing that obese diabetic db/db mice have severe macrophage infiltration in visceral WAT, while gene expression of NO synthase (iNOS) was increased, and the adiponectin expression was decreased.

A S-nitrosylation in PPARγ2 (C139) was described [38] after observation of the effects of NO in bone-marrow-derived MSCs, precursor cells for adipocytes and osteoblasts [229]. The S-nitrosylation residue (C139) was suggested by the predicted acid-based nitrosylation conservative motif [299] and confirmed through single point mutations [38]. Treatment of HEK-293 T cells with a GSNO decreased PPARγ activity, which was not completely recovered after rosiglitazone treatment. Animal model denitrosylases GSNOR deficient (GSNOR−/−) presented decreased adipogenesis, with smaller adipocytes, lower body weight, and fat mass, with an

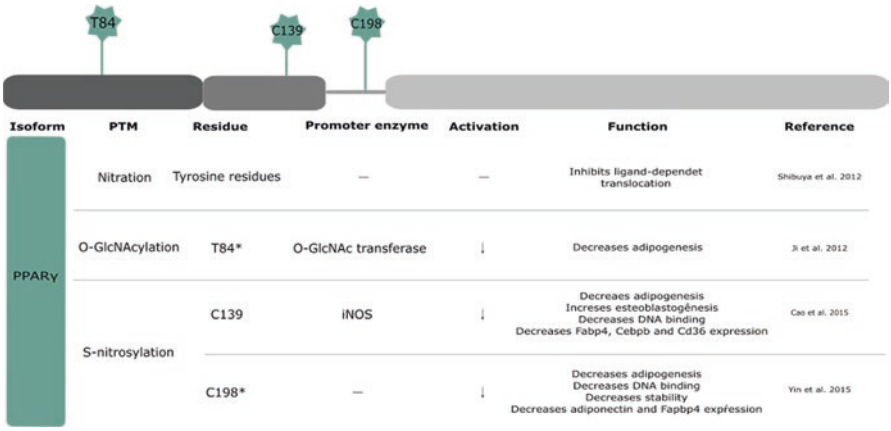
increased proportion of lean mass. Moreover, these animals presented increased osteoblastic differentiation with augmented osteoclastic effects [38]. S-nitrosylation of PPARγ increased 50% in MSCs of GSNOR−/− compared to wild type, suggesting the PPARγ role in the observed effects. In cell culture, GSNOR−/− MSCs showed decreased differentiation and expression of PPARγ target genes involved in adipocyte differentiation (*Cebpb, Fabp4, Cd36*), while adiponectin expression remained the same as wild type. These effects were also observed in wild-type cells treated with GSNOR inhibitor. PPARγ decreased activity observed in reporter gene assays was not followed by a decrease in PPARγ mRNA expression, but it was observed decreased binding affinity by CHIP assays to FABP4 promoter region [38].

Another modification caused by nitrosative stress is the irreversible nitration of tyrosine residues. In this case, the tyrosine amino acid of target proteins reacts with the cytotoxic oxidant peroxynitrite (OONO−), generated from NO and superoxide. This reaction leads to a covalent addition of a nitro group (-NO2) to one of the two equivalent ortho-carbons of the tyrosine residues aromatic ring [286, 306]. This covalent modification affects protein function and structure, including a change in the proteolytic degradation rate and protein activity loss [286]. Nitrosative stress is also present in inflammation; therefore, tyrosine residues' nitration is considered a marker of inflammation [286, 306]. Macrophages are considered key players in inflammation and highly express PPARγ, which also plays a role in the control of inflammation, particularly modulating the production of inflammatory mediators [209]. PPARγ nitration was identified in macrophage-like cell line RAW 264 stimulated by peroxynitrite, LPS, or tumor necrosis factor-K (TNF-K). This nitration inhibits ligand-induced translocation into the nucleus, which might change the PPARγ function [290]; however, the key tyrosine residues that suffer nitration were not identified yet.

21.2.5.2 PPARγ O-GlcNAc

The addition of a single residue of O-linked *N*-acetylglucosamine (O-GlcNAc) is a PTM that occurs in the nuclear and cytosolic compartments of eukaryotic cells [99]. In mammals, this modification is dynamically regulated by two highly conserved enzymes: the glycosyltransferase, named O-linked N-acetylglucosaminyltransferase (GlcNAc transferase, OGT), and the antagonistic enzyme β-*N*-acetylglucosaminidase (O-GlcNAcase, OGA). Analogous to the other PTMs, evidence indicated that O-GlcNAc modification of protein could regulate its activity. O-GlcNAc addition has been mapped to modify serine/threonine (S/T), which are the same sites for phosphorylation addition by kinases, implying that the two modifications might compete for the same site [99].

PPARγ O-GlcNAc modifications were reported in 3 T3-L1 adipocytes [145], by using immunoprecipitation and western blotting techniques (Fig. 21.8). Both PPARγ1 and γ2 were reported to have O-GlcNAc in this cell type, although only PPARγ1O-GlcNAc modification was significantly increased in high glucose condi-



Isoform	PTM	Residue	Promoter enzyme	Activation	Function	Reference
PPARγ	Nitration	Tyrosine residues	–	–	Inhibits ligand-dependet translocation	Shibuya et al. 2012
	O-GlcNAcylation	T84*	O-GlcNAc transferase	↓	Decreases adipogenesis	Ji et al. 2012
	S-nitrosylation	C139	iNOS	↓	Decreaes adipogenesis Increses esteoblastogênesis Decreases DNA binding Decreases Fabp4, Cebpb and Cd36 expression	Cao et al. 2015
		C198*	–	↓	Decreases adipogenesis Decreases DNA binding Decreases stability Decreases adiponectin and Fapbp4 expression	Yin et al. 2015

Fig. 21.8 Others PTMs in PPARs. (Top) Primary structure with representative of other PTMs in PPARs. (Bottom) Table summarizing identified other PTMs (S-nitrosylation, Nitration, O-GlcNAc) sites in PPARs and their effects. Residues are numbered after PPARγ2 isoform. There is an asterisk for PTM only described for y1 isoform. Effect on PPAR activation is described as upward arrow for activation and downward arrow for repression

tions. This modification was not identified for PPARα and PPARδ/β isotypes. Since O-GlcNAc modification for PPARγ2 was not increased under high glucose conditions, this modification was further investigated for the isoform PPARγ1. Protein digestion and single point mutations assays showed that the residue which suffers O-GlcNAc-modification PPARγ1 is T54, present in the AF-1 domain. Through a reporter gene assay in HeLa cells, using T54A mutant protein and OGA inhibitor, it was demonstrated that the presence of O-GlcNAc reduces PPARγ1 activation, which is not rescued by rosiglitazone treatment [145]. Regarding physiological conditions, it was previously described that a general increase in O-GlcNAc is observed in adipocyte differentiation and that inhibition of this PTM decreases adipogenesis [134, 142]. Treatment of 3 T3-L1 adipocytes with OGA inhibitor resulted in more O-GlcNAc modifications of PPARγ1 and reduced transcriptional activity in this cell type, indicating that, although proteins level of PPARγ1 targets in adipocyte differentiation were not changed, the decrease in adipocyte differentiation might be due to the decrease in this receptor activity through O-GlcNAc modification [145].

21.3 Crosstalk of PPARγ PTMs

Reports of crosstalk are usually found between proteins of a signaling cascade; however, PTM crosstalk can occur within a single protein [60, 327]. In general, the PTM crosstalk can be classified into two forms: positive or negative [327]. In positive crosstalk, one PTM can signalize for the addition of a second PTM at the same site or for recognition by a binding protein that carries out a second modification

(e.g., phosphorylation-dependent SUMOylation) [304, 357]. In this second case, the first PTM can induce conformational changes in the protein that enable access for the protein effector of the second PTM. In negative crosstalk, direct competition for modifying a single residue in a protein can occur, or indirectly by masking the recognition site for a second PTM [327]. An example of direct negative crosstalk is the addition of O-GlcNAc mapped to modify serine/threonine (S/T), which are the same sites for phosphorylation modifications by kinases [99, 115, 341]. Furthermore, lysine residues were reported to suffer not only acetylation but SUMOylation and ubiquitination as well, where the latter plays an important role signaling for protein degradation by proteasome pathway and the former is important for regulating cellular processes, including cell cycle, apoptosis, DNA repair, and signal transduction pathways [120].

In the case of PPARγ, direct negative crosstalk was reported in many sites that are a target for different modifications, for example, lysyl residues, which are both targets for acetylation and SUMOylation or ubiquitination. Some positive PTM crosstalks were also reported, such as acetylation, which may induce phosphorylation, phosphorylation-mediated SUMOylation, and ubiquitination. These combinatorial actions of PTMs provide a fine-tuning mechanism in regulating protein function. So far, no PTM crosstalk was identified for the other PPAR isotypes.

21.3.1 Negative Crosstalk at PPARγ K184/K185 and K268/K293 Which Are Shared Residues for Acetylation and Ubiquitination

Examples of direct negative crosstalk that might occur at PPARγ2 K184/K185 are acetylation and ubiquitination. Acetylation in PPARγ1 K154/K155 (which corresponds to PPARγ2 K184/K185) was described, and its acetylation mimetics mutants (K154Q/K155Q) were very similar to wild type in lipogenic differentiation, whereas acetylation-defective (K154R/K155R) mutants decreased lipogenic differentiation, indicating repression of PPARγ1 activity [314]. MKNR1 ubiquitination of these residues in PPARγ2 K184/K185 also reduced basal and ligand-dependent activation, targeting the receptor for proteasome degradation [160]. These results allow us to speculate that the deacetylation of K154/K155 allows for PPARγ1 ubiquitination, targeting for proteasome degradation and reducing protein activity; however, this hypothesis still needs to be addressed experimentally.

Another pair of acetylated residues, PPARγ2 K268/K293, is upregulated in obesity and was reported to favor lipid storage in adipocytes and cell proliferation, in agreement with acetylation mimetics mutants (K268Q/K293Q), increasing expression of insulin-resistant genes on WAT of HFD-obese mice [260]. Deacetylation of K268 and K293 residues led to the browning of WAT and repression of insulin resistance and adipogenic genes [260], in agreement with PPARγ1 K154/K155 deacetylation results of decreased lipogenic differentiation and decreased PPAR

activation with consequent protein degradation [160]. Corroborating the hypothesis of K268/K293 being shared residues for acetylation and ubiquitination, one report of ubiquitin-mediated degradation of AhR transcription factor shows the same lysine residues acting as the substrate and receptor for CRL4B E3 ligase.

Taken together, these reports suggest that PPARγ deacetylation in K184/K185 and K268/K293 pairs both allows ubiquitination and targets for protein degradation, reducing lipogenic differentiation.

21.3.2 Negative Crosstalk at PPARγ K107, Which Is a Shared Residue for SUMOylation and Acetylation

PPARγ2 K107 is a shared residue for SUMOylation and acetylation. This residue's SUMO modification is the most studied PPAR SUMOylation and strongly represses PPARγ, repressing inflammatory genes in macrophages [143]. DeSUMOylating of this residue was shown to increase the receptor activity, increasing expression of some PPARγ target genes, such as *Fabp3* and *Cd36*, both in the absence and presence of rosiglitazone, in rat C2C12 myotubes [51]. K107 was strongly acetylated in a mass spectrometry analysis of 3 T3-L1; however, no studies were done about its physiologic effects [260]. Based on the effects of acetylation in other PPARγ residues (K154/K155, K268/K293), acetylated mimetics mutants of PPARγ had effects very similar to native, whereas deacetylation of these residues was shown to decrease lipogenic differentiation, and to promote expression of "browning" genes and adiponectin in WAT [260, 314]. Extrapolating to K107 deacetylation, its effect could also be of decreased lipogenic differentiation, which would agree with SUMOylation effects of PPARγ repression, suggesting that when K107 is deacetylated, it could be SUMOylated. Although it is clear that this same residue can suffer two types of PTM, the molecular basis of this alternance is not determined yet. It will be of interest to determine if there is a reciprocal regulation of acetylation and SUMOylation at this site during browning or adipogenesis.

21.3.3 Positive Crosstalk of PPARγ1 T74 and PPARα S73 Phosphorylation-Dependent Ubiquitination

PPARγ1 T74 and PPARα S73 were reported to be target of phosphorylation-dependent ubiquitination, signaling for protein degradation [102, 350]. Y74 phosphorylation occurs more frequently in colonic cancer tissues, where PPARγ phosphorylation leads to ubiquitination by MDM2, signaling for protein degradation. Y74 phosphorylation seems important to colonic cancer cell survival and proliferation since Y74A mutants decreased tumor-associated gene expression (c-MYC,

COX-2, and IL-6), inhibited cell proliferation, colony formation, and anti-apoptotic gene expression [350].

In the case of PPARα S73 phosphorylation, a BVRA-KO mouse model addressed this phosphorylation's effects in the liver. In the BVRA-KO mice, upregulation of S73 phosphorylation was observed together with hepatic steatosis and decreased expression of PPARα target genes in the liver (*Fgf21, Cpt1a, Cd26*), suggesting that the phosphorylation decreased PPARα activity [127]. Lower levels of ubiquitination observed with S73A PPARα indicates that the reduced activity of the receptor after phosphorylation might be due to increased ubiquitination and protein degradation [127].

21.3.4 Positive Crosstalk of PPARγ K268/K293 Acetylations and S273 Phosphorylation

In PPARγ, K268/K293 acetylation might be an example of positive crosstalk, whereas the presence of a modification induces the occurrence of a second one. S273 phosphorylation was reported to correlate with K268 and K293 acetylation because this phosphorylation was increased in the presence of K293 acetylation mimetic mutant (K293Q) [260]. Both modifications were reported independently to have overlapping effects in adipogenesis and browning: deacetylation mimetics mutants (K268R/K293R) and S273 phosphorylation-inhibition by rosiglitazone increased expression of adipokine and "brown genes" in WAT [45, 236, 260]. However, phospho-defective mutation (S273A) alone did not promote upregulation of "brown genes", whereas the combined deacetylation and dephosphorylation mimetic (S273A/K268R/K293R) had a similar result as the deacetylation mimetic (K268R/K293R) [260]. This upregulation of "brown genes" in both deacetylation mimetics was more pronounced together with rosiglitazone treatment, [260], indicating that PPARγ activation and deacetylation have a combined effect increasing PPARγ browning activity.

These residues' structural proximity might explain this positive crosstalk among S273 phosphorylation and K268/K293 acetylation. S273 is buried within the grooved, lined by K268 and K293, and it was suggested that the acetylation state of these two residues could induce conformational changes in the protein structure, which affect access to related kinases or phosphatases, to the phosphorylation site of S273 [260]. In some cases, deacetylation of proteins was reported to increase their phosphorylation [258, 259]; however, in this case, it was suggested that mainly K293 acetylation would induce conformational changes allowing S273 phosphorylation. This hypothesis is addressed because K293 acetylation mimetic mutant was reported to increase S273 phosphorylation and their deacetylated and dephosphorylated states were shown to have the same effects inducing browning of WAT and decreasing lipogenesis [260].

Nevertheless, another work showed that deletion of SIRT1 from adipocytes leads to increased PPARγ acetylation under short-term (5 weeks) and long-term (15 weeks) HFD, but S273 phosphorylation levels, which were higher in the SIRT1-KO animals, decreased after long-term HFD compared to wild-type animals [210]. These results indicate that more research is necessary to understand the fine-tuning modulation of PTM crosstalk regulating PPARγ functions in metabolic syndrome.

21.3.5 Positive of Crosstalk of PPARγ S112 Phosphorylation-Dependent SUMOylation of K107

A phosphorylation-dependent SUMOylation motif (PDSM) ΨKxExxSP has been identified in NRs, including PPARγ and thyroid hormone receptor (TRβ) [126]. In PPARγ2, S112 phosphorylation promoted a phosphorylation-dependent SUMOylation of K107, as demonstrated by decreased SUMOylation in the presence of phosphorylation defective mimetic S273A [353]. S112 and K107 are close in the PDSM, and the proposed model is that S112 phosphorylation induces a conformational change in PPARγ that allows for the SUMO-1 SUMOylation of K107 [323]. Both phosphorylation and SUMOylation at these residues cause a decrease in the receptor activity, with impaired coactivator and increased corepressor recruitment, and activation of the adipogenic gene expression pathway [122, 135, 150, 287, 291, 353]. The blockage of these PTMs resulted in the improvement of insulin sensitivity [150, 265].

21.4 Modulation of PPARs PTMs

Modulations of PPAR posttranslational modifications (PTMs) were reported in the literature in two major ways. The most common is through the use of a PPAR ligand that binds in the LBD and induces conformational changes that allow or decrease the occurrence of a PTM. Another way described in the literature for PTM characterization is using an activator or inhibitor of the protein responsible for the PTM addition. However, as will be discussed further in this topic, kinases, acetylases, and other proteins involved in the addition of a posttranslational modification at PPARs are also involved in modulating other pathways. Therefore, pharmaceutical inhibition or activation of these effectors seems not to be the best strategy for the specific modulation of PPARs PTMs.

On the other hand, the use of PPARs LBD ligands to inhibit or promote a specific PTM could lead to undesired side effects resulting in receptor activation or inhibition, for example, the use of rosiglitazone for the inhibition of PPARγ S273 phosphorylation. Aiming to block the S273 phosphorylation effect of insulin resistance,

the agonist rosiglitazone activates the receptor, leading to adipogenic and osteoclastic effects. This constant activation of PPARγ by rosiglitazone in diabetic patients resulted in the side effects of weight gain and bone loss, which resulted in the removal of the drug in the treatment of T2D. In this topic, we are going to present the molecules that can regulate the addition of PTM in PPARs, either by promoting structural changes in the receptor LBD or by activating/inhibiting the effectors proteins of these modifications. Moreover, we are going to discuss the positive and negative aspects of each modulation, discoursing about the perspectives of the clinical use of molecules to modulate PPAR function through PTM regulation.

21.4.1 Modulation by PPAR Ligands

In this section, we are going to focus on ligands that bind in the LBP of the PPAR-LBD and promote conformational changes, interfering directly or indirectly in the PTMs occurrence. The binding site cavity for the three PPAR isotypes is very similar and is located in their protein cores [75, 254]. The Y-shaped LBP is mainly formed by hydrophobic residues and presents a large volume of ~1300 Å3, which allows the interaction of single and multiple branched ligands in different conformations [59]. This pocket is flanked by helix (H) 3, 5, 7, and 10 and by an antiparallel beta-sheet. The space between H3 and beta-sheet is the ligand-entry site, whereby different ligands can access the PPAR LBP, promoting structural changes mostly in H12.

21.4.1.1 PPARα

In PPARα, three PTMs seem to have their presence regulated by PPAR ligand: a polyubiquitination enhanced by WY-14643 [102]; a K185 SUMOylation, in the hinge domain, reduced in the presence of GW-7647 [257]; and K358 SUMOylation, in the LBD, increased in the presence of WY-14643[185]. No sites for the polyubiquitination by MDM2 were identified, but this modification at the A/B domain of PPARα decreased its basal activity, promoting its degradation, and this process was enhanced by WY-14643 [102].

Both SUMOylations promoted repression of PPARα, as defective mutations of K165A and K358A increased the receptor activity compared to the native protein. This inhibited activity could be explained by increased recruitment of NCoR in the case of K165 and GA-binding proteins and histone deacetylases in K385 [185, 257]. However, these two SUMOylations differ regarding the effect of PPARα agonists. Treatment with GW-7647 reduced specifically PPARα SUMOylation; however, it is not clear if ligand binding impairs the SUMOylation of PPARα or promotes its deSUMOylation [257]. On the other hand, treatment with WY-14643 increased PPARα SUMOylation because the agonist induces conformational changes in the LBD, in which K358 is presented at the surface and therefore available for

SUMOylation, in contrast to the antagonist-induced conformation, in which case K358 is hidden [185]. This agonist-induced conformational change that exposes K358 for acetylation might also hide K165, protecting it from SUMOylation.

Regarding the others PPARα PTMs, ligands such as oleic acid and WY-14643 were reported only to modulate the activity of the receptor bearing phosphorylations at S12/S21 and S73, and studies analyzing the effects of the ligands in the modification per se are still required [17, 147, 300]. S12/S21 phosphorylation increased basal PPARα transactivation, which was enhanced in the presence of the ligand oleic acid or insulin [17, 147]. Downregulation of these phosphorylations was observed in TTD mice, which showed downregulation of *Cyp4a1* and *Pacoth* in the liver [55]. On the other hand, phosphorylation of S73 was reported to decrease PPARα activation induced by ligand WY-14643 [127]. Downregulation of *Cyp4a12* and *Cpt1a* was observed in BVRA KO mice bearing increased S73 phosphorylation [127]. These results indicate that S12/S21 and S73 phosphorylations have antagonist effects in hepatocytes.

However, since S12/S21 and S73 phosphorylations are in the AF-1 domain, an activation domain independent of ligand, it might be improbable that ligands bound to the LBD could induce conformational changes to inhibit or increase the occurrence of phosphorylation on AF-1 domain. On the other hand, conformational modifications or other allosteric-like effects might happen, but further studies in this field are necessary to elucidate these mechanisms.

21.4.1.2 PPARδ/β

Until now, few PTMs are related to the subtype PPARδ/β. Unlike most nuclear receptors that are degraded upon ligand binding, PPARδ/β ligands (L-165,041, GW501516, and PGI2) were reported to inhibit the ubiquitination of this receptor, thereby preventing its degradation [97, 275]. In this case, the ligand-mediated ubiquitination might be influenced by PPARδ/β protein levels, as was observed in mouse fibroblasts transfected to overexpress PPARδ/β, where the agonist GW-501516 strongly inhibits the ubiquitination and degradation of PPARδ/β. However, this effect was not observed at moderate protein levels, indicating that the process is not influenced by the ligand presence, but by the protein level [275].

21.4.1.3 PPARγ

As mentioned before, the PPARγ is the most studied isotype due to its role and relevance in obesity, diabetes, and other metabolic disorders. Ligand effect on PTM modulation was not reported for modifications occurring at AF-1 and DBD (phosphorylations at S46, S51, Y74, and Y78 and PPARγ1 acetylations at K154/K155). However, the influence of ligands in PTMs that occur in the LBD was extensively reported, especially for phosphorylation at S273 [45].

Rosiglitazone is the PPARγ ligand used for many years for T2D treatment since it acts as an insulin sensitizer by blocking phosphorylation at S273, whereas its full agonism activates the nuclear receptor and promotes the transcription of genes related to adipogenesis. However, its use causes side effects, such as weight gain and others related to fluid retention and cardiac hypertrophy. Thus, this molecule was removed from the market [45, 93, 339]. Besides this, rosiglitazone is still used as a classical ligand for PPARγ function studies, mainly on in vitro and in vivo assays.

The only PPARγ phosphorylation reported to be modulated by rosiglitazone is at the residue S273 [45]. Treatment with this and other PPARγ ligands blocks S273 phosphorylation and results in improving insulin sensitivity, as will be discussed in detail in the next topic.

Rosiglitazone treatment also decreased K268/K293 acetylation in a SIRT1-dependent manner [260]. Furthermore, acetylation defective animals for both K268R/K293R, treated with rosiglitazone, maintained the insulin-sensitizing, glucose-lowering response to TZDs, while not showing the TZDs adverse effects on fat deposition, bone density, fluid retention, and cardiac hypertrophy [171]. The crosstalk among S273 unphosphorylated state and K268/K263 deacetylation indicates that treatment with rosiglitazone promotes insulin sensitivity improvement due to inhibition of S273 phosphorylation, with no collateral effects of PPARγ activation, which may be the resulted of the K268/K293 deacetylated state. Drugs that inhibit both S273 phosphorylation and K268/K293 acetylation could be interesting to the treatment of T2D and obesity.

Regarding SUMOylations, rosiglitazone was reported to enhance this PTM at K395 [12, 245] and to activate PPARγ SUMOylation in HK-2 cells [199]. Ligand-dependent increase in K395 SUMOylation might be explained by structural analysis: crystal structures of apo and rosiglitazone-bound forms of PPARγ1 indicate that K365 is oriented toward the interior of the LBD in the apo form but is solvent-exposed in the rosiglitazone-bound form, allowing for covalent attachment of SUMO [245]. However, rosiglitazone and another ligand, GW1929, negatively regulate SUMOylation of K395 by intramolecular communication between the C-terminal LBD and the N-terminal AF1 domain [67].

Rosiglitazone and other TZD ligands (troglitazone and pioglitazone), although increasing PPARγ activity, were reported to enhance the receptor ubiquitination and degradation [119, 158], but interestingly pioglitazone was also reported to inhibit EGFR/MDM2 signaling-mediated PPARγ degradation, suppressing cancer cell chemoresistance [289].

21.4.1.4 Modulation of PPARγ S273 Phosphorylation

Phosphorylation of S273 is one of the most studied PPARγ PTM due to its involvement in insulin resistance in obese and diabetic humans. As mentioned before, rosiglitazone is a member of the TZD class, together with pioglitazone and troglitazone, acting as PPARγ agonists and binding directly in the LBD of the protein, stabilizing

H12, and recruiting coactivators that will promote expression of PPARγ target genes. It was also reported that rosiglitazone and pioglitazone were able to block the S273 phosphorylation [45], although this effect has not been reported yet to troglitazone. This inhibition, mainly by rosiglitazone, was the cause of the improvement of insulin sensitivity, reducing fasting glucose and insulin levels, and rosiglitazone was used for many years for the treatment of T2D. However, treatment with this drug resulted in several side effects, which are related to the strong PPARγ activation caused by this molecule, which leads to the expression of genes related to adipogenesis, promoting weight gain, increase on hepatic steatosis, and fluid retention, among others side effects [45, 92, 204]. Moreover, phospho-defective (S273A) $PPAR\gamma^{A/A}$ did not show innately any of the TZD-associated side effects (bone loss, fluid retention, and increase in adipocyte size) [108], corroborating the hypothesis that these effects are associated with PPARγ full agonism.

It is important to highlight this phosphorylation mechanism, which involves strong interaction between the kinase, specifically CDK/p25, and their specific substrate [65, 247]. Two elements guide this mechanism: the first one is the recognition of the phosphorylation motif by the catalytic site of the kinase, and the second, mainly involved in S273 phosphorylation, is the substrate recruitment, involving an increase of encounters between the enzyme and the substrate through distal docking sites [65, 311]. In PPARγ there is a noncontiguous recognition site (K261, K263, and K265), located at the H2'-H3 loop of the LBD region, being essential for CDK5 and PPARγ interaction [273]. However, these residues seem not to be involved in inhibiting S273 phosphorylation by PPARγ ligands, since these molecules do not interact with the H2'-H3 loop [273]. It was described that another residue, I341, may be involved in this process, mainly due to a structural shift promoted by ligand interaction with the receptor, stabilizing H2′ and part of the H2-H2′ loop and impacting its association with CDK5 [273]. Here it is shown PPARγ LBP ligands that promote insulin sensitization, with lower activation and, except for a few cases, experimentally confirmed blocking of S273 phosphorylation.

YR4-42 In 2019, a PPARγ agonist was reported to block S273 phosphorylation: YR4-42, a tetrahydroisoquinoline derivative [137]. This ligand showed weaker affinity and equivalent activation of PPARγ compared to pioglitazone [137]. In 3 T3-L1 adipocytes, YR4-42 promoted fewer lipids droplets than TZDs, same triglyceride levels as the control group, and blockage of S273 phosphorylation. Moreover, through a diet-induced obese (DIO) mouse model, it was shown that YR4-42 could control blood glucose and improves insulin sensitivity, with results similar to pioglitazone, also decreasing serum triglycerides, total cholesterol, and FFA, with lower body weight and an improvement on hepatic steatosis. Regarding gene expression, it was observed upregulation in genes involved in glucose metabolism and on thermogenesis, as *Cidea* and *Ucp1* [137].

WSF-7 Another PPARγ agonist was discovered at the end of 2019, called WSF-7 (5,5,7-trimethyl-3-(p-tolyl)- 3,3a,4,5,6,7-hexahydro-4,6-methanobenzo[c]isoxazol-7-ol), which is derived from natural monoterpene α-pinene [365]. This molecule

was detected by a screening for PPARγ agonists with the capacity of inhibiting S273 phosphorylation. In vitro studies demonstrated its ability of binding to the LBD of this receptor and activate it, also promoting adipogenesis, but it is less potent than rosiglitazone. Moreover, this ligand upregulated adiponectin expression and its oligomerization, increased insulin-stimulated glucose uptake, and SLC2A4 protein expression. They also confirmed its effects on inhibiting S273 phosphorylation in 3 T3-L1 cells, indicating WSF-7 as a potential insulin sensitizer and drug for T2D treatment [365].

EPA-PC and EPA-PE Sea cucumber phospholipids were also detected as PPARγ agonists, mainly the phosphatidylcholine (EPA-PC) and phosphatidylethanolamine (EPA-PE) ones, binding to this receptor with high affinity, as well as to PPARα [316]. In vitro assays demonstrated that these compounds promoted adipocyte differentiation and lipid accumulation, promoting an increased expression of *Pparg, Fabp4, Fas*, and *Cebpa*. EPA-PC and EPA-PE also activated hepatic fatty acid β-oxidation in HepG2 cells. In addition, in vivo experiments revealed that EPA-PC and EPA-PE treatment slightly decreased S273 phosphorylation, but increased the protein expression of CD36 and FABP4, also suppressing the increase in iWAT and eWAT weight, reducing adipocytes size and lipid droplets. These molecules ameliorate glucose intolerance and insulin resistance in mice, with a significant reduction in triglycerides, cholesterol, and non-esterified fatty acids (NEFA), suggesting its role on improving metabolism and as a new therapeutic approach on T2D treatment [316].

In order to avoid the undesirable side effects of PPARγ full activation (adipogenesis, bone loss, etc.), other researchers are focusing on molecules that act as S273 blockers, but not as receptor full activators, acting as partial agonists or non-agonists. Some of them are presented here, showing their physiological roles related to this PTM inhibition (Fig. 21.9).

nTZDpa In this context, in 2003, 5-Chloro-1-[(4-chlorophenyl)methyl]-3-(phenylthio)-1H-indole-2-carboxylic acid (nTZDpa) was first described as potent and selective PPARγ partial agonist, antagonizing the effects of full agonists [23]. On in vitro assays, this molecule treatment reduced the lipid content of fully differentiated adipocytes, causing alterations in *Fabp4* expression. In a few experiments in vivo with DIO mice, nTZDpa effects on attenuating insulin resistance and hyperglycemia, decreasing weight gain, and increasing adiponectin expression were also observed [23]. After, in 2010, when S273 phosphorylation was reported, this molecule was described as a blocker of PPARγ phosphorylation [45].

2-BABAs After that, in 2004, it was reported another class of molecules, the 5-substituted 2-benzoylaminobenzoic acids (2-BABAs), which binds to PPARγ without direct interaction with H12, although activating the receptor, herein being classified as partial agonists [239]. Among them, the compound BVT.13 was evaluated in ob/ob mice, in which, although resulting in a significant reduction in fasting plasma glucose, triglycerides, plasma insulin, and FFA, the treatment led to weight

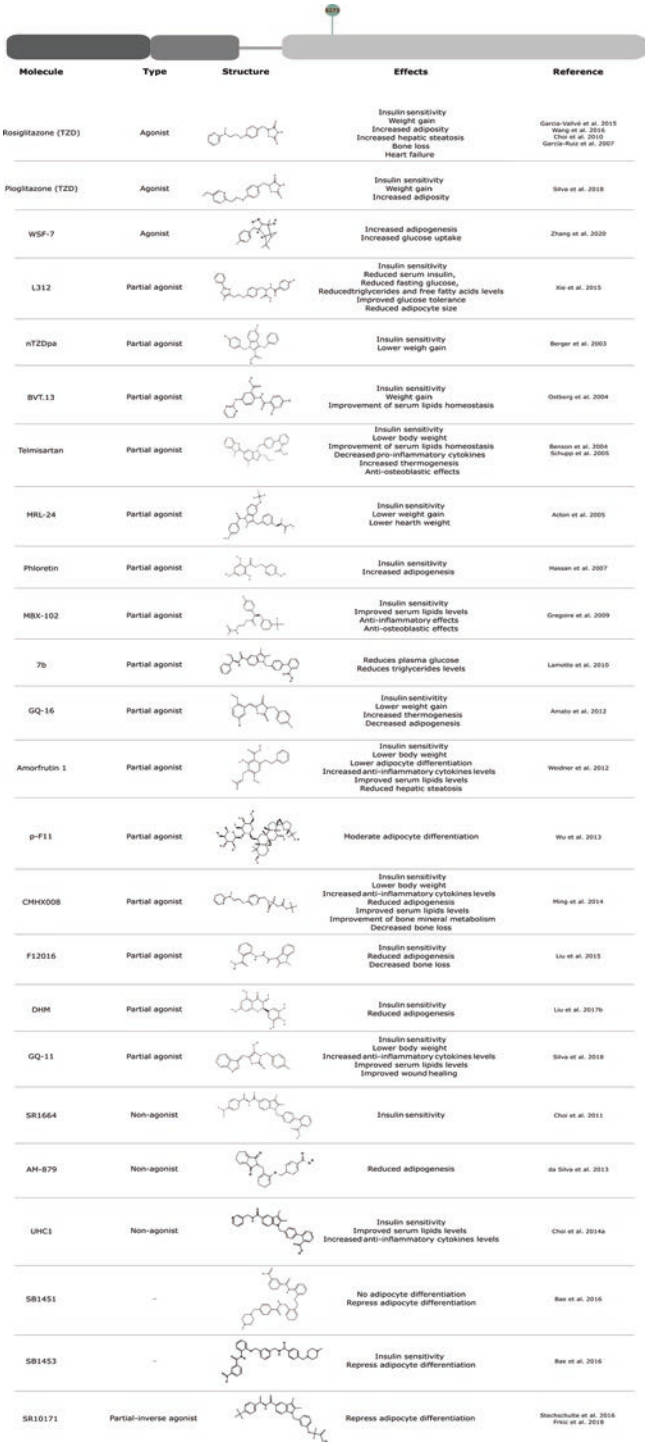


Fig. 21.9 PPARγ ligands that prevents S273 phosphorylation and their main effects. Here is listed only ligands with retrieved structural information

gain [239]. This compound was further investigated in vitro, where the ligand showed moderate transcriptional activity and confirmed its lack of interaction with H12 [33], also acting as a blocker of S273 phosphorylation [45].

TEL In 2004, an angiotensin II receptor blocker called telmisartan (TEL), was reported as a partial agonist of PPARγ [22, 283], and molecular docking analysis indicated that TEL interacts with the receptor by H3, H6, and H7, making strong hydrophobic interactions, and it does not appear to make contact with AF-2 region or histidine adjacent regions [22]. Although in vitro assays showed that TEL promoted adipogenesis in 3 T3-L1 cells, this result was not compared to tosiglitazone's effects on differentiation [22]. In adipocytes, TEL upregulates PPARγ target genes related to adipogenesis but in a less extent than rosiglitazone and decreased *Cfd* expression [166, 283]. Cellular assays also demonstrated that TEL upregulates thermogenic genes, did not have anti-osteoblastic activity, and decreased S273 phosphorylation levels [166]. In the cellular model of insulin resistance condition through TNFα treatment, TEL was able to reduce the TNFα- increased PPARγ S273 phosphorylation, reverse the decrease on glucose uptake, partially restore expression levels of *Adipoq, Cfd*, leptin (*Lep*), and *Slc2a4*, and decrease the expression of *Fabp4* [74].

TEL treatment in three DIO rodent models (C57BL/6 J mice, OLETF rats, and Male Sprague-Dawley rats) and one obese diabetic yellow agouti Avy/a mice resulted in lower weight gain and decrease in glucose, insulin, and lipids levels [22, 166, 283, 367]. Besides, in Avy/a mice, TEL did not affect the volume and structure of trabecular bone, with no fat accumulation in the marrow, and the combination of TEL and rosiglitazone treatments resulted in partial protection against bone loss [166]. TEL treatment in diabetic mice also induced the expression of beige markers, increased oxygen consumption, and carbon dioxide production, increasing respiration rate and confirming its action on energy expenditure [166]. Moreover, this molecule also provoked a decrease of pro-inflammatory cytokines and leptin levels, as well as an increase in adiponectin and a significant reduction in insulin resistance in OLETF rats [367]. Preliminary human studies showed that TEL improved insulin resistance in hypertensive and T2D, whereas no significant changes observed in adiponectin were upregulated only in high doses of TEL body weight, fasting plasma glucose, and plasma lipids levels [224, 321].

MRL24 A benzoyl indole called MRL24 was discovered in 2005 as a poor agonist of PPARγ with significant anti-diabetic effects, reducing weight gain, heart weight, and glycemia, when compared to rosiglitazone, in db/db mice [1]. This ligand also improved insulin sensitivity and glucose tolerance, as well as fasting insulin levels on mice fed HFD, mostly due to its action on reducing CDK5-mediated phosphorylation [45].

Phloretin In 2007, based on researches focused on the effects of flavonoids and chronic diseases, the chalcone phloretin was identified as PPARγ ligand [117]. Using 3 T3-L1 cells, it was observed that phloretin treatment resulted in an increase

on lipid accumulation, an increase on the triglycerides content, and in adiponectin expression and secretion, together with an increase on the expression of adipogenic markers, such as *Pparγ, Cd36,* lipoprotein lipase *(Lpl),* and *Cebpa* during the process of differentiation, indicating its effects on adipogenesis and suggesting its possible role on insulin sensitivity [117]. Later on, microarray analysis of phloretin treatment on adipocytes identified an upregulation of genes associated with carbohydrate and lipid metabolism, as well as of genes encoding adipokines and transcriptional regulators associated with adipocyte phenotype, confirming the previous results [118].

Phloretin also affected the insulin signaling pathway, mainly by increasing phosphor-Akt and phosphor-GSK3β, despite no effect on the AMPK pathway, revealing its adipogenesis role to the PI3K-AKT signaling pathway [292]. In vivo experiments with C57BL BKS-DB mice showed that treatment with phloretin increased food consumption with no effect on body weight, decreasing blood glucose and cholesterol levels and improving glucose tolerance. It was also observed an increase in PI3K and AKT's activity on mouse adipose tissue, besides an increase in proteins of adipogenic markers, as SLC2A4 (former GLUT4) and CD36 confirming the in vitro results [292].

Other in vivo experiments were performed to evaluate phloretin's effects on glucose metabolism, and it was observed that the treatment with this flavonoid protects mice from HFD-induced obesity, with no weight gain, loss of fat mass, and smaller WAT, suppressing lipid accumulation on this tissue. It was also detected an increase in *Adipoq* expression, decreased fat content on the liver due to a reduction in the expression of PPARγ, and a decrease in glycemia and an improvement in insulin sensitivity [6]. Molecular docking and molecular dynamics analysis showed that the PPAR-phloretin complex was formed by three hydrogen bonds and six hydrophobic interactions, suggesting that phloretin was effectively bound to PPARγ [174]. All these positive effects of phloretin on glucose homeostasis and insulin sensitivity, as well as its binding site in an activated conformation of PPARγ, suggest that this ligand decreases S273 phosphorylation [174]; however, more experiments are required to confirm the phosphorylation modulation.

MBX-102 Another PPARγ partial-agonist group, MBX-102, was described in 2009 [104]. This compound belongs to the halofenate compounds family, which are a racemic mixture of (−)- and (+)-[2-acetoaminoethyl (4-chlorophenyl) (3-trifluoromethylphenoxy) acetate]. These compounds have already been clinically tested in the 1970s, revealing its actions as hypolipidemic and hypouricemic agents [13]. MBX-102 is an enantiomer of halofenate, a pro-drug ester that is wholly modified in vivo by nonspecific serum esterases to the mature free form MBX-102 acid, which is the circulating form of the molecule. In vitro assays showed its capacity to bind to PPARγ and activate it in a dose-dependent manner, but with lower efficiency than rosiglitazone, also having the ability to antagonize rosiglitazone-dependent PPARγ activation. It was shown that the interaction of MBX-102 with LBD of PPARγ occurs distinctly from TZDs. Moreover, this ligand has the capacity of dis-

placing the corepressors NCoR and SMRT and has a reduced ability to recruit coactivators, explaining its partial agonism [104].

Regarding the physiological effects of MBX-102, in vitro studies using 3 T3-L1 adipocytes revealed its ability to enhance insulin-stimulated glucose membrane translocation and a decreased ability to stimulate adipocyte differentiation [104]. In vivo studies with three T2D rodent models (ob/ob and db/db mice and Zucker fatty diabetic (ZDF) rats) demonstrated that the treatment with this ligand promoted a reduction on fasting plasma glucose [41, 104]. ZDF treated with MBX-102 also showed reduced plasma insulin levels and increased glucose infusion rate and glucose disposal rate on the hyperinsulinemic-euglycemic clamp, suggesting its role on insulin sensitivity. Long-term treatment with MBX-102 revealed an improvement in glucose tolerance and an increase in adiponectin levels, with no increase in body and heart weight, side effects observed with rosiglitazone treatment [104]. Another study using ZDF rats revealed positive effects of MBX-102 on reducing triglycerides, FFA, and cholesterol levels [41].

MBX-102 also reduced osteoblastic differentiation in vitro and decreased the levels of LPS-stimulated pro-inflammatory cytokines (such as monocyte chemoattractant protein-1 (MCP-1) and interleukin 6 (IL-6) in mouse primary peritoneal macrophages, revealing its promising therapeutic potential on the treatment of T2D [104], also acting as a blocker of S273 phosphorylation [45]. MBX-102 anti-diabetic effects were addressed in humans in phase 2 and 3 clinical trials (identifiers NCT00814372, NCT00353587), but up to 2020, no results were published yet.

7b In 2010, a potent PPARγ partial agonist, called 7b, was developed after modifications on TEL structure and showed a high affinity to the NR and binding mode that differs from the full agonists [178]. On this molecule, the molecule's carboxylic acid binds at the opposite end of the active site and hydrogen bonds with R288 and S342, allowing the amide group to donate and accept hydrogen bonds to S289 and Y327, respectively. This compound was used for in vivo assays with ZDF rats, being evaluated as suitable for chronic administration. A reduction in plasma glucose and triglycerides levels was observed with fewer side effects than full agonists, but more experiments are necessary to confirm this compound's efficacy and confirm if this molecule may act as a blocker of S273 phosphorylation [178].

GQ-16 Two years later, other PPARγ partial agonist, GQ-16, was reported to reverse the impairments on insulin signaling that HFD promotes [7]. GQ-16 treatment increased insulin receptor expression, insulin receptor substrate 1, and protein kinase B, among others. In addition, HFD mice showed an improvement in insulin sensitivity due to an increase in glucose disappearance rate (KITT) and glucose tolerance, with a lower increase in body weight and a decrease in fat mass. It was showed that this molecule could block S273 phosphorylation in a concentration-dependent manner, binding to PPARγ in an axial orientation, parallel to H3, making no contact with residues of H12. This interaction protects the lower half of LBD, stabilizing the H11-12 loop, H3, and β-sheet/S273 regions, more efficiently than rosiglitazone does [7].

Mice treated with GQ-16 exhibited a slight decrease in hepatic triglycerides and attenuation on adipocyte hypertrophy and a decrease in interscapular BAT, with small lipid droplets. Interestingly, this ligand promoted an increase on the expression of *Ucp1*, *Cidea*, and *Prdm16* in BAT and epididymal WAT, which are genes related to thermogenesis, indicating the potential role of this molecule on the treatment of T2D and obesity [52].

Amorfrutins In 2012, another group of molecules belonging to the family of natural products, amorfrutins, was described as partial-agonist PPARγ. They were identified by screening a library containing 8000 pure compounds, which revealed 90 potential PPARγ ligands. Amorfrutins, a family of isoprenoid-substituted benzoic acid derivatives without any stereocenters, were selected as structurally new molecules with a high binding affinity to the NR [343]. It was reported that these compounds induce partial recruitment of coactivators, as CBP, PGC1α, TRAP220/DRIP, and PRIP/ RAP250, as well as disrupt the recruitment of the corepressor NCoR. It was also shown that these molecules interact with PPARγ LBD by contact between H3 and the β-sheet, stabilizing these structures [343].

Regarding the physiological effects of amorfrutins, in vitro assays using 3 T3-L1 adipocytes showed an upregulation of PPARγ target genes, as *Fabp4, Slc2a4*, and LXRα (*Nr1h3*), but in a lower degree than rosiglitazone. Moreover, it was observed a less pronounced adipocyte differentiation and an upregulation of genes involved in cholesterol biosynthesis, fatty acid elongation, and oxidation, in contrast to a downregulation of inflammatory genes. Besides these results, in vivo studies using DIO C57BL/6 mice revealed that the treatment with amorfrutin 1 reduced insulin resistance, enhanced glucose tolerance, and decreased plasma triglycerides, FFA, insulin, and glucose in the same levels as rosiglitazone did. Although it promoted an increase in food intake, body weight gain was significantly reduced, which was related to an increase in plasma levels of thyroxine (T4), a marker of increased energy expenditure [343].

The anti-diabetic effects of amorfrutin 1 were also evaluated in db/db mice, showing no weight gain, with a reduction in plasma insulin levels, as well as glucose, triglycerides, and FFA compared to vehicle. As S273 dephosphorylation was described as a mechanism for improvement on insulin sensitivity, this parameter was checked, and it was observed a reduction in S273 phosphorylation on WAT of DIO mice, increasing the expression of gene related with this PTM, as *Nr1d2, Selenbp1, Adipoq,* and *Cfd*. In the liver of DIO mice, amorfrutin 1 treatment reduced hepatic triglycerides and induced FAO by upregulating *Fabp4, Pgc1a*, and *Cpt1a* compared to a vehicle with reduced TNFα protein concentration and higher glycogen content. Besides, amorfrutin 1 leads to decreased inflammation and macrophage accumulation in the liver and WAT. All these effects could indicate the potential action of amorfrutins on glucose metabolism and lipid profile, improving insulin sensitivity and acting as a molecule against T2D [343].

p-F11 In 2013, pseudoginsenoside F11 (p-F11), an ocotillol-type ginsenoside isolated from the roots and leaves of *Panax quinquefolium* L. (American ginseng), was

identified in a screening for partial PPARγ agonists. p-F11 was described with moderate adipogenic activity in vitro [347]. In a gene reporter assay, p-F11 was shown to dose-dependently increase PPARγ activation, although higher concentrations of p-F11 promoted an activation increase smaller than lower concentrations of rosiglitazone, corroborating its action as a partial agonist. This ligand increased the mRNA expression and protein level of both PPARγ and adiponectin, as well as adiponectin oligomerization and secretion in 3 T3-L1 adipocytes, indicating that this adiponectin upregulation is through PPARγ activation during the differentiation of 3 T3-L1 preadipocytes. Regarding S273 phosphorylation, p-F11 decreased this PTM occurrence in 3 T3-L1 adipocytes in the same extent than rosiglitazone [347].

CMHX008 In the following year, other PPARγ partial agonist was discovered through docking methods. CMHX008 presents a different binding mode to interact with the receptor, forming hydrophobic interactions with L255, I281, M348, and I341 in the receptor entrance [219]. Compared to rosiglitazone, this molecule showed, in vitro, a decreased activation of PPARγ, reduced adipocyte differentiation and lipid accumulation, and an increase on *Adipoq, Slc2a4,* and *Fabp4* expression and on adiponectin secretion [219].

To confirm these effects, in vivo studies were performed using DIO mice, in which CMHX008 treatment showed a decrease in body weight, together with a reduction in adipocyte size and WAT weight and reduction on triglycerides and LDL-cholesterol levels [219]. These animals also improved glucose tolerance with reduced glycemia and reduced insulin plasma levels, indicating CMHX008 antidiabetogenic effects. These effects were justified as a result of S273 phosphorylation blockage and alteration on the ability of PPARγ to interact with coactivators, promoting differential recruitment of the receptor to the promoter of its target genes [219].

Regarding inflammation, DIO mice treated with CMHX008 showed reduced serum IL-6 and TNFα and increased IL-10, a cytokine with anti-inflammatory properties [219]. CMHX008, as well as with rosiglitazone, switched macrophage polarization from pro-inflammatory M1 to anti-inflammatory M2 dominant [219]. Studies to compare the effects of CMHX008 with rosiglitazone, concerning one of its well-known side effects, bone mass loss, showed that CMHX008 promoted a decrease in trabecular bone, but in a small proportion. Moreover, treatment with this molecule displayed a more mineralized matrix during differentiation into osteoblasts [132]. Altogether, these results indicate the effects of CMHX008 in glucose, lipid, and bone mineral metabolism.

L312 In 2014, another PPARγ partial agonist was described as a potent molecule on insulin sensitivity with low side effects. It was called L312 ((S)-2-(4-chlorobenzamido)-3-(4-(2-(5- methyl-2-phenyloxazol-4-yl)ethoxy)phenyl) propanoic acid), and it had a similar affinity to PPARγ-LBD as pioglitazone with a less extent transcriptional activity [349]. In vitro assay demonstrated its effects on the recruitment of CBP coactivator and NCoR displacement, besides a weak adipogenic activity. Moreover, in vivo experiments revealed that L312 improves insulin resis-

tance in a dose-dependent manner and improves glucose tolerance, reducing serum insulin, fasting glucose, triglycerides, and FFA levels. This molecule also reduced adipocyte size and results in a lower increase on body weight when compared to pioglitazone. All these effects were related to the inhibition of S273 phosphorylation in vitro and in vivo, attenuating the expression of several genes regulated by this PTM, which makes L312 a potential drug on T2D treatment [349].

Chelerythrine In 2015, a molecule called chelerythrine, which is derived from *Chelidonium majus* (greater celandine) and is already used as a medical therapy with antiviral, antitumor, antifungal, and anti-inflammatory activity, was revealed as a selective modulator of PPARγ [368]. It was shown that this compound has weak activity on PPARγ-LBD but exhibited a high binding potency, promoting the recruitment of coactivators as SRC1 and PGC1α, and displacement of SMRT corepressor with less efficiency than rosiglitazone, being considered a partial agonist. In vivo experiments demonstrated its effects on improving glucose tolerance and insulin sensitivity with no weight gain. In addition, chelerythrine promoted a reduction on serum glucose, insulin, and cholesterol levels and a decrease on the expression of *Cd36*, *Il1b*, interferon gamma (*Ifng*), and *Tnfa*. Despite its lower transcriptional activity, this molecule exhibited a higher capacity on blocking S273 phosphorylation when compared to rosiglitazone, which confirms its potential role on the treatment of diabetes and obesity [368].

F12016 Another PPARγ partial agonist and an S273 phosphorylation blocker, F12016, was reported in 2015. This benzamide derivate is structurally different from TZDs, and it was shown to promote the transcriptional activity of the receptor through the binding to its LBD, but with moderate intensity when compared to rosiglitazone [193]. This interaction includes binding through two hydrogen bonds, a π-π stacking interaction, and several van der Waals forces with surrounding amino acids, such as C285, M364, I326, L330, M329, and I281 [193]. F12016 showed moderate activation of PPARγ, with impaired coactivator and improved corepressor recruitment compared to rosiglitazone, suggesting its partial agonism [193].

The effects of F12016 in vitro using 3 T3-L1 and hepatocytes showed that this compound enhanced insulin-stimulated glucose uptake, increasing insulin sensitivity and promoting glucose transport. Besides, F12016 presented low potency to induce the formation of lipid droplets, also reducing triglycerides content inside the cells [193]. As expected from partial agonists, F12016 differentially regulates a set of genes involved in adipogenesis, decreasing expression of *Fabp4* and *Cd36*, among others, and increasing the expression of *Adipoq*. Still using cell models, researchers found that this molecule caused less reduction of bone cells' calcification than rosiglitazone, suggesting F12016 would cause less osteoporosis. Besides this, in the KK-Ay murine diabetes model, F12016 promoted a reduction in fasting glucose levels and improved glucose tolerance and insulin sensitivity, with no weight gain. In conclusion, this ligand demonstrated various advantages as insulin sensitizer without showing side effects [193].

DHM Still, on the PPARγ of partial agonists class, many flavonoids are reported to act on glucose and lipid metabolism. One example is dihydromyricetin (DHM), described in 2016, that promoted less weight gain than rosiglitazone in ZFD rats and significantly reduced fasting blood glucose, improving the insulin/glucagon ratio [194]. Moreover, DHM reduced insulin resistance by 50% in comparison to rosiglitazone. On lipid profile, it was observed an improvement on serum lipids levels compared to control and a significant reduction on visceral and total fat mass, reducing adipocytes size more efficiently than rosiglitazone, also promoting an increase in adiponectin protein levels in adipose tissue [194]. DHM reduced PPARγ phosphorylation in vivo more potentially than the rosiglitazone [194]. In 3 T3-adipocytes, DHM also showed a decrease in lipid accumulation and the expression of the adipogenic marker *Fabp4*, and in combination with dexamethasone, DHM increased glucose uptake by 90% in cells, also improving adiponectin and FGF21 secretion by adipocytes [195]. In normal adipocytes, DHM treatment decreased CDK5 activation and ERK phosphorylation, reducing insulin-resistant adipocytes, through the prevention of S273 phosphorylation, further elucidating the mechanisms of anti-diabetic properties of DHM [195].

GQ-11 In 2018, the compound GQ-11 was described as a partial agonist of PPARα and PPARγ. Using docking studies, it was shown that this molecule interacts with the hydrophobic residues F282 and L469 of PPARγ arm I and forms a hydrogen bond with S289, also interacting with PPARα [295]. The pharmacological effects of this molecule were evaluated in LDL receptor-deficient mice (LDLr−/−) fed on a diabetogenic diet, showing positive effects on insulin sensitivity, decreasing fasting glucose and insulin levels, and improvement of glucose tolerance compared to control. Furthermore, GQ-11 treatment resulted in lower body weight and did not modify adipose mass, differing from pioglitazone [295]. On PPARγ modulation, GQ-11 is involved in improving adipokines levels, as adiponectin, with a concomitant increase on the expression of *Slc2a4*, followed by a decrease in serum leptin. This molecule also affects the animals' inflammatory state, increasing IL-10 in adipose tissue, with a decrease in MCP-1, suggesting its influence on local and systemic inflammation. Related to lipid profile, GQ-11 treatment promoted a decrease in VLDL cholesterol levels and serum triglycerides, with an increase in HDL-cholesterol levels compared to control [295]. GQ-11 was also reported as an anti-diabetic compound because it induces the upregulation of anti-inflammatory cytokines and growth factors gene involved in tissue repair in db/db and non-diabetic mice. This ligand improved wound closure in db/db mice compared to control or pioglitazone groups, increasing collagen deposition and decreasing macrophage infiltration in this lesion [295].

Another possible approach to modulate PPARγ action would be to prospect molecules that act as non-agonists of PPARγ. These compounds should bind to the receptor but do not promote its transcriptional activity, and some of them were reported to block S273 phosphorylation, promoting insulin sensitivity.

SR1664 In this context, in 2011, after discovering compound 7b, some analogs of this molecule were developed, of which the most efficient one was SR1664 [46]. In silico docking studies revealed that SR1664 increases the conformational mobility of C-terminal end of H11, a helix that abuts H12, which could be explained by the interaction of phenyl substituted nitro group of the molecule with hydrophobic side chains of H11, such as L452 and L453 of the loop N-terminal to H12 [46]. Following this, it was evaluated its capacity to recruit cofactors and DNA binding ability, observing that SR1664 did not influence SRC1 recruitment or the occupancy of PPARγ [46]. On the first in vitro assays with SR1664, its effects on adipocyte differentiation were evaluated, resulting in no changes in lipid accumulation or the fat cells' morphology, with little or no change on fat cell gene expression. It is already known that TZDs affect bone formation, and, using MC3T3-E1 cells, it was seen that SR1664 did not affect the extent of calcification or the expression of osteoclastic genes [46].

Subsequently, the anti-diabetic properties of SR1664 were analyzed in vivo using DIO mice, and the treatment promoted a decrease in S273 phosphorylation, as well as on glucose and fasting insulin levels, with an improvement on insulin sensitivity, without changing body weight. These same results were observed in a more severe animal model, ob/ob mice, confirming the beneficial effects of SR1664 on glucose metabolism and insulin sensitivity, with no side effects due to its non-agonism characteristics [46].

AM-879 Another PPARγ non-agonist, discovered in 2013 on a study that used a structure-based strategy to search for new ligands, was AM-879 [61]. On the initial screening, this molecule was selected due to its ability to ensure increased thermal stability for the receptor over the unbound one, proving its capacity to bind to the PPARγ. After, in vitro transactivation assays presented its capacity to decrease the basal transcription of this receptor [61, 272]. Further, this ligand did not favor coactivator recruitment, did not induce corepressor release, and did not induce a significant increase in lipid accumulation or adipocyte differentiation in adipogenesis assays, decreasing expression levels of *Adipoq*, *Cfd*, and *Cd36* [272]. Finally, this study showed that AM-879 reduced S273 phosphorylation more effectively than rosiglitazone, indicating its potential anti-diabetic role and the requirement of further studies [272].

UHC1 Another PPARγ non-agonist, described in 2014, was UHC1, which showed in vitro assays not to stimulate lipid accumulation and not increase some classical adipogenic markers but influencing the expression of genes regulated by S273 phosphorylation [47]. In addition, in vivo studies with HFD mice showed that UHC1 induced an improvement in glucose tolerance and reduced fasting glucose and insulin levels. On lipid profile, UHC1 promoted a reduction in serum triglycerides, cholesterol, and FFA and upregulated the expression adiponectin and adipsin. Regarding the inflammation process, UHC1 showed a potential role in the inflammatory process, as it inhibited the TNFα-stimulated pro-inflammatory responses in 3 T3-L1 adipocytes and reduced the mRNA levels of the pro-inflammatory cytokine *Il6* and

increased the levels of anti-inflammatory markers as *Il10* and arginase in HFD mice. All these data indicate that UHC1 exerts potent anti-diabetic effects, positively influencing inflammation, without causing side effects as TZDs [47].

SB1451 and SB1453 Afterward, in 2016, two new molecules were synthesized based on previous compounds that bind to PPARγ and block S273 phosphorylation: SB1451 and SB1453 [15]. These ligands contain hydrophilic piperazine moieties attached to the other benzene rings to improve their solubility. In vitro assays confirmed their ability to block CDK5-mediated S273 phosphorylation. Further, these molecules have shown a low activation of the receptor and did not trigger adipogenesis in 3 T3-L1 cells. To evaluate the anti-diabetic effects, DIO mice model showed that SB1453 was more effective in reducing S273 phosphorylation than SB145, as well as altered the expression of 10 out of 17 affected genes by this PTM and improvement on glucose tolerance. Possible side effects of SB1453 were studied, and no significant changes were seen on the markers for heart failure (natriuretic peptide B) and hypertrophy (myosin heavy chain β). Finally, the crystal structure of SB1453 with PPARγ-LBD showed that this compound was covalently bound to C313 on H3 and occupied the hydrophobic region between H3 and β3-β4 sheets, which is closely related to the inhibition of S273 phosphorylation [15].

SR10171 An inverse agonist of PPARγ was also described, binding to the receptor as an agonist but promoting an opposite response. SR10171 is considered a partial inverse agonist since it partially represses the NR's basal transcriptional activity [301]. This molecule reduces S273 phosphorylation, resulting in induced pro-osteoclastic activity, increased osteoclastogenesis, and cortical bone thickness, at the same time that it enhances insulin sensitivity in DIO mice [87, 301]. In addition, this same ligand promotes preferential recruitment of corepressors than coactivators, due to its interaction with H12 of the receptor, favoring the antagonist conformation [87].

21.4.2 Modulating the Activity of Enzymes Responsible for PTMs

In order to characterize a PPAR PTM, its addition or removal is often modulated by an activator or inhibitor of the enzyme responsible for the modification. Although useful in isolated systems such as in vitro cell culture or knock-in/knockout animals, this approach may not correlate well in the clinical trials. Modulation of a PPAR PTM occurrence requires a fine-tuning adjustment to avoid unspecific activation or blockage of other signaling pathways and to avoid undesirable side effects.

In this topic, strategies used in the research laboratories to inhibit or stimulate the addition of a PTM in PPAR using PTM enzyme modulators will be presented.

Moreover, we are going to discuss the applicability of this strategy in the clinic to modulate the PPAR function.

21.4.2.1 Phosphorylation

Phosphorylation in PPARα seems to have antagonistic effects as increased S12/S21 and decreased S73 phosphorylation lead to increased activity of the receptor, resulting in a protective effect for hepatic steatosis [55, 128]. However, the reports with kinases and phosphatases activators and inhibitors to modulate these phosphorylations are rare. In the only report of kinase modulation, S12/S21 phosphorylation is inhibited by MAPK inhibitor PD98059, decreasing the receptor activity [147]. In order to stimulate the PPARα activation and its protective effect in hepatic steatosis, it would be interesting to induce S12/S21 phosphorylation and/or to reduce the one at S73. GSK3β phosphorylates the later serine, and the phosphatase that mediates this dephosphorylation was not yet described [127].

PPARγ phosphorylations can be increased upon stimulation of the kinases MAPK, ERK, and CDK5 pathways with growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), TGFβ, insulin, prostaglandin F2α (PGF2 α), TNFα, cellular stress (UV, TPA, and anisomycin), IL-6, or FFAs [2, 37, 45, 135, 270, 361]. PPARγ phosphorylation in the LBD (S273) does not change transcriptional activity, but inhibition of this phosphorylation increases insulin sensitivity, whereas PPARγ phosphorylation in AF-1 and DBD decreases the receptor activity (S112, Y74, Y78, S16/S21). Therefore, with the objective to revert the insulin resistance mediated by S273 phosphorylation without activating the NR and inducing weight gain, hepatic steatosis, and bone loss, a good strategy would be to block the kinase or activate the phosphatase responsible to respectively phosphorylate and dephosphorylate the S273 residue, while promoting the phosphorylation of serines and tyrosines present in AF-1 and DBD. However, these residues are phosphorylated by different kinases: casein-kinase II (S16/S21 in PPARγ1), c-SRC (Y78 PPARγ2), EGFR kinase (Y74 PPARγ1), and MAPK, CDK7, CDK9, and ERK for S112 in PPARγ2, making this a hard approach.

Some protein kinases, such as WEE1 and MEK, are specific and perhaps phosphorylates only one or two distinct protein targets [320]. However, many other protein kinases have a broader specificity and are likely to phosphorylate hundreds of distinct proteins within cells.

Casein-kinase II (CK2), which phosphorylates PPARγ1 S16/S21, is a ubiquitous serine/threonine kinase that has over 100 potential physiological targets [192], including growth-related proteins, NOPP140, tumor protein p53, Fas-associated factor-1 (FAF-1), topoisomerase II and CD5, and potential CK2 regulators such as fibroblast growth factor-2 (FGF-2) [9, 28, 29, 80, 144, 186, 263]. Due to its role in cell fate determination in cancer cells, there is an increasing interest in the development of CK2 modulators for cancer therapies [103, 111, 261].

Y78 phosphorylation can be inhibited with a c-SRC kinase inhibitor, PP2, aggravating insulin resistance in obese mice and dysregulating the gene expression of

cytokines and chemokines in adipocytes [49]. However, the non-receptor tyrosine kinase c-SRC, the first proto-oncogenic protein ever described [208, 330], is ubiquitously expressed in all cell types, and pharmaceutical modulation of its activity can interfere in other pathways which regulate cell growth, differentiation, and cytoskeletal regulation [77, 313].

Differently, EGFR is a transmembrane receptor for EGF with a tyrosine kinase domain and member of the ErbB family of receptors [362]. The kinase domain of EGFR can cross-phosphorylate tyrosine residues of other receptors with which it is aggregated, for example, PPARγ 1 Y74 [350], and can be itself activated in that manner.

PPARγ S112 was shown to be phosphorylated by several kinases (MAPK, CDK7, CDK9, ERK), and treatment with a MEK inhibitor, U1026, reduced phosphorylation and the receptor degradation [83].

The best strategy to modulate insulin resistance would be to only block S273 phosphorylation, with a CDK5 or ERK inhibitor, or to promote its dephosphorylation with the activation of PPM1A phosphatase. For example, treatment with roscovitine, a selective CDK5 inhibitor, prevents S273 phosphorylation [45]. However, CDK5 does not seem to be a simple drug target for specific PPARγ modulation because its pharmaceutical activation could deregulate the different pathways coordinated by this kinase, resulting in undesirable side effects.

CDK5, unlike other members of the cyclin-dependent kinases family, is not typically activated upon binding with cyclin and does not require T-loop phosphorylation for activation. CDK5 is activated by binding of p35 or its cleaved form p25 [65, 129, 247]. Additionally, CDK5 has functions in both terminally differentiated and proliferating cells [206] and, as others CDKs, is highly expressed in mitotic cells [285]. CDK5 plays a vital role in the central nervous system but also has a function in the immune system by increased interferon γ-induced programmed death-ligand 1 (PD-L1) expression, in insulin secretion, in angiogenesis promotion, in cell cycle by increasing expression of cyclins and other CDKs, and in cancer progression [294]. Due to its biological and clinical relevance in multiple cell types, CDK5 presents an attractive therapeutic target for treating various conditions such as diabetes, cancer, and neurodegeneration. Two CDK5 inhibitors are in clinical trials for cancer treatment, dinaciclib and seliciclib, whereas roscovitine, widely experimentally used to inhibit CDK5 activity, is being intensively examined as clinical cancer therapeutics [26, 85, 345].

To modulate the phosphate addition without interfering in the other targets of CDK5 phosphorylation, a different approach has been proposed: the development of ligands or peptides targeting the interface region between PPAR and this kinase [273]. Computational and biophysical analysis of PPARγ and CDK5 structures resulted in a model of interface interaction, which was validated with single point mutations experiments with purified proteins and in cell culture. At PPARγ, the CDK5 phosphorylation occurs in a noncontiguous motif, where P0 is S245, P + 1 is P246, P + 2 is F247, and K261 structurally occupies P + 3 position [273]. These computational analyses identified PPARγ K261, K263, and K265 as anchor

residues in the CDK5/p25 interaction, and single point mutations of these lysines resulted in decreased interaction with CDK5, decreasing the NR phosphorylation [273]. These results suggest that inhibition of CDK5-mediated phosphorylation of PPARγ with ligands that bind in the LBP might occur due to conformational changes in the receptor H2′ (residues 254 to 259), which loses its flexibility after ligand binding, probably blocking CDK5 anchorage to its recognition interface. It was hypothesized that peptides targeting the residues in the interaction interface could also block PPARγ phosphorylation, being an attractive therapeutic approach to treat T2D. This approach could also be expanded to the other PPARs PTMs, with the use of computational biology and biophysics experiments being used to propose and validate sites for protein anchorage to promote acetylation (and deacetylation), SUMOylation, phosphorylation, and other PTMs, which further the development of inhibitors of these specific interactions and with specificity to the desired PPAR isotype.

21.4.2.2 Acetylation/Deacetylation

In order to map and study PPARγ acetylation, the acetyl addition was promoted by treatments with acetyltransferase CBP or with HDAC inhibitors like trichostatin A (TSA) and nicotinamide (NAM), which is also a SIRT1 inhibitor [260, 314], suggesting that basal levels of PPARγ acetylations are very low.

The importance of PPARγ acetylation state in the balance between the beneficial and adverse effects of TZDs raises scientific interest in targeting this modification together with S273 phosphorylation. Deacetylation of PPARγ was promoted with purified deacetylase together with deacetylase co-factor nicotinamide adenine dinucleotide (NAD), and in cells with RSV, an activator of the deacetylase SIRT1, resulting in decreased expression of adipogenic genes [260, 314]. Consequently, inhibition of SIRT1 by NAM treatment prevented PPARγ deacetylation [314].

The modulation of SIRT1 seems an interesting approach to maintain the deacetylated state of PPARγ in a research laboratory isolated set up of experiments. However, treating obese patients with SIRT1 activator would be seen with caution once this deacetylase regulates many other relevant pathways in humans. SIRT1 is a member of the sirtuin family and can deacetylate various substrates and is, therefore, involved in a broad range of physiological functions, including control of several cardiometabolic and aging-related pathways [262, 340]. Besides PPARγ, other SIRT1 substrates are the tumor suppressor protein p53, members of the Forkhead box factors regulated by insulin/Akt (FoxO) family, hairy and enhancer of split 1 (HES1), hairy/enhancer-of-split related with YRPW motif 2 (HEY2), COUP-TF-interacting protein 2 (CTIP2), p300, PGC1α (PPARγ coactivator), and NF-κβ [106, 215, 253, 352].

Because of its many substrates, among other effects, SIRT1 is reported to regulate energy and lipid homeostasis, hepatic lipid homeostasis [352], DNA damage

repair and genome integrity [338], and chronic inflammation [281]. In these last two examples, insulin sensitivity and lipid accumulation in adipocytes regulate PPARγ activity [249, 250, 305].

SIRT1 has a controversial effect on cancer; it is upregulated and serves as a tumor promoter in human prostate cancer [138], acute myeloid leukemia [31], and primary colon cancer [191, 201, 303]. However, it is downregulated in other cancer types such as glioblastoma, bladder carcinoma, prostate carcinoma, and ovarian cancers when compared to the corresponding normal tissues [338], and its overexpression, in these cases, reduces tumor development [64, 81, 107].

Because of its involvement in many pathways, SIRT1 does not seem a simple drug target for specific PPARγ modulation through deacetylation. However, the use of sirtuin modulators has been described to treat diabetes, fatty liver diseases, obesity-induced insulin resistance, and inflammation [190, 340].

Promising SIRT1 activator compounds include the natural polyphenol RSV and SRT1720 [76, 133, 218, 296]. Due to the RSV low bioavailability, some derivatives were developed to improve this characteristic and have already been tested in clinical trials [328]. resVida®, a nutraceutical formulation of RSV, demonstrated beneficial effects in healthy obese men, decreasing intrahepatic lipid content, circulating glucose, triglycerides, alanine-aminotransferase, and inflammation markers, and mimicking the effects of calorie restriction [317]. However, another nutraceutical formulation, Longevinex®, did not modify blood pressure, insulin resistance, lipid profile, or inflammatory markers [89]. SRT501, a commercial micronized RSV formulation, enhanced mitochondrial biogenesis, improved metabolic signaling pathways, and blunted pro-inflammatory pathways in mice fed a high-calorie diet [296]. Moreover, it was shown to lower blood glucose and to improve insulin sensitivity in patients with T2D in a Phase IIa trial [133], requiring further clinical studies.

SIRT1 activators structurally unrelated to RSV were also developed, and some of them, for example, SRT1720, activate the deacetylase more potently than the former [76]. Its therapeutic potential to treat insulin resistance and diabetes was tested in three in vivo models of T2D, and it was able to promote insulin sensitization via metabolic adaptations simulating low energy levels [76, 218, 296]. Administration of SRT1720 reduced fed glucose levels, partially normalizing elevated insulin levels, and reduced fasting blood glucose to near normal levels in mice on HFD, strongly protecting mice from DIO and insulin resistance by enhancing oxidative metabolism in skeletal muscle, liver, and BAT [76, 218, 296].

Another synthetic SIRT1 activator, SRT2104, was tested in a Phase IIa trial in patients with metabolic, inflammatory, and cardiovascular diseases and was shown to significantly attenuate LPS-induced IL-6 and interleukin 8 (IL-8) release and activation of coagulation [328]. The cardiometabolic effects were also evaluated in a clinical trial with T2D, where treatment with SRT2104 resulted in weight loss and deterioration in glycemic control [235].

Nevertheless, these compounds' tissue-specific effects need to be carefully evaluated to avoid undesirable side effects due to the broad spectrum of pathways modulated by SIRT1.

21.4.2.3 SUMOylation

PPARs SUMOylations decreases the activity of the receptors in all isoforms. For PPARβ/δ, SUMOylation physiological effects are not clear yet [168], whereas PPARα-reduced activity is related to hepatic steatosis [185, 257] and PPARγ-decreased activity is desired in the context of metabolic disorders since its activation leads to adipogenesis [216, 353]. Increased deSUMOylation of PPARγ was observed in a report describing the receptor ligand modulating deSUMOylation effects by targeting a SUMO-specific protease, SENP2. Treatment with saturated FA, like palmitate, led to NF-κβ-mediated increase in the expression of SENP2, resulting in increased PPARγ deSUMOylation, and consequently increased PPARγ activity and upregulation of some target genes, such as *Fabp3* and *Cd36* [51].

SUMOylation is a PTM that regulates several biologic processes, including transcription, cell cycle, DNA repair, and innate immunity [24, 284]. This modification is involved with the immune system and inflammatory responses, cancer progression, and Alzheimer's disease, and the modulation of SUMO addition has been described for many therapeutics claims [86, 155, 207, 356].

Inhibition of SUMOylation has been achieved by several natural products, such as ginkgolic and anacardic acids, curcumin, α-lipoic acid, and flavone 2-D08 (flavonoids) [207]. Ginkgolic acid [90], kerriamycin B [91], davidiin [308], and tannic acid [307] are natural products confirmed to inhibit SUMO E1 by blocking formation of SUMO E1–SUMO intermediate. A SUMO E2 protein, UBC9, is increased in many cancers [284], including advanced melanomas, head and neck tumor, lung tumor, HCC, colon cancer, breast cancer, and glioblastoma [4, 44, 51, 182, 225, 226, 318, 346, 354]. UBC9 inhibition is interesting as anticancer therapeutics and GSK145A (doi: https://doi.org/10.1089/adt.2012.501), 2-D08 (2′,3′,4′-trihydroxyflavone) [162], and spectomycin B1, an antibiotic against gram-positive bacteria [298], have been described as UBC9 inhibitors. SUMO-activating enzymes (SAE) 1/2 inhibitors, such as ML-792, have been shown to potently inhibit SUMOylation with a promising application in treating MYC-amplified malignancies [121, 279]. PIAS1 is a SUMO E3 which enhances the SUMOylation of many proteins, including PPARγ. Although it has a potential drug target for cancer therapy, and perhaps obesity-related diabetes, no small-molecule inhibitor has been designed so far for its inhibition [356].

Topotecan, a drug with approval of the US Food and Drug Administration (FDA_ for the treatment of several cancers (e.g., small cell lung cancer, cervical, ovarian) [32, 256], is primarily a DNA topoisomerase I inhibitor; however, it also modulates the SUMOylation status of this protein [68, 222].

Another strategy to modulate the SUMOylation state is to inhibit the deSUMOylates SENPs. Except by SENP2, which is decreased in bladder cancer [309] and HCC [288], other SENP members, such as SENP1, SENP3, and SENP5, are upregulated in various cancers, including neuroblastoma, multiple myeloma, gastric cancer, oral squamous cell carcinoma, and breast cancer [351, 356, 371]. Experimentally, SENP1 inhibitors have exhibited anticancer activities in vitro, including benzodiazepine-based peptidomimetic covalent compounds,

SUMO-derived peptide-based covalent inhibitors, noncovalent 2-(4-chlorophenyl)-2-oxoethyl 4-benzamidobenzoates, 1-[4-(N-benzylamino) phenyl]-3-phenylureas, triptolide, and Momordin Ic [356], showing that SENP1 could serve as a drug target for developing new cancer therapeutics.

SENP2, reported to deSUMOylate PPARδ/β and PPARγ, also regulates SUMOylation levels of the tumor suppressor p53 and ERK5 [173], and it could serve as a drug target to atherosclerotic plaque formation [123] and hepatocellular carcinoma cell growth [288] and cardiac dysfunction [159].

21.4.2.4 Ubiquitination

All PPAR isoforms suffer ubiquitination, and this PTM, in general, regulates the protein level and decreases receptor activity by targeting for protein degradation. The ubiquitin proteasome system (UPS) is composed by sequential actions of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3), which promote ubiquitin addition in internal lysine residues or, more uncommon, in the N termini of target proteins, directing them to proteasomal degradation [363]. There are reports of modulation of this PTM in PPAR by either inhibiting the ubiquitin ligases or the proteasome machinery. Starting with the beginning of the UPS cascade, inhibition of the E1 by E1-I leads to an inhibition of ubiquitination that returns PPARγ protein to control levels even in the presence of rosiglitazone, which usually induces ubiquitin-mediated degradation of PPARγ [158].

UPS is also involved in regulating proteins involved in several pathways, being the dysfunction of its components observed in many pathological disorders, including cancers, cardiovascular diseases, viral diseases, neurodegenerative disorders, and congestive heart failure [19, 348]. For this reason, selective inhibition of the UPS components has significant therapeutic potential. At the beginning of the UPS cascade, inhibitors such as E1-I were developed to inhibit E1 Ub activating enzymes. PYR-41, an irreversible pyrazone derivative inhibitor, was identified to selectively inhibit ubiquitin-like modifier-activating enzyme 1 (UBA1), without affecting E2 and E3 proteins [355]. PYR-41 also partially inhibits NEDD4 [344]. TAK-243, an inhibitor to UBA1, was in 2019 in a clinical trial (phase 1) for advanced solid tumors [140, 217]. Although E1 inhibitors have shown efficacy, an obvious drawback of such compounds is that they influence generically many proteins/cellular networks, which renders their toxicity [348].

The second step of the ubiquitination cascade is controlled by E2 Ub conjugating enzymes (~20), which have been linked to head and neck carcinoma [203], lung cancer [113], and tumor formation [324]. CC0651 is a highly selective inhibitor of the CDC34 E2 enzyme, and its treatment caused accumulation of the cell cycle inhibitor p27 and cyclin E in cells and inhibited proliferation of human cancer cells [39].

At the last step of ubiquitination, E3 Ub ligases transfer the Ub from E2 to the substrate, and there are many kinds of these enzymes since E3 ligases can be divided

in N-end rule family E3α, homologous to the E6AP carboxyl terminus (HECT) domain family (~30), multi-subunit complex family (e.g., SCF complex and anaphase-promoting complex (APC)), and the fascinating new gene (RING) finger ligases (~600), which contains MDM2 and CRL4B [73, 348]. These RING ligases can be separated into single subunit ligases, which can be targeted by enzymatic inhibitors, and into multi-subunit RING finger ligases, which should be targeted in their substrate-specific adaptor bound to the catalytic core. This strategy resembles the one described to inhibit CDK5 phosphorylation: the use of small molecule inhibitors that disrupts the protein-protein interaction between that adaptor and its target protein; however, such specificity can be challenging to achieve.

Among several E3 ligases inhibitor, we are going to focus on molecules described to inhibit proteins involved in PPAR ubiquitination. Nutlin-2 is an inhibitor that disrupts the interaction between MDM2 and p53 by binding directly to the interface of MDM2-p53 contact [326]. Therefore, this inhibitor stabilizes p53 and has a significant anti-tumor effect and has advanced to clinical trials for solid tumors and leukemia [53]. Four MDM2 inhibitors were under clinical trials in 2020: avadomide (CC-122) [267], iberdomide (CC-220) [25], APG-115 [266], and CGM097 [130]. Inhibitors for other E3 ligases that target PPARs were also described: HS-152 inhibitor of SMURF1 [315] and small-molecule covalent inhibitors of NEDD4–1 [151].

Another approach to modulate reversible ubiquitination is to target deubiquitinating enzymes (DUBs) (~100); such inhibitors have been developed to target severe acute respiratory syndrome coronavirus (SARS-CoV), the papain-like protease DUB PLpro [72, 268], USP1 [43], USP7 [42, 271], and USP14 [180].

In the final steps of the UPS cascade, the use of proteasome inhibitors experimentally prevented PPAR degradation. For example, treatment with MG132, a proteasome inhibitor, has demonstrated to increase the level of ubiquitinated PPARα and PPARγ and to inhibit their degradation, therefore increasing their activity [27, 119, 157, 158]. This treatment also partially prevented the decrease of PPARγ levels after C168 S-nitrosylation, suggesting that for this modification, the proteasome-dependent degradation might account for the impaired PPARγ stability [358]. Moreover, the use of the PS341, a selective proteasome inhibitor, inhibited PPARδ/β proteolysis, increasing the half-life of the DNA-bound receptor and therefore increasing its activity [97]. The 26S proteasome is a 2.4 MDa multifunctional ATP-dependent proteolytic complex, which degrades a large variety of cell proteins and is essential for many cellular regulatory mechanisms, that includes cell cycle progression, by the proteasomal degradation of cyclins and inhibitors of CDKs [165], transcriptional regulators (such as c-JUN, E2F-1, and β-catenin) [124], and kinases (such as SRC and protein kinase C (PKC)) [114, 200], terminating specific signal transduction cascades. Furthermore, the ubiquitin-proteasome pathway also plays an essential role in immune surveillance [142], muscle atrophy [221], regulation of metabolic pathways [109, 227], acquisition of long-term memory [40], inflammatory response [212, 242], and in the regulation of circadian rhythms [230] and tumor progression [124, 240].

Due to its involvement in several pathways, inhibition of the 26S proteasome results in a decrease of overall rates of protein breakdown in cells [57, 276], increasing the levels of ubiquitin-conjugated protein, as well as of misfolded and damaged proteins [79, 213, 280, 329]. The long-term exposition to proteasomal inhibitors is toxic for most cells and leads to death by apoptosis [70, 198, 214, 277]. Nonetheless, these undesirable side effects can have a bright side. For example, the accumulation of unfolded polypeptides incites the expression of heat shock proteins, which protects the cells against toxic conditions, including increased temperature or oxygen radicals [181]. Moreover, the ability of some inhibitors to inhibit cell proliferation and selectively induce apoptosis in proliferating cells, together with their ability to inhibit angiogenesis [70, 238], makes these molecules attractive candidates as anticancer drugs [164]. For example, PS341, a potent selective 26S proteasome inhibitor, was the first drug (generic name Bortezomib) targeting the UPS approved by the FDA in 2003, and it is used for patients with multiple myeloma [148]. Two other drugs received FDA approval, carfilzomib (PR-171) [161] and ixazomib [176], and three others were in clinical trials in 2020: oprozomib, delanzomib, and marizomib [36, 176, 255, 348].

21.4.2.5 S-nitrosylation

S-nitrosylation modifications were identified in the AF-1 and DBD of PPARγ and were reported to decrease the receptor activity. There is one report about modulation of PPARγ denitrosylation by inhibiting the GSNOR with 4-[[2-[[(2-cyanophenyl) methyl]thio]-4-oxothieno-[3,2d] pyrimidin-3(4H)-yl]methyl]-benzoic acid [38]. This blockage of GSNOR led to decreased adipocyte differentiation and a decreased expression of PPARγ target genes involved in adipocyte differentiation, indicating that the observed effects are due to the maintenance of the S-nitrosylated state of C139.

S-nitrosylation is a ubiquitous mediator of nitric oxide (NO) signaling and, therefore, is a PTM that occurs in many proteins involved in several physiological processes, including neuronal development and survival, blood pressure regulation, smooth muscle constriction, G-protein-coupled receptor (GPCR) signaling, and endothelial permeability [125, 286, 306]. Dysregulation of this PTM may compromise cell function and cause neurodegenerative diseases, heart failure, and dystrophic-like phenotype in the muscle [21, 101, 231].

To modulate the effects of excessive S-nitrosothiols (SNOs) formation and SNO-proteins, one therapeutic approach would be pharmacological inhibition of NO synthases (NOS) and/or application of antioxidants. Some NOS inhibitors and antioxidant treatments showed limited clinical trial results due to the nonspecificity of NOS inhibitors to NOS isoforms and mixed outcomes with antioxidants in neurodegenerative disorders [231]. Another approach would be to regulate protein denitrosylation with GSNOR modulators as it was reported that GSNOR inhibitor treatment seems to maintain the S-nitrosylated state of PPARγ C139 [38]. However,

in the case of PPARγ, S-nitrosylation would be interesting to use as GSNOR activator to increase denitrosylation and decrease PPARγ adipogenic effects.

However, both approaches have the limitation of being unspecific for the SNO-protein target. About 3000 SNO-proteins have been described both under physiological and pathological conditions [112, 241], making it difficult to modulate an SNO addition in one specific protein with a modulator of a promiscuous enzyme.

21.4.2.6 O-GlcNAc Addition

Generally, an increase in O-linked beta-N-acetylglucosaminylation (O-GlcNAc) is observed during adipocyte differentiation [134, 142], and this differentiation can be blocked by a general decrease in intracellular O-GlcNAc modification induced by pharmacological inhibition of glutamine by fructose-6-phosphate amidotransferase (GFAT) [134, 142]. The compound 6-diazo-5-oxo-L-norleucine (DON) inhibits GFAT, and it is commonly used to decrease intracellular O-GlcNAc modification level [142]. On the other way of O-GlcNAc PTM modulation, treatment with the O-GlcNAcase inhibitor, NButGT, increased O-GlcNAc modifications of PPARγ1 in 3 T3-L1 adipocytes, reducing the nuclear receptor transcriptional activity [145]. However, it must be considered that reduced intracellular levels of O-GlcNAc may affect other glycosylation reactions [335, 366], for example, exacerbating the side effects of Alzheimer's disease and frontotemporal dementia and parkinsonism [359].

O-GlcNAc levels work as a nutrient sensor, and glycosylation reactions regulate not only PPARγ activity in adipocytes but virtually all functional classes of protein since O-GlcNAc addition modulates nearly every cellular process, including signaling, transcription, translation, cytoskeletal functions, and cell division [359]. O-GlcNAc levels are critical in chronic diseases of aging, including diabetes, cancer, neurodegeneration, and cardiomyopathies [78, 205, 233, 244]. Only two proteins, glycosyltransferase OGT and the antagonistic one OGA, regulate O-GlcNAc addition in many proteins in the human body. Their modulation by ligands can deregulate the desired protein target and several other proteins that suffer this modification.

21.5 Perspectives

PPARα has been reported to be a target of phosphorylation, SUMOylation, and ubiquitination. Apart from S12/S21 phosphorylation, all PTMs were reported to decrease the activity of the receptor. Modulation of PTMs with PPARα ligands was described: WY-14643 enhanced polyubiquitination and K358 SUMOylation in the LBD, whereas GW-7647 reduced K185 SUMOylation in the hinge domain.

PPARδ/β is target only of ubiquitination in non-specified residues, signaling for degradation, and SUMOylation at K104, where deSUMOylation of this residue was

reported to increase the receptor activity. Modulation of ubiquitination was reported with PPARδ/β ligands (L-165,041, GW501516, and PGl2), which prevented the ubiquitination of this receptor, thereby decreasing its degradation.

Most efforts have been applied in studying PPARγ due to its involvement in adipogenesis, energetic metabolism, and insulin resistance induced by obesity. Until now, many residues were identified to suffer PTMs, such as phosphorylation, acetylation, SUMOylation, ubiquitination, nitration, S-nitrosylation, and glycosylation. PPARγ acetylation mimetics showed the same effects as the native protein, whereas its deacetylation decreases lipogenic differentiation and promotes the expression of "browning genes" and adiponectin in WAT. SUMOylation, S-nitrosylation, nitration, and O-GlcNAcylation decrease transcriptional activity and so does PPARγ phosphorylation in AF-1 and DBD (S112, Y74, Y78, S16/S21). Although PPARγ phosphorylation in the LBD (S273 phosphorylation) does not change transcriptional activity compared to wild type, the occurrence of this modification is associated with insulin resistance and the recruitment of corepressors. TZDs molecules are PPARγ agonists that received FDA approval to treat type 2 diabetes by targeting the receptor and blocking phosphorylation of S273. However, adverse cardiometabolic effects were reported after rosiglitazone (trade name Avandia) use, and in 2010 it was withdrawn from the market in the UK, Spain, Brazil, and India and some other countries later. The reports of TZDs side effects were associated with full PPARγ activation, and therefore research efforts have been focused on the development of a ligand able to block S273 phosphorylation without fully activating the receptor. In our knowledge, 21 PPARγ ligands (partial agonists and inverse agonists) were reported experimentally to block this phosphorylation with none or reduced side effects compared to TZDs; however, none of them reach and succeed in clinical trials.

Other approaches have been explored to modulate PTMs, for example, the use of modulators of enzymes responsible for the addition or removal of modifications. Nevertheless, as said before, these enzymes target several proteins in many cellular pathways, and its general modulation could have the side effect of dysregulating other cellular mechanisms instead of a specific protein. Despite this nonspecificity, some clinical trials and approved drugs for modulation of SIRT1, CDK5, MDM2, and 26S proteasome at the same time, are under studies.

Until this moment, no treatment has been approved to modulate PPARs PTMs, besides PPARγ S273 phosphorylation. However, the research and understandings of PPARs PTMs and their modulation have primary importance for improving therapeutics' development with more specificity and fewer side effects. For example, it is already known that an ideal drug for diabetes targeting PPARγ should inhibit S273 phosphorylation without a full activation of the receptor. However, recent reports suggest that inhibition of K268/K293 acetylation could prevent the collateral side effects triggered by rosiglitazone activation of PPARγ.

Besides the development of PPAR ligands that inhibit certain PTMs from having the desired effect of insulin sensitization, new drugs for this claim can be developed approaching to inhibit an addition of a PTM by blocking the protein-protein

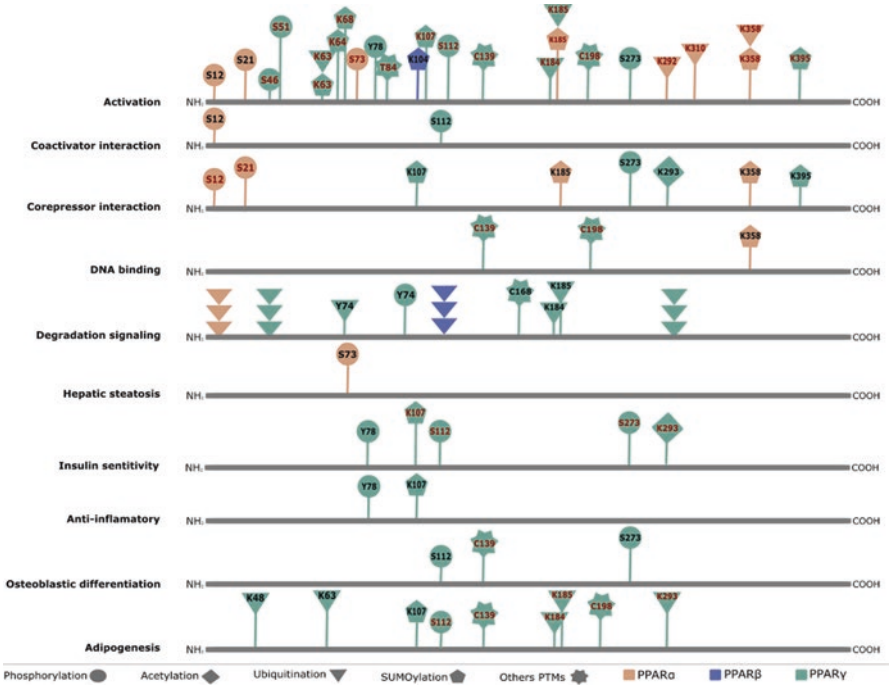


Fig. 21.10 Schematic representation of PPARs posttranslational modification sites grouped by their roles. For PPARγ, the amino acid number is representative of the PPARγ2 isoform

interface with molecules designed specifically for the desired target. Finally, the understanding of PTMS modulation of PPARs is not restricted to the scope of metabolic diseases, as the strategies learned from this receptor can be applied to the development of modulators for other proteins that undergo PTM (Fig. 21.10).

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Chapter 22
Developing Inhibitors to the Amino-Terminus Domains of Steroid Hormone Receptors

Jon K. Obst, Amy H. Tien, and Marianne D. Sadar

Abstract Steroid hormone receptors are ligand-activated transcription factors, acting as master regulators of gene expression. Steroid receptors mediate formation of large protein complexes by recruiting coregulatory proteins and transcriptional machinery to specific genomic regions. Unlike the structured ligand-binding or DNA-binding domains, the N-terminal domain (NTD), where many of these protein-protein interactions occur, contains extended regions of intrinsic disorder. Interactions in the NTD and allosteric binding elsewhere induce temporary and reversible changes in the NTD structure, substantially influencing the repertoire of potential binding partners. This structural plasticity is key for the steroid receptors to coordinate intra- and intercellular signals into a tissue specific response. Designing small molecule inhibitors against intrinsically disordered proteins (IDP) in general has proven difficult as structural information is limited. While some progress has been made in this area, only recently has any molecule targeting IDPs progressed beyond the preclinical stage. Here we summarize the discovery and development of sintokamides, niphatenones, and EPI compounds which target the intrinsically disordered NTD of the androgen receptor. These are the first drugs to target the NTD of any steroid receptor, and EPI-506 and EPI-7386 remain the only compounds that bind to an IDP to have been tested in clinical trials.

Keywords Androgen receptor · Androgen receptor variant · Intrinsically disordered protein · Amino-terminus domain

J. K. Obst · A. H. Tien · M. D. Sadar (✉)
Canada's Michael Smith Genome Sciences Centre at BC Cancer, Vancouver, BC, Canada
e-mail: msadar@bcgsc.ca

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Abbreviations

AF	Activation function
AR	Androgen receptor
CRPC	Castration-resistant prostate cancer
DBD	DNA-binding domain
ER	Estrogen receptor
GR	Glucocorticoid receptor
IDP	Intrinsically disordered protein
LBD	Ligand-binding domain
MR	Mineralocorticoid receptor
NTD	N-terminal domain
PR	Progesterone receptor

22.1 Steroid Hormone Receptors Are Modular, Ligand-Activated Transcription Factors

The steroid hormone receptors are members of the nuclear receptor superfamily and exert control over a large range of critical biological functions. These proteins include the androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR). All are soluble, ligand-activated transcription factors which function as master regulators of gene expression [38, 42, 96, 162]. These receptors are modular and consist of four major functional domains: the N-terminal domain (NTD or A/B domain), a DNA-binding domain (DBD or C domain), a flexible hinge region (D domain), and a C-terminal ligand-binding domain (LBD or E/F domain) [31]. Fat-soluble steroid hormone ligands which diffuse across the cell membrane bind the LBD of the cognate receptor. Ligand binding induces conformational changes typically releasing the receptor from chaperones and mediates the translocation of the receptor to the nucleus. There, the DBD recognizes specific genomic regions, and the receptor mediates the regulation of target genes through recruitment of transcriptional regulatory proteins [11, 31, 38, 89, 96, 162]. This is done primarily through the activity of two specialized subregions termed activation functions 1 and 2 (AF-1 and AF-2) located in the NTD and LBD, respectfully [22, 34, 66, 85, 107]. These mediate transcriptional activity by providing interaction surfaces which recognize and bind coregulators and transcriptional machinery.

22.2 Steroid Hormone Receptor: The Ligand-Binding Domain

The LBD is a highly structured domain which contains a ligand-binding pocket, a dimerization interface for homo- or heterodimerization, and the transcriptional regulatory region AF-2. This region is ligand-dependent and mediates coregulator

interaction with the steroid receptor [15, 91]. The crystal structures of LBDs have been resolved for all steroid receptors and are publically available in the Protein Data Bank. The LBD shares modest sequence homology across the steroid receptors, although is less conserved compared to the DBD, particularly in the ligand-binding pocket which allows for ligand specificity. Even the two isoforms of ER, ERα and ERβ, show 56% sequence homology in their LBD, whereas they have 97% sequence homology in the DBD [91, 165].

Despite differences in sequence homology, the LBDs of steroid receptors share similar structural patterns and function. Namely, they are folded similarly in 3 layers to form an anti-parallel α-helical sandwich containing up to 12 α-helices and 1–4 short β-strands that may form β-sheets [15, 54, 91, 137, 163]. ER has 12 helices (H1-H12), while other steroid receptors lack helix 2 [54]. In general, binding with an agonist induces conformational changes such that helix 12 stabilizes and covers the ligand-binding pocket, forming a hydrophobic cleft and exposing the AF2 region. This creates a binding interface to interact with LxxLL motifs of coactivators such as steroid receptor coactivators (SRCs) [52, 54, 91, 125].

Additionally, the LBD seems to negatively regulate the transcriptional activity of the NTD in the absence of ligand. Deletion of the LBD in the PR, GR, and AR results in a constitutively active receptor, albeit one with reduced overall transcriptional activity relative to wild-type receptor [6, 22, 57, 65]. Deletion of LBD in ER resulted in a 95% decrease of its transcriptional activity relative to wild-type full-length ER activity [92], whereas deletion of LBD in AR resulted in only a 25% reduction in transcriptional activity [65] demonstrating the heterogeneity with which each steroid receptor relies upon the AF-2 for full transcriptional activity. Furthermore, the ability of the LBD to influence transcriptional regulation supports that agonist binding induces unique allosteric changes not only locally but also in distant regions within the receptor as well. This concept will be explored in greater detail in the context of intrinsic disorder within the NTD. Taken together with the many possible binding partners which exist for the steroid receptors, numerous transcriptional outputs are possible depending on the particular cellular context, allowing for exquisite control over targeted gene expression [80, 84].

22.3 Steroid Hormone Receptors: The DNA-Binding Domain and Hinge Region

The DBD is highly conserved among the steroid receptors (56%–79% sequence homology) [38] and directs the receptor to specific genomic regions termed hormone response elements contained within the promoter or enhancer regions of target genes. Like the LBD, the crystal structure of the DBD has been resolved and contains three α-helices which form a hydrophobic core. The key feature of this domain revolves around two highly conserved zinc finger motifs, which each coordinate binding of a single zinc atom. These structures form the main DNA-binding interface and facilitate binding in the major groove [74, 89, 173]. In this way, the DBD is split into two interdependent subdomains, each containing a single zinc

finger which fold together as a unified globular domain [45]. The amino terminal zinc finger and three associated amino acids form a "P-box" which is largely responsible for recognition of specific genomic sequences. This includes both general response elements which are recognized by the majority of steroid receptors (AR, GR, PR, MR) [144] and receptor-specific response elements [20, 25, 41, 64, 162]. The second zinc finger along with associated amino acids forms the "D-box" which functions primarily to stabilize the protein-DNA interaction and mediates homo- and heterodimerization with other transcription factors [123].

The region between the DBD and LBD is commonly referred to as a hinge region. The hinge region contains a nuclear localization sequence (NLS), which is sequestered in the absence of ligand binding [116]. The hinge region also contains the C-terminal extension (CTE) which is considered part of the DBD as it participates in DNA-receptor interactions [54]. Largely disordered with little sequence homology across steroid receptors, the CTE does share some functional features. The CTE interacts with the minor groove of DNA and has been proposed to assist in recognition of response elements with weak half-sites, increasing the overall number of target genes able to be transcribed [54, 123]. DNA binding also induces conformational changes within the CTE, stabilizing intramolecular interactions [54] and priming the CTE to serve as a binding site for coregulatory proteins [13, 119]. A common feature among the steroid receptors is their ability to distort and alter DNA conformation following binding. It is postulated that this may assist in facilitating the assembly of multi-protein complexes at enhancer or promoter regions of target genes [54].

22.4 Steroid Hormone Receptors: The N-Terminal Domain

Despite being the least conserved domain with respect to both amino acid sequence (<15%) [6] and length, the NTD shares structural and mechanistic features among the steroid receptors. Within the NTD of all steroid receptors lies a major transactivation domain termed AF-1 [83]. Much like its AF-2 counterpart in the LBD, the AF-1 provides a binding interface and mediates protein-protein interactions with coregulatory proteins and transcriptional machinery [53, 67, 86, 92]. With the exception of the ER, the NTD contributes the majority of transcriptional activity, and its presence is necessary for full transcriptional activity [6, 22, 57, 66, 85]. Nonetheless, while mutational and deletion experiments have shown that each activation function is capable of acting independently, maximum activity is found when both can cooperate in concert [86, 104]. The NTD is characterized by large regions of intrinsic disorder which allows it to sample many unique structural conformations, dramatically increasing the number of potential binding partners [51]. Intrinsic disorder is intimately related to the ability of steroid receptors to mediate the coordination and assembly of large protein complexes in a tissue-specific manner under a wide range of cellular contexts. While the structure and function of the NTD will

be explored in greater detail in later sections, it is necessary to briefly discuss the concept of intrinsic disorder and its impact upon cellular signaling networks.

22.5 Properties of Intrinsically Disordered Proteins

Unlike ordered proteins which are restricted to well-defined tertiary structures, intrinsically disordered proteins (IDP) and proteins which contain localized regions of intrinsic disorder are characterized by their inability to spontaneously form into stable globular three-dimensional structures. This is primarily due to an amino acid sequence largely made up of charged and polar residues with a low percentage of hydrophobic bulky side chains [29, 150, 157]. These features have allowed the existence of IDPs to be predicted using algorithms such as PONDR or GlobPlot [49, 161].

All IDP have significant structural heterogeneity and exist between rapidly changing structural conformations [19, 26, 35, 105] allowing them to recognize binding partners with high specificity but low affinity. This endows IDPs the ability to interact with a plethora of potential binding partners under varying cellular contexts [28, 29, 171]. Generally IDPs are thought to undergo a disorder-to-order transition upon interaction with their binding partner as understood by the induced fit model, where folding and binding are coupled [29, 75, 76, 101, 105, 157–159], although there are also examples of IDPs remaining disordered despite binding [101, 132, 159].

Sites involved in posttranslational modifications are predominantly found in regions of disorder as compared to structured domains [5, 23, 60, 172]. IDPs are particularly sensitive to electrostatic effects, and modifications such as phosphorylation or acetylation can have profound impact on intra- and intermolecular interactions [5]. These often influence local stabilization or destabilization of secondary structure (and in some cases determine global conformational changes), protein activity, turnover, and binding partner preference, among others [23, 50, 172]. Combined with their structural plasticity, IDPs are perfectly positioned to perform a variety of vital cellular functions including regulation of transcriptional processes, cell cycle, mRNA processing scaffolding, and apoptosis [27, 29, 35, 101, 117, 150, 157, 168]. Unsurprisingly, IDPs are commonly found functioning as hubs within signaling networks, and those proteins which promote supramolecular complexes [27, 75, 101, 131, 149, 150, 157]. In following, approximately 70% of transcription factors have been estimated to contain extended regions of disorder [100]. Further evidence for the importance of IDPs is provided by the fact that they are ubiquitously found throughout nature, with concentration increasing from prokaryotes to higher species [117, 160, 168].

22.6 Intrinsic Disorder in the Steroid Hormone Receptor NTD Is Vital for Function

Large regions of the steroid hormone NTDs preferentially exist in a disordered state as random coils with limited secondary or tertiary structure (Fig. 22.1). However, they are capable of adopting transient stable structures following interaction with DNA or other proteins [34, 81, 90, 152, 154]. As a result while the crystal structures of the LBD and DBD of many of the steroid receptors have been solved [32, 45, 71, 106, 128, 129, 138], detailed structural information of the NTD is lacking. Nonetheless extensive work has been undertaken to better understand and describe intrinsically disordered proteins as a whole and NTD of steroid receptors, specifically.

Integral to the capability of the steroid hormone receptors to function as master regulators of gene expression in a tissue and a cellular context-specific manner, is their ability to interact with a wide array of protein cofactors and participate as hubs in complex signaling networks [51, 78, 130]. Mutational and deletion studies have

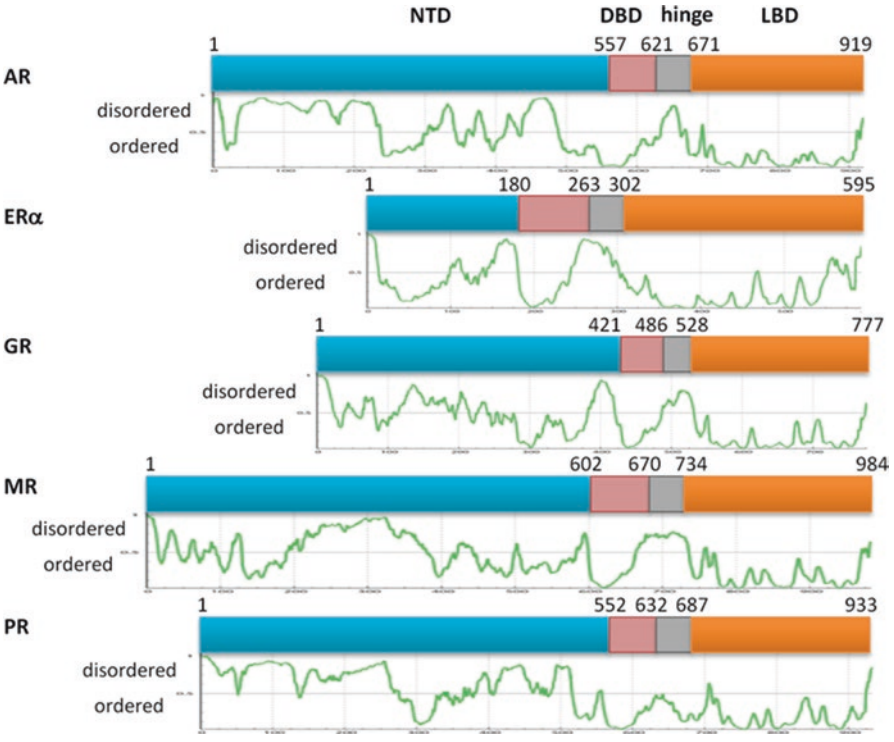


Fig. 22.1 Predicted disorder in different domains of the steroid hormone receptor family. Each steroid hormone receptor consists of four functional domains: NTD, DBD, hinge, and LBD. The disorder disposition was predicted by PONDR-FIT (DisProt)

identified an AF-1 region within the NTD which contributes a substantial amount of transcriptional activity, although the relative "strength" varies between the steroid hormone receptors [6, 34, 39, 57, 107, 133] and appears to be related to overall sequence length [47, 84]. While the AF-2 contained within the LBD is ligand dependent, the AF-1 appears to be able to retain transcriptional activity despite the absence of ligand, including even when the LBD itself is absent [6, 22, 57, 66, 85]. In contrast to the AF-2 which has a well-defined LxxLL-binding motif, no equivalent has yet been found for AF-1 [88]. This is in agreement with the fact that IDP regions in general lack a discrete interaction motif despite interacting with a large number of protein partners.

The NTD of steroid receptors have been experimentally shown to undergo a disorder-to-order transition, increasing in both secondary and tertiary structure in the AF-1 region following treatment of naturally occurring osmolytes such as TMAO or trehalose [55, 72, 85, 87]. Osmolytes are produced within the cell to protect against extreme conditions such as heat shock or excessive osmolarity and function by inducing structure in proteins which fail to fold spontaneously [14]. Allosteric effects elsewhere in steroid hormones may influence NTD structure and stability. For instance, deletion studies have shown that two domain constructs consisting exclusively of the NTD and DBD tend to be constitutively active in several of the steroid receptors; implying that in the unbound state the LBD has an inhibitory effect [22, 57, 66]. Binding of ligand to LBD relieves this repression, if not through a direct conformational change in the NTD, then by facilitating interaction with coregulatory proteins.

Interestingly DNA itself has been proposed to act as an allosteric ligand, as DBD interaction with genomic response elements has also been shown to induce tertiary structure and α-helical content of the NTD/AF-1, and subsequently promote protein-protein interaction with cofactors and transcriptional activity [6, 102, 103]. While the DBD functions to guide the receptor to specific regions of the genome through recognition of consensus sequences, it also serves to prime the NTD for interactions with specific coregulatory proteins. Thus it would appear that the genomic sequence of a particular response element exerts some influence over the degree of the transcriptional response [55].

NTD-interacting proteins such as TBP [34, 88, 90, 154], CBP [82], RAP74 [82, 122], and JDP2 [154] have a similar effect on stabilizing the NTD by increasing α-helical content, preparing the receptor for further protein interaction. Indeed simply modifying the concentration of coregulatory proteins has been shown to influence relative agonist activity and reflects the ability of cofactor binding to modulate additional protein-protein interactions [134, 164]. In addition, local structural changes in AF-1 may also induce unfolding of adjacent structured regions [113], further impacting potential binding partners and allowing the steroid receptors to interact with a staggering combination of binding partners [53, 78, 167].

The intrinsically disordered NTD of the steroid receptors is a hotspot for post-translational modifications which play a major role in cellular and tissue-specific activity. The majority of phosphorylation sites are found in the NTDs of all steroid hormone receptors, with many of these localized to the AF-1 region [4, 33, 40, 63,

73]. Regulation of phosphorylation of the AF-1 region in several of the steroid receptors has been experimentally shown to be able to promote a disorder-to-order conformational transition, with a subsequent increase in interaction with coregulatory proteins and transcriptional activity [4, 37, 40, 73]. However, phosphorylation can also have an opposing inhibitory effect on receptor stability by triggering ubiquitination and proteolytic degradation as has been shown in the AR and GR [99, 156]. In general, the NTD region of the steroid receptors is susceptible to proteolysis due to ease of access by proteolytic enzymes as compared to the structured DBD and LBD domains [6, 7, 72]. Studies of NTD-DBD constructs of the AR, GR, and PR have shown that increased NTD folding and stability occurs following DNA interaction, leading to resistance against proteolytic degradation [6, 7, 81]. Thus, modulating NTD structure through local and/or allosteric effects following ligand binding in the LBD, DNA binding, interaction with coregulatory proteins, or posttranslational modifications has a large impact on receptor activity, overall stability, and receptor turnover.

Collectively, steroid hormone receptors function as major signaling hubs coordinating large numbers of coregulatory proteins and transcriptional machinery. These proteins are responsible for the strict regulation of hundreds of gene targets [24] and function as master regulators over a host of critical and diverse transcriptional programs. This ability is largely due to the NTD and the AF-1 region in particular. The AF-1 is unique from the AF-2 in that it does not rely upon a core motif or minimal sequence for function. Rather, the AF-1 contains multiple regions capable of adopting temporary stable, yet reversible structures which favor different binding partner combinations and can be tailored to a wide variety of cellular cues [85, 154, 155].

Abnormal regulation of steroid receptors is associated with many human diseases including cancer. Therefore, extensive research has delved into the development of antagonists which can abrogate transcriptional activity by disrupting receptor/DNA and/or receptor/coregulator interactions. Due to both the lack of structural information of the NTD, and the high sequence homology of the DBD between the steroid receptors, most of this effort was focused on designing drugs which compete with ligand binding in the LBD or prevent interaction of cofactors with AF-2 [16, 67]. This however has proven to be inadequate as this strategy does not wholly inhibit AF-1 transcriptional activity, in part due to its ability to function semi-autonomously from AF-2 and ligand stimulation [6, 22, 57, 66, 85]. Additionally at least in the case of prostate cancer, AR-splice variants exist which lack the LBD entirely, yet retain transcriptional activity and are clinically relevant [3].

The NTD contributes the majority of transcriptional activity for many steroid receptors. Coregulators, DNA, and posttranslational modifications have the ability to influence transcriptional activity by facilitating allosteric effects upon distant regions of the NTD. This raises exciting prospects for the development of small molecule antagonists targeting these critical regulatory regions. Developing antagonists to the NTD of steroid receptors represents a novel strategy which could have wide-ranging implications for a number of human maladies.

22.7 The Challenge of Targeting Intrinsically Disordered Proteins

The expression of IDPs is strictly controlled, owing to their prevalence in mediating critical biological functions and regulating signaling pathways [43, 148]. Thus, the misprocessing, mismodification, misexpression, or otherwise deregulation of IDPs is often associated with pathological disease states. This phenomenon has been termed "disorder within disorders" or D^2, and IDPs or proteins containing long regions of disorder have been associated with several neurodegenerative diseases, cardiovascular disease, cystic fibrosis, type II diabetes, AIDS, and various cancers [79, 146, 147, 151]. Indeed the majority (~80%) of proteins associated with human cancers are predicted to contain continuous regions of disorder spanning 30 or more residues [59].

IDPs are thus attractive therapeutic targets; however, developing small molecules which target these regions has proven difficult. This is owing primarily to the fact that IDPs exist as ensembles of rapidly changing conformations, preventing the formation of a stable three-dimensional structure which can be modeled [19, 26, 35, 105]. Additionally many unique protein-protein interactions are possible and occur over relatively large and dynamic interfaces, while small molecule inhibitors typically interact with only a small portion of the target [101, 157]. Therefore, potential candidates are typically discovered through large-scale chemical screens and computational tools, rather than structure-based rational drug design. Another concern relates to the high degree of conformational flexibility inherent in IDPs which allow transient interaction with many binding partners, including small molecules. This promiscuity does indeed make it easier for high throughput screens to identify initial hits; however, drugs must not only show sufficient affinity for the target but also not interact with other proteins or alternative IDPs [12]. While numerous compounds have been discovered through high-throughput chemical screens, the lack of IDP inhibitors which have advanced to the clinic reflects this challenge of reducing off-target effects and drug-related toxicity [12, 30, 115]. Despite these challenges, much progress has been recently made in the discovery and development of small molecule inhibitors targeting IDPs.

22.8 Examples of Targeting IDPs of Transcriptional Regulators in Human Cancers

Transcription factors play pivotal roles as master regulators of gene expression capable of both positively and negatively controlling gene transcription and are critically important to the normal function of the cell. These proteins receive a host of extra- and intracellular signals, distilling them into a singular and coordinated response. This allows the cell to rapidly respond to an ever-changing environment. Underpinning their ability to exert such influence lies with extended regions of

intrinsic disorder, a hallmark of the vast majority of transcription factors which increases with organism complexity [100, 109]. Unsurprisingly, mutations and deregulation of transcription factors are associated with many human maladies ranging from developmental disorders, to obesity, to infertility, to cancer [98, 117, 147].

As yet apart from the EPI, sintokamide, and niphatenone compounds discussed in detail later on, no inhibitors of any steroid receptor NTD has been published to date. The following therefore highlights the recent progress which has been made in the discovery and development of inhibitors targeting various IDPs in general. Specifically, studies focusing on targeting transcriptional regulators in human cancers are briefly discussed. While not exhaustive, the works summarized provide a general roadmap for the development of compounds targeting IDPs, as well as serving to illustrate some of the challenges associated with targeting regions of disorder.

c-Myc is a transcription factor directly involved in the regulation of cellular growth and differentiation, apoptosis, and metabolism. Deregulation of c-Myc is commonly found in many human cancers [21, 135]; thus, many groups have focused on developing small molecule antagonists against it. c-Myc functions through interactions with its binding partner Max via complimentary helix-loop-helix-leucine zipper (bHLHLZip) domains. As a monomer, c-Myc exists in an unfolded and disordered state and only undergoes coupled folding following interaction with Max [18, 44, 166, 169]. The resulting structured heterodimer is capable of binding DNA and regulating the transcription of target genes.

Some of the first inhibitors to be discovered which target IDPs were against c-Myc [12], and several unique strategies to identify potential compounds have been employed. These were identified following a large chemical screen of 7000 compounds using fluorescence resonance energy transfer (FRET) to discover potential hits by their ability to disrupt heterodimerization of recombinant fluorescent c-Myc and Max constructs [12]. Hits were confirmed using ELISA and electrophoretic mobility shift assay (EMSA). Two compounds identified by the screen, IIA4B20 and IIA6B17, were capable of disrupting Myc-induced transformation of CEF cells. However IIA6B17 and to a lesser extent IIA4B20 also had activity against Jun-induced transformation as well [12]. This proof of principal study confirmed the potential for disrupting protein-protein interactions of IDPs, however also highlighted the challenge of ensuring on-target specificity using this strategy.

Subsequent studies investigating c-Myc inhibitors have also exploited large-scale screens of chemical libraries, identifying candidates using a protein fragment complementary assay (~400,000 compounds) [18, 46] and yeast two-hybrid (10,000 compounds) [44, 166] systems. From the latter, seven small molecules were discovered using circular dichroism (CD) and NMR studies, and three binding sites within the bHLHLZip domain were resolved [44]. A follow-up study exploiting the newly discovered binding sites used a structure-based approach. In this case, a virtual screen was used to identify potential compounds by modeling predefined binding sites within typical c-Myc conformations [169]. Two hundred and seventy-three potential compounds were identified which were predicted to bind to one of these binding sites within the bHLHLZip domain on c-Myc. These were initially tested

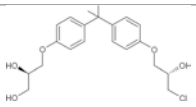
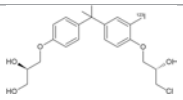
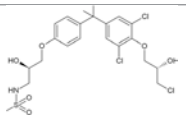
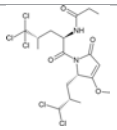
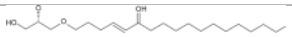
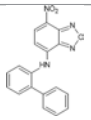
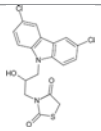
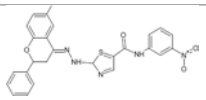
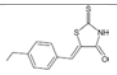
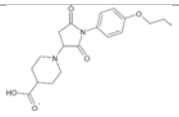
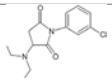
using CD and confirmed using NMR. Four compounds bound the c-Myc bHLHL-Zip domain, inhibited c-Myc/Max heterodimerization, and displayed biological activity that inhibited cancer cell growth in the micromolar range. Thus, computer-aided drug design is feasible for discovering compounds which bind to IDPs; however, this is reliant upon the prior discovery and characterization of potential binding sites [169]. Another strategy involves the design of synthetic peptidomimetics which act as decoy molecules preventing protein-protein interactions. The *MLL* gene is often involved in a reciprocal translocation with *AF4* especially in acute lymphoblastic leukemia in infants [136]. AF4 has been shown to directly interact with AF9, both of which are both intrinsically disordered. A yeast two-hybrid system was utilized to map a 10-residue AF9 binding domain located within AF4. The PFWT peptide corresponding to the AF9 recognition sequence was shown to compete with AF4 for AF9 binding and impeded AF4-AF9 interaction both in cell-free and cellular assays. PFWT was also capable at inhibiting the growth of several leukemia cell lines containing *MLL* rearrangements [136].

Similarly, the EWS-FLI1 fusion product is a common occurrence in Ewing's sarcoma family tumors (ESFT) and promotes tumor formation and maintenance through interaction with RNA helicase A (RHA) [30, 145]. EWS-FLI1 is an IDP [145], and disruption of EWS-FLI1 binding with RHA using a synthetic peptide was sufficient to disrupt ESFT cellular growth [30]. A subsequent large chemical screen of 3000 small molecules using surface plasmon resonance (SPR) initially identified NSC635437 as a promising candidate. Optimization of NSC635437 chemistry yielded YK-4-279 which disrupted EWS-FLI1 and recombinant RHA interaction, induced apoptosis in several ESFT cell lines expressing the EWS-FLI1 fusion, and also had antitumor activity in vivo [30]. However, YK-4-279 did show toxicity in A673 cells, a line which has low EWS-FLI1 activity possibly indicating off-target effects [30].

Many investigations into the discovery of novel compounds targeting IDPs exploit the fact that a single, specific binding partner is well-known and that blocking this interaction results in loss of biological activity. In these cases, screens using a yeast two-hybrid, FRET, PCA, or SPR can be employed to identify candidates, and additional techniques such as NMR or CD are used to confirm binding. Furthermore structural information following partner binding can be obtained, allowing a degree of structure-based rational drug design to be employed to better optimize initial hits as was seen with PFWT [30]. This is also exemplified by c-MYC/Max [12, 18, 44, 46, 69, 166, 169], EWS-FLI1/RHA [30], and AF4/AF9 [136] interactions as described above. Other examples exist as well, and compounds disrupting NUPR1/MSLI1 (trifluoperazine and ZZW-115) [115, 127], HIF-1α/p300 (chetomin, gliotoxin, and chaetocin) [93, 121], and $p27^{Kip1}$/Cdk2 (SJ-710 and SJ-403) [61] interactions have been published (Table 22.1). Many of these have shown favorable activity in a variety of different cancer cell lines, and several also show antitumor activity in vivo [30, 46, 69, 77, 93, 115, 121, 127].

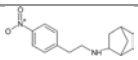
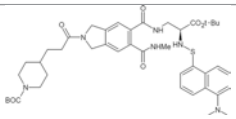
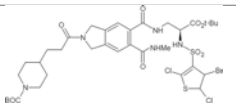
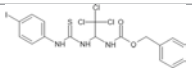
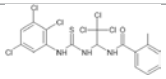
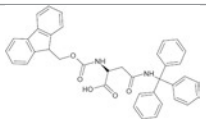
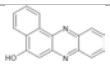
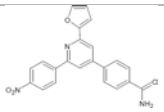
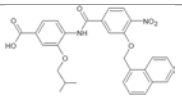
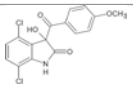
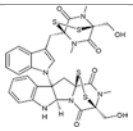
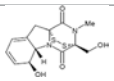
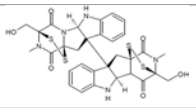
Despite these advances, with the exception of the EPI compounds discussed in detail below, no drug that directly binds to an IDP has yet progressed beyond the preclinical stage. Still, significant progress has been made in this area. As more light

Table 22.1 List of compounds targeting regions of intrinsic disorder in human cancers

Target	Compound	Structure	Detection Method	Disease model (cell lines)	Refs
AF9	PFWT peptide	pen-LWVKIDLDLLSRV	RD	Leukemia (RS4;11, MV4-11, B1)	[136]
AR (NTD)	EPI-002		htCLS (Luc-reporter)	Prostate (LNCaP, LN95, VCaP, 22RV1) – NCT02606123	[1, 114, 118, 164]
	I-EPI-002		RD	Prostate (LNCaP, LN95)	[62]
	EPI-7170		RD	Prostate (LNCaP, LN95, VCap, C4-2B)	[8, 56]
	SINT1		htCLS (Luc-reporter)	Prostate (LNCaP, LN95)	[10, 126]
	Niphatenone B		htCLS (Luc-reporter)	Prostate (LNCaP)	[9, 108]
c-MYC	10074-G5		htCLS (yeast two-hybrid)	c-MYC overexpression (Rat1a-c-Myc)	[12, 166]
	10074-A4		htCLS (yeast two-hybrid)	c-MYC overexpression (Rat1a-c-Myc), Leukemia (HL-60)	[12, 44, 166]
	10050-C10		htCLS (yeast two-hybrid)	c-MYC overexpression (Rat1a-c-Myc)	[12, 166]
c-MYC	10058-F4		htCLS (yeast two-hybrid)	c-MYC overexpression (Rat1a-c-Myc), Leukemia (HL-60, Ramos, KG1a), NSCLC (NCI-H460),	[44, 46, 166, 169]
	10031-B8		htCLS (yeast two-hybrid)	c-MYC overexpression (Rat1a-c-Myc)	[12, 166]
	10075-G5		htCLS (yeast two-hybrid)	c-MYC overexpression (Rat1a-c-Myc)	[12, 166]

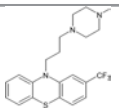
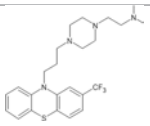
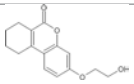
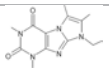
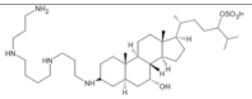
(continued)

Table 22.1 (continued)

Target	Compound	Structure	Detection Method	Disease model (cell lines)	Refs
	10009-G9		htCLS (yeast two-hybrid)	c-MYC overexpression (Rat1a-c-Myc)	[12, 166]
	IIA4B20		htCLS (FRET)	CEF	[135]
	IIA6B17		htCLS (FRET)	CEF	[135]
	PKUMDL-YC-1203		VS	Leukemia (HL-60)	[44]
	PKUMDL-YC-1204		VS	Leukemia (HL-60)	[44]
	PKUMDL-YC-1205		VS	Leukemia (HL-60)	[44]
c-MYC	sAJM589		htCLS (PCA)	Leukemia (HL-60, Ramos, KG1a)	[18]
	KJ-Pyr-9		htCLS (FP)	B Cell Lymphoma (P493-6), NSCLC (NCI-H460), TNBC (MDA-MB-231, SUM-159PT)	[46]
	4da (JKY-2-169)		RD	Leukemia (HL-60), NSCLC (NCI-H460)	[69]
EWS-FLI1	YK-4-279		htCLS (SPR)	Prostate (PC3), Ewing Sarcoma (CHP-100, TC71)	[30]
HIF-1α	Chetomin		htCLS (yeast two-hybrid)	Hepatocellular carcinoma (Hep3B, HepG2), Prostate (PC3, DU-145), Colorectal (HCT116)	[93, 121]
	Gliotoxin		RD	Prostate (PC3, DU-145), Colorectal (HCT116)	[121]
	Chaetocin		RD	Prostate (PC3, DU-145), Colorectal (HCT116)	[121]

(continued)

Table 22.1 (continued)

Target	Compound	Structure	Detection Method	Disease model (cell lines)	Refs
NUPR1	Trifluoperazine		htCLS (FTD)	Pancreatic (MiaPaCa-2)	[115]
NUPR1	ZZW-115		RD	Pancreatic (MiaPaCa-2, LIPC, Foie8b, 02-063, HN14)	[127]
$p27^{Kip1}$	SJ-710		htCLS (NMR)	n/a	[61]
	SJ-403		htCLS (NMR)	n/a	[61]
PTP1B	MSI-1436		RD	Breast (BT474)	[79]

Abbreviations: *FP* fluorescence polarization, *FRET* fluorescence resonance energy transfer, *FTD* fluorescence thermal-denaturation, *htCLS* high throughput chemical library screen, *PCA* protein-fragment complementation assay, *RD* rational design, *SPR* surface plasmon resonance, *VS* virtual screen

is shed on the interaction between IDPs and specific binding partners, elements of rational-based drug design can be applied to design compounds which inhibit these interactions [69, 121, 127, 136]. Additionally, initial hits discovered from chemical screens have been successfully modified to increase potency and on-target specificity [30, 44, 127, 169], recently culminating in the first clinical trials of any drug to target an IDP [17, 110]. While the number of IDP targets and corresponding antagonists is currently limited, the rapid development of novel tools capable of probing the molecular features of IDPs suggests both will increase dramatically in the near future.

22.9 A Rationale for Targeting the AR-NTD in Prostate Cancer

The AR is a steroid hormone receptor and has long been recognized as a key driver of prostate cancer [58]. As has been described, while the C-terminal LBD is structured, the NTD is characterized by large regions of intrinsic disorder, and the crystal structure has not been resolved [83, 97]. The AR is capable of interacting with ~160 binding partners, most of which occur through the NTD [51]. Like other steroid hormone receptors, the AR-NTD contains the AF-1 motif which contributes the

majority of transcriptional activity to the AR through two distinct subregions: transcriptional activation unit 1 (Tau-1) and Tau-5 [65]. Tau-1 comprises amino acid residues 101–370, whereas Tau-5 spans residues 360–485. AF-1 mediates AR transcriptional activity both in the presence and absence of androgen ligand, and its presence is vital for the AR to properly function [66].

In addition to interacting with coactivators, the AF-1 can interact with the AF-2 either directly or indirectly through coactivators, although this remains dependent upon ligand activation [86, 140]. Moreover, He and colleagues demonstrated that this N/C interaction in the AR was required to stabilize helix 12 and slow down the dissociation of androgens, allowing for sustained transcriptional activity [48]. The N/C interaction is also important for stabilizing dimerization and overall receptor stability [137]. Disruption of N/C interaction due to mutations V889M and R752Q increased androgen dissociation rate, induced AR instability, and accelerated AR degradation [95].

Abnormal regulation of steroid receptors is associated with many human diseases including cancer. Therefore, extensive research has delved into the development of antagonists which can abrogate transcriptional activity by disrupting receptor/DNA and/or receptor/coregulator interactions. Due to both the lack of structural information of the NTD and the high sequence homology of the DBD between the steroid receptors, most of this effort was focused on designing drugs which compete with ligand binding in the well-defined LBD or prevent interaction of cofactors with AF-2 [16, 67]. This however has proven to be inadequate as this strategy does not wholly inhibit AF-1 transcriptional activity, in part due to its ability to function semi-autonomously from AF-2 and ligand stimulation [6, 22, 57, 66, 85]. In addition, constitutively active AR-splice variants which lack the AR-LBD and gain-of-function mutations in the LBD are clinically relevant [2, 3, 68, 139]. Small molecules targeting the intrinsically disordered AR-NTD have the potential to inhibit both ligand-dependent and ligand-independent AR transcriptional activity and could thus overcome multiple resistance pathways to existing therapies for lethal castration-resistant prostate cancer (CRPC).

22.10 Decoys and Chemical Libraries

Early studies in the 1990s showed that the transcriptional activity of AR resides within the AF-1 contained in the NTD and that deletion of the LBD yielded a constitutively active truncated receptor [65]. Research efforts to discover inhibitors of the AR NTD were prompted by these studies together with the finding that androgen-independent activation of AR could occur through crosstalk with signaling pathways initiated by bone-derived factors, cytokines, and protein kinases which converged upon the AR-NTD [124, 142, 143].

Evidence supporting the AR-NTD as a therapeutic target was provided with proof-of-principal studies using a decoy molecule corresponding to the AR-NTD ($AR_{1–558}$) [120]. These studies showed that $AR_{1–558}$ was capable of inhibiting

endogenous AR^{FL} signaling presumably by sequestering coregulatory proteins required for its transcriptional activity [120]. In spite of the decoy encoding the entire AR NTD, there was considerable specificity with little to no inhibitory effects on agonist-induced transcriptional activities of GR or ER-α; however, decoy $AR_{1–558}$ blocked agonist-induced PRβ transcriptional activity by 90% [120]. Impressive in vivo antitumor activity against human CRPC xenografts in castrated hosts was measured using viral delivery of the decoy to established tumors and in prostate cancer cells engineered to stably express abundant levels decoy [120]. These studies provided evidence that protein-protein interactions with AR NTD were essential for AR transcriptional activity and growth of CRPC tumors and, if targeted, would mediate a therapeutic response [120].

To find a small molecule inhibitor that could duplicate the therapeutic response achieved with the decoy, libraries of rationally pre-selected pharmacophores as well as extracts of marine sponge were screened in a series of specialized cell-based assays [125]. No hits were detected from the 52,000 compounds tested in the ChemBridge DIVERSet and NCI Diversity set, whereas there were approximately 30 hits from unique marine sponge extracts. Of these 30 hits, three different chemical scaffolds have been published that directly interact within AF-1 of the AR NTD. These are the EPI compounds (ralaniten), sintokamides, and niphatenones. The remaining active extracts and unique scaffolds continue to be characterized.

22.11 EPI Compounds (Ralaniten)

The EPI molecules are the best described and have generated two clinical candidates thus far. As first-in-class compounds, the USAN council granted a new generic stem name “aniten” to distinguish their unique mechanism of action from antiandrogens with the stem name “lutamides.” EPI-001 is a mixture of four stereoisomers (EPI-002 to EPI-005). These compounds directly bind to Tau-5 within AF-1 of AR-NTD to prevent essential protein-protein interactions required for both ligand-dependent and ligand-independent AR transcriptional activity [1, 111]. EPI-001 is a derivative of bisphenol A diglycidyl ether (BADGE) isolated from the marine sponge *Geodia* lindgreni and has a unique chemical structure compared to antiandrogens. Increasing concentrations of androgen has no effect upon the ability of EPI-001 to antagonize AR^{FL} transcriptional activity [114], nor can high concentrations of EPI-001 displace fluoromone ligand from AR-LBD as demonstrated by a fluorescence polarization assay [1]. These studies suggested that EPI-001 did not inhibit AR transcriptional activity through competition with androgen for the ligand-binding pocket in the C-terminus LBD. Instead, EPI-001 has a unique mechanism from antiandrogens.

The first data to provide evidence that EPI-001 directly interacted with AF-1 in AR NTD was using a cell-free assay employing fluorescence emission spectroscopy [1]. These studies exploited the presence of tryptophan and tyrosine residues within recombinant AF-1 protein to reveal a change in conformation upon binding of

EPI-001 [1]. Additional studies using biotinylated recombinant AF-1 with various EPI probes revealed that approximately 1% of the AF-1 population formed irreversible bonds with EPI thereby allowing application of SDS-PAGE to detect direct binding to biotinylated compounds [114]. Due to the potential for an intrinsically disordered protein in a cell-free assay to not acquire the structural conformations present in vivo, it was important to examine direct binding of EPI to endogenous AR in cells with the physiological concentrations and repertoire of interacting proteins. These studies employed radiolabeled and biotinylated EPI analogs and probes with the endogenous full-length AR in LNCaP cells and transiently transfected fragments of AR [62, 114]. This seminal work was the first to show direct interaction of a small molecule to an intrinsically disordered target in living cells. Importantly these studies also provided evidence for the specificity of EPI compounds to bind to AR with little to no binding to other proteins [62, 114].

No reactivity of the chlorohydrin group within the scaffold of EPI-001 and its stereoisomers was detected at physiological pH as indicated with radiolabeled analogs in vitro, in vivo SPECT-imaging, nor in patient samples, and no adduct formation with glutathione [9, 62, 118, 153]. In 2016, the binding site for EPI-001 was mapped by NMR using a fragment of the AF-1 region ($AR_{142–448}$). EPI-001 was shown to preferentially bind residues spanning 354–448 and which are part of the Tau-5 region within the AR-NTD[58]. While three regions (341–371; 391–414; and 426–446) had large chemical shifts, EPI-001 failed to bind when peptides corresponding to these regions were used independently. This suggests that the specificity of EPI-001 for Tau-5 is dependent upon the simultaneous presence of $AR_{354–448}$ and is related to a particular structural conformation of Tau-5 [111]. Thus EPI-001 does not bind linear amino acid sequences but instead binds within a pocket, supporting the specificity of EPI-001 and its stereoisomers against the AR. The site of EPI-001 interaction mapped by NMR was consistent with previous studies showing EPI-blocked protein-protein interactions with CBP and RAP74 [1, 164] which both interact with Tau-5 shown in Fig. 22.2 [94, 112]. K_D binding affinities for TFIIF (RAP74) binding to Tau-5 are influenced by phosphorylation within this region going from 1749 μM to 702 μM with phosphorylation of S424 [112]. These binding affinities in the mM and high μM range highlight important differences in terms of drug development between targeting protein-protein interactions within Tau-1 or Tau-5 versus developing an inhibitor to AR-LBD such as an antiandrogen. Ralaniten with an IC50 of 10 μM was sufficient to mediate in vivo efficacy both in patients and in vivo in preclinical models, whereas antiandrogens that target the AR-LBD are required to have affinities in the low nanomolar range in order to compete with DHT. As expected, ralaniten did not block interaction with the p160 family of SRC proteins [164] that interact within residues 1–233 of the AR-NTD [142, 170] that is outside of Tau-5 (residues 360–485).

Initial in vitro experiments describing the mechanism of EPI-001 binding to Tau-5 indicate a rapid reversible interaction, followed by a slow covalent binding step, requiring the secondary hydroxyl group on C20 [111, 114]. Later a study with mass spectrometry determined cysteine residue 404 was the major residue in AF-1 involved in the slow covalent binding step with EPI-001 [111]. A subsequent study

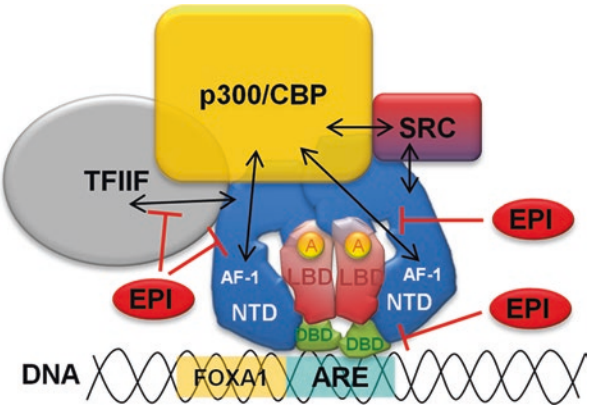


Fig. 22.2 Binding of ralaniten to AR NTD prevented N/C interaction, reduced the interaction between RAP74 (TFIIF) and CBP with AF-1, and decreased AR binding to DNA (red T bars indicate the interrupted interactions). However, ralaniten did not block the interaction between SRC-1 and AR1–233. Upon ralaniten binding, local conformation may be induced in NTD, leading to interrupted interactions

investigating a radiolabeled EPI analog for potential development as an imaging agent to identify the presence of AR-splice variants in metastatic lesions, suggested that covalent binding may not occur in vivo. Biodistribution data in mice indicated a relatively fast turnover of approximately 3 hours, and thus the slow covalent binding step observed only in closed in vitro systems was likely not permitted [62].

EPI-001 has two chiral carbons (C1 and C20). The stereoisomer EPI-002 (2R,20S) was given the generic name "ralaniten" and chosen as a lead candidate for further study due to its potency and low toxicity profile [114]. Ralaniten demonstrated on-target activity in a variety of CRPC models [56, 62, 114, 118, 164] and importantly retained efficacy against constitutively active AR-splice variants lacking the LBD in vitro and in vivo [8, 62, 70, 164]. Ralaniten is well-tolerated in animals and does not inhibit the transcriptional activity of other highly related steroid receptors (ER, GR and PR) [1, 114]. As a result, a phase I/II clinical trial was initiated in November 2015 (NCT02606123) to test the efficacy of ralaniten acetate (EPI-506, the orally administered prodrug of ralaniten) in patients who had progressed on abiraterone or enzalutamide. In vivo, ralaniten acetate is converted within minutes to the active compound, ralaniten. This clinical trial represented the first time that a drug which directly interacts with an IDP had ever advanced to the clinic. Ralaniten was well-tolerated and a PSA response, and stable disease were reported in some patients. Unfortunately the trial was ultimately terminated due its poor pharmacokinetic profile [17, 110].

Despite this setback, analogs of ralaniten have been synthesized with improved potency and optimized pharmacokinetic profile as a direct result of the lessons learned from the first clinical trial. The primary functional groups responsible for the metabolism of ralaniten have been revealed, and modification of these groups was sufficient to decrease metabolism [8, 56, 118]. Potency has been improved by

over tenfold while maintaining specificity with the discovery of the addition of halogens to the phenyl groups as shown with I-EPI-002 and EPI-7170 [8, 56, 62]. Data has been published using the newest EPI analogs and confirms that these compounds inhibit both AR^{FL} and AR-V and show synergistic effect when combined with existing antiandrogen or radiotherapies [8, 56]. The newest lead compound, EPI-7386, has been tested in several prostate cancer xenograft models and shows substantial superiority to EPI-002 in terms of potency, metabolic stability, and pharmacokinetics. EPI-7386 is currently being tested in a phase I clinical trial (NCT04421222) for men with CRPC, which began in June 2020.

22.12 Sintokamides and Niphatenones

Additional antagonists of the AR-NTD have been discovered from natural compound libraries, and work is ongoing to develop and further refine these alternative classes of compounds. The sintokamides were first identified as bioactive compounds extracted from the marine sponge *Dysidea* sp. in 2008. Five sintokamides of interest were studied (sintokamide A–E), all members of a family of chlorinated peptides [126]. Sintokamide A (SINT1) blocked transactivation of the AR-NTD and prevented AR-dependent proliferation in vitro [126]. The mechanism of action involved SINT1 binding to the AF-1 region in the AR-NTD and did not compete with androgen binding to LBD similar to ralaniten [10]. SINT1 was specific for blocking AR transcriptional activity and did not inhibit other structurally related steroid receptors.

Despite also binding to the AF-1 region like ralaniten, differences between these two classes of compounds are apparent. Stimulation with IL-6 induces AR transcriptional activity via STAT3-AR interaction through the NTD in a ligand-independent manner. The STAT3 binding site has been mapped to a region encompassed by amino acids 234–558 [143] which overlaps with the ralaniten binding site (354–448). Accordingly, ralaniten blocks STAT3 interaction with the AR-NTD [1, 10]. However, SINT1 had no effect upon IL-6 stimulation of the AR and failed to block STAT3 interaction with AR NTD [10]. Taken together with the fact that combination treatment of SINT1 and ralaniten resulted in an additive effect, these results suggest that the mechanism of binding is unique for these classes of AR-NTD antagonists despite both targeting the AF-1 region.

The binding site of SINT has been mapped between residues 142–485 and is likely toward the N-terminus relative to the ralaniten binding site (Fig. 22.3), possibly within Tau-1 (residues 101–370) [10]. Unlike Tau-5 that contains a core unit $_{435}$WHTLF$_{439}$, Tau-1 lacks a singular core motif, and its transcriptional activity has not been mapped to a discrete region [65]. This means that to inhibit Tau-1, a large region would need to be disrupted. When the LBD is deleted, there is a shift from the location of transcriptional activity from Tau-1 to Tau-5 [65]. This shift in location of transactivation has implications for developing drugs that target AF-1 to block full-length AR in response to androgen (employs Tau-1) and also to block

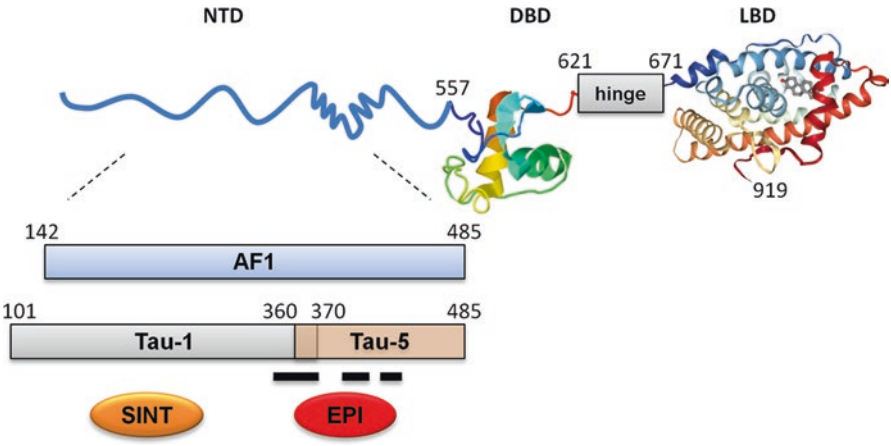


Fig. 22.3 Binding and potential mechanisms of AR NTD inhibitors. AR consists of four functional domains: NTD, DBD, hinge, and LBD. The NTD contains activation function-1 (AF-1) region where the majority of transcriptional activity occurs. Transactivation units (Tau) 1 and 5 are located in NTD. Ralaniten (EPI) makes direct contact with three small regions (black lines) of a pocket within Tau-5, whereas sintokamides (SINT) are proposed to bind within Tau-1. The DBD and LBD are both folded domains, and they are separated by a hinge region that contains nuclear translocation signal. (RCSB Protein Data Bank: 1E3G for LBD; RCSB Protein Data Bank: 1R4I for DBD)

constitutively active AR-V that lacks LBD (employs Tau-5). Thus an approach that combines a Tau-1 inhibitor with a Tau-5 inhibitor may yield superior therapeutic effects. In spite of SINT1 possibly being a Tau-1 inhibitor, it has antitumor activity against several models of prostate cancer including LNCaP95 xenografts which are driven primarily by the AR-V7 variant that lacks LBD. While development and characterization of the sintokamides is still in the preclinical stage, synthetic analogs have been created [10]. IL-6 plays an important role in metastatic CRPC bone lesions and transactiving AR [143], therefore application of sintokamides as a monotherapy for this stage of prostate cancer is not recommended due to its mechanism of action of not blocking STAT3 interaction. Instead, sintokamides could be used in combinations with other inhibitors of AR-NTD such as ralaniten analogs or developed as an imaging agent. Investigations continue with the goal of progressing these and other classes of compounds to the clinic.

Extracts from the marine sponge *Niphates digitalis* has revealed the glycerol ethers niphatenones A and B. Like the EPI and sintokamide compounds, the niphatenones bind the AR AF-1 region yet are structurally distinct from other AR antagonists. Thus, these molecules represent a potential new lead for further development of inhibitors of the AR-NTD. Initial characterization studies focused on niphatenone B, and its synthetic analogs showed on-target activity and antiproliferative effects in prostate cancer cells [108]. Subsequent studies confirmed the ability of niphatenone B enantiomers to prevent transactivation of the AR-NTD and were capable of inhibiting both AR^{FL} and AR-variant transcriptional activity [9]. However,

unlike the sintokamides or EPI compounds, niphatenone B binds to the AF-1 region of GR and also forms adducts with glutathione indicating that this compound is a reactive alkylating agent [9]. Additionally at higher concentrations, there was evidence that niphatenone B may interfere with ligand binding to the AR, PR, and GR again suggesting off-target activity [9]. As a result, the niphatenone class of compounds was deemed to be too reactive with insufficient target specificity to justify continued development. These results highlight the challenges of drug discovery and development from large-scale screens and the importance of detailed characterization studies which must be undertaken.

22.13 Summary

Steroid hormone receptors function as signaling hubs coordinating the formation of large protein complexes through intricate protein-protein interactions. A key component in their ability to function relates to extended regions of intrinsic disorder in their NTD which allows for rapid interactions with a wide range of binding partners, occurring with high specificity but low affinity [26, 54, 157]. IDPs are thought to undergo induced or coupled binding, adopting local regions of structure upon interaction with their binding partner. Protein-protein or protein-DNA interaction, post-translational modification, and ligand binding all are capable of modulating NTD structure through allosteric effects, and by extension, influence binding partner preference [6, 7, 53, 78, 81, 167]. As ligand-activated transcription factors, the steroid receptors play vital roles as master regulators of key cellular processes. Thus, misregulation or mutation of these proteins is associated with many human diseases ranging from cardiovascular disease to developmental and neurological disorders to cancer [98, 141]. Due to their overrepresentation in signaling networks and disease, much interest has been generated in developing novel inhibitors to steroid receptors and to IDPs in general.

The approaches to drug discovery outlined can be divided into two main strategies: (1) utilizing large-scale screens to "fish" for small molecules which are capable of binding an intrinsically disordered region of a target protein and (2) where short regions of the intrinsically disordered protein sequence are known or predicted, inhibitors are designed to inhibit interaction with a known binding partner. Indeed once hits are identified through large-scale screens, further characterization studies allow better understanding and modeling of the binding site to permit the second strategy to be more effectively applied [56, 111, 118, 169]. With this in mind, much of the focus so far has been on inhibiting well-defined protein-protein interactions which are essential for activity. This is seen in the case of c-MYC/Max [12, 18, 44, 46, 69, 166, 169], EWS-FLI1/RHA [30], AF4/AF9 [136], NUPR1/MSLI1 [115, 127], HIF-1α/p300 [93, 121], and $p27^{Kip1}$/Cdk2 [61].

For the discovery of inhibitors specific to the AR-NTD, the first approach was required, as a specific and singular protein-protein responsible for the majority of transcriptional activity has not been found. Rather a ligand-based approach to

identify a general inhibitor of transcriptional activity that bound specifically to the AR-NTD was sought. This led to the discovery of the EPI, sintokamide, and niphatenone compounds which remain the only inhibitors targeted against the NTD of any steroid receptor. The binding site of EPI-001 has been resolved using NMR and mapped to the Tau-5 region within the AF-1 [1, 112, 164], and a general model for binding has been proposed [112, 114]. Functional studies have also shed insights into the structure and activity relationship, thereby allowing for functional groups to be altered and optimized [8, 56, 62, 118]. While the clinical trial of ralaniten acetate was ultimately unsuccessful due to poor pharmacokinetics, the data from that trial has influenced the design of second-generation EPI compounds and led to the generation of the clinical candidate (EPI-7386) which is currently in clinical trials (NCT04421222).

The sintokamide and niphatenone compounds represent alternative leads into compounds targeting the AR-NTD, although development of the niphatenones is no longer ongoing. SINT1 like the EPI compounds binds the AF-1 region, though in a unique binding site and with a different mechanism of action [10]. This is not unsurprising as three unique binding sites were discovered in the bHLHLZip domain in c-MYC, and substantial differences in structure exist between these various inhibitors [36, 44, 127]. An important consideration is that small molecule inhibitors may not necessarily induce global structural changes and induce rather only local changes in structure near the binding site of the inhibitor. This was seen with c-Myc [36, 44] and $p27^{Kip1}$ [61] where both of the targeted proteins generally remained disordered following binding with inhibitor. As such, the inhibitor may be able to restrict a subset of possible protein-protein interactions yet permit others to occur. As SINT1 and EPI-002 target different regions and show an additive effect, it is likely that a similar trend is occurring in this case as well (rather than inducing wide spread structural changes in the AR NTD). Nonetheless, optimization of the EPI scaffold has yielded second-generation inhibitors with substantially improved potency [8, 56]. Whether these new compounds block an abundance of protein-protein interactions, or merely a few key interactions, remains to be determined.

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Chapter 23
Redirecting the Cellular Waste Disposal Machinery to Target Transcription

Helen H. Yu and Kathleen M. Sakamoto

Abstract This chapter focuses on the development of therapeutics that work to induce the degradation of nuclear receptors. These therapeutics, unlike traditional small molecule inhibitors, work by redirecting the cellular waste disposal machinery to remove a therapeutic target. They are advantageous because they work through a catalytic mechanism, raising the possibility of drugs with enhanced potency. Current work in the field is toward identifying novel molecular "glues," in the hopes of bringing proteolysis targeting chimeras (PROTACs) to the clinic. However, translating it from a concept dreamed up in a laboratory to a clinically viable tool took almost 20 years of basic research.

Keywords PROTACs · Degradation · Nuclear receptor · Druggable · Transcription factor

23.1 Introduction

Redirecting the cellular degradation machinery to remove a therapeutic target is currently one of the most promising areas of drug discovery research. Almost every major pharmaceutical company has dedicated resources toward developing these drugs termed proteolysis targeting chimeras (PROTACs). Drugs that work through catalyzing degradation have been shown to be much more potent and effective against a much larger scope of targets than traditional small molecule inhibitors and hold the promise to make an impact on human health. However, it took almost 20 years of engineering and basic biochemical research before this therapeutic strategy could be deployed on a mass scale.

H. H. Yu (✉)
Computer Science Department at Stanford University, Palo Alto Ca, Sillicon Valley, CA, USA

K. M. Sakamoto
Division of Hematology/Oncology, Stanford School of Medicine, Stanford, CA, USA

Department of Pediatrics, Stanford School of Medicine, Stanford, CA, USA

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23.2 Overview of the Ubiquitin-Proteasome System

One of the primary pathways to degrade proteins is the ubiquitin proteasome system. Unwanted cellular material is removed by the proteasome, which is a 2 mDa enzymatic complex that digests proteins by first unfolding them then looping them into its central chamber to cleave into smaller peptides (Fig. 23.1e) [1, 2]. The cell can selectively direct the enzymatic activity of the proteasome toward unwanted proteins, by labeling them with a minimum of four ubiquitin moieties (Fig. 23.1e) [3, 4]. Ubiquitin is an 8 kDa protein that is appended onto proteins through an isopeptide linkage between the C terminus of ubiquitin and a lysine on the target protein [5]. Multiple enzymes coordinate the attachment of ubiquitin onto the target protein and polymerize it into a chain long enough for the receptors on the proteasome to recognize for degradation (Fig. 23.1a–d) [6, 7]. Therefore, for this therapeutic strategy to be successful, ubiquitin would need to be specifically conjugated to the target protein.

Redirecting the specificity of ubiquitination has been the rate-limiting step in the development of this therapeutic strategy. Ubiquitin is first converted into a more reactive thioester by acylating it onto a cysteine on an E1 ubiquitin-activating enzyme (Fig. 23.1a) [4, 8]. The reactive thioester is directed toward specific substrates by E3 ubiquitin ligase enzymes. E2 ubiquitin-conjugating enzymes serve as intermediaries that rapidly deliver a reactive thioester to E3s (Fig. 23.1b) [9, 10]. Hundreds of E3 ubiquitin ligases target subsets of the proteome, ultimately dictating the specificity of the ubiquitin proteasome system (Fig. 23.1c). Although this strategy would only be amenable toward intracellular targets, there was a more obvious route to repurpose this pathway to remove a therapeutic target. A drug that aims to change the specificity of ubiquitination needs to alter the specificity of an E3 ligases.

Identifying the correct E3 ubiquitin ligase is critical toward development of a therapeutic modality. E3 ubiquitin ligases bring the target substrate near thioesterified ubiquitin. Redirecting them to ubiquitinate a therapeutic target necessitates being able to bind to the target in a way that does not disrupt catalysis. One class of E3 ubiquitin ligases that are particularly amenable toward having their specificity redirected without influencing catalysis are cullin-RING ligases [10]. This is a class of multi-subunit E3 ubiquitin ligases that are comprised of an interchangeable subunit that recognizes substrate (substrate receptor) and combine with the catalytic core bound to the charged E2 ubiquitin thioester.

A pathway in nature was known to redirect the specificity of an E3 ubiquitin ligase with a small molecule. Indole-3-acetic acid regulates growth in plants by changing the binding surface of the cognate E3 ubiquitin ligase (SCF^{TIR1}). This enables the E3 ligase to bind to substrates that enable it to dynamically respond to changing environments [11]. Although it was a promising start, it was unclear if it would be amenable to building drugs in a modular fashion that would enable the same E3 ligase to be redirected toward catalyzing the degradation of multiple different therapeutic targets.

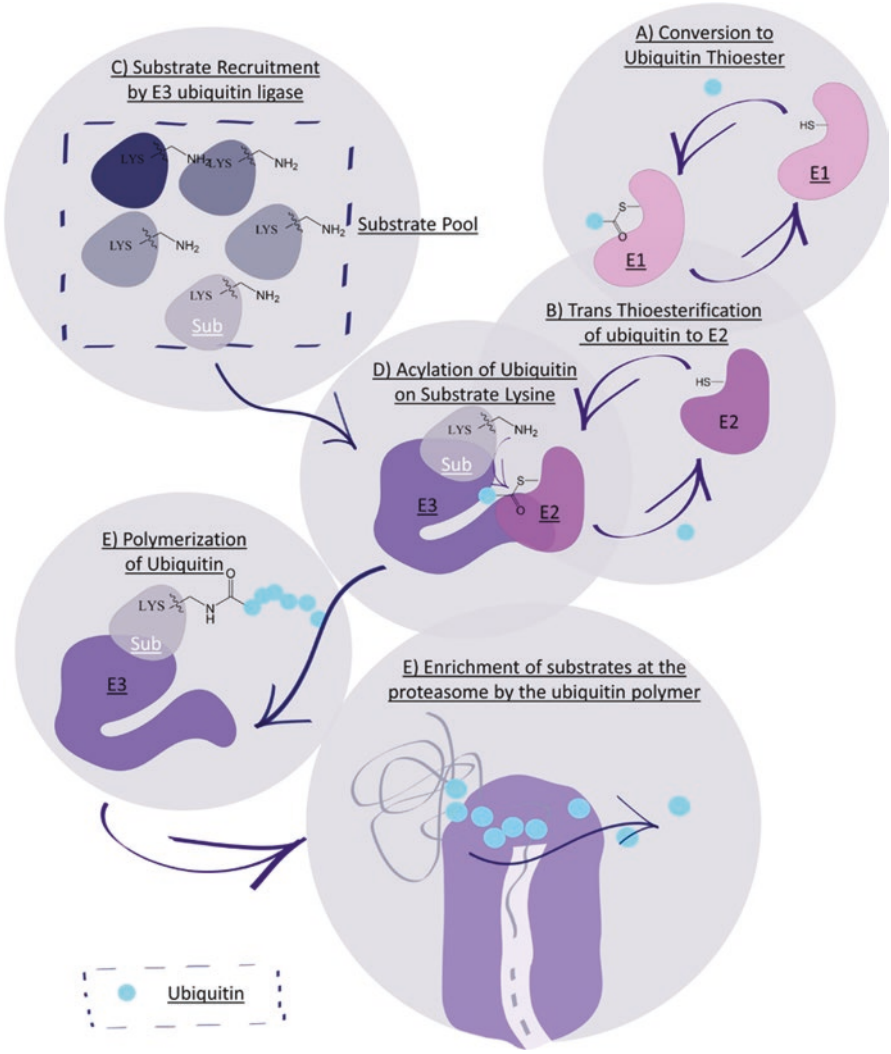

Fig. 23.1 (**a**) The C terminal carboxylic acid is first converted to a reactive thioester by an enzyme called E1. (**b**) The ubiquitin thioester is then transthioesterified onto E2, to enable proper orientation of the reactive thioester. (**c**) cullin-RING domain E3 ubiquitin ligases bring the lysine on the target protein in close proximity to the reactive thioester, by binding to both the substrate and the E2 thioester. (**d**) Since the receptors on the proteasome can only bind to ubiquitin polymers with at least 4 monomeric units, the ligase must create a chain long enough for the proteasome to grab onto. (**e**) The ubiquitinated protein is recruited by receptors on the proteasome, before being removed en bloc by deubiquitinases on the proteasome. The protein is subsequently unfolded by ATPases and digested into smaller peptides

An alternative mechanism that could be directed toward cellular waste disposal is autophagy, which is traditionally considered the most effective way to convert biomass into simple macromolecules. The target is enclosed in a double membrane bilayer before fusion with the lysosome, which has digestive enzymes to convert it into simple macromolecules. Although this would be effective toward both intracellular and extracellular proteins (that become internalized through endocytosis), one challenge is finding a way to selectively direct it to the protein of interest without triggering autophagy [12]. Additionally, there would be no way to selectively remove one subunit of a macromolecular complex or a protein embedded within an organelle.

23.3 Advantages of PROTACs Over Other Targeted Therapeutics

23.3.1 Small Molecule-Based Approaches

The proteome can be thought of as the metaphorical ship of Theseus, where old decayed components are constantly being removed and replaced by a new component. Over time, a cell is constructed from completely resynthesized "parts." This analogy is a useful framework for systematically evaluating different therapeutics, to highlight the advantages of using a degradation-based therapeutic strategy.

Small molecule inhibitors have been traditionally considered the workhorses of the pharmaceutical industry. An example that illustrates the utility as well as some of the limitations of small molecule-based therapeutics is a drug like abiraterone acetate. Abiraterone acetate has been shown to be useful in a clinical setting because it can block the biosynthesis of biological androgens [13, 14]. Although deployment of this drug has been shown to be effective in patients with castration-resistant prostate cancer, there are limitations to the efficacy of the tool. Only a third of the patients respond to treatment, and patients often end up with a form of cancer that is more lethal and more aggressive.

A key limitation is that the efficacy of small molecule inhibitors depends on the fractional occupancy of the cognate hormone; only a few mutations in the binding site are enough to become resistant to existing treatments. Additionally, because first-line inhibitors work because the target contains a well-defined ligand binding pocket, this necessarily excludes proteins like transcription factors as therapeutic targets (Fig. 23.2b–c). An example of a protein that has been shown to have a lot of promise as a therapeutic target is the bromodomain and extra terminal family protein (BRD4). Although evidence from knockdown studies have indicated that this protein would be a promising therapeutic target, the small molecules that have been developed against this target do not completely recapitulate all the effects of a knockdown due to incomplete suppression from of the reversibility of the binding kinetics [15, 16]. Adding a further level of complication, treatment with a small

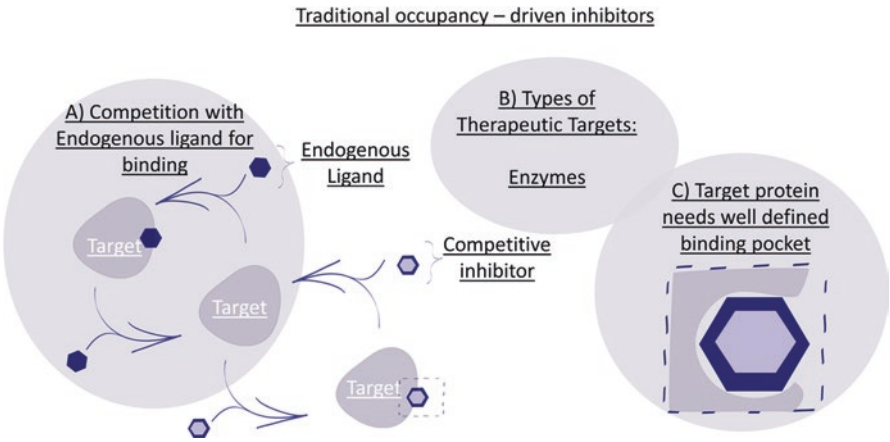

Fig. 23.2 (**a**) Traditional inhibitors need to compete with the endogenous ligand for binding. (**b**) The types of proteins that can be targeted tend to be enzymes. (**c**) The key is that these inhibitors require a protein with a well-defined binding pocket

molecule inhibitor results in increased steady-state levels of proteins, illustrating the need for new tools that can be deployed clinically [17]. Both examples above illustrate that although traditional small molecule inhibitors have had a lot of utility within a clinical setting, there are major limitations to the scope of the proteins that can be targeted and the potency of the drugs.

23.3.2 Nucleic Acid-Based Approaches

An alternative strategy that would open every single protein in the proteome is knockdown through a nucleic acid-based approach. Unfortunately, the realities of translating the idea clinically limit its versatility as a treatment option. Small molecules can readily access the target biochemical machinery, because they can readily diffuse across the lipid bilayer as they tend to be small and hydrophobic. As a result, they can target most cell types, and the effects are exerted almost immediately. Nucleic acids on the other hand rely on a delivery vehicle like liposomes or viruses to inject them into cells because they are highly charged [18]. Because some cell types (e.g., post-mitotic neurons) have a much lower rate of transduction than others, nucleic acid-based technology cannot be assumed to work as broadly as small molecule inhibitors. Finally, there are some intrinsic design flaws associated with targeting the rate of protein production as a therapeutic strategy. The length of time it takes for a protein to reach a new equilibrium depends on the rate of protein degradation. Because of the large variability in the rate of degradation that can run the gamut from seconds to months, it is a great strategy for proteins that are

unstable, but not as great of a strategy for proteins with a long half-life, especially proteins that exist in multiprotein complexes [19].

23.3.3 Protein Degradation

An alternative chemical strategy for nuclear receptor inhibition is modifying endogenous nuclear receptor ligands such that their binding directs the nuclear receptor for degradation. As a general design strategy, there are several advantages to catalyzing the rate of decay as a therapeutic strategy. Because the time it takes a protein to reach a new equilibrium depends on the rate of decay, it could potentially work more rapidly than drugs that normally work through targeting the rate of production [10]. Additionally, it would be generalizable toward proteins that have a long half-life.

Finally, because this strategy would entail using a small molecule, it would bypass the necessity of designing the appropriate delivery vehicle that a nucleic acid-based strategy would need. It would also not have the limitations of small molecule inhibitors primarily since the role of each molecule is to catalyze the degradation of the target protein, meaning that the potency of the drug would not depend upon the fractional occupancy at equilibrium (Fig. 23.3c) [20, 21]. As a result, it also means that drugs could be used in lower dosages, minimizing effects from off-target toxicity [22]. Finally, the biological effects of these alternative therapeutics would be exerted over a longer time frame because its therapeutic window depends on the length of time to resynthesize the protein instead of biomolecular dissociation. Since catalysis is not as dependent on equilibrium kinetics, they also hold the potential to become more resistant to binding pocket mutations.

In conclusion, designing a strategy that is focused on catalyzing the removal of proteins by redirecting the cellular waste disposal machinery to remove a therapeutic target is a versatile therapeutic strategy where there is a pressing need for new tools.

23.3.4 Roadblocks to PROTAC Development

The architecture of E3 ubiquitin ligases created several roadblocks to drug discovery. Since the catalysis is achieved by enhancing the proximity of substrates to ubiquitin thioesters, the active sites of E3 ubiquitin ligases are flat, solvent-exposed surfaces [10]. One strategy that had been deployed to target specific enzymes is to synthesize a small molecule inhibitor that is a mimic of a reactive intermediate [23]. This strategy is not a viable for E3 ubiquitin ligases, because the reactive intermediate is an E2 ubiquitin thioester. Each E2 can combine with multiple E3 ubiquitin ligases. Catalysis is dependent on the proximity of the substrate's target lysine to the reactive intermediate, adding an additional layer of complexity. As a result, the

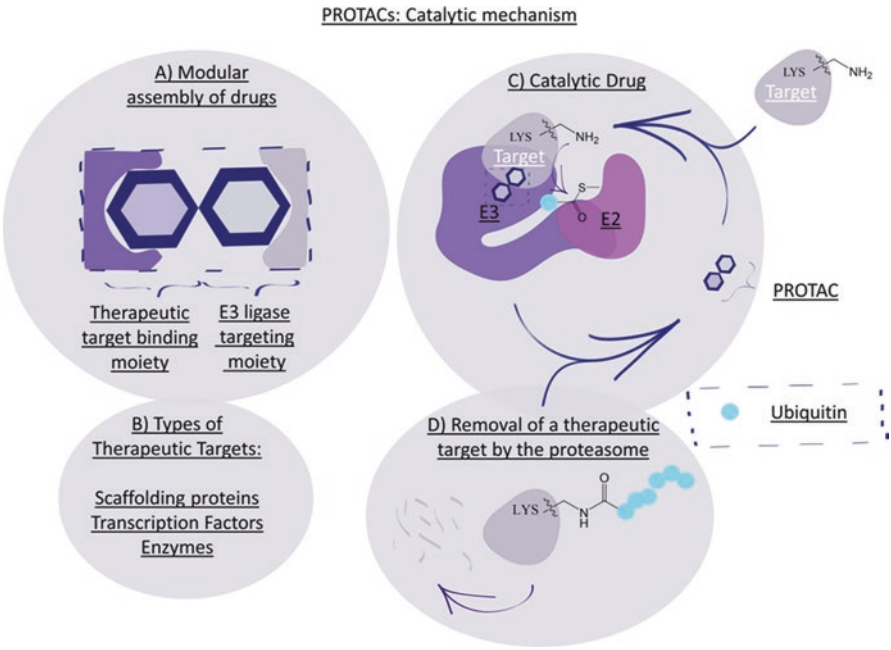

Fig. 23.3 PROTACs are a class of drugs that are uniquely poised to revolutionize the small molecule inhibitors. (**a**) The modular nature of their assembly makes it easy to repurpose the same E3 targeting moiety to a number of different targets. (**b**) The scope of the proteins that can be targeted means that proteins that were previously considered undruggable because they contained an exposed active site like scaffolding proteins and transcription factors could now be targeted with a small molecule. (**c**) The drug recruits the target protein to an E3 ligase complex, meaning that one molecule of the drug can be used multiple times before (**d**) removal of the target protein by the proteasome

ligands that were identified that were shown to be promising E3 ligase "glues" were discovered either through deliberately engineering a small molecule that could mimic the binding of an endogenous substrate or serendipity.

An overview of the Herculean engineering challenges that went into fashioning the first E3 ligase targeting ligand underscores why it took 20 years before redirecting the waste disposal machinery was ready for prime time. Engineering an E3 ligase ligand entailed exploiting the differential selectivity that E3 ubiquitin ligases have for different substrates (Fig. 23.3a). E3 ubiquitin ligases target an amino acid subsequence within a protein, which is referred to as degron [24]. The goal was to repurpose the amino acid subsequence to direct an E3 ligase to target a novel protein, through engineering a chimeric molecule where it was fused to a ligand that bound to the protein of interest. There were two key engineering challenges: selecting a degron to engineer into a chimeric molecule and identifying a suitable model substrate.

Because the interaction between the degron and the E3 ligase needed to be well characterized, it artificially restricted the pool of possible pairs to ones that

mediated physiological responses. One complex that was known at the time was a E3 ligase with a core enzymatic complex comprised of Skp-1, Cul1, and Hrt1 that could combine with β-TRCP, the subunit that was shown to recruit IκBα, a protein involved in mediating the inflammation response to catalyze its degradation [25]. At the time that this work was being done, the Deshaies Lab had just reported which components were needed for proteins to be ubiquitinated and degraded by cullin-RING ligases [26]. A 10 amino acid subsequence was shown to be phosphorylated, enabling the ubiquitination and subsequent degradation of the target protein.

Next, the appropriate target substrate needed to be identified. Ascertaining that the source of observed ubiquitination was the engineered chemical and not an endogenous source necessitated selecting a protein that showed little to no ubiquitination and was not a cullin-RING ligase substrate. Additionally, the target protein would need to bind with a known ligand that had high enough of affinity to selectively recruit the target protein. A protein that fit this description was MetAP-2, which is a protein that was believed to play a role in regulating the progression of the cell cycle [27]. MetAP-2 was serendipitously characterized as the target of fumagillin and ovalicin [27, 28]. The interaction between MetAP-2 and ovalicin had also been shown to be stable enough to enable isolation of a heterodimeric complex that would be assembled by the PROTAC, an experiment that would be critical toward establishing the core mechanism [29].

The authors were able to demonstrate that this chimeric molecule, where ovalicin, a MetAP-2 ligand, was fused to a 10 amino acid ligand, was able to recruit MetAP-2 to the E3 ubiquitin ligase complex within a controlled in vitro setting. Supplementing the in vitro reaction with E1, E2, and ubiquitin enabled identification of the ubiquitinated intermediate, showing that the engineered chimeric molecule could redirect the E3 ubiquitin ligase to catalyze the ubiquitination of a neosubstrate. Because peptides do not easily passivate lipid bilayers, demonstrating that an engineered small molecule could catalyze the degradation of a target compound with the levels of proteins that would be typical inside of the cell required the work to be done within xenopus egg extracts [29].

For PROTACs to be a generalizable drug paradigm that could be deployed across different types of diseases, it would be critical to establish that changing the identity of the molecule connected to the E3 targeting ligand would enable targeting of the E3 ubiquitin ligase receptor to new targets within a live cell. At the time, it was known that signaling through a nuclear receptor through steroids like androgen or estrogen promoted the growth of cells during prostate cancer because of the availability of clinically deployed inhibitors that blocked signaling through both pathways. Therefore, redirecting an E3 ligase toward the androgen receptor (AR) or estrogen receptor (ER) was attractive because of the possibility of crafting a drug that could be deployed within a clinical setting.

To demonstrate that this class of drugs could work in a modular fashion, the authors synthesized an IκBα phosphopeptide fused with estradiol or dihydrotestosterone to target it to the estrogen receptor or androgen receptor, respectively. In the in vitro ubiquitination experiments, the authors observed that both peptides were able to catalyze the ubiquitination of estrogen receptor and androgen receptor.

Additionally, the authors were able to observe the intramolecular "hook effect," a byproduct of forming an intermolecular complex where a bivalent ligand becomes autoinhibitory at high concentrations, a key characteristic that has been observed in almost every PROTAC that has been synthesized [30].

Ubiquitin can be linked in a variety of different ways. Different linkages can result in different outcomes [31]. The next hurdle was to show that the ubiquitinated conjugates generated by PROTACs had the correct linkage to enable their identification and degradation by the proteasome. An in vitro proteasome degradation reaction where proteasome was added to the ubiquitinated conjugates showed that the conjugates were able to catalyze its degradation.

Although this demonstrated that this class of drugs could work in an in vitro setting, it was unclear if this would translate into cells. One of the biggest challenges was that a charged phosphopeptide degron was not cell permeable. Therefore, what was needed was a delivery mechanism that would enable its delivery across the cell surface. Microinjection enabled delivery of heterodimeric peptides into live cells, which induce degradation of the target protein, showing that this drug is able to redirect the cellular degradation machinery in a live cell.

These pioneering efforts were the first example that demonstrated that it was possible to engineer a molecule that could redirect an E3 ubiquitin ligase to catalyze the degradation of a therapeutic target. Critically, this work also showed that these drugs could build modularly, which meant that one E3 ligase could catalyze the degradation of almost any protein by changing the identity of the targeting moiety. These experiments provided a roadmap that would enable this class of drugs to be applicable to any disease. Additionally, these drugs could be redirected toward the androgen receptor and the estrogen receptor, providing a roadmap toward utilizing these drugs to treat human disease.

A lot of additional work needed to be done to convert the first peptide-based PROTAC into an orally available drug. Orally administered drugs need to be small enough to efficiently diffuse into the target tissue (<500 Da), which is problematic given the high molecular weight of most peptides [32]. Additionally, drugs need to have enough hydrogen donors and acceptors for them to be soluble in water, but not so many that there would be problems with crossing the lipid bilayer to access the target biochemical machinery. Finally, peptides have amide bonds which make them susceptible to proteolytic degradation, meaning that the peptide-based drugs often have poor pharmacodynamics. Additionally, they cannot be administered orally, as they would not survive cleavage by gastrointestinal enzymes [33].

Subsequent work demonstrated that using a peptide that was modeled on a HIF-1α degron, an interaction was not reliant upon charged amino acids, was able to induce the degradation of androgen receptor or estrogen receptor when fused to estradiol or dihydrotestosterone. This work was critical because it showed that changing the identity of the degron could change the identity of the E3 ubiquitin ligase that was recruited to the target molecule [34]. Almost 10 years of engineering by synthetic chemists would have to occur before the proof-of-concept molecules were crafted into a small molecule that could be deployed within a clinical setting.

23.3.5 *Crafting an E3 Ligase Targeting Moiety from Known Degrons*

Engineering an E3 targeting ligand from a known peptide took a feat of synthetic organic chemistry. The degron would need to be sourced from an interaction that was not dependent upon charged amino acids to maximize the probability of ending up with a small hydrophobic molecule (Fig. 23.4a). A famous example in the literature is hypoxia-inducible factor 1 Subunit – α (HIF-1α). This is a transcription factor whose protein levels are oxygen dependent, enabling the cell to sense and respond to levels of oxygen [35]. Research directed toward uncovering the molecular mechanisms behind regulating HIF -1α as an area of intense research because tumors need to grow to a certain size before they run out of oxygen. Therefore, they often adapt by promoting the growth of new blood vessels. The ability to detect changes in oxygen levels are critical to this process.

At a critical threshold level of oxygen, Hif-1α is hydroxylated at P564 by a proline hydroxylase, triggering a negative feedback loop that enables HIF-1α to be identified and targeted for degradation by the corresponding E3 ubiquitin ligase comprised von Hippel-Lindau (VHL), Cullin2 (Cul2), and Elongin BC [34–39]. The hydroxylated HIF-1α degron is a particularly promising candidate to be repurposed for drug discovery, because it does not contain any charged amino acids, and the key interaction is binding to a hydroxylated amino acid, meaning that the entire degron could potentially be simplified to a mimetic of that single amino acid.

Initial attempts demonstrated that HIF-1α degron could be made cell permeable by hijacking the endogenous mechanism that viruses use to invade the cell. Since the cell surface is normally covered with charged lipids and carbohydrates, it would be difficult for large macromolecular complexes like viruses to enter the cell through passive diffusion. Viruses like HIV get around this complication by using a peptide that is comprised of positively charged amino acids, specifically exploiting the charged guanidinium head group in arginine [22, 40, 41]. A chimeric molecule comprised of both the polyarginine and the PROTAC was engineered and was demonstrated to be able to successfully catalyze the degradation of the androgen receptor at 25 μM [42].

Although it was able to overcome the hurdle of getting a large peptide across the cell surface, the resulting molecule was still extremely heavy (~2 kDa) and contained peptide bonds that would make it vulnerable to hydrolysis mediated by extracellular proteases. The crystal structure was published a year after the first SCF-based PROTACs were synthesized and revealed that the key interaction was mediated by a hydroxylated proline. Crews and coworkers were able to simplify the structure to a small molecule using a hydroxylated proline as a chemical handle to develop an analog with submicromolar affinity [22, 43]. Subsequent work showed that this small molecule could be used to remove estrogen-related receptor alpha (ERRα), an a orphan nuclear receptor, in both cell culture and in in vivo mouse models under normoxic conditions, indicating that this scaffold would be promising for clinical applications [22]. Critically, the authors found that the estrogen receptor was

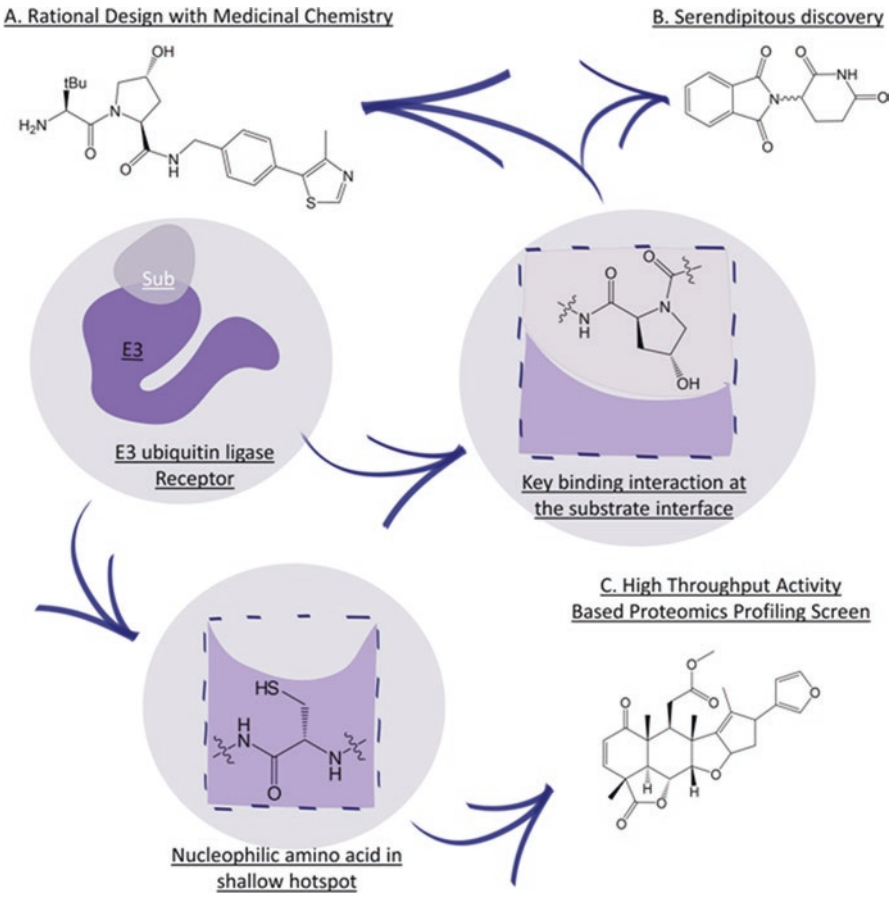

Fig. 23.4 There are multiple different strategies for discovering an E3 targeting ligand. One strategy is to exploit interactions between the E3 ligase receptor and the cognate substrate through (**a**) rationally designing small molecules that recapitulate the key binding interactions or (**b**) serendipitously discovering a known drug works through targeting the substrate-binding interface. (**c**) An alternative is a higher-throughput approach to target a nucleophilic hotspot outside of the substrate binding interface through an activity-based proteomics profiling screen

completely degraded after 4 hours, almost a tenfold increase over the known half-life of 60 hours. This core observation was critical as it demonstrated that PROTACs could potentially induce a much more rapid response than a traditional inhibitor.

Although PROTACs should theoretically be able to operate sub-stoichiometrically, it was unknown how metabolism of a small molecule would affect the number of times a single molecule could be turned over. The second key experiment from the paper measured the number of molecules of protein each PROTAC was able to catalyze the ubiquitination of. The core finding is that each molecule could catalyze the degradation of multiple proteins, showing that these PROTACs were catalytic (Fig. 23.3c). What this meant is that PROTACs could potentially be drugs that could

be used at lower dosages and have more potent effects. What is needed now is a test case that would enable a head-to-head comparison between the small molecule and the PROTAC version of the same drug.

23.3.6 Repurposing Known Inhibitors: How PROTACs Improve Existing Targeted Therapeutics

Since it was known that a degron could be redirected to catalyze the ubiquitination of the androgen receptor and the estrogen receptor by fusing the degron with the endogenous ligand, an obvious test case would be comparing the minimized degron fused to an inhibitor against the regular inhibitor. A small molecule inhibitor against the androgen receptor that was commonly used in the clinic is eluzatanib. Eluzatanib relies on high fractional occupancy to be efficacious, which mean that mutations in the active site that expand the selectivity of the receptor enable cancer cells to bypass inhibition by activation with alternative ligands [44].

Crews and coworkers synthesized a panel of the Hif-1α ligand fused to enzalutamide with different linkers of different lengths [45]. In a head-to-head comparison, the most potent PROTAC was more robust to competitive inhibition with high concentrations of endogenous androgens and was also effective toward truncated isoforms that retained the signaling activity. This finding demonstrated that PROTACs could be used in treatment regimens that were resistant to the parental inhibitor. Additionally, the most potent compound, ARCC-4, was more effective than the parent compound at inducing apoptosis and stopping the growth of cell lines, showing that converting existing drugs into a PROTAC could substantially enhance their existing efficacy by changing the mechanism of action.

The next question is could PROTACs be used to make proteins that had been previously considered undruggable accessible like transcription factors? An example of a protein considered undruggable is BRD4, which was known to epigenetically regulate genes that are directly targeted by the androgen receptor [17]. Because BRD4 has a poorly defined ligand-binding site, inhibitors designed against BRD4 are particularly sensitive to the reversibility of ligand binding, resulting in incomplete suppression of the oncogene [15, 16]. Critically, BRD4 inhibitors cannot replicate downstream suppression of an oncogene shown to be critical to its activity (c-MYC). The PROTAC version was able to replicate this effect, showing the potential of PROTACs to redefine the type of drugs that are considered druggable [15, 46]. Additionally, this work showed that a small molecule inhibitor could have the same effect as an siRNA knockdown. A head-to-head comparison with a PROTAC showed that it was able to inhibit transcription over a longer window of time.

Even using the optimized E3 targeting domains, many of the PROTACs have a molecular weight of 800–1000 Da. In order for a drug to be bioavailable and have a good adsorption profile, there are upper limits on the molecular weight [47, 48]. One workaround is to split the drug into two halves using biorthogonal chemistry.

Astex Pharmaceuticals was able to use tetrazene ligation in order to design a PROTAC that rapidly assembled in the cell through click chemistry [49]. These technologies indicate the critical role that chemical biologists will play in the future of PROTACs.

23.3.7 Identification by Serendipity: Repurposing Existing Inhibitors to Redirect the Protein Degradation Machinery

Because of the challenges associated with directly engineering an E3 targeting ligand, serendipity is an important engine for discovery novel moieties (Fig. 23.4a). An example that showcases how powerful serendipity can be is thalidomide. Thalidomide is a drug that was originally prescribed as a sedative for morning sickness, which was pulled off the market for its teratogenic effects [50]. Subsequent work revitalized interest in the drug, when it was shown to be effective toward the treatment of multiple myeloma. However, although it was a commercially successful drug, the mechanism of action was unknown at the time.

All of this changed when a paper in early 2000 when Hiroshi Handa and coworkers serendipitously identified cereblon (CRBN), the substrate recognition subunit of an E3 ubiquitin ligase complex, as its key binding partner [51]. This interaction was stable enough isolation of the protein complexes by immunoprecipitation with thalidomide, indicating that this drug could be repurposed as an E3 ligase targeting ligand. Thalidomide and its derivatives have been repurposed to catalyze the degradation of proteins previously believed to be undruggable like BRD4 [15]. However, this example also highlights some of the challenges associated with discovering new E3 targeting moieties. Although there are >600 E3 ubiquitin ligases, there have only been a couple of ligands that have been shown to be effective for being repurposed into PROTACs [52]. The rate of a serendipitous discovery depends in part upon the frequency of stochastic collisions between a novel compound and a target protein. However, when the pool of potential compounds is restricted to already commercially viable compounds, the rate of discovery is artificially deflated from under sampling of the possible chemical search space. What is needed is a more systematic and higher throughput way to screen interactions between potential E3 targeting ligands and E3 ligases.

One technology that can open up the bottleneck is activity-based proteomics profiling (ABPP) [53]. ABPP maps out nucleophilic sites on target proteins that can be exploited for covalent ligand development. Covalent ligands are uniquely suited toward engineering an E3 ligase targeting moiety. Because they target a hotspot within the target molecule, lack of a well-defined binding pocket does not create the same type of issues that are created for traditional small molecule inhibitors. Libraries of potential ligands can be screened by competing against reactive probes, enabling high throughput screening of potential electrophilic ligands and

nucleophilic pairs (Fig. 23.4c). An example that illustrates how this technology has significantly accelerated the rate of discovery of novel E3 ligase targeting moieties is the discovery of nimbolide, an E3 ubiquitin ligase RNF114 targeting ligand, by Dan Nomura and coworkers [54]. Because nimbolide started out as a hydrophobic molecule, the length of time it would take to convert nimbolide to an orally available drug would be substantially shorter than the 20 years it took to engineer HIF-1α ligand into a small hydrophobic molecule. ABPP holds the promise to rapidly accelerate the rate of identification of novel E3 ligase targeting moieties, as well as the rate of translating a novel discovery in the laboratory to the clinic.

23.4 Using PROTACs with Covalent Inhibitors

23.4.1 Covalent Inhibitors: A Potentially Promising New Source of Potent Therapeutics

Covalent ligands have also been shown to be effective toward directly targeting "undruggable" proteins. An example of a protein that was previously considered undruggable and inaccessible that was successfully targeted with covalent inhibitors is the Kirsten rat sarcoma viral oncogene homolog (KRAS). The KRAS gene is known to be mutated in ~20% of human cancers [55]. KRAS's enzymatic activity is regulated through binding to nucleotides: the enzymatically active GTP-bound state can induce cellular proliferation downstream [56]. As a consequence, many cancers harbor mutations that cause a gain of function type phenotype [57]. A common mutation is the G12C mutation that enables accumulation of the active, GTP-bound enzyme [58]. Therefore, a drug that could target KRAS would have a lot of clinical utility.

Because KRAS has a picomolar affinity for its endogenous ligand, it is difficult to target directly with a competitive inhibitor [59]. KRAS also does not have any obvious allosteric regulatory sites. However, the common G12C gain-of-function mutation creates a chemical handle that enables identification of a covalent ligand by a chemoproteomic platform. Shokat and coworkers were able to use this strategy to identify compounds that were able to selectively react with a the soft nucleophile generated by the mutation, enabling identification of a compound that was able to selectively bind to KRAS and shift it to the inactive GDP-bound form [60]. Subsequent work by a number of pharmaceutical companies have used this strategy to develop an orally available drug [61]. However, KRAS also showcases some of the limitations of using a covalent ligand as a drug. Cancer cells can bypass KRAS inhibition through either modifying feedback mechanisms or through temporarily entering a quiescent state within 24 hours, illustrating the pressing need to develop new tools that can increase the therapeutic window [62, 63].

Using a covalent ligand as a PROTAC warhead ameliorates many of the problems raised by the first generation of covalent ligands. First, covalent ligands are

particularly susceptible to mutagenesis, as a single point mutation at the druggable hotspot can change the binding mechanism from a covalent bond to reversible binding. Because PROTACs primarily work as a catalyst, a PROTAC would be more robust to this mechanism of resistance. Several challenges can complicate converting covalent inhibitor into a PROTAC. The work done by Nathanial Gray and coworkers toward designing a KRAS inhibitor highlights some of the problems. Although they were able to identify a KRAS PROTAC using a FACS-based assay against GFP-KRAS, the lead compound was unable to catalyze the degradation of endogenous KRAS [64]. Subsequent experiments showed that the GFP fusion changed the subcellular localization of KRAS, highlighting an additional layer of complexity when designing a protein degrader. Subsequent work by Craig Crews showed that changing the E3 targeting ligand to a VHL ligand enabled degradation of KRAS across multiple cell lines [65]. This work highlights the importance of identifying additional E3 targeting ligands toward opening the entire proteome for druggability.

23.5 Recent Work Improving the Specificity of PROTACs

Although substantial progress has been made toward increasing the accessibility of the proteome, substantial work remains to be done to increase the specificity and selectivity of PROTACs. A drug that illustrates some of the limitations of the existing technology is ARV-771. Although the target cell type are prostate cells, because both the E3 ubiquitin ligase and the target protein are expressed in the skin, it can often result in unwanted and unpleasant side effects [66]. This example highlights the pressing need to further sharpen the specificity of a molecular scalpel.

One tool that has been shown to be useful for neuroscientist to manipulate spatially subsets of neurons is light. Light is an ideal tool because of the ease of manipulation of the dosage and timing for when each treatment is dispensed [67]. However, to use light as a molecular scalpel, chemists would need to craft a new molecule from scratch. One possible route is designing a reaction that exploits the sensitivity PROTACs have to the distance between the ligand and the E3-ligase binding moiety [22, 68]. Subtle changes in the number of methylene linkers result in dramatic differences in reactivity, meaning that a chemical reaction that adjusts the intermolecular distance can turn a PROTAC on and off. A chemical reaction that could be repurposed to adjust the intermolecular distance between the ligand and the E3 ligase binding moiety is a cis-trans isomerization. The change between the cis and the trans isomer changes the intermolecular distance between distal substituents 3-4 Å for azobenzene, roughly the same effect of eliminating a methylene group on a linker [69]. Crews and coworkers showed that they were able to utilize this isomerization reaction to create a PROTAC that was able to be selectively turned on by light, enabling light to trigger ubiquitination of a target molecule [70] .

Although subsequent work by many other groups have since expanded the toolkit of possible reactions to choose from, most would have a poor penetration depth

based upon the wavelength of light required to trigger the reaction [71, 72]. Substantial work still needs to be done to redshift existing photoswitchable PROTACs to increase the possible penetration depth to enable access to tissue deep within the body [73]. An alternative strategy that would circumvent this problem is to use an antibody to target the PROTAC to the cell type of interest. Proteins are normally digested into small peptides by the proteasome, which are displaced on the cell surface by MHC receptors [74]. Therefore, proteome wide changes in the expression level of different proteins would be reflected in the identity of antigens displayed on the cell surface, creating a chemical handle that can be exploited by an antibody [75].

The antibody would bind to the target antigen, enabling internalization of the antibody-drug conjugate through a receptor-mediated endocytosis [76, 77]. Once the antibody-drug conjugate is internalized in an endosome, differences in the redox potential inside of an endosome are able to reverse the covalent modification, enabling the cargo to then be released into the cell [78, 79]. Repurposing this strategy requires the appropriate chemistry to tether to the target antibody and identifying the correct antigen to target. Previous work was able to show that this strategy was effective for selectively targeting a BRD4 PROTAC to a HER2-expressing cell line [80].

Because PROTACs are catalytic, very few molecules of the drug need to be successfully delivered into the cell type for it to be effective, making them the ideal cargo for an antibody-drug conjugate. Additionally, because PROTACs have a lower intrinsic cytotoxicity than cytotoxic drugs, clinicians do not need to worry as much about balancing the bystander effect, where the endocytosed drug can diffuse to neighboring cells and wreak havoc [81]. Antibody-drug conjugates, photoactivatable PROTACs, hold the promise to design the second generation of cell-type-specific PROTACs.

23.6 Translation into Clinics

In 2017, Arvinas, Inc., was able to develop the first bioavailable androgen degrader, ARV-110 [82]. In mouse xenograft studies, the authors were able to show that ARV-110 outperformed the parent compound eluzatanib and was able to be effective in an eluzatanib-resistant model. On May 13, 2020, almost 20 years after the first PROTAC was synthesized, through a personal communication on Twitter, Craig M. Crews announced that PROTACs worked in humans. Two out of eight patients showed a response to ARV-110 despite failing prior treatments with conventional inhibitors. PROTACs had gone from a concept dreamed up in a laboratory to a drug that made an impact on human health.

23.7 Conclusion

What started out as a peptide and became a drug will make an impact on human health for patients across the world. The scope of this technology cannot be understated – it can be applied to almost every target in the proteome. This finding provides a path for old inhibitors that had fallen out of use to make their way back to the clinic.

Designing the first PROTAC would not have been possible without the painstaking work to fundamentally advance underlying biochemistry of the E3 ligase complex. Without fundamental understanding of how the underlying biochemistry worked, the first PROTAC could not have been made, and characterization of compounds like thalidomide and sulfonamides as E3 targeting ligands would not have been possible.

Without the knowledge gleaned by the pharmaceutical industry in converting a small molecule into a drug that could be properly absorbed and excreted, ARV-110 might never have been discovered. What started out as a drug that was designed to target diseases caused by aberrant signaling through the nuclear receptor provided the blueprint to change the way every drug is designed.

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