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

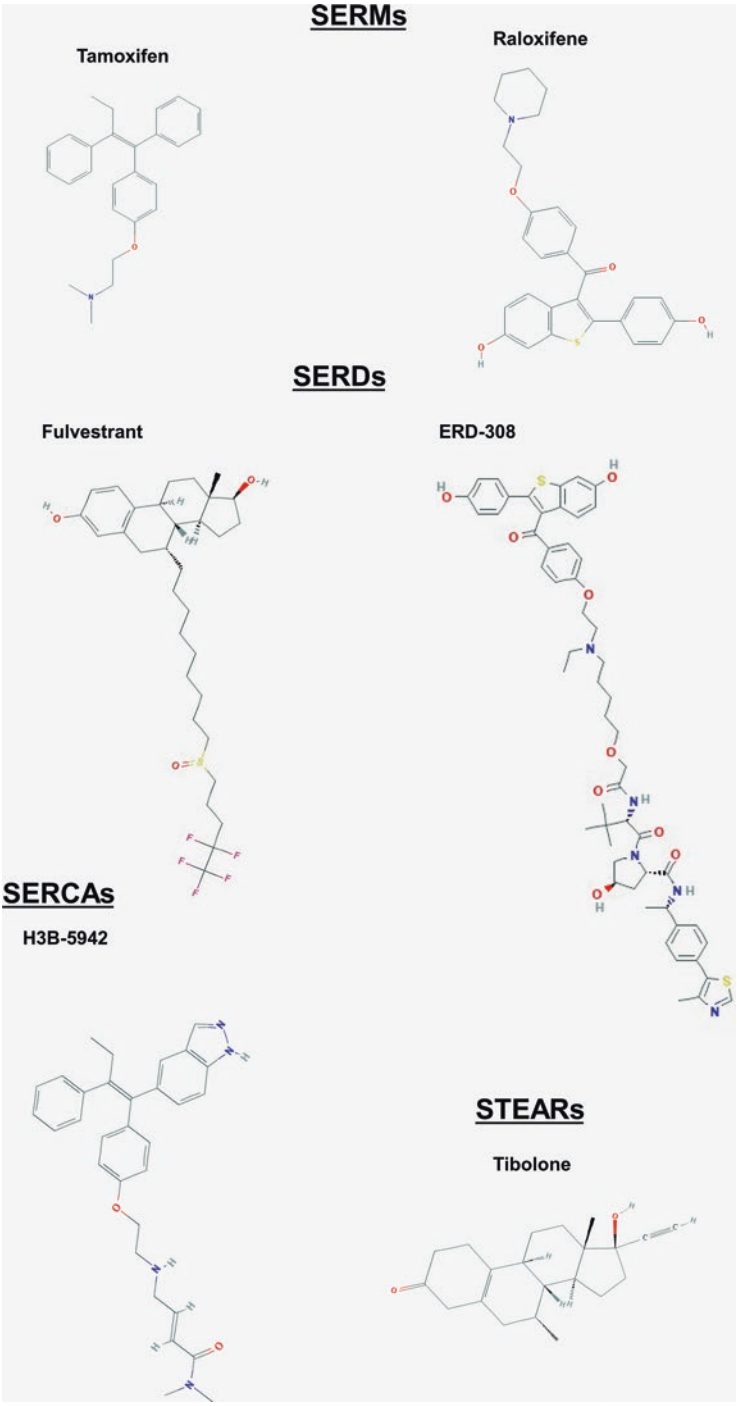


Fig. 9.1 Chemical structures of representative SERMs, SERDs, SERCAs, and STEARS

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

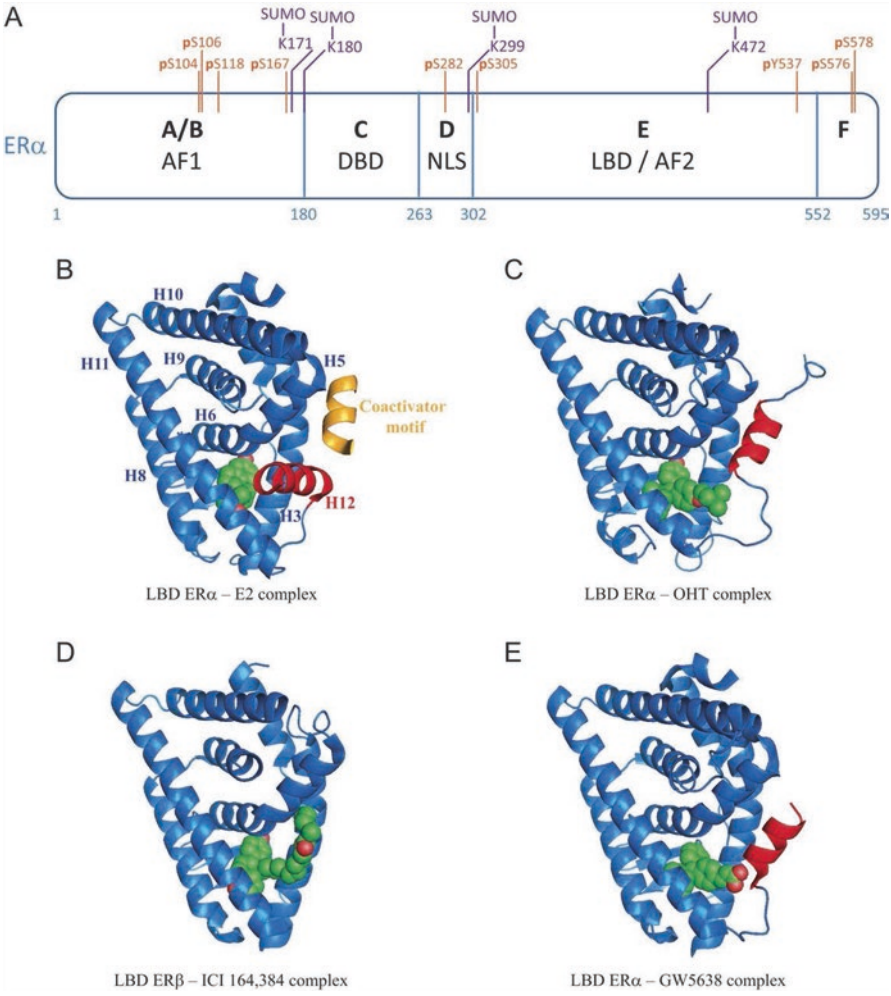


Fig. 9.2 ERα structure, post-translational modifications, and conformational changes induced by different ligands. (**a**) Schematic representation of ERα structure. AF1/AF2, activation function 1/2; DBD, DNA-binding domain; NLS, nuclear localization signal; LBD, ligand-binding domain. SUMOylation sites 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

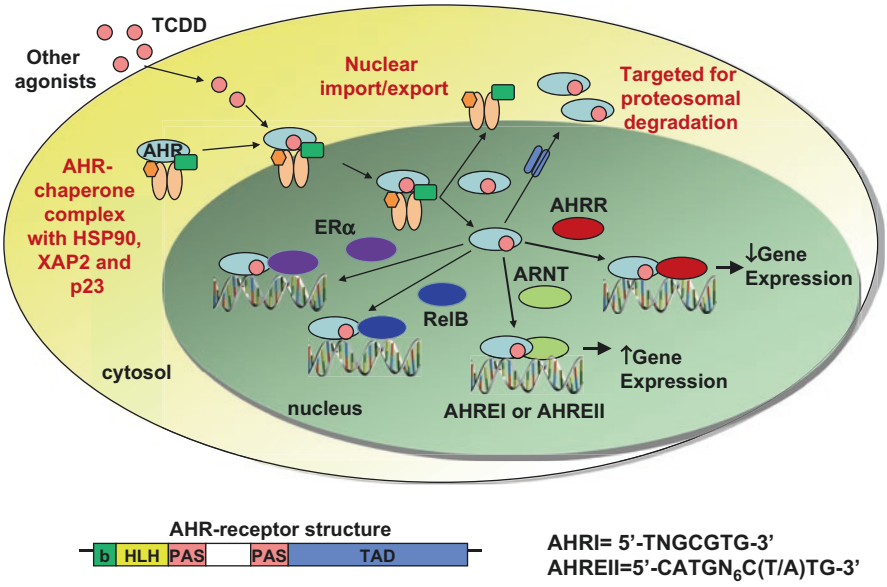


Fig. 9.3 Events initiated upon agonist activation of the AHR. Binding of agonist (e.g., TCDD) induces nuclear translocation of the AHR complex. Within the nucleus, the AHR dimerizes with ARNT or other transcription factors such as ERα or RelB, thereby altering gene transcription. The AHR/ARNT heterodimer 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

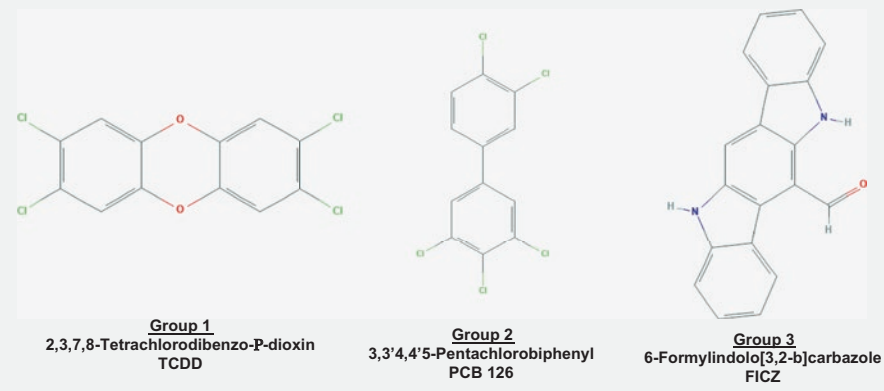


Fig. 9.4 Chemical structures of AHR agonists representing of three different groups

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

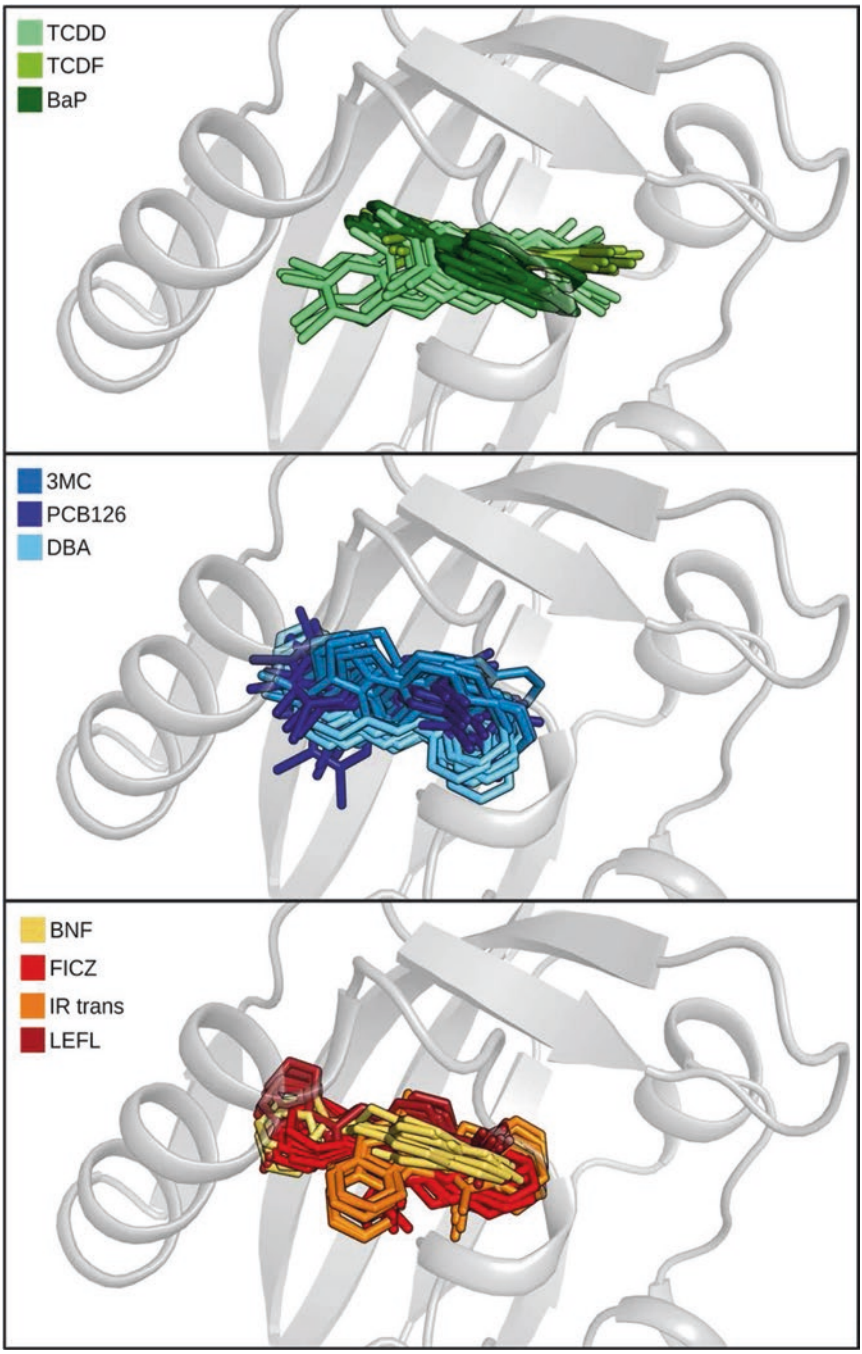


Fig. 9.5 Occupancy of different sites within the AHR cavity by the three groups of AHR ligands as determined by computational molecular docking analyses. Group 1 is depicted in the upper panel, Group 2 in the middle panel, and Group 3 in the lower panel. The ligands are depicted as sticks. (Reproduced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

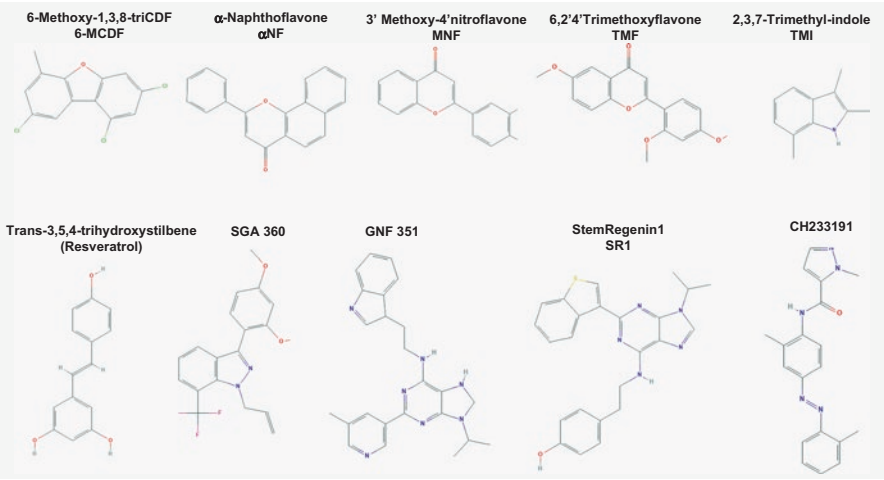


Fig. 9.6 Chemical structures of a variety of SAHRMs

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

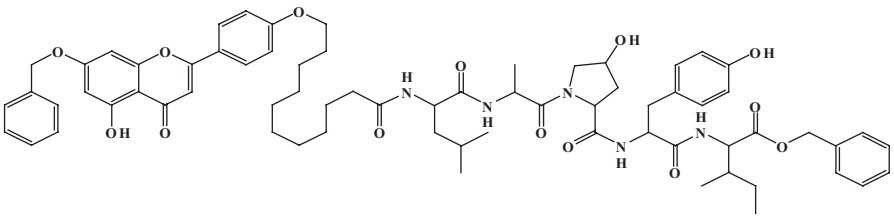
indole moiety. Recently, a recent screen of methylated and methoxylated indoles has offered insights into the rules that may determine how indoles may act as either AHR agonists or antagonists [73]. Here, indoles that exerted high agonist activity were 4-Me-indole and 7-Meo-indole, whereas those with 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

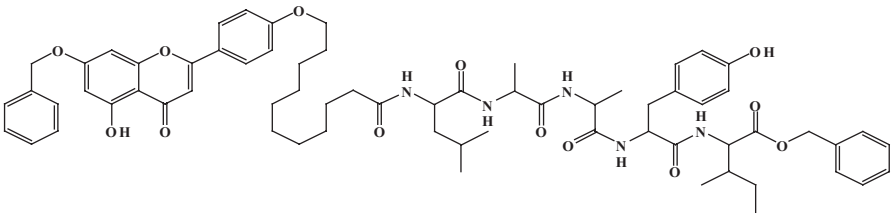
Interest in stilbenes as AHR antagonists was initiated by reports that resveratrol (trans-3,5,4-trihydroxystilbene) (Fig. 9.6) could inhibit the ability of TCDD to activate genes [12] and act as a competitive antagonist [9] that inhibited AHR recruitment at the CYP1A1 promoter [3]. Subsequent efforts 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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