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Small-molecule modulators of PXR and CAR

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Abstract

Two nuclear receptors, the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), participate in the xenobiotic detoxification system by regulating the expression of drug-metabolizing enzymes and transporters in order to degrade and excrete foreign chemicals or endogenous metabolites. This review aims to expand the perceived relevance of PXR and CAR beyond their established role as master xenosensors to disease-oriented areas, emphasizing their modulation by small molecules. Structural studies of these receptors have provided much-needed insight into the nature of their binding promiscuity and the important elements that lead to ligand binding. Reports of species- and isoform-selective activation highlight the need for further scrutiny when extrapolating from animal data to humans, as animal models are at the forefront of early drug discovery.

Keywords

PXR; CAR; small molecule; xenobiotic; detoxification; binding promiscuity

1. Introduction

Organisms are constantly exposed to environmental chemicals that can profoundly affect their wellbeing, and they have developed mechanisms to metabolize and excrete these xenobiotics through the concerted transcriptional activation of an arsenal of genes [1]. The xenobiotic detoxification strategy involves nuclear receptors (NRs), such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which serve as xenosensors and coordinate the expression of metabolizing enzymes and transporters [2]. Although these chemicals can be eliminated unchanged, the majority first undergo biotransformation through a series of processes that include oxidation and conjugation reactions.

Some of the metabolites that result from the degradation of exogenous substances have themselves the potential to cause inadvertent harmful effects, including toxicity [3]. In

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addition, the activation of this detoxification and disposition system can reduce the efficacy of concurrently administered drugs or exacerbate their side effects [2]. Importantly, these xenosensors act on endogenous chemicals to maintain appropriate physiologic levels. Among the members of the large NR superfamily, PXR and CAR are predominant in their ability to bind a vast array of chemicals, thereby leading to the upregulation of genes involved in biotransformation and homeostasis [4].

The molecules that can modulate PXR and CAR are diverse and pervasive, ranging from small synthetic organic compounds to large natural products [1]. Because the binding of these molecules to these NRs can result in important biologic effects, this review aims to summarize the relevant findings regarding the modulation of PXR and CAR by small molecules. Emphasis is placed on cross-species differences in PXR and CAR activation, the structural understanding of ligand binding, and the potential clinical impact of these modulators.

2. PXR

PXR (NR1I2), a member of the NR superfamily, is well characterized as a xenobiotic sensor because it binds to structurally diverse chemicals, including numerous clinical drugs and exogenous and endogenous substances [1]. PXR transcriptional activation regulates the expression of its target genes, which encode proteins involved in xenobiotic detoxification and endobiotic metabolism, such as drug-metabolizing enzymes and transporters [5]. PXR transcriptional activation is regulated through either direct binding by a ligand or indirect post-translational modification at key amino acid residues, for example, by phosphorylation [6–10]. Four major structural domains of PXR are involved in its function: a sequence-specific DNA-binding domain (DBD), a flexible hinge, a ligand-binding domain (LBD), and an activation function 2 (AF-2) domain located in the LBD [11]. Upon binding a ligand, the PXR LBD changes its conformation, allowing the AF-2 domain to dissociate from the co-repressor. The subsequent hPXR-co-activator interaction facilitates the binding of the DBD to site-specific DNA sequences, leading to the initiation of transcription of target genes. The heterodimerization of PXR with retinoid X receptor α (RXRα) is also required for ligand-induced PXR transcriptional activation [12].

One of the main physiologic functions of PXR is to regulate the expression of genes encoding drug-metabolizing enzymes and transporters in response to xenobiotic exposure [4, 5]. Therefore, PXR is known as a master regulator of the xenobiotic detoxification system. However, the unwanted activation of PXR by xenobiotics during pharmacotherapy may lead to deleterious reactions, such as adverse drug-drug interactions (DDIs), cancer drug resistance, and liver toxicity [13–15]. Therefore, researchers have long been interested in identifying and investigating the small molecule modulators that can control PXR transcriptional activation.

2.1 Species-selective PXR modulation

Ligand-induced PXR activation displays marked species selectivity. The LBDs of human and mouse PXR differ markedly in their amino acid sequences [16]. Therefore, ligands can selectively bind to the PXR of different species. For example, rifampicin and SR12813 can

strongly bind to human PXR (hPXR) but not to mouse PXR (mPXR), whereas 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) is a poor ligand for hPXR but a strong agonist for mPXR [17]. The species selectivity of PXR complicates the relevance of using mouse models to evaluate the PXR activation of a given hPXR ligand *in vivo* and to investigate the *in vivo* function of hPXR. To overcome the species-related differences in ligand recognition and to enable functional studies of hPXR to be conducted in mice, several humanized mouse models of PXR have been developed for investigating the *in vivo* activation of hPXR by its ligand. The first-generation hPXR mouse model was developed by randomly inserting the hPXR gene into a mouse genome from which the mPXR gene had been deleted. In this transgenic mouse model, the expression of the hPXR gene was under the control of either the liver-specific albumin promoter [18] or the rat fatty acid–binding protein promoter [19]. Similarly, the second-generation hPXR mouse model was also developed using a transgenic approach. A genomic fragment containing the entire hPXR gene and its promoter was cloned and randomly integrated into a mouse genome with a *Pxr*-null background [20]. To further improve this model, a double transgenic mouse model expressing hPXR and cytochrome P450 3A4 (CYP3A4) was generated by using bacterial artificial chromosome transgenesis in *Pxr*-null mice. In this model, rifampicin treatment robustly induces CYP3A4, mimicking the human response to rifampicin [21]. To precisely control the copy number and locus of hPXR genes inserted into the mouse genome, the third-generation hPXR mouse model was developed through knock-in strategies by simultaneously replacing the mPXR gene with the hPXR gene under the control of the endogenous mouse promoter [22]. The same knock-in strategy was used to develop the latest humanized PXR mouse model, in which the genomic sequence of the LBD of hPXR was homologously knocked into the mPXR gene, resulting in the expression of a chimeric PXR (mDBD-hLBD) [23]. This chimeric PXR mouse model was intended to negate the effects of potential differences between hPXRs and mPXRs in terms of the DNA binding affinity of their DBDs. These humanized PXR models have broad application in basic biology research for demonstrating the functions of PXR and in translational research for evaluating the *in vivo* toxicity and pharmacokinetics of PXR ligands during drug development.

2.2 Potential clinical use of PXR functional modulators

PXR agonists have been extensively investigated and are well documented; they include clinical drugs, phytochemicals, dietary constituents, and endogenous substances. In the clinic, people are cautioned about using PXR agonists because they may cause adverse drug-drug or diet-drug interactions during drug therapy. However, recent clinical and preclinical evidence suggests that some PXR agonists can be used to treat certain diseases, such as inflammatory bowel disease (IBD), through the activation of PXR transcriptional function [24]. In this section, we will highlight the beneficial effects of PXR agonists in treating IBD.

IBD is a group of chronic or recurring conditions characterized by an immune response and inflammation of the gastrointestinal tract. Early association studies of the pathogenesis of IBD revealed that the expression and activity of PXR and the expression of PXR transcriptional target genes were substantially reduced in the intestines of patients with IBD [25]. Moreover, genetic variation in the gene encoding PXR was associated with altered activity of PXR and was strongly associated with susceptibility to adult IBD [26]. By using a

Pxr-null mouse model, Cheng and colleagues clearly showed that a PXR-dependent mechanism was involved in dextran sulfate sodium (DSS)–induced IBD and that treatment with PCN, an mPXR agonist, can protect mice from DSS-induced colitis (Cheng et al. 2010). Using a humanized PXR mouse model, the same group further demonstrated that rifaximin, a selective hPXR agonist, exhibited preventive and therapeutic effects on IBD induced by DSS and 2,4,6-trinitrobenzene sulfonic acid (TNBS) in mouse models [27]. Further clinical evidence indicated that rifaximin alone or in combination with other antibiotics was effective at alleviating active IBD, supporting the therapeutic value of PXR agonists in treating IBD [28]. The underlying mechanism by which PXR agonists treat IBD mainly involves reciprocal crosstalk between PXR and NF-κB. Upon PXR activation, the NF-κB signaling cascade can be inhibited, resulting in the suppression of the NF- κB–mediated proinflammatory response [29].

In pursuing the PXR activation approach to treating IBD, several PXR agonists derived from natural products have been identified, and their potential use in IBD therapy has been assessed. Solomonsterol A, isolated from the marine sponge *Theonella swinhoei*, is a potent PXR agonist and effectively protects humanized PXR mice from developing the clinical signs and symptoms of colitis through PXR-dependent inhibition of NF-κB activation [30]. Artemisinin, a natural compound used to treat malaria, was identified as a potential PXR agonist [31], and a recent report indicated that this drug can prevent and reduce IBD development in a mouse model of DSS-induced IBD by a mechanism associated with the transcriptional activation of PXR [32]. Chrysin, a flavonoid compound that can be extracted from many plants, can activate hPXR and mPXR in reporter gene assays and upregulate xenobiotic detoxification genes in the colon but not in the liver. Chrysin can ameliorate chemical-induced colitis in a mouse model through a PXR/NF-κB–dependent mechanism [33]. Isorhamnetin, an activator of the human PXR, has also been reported to attenuate TNBS-induced colitis in mice [34]. Because flavonoids are important components in many plants, foods, herbal medicines, and health supplements, a recent study systemically examined the effect of 3-hydroxyflavone and its structurally related analogs on PXR activation [35]. By using structure-activity relationship analysis, this study found that flavonols activated PXR in a species-dependent manner and identified 3-hydroxyflavone, galangin, quercetin, isorhamnetin, and tamarixetin as hPXR agonists; this scaffold may serve as a starting point for developing nutraceuticals to treat PXR-associated intestinal diseases, such as IBD.

Studies using genetically modified PXR-related animal models have established PXR as a therapeutic target for IBD. However, rifampicin, a well-known hPXR agonist, has no effect on experimentally induced IBD [27]. One possible explanation for this is that rifampicin is highly bioavailable, and its distribution in the liver can markedly reduce hepatic expression of stearoyl-CoA desaturase-1, leading to lower levels of anti-inflammatory unsaturated fatty acids in the plasma, which counteract the anti-inflammatory function of PXR in the gut [27]. Unlike rifampicin, rifaximin is a gut-specific agonist of hPXR [36]. It is concentrated in the intestine, and less than 0.2% of rifaximin is transported into the liver and kidneys. This suggests that the discovery and development of gut-specific hPXR agonists is needed to treat IBD. In evaluating the therapeutic efficacy of PXR agonists against IBD, both humanized

PXR and *Pxr*-null mouse models should be used to ensure the on-target effect of the agonists.

PXR activation has been linked to several clinical phenomena, such as adverse reactions caused by DDI, drug-induced liver injury (DILI), chemoresistance in cancer patients, and increased risk of Type-2 diabetes [37]. Therefore, there is ongoing interest in developing modulators that can counteract PXR activation. In this review, we define this group of PXR modulators as PXR antagonists. It is anticipated these antagonists will be able to prevent the onset of PXR activation–related clinical symptoms and/or enable their progression to be managed.

Ecteinascidin 743 (ET 743), a natural product derived from the Caribbean marine tunicate *Ecteinascidia turbinata*, was the first compound reported to have a repressive effect on PXR agonist–mediated activation of PXR target genes, such as CYP3A4 and multidrug resistance protein 1 (MDR1) [38]. However, ET 743 was reported to bind to the minor groove of DNA [39] and can, therefore, interfere with DNA repair pathways, leading to perturbation of the cell cycle and microtubule disorganization [40]. Thus, it remains unclear whether ET 743 negatively modulates PXR activation through direct interaction with the receptor or through another, indirect mechanism(s). The clinical use of ET 743 as a PXR inhibitor is also limited because of its potent cytotoxic properties.

Phytochemicals in the daily diet or herbal medicines are thought to have low or no cytotoxicity and to be relatively safe for potential clinical use. Therefore, great efforts have been made to identify PXR antagonists among such compounds. Sulforaphane (SFN), a major component of certain cruciferous vegetables, was the first naturally occurring PXR antagonist to be reported. In cell-based assays, this compound has IC_{50} values of 12 to 14 μM against ligand-induced PXR transcriptional activation. SFN has been inferred to bind to the PXR LBD on the basis of scintillation proximity assays, yielding a K_i of 16 μM, and this is further evidenced by its disruption of co-activator recruitment when evaluated using the mammalian two-hybrid assay [41]. Computational results suggest that this molecule may also interact with the outer surface of PXR at the AF-2 domain [42]. Thus, the molecular mechanism and ligand-receptor binding model of SFN action on PXR are still exclusive. Moreover, a recent human clinical study suggested that this compound was not an effective antagonist of the hPXR *in vivo* [43]. Considering the antagonistic effect on PXR of SFN at relative high concentrations *in vitro* and its other pharmacologic activities, such as histone deacetylase inhibition [44], achieved at relatively lower concentrations, further investigation of the on-target effect of SFN against PXR is warranted, especially at physiologically relevant concentrations.

Coumestrol, a phytoestrogen prevalent in legumes and soy beans, is another naturally occurring chemical that affects PXR transcriptional activation [45]. Very similar to SFN, this compound can elicit an antagonistic effect on PXR activation at relative high concentrations, with an IC_{50} value of 12 μM in a PXR promoter reporter assay and a K_i value of 13 μM in competitive ligand binding assays of the PXR LBD [45]. Further mutagenesis studies have shown that the compound binds to the outer surface of the PXR LBD. Collectively, this evidence suggests that the binding of coumestrol to the PXR ligand binding pocket is weak

[42, 45]. Other phytochemicals, including sesamin (a lignan found in sesame seeds) and camptothecin (a quinoline alkaloid isolated from the plant *Camptotheca acuminata*), have also been reported to functionally block PXR activation–mediated gene expression [46, 47]. However, there is still no clear evidence of their direct engagement with PXR. Therefore, this class of compounds can be regarded as functional blockers of PXR, as opposed to PXR antagonists that can directly affect the binding of an agonist to this target.

The most investigated synthetic inhibitors of PXR transactivation are the azole class of chemicals, including ketoconazole and its derivatives [48]. A pioneer study found that ketoconazole, an antifungal drug, was active in repressing ligand-induced PXR activation, and it can also decrease the interaction of PXR with the co-activator SRC-1 and co-repressor SMRT in a mammalian two-hybrid assay in HepG2 cells [49]. One group used biochemical and cell-based assays and molecular docking computations to gain further molecular and structural insights into the action of ketoconazole in regulating the transcriptional activity of PXR. They concluded that ketoconazole can bind at the AF-2 surface of PXR, rather than in the ligand-binding pocket, resulting in PXR transcriptional regulation [50–52]. By using a yeast two-hybrid screening strategy together with computational docking analysis, the same group recently identified a putative binding region on the AF-2 domain of PXR that allows the binding of ketoconazole to this receptor. The binding region includes Gln-272, Phe-264, and Ser-208 of PXR, which could be critical for disrupting the PXR–SRC-1 interaction due to the binding of ketoconazole to PXR [53]. Because ketoconazole is a well-documented inhibitor of the enzymatic activity of CYP3A4 and can somehow cause hepatic toxicity, a group of its analogs were synthesized in order to develop a safer PXR activation blocker with fewer off-target effects [54]. FLB-12, a ketoconazole analog, was discovered as a result of a structure-activity relationship study. FLB-12 exhibited relative selectivity against ligand-induced PXR activation *in vitro* and *in vivo*, no inhibition of CYP3A4 activity, and less toxicity than ketoconazole. In pharmacologic studies, FLB-12 abrogated the PXR-mediated resistance to 7-ethyl-10-hydroxycamptothecin (SN-38) of colon cancer cells *in vitro* and attenuated PXR-mediated acetaminophen hepatotoxicity *in vivo* [55]. In light of these studies, developing selective PXR antagonists appears to be a feasible approach for managing PXR-related adverse DDIs and cancer drug resistance.

PXR antagonists could, theoretically, act more selectively by directly competing with the binding of agonists to the ligand-binding pocket of PXR. However, no such compound has yet been reported, at least in part because of the promiscuous nature of this receptor for ligand binding. Because the PXR ligand-binding cavity is comparatively large, this receptor can bind compounds with diverse chemical structures. Therefore, it could be challenging to discover a compound that specifically and directly competes with the binding of structurally diverse PXR agonists to the ligand-binding pocket of PXR. However, our recent experience leads us to believe that large-scale high-throughput screening, using a large collection of structurally diverse compounds, might be an effective approach to discover this novel class of PXR antagonists characterized by 1) direct binding at the PXR binding pocket, 2) effective antagonism against variable well-characterized PXR agonists, 3) on-target efficacy in a humanized PXR mouse model, and 4) no obvious toxicity *in vitro* or *in vivo*.

3. CAR

Originally identified as MB67 [56], CAR belongs to the Type I NR family and is denoted as NR1I3. The structural domains of CAR are arranged much like those of other NRs, and the function of CAR is determined by its domains; the DBD, the dimerization domain, and the LBD. CAR is a transcription factor that binds to specific response elements in the promoter regions of CAR target genes. Activated CAR uses RXRs as heterodimeric partners for DNA binding [56, 57]. The best described CAR response element is the phenobarbital-responsive enhancer module (PBREM), which is present in the promoter regions of CAR target genes. For example, PBREM is found in the promoter region of the *CYP2B6* gene, approximately 1.8 kb upstream of the transcriptional start site, and harbors two direct repeat separated by 4 nucleotides (DR4) motifs [58]. CAR also binds DNA at the xenobiotic responsive enhancer module (XREM), which is also found in the *CYP2B6* distal regulatory regions and contains one DR4 motif surrounded by half sites [59]. CAR shares these binding sites with PXR [18, 60]. This commonality of DNA response elements, among other factors, allows CAR and PXR to co-regulate a subset of their target genes, though they also separately regulate other genes [61].

CAR is referred to as a xenobiotic sensor owing to its broad specificity for both endogenous and exogenous ligands with varying chemical structures. Solving the structure of the CAR LBD has enabled researchers to understand this phenomenon and has suggested that the large hydrophobic binding region of the CAR LBD sanctions binding to diverse chemical structures. The AF-2 region in the CAR LBD appears to be rigidly fixed in an active conformation, even in the absence of ligands, and ligand docking further supports the idea that conformational changes affect the transactivation activity of CAR [62].

3.1 CAR functions as a transcription factor

CAR expression is modulated by other NRs, such as hepatocyte nuclear factor 4 alpha (HNF4α), peroxisome proliferator-activated receptor alpha (PPARα), glucocorticoid receptor (GR), and possibly others [63–65]. It is well documented that glucocorticoids (such as dexamethasone) increase the amount of CAR mRNA in the liver [65]. CAR expression is increased by a variety of xenobiotics; BMS-665351 is an inhibitor of insulin-like growth factor 1 receptor (IGF-1R) that selectively induces CAR expression without activating GR, PPARα, or HNF4α, implying the involvement of as yet unknown mechanisms in the transcriptional regulation of CAR [66].

Activated CAR regulates the expression of genes involved in the metabolism and efflux of xenobiotics and endobiotics. These target genes are often phase I, II enzymes and transporters that regulate the metabolism and secretion of endogenous and exogenous signaling molecules, including many members of the CYP family, SULTs, UGTs, GST, membrane transporters, and others. Among the phase I enzymes, *CYP2B6* is considered a primary target gene for CAR activation, as evidenced by the well-established CAR-binding promoter regions. *CYP2B6* is also co-regulated by PXR. Additionally, even though *CYP3A4* is thought to be mainly regulated by PXR, activated CAR can increase CYP3A4 levels [67, 68]. Studies have revealed that, together, CAR and PXR play key roles in xenobiotic and endobiotic metabolism, especially in liver and intestinal tissues, and in maintaining cellular

and systemic homeostasis. Bilirubin breakdown and subsequent heme reabsorption is regulated by CAR through UGTs and membrane transporters that promote bilirubin excretion [69–71].

CAR activation also appears to change the metabolic environment in the liver in response to drugs. Phenobarbital (PB) activation of CAR causes a repression of hepatic gluconeogenic enzymes, such as phosphoenoylpyruvate carboxykinase 1 (PEPCK1) and glucose-6-phosphatase (G6Pase) [72], suggesting that CAR plays a critical role in regulating hepatic energy metabolism in response to drug exposure. CAR helps maintain hormone homeostasis by responding to androgens, estrogens, and other hormones that are metabolized by the CAR target genes SULTs and UGTs [73, 74]. CAR also directly regulates the synthesis of the thyroid hormone [75]. Such crosstalk between CAR and other NRs may explain the endocrine disruptions that result from exposure to certain xenobiotics. Thus, as a xenosensor, CAR can regulate several important physiologic processes that maintain systemic homeostasis.

3.2 Species selectivity of CAR

CAR exhibits remarkable species selectivity in its ligand binding and activation profiles. Human and rodent CARs share several common characteristics, such as nuclear translocation on direct or indirect activation and DNA response element sequences that recruit CAR. On the other hand, in contrast to most mammalian CARs, the activation of the *Xenopus* CAR seems to be translocation independent, as these receptors accumulate in the nucleus, even in the absence of activators [76]. CAR is also absent in most fish species [76]. Compared to the more than 20 functional hCAR splice variants identified, far fewer CAR variants have been detected in other species, and only approximately five in the rat [77] and pig [78], most of which generate truncated protein products. Comparative genomic analyses show that mice, rats, and marmosets do not have a conserved CAR2 site and are thus incapable of generating a protein analogous to hCAR2. CAR2 in human tissue has been shown to bind di(2-ethylhexyl) phthalate (DEHP) and acts as the main human DEHP receptor [79]. This means that rodent models of DEHP toxicity will not accurately profile potential human toxicity because of their inability to generate a CAR2-like transcript. Porcine CAR (pgCAR) might be more homologous to human CAR than is rodent CAR, as is evidenced by the responses of pgCAR and hCAR to ligands being more similar than are those of hCAR and mCAR [78].

All things considered, drug metabolism data generated from rodent models may not reflect human CAR functions accurately, and caution is necessary when directly extrapolating from mouse/rat data to humans. In a classic example of species selectivity, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), an mCAR agonist, activates mouse but not human CAR, and pharmacologic concentrations of androstanol repress mouse but not human CAR [80, 81]. CITCO, an hCAR agonist, binds and activates hCAR but has no effect on mCAR. There is also an important species-related difference in hepatic energy metabolism between hCAR and mCAR. Whereas hCAR activation selectively inhibits gluconeogenesis without suppressing fatty acid synthesis, mCAR activation results in the downregulation of genes associated with gluconeogenesis (G6Pase and PEPCK1), fatty acid synthesis (Fas and

Acc-α), and lipogenesis [sterol regulatory element-binding protein 1c (SREBP-1c) and Scd-1] [82].

The apparent species-specific differences between human CAR and CAR of other animals are particularly important when choosing animal models for analyzing the pharmacologic effect of new drugs on CAR induction and the subsequent activation of downstream CYPs. Rodent models are commonly used to evaluate the potential of clinical drugs and environmental agents for being tumor initiators or tumor promoters in humans. In the case of CAR, particular attention is needed when evaluating the carcinogenic potential of chemicals in animal models and humans, considering the reduced interspecies homology of CAR LBDs. For example, PB and other CYP2B inducers can act as nongenotoxic carcinogens and tumor promoters in mice and rats but appear to have a low likelihood of producing liver tumors in humans. In fact, Lake and colleagues postulated that human CAR activators do not increase replicative DNA synthesis in human hepatocytes, whereas the activation of rodent CAR results in liver hypertrophy, increased replicative DNA synthesis, altered hepatic foci, and liver tumors [83, 84].

3.3 Isoform selectivity of CAR modulation

The liver expresses high levels of CAR, and a lower level of CAR expression has been detected in brain, intestinal, heart, adrenal, testis, prostate, and kidney tissues [56, 85, 86]. CAR exists in many alternatively spliced variants [57, 87, 88] with diverse tissue expression profiles [85, 89–92], heterodimerization properties [89, 93], and ligand specificities [79, 94–98]. The organ-specific expression of CAR splice variants requires further exploration in order to understand the variations in drug metabolism and DDIs. The best studied human CAR variant is constitutively active when expressed in immortalized cells and is denoted as hCAR1 or simply CAR1 (348aa; NM_005122) by most researchers. Table 1 lists all annotated CAR isoforms identified so far.

The investigation of hCAR activation by flavonoids revealed interesting information regarding ligand specificities for some hCAR splice variants. 3-hydroxyflavone indirectly activated the hCAR2 (denoted SV23; NM_001077480.2) and hCAR3 (denoted SV24; XM_005245697.3), but without transactivation through LBD binding or recruitment of steroid receptor co-activators (SRCs). 3-hydroxyflavone can also activate wild-type (WT) hCAR by directly binding to its LBD, along with other flavonols, such as galangin, quercetin, and tamarixetin, but has no effect on full-length hCAR (denoted SV1; NM_001077482.2) [98]. The DEHP is a highly potent and selective agonist of hCAR2 [79, 99]. The antimalarials artemisinin, artemether, and arteether activate hCAR2 (SV23) in the absence of fetal bovine serum, but the direction of their effect can be reversed if serum-free conditions are used during cell culture [95]. The antibacterial agent triclosan (2,4,4'-trichloro-2'-hydroxydiphenylether) is an inverse agonist of hCAR1 but a weak agonist of hCAR3 [100]. The antiretroviral drugs rilpivirine and etravirine activate hCAR in an isoform-dependent manner. Both drugs activate the constitutively expressed WT hCAR without direct binding to the LBD or SRC recruitment [97]. Most probably, this phenomenon is a result of indirect CAR activation mechanisms, as in the case of PB and phenytoin. Interestingly, however, neither drug has any effect on the inducible variants

hCAR2 and hCAR3. In addition to splice variants, various clinical variants resulting from single-nucleotide polymorphisms in the CAR gene have also been identified [101], and these might confer differential functional effects, such as changes in bone mineral density or in the pharmacokinetics of the anti-HIV drug efavirenz [102, 103].

In one study of the splice-variant profile of CAR in the liver, researchers observed interethnic and interindividual variations among human liver samples from Koreans and Caucasians, including four novel splice variants in Korean livers [104]. The most predominant splice variant in the 30 Korean livers analyzed was, surprisingly, not the WT hCAR. These results suggest that there is a range of expressional variation of hCAR splice variants among different ethnic groups. Variations in CAR activity that result from genetic polymorphisms or from alternative splicing may influence the expression of CAR target genes and contribute to the overall interindividual variability in drug metabolism.

3.4 Indirect activation of CAR

Indirect activation of CAR with PB or phenytoin has been well documented [105, 106] and is now understood to be a common mechanism of CAR activation, with an increasing number of indirect activators having been identified [e.g., flavonoids [107], triclosan [108], or acetaminophen [109]]. These activators do not bind directly to CAR; instead, they activate CAR by stimulating its nuclear translocation in a ligand-independent manner. In rodent hepatocytes, a PB-mediated increase in CYP2B mRNA had been documented before the discovery of CAR, but the latter shed light on the mechanism of action underlying this phenomenon [58]. In the native hepatocyte environment, CAR is sequestered in the cytoplasm as part of a multiprotein complex that includes the heat-shock protein 90 [110]. Upon stimulation by indirect activators or direct ligand-binding, CAR disassociates from the cytoplasm-localized protein complex and moves into the nucleus, subsequent to dephosphorylation at Thr-38. The pathway of indirect CAR activation by PB involves the EGF receptor (EGFR) signaling pathway, in which PB binding to EGFR antagonizes EGF activation of EGFR, which eventually leads to the dephosphorylation of CAR at Thr-38 [111, 112]. Dephosphorylation of CAR activates nuclear translocation, and transcriptional activity ensues [112], even in the absence of direct ligands. Similarly, small molecule modulators of various kinase pathways (growth hormones and cytokines) that converge in changing the phosphorylation status of CAR (via ERK, MAPK, PP2A, etc.) are all capable of regulating CAR nuclear translocation and, ultimately, CAR function [113–116]. It should be noted that PB can indirectly activate NRs other than CAR, PXR [63], and PPARα [64], and hence the indirect method is not a specific mechanism of receptor activation.

The localization status of CAR in its —non-active state might be ambiguous. True to its name, CAR is indeed constitutively active when exogenously expressed in immortalized cell lines. Many research groups have shown that CAR is predominantly cytoplasmic in liver tissue of animals in the absence of ligands [66, 113, 117, 118]. While CAR spontaneously accumulates in the nucleus in immortalized cells, Lynch et al. have illustrated by using adenovirus expressing fluorescent-hCAR in human primary hepatocytes, that CAR is predominantly retained in the cytoplasm prior to chemical stimulation. However a small percentage (~7%) of total CAR protein was detected in the nucleus [119]. It is difficult to

assess the transcriptional function of this low level of nuclear CAR, especially when using the expression of CAR target genes to monitor the activity of CAR, as these genes may be regulated by multiple transcription factors (e.g., CYP2B6 can be regulated by CAR, PXR, HNF4α and others). Moreover, CAR has been shown to be sensitive to the activation states of various signaling pathways including EGF, IGF , AMPK, and ERK-MAPK [120]. These pathways regulate the phosphorylation status of CAR, and dephosphorylation of a single CAR residue at Thr38 induces the nuclear translocation of CAR and stimulates its transcriptional activity [111]. This, as discussed earlier, has been shown to be the main mechanism by which indirect activators, such as PB, stimulate CAR function even in the absence of direct binding ligands. Translocation of CAR to the nucleus drives its transcriptional activation. It is conceivable that the ratio of CAR distributed between the cytoplasmic and nuclear compartments in the absence of ligands, is dependent on a fine balance of signaling molecules and hence needs to be taken into consideration when discussing the CAR localization.

3.5 Potential clinical use of CAR modulators

3.5.1 CAR in metabolic syndromes—Several reports have delineated the antilipogenic properties of CAR. CAR activation suppresses lipid metabolism and lowers serum triglyceride by inducing Insig-1, which in turn reduces the levels of the lipogenic transcription factor SREBP-1 [65]. CAR activation in mice is associated with an improved serum lipid profile and increased insulin sensitivity. The mouse CAR activator TCPOBOP markedly suppresses adipose deposition and weight gain induced by a high-fat diet in mice [121–123]. Previously, in human studies, it has been noted that PB decreases plasma glucose levels and improves insulin sensitivity in diabetic patients [124]. The anti-epileptic drug phenytoin, which is also an indirect activator of CAR, increases the levels of high-density lipoprotein (HDL) cholesterol [125, 126]. CAR activation in mice decreased HDL and plasma apolipoprotein A-I, suggesting a protective role for CAR in atherosclerosis, cardiovascular ailments, and obesity [60]. Because CAR plays a role in reducing insulin resistance, Masuyama and Hiramatsu considered its role in the pathogenesis of preeclampsia (a complication of pregnancy that is characterized by high blood pressure and proteinuria) in individuals with insulin resistance and adipocyte dysfunction. In pregnant mice in which obesity had been induced by a high-fat diet, CAR activation with TCPOBOP resulted in improved glucose tolerance, with significant changes in the expression of gluconeogenic, lipogenic, and adipocytokine genes and reduced hypertension and proteinuria [127]. This suggests that CAR is a potential therapeutic target for obese patients with preeclampsia and insulin resistance, though no parallel studies in humans have yet been conducted.

Much effort has been exerted to understand the role of CAR activation in bile acid homeostasis in mice. Increased bile acid metabolism and detoxification, together with reduced bilirubin and bile acid serum levels, have been observed in healthy mice upon CAR activation [128, 129]. Hepatic necrosis, increased alanine aminotransferase levels, and a corresponding increase in serum concentrations of bilirubin have been observed in CAR KO mice, suggesting an important role for CAR in hepatic maintenance [129]. CAR agonists could potentially be used to treat cholestasis in patients with bile acid dysregulation.

3.5.2 CAR in cancer—The function of CAR in tumor maintenance and the development of chemoresistance is less clear than are its roles in other processes. In rodent models, it is accepted that CAR activators may act as nongenotoxic carcinogens and tumor promoters. PB activation of CAR induces the *Gadd45b* gene in mouse liver. GADD45B represses apoptosis, and CAR interacts with GADD45B to repress TNFα-induced JNK1 phosphorylation and cell death, thus promoting the development of hepatocellular carcinoma in mice [130]. However, studies of human CAR have attributed no carcinogenic properties to CAR activators [131]; in fact, some reports suggest that chemotherapeutic efficacy may be increased upon CAR activation. For example, the chemotherapeutic agent cyclophosphamide is converted from the prodrug to the active form by the enzyme CYP2B6, which in turn is a target of CAR activation, thus making it advantageous to use CAR agonists to improve chemotherapeutic efficacy [132]. Similarly, CAR agonists modestly increased paclitaxel-induced tumor depletion in five human lung cancer cell lines [133]. The growth and expansion of brain tumor stem cells was inhibited by the human CAR agonist CITCO in isolated human gliospheres and in xenograft mouse studies by inducing cell cycle arrest and apoptosis [134]. Conversely, CAR is known to regulate the expression of MDR1, which functions as a transmembrane efflux pump for eliminating anticancer agents and plays a major role in the development of chemoresistance. In four ovarian cell lines expressing CAR, treatment with the CAR activator CITCO and an anticancer agent caused an upregulation of MDR1 and UGT1A1, decreased the efficacy of the anticancer agent, and reduced the levels of apoptosis to a greater extent than did the anticancer drug alone [135]. Similar observations were made for multiple anticancer agents. CAR inhibition reversed these effects and increased both chemotherapeutic efficacy and apoptosis.

In summary, the data from these investigations has advanced our understanding of the role of CAR: once merely a well-known xenobiotic sensor and endobiotic modulator, CAR is now seen as a promising drug target for therapy of metabolic disorders and cancer.

4. Structural features of the PXR and CAR LBDs

The PXR and CAR LBDs represent a compact α-helix sandwich fold (Figure 1), with the PXR LBD consisting of three sets of α-helices: α1/α3, α4/α5/α8/α9, and α7/α10 [136]. The CAR LBD consists of 11 α-helices [62, 137], with the two 3_{10} helices α2 and α2′ being believed to contribute to the hydrophobic character of the binding pocket while possibly forming part of the ligand access gate [138]. In contrast to the three β-strands present in CAR, PXR contains a layer of five-stranded antiparallel β-sheets, which include the two novel β-strands (β1 and β1′) that are not observed in other NRs. It encompasses an insert of approximately 60 amino acids between the α1 and α3 helices, which cover β1, β1′, and the novel helix α2. There is evidence that PXR homodimerizes through the terminal β1′ strands from each monomer by interlocking the corresponding Trp-223 and Tyr-225 residues [139]. Trp-223Ala and Tyr-225Ala double mutants prevent homodimerization, with an associated reduction in CYP3A4 induction.

Co-activators and co-repressors, such as SRC-1 and SMRT, bind the AF-2 surface of the LBD through the Leu-Xxx-Xxx-Leu-Leu (for co-activators) and Ile/Leu-Xxx-Xxx-Ile/Val-Ile (for co-repressors) motifs [140]. The interaction of the agonist with the LBD leads to the

exposure of a hydrophobic surface for co-activator binding (Figure 1). Crystal structures show that the SRC-1 peptide is buried in a groove on the surface of the PXR LBD that is composed of the AF-2 helix, α3, and α4, with all the Leu residues of the peptide being in contact with the surface of the protein [141]. Polar interactions involving Leu-259 and Glu-427 of hPXR contribute to the formation of a —charge clamp that is believed to stabilize contacts with the co-activator peptide. The co-activator recruitment appears to play a vital role in fixing the ligand in the correct arrangement. The structure of PXR when binding the cholesterol-lowering drug SR12813 in the absence of SRC-1 reveals that the ligand occupies the binding cavity at three distinct positions [136], which corroborates the flexible nature of the LBD. However, in structures consisting of PXR-SR12813 in complex with an SRC-1 peptide, the agonist is constrained to a single binding orientation [141]. The idea that SRC-1 is necessary in order to restrict the bound ligand to a single active conformation led to the generation of binding proteins constructed using the fibronectin type III domain (Adnectins) that attach in the same location as do SRC-1 peptides, in the belief that these biologics would provide additional advantages over the SRC-1 peptides as chaperones for crystallographic studies [142]. It was anticipated that problems arising from the lack of electron density resolution observed in some structures when using small peptides would be ameliorated by the use of the larger Adnectins, and their higher affinity for PXR would increase the chances of trapping the ligand in a single binding mode.

Even though agonist binding to the LBD forces the AF-2 helix to be positioned in the active conformation for co-activator recruitment, a review of the large set of available hPXR and CAR structures revealed that not all agonists interact directly with residues in the AF-2 helix. In the case of PXR, TO901370 and compound 1 interact with Met-425 [142, 143], and PNU-142721 and estradiol interact with Met-425 and Phe-429 [144, 145]. In addition to Met-425 and Phe-429, SR12813 makes contact with Phe-420, which is adjacent to the AF-2 helix [141]. Colupulone also interacts with Phe-420 in addition to Met-425 [146]. However, no interaction has been observed with hyperforin [147]. When in complex with hCAR, 5β-pregnanedione and CITCO did not form direct contacts with the AF-2 helix [62], which was blocked from the binding cavity by a barrier composed of the residues Phe-161, Asn-165, Phe-234, and Tyr-326 [62]. Interestingly, the mCAR superagonist TCPOBOP penetrated this barrier, allowing it to interact with the AF-2 residue Leu-353 and the linker helix residues Leu-346 and Thr-350 [137].

Important features of the constitutive nature of CAR include the short and rigid AF-2 helix with a missing C-terminal extension, as is found in other NRs [62, 137, 138], which results in further stabilization of the active AF-2 conformation due to an interaction between the free carboxylate and Lys-195 (hCAR). A short —helix x composed of only four to six residues replaces the extended loop linking α10 and the AF-2 (Figure 1). Because only a single amino acid separates helix x from the AF-2 helix, the latter is restricted to favoring the active orientation. This hypothesis requires validation, as other NRs exhibit a comparable helix with no basal activity [148]. The structure of mCAR when in complex with the inverse agonist androstenol provides additional insights about its constitutive activity and its repression by this inhibitor [138]. In the active conformation, CAR exhibits α10 and α11 as a single continuous helix, but androstenol causes the formation of two distinct helices separated by a —kink that ultimately leads to the destabilization of the AF-2 helix [138].

RXR heterodimerizes with a number of NRs, including PXR and CAR. Association with RXR was shown to increase the affinity of CAR for ligands and the co-activator [137]. It has been suggested that the large heterodimerization surface contributes to the constitutive nature of CAR by stabilizing the active conformation [149]. The affinity of the heterodimeric PXR and RXR duplex for the co-activator peptide is greater than that of either individual receptor, but this cooperativity does not apparently involve significant structural changes during heterodimerization [150]. Ligand binding appeared to stabilize the complex and enhance co-activator recruitment. Therefore, NR activation involves more than just a sequence of events starting with ligand binding; instead, there is dynamic interplay among all the participating elements.

Unlike other NRs that have evolved to bind very selective ligands, PXR and CAR do not discriminate between molecules on the basis of size or chemical composition. This is largely due to the highly flexible and fluid LBD. The binding cavity of PXR is considerably larger than that of other NRs. The volume of the binding cavity of the apo-PXR is approximately 1150 $Å^3$ [136]. Ligand binding changes the shape of the pocket to fit the bound molecule, which is generally accompanied by an overall volume expansion. The size of the binding cavity increases to 1544 $Å^3$ with hyperforin [147] and to 1344 $Å^3$ with SR12813 [141]. However, the particular area that is in direct contact with SR12813 shrinks when the SRC-1 peptide is bound to PXR, thus constraining the ligand to being positioned in a single orientation, in contrast to the multiple modes observed in the absence of the co-activator. The change in volume upon ligand docking follows an internal rearrangement of certain structural features, such as helix α2. The observation that CAR is less promiscuous than PXR could be explained, to a large extent, by its smaller ligand pocket size, which ranges between 525 $Å^3$ and 675 $Å^3$ [62, 137, 138].

The ligand pockets of PXR and CAR are lined by mostly hydrophobic residues, but there are a few polar amino acids present that can form significant interactions with ligands. The cavity of PXR encompasses 28 amino acids, of which eight have polar or charged side chains [136]. Similarly, the 27 residues of the CAR pocket create a highly hydrophobic environment, with only a quarter of them being polar [138]. In both receptors, amino acid charge effects are further neutralized in the interior or vicinity of the ligand site by salt bridges, such as those between Glu-321 and Arg-410, between Asp-205 and Arg-413 in hPXR [136], or between the pairs Asp-238/Arg-156 and Glu-225/Lys-235 in mCAR [138].

Because the binding pocket is composed mainly of nonpolar residues, it is no surprise that hydrophobic and van der Waals forces play crucial roles in the interactions of the ligand with PXR and CAR protein residues. In the case of PXR, other important pairwise contacts have been observed in many of the structures elucidated thus far, as illustrated in Figures 2 and 3 [141–143, 145–147, 151]. In several cases, such as with hyperforin, SR12813, and rifampicin, Ser-247 and His-407 in hPXR participate jointly in hydrogen bonding. Gln-285 forms direct hydrogen bonds with hyperforin, TO901370, rifampicin, compound 1, and via a water molecule with colupulone. Far less common involvement was noticed for Arg-410 (interacting with 17β-estradiol) and His-327 (coupling to TO901370). Aromatic side chains have been implicated in forming additional attractive forces: Trp-299 forms π-stacking contact with SR12813, TO901370, and compound 1, with Phe-288 and Tyr-306 participating

with less frequency. In sharp contrast to PXR, hydrogen bonding between ligands and residues lining the cavity of CAR is not that common: CITCO [62] and TCPOBOP form no hydrogen bonds [137], 5β-pregnanedione participates in a single hydrogen bond with His-203 in hCAR [62], and androstenol forms a direct bond with Asn-175 and a water-mediated interaction with His-213 in mCAR [138]. Hence, most of the CAR ligands co-crystallized thus far depend heavily on hydrophobic contacts for binding.

The initial PXR structure provides clues to the importance of certain residues in species-selective PXR activation. SR12813 is an efficient agonist of hPXR but not of the mouse ortholog. On the other hand, PCN preferentially activates mPXR over hPXR. Based on the interactions of hPXR with SR12813, four amino acids were mutated in the mPXR expression system to the corresponding hPXR residues (Leu-206, Ser-208, His-407, and Arg-410), resulting in a reversed response profile: SR12813 became an agonist, whereas PCN had no noticeable effect [136]. This would explain the species-selective responsiveness to xenobiotics and the interesting fact that few residues are critically involved in this process.

The synergistic activation of PXR has been observed for the pesticide trans-nonachlor (TNC) and the active ingredient of contraceptive pills 17α-ethinylestradiol (EE2) [152]. Although each of these chemicals displays weak potency by itself, their simultaneous use results in a much stronger biological response, which correlates with cooperativity, as opposed to merely additive effects. Structural comparisons of PXR-EE2, PXR-TNC, and PXR-EE2-TNC revealed unambiguous binding of EE2 in both the binary and ternary complexes, but TNC could be observed only when bound in combination with EE2. Even after EE2 binding, a substantial portion of the ligand-binding site remains unoccupied, and this space is large enough to accommodate ligands of the size of TNC. In fact, the space filled by the EE2-TNC pair appears to overlap with that occupied by SR12813 or rifampicin. In addition to nonpolar contacts between TNC and PXR, TNC is further stabilized by van der Waals interactions with EE2. The notion of functional enhancement as a result of interligand interactions within the pocket leads the authors to propose the concept of a — supramolecular ligand. Because of the large size of the PXR ligand pocket, it is plausible that more of these multiligand assemblies will be discovered, particularly as other reports of crystal structures have shown that large portions of the pocket are left unoccupied by the bound ligand. For instance, 17β-estradiol and TO901317 leave vacant volumes of 981 $Å^3$ and 442 $Å^3$, respectively [143, 145].

5. PXR and CAR inhibitors

There is great impetus to develop PXR and CAR inhibitors in order to prevent the reduction of drug efficacy and prevent drug-induced toxicity. Several classes of chemicals have been found to mitigate the activity of PXR and CAR (Figure 4), but they all have major drawbacks, including cytotoxicity, poor pharmacologic properties, lack of selectivity, or poor potency.

NR activity can be repressed by a number of mechanisms. In addition to displacing an agonist by directly positioning itself at the ligand pocket, an inhibitor can attach to the outer surface of the protein. This directly or allosterically blocks the recruitment of partner

proteins, such as co-activators or RXR, or it strengthens the interactions with a co-repressor. It is also foreseeable that upstream events can be modulated by, for example, posttranslational modifications that mitigate the activity of the NR.

Given the large and flexible pockets of PXR and CAR and the extensive collection of agonists discovered so far, it is not surprising that a relatively large number of inhibitors have also been reported. However, based on biochemical assays that measure displaced radiolabeled ligands for PXR, it is believed that only a few of these inhibitors, such as SFN [41], polychlorinated biphenyls [153], and coumestrol [45], bind at the ligand pocket. The antifungal drug clotrimazole was reported to attenuate the activity of hCAR by directly competing with the agonist [17]. Coumestrol not only competes with a PXR agonist for binding to the ligand-binding cavity, but also likely binds to a surface outside the ligand binding pocket [45]. Computational studies cannot definitely confirm the binding of the inhibitor to the ligand cavity, but they can provide a plausible scenario in which this event can occur. This approach was applied to the hCAR inverse agonist S07662 by using molecular dynamics simulations [154].

Inhibitors that probably interact with the outer surface of PXR ligand binding pocket include ketoconazole [48], SPB03255, and SPB00574 [42], which are thought to reside at the AF-2 surface, preventing the recruitment of co-activators. Most of the remaining reported inhibitors cannot be assigned a particular inhibition modality, because only cell-based data is available, with no confirmation of direct PXR binding. Crystal structures would provide definite validation of the mechanism behind the antagonism, but the only structure obtained so far for an inhibitor in complex with either NR is for the mCAR inverse agonist androstenol; this provides insight into the structural features that lead to decreased basal activity [138].

A number of PXR and CAR inhibitors are of natural origin. Some of these compounds, such as sesamin [46], are present in a regular diet and, therefore, are not thought to be highly toxic. However, others exhibit potent cytotoxicity, such as ET-743 [40]. These natural products range widely in size and chemical structure complexity, as is evident when the structure of ET-743 is compared with that of SFN (Figure 4).

Some of the reported PXR inhibitors were previously described as possessing other biological activity. Leflunomide was portrayed as the first FDA-approved compound to be repurposed for PXR inhibition [42], which is now approved for treating rheumatoid arthritis and psoriatic arthritis. Metformin is a marketed biguanide compound used as an antihyperglycemic agent to treat diabetes [155]. A-792611 is an HIV protease inhibitor, which not only suppresses PXR activity but is also metabolized by and inhibits CYP3A4 [156]. These types of novel PXR inhibitors, which have proved potent in preclinical studies for other indications, would have the added benefits of their established safety and desirable PK/PD profiles. However, their non-PXR related activities indicate that they are not specific for PXR.

Small-molecule modulators of PXR can most likely affect the activity of CAR, and vise versa. The difficulty in obtaining modulators specific to one of the xenobiotic receptors is by

and large due to their ligand promiscuity. For instance, most of the reported CAR inhibitors have been shown to be medium to potent activators of PXR. Hence, these inhibitors would be bound to be ineffective in cases where both receptors are present, as the same sets of genes to be silenced by one of the receptors would be activated by the counterpart. Recently, the chemical CINPA1 and its analogs were identified as potent CAR selective inhibitors that do not activate PXR [157, 158]. This novel class of compounds enables receptor-selective inhibition to address the biology of a particular receptor without interference from other NRs.

6. Conclusion

PXR and its close relative CAR are recognized as master xenobiotic sensors and are notorious for their characteristic ligand promiscuity. Indeed, their reported agonists differ greatly in molecular size, structure, and physiochemical properties. The ability of PXR and CAR to regulate the expression of a number of overlapping or distinctive sets of genes that are responsible for xenobiotic detoxification can be seen as a double-edged sword. Foreign chemicals that can harm the organism are degraded and excreted out of the system, and endogenous substances are maintained at appropriate levels. However, PXR and CAR activation can also lead to undesirable DDIs, and xenobiotic metabolites (including therapeutics) can result in the generation of toxic by-products.

These ligands have displayed species and—mainly for CAR—isoform preference. Investigating cross-species preference is vital in order to be able to extrapolate *in vivo* findings of DDIs from animal models to humans, and organ-specific expression of the variants would enhance our understanding of variations in drug metabolism. The discovery of novel agonists and antagonists with species and isoform selectivity would provide important tools to greatly broaden our knowledge of these issues and would serve as a basis for developing therapeutic agents to counter adverse reactions in drug coadministration.

Crystal structures provide insights into the molecular basis of promiscuity for PXR and CAR, and the constitutive character of CAR, laying the groundwork for developing antagonists to be used as cotherapeutics. An increase in the number of known structures with different classes of ligands would greatly improve the prediction of potential PXR or CAR activators, enabling the identification of compounds that may cause adverse drug effects. All of the structures reported so far involve only the LBD, but studies encompassing full-length proteins would provide an essential understanding of the interplay among ligands, target DNA, and partner proteins. Structural characterization on the other isoforms is lacking, particularly when the activity of CAR vary across isoforms.

Although there is convincing evidence to support the use of PXR and CAR as therapeutic targets, their roles beyond being xenobiotic receptors need to be expanded to include their effects on health and diseases such as inflammation, diabetes and cancer.

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ABBREVIATIONS

AF-2	activation function 2
CAR	constitutive androstane receptor
CYP	cytochrome P450
DBD	DNA-binding domain
DDI	drug-drug interactions
DEHP	di(2-ethylhexyl) phthalate
DILI	drug-induced liver injury
DR4	direct repeat separated by 4 nucleotides
DSS	dextran sulfate sodium
EE2	17α-ethinylestradiol
EGFR	EGF receptor
G6Pase	glucose-6-phosphatase
GR	glucocorticoid receptor
GST	glutathione S-transferase
HDL	high-density lipoprotein
HNF4α	hepatocyte nuclear factor 4 alpha
IBD	inflammatory bowel disease
IGF-1R	insulin-like growth factor-1 receptor
LBD	ligand-binding domain
MDR1	multidrug resistance protein 1
NR	nuclear receptor
PB	Phenobarbital
PBREM	phenobarbital-responsive enhancer module
PCN	5-pregnen-3β-ol-20-one-16α-carbonitrile
PEPCK1	phosphoenoylpyruvate carboxykinase 1
PPARα	peroxisome proliferator-activated receptor alpha

PXR	pregnane X receptor
RXR	retinoid X receptor
SFN	Sulforaphane
SN-38	7-ethyl-10-hydroxycamptothecin
SRC	steroid receptor co-activator
SREBP-1	sterol regulatory element-binding protein 1
SULT	sulfotransferase
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNC	trans-nonachlor
UGT	UDP-glucoronyltransferase
Wild-type	WT
XREM	xenobiotic responsive enhancer module

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HIGHLIGHTS

- PXR and CAR are the major xenobiotic sensors that initiate detoxification.
- PXR and CAR bind diverse chemical classes and exhibit species and isoform selectivity.
- Crystal structures reveal the molecular basis of binding promiscuity.
- Structure analysis enables prediction of modulators of PXR and CAR.
- PXR and CAR are important therapeutic targets in a number of diseases.

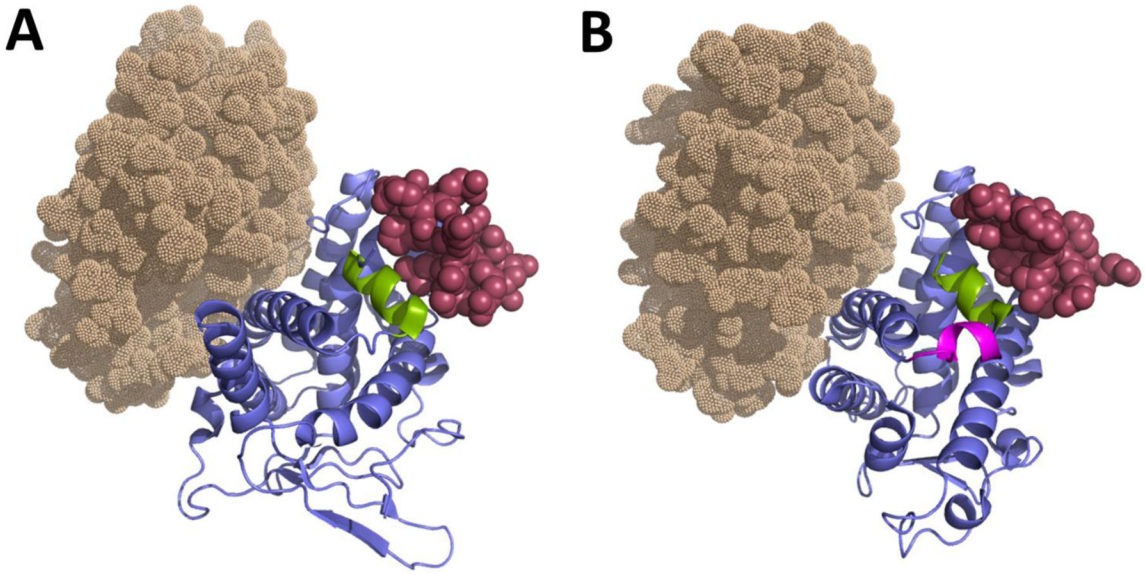

Figure 1.

Crystal structures of hPXR LBD (PDB code 4J5X) and hCAR LBD (PDB code 1XV9). The LBDs of PXR **(A)** and CAR **(B)** are depicted in blue (cartoon representation), showing their respective AF-2 helices in green and the co-activator SRC-1 peptide as maroon spheres. The —helix x that is notable in the CAR structure is shown in pink. The RXRα LBD is shown as light brown dots. Images of all the protein structures have been generated using the program Pymol.

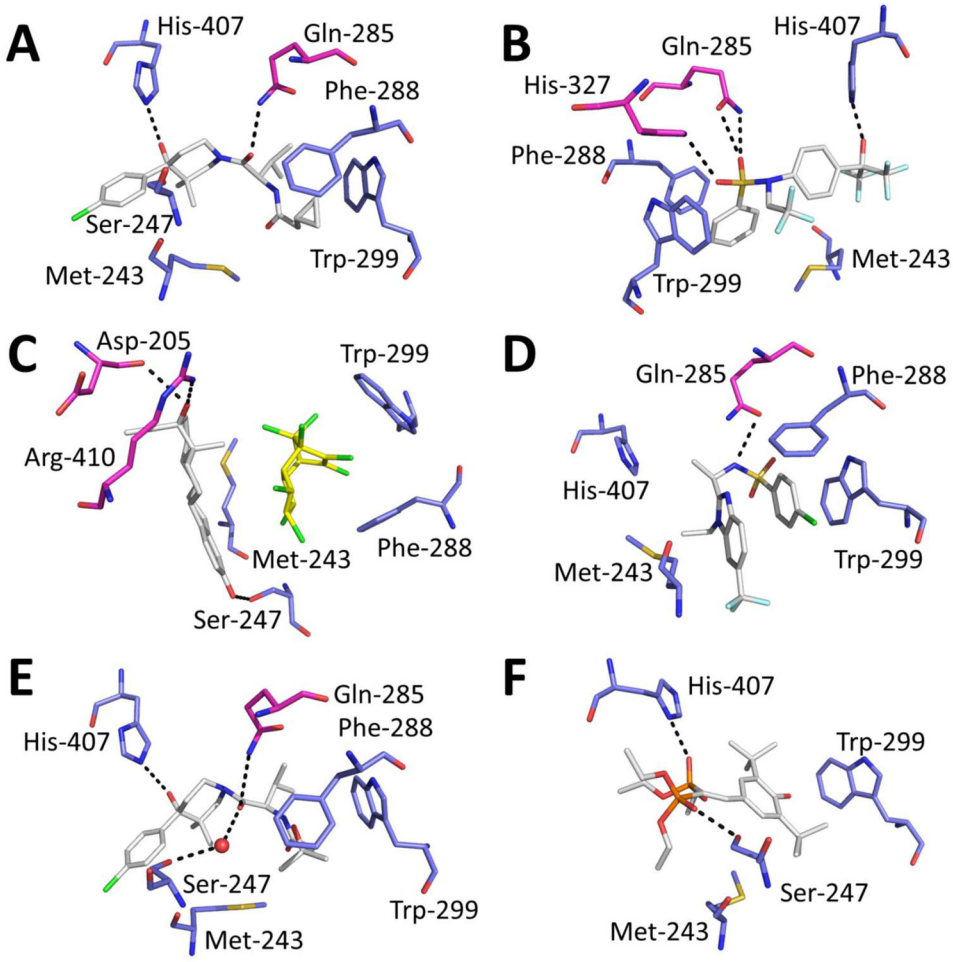

Figure 2.

Crystal structure of agonists in complex with hPXR. **(A)** Compound 1, PDB code 4XHD; **(B)** TO901317, PDB code 2O9I; **(C)** 17α-ethinylestradiol (white) and trans-nonachlor (yellow), PDB code 4X1G; **(D)** a sphingosine 1-phosphate receptor antagonist, PDB code 5A86; **(E)** BMS817399, PDB code 4NY9; and **(F)** hPXR-SR12813, PDB code 1NRL. Color code: white: ligand carbon; red: oxygen; blue: nitrogen; green: chlorine; pale blue: fluorine; and yellow: sulfur. The protein residues represented in blue (carbon) are commonly found in most reported crystal structures. The residues shown in pink are less frequently observed in other structures but participate in hydrogen bonding (indicated with a dashed line).

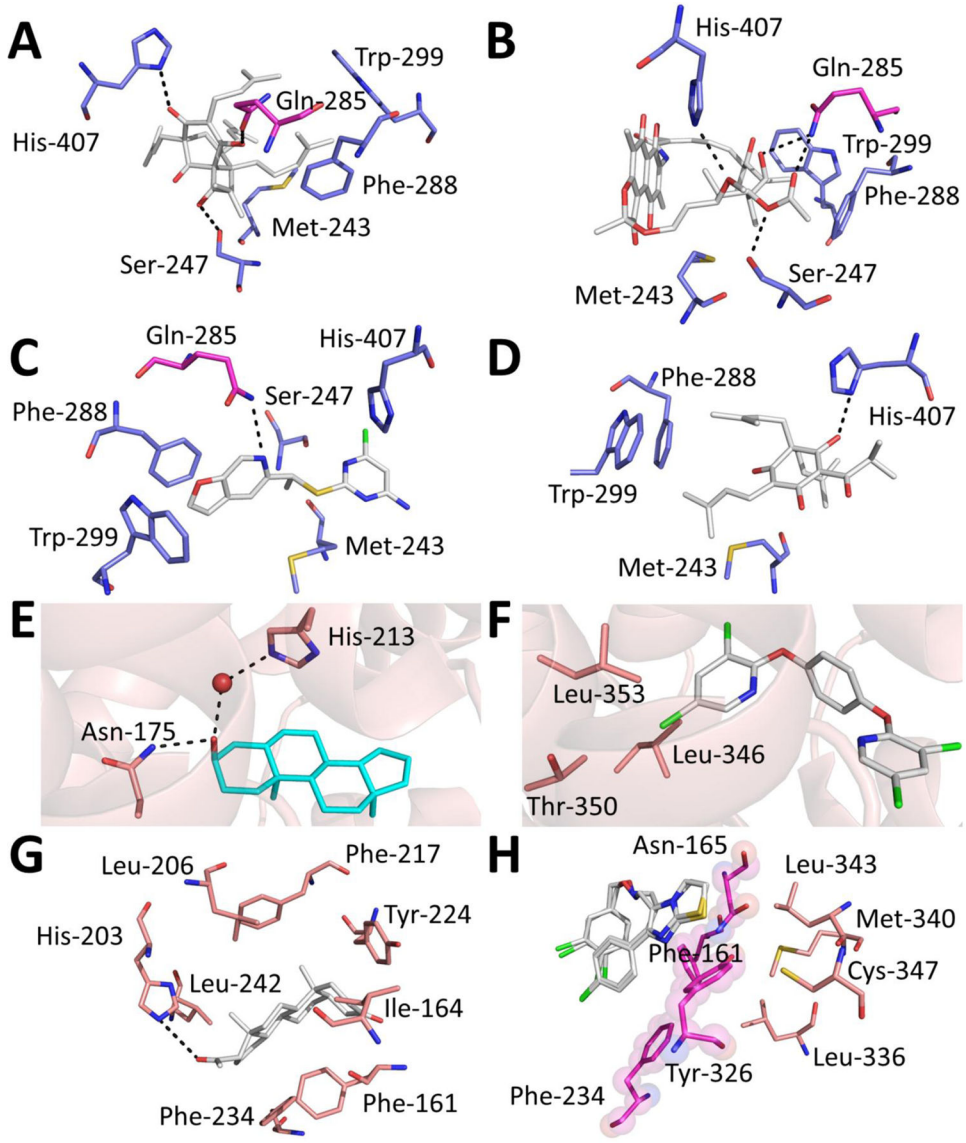

Figure 3.

(A–D) hPXR in complex with agonists (continued from Figure 2): **(A)** hPXR-hyperforin, PDB code 1M13; **(B)** hPXR-rifampicin, PDB code 1SKX; **(C)** hPXR- PNU142721, PDB code 3R8D; and **(D)** hPXR-colupulone, PDB code 2QNV. **(E)** mCAR complexed with the inverse agonist androstenol (PDB code 1XNX), showing direct and water-mediated hydrogen bonding. **(F)** mCAR complexed with the superagonist TCPOBOP (PDB code 1XLS) interacting with residues at the AF-2 helix. **(G)** hCAR complexed with the agonist 5β-pregnanedione (PDB code 1XV9) surrounded by interacting protein residues. **(H)** hCAR complexed with the agonist CITCO (PDB code 1XVP), showing the barrier residues (pink) that block access to residues in the AF-2 helix (orange). CITCO is depicted in the two modeled conformations.

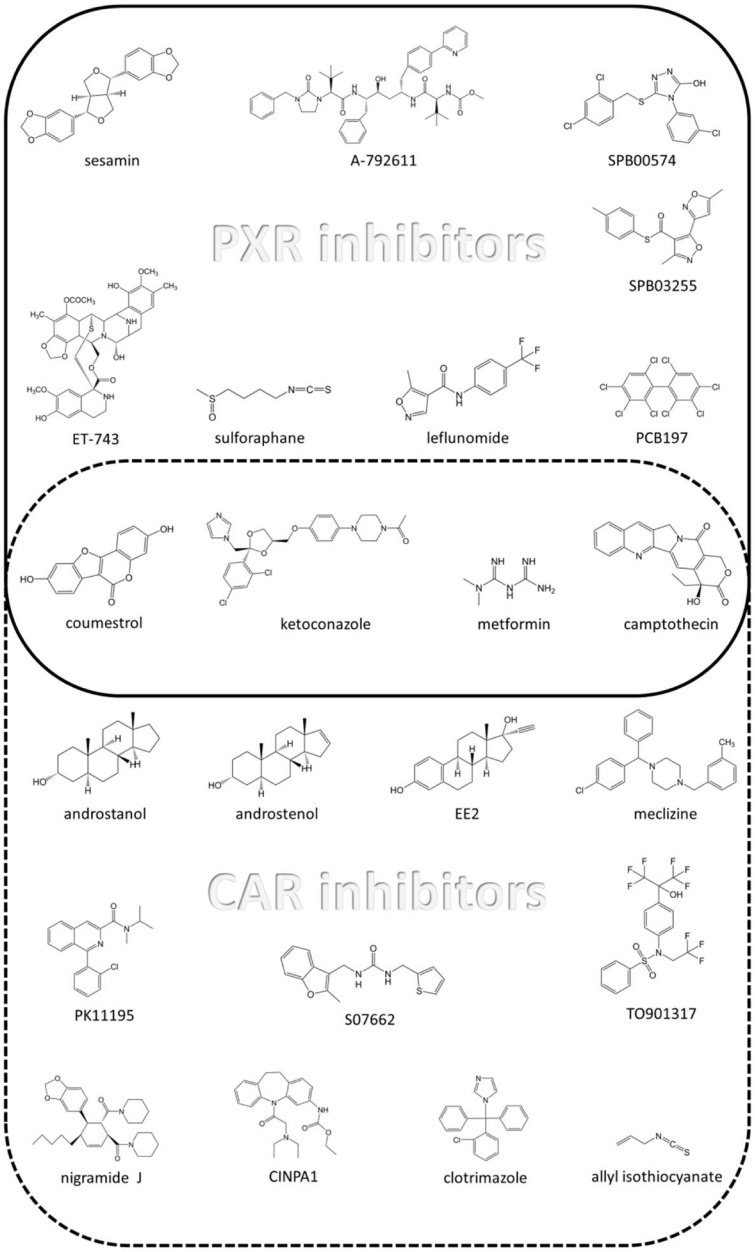

Figure 4.

Reported PXR (top) and CAR (bottom) inhibitors. Compounds described as inhibiting both NRs are enclosed in the center.

Table 1

List of annotated human CAR variants from the NCBI and Ensembl databases

	CAR splice variant	Transcript variant	NCBI ID	Transcript ID on Ensembl*	Transcript length (nt)	Protein length (aa)	Annotated coding sequence	Common name
1	NR1I3-036; NR1I3-201	1	NM_001077482.2	ENST00000367980	1074	357	CCDS41429.1	SV25
2	NR1I3-002	2	NM_001077480.2	ENST00000367982	1059	352	CCDS41430.1	hCAR2; SV23
3	NR1I3-001	3	NM_005122.4	ENST00000367983	1047	348	CCDS1228.1	hCAR1; WT; REF; SV0
4	NR1I3-012	4	NM_001077481.2	ENST00000367985	945	314	CCDS41428	
5	NR1I3-011	5	NM_001077471.2	ENST00000367984	930	309	CCDS44262	
6	NR1I3-031	6	NM_001077469.2	ENST00000428574	1023	340	CCDS44261.1	
7	NR1I3-029	7	NM_001077478.2	ENST00000442691	1020	339	CCDS 44260.1	
8	NR1I3-032	8	NM_001077474.2	ENST00000505005	985	296	CCDS53405	
9	NR1I3-019	9	NM_001077472.2	ENST00000367981	975	324	CCDS41427.1	
10	NR1I3-015	10	NM_001077479.2	ENST00000511676	960	319	CCDS53409	
11	NR1I3-014	11	NM_001077470.2	ENST00000504010	944	280	CCDS53407	
12	NR1I3-030	12	NM_001077473.2	ENST00000412844	948	315	CCDS53411	
13	NR1I3-033	13	NM_001077476.2	ENST00000508740	977	311	CCDS53410	
14	NR1I3-025	14	NM_001077477.2	ENST00000437437	973	306	CCDS53408.1	
15	NR1I3-024	15	NM_001077475.2	ENST00000512372	861	267	CCDS53406	
16	NR1I3-X01	X1	XM_005245693.3	--	1290	429	CDS from 236..1525	
17	NR1I3-X02	X2	XM_005245694.3	--	1275	424	CDS from 235..1509	
18	NR1I3-X03	X3	XM_011510237.1	--	1161	386	CDS from 234..1394	
19	NR1I3-X04	X4	XM_005245697.3	--	1062	353	CDS from 87..1148	hCAR3; SV24
20	NR1I3-008	--	--	ENST00000515452	1093	238	Protein coding	
21	NR1I3-009	--	--	ENST00000506209	1441	297	Protein coding	
22	NR1I3-013	--	--	ENST00000508387	634	118	Protein coding	
23	NR1I3-016	--	--	ENST00000502985	774	147	Protein coding	
24	NR1I3-021	--	--	ENST00000515621	1130	273	Protein coding	
25	NR1I3-026	--	--	ENST00000511944	620	147	Protein coding	
26	NR1I3-027	--	--	ENST00000511748; ENST00000628566	357	118	Protein coding	

	CAR splice variant	Transcript variant	NCBI ID	Transcript ID on Ensembl*	Transcript length (nt)	Protein length (aa)	Annotated coding sequence	Common name
27	NR1I3-010	--	--	ENST00000506018	1380	274	Nonsense- mediated decay	
28	NR1I3-017	--	--	ENST00000512340	891	90	Nonsense- mediated decay	
29	NR1I3-018	--	--	ENST00000510951	774	90	Nonsense- mediated decay	
30	NR1I3-020	--	--	ENST00000502848	914	119	Nonsense- mediated decay	
31	NR1I3-023	--	--	ENST00000507215	620	90	Nonsense- mediated decay	
32	NR1I3-004	--	--	ENST00000488651	580	No protein	Processed transcript	
33	NR1I3-005	--	--	ENST00000464422	480	No protein	Processed transcript	
34	NR1I3-006	--	--	ENST00000479324	764	No protein	Processed transcript	
35	NR1I3-035	--	--	ENST00000503547	535	No protein	Processed transcript	
36	NR1I3-003	--	--	ENST00000491193	1435	No protein	Retained intron	
37	NR1I3-034	--	--	ENST00000505944	865	No protein	Retained intron	

* http://www.ensembl.org/