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Targeting xenobiotic receptors PXR and CAR in human diseases

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Abstract

Nuclear receptors such as the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are xenobiotic receptors regulating not only drug metabolism and disposition but also various human diseases such as cancer, diabetes, inflammatory disease, metabolic disease and liver diseases, suggesting that PXR and CAR are promising targets for drug discovery. Consequently, there is an urgent need to discover and develop small molecules that target these PXR- and/or CAR-mediated human-disease-related pathways for relevant therapeutic applications. This review proposes approaches to target PXR and CAR, either individually or simultaneously, in the context of various human diseases, taking into consideration the structural differences between PXR and CAR.

Keywords

PXR; CAR; drug discovery; drug targets; human diseases; nuclear receptors

Introduction

Nuclear receptors (NRs) are ligand-dependent transcription factors [1] that regulate many biological events by inducing gene transcription [2]. Binding of an agonist ligand to a NR causes conformational change of the ligand-binding domain (LBD), dissociation with co-repressors and association with co-activators, leading to activation of gene transcription. These events contribute to regulation of signal transduction pathways under physiologic and pathologic conditions, and during human disease development. For this reason, NRs have been therapeutic targets for the development of new drugs [3] for various diseases, including asthma, type 2 diabetes, atherosclerosis, osteoporosis and cancer [3,4]. Structural analysis of NRs shows that NRs consist of common structural domains with a variable N-terminal domain, a conserved DNA-binding domain (DBD) and a C-terminal LBD that can be targeted in NR drug discovery [5]. NRs have been an established therapeutic target class with many prescribed drugs already on the market [6]. Thus, understanding the network of

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target proteins associated with NRs and their contributions to the development of diseases will advance the development and expand the utilization of NR-targeted small molecules to cure human diseases [6].

Pregnane X receptor (PXR) and constitutive androstane receptor (CAR) belong to the NR superfamily. These xenobiotic receptors are known to bind various structurally diverse chemicals [7,8]. Because they regulate an overlapping set of target genes, it is difficult to dictate their specificity in gene regulation and associated biological functions [9]. PXR is expressed in normal tissues such as liver, intestine, colon, kidney, brain, breast, prostate, peripheral mononuclear blood cells, heart, bone marrow, spinal cord, stomach, ovary, placenta and immune cells; as well as in many human cancers, including breast, prostate, colon, osteosarcoma, ovarian and endometrial cancers, with elevated expression in some cancers [10,11]. Xenobiotic receptors are not only involved in drug metabolism but they are also involved in regulating many other signal transduction pathways and related physiological processes. Nevertheless, altered expression of PXR and CAR can lead to bone disorders, hepatic steatosis, inflammatory bowel disease and cancer [12,13]. Moreover, PXR and CAR can interact with co-activators or co-repressors, depending on the ligands the receptors bind to. Binding of agonistic ligands causes receptor conformational changes that expose the hydrophobic surface within the LBD for co-activator binding. By contrast, antagonistic ligands induce co-repressor binding, resulting in receptor deactivation [8].

Because PXR and CAR bind to many drugs and have potential roles in many physiologic and pathologic processes, defining and distinguishing the following interactions will be crucial in validating PXR and CAR as potential therapeutic targets: (i) the interactions between drugs and the signaling pathways these drugs modulate; (ii) the interactions between drugs and PXR and CAR; (iii) the interactions between PXR and CAR and the signaling pathways modulated by the drugs that also modulate PXR and CAR.

PXR and CAR in drug metabolism and drug–drug interactions

Xenobiotic receptors such as PXR and CAR have important roles in drug metabolism and drug–drug interactions (DDIs) by regulating the expression of genes encoding drug-metabolizing enzymes and transporters [14,15]. The unique structure of the ligand-binding pocket of PXR enables the pocket to accommodate molecules of various shapes and sizes [14,16]. CAR is also promiscuous in ligand binding, although to a lesser extent than PXR. PXR and CAR are highly expressed in the liver, where drug metabolism and clearance occur [13]. PXR and CAR regulate the expression of genes encoding drug-metabolizing enzymes such as cytochrome P450 (CYP)3A4, CYP2B6, CYP2C9 and CYP2C19, which are responsible for metabolizing more than 80% of clinically prescribed drugs [13]. PXR and CAR also have important roles in glucose, lipid and bile acid metabolism [17]. CYP3A4 is most highly expressed in the human liver, and its induction by PXR and CAR enhances drug metabolism. This, in turn, can contribute to DDIs, which can cause an undesired decrease in the bioavailability of administered drugs, increased hepatic clearance or accelerated formation of reactive metabolites, resulting in toxicity [18]. Agonists of human PXR (hPXR) such as rifampicin increase the metabolism of the antihypertensive drug verapamil by inducing CYP3A4 [18,19]. Long-term treatment of patients with rifampicin caused a

reduction in the oral bioavailability of (*S*)-verapamil by 96% and abolished its antihypertensive effect [19]. Thus, PXR antagonists might be useful in preventing DDIs induced by PXR agonists such as rifampicin. Recent studies showed that treatment of rifampicin causes toxicity and liver injury [20]. Several PXR antagonists, such as ET-743, ketoconazole, FLB-12, sulforaphane, A-792611, polychlorinated biphenyls, coumestrol, aryl sulfonamides and allyl isothiocyanate, have been reported [21–29]. CAR antagonists or inverse agonists such as meclizine, clotrimazole and PK11195 have also been discovered [30,31]. Because PXR and CAR regulate the expression of an overlapping set of genes, antagonists that regulate PXR and CAR might be useful. PXR and CAR can also regulate distinctive sets of genes. For example, phase 1 *CYP* genes encoding CYP7A1 and CYP4F12 are regulated by PXR, whereas phase 1 *CYP* genes for CYP1A1 and CYP1A2 are regulated by CAR [13]. The overlapping ligands and transcriptional target genes of PXR and CAR suggest possible collaborative mechanisms for detoxifying toxic compounds [13,14,32–34], an interesting topic that will be discussed below.

Role of PXR in human diseases

Regulation of xenobiotic metabolism provides a major pathway for detoxification and clearance of various compounds. PXR has been strongly associated with diseases such as cancer, as well as metabolic and inflammatory diseases. PXR polymorphisms have been shown to have significant effects on disease risk in patients with ulcerative colitis and Crohn's disease [35]. Andersen *et al*. reported that the G allele of the PXR polymorphism rs6785049(A/G) was associated with a higher disease risk of ulcerative colitis in a case-control study of 495 Danish ulcerative colitis patients [35]. Interestingly, the A allele of the PXR polymorphism rs6785049(A/G) was associated with an increased risk of inflammatory bowel disease (IBD) in the 422 Irish patients, suggesting a positive correlation between PXR polymorphisms and interindividual susceptibility to IBD [35,36]. Mechanistically, Zhou *et al.* demonstrated inhibitory cross-talk between PXR and nuclear factor (NF)-κB proinflammatory pathways (Figure 1a) [37]. In human intestinal epithelial LS180 cells, the human PXR agonists clotrimazole, rifampicin and RU486 all suppressed tissue plasminogen activator (TPA)- and tumor necrosis factor (TNF)-α-induced expression of NF-κB proinflammatory target genes interleukin *(IL)-2*, cyclooxygenase *(COX)-2*, and *TNF-α* [37]. In mouse primary hepatocytes, the presence of TNF-α suppressed pregnenolone-16α-carbonitrile (PCN)-induced *Cyp3a11* expression mediated by mouse PXR (mPXR) [37]. However, there were no changes in PXR expression, suggesting that the TNF-α-mediated effects seen were not the result of modulation of PXR expression [37]. In addition, PXR knockout (KO) mice had more proinflammatory infiltration within jejunal tissues than wild-type mice, demonstrating PXR-mediated cross-talk with NF-κB proinflammatory signaling *in vitro* as well as *in vivo* [37]. Interestingly, the authors reported lower mRNA levels of TNF-α in liver samples of patients treated with phenytoin, which positively correlated with an increase in *CYP3A4* levels [37]. Shah *et al.* later found that activation of PXR has a protective role in a mouse model of IBD by repressing NF-κB target genes, suggesting the therapeutic potential of targeting PXR [38].

PXR activation has also been linked to metabolic diseases such as diabetes, owing to the effects of PXR on glucose metabolism. A randomized open, placebo-controlled, crossover

trial in healthy volunteers provided insight into postprandial glucose homeostasis and the associated effects in patients that received the human PXR agonist rifampicin versus placebo [39]. A total of 12 healthy volunteers received 600 mg of rifampicin or placebo once daily for 7 days and the oral glucose tolerance test was performed on day 8. Rifampicin treatment increased the total area under the curve for insulin by 40% and glucose by 16%, compared with placebo. The authors performed a concurrent study in rats and observed a similar increase in glucose levels during the oral glucose tolerance test in rats following administration of the rodent PXR agonist PCN compared to vehicle control [39]. Interestingly, three PXR-regulated genes involved in glucose metabolism were downregulated in the rat model that included glucose transporter 2, pyruvate dehydrogenase kinase isoenzyme 2 and glucokinase, in addition to phosphoenolpyruvate carboxykinase 1. The results corroborate previous studies reporting downregulation of phosphoenolpyruvate carboxykinase 1 with agonists of PXR and CAR [40]. Nevertheless, the authors suggested that PXR-mediated downregulation of these genes contributes to postprandial hyperglycemia and could potentially have implications in diabetes; however the clinical implications warrant further investigation.

Altered PXR expression and localization have been implicated in Barrett's esophagus and esophageal adenocarcinoma [41]. In a previous study, van de Winkel *et al.* reported significant increased risk of esophageal adenocarcinoma associated with the G allele of the PXR polymorphism rs6785049(A/G), as well as the T allele of the PXR polymorphism rs2276707(C/T). Moreover, PXR mRNA levels were significantly higher in adenocarcinoma tissues and Barrett's esophagus, suggesting a predictive correlation between PXR polymorphisms, expression and disease risk [41].

Castaño *et al.* reported that the G allele of PXR polymorphism rs2461823(A/G) was significantly associated with intrahepatic cholestasis of pregnancy, a disease characterized by pruritus and increased serum bile acid levels usually occurring during the third trimester of pregnancy in human patients [42]. By contrast, several studies have shown a hepatoprotective role of PXR in liver injury and cholestasis [43]. In a study reported by Staudinger *et al.*, mouse PXR activation by the cognate ligand PCN protected mice from lithocholic-acid-induced injury that was not seen in PXR KO mice [43]. They reported that the protective effect of PXR activation was attributed to PXR-mediated repression of *Cyp7a1* and induction of Na(+)-independent organic anion transporter *(Oatp)2* [43]. In a similar study, Teng and Piquette-Miller showed that PXR hepatoprotection was mediated by the induction of multidrug-resistance-associated protein 3 (MRP3) and *Cyp3a11* by PXR [44].

Interestingly, PXR is also known to regulate multidrug resistance protein (MDR)1, leading to chemoresistance and contributing to tumor progression (Figure 1b) [22,45]. Furthermore, Wang *et al.* found a link between PXR and colon cancer, in which PXR activation enhanced tumor cell growth through upregulation of fibroblast growth factor *(FGF)19* [46]. Upon PXR activation, transcriptional upregulation of *FGF19* can promote colon tumor progression (Figure 1b) [46]. Thus, these reports suggest the therapeutic potential of targeting PXR in various human diseases. However, the therapeutic approach of targeting PXR is disease specific. For inflammatory diseases, PXR activation has suppressive effects

on NF-κB-mediated IBD, implying the therapeutic potential of PXR agonists in treating IBD. Moreover, for other benign diseases such as cholestasis, the observed association of PXR activation and hepatoprotection suggests the therapeutic potential of PXR agonists in the treatment of cholestasis. However, for malignant diseases such as colon cancer, PXR antagonists are of potential therapeutic utility owing to the role of PXR in promoting tumor cell growth, chemoresistance and malignancy. Lastly, the identification of PXR polymorphisms in various disease types will be useful in predicting disease risk, prognosis and treatment response. Figure 1a,b summarizes the regulation of gene expression, protein function and signaling pathways by PXR that are relevant to human disease.

Role of CAR in human diseases

CAR plays a part in regulating bile acid detoxification via the induction of drug-metabolizing enzymes and transporters [47,48]. Wagner *et al.* found that activation of CAR by phenobarbital and 1,4-*bis*-[2-(3,5-dichlorpyridyloxy)]benzene reduced bilirubin and bile acid serum levels in healthy mice and increased bile acid metabolism and excretion as shown by increased serum and urine levels of polyhydroxylated bile acids in cholestatic mice [49]. In a previous study, Zhang *et al.* reported that CAR plays a part in bile acid detoxification by inducing *Cyp3a11* and *MRP3* [50]. Specifically, they utilized PXR, CAR and PXR/CAR double KO mice to investigate the roles of PXR and CAR in coordinating the function of bile acid detoxification [50]. Interestingly, CAR KO mice had more hepatic necrosis than PXR KO mice. Moreover, alanine aminotransferase (ALT) levels were approximately twofold higher in CAR KO mice than in PXR KO mice [50]. The ALT measurements corresponded to an approximate fourfold increase in total serum bilirubin levels in CAR KO over those seen in PXR KO mice [50]. Nevertheless, these findings suggest an important role of CAR in bile acid detoxification and hepatoprotection, as well as the rationale for discovery of CAR agonists that could have therapeutic potential in treating cholestasis. The potential utilization of CAR activators in treating human diseases is further demonstrated in a few recent studies. Lynch *et al.* reported inhibition of hepatic gluconeogenesis by novel activators of human CAR [51]. Wang *et al.* showed that selective activation of CAR and subsequent induction of CYP2B6 in human primary hepatocytes enhanced the bioactivation of chemotherapeutic prodrug cyclophosphamide. Therefore activator of human CAR might improve the efficacy of cyclophosphamide-based chemotherapy [52].

Growth arrest and DNA-damage-inducible beta (GADD45B) is another signaling molecule that can be upregulated in response to inflammation and has been implicated in the apoptotic suppressive functions of NF-κB [53]. Recent studies identified the role of CAR in liver tumor promotion and in promoting resistance to TNF-α-induced cell death [54,55]. Yamamoto *et al.* found that, although phenobarbital can induce CAR-mediated *Gadd45b* gene expression, CAR forms a protein–protein complex with GADD45B (Figure 1c), preventing TNF-α phosphorylation of c-Jun N-terminal kinase (JNK)1, resulting in resistance to apoptosis in primary hepatocytes from mice treated with TNF-α and actinomycin D [54].

CAR has also been shown to prevent liver injury and apoptosis through another TNF receptor superfamily member: Fas (CD95/APO-1) [56]. Baskin-Bey *et al.* found that mice treated with the CAR agonist 1,4-*bis*[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) had decreased liver injury and hepatocyte apoptosis [56]. The antiapoptotic effect was mainly induced by depleted proapoptotic Bak and Bax and increased expression of myeloid cell leukemia factor-1 mediated by direct CAR activation by TCPOBOP [56]. The antiapoptotic mechanism was not seen in CAR KO mice, suggesting the direct involvement of CAR-induced hepatoprotection [56].

Similar to PXR, CAR has also been shown to be modulated by inflammation (Figure 2a). Pascussi *et al.* demonstrated the inverse relationship between inflammatory mediators, such as IL-6, and PXR and CAR mRNA expression in primary human hepatocytes [57]. Interestingly, the presence of IL-6 significantly reduced phenobarbital-mediated induction of CAR, as well as dexamethasone-mediated PXR mRNA expression, resulting in a significant decrease in the expression of *CYP2B6* and various other *CYP* genes [57]. These findings aided in identifying a mechanism supporting the downregulation of liver drug metabolism during inflammatory diseases. Similar to PXR, the therapeutic approach to targeting CAR is disease-specific and dependent on the specific therapeutic purpose. For example, CAR agonists might have therapeutic potential in treating diseases such as cholestasis and associated liver injury, diabetes and obesity, as well as in improving the efficacy of certain prodrugs that rely on CYP2B for their bioactivation. By contrast, CAR inhibitors (antagonists or inverse agonists) might have therapeutic potential in malignant diseases such as hepatocellular carcinoma.

Crosstalk between PXR and CAR

PXR and CAR have significant roles in regulating the expression of drug-metabolizing enzymes and xenobiotic biotransformation. However, when targeting either CAR or PXR for novel drug discovery, the signaling crosstalk between PXR and CAR must be considered. It is known that CAR and PXR share ligands that act as dual activators of the expression of corresponding genes [32–34,58]. For example, phenobarbital, phenytoin and TCPOBOP are activators of PXR and CAR [15,58]. Furthermore, CAR and PXR modulate the gene expression of CYPs, MDR1, GSTs, UGTs and SULTs [13]. More specifically, PXR and CAR can bind to NR1, NR2, DR3, ER6, DR4, DR5 and gtPBREM motifs within the promoter regions of their targeted genes [59,60], resulting in the possibly daunting task of targeting PXR and CAR in human diseases. Recent studies have revealed the opposing effects of CAR and PXR activation in treating diabetes by modulation of hepatic gluconeogenesis. The CAR indirect activator phenobarbital was reported to enhance insulin sensitivity by decreasing glucose levels in diabetic patients [61]. A similar mechanism was reported utilizing the mouse PXR agonist PCN via suppression of phosphoenol-pyruvate carboxykinase and glucose-6-phosphatase expression [40]. Interestingly, PXR activation induced hepatic steatosis and increased serum corticosteroid levels, which was not reported with CAR activation [62,63]. However, these effects were reported primarily in mouse models, which raises questions about the translational aspect of PXR agonists *in vivo* and ultimately as a novel treatment modality for diabetes. Other studies have shown that the CAR indirect activator phenobarbital downregulated gluconeogenesis genes [64,65]. A

recent study further showed that activation of human CAR led to inhibition of hepatic gluconeogenesis [51]. Kodama *et al*. observed crosstalk between PXR, CAR and forkhead transcription factor O1 (FOXO1) (Figure 2b) [40]. FOXO1 transcriptionally regulates phosphoenolpyruvate carboxykinase 1, glucose 6-phosphate and insulin-like growth factor-binding protein 1 [66–69]. FOXO1 was shown to be a co-activator of CAR by directly binding to CAR and enhancing the expression of *CYP2B6*, as shown by luciferase reporter activity [40]. Inversely, activation of CAR by ligands was shown to repress FOXO1 activity, providing further insight into the role of CAR in regulating metabolic diseases, particularly hyperglycemia and gestational diabetes [40]. Masuyama and Hiramatsu reported that pregnant mice treated with the CAR ligand TCPOBOP had greater glucose tolerance and suppressed hypertension and proteinuria [70]. Taken together, PXR and CAR are major regulators of genes involved in drug metabolism and transport, as well as disease progression; therefore, identifying compounds that are effective, and potentially possess dual functionality for PXR and CAR, is crucial when targeting these xenobiotic receptors involved in metabolic, benign and malignant diseases. Figure 2a,b summarizes the regulation of signaling pathways and cellular function by PXR and CAR; and the possible link of such regulation to relevant human diseases.

Structural view of small molecule modulators binding to PXR and CAR

PXR and CAR have been shown to be modulated by a diverse range of structurally different molecules. The LBD of PXR contains three sets of α-helices, including α1/α3, α4/α5/α8/α9 and α7/α10, and a layer of five stranded antiparallel β-sheets [71]. PXR has two extra β-strands that comprise 60 additional residues that are absent in other NRs. The short AF-2 helix (αAF) allows co-activator binding [71]. Structural analysis showed that PXR has a large and flexible LBD that is highly hydrophobic, including a few polar amino acid residues, enabling it to accommodate various structurally diverse ligands that can bind in different orientations [71]. There are several X-ray crystal structures available for PXR LBD alone or PXR LBD with its agonists, such as hyperforin, colupulone (from hops), 17β-estradiol, SR12813, T1317, rifampicin and anti-HIV drug PNU-142721 [71–75]. The crystal structure of PXR LBD bound to rifampicin is shown in Figure 3a, and the residues important for binding are shown in Figure 3b. Structural analysis has shown that hydrophobic residues along with a few polar residues have important roles in ligand binding [75]. Although no crystal structure has been reported for PXR LBD with PXR antagonist, several reports have demonstrated how the putative antagonists might bind to PXR [76–79]. Recently, based on a novel yeast-based analysis and docking studies, Li *et al*. [76] proposed that a PXR antagonist such as ketoconazole might bind at the outer surface of PXR [76]. However, an antagonist specific to PXR with less cytotoxicity and with a co-crystal structure with PXR LBD is still lacking. In addition to modulating PXR, the diverse ligands (agonists and antagonists) of PXR are involved in regulating various signaling pathways. It is therefore possible that binding of these ligands to PXR could cause pleiotropic biological effects.

Contrary to PXR, which is highly inducible by agonists, CAR is constitutively active. Additionally, there are fewer agonists available for CAR. However, it has been found that some human CAR agonists are not mouse CAR agonists. Some of the CAR activators reported are phenobarbital (indirect activator), CITCO, di(2-ethylhexyl)phthalate and 6,7-

dimethylesculetin [61,80–82]. CAR antagonists or inverse agonists have also been reported, such as PK11195 and meclizine [30,31,58]. X-ray crystal structures of CAR in complex with retinoid X receptor α (RXRα) and either of the two agonists CITCO and 5β-pregnanedione have been reported; and they revealed an unusual mechanism of constitutive activation of CAR by small molecules [83]. The crystal structure of CAR bound to CITCO is shown in Figure 3c. Residues of CAR important for this binding are shown in Figure 3d. From the structural analysis, it was seen that hydrophobic residues play an important part in ligand binding. However, no specific hydrogen bonds were observed between the ligand and the receptor. The imidazothiazole heterocycle of the CITCO molecule occupies a relatively polar region of the active site formed by residues such as Asn165, Val199, Cys202, His203 and Tyr326, and makes weak electrostatic interactions with His203, Asn165 and Tyr326 [83]. The crystal structure of CAR reveals a very short helix (helix X) between α10 and the AF-2 helix. The short and rigid AF-2 helix lacks the C-terminal extension present in other NRs, thus allowing the interaction between the free carboxylate and Lys195 of human CAR, leading to further stabilization of the active AF-2 confirmation and the constitutive activity of CAR. In addition, the CAR LBD is formed by helices α2-α7, α10 and β-strands 3 and 4 [83]. Because PXR and CAR share overlapping ligands and target genes, antagonists that target PXR and CAR will be useful for mechanistic investigations and clinical applications, such as effectively preventing DDIs and improving drug efficacy. The ligand-binding cavity of PXR LBD (>1150 $Å^3$) is larger than that of CAR LBD (~675 $Å^3$) [71,83,84]. However, whether or not binders of PXR LBD can function as PXR antagonists remains to be determined.

PXR and CAR as therapeutic targets in small-molecule-based drug discovery and development

The roles of PXR and CAR in various human diseases, as discussed above, suggest their potential as promising therapeutic targets. To validate PXR and CAR as clinically relevant and druggable targets, it is crucial to develop compounds that are effective and either selective for each receptor or dual-functional for both, based on the signaling pathways and diseases they are involved in, to minimize side-effects and improve efficacy. Tables 1 and 2 summarize the targets, signaling pathways and potential clinical implications relevant to the functions of PXR and CAR. We have also summarized the challenges associated with the particular assay described.

Among the targets and pathways summarized in Tables 1 and 2 is NF-κB. It has been reported that PXR plays an important part in modulating inflammation by regulating the NF-κB pathway [37]. Activation of PXR by agonists is expected to inhibit the NF-κB pathway, resulting in decreased expression of proinflammatory cytokines, therefore suggesting PXR as a novel target for IBD therapy. Because NF-κB directly interacts with RXR, which is a heterodimeric partner of PXR, inhibition of NF-κB to control inflammation could be achieved by compounds that activate either PXR or RXR or directly inhibit NF-κB [85]. PXR or CAR can also be involved in metabolic diseases such as type 2 diabetes mellitus via regulation of FOXO1. Type 2 diabetes mellitus has been characterized by impaired β cell function and insulin resistance [86]. Interestingly, whereas FOXO1 functions as a co-

activator to modulate the transcriptional activity of PXR and CAR, PXR and CAR act as co-repressors of FOXO1-mediated transcriptional activity [40]. Therefore, PXR/CAR dual agonists might be therapeutically useful compounds in preventing FOXO1-mediated gluconeogenesis in diabetic patients. Recently, PXR was shown to interact with and be inhibited by wild-type p53, possibly leading to compromised drug efficacy in tumors with loss-of-function p53 [87]. Although the mechanism responsible for mutated p53 interacting with but failing to inhibit PXR is still unknown, compounds that can restore the inhibitory effect of mutated p53 on PXR might be useful in enhancing drug efficacy and reducing drug resistance. By contrast, activation of PXR induces the level of FGF19 to enhance colon cancer growth [46]. It is therefore useful to discover PXR antagonists that downregulate *FGF19* and reduce colon tumor growth. To develop modulators of PXR and CAR with mechanistically and physiologically relevant properties, *in silico*, such as structure-based drug design, and *in vitro*, such as small-molecule-based HTS, approaches can be utilized (Figure 4). Enabled by the available crystal structures of PXR LBD, virtual screening approaches to identify novel modulators for PXR have been reported, although the ligand promiscuity of PXR might have been one of the limitations of such efforts [78,88,89]. Unbiased HTS might therefore provide an alternative and potentially complementary approach to identifying novel modulators of PXR and CAR [90,91]. Biochemical- and cell-based HTS approaches can be utilized and have been used previously to identify PXR modulators [92,93]. Recently, CAR activators have been identified using docking studies [94]. Although the agonist-bound structural model for PXR was not particularly useful for designing novel antagonists for PXR [79,95,96], molecular docking, ligand-based pharmacophore and *in vitro* studies showed that antagonists could be interacting at the AF-2 site or surrounding region on the outer surface of PXR [77–79,97]. Recent studies found a new ketoconazole-binding site near residue Ser208 which is distant from the AF-2 site, thus describing a potential second antagonist-binding site [76], which will be useful for the structure-based approach to reveal a PXR antagonist. To discover a compound that dually modulates PXR and CAR, a two-step process might be used. Because PXR appears to be more promiscuous than CAR, it might be reasonable and effective to identify PXR modulators first, followed by testing the effect of such PXR modulators on CAR.

Concluding remarks and future directions

Targeting PXR and CAR individually or simultaneously in relevant signaling pathways and human diseases presents one of the most promising yet challenging strategies for developing novel therapeutics. PXR and CAR are involved in regulating various physiological processes in addition to drug metabolism and disposition, and can contribute to the development of various human diseases. To target PXR and CAR effectively in the context of specific and relevant pathways and diseases, it is crucial to understand their disease-specific roles and the specific therapeutic purpose, and design the appropriate assays to discover small molecules with desirable properties. Overall, the ultimate goal is to develop drugs with high specificity and potency.

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Highlights

- PXR and CAR regulate drug disposition and human diseases
- PXR and CAR are potential drug targets
- Small molecules modulating specific function of PXR or CAR are needed
- We propose approaches to target PXR and CAR
- We discuss the challenges in targeting PXR and CAR

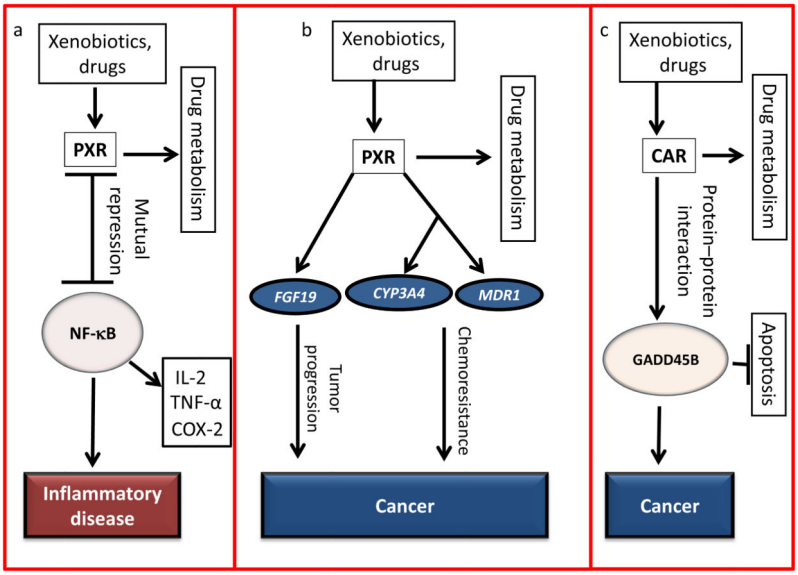

Figure 1.

Major human diseases involving pregnane X receptor (PXR) or constitutive androstane receptor (CAR). Various proteins can regulate PXR-mediated signaling pathways and thereby contribute to human diseases. PXR and CAR also regulate drug metabolism. **(a)** PXR activation can suppress the expression of nuclear factor (NF)-κB proinflammatory target genes interleukin *(IL)-2*, cyclooxygenase *(COX)-2*, and tumor necrosis factor *(TNF)-α*. Inversely, activation of NF-κB signaling and the presence of NF-κB-regulated proinflammatory mediators can suppress gene expression mediated by PXR [37]. **(b)** PXR activation leads to transcriptional upregulation of cytochrome P450 (CYP)3A4 and/or multidrug resistance protein (MDR)1, potentially contributing to chemoresistance [22,45]; however, PXR-mediated transcriptional upregulation of fibroblast growth factor (FGF19) can promote tumor progression, thus contributing to cancer development [46]. **(c)** CAR can bind to the antiapoptotic protein growth arrest and DNA-damage-inducible beta (GADD45B) to suppress apoptosis and contribute to cancer development [54]. Arrows indicate activation, stop bars indicate suppression.

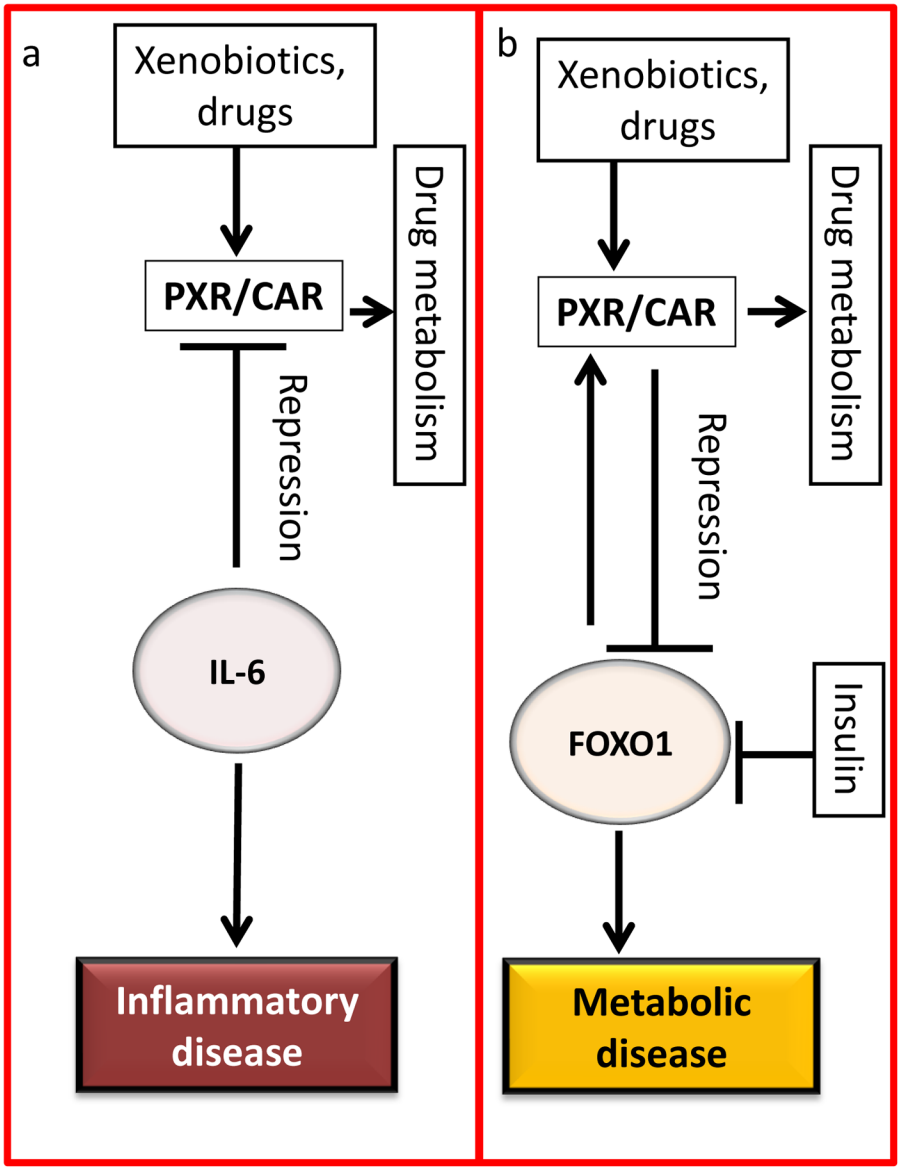

Figure 2.

Major human diseases involving constitutive androstane receptor (CAR) and pregnane X receptor (PXR). Various proteins can regulate CAR and PXR-mediated signaling pathways and thereby contribute to human diseases. PXR and CAR also regulate drug metabolism. CAR and PXR activation has been shown to be modulated by inflammation. **(a)** The presence of inflammatory mediators such as interleukin (IL)-6 significantly reduces the expression of PXR and CAR and their target genes [38,57]. IL-6 contributes to inflammatory disease. **(b)** CAR and PXR activation can downregulate gluconeogenesis-associated genes via crosstalk between CAR, PXR and the transcription factor forkhead box protein O1 (FOXO1), as proposed by Kodama *et al.* [40]. FOXO1 is suppressed in the presence of insulin. FOXO1 is a co-activator of CAR and PXR. Inversely, CAR and PXR can repress FOXO1 activity, reducing the expression of FOXO1-mediated downstream

targets providing further insight into the role of CAR in regulating metabolic diseases [40]. Arrows indicate activation, stop bars indicate suppression.

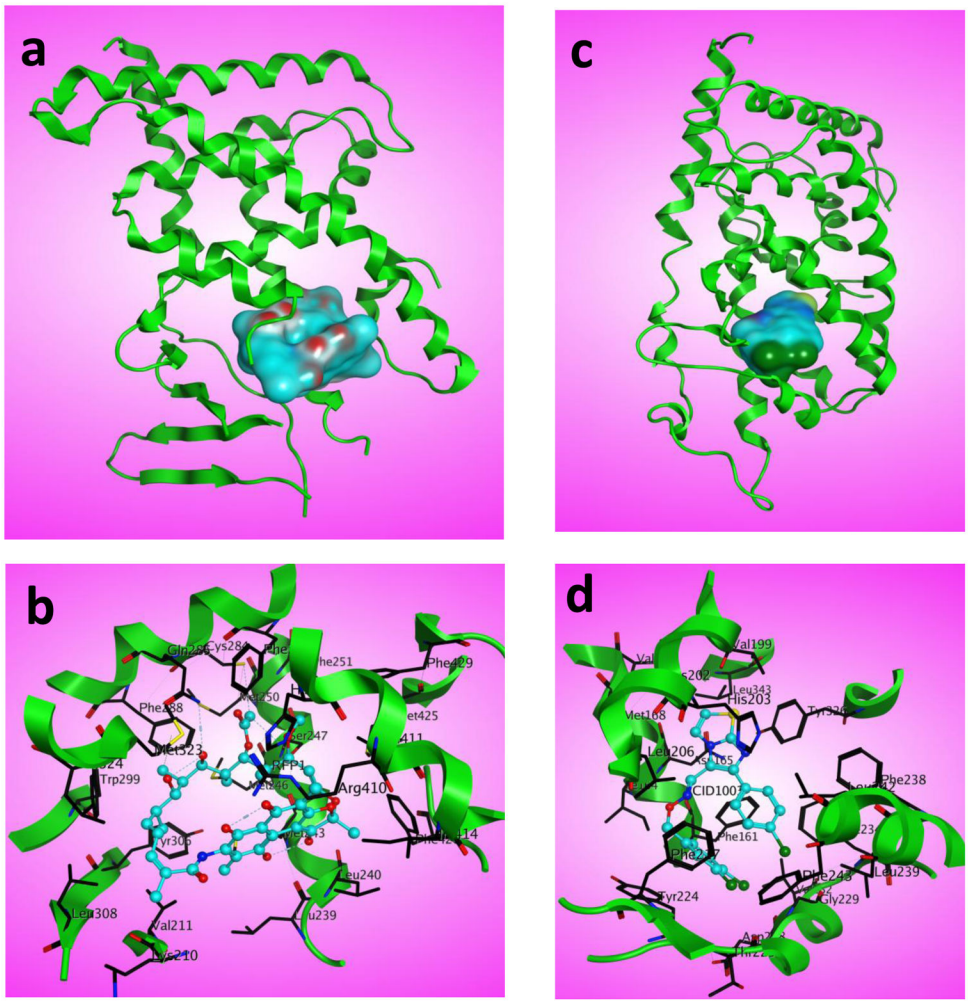

Figure 3.

Structural analysis of human pregnane X receptor (PXR) and human constitutive androstane receptor (CAR) ligand-binding domain (LBD). **(a)** X-ray crystal structure of PXR LBD with bound rifampicin (cyan surface) (PDB: 1SKX). **(b)** Residues of PXR LBD important for binding to rifampicin (cyan) are shown. **(c)** X-ray crystal structure of CAR LBD with bound CITCO (cyan surface) (PDB: 1XVP). **(d)** Residues of CAR important for binding to CITCO (cyan) are shown. Figure 3 was prepared using MOE software.

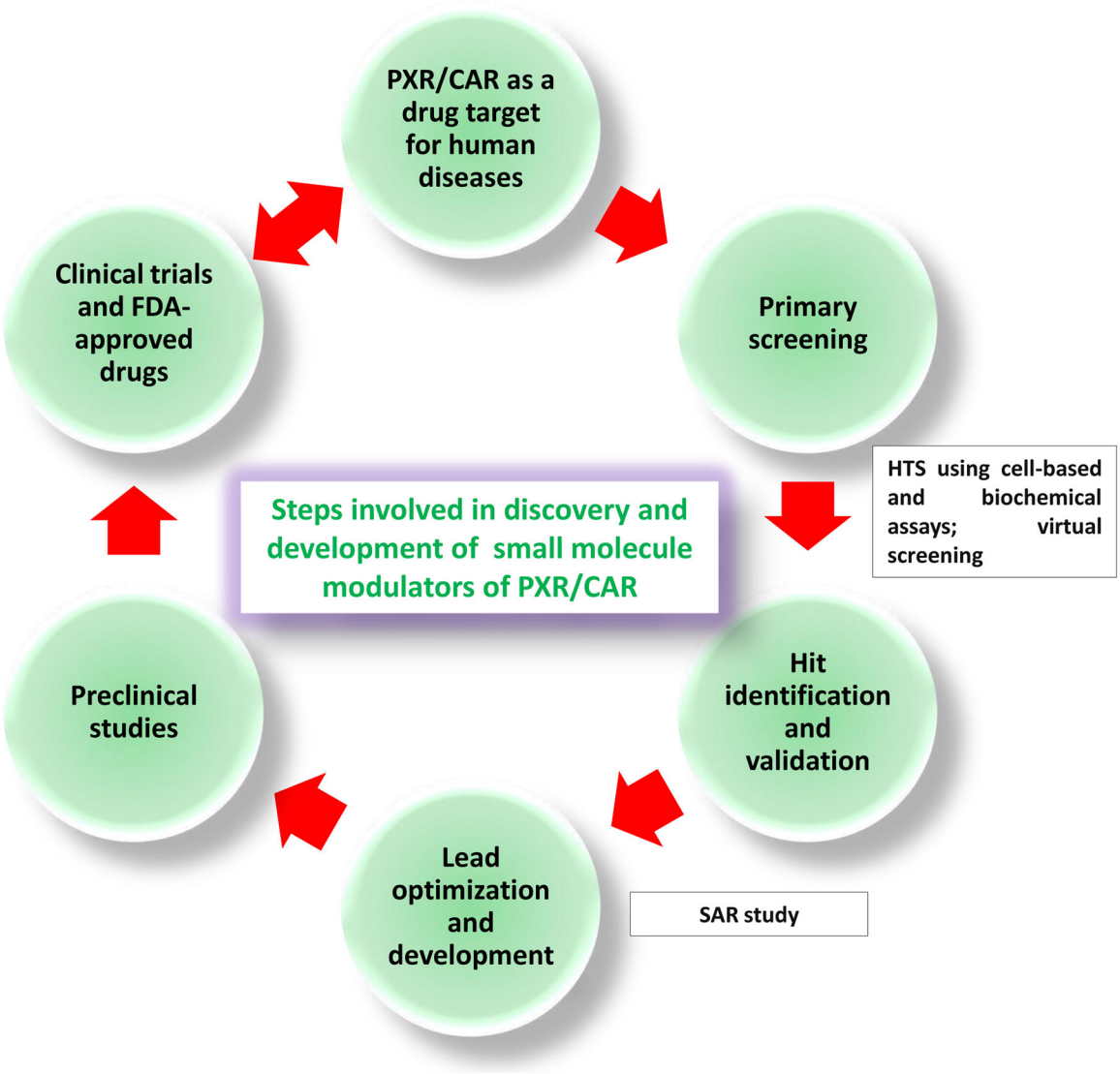

Figure 4. Pregnane X receptor

(PXR)- and constitutive androstane receptor (CAR) -targeted drug discovery process. The proposed process involves the identification and validation of PXR and CAR as drug targets, the discovery and development of modulators of PXR and CAR, preclinical studies and clinical trials.

Table 1

Summarized for pregnane X receptor (PXR) or constitutive androstane receptor (CAR): critical target genes or protein-protein interaction partners, clinical implications, small molecule targeting approach, assay design, assay development and possible challenges associated with the drug discovery process

PXR or CAR	Target gene or protein– protein interaction partner of PXR or CAR	Clinical implications	Targeting approach	Assay design and development	Significance	Challenges
PXR	**Cytorchrome P450 (CYP)3A4**	Drug metabolism Drug–drug interactions (DDIs)	PXR agonists to enhance drug metabolism PXR antagonists to decrease drug metabolism and associated DDIs	Small molecule HTS using biochemical time-resolved fluorescence resonance energy transfer (TR-FRET) assays [93] and cell-based luciferase reporter assays [71,92] Virtual screening of small molecules using PXR crystal structures [78,89]	Potent and/or selective PXR modulators to regulate CYP3A4 levels	Finding potent and selective compounds Lack of X-ray crystal structure for PXR bound to antagonist
PXR	**p53**	Compromised drug efficacy in cancer cells with mutated p53	Small molecules that restore the inhibitory effect of mutated p53 on PXR	Cell-based reporter assays in the presence of either wild-type or mutated p53 Biochemical assays to investigate the binding of compound to PXR or p53	Small molecules that restore the inhibitory effect of mutated p53 on PXR might enhance drug efficacy and reduce drug resistance	No specific site known for PXR–p53 interaction No crystal structure or mechanism available on how p53 binds to PXR
PXR	**Nuclear factor (NF)-κB**	Inflammatory diseases	PXR agonists	Cell-based luciferase reporter assays to identify compounds that inhibit NF-κB activity in a PXR- dependent manner [37]	Potent and/or selective PXR agonists to inhibit NF-κB with possible therapeutic implications in treating diseases such as inflammatory bowel disorder (IBD)	Identifying specific PXR activators that are also NF-κB inhibitors
PXR	**Fibroblast growth factor (FGF)19**	Tumor aggressiveness	PXR antagonists	Cell-based luciferase reporter assay to identify PXR antagonists that reduce the levels of FGF19 in a HTS format [46]	Potent and selective PXR antagonists to inhibit FGF19 expression with potential anticancer properties	Identifying selective antagonists to regulate FGF19 in a tumor-specific manner
CAR	**CYP2B6**	Drug metabolism DDIs	CAR agonists to enhance drug metabolism CAR antagonists or inverse agonists to decrease drug metabolism and associated DDIs	HTS using cell-based luciferase reporter assays [40] Virtual screening of small molecules using CAR crystal structures [94]	Potent selective CAR modulators to regulate CYP2B6	Finding potent and selective compounds Lack of crystal structure for CAR bound to antagonist or inverse agonist
CAR	**Growth arrest and DNA-damage- inducible beta (GADD45B)**	Resistance to apoptosis mediated by CAR-GADD45B interaction	CAR inverse agonist that disrupts GADD45B binding [54]	*Fluorescence polarization assay using GADD45B peptide.	Disruption of CAR-GADD45B interaction can increase chemsensitivity to anticancer drugs	No specific site known for CAR – GADD45B interaction

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Table 2

Pregnane X receptor (PXR) and constitutive androstane receptor (CAR): target genes or protein–protein interaction partners, clinical implications, small molecule targeting approach, assay design, assay development and possible associated challenges in the small molecule drug discovery process

Target gene or protein–protein interaction partner of PXR and CAR	Clinical implications	Targeting approach	Assay design and development	Significance	Challenges
Forkhead transcription factor O1 (FOXO1)	Metabolic disease Diabetes	PXR/CAR agonists to inhibit FOXO1-mediated gluconeogenesis for insulin-resistant diabetic patients [40]	Cell-based luciferase reporter assays [40] Mammalian two-hybrid assays [40]	PXR/CAR agonists downregulate FOXO1 potentially to benefit insulin-resistant diabetic patients [40]	Identifying selective agonists of PXR and CAR
Multidrug resistance protein (MDR)1	MDR1, a PXR/CAR transcriptional target, contributes to drug resistance	PXR/CAR inhibitors to decrease MDR1 levels	HTS using biochemical and cell-based assays	Inhibition of PXR/CAR-mediated induction of MDR1 can increase drug efficacy and decrease drug resistance	Selective PXR/CAR inhibitors that decrease MDR1 levels
Retinoid X receptor (RXR)	Drug metabolism Drug–drug interactions (DDIs)	PXR/CAR modulators that target the PXR–RXR or CAR–RXR interactions: activators to enhance drug metabolism; inhibitors to decrease drug metabolism and associated DDIs	Cell-based assays to identify modulators for PXR/CAR that target their interactions with RXR Virtual screening of small molecules that bind to PXR or CAR surface resulting in RXR disruption CAR–RXR heterodimerization surface has been reported [83] PXR–RXR heterodimerization surface has recently been published [89]	Modulators targeting the interactions of PXR/CAR with RXR can regulate PXR/CAR activity	Identifying specific modulators for PXR–RXR or CAR– RXR interactions
Cytochrome P450 (CYP)3A4 and CYP2B6	See Table 1				