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Regulation of PXR and CAR by protein-protein interaction and signaling crosstalk

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Abstract

Introduction—Protein-protein interaction and signaling crosstalk contribute to the regulation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) and broaden their cellular function.

Area covered—This review covers key historic discoveries and recent advances in our understanding of the broad function of PXR and CAR and their regulation by protein-protein interaction and signaling crosstalk.

Expert opinion—PXR and CAR were first discovered as xenobiotic receptors. However, it is clear that PXR and CAR perform a much broader range of cellular functions through protein-protein interaction and signaling crosstalk, which typically mutually affect the function of all the partners involved. Future research on PXR and CAR should, therefore, look beyond their xenobiotic function.

Keywords

pregnane X receptor; constitutive androstane receptor; protein-protein interaction; signaling crosstalk

1. Introduction

The liver plays a major role in the homeostasis of endogenous compounds (endobiotics) such as fatty acids, steroids, leukotrienes, prostaglandins, bile acids, biogenic amines, and fat-soluble vitamins, as well as in the metabolism of xenobiotics such as therapeutic drugs and an immense array of environmental contaminants. The liver has mechanisms to induce hepatic enzymes and transporters, leading to the detoxification and elimination of foreign or xenobiotic chemicals. Cytochrome P450s (CYPs) and specific transferases, i.e., xenobiotic metabolizing enzymes, as well as drug transporters, are induced by the aforementioned

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Declaration of Interest

The authors declare no conflict of interest.

compounds. Thus, CYPs, transferases, and transporters are important in maintaining steady-state levels of many compounds involved in diverse cellular processes, and they facilitate the excretion of xenobiotics. Acting as master xenobiotic-regulated transcription factors are the nuclear receptors (NRs) pregnane X receptor (PXR; NR1I2) and constitutive androstane receptor (CAR; NR1I3), which form complexes with retinoid X receptor (RXR). They regulate the induction of their target genes, such as CYP genes, by binding to the xenobiotic- and phenobarbital (PB)-responsive enhancer modules XREM and PBREM in response to compounds that are either specific to each receptor or shared by both receptors [1–8].

PXR and CAR are members of the NR superfamily, which includes the steroid, retinoid, and thyroid hormone receptors. Members of this family function as ligand-activated transcription factors and play critical roles in nearly every aspect of development and adult physiology. The family members share a common domain structure that includes a highly conserved DNA binding domain (DBD) with two zinc fingers. By means of this DBD, the receptor targets short stretches of DNA, termed response elements, in the regulatory regions of target genes. The carboxy-terminal region of the nuclear receptors includes the conserved ligand-binding domain (LBD). The LBD serves as the docking site for ligands and also contains dimerization motifs and transcriptional activation domains, such as the activation function 2 (AF-2) helix. The binding of a ligand to the LBD results in a conformational change in the AF-2 helix, and this change allows the nuclear receptor to interact with accessory proteins and regulate the expression of target genes (reviewed in references [9, 10]).

PXR was originally identified as a xenobiotic sensor that is highly expressed in the liver, intestine, and colon. This identification was an important step toward understanding the body's xenobiotic defense mechanism. PXR is involved in drug metabolism, bile acid transport, cancer, cholesterol metabolism, and inflammation [3, 11–14]. PXR is similar to other NRs in its structure; however, in structural studies, Watkins *et al.* demonstrated that PXR has a larger and more flexible ligand-binding pocket than do other NRs; this accounts for the promiscuity of PXR with respect to a wide spectrum of structurally diverse ligands [15]. It is noteworthy that PXR has species ligand specificity. For instance, rifampicin is a potent human PXR (hPXR) activator, whereas pregnenolone-16α-carbonitrile (PCN) is a rodent-specific PXR agonist. Upon ligand binding, PXR forms a heterodimer with RXR and binds to xenobiotic responsive elements in the promoters of its target genes. PXR also undergoes a conformational change that facilitates cofactor recruitment to modulate the transcription of its target genes (reviewed in references [16, 17]). In recent years, PXR has been implicated as an endobiotic sensor that regulates energy homeostasis (reviewed in reference [18]), in physiologic processes such as cell proliferation and apoptosis [19, 20], and in pathophysiologic processes such as inflammatory bowel disease, tumor development and drug resistance [21–24].

CAR, like PXR, was initially identified as a xenobiotic nuclear receptor that shows enriched expression in the liver and mediates the hepatic detoxification of foreign chemicals [4, 25]. CAR is unique in its constitutive activity; its AF-2 is constantly fixed in an active conformation because of the presence in its structure of a very stable helix H11 [26]. Interaction with agonists further enhances the activity of the receptor. CAR is mainly regulated by interaction with cofactors, post-translational modifications such as

phosphorylation and acetylation, and the mechanism that regulates its subcellular localization (reviewed in reference [27]). Microarray analysis has shown that CAR not only induces drug-metabolizing genes but also regulates the expression of genes involved in glucose and lipid metabolism, as well as those genes responsible for hepatocyte proliferation [7, 28, 29].

Considerable efforts have been made to understand the regulation of PXR and CAR mainly by their cognate ligands; however, additional regulatory mechanisms modulate these receptors. One such mechanism is the interaction with other proteins and the crosstalk with different signaling pathways. This review highlights the recent progress in our understanding of the molecular mechanisms regulating PXR and CAR activity. We focus on signaling crosstalk and protein-protein interactions that affect PXR- and CAR-mediated signaling.

2. Regulation of PXR through protein-protein interaction and signaling crosstalk

2.1 SMRT

SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) is a nuclear protein that interacts with transcriptional regulators, including nuclear receptors, by means of two receptor-interacting domains, ID-1 and ID-2, which contain extended L/I-X-X-I/V-I corepressor motifs [30, 31]. Johnson *et al.* demonstrated that PXR interacts with SMRT and, more importantly, that the C-terminal domain of PXR is essential for interaction between PXR and the ID-2 domain of SMRT *in vitro* [32]. This finding was corroborated by structural studies showing that the PXR LBD specifically binds the SMRT ID-2 [33]. Rifampicin (RIF), a potent PXR agonist, disrupted PXR-SMRT interaction in the micromolar to millimolar range *in vitro* and in a yeast two-hybrid system, suggesting that SMRT preferentially interacts with unliganded PXR. It is noteworthy that disruption by RIF is specific for PXR-SMRT interaction. Furthermore, PXR-SMRT interaction was confirmed in mammalian cells, because PXR colocalized with SMRT in a distinct punctate distribution in nuclei [32]. The same study established the significance of PXR-SMRT interaction as a mechanism for repressing PXR transcriptional activity [32] Interestingly, PXR-SMRT interaction might also be responsible for PXR repressing the activation of the *CYP24A1* promoter by vitamin D3 [34].

2.2 Protein kinase A

Forskolin, a purified extract from the herb *Coleus forskohlii*, is able to increase cAMP levels, thereby activating protein kinase A (PKA); however, forskolin induces CYP3A expression in rat hepatocytes independent of cAMP [35]. Ding and Staudinger showed that forskolin can function as an agonist for PXR and facilitate PXR-PKA interaction [36]. In *in vitro* kinase assays performed using a catalytically active purified PKA and purified human GST-PXR fusion proteins, both the DBD and LBD of PXR were phosphorylated by PKA [36]. Mouse PXR (mPXR) was similarly phosphorylated by PKA *in vitro*. In addition, the activation of PKA signaling with 8-Br-cAMP increases the strength of the mPXR-coactivator protein-protein interaction in cell-based assays. Activation of the PKA signaling pathway also potentiates the induction of CYP3A by PXR agonists in cultured mouse

hepatocytes. These data suggest that phosphorylation of PXR by PKA plays a key role in regulating the induction of *CYP3A* gene expression in mouse hepatocytes, in part through modulating PXR–protein cofactor interaction [36]. It is important to note that the effects of PXR-PKA interaction and the crosstalk of the PXR and PKA pathways are species-specific: PXR and PKA were synergistic in mouse hepatocytes, whereas their interaction repressed PXR transcriptional activity in rat and human hepatocytes [37]. These results indicate that PKA signaling has an important effect on the induction of PXR target-gene expression through the phosphorylation of PXR.

2.3 Protein kinase C

Protein kinase C (PKC) is a key component of the cytokine signaling pathway in the liver. After cytokines are released, PKC represses *CYP* gene expression in the liver [38–40]. PXR was shown to be transcriptionally repressed after PKC signaling was activated by phorbol 12-myristate 13-acetate (PMA) in cell-based reporter gene assays and in cultured mouse hepatocytes, whereas the PKC-inactive phorbol ester 4a-phorbol 12-myristate 13-acetate (4a-PMA) had no effect on PXR transcriptional activity [41]. Interestingly, in primary hepatocytes treated with okadaic acid (a PP1/PP2A inhibitor), ligand-dependent PXR activity was completely abolished. Mammalian two-hybrid analysis revealed that treatment with PMA increased the strength of the interaction between PXR and the nuclear receptor corepressor (NCoR) and also inhibited ligand-dependent interaction between PXR and steroid receptor coactivator 1(SRC-1), whereas treatment with the PKC-inactive phorbol ester 4α-PMA had no similar effects [41]. These findings suggest that, through activating the PKC signaling pathway, the phosphorylation status of PXR or PXR interaction with cofactors is altered and that this modulation of PXR-cofactor interaction ultimately regulates PXR transcriptional activity. Sugatani and colleagues performed mutagenesis studies of Thr57, Ser180, Ser192, Ser208, Ser230, Ser274, Thr290, Ser305, Ser350, and Thr408 of PXR on the basis of a report by Lichti-Kaiser *et al.*[42] and computer predictions of consensus kinase sites. Sugatani *et al.* showed that phosphomimetic mutations at Thr57, Thr290, Ser350, and Thr408 of PXR strongly attenuated RIF-induced UGT1A1 expression, and they speculated that Thr290 might be the site for PKA and PKC phosphorylation of PXR [43].

2.4 S6K

The Group-Based Phosphorylation Scoring Method (GPS) predicted Thr57 on PXR to be the target for ribosomal protein S6 kinase (S6K). In an *in vitro* kinase assay, the reconstituted complexes of the purified 70-kDa form of S6K (p70 S6K) directly phosphorylated purified hPXR and conferred negative regulation of both basal and RIF-induced PXR transcriptional activity [44]. To further implicate Thr57 in PXR function, the same group performed mutation studies in which Thr57 was mutated to Asp (PXR T57D) in order to mimic phosphorylation. They observed that PXR T57D colocalized with the corepressor SMRT, but interestingly, knockdown of SMRT did not rescue the impaired function of PXR T57D, suggesting that SMRT is not involved in the PXR loss of function caused by the phosphomimetic mutation [44]. Furthermore, Thr57 mutation of PXR did not affect the interaction of PXR with SRC-1 [44]. These results suggest that Thr57 of PXR may not be involved in cofactor recruitment. Upon further investigation, the same group

discovered that Thr57 of PXR is important for the binding of PXR to the promoters of target genes, highlighting another level of PXR regulation by phosphorylation [44].

2.5 PRMT1

The protein arginine methyltransferases PRMT1, PRMT2, and PRMT4 (CARM1) have been described as nuclear receptor coactivators [45–48]. These enzymes can methylate histones [45, 47], suggesting nucleosome remodeling as a possible mechanism for their action. Histone methylation could cooperate with other types of histone modification by coactivators, including acetylation and phosphorylation, suggesting that large multi-subunit enzyme complexes containing multiple histone- and non–histone-modifying activities work concomitantly with other chromatin-remodeling machinery to regulate gene transcription (reviewed in reference [49]). In a coimmunoprecipitation assay in a HepG2 cell line and transgenic mouse liver tissues, PRMT1 was identified as a major histone methyltransferase that specifically associated with PXR, and PXR activation was found to be important for the association. The interaction of PRMT1 with PXR was further supported by a mammalian two-hybrid assay [50], suggesting that the association may be direct. The LBD of PXR was mapped as the interactive domain of PXR that is responsible for its association with PRMT1 [50]. Interestingly, by using mouse embryonic stem cells and a HepG2 cell line, the authors showed that PXR requires PRMT1 for full transcriptional activity [50]. Furthermore, the PXR-PRMT1 interaction was also shown to be important for PRMT1 subcellular localization, which may affect the activity of PRMT1 [50]. Recently, Li *et al.* demonstrated that the PXR-mediated overexpression of multidrug resistant gene 1 (MDR1) regulated PRMT1 in breast cancer cells [51]. Altogether, these data indicate that the direct interaction of PXR and PRMT1 plays a role in recruiting PRMT1 to the promoters of PXR target genes, where it regulates transcription by methylating chromatin. PRMT1 may directly modify PXR through methylation, hence altering its transcriptional activity. These are, however, conjectures that require further investigation.

2.6 SRC-1, RIP140, SUG1, GRIP1, and PBP

SRC-1 was originally discovered as an agonist-specific protein that stimulated steroid receptor transcriptional activity [52]. In an *in vitro* binding assay, SRC-1 directly bound progesterone receptor (PR) and augmented the transcriptional activity of PR, estrogen receptor (ER), glucocorticoid receptor (GR), thyroid hormone receptor (TR), and RXR in response to their cognate ligands [52], hence the namesteroid receptor coactivator 1. Multiple coactivators interact with steroid receptors in a ligand-dependent manner, including RIP140 [53] and the human homolog of yeast SUG1 called TRIP1 [54]. Masuyama *et al.* [55] showed that PXR interacted with SRC-1 and RIP140 in a ligand-dependent manner, an observation similar to that reported for steroid receptors. In addition, they demonstrated that endocrine-disrupting compounds—phthalic acid and nonylphenol—enhanced the interaction between PXR and SRC-1 or RIP140 and PXR transcriptional activity, suggesting that phthalic acid and nonylphenol enhanced PXR-mediated transcription through the interaction of PXR with the coactivators. In other studies, the mycoestrogen zearalenone, forskolin, and 1,9-dideoxyforskolin decreased the interaction between PXR and the corepressor protein NCoR while enhancing the interaction between PXR and SRC-1 in cells [36, 56]. Surprisingly, however, phthalic acid and nonylphenol did not affect the interaction between

PXR and SUG1, an interaction that was enhanced by progesterone [55]. An alternate concept of PXR interaction with cofactors was suggested by Navaratnarajah and colleagues showing that in *in vitro* assays, RIF did not alter the thermodynamic and kinetics of PXR-LBD interaction with peptide fragments of SRC-1 or SMRT [57]. This concept shows uniqueness in PXR interactions with coregulators; however, the results of a similar study differs in a cellular context in which many more factors are involved [32]. Together these findings suggest that different ligands may confer different conformational changes on PXR, thereby selecting for distinct cofactors or combinations thereof, depending on the physiologic conditions.

Zearalenone, a known ER agonist, was shown to activate human PXR selectively, as compared to mouse PXR. To determine the molecular basis of PXR activation by zearalenone, Ding *et al.*, used a mammalian two-hybrid system to demonstrate that, in response to zearalenone, PXR dissociated from the corepressor NCoR and recruited the coactivators SRC-1, PPAR- binding protein (PBP), and glucocorticoid receptor–interacting protein 1 (GRIP1, also known as SRC-2) [56]. Another study showed that forskolin and 1,9-dideoxyforskolin also enhanced the interaction between PXR and PBP [36].

2.7 SREBP1

Sterol regulatory element–binding proteins (SREBPs) are transcription factors of the basic helix-loop-helix-leucine zipper (bHLH-zip) family that play major roles in the synthesis of cholesterol and triglyceride. The bHLH-zip of SREBPs is located in the N-terminal region, with which it binds DNA, and the C-terminal region performs the important regulatory functions. The SREBP family has three members, namely SREBP1a, SREBP1c, and SREBP2. SREBP1a and SREBP1c, which are transcriptional variants, mainly regulate triglyceride and fatty acid synthesis, whereas SREBP2 regulates cholesterol biosynthesis (reviewed in references [58, 59]). The activation or overexpression of SREBP1 in primary human hepatocytes greatly repressed drug-mediated induction of *CYP* genes by PXR and CAR. SREBP1 was recruited to the promoter of PXR or CAR target genes, but did not bind the promoter elements. The molecular mechanism of this inhibitory crosstalk was delineated by GST pull-down assays that showed a direct protein-protein interaction between purified GST-tagged SREBP1 and *in vitro*–translated PXR or CAR [60]. The interaction of PXR or CAR with SREBP1a was significantly stronger than the interaction with SREBP1c [60]. Furthermore, a cofactor recruitment assay showed that PXR-SREBP1 interaction interferes with SRC-1 recruitment to PXR or CAR, thereby decreasing transcriptional activity.

2.8 PGC-1α

The peroxisome proliferator–activated receptor 1 (PGC-1) family of coactivators has three members, namely PGC-1α, PGC-1β, and PRC (PGC-1–related coactivator). Even though PGC-1s have no intrinsic histone acetyltransferase (HAT) activity, they recruit coactivators that possess HAT activity, such as SRC-1, p300, and TRAP/DRIP (a complex of the thyroid hormone receptor [TRAP] and the vitamin D_3 receptor [DRIP]). PGC-1s are ubiquitously expressed in various tissues, including heart, muscle, liver, brain, and kidney, where they play important roles in glucose, lipid, and energy metabolism. PGC-1 coactivators are highly versatile and are able to interact with many different transcription factors and, notably, with

PXR and CAR, thereby activating distinct biological activities in a variety of tissues (reviewed in references [61–63]). Among the coactivators that affect xenobiotic signaling pathways, PGC-1α plays a critical role. In a coimmunoprecipitation assay, PXR was associated with PGC-1 in mouse liver nuclear extract, and an *in vitro* binding assay showed the interaction to be direct [64]. The PXR–PGC-1 interaction was modulated by ligand, because RIF enhanced the association of the two proteins in HepG2 and COS-1 cells, as shown by coimmunoprecipitation and subcellular colocalization, respectively. Further analysis using the truncated form of PXR demonstrated that the LBD of PXR interacted with PGC-1 [64]. The interaction of PXR with PGC-1 enhanced PXR transcriptional activity, which was attenuated by HNF-4. This suggests that PGC-1 is a common cofactor for PXR and HNF-4 and that competitive recruitment of the coactivator results in the mutual antagonism of PXR and HNF-4 [64].

2.9 TLR

Maintaining the integrity of the intestinal mucosal lining is important to prevent intestinal injury and maintain the gut barrier function. It is now clear that, despite the usual insults to the intestinal lumen, microbes, food, and metabolites regulate the gut barrier function through immune recognition (reviewed in reference [65]). It has become apparent that intestinal epithelial barrier dysfunction is associated with a number of diseases, including inflammatory bowel disease, intestinal ischemia, graft-versus-host disease, Crohn disease, ulcerative colitis, and celiac disease [66–73]. Recently, PXR was shown to be a mediator in maintaining gut barrier integrity. Indole 3-propionic acid (IPA), an indole metabolite that is exclusively produced by gut microflora [74], was shown to be a possible physiologic ligand for PXR that downregulated the inflammatory response cytokine TNFα while upregulating cell-cell junctional complex markers such as occludin, ZO-1, E-cadherin, and claudin-7 in the mouse small intestine. Claudin-2, a known inducer of barrier defects, remained unchanged in this study. This observation was supported by transmission electron microscopy of NR1I2−/− mouse intestinal epithelial cells, which showed the microvilli to be shorter and more loosely packed than those of the corresponding cells of NR1I2+/+ mice [75]. Interestingly, kinases downstream of the Toll-like receptor (TLR) pathways were activated in NR1I2−/− mice, suggesting that crosstalk in the TLR and PXR pathways regulates gut barrier function. Through inhibition studies in mouse enterocytes and mouse knockout studies, TLR2 and TLR4 were identified as being important in maintaining the intestinal barrier; however, TLR4 was demonstrated to be essential for maintaining barrier integrity [75]. These studies implicate PXR as a physiologic regulator of TLR-mediated control of intestinal barrier function. Interestingly, Shah and colleagues implicated CAR in the TLR2 pathway in the lipoteichoic acid–dependent downregulation of drug-metabolizing enzymes and drug transporters [76]. In another recent study, Ghose *et al.* showed that the activation of human *CYP3A4* promoter by RIF and the activation of the mouse *Cyp3a11* gene in response to paclitaxel were significantly attenuated by TNFα and LPS, respectively [77]. Thus, these findings indicate that there is important crosstalk between PXR and CAR-mediated signaling in inflammatory responses, and TLRs in xenobiotic responses.

2.10 p53

When cells are exposed to genotoxic stress, their DNA can be damaged. The damaged DNA is then repaired or the cells undergo apoptosis. The well-known tumor suppressor p53 has critical functions in carcinogenesis, particularly in regulating apoptosis. Interestingly, p53 interacts with PXR, and this interaction exerts an inhibitory effect on PXR transcriptional activity. The DBD and AF-2 domains of PXR are critical for PXR interaction with p53 [78]. To further test the effect of p53 on PXR activity, a p53 mutant carrying a point mutation at amino acid 175 (R175H) in its DBD was used to show that p53 R175H and PXR interacted; however, there was no inhibitory effect on PXR-regulated *CYP3A4* promoter activity [78]. In the colon cancer cell lines LS180 and HCT116 and in normal mouse colon epithelium, PXR inhibited deoxycholic acid (DCA)-induced apoptosis while downregulating p53, together with another proapoptotic gene, Bcl2-antagonist/killer 1 (BAK1) [79]. Robbins and colleagues recently showed that the expression of PXR reduced p53-mediated transactivation by decreasing the recruitment of p53 to the promoters of its target genes in colon cancer cell lines expressing wild-type PXR [80]. Interestingly, in the HT29 colon cancer cell line, which carries a p53 mutation, PXR overexpression did not alter the p53 protein level but led to increased expression of *CDKN1A* (encoding p21), a downstream target of p53, which contributed to G_0/G_1 cell cycle arrest and the suppression of cancer cell proliferation [81]. In another study, Verma and colleagues showed that the activation of PXR in the breast cancer cell lines MCF-7 and ZR-75-1 induced cell cycle arrest and apoptosis by inducing the expression of p53 and the p53 target genes *CDKN1A, BAX*, and *BBC3* (encoding PUMA) [82]. In breast cancer models, one proposed mechanism of PXR-mediated apoptosis is p53 stabilization in response to cellular stress, because PXR agonists increased iNOS mRNA levels in MCF-7 and ZR-75-1 cells [82]. This was consistent with earlier reports that p53 could be activated or stabilized in response to DNA damage or cellular stress, such as that caused by an accumulation of reactive oxygen species or reactive nitrogen species (RNS) in cells. Additionally, RNS-stabilized p53 upregulates both p21 and BAX expression [83, 84]. Therefore, the cellular effect resulting from the interaction of PXR with p53 may be dictated by the status of the p53 (i.e., wild-type vs. mutated) and the cellular and tissue context (i.e., colon vs. breast cancer). As pointed out by Robbins and colleagues in a recent editorial [85], the mutual inhibitory effect of PXR-p53 interaction suggests a tumor-suppressive function of p53 and an oncogenic function of PXR. Whereas p53 increases cancer cell death in response to chemotherapy by inducing apoptosis and inhibiting PXR to decrease drug metabolism and enhance drug efficacy, PXR contributes to drug resistance by enhancing drug metabolism to decrease drug efficacy and inhibiting p53 to decrease apoptosis. Because both PXR and p53 can be regulated by many xenobiotics, the complex regulation of PXR-p53 interaction warrants further investigation.

2.11 Interleukin-6 and NF-κB

The interest in the role of PXR in the immune response emanated from the immunosuppressive role of RIF, which has been observed in many clinical treatments since the 1970s [86]. The pro-inflammatory cytokine interleukin 6 (IL-6) was shown to specifically inhibit RIF- and PB-mediated induction of the *CYP2B6, CYP2C8/9*, and *CYP3A4* genes. This was due to the IL-6–dependent repression of PXR and CAR mRNA levels. Surprisingly, the promoter activities of the receptors were not affected by IL-6, as

demonstrated in reporter assays [87]. This finding was later corroborated by another study showing that PXR was required for IL-6–dependent repression of *CYP3A4* in human hepatocytes [88]. The regulation of PXR in the inflammatory response was further addressed when mutual inhibitory crosstalk between the PXR and NF-κB signaling pathways was reported [14, 89–91]. Treatment with RIF was shown to repress NF-κB–targeted pro-inflammatory genes, such as *IκBα, cox-2, TNFα, ICAM-1*, and those encoding several interleukins. RIF also inhibited p65 and NF-κB activities in a dose-dependent manner. Furthermore, in the same study, increased inflammation was observed in the small bowel of PXR-null mice [91]. Conversely, NF-κB activation by LPS and TNFα plays an important role in *CYP3A4* downregulation [89, 91] which is dependent on NF-κB interaction with the heterodimer of PXR and RXR [89]. p65 was observed to interact directly with the RXR DBD and interfered with PXR-RXR dimerization on PXR response-element (ER6) consensus sequences in the *CYP3A4* promoter. The p65-RXR interaction thereby inhibited PXR-regulated gene expression [89]. These findings explain the clinically observed mutual suppression of xenobiotic metabolism and the immune response. The crosstalk between NF-κB, IL-6, and PXR represents an important nexus between the inflammatory response and drug metabolism.

2.12 FoxO1

Forkhead box protein O1 (FoxO1) is a forkhead transcription factor that plays important roles in regulating gluconeogenesis by insulin signaling. It directly binds to the insulin response sequence (IRS) to regulate some gluconeogenic genes, such as *PEPCK1*and glucose-6-phosphate (*G6P*), in the absence of insulin. In response to insulin, FoxO1 is phosphorylated and inactivated through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway [92]. It was found that FoxO1 crosstalks with CAR- and PXR-related drug metabolism. FoxO1 was shown to directly bind to CAR and PXR in a ligand-dependent manner to enhance CAR- and PXR-mediated expression of *CYP3A* [93]. Interestingly, this interaction was also repressively regulated by the insulin-PI3K-Akt pathway. In contrast, CAR and PXR can repress FoxO1-IRS activity, thus disrupting gluconeogenesis [93]. Glucose-6-phosphatase (G6Pase) is a critical enzyme in glucose metabolism. It was demonstrated that not only FoxO1 [93] but also hepatocyte nuclear factor 4 (HNF4) and CRE (cAMP response element)-binding protein (CREB) have binding motifs in the promoter region of the *G6Pase* gene. PXR strongly repressed HNF4-activated *G6Pase* promoter activity in a RIF-dependent manner [94]. GST pull-down revealed that PXR directly binds to the CREB DBD, consequently decreasing CREB binding to the *G6Pase* promoter region and repressing the transcriptional activity of CREB, and thus downregulates gluconeogenesis [94].

The PXR-CREB interaction–dependent repression of gluconeogenesis may not be dominant in human cells. Other studies showed that, in a human liver cancer cell line, RIF-activated PXR increased the expression of G6pase, concomitant with an increase in the level of serum- and glucocorticoid-regulated kinase 2 (SGK2) mRNA [95, 96]. These studies demonstrated that PXR binds to two PXR response elements in the SGK2 promoter region when treated with RIF. Knockdown experiments revealed that SGK2 is required for the PXR-mediated expression of *G6Pase* and *PEPCK1* and that PXR is also required for SGK2-

dependent G6Pase expression in response to RIF [96]. Further studies revealed that the dephosphorylation of SGK2 at Thr193 by PP2Cα in response to statin increased the recruitment of PXR and RXRα to the *G6pase* promoter, thereby increasing the level of G6Pase mRNA [95]. Although SGK2 is important in gluconeogenesis, PXR is required for transcriptional regulation of *G6Pase*.

The crosstalk between PXR and another member of the FoxO family, FoxO3, was reported to stimulate hepatocyte proliferation. PXR activation by PCNinhibits FoxO3-mediated transcriptional regulation of cell-cycle suppressor genes such as *Rbl2*, thus promoting hepatocyte proliferation [97]. On the other hand, overexpressed FoxO3 inhibits the PXR-mediated enhancement of hepatocyte proliferation [97]. The regulation of hepatocyte proliferation by PXR has previously been reviewed by Pondugula *et al.* [98].

2.13 HNF4α and SHP

Several cofactors are involved in PXR-mediated transcriptional regulation. Hepatocyte nuclear factor 4-alpha (HNF4α) plays a vital role in liver development and in the regulation of bile acid synthesis, lipid homeostasis, and xenobiotic responses. Although HNF4α harbors a motif for binding to the *CYP3A4* promoter, it can activate the *CYP3A4* promoter without binding to it. HNF4α induces PXR expression in fetal liver and synergistically enhances PXR-induced CYP3A4 [99]. A GST pull-down assay showed that RIF treatment enhanced the binding between PXR and HNF4α; however, this interaction was disrupted by the small heterodimer partner (SHP, NR1I0) [100]. SHP is the functional partner of farnesoid X receptor (FXR) that regulates bile acid and lipid homeostasis. SHP functions as a corepressor binding partner of FXR or PXR, resulting in the downregulation of *CYP3A4* [100]. The interaction between PXR and SHP is ligand dependent, as it was enhanced by RIF treatment. Interestingly, a GST pull-down assay showed that HNF4α and SHP competed for interaction with PXR. A ChIP assay demonstrated that SHP did not block the binding of PXR to HNF4α but affected PXR recruitment of HNF4α to the *CYP3A4* promoter [14].

The transcriptional regulation of *CYP3A5*, a member of the CYP3A subfamily, is also controlled by PXR and CAR [101]. Recently, *CYP3A5* was shown to mediate resistance to tyrosine kinase inhibitors (erlotinib and dasatinib) and paclitaxel in all subtypes of pancreatic ductal adenocarcinoma (PDAC) [102]. By using an siRNA knockdown assay, Noll *et al.* showed that the basal expression of CYP3A5 was controlled by HNF4α; however, the drug-induced upregulation was regulated by PXR. Therefore, knockdown of both HNF4α and PXR maximally sensitized exocrine-like pancreatic adenocarcinoma to drug treatment [102]. CYP3A5 was shown to have a minor role in normal physiology [103], and together with the recent finding, this makes CYP3A5 an attractive target for overcoming basal and acquired drug resistance in PDAC. Because both HNF4α and PXR regulate CYP3A5 expression, interfering with these regulatory mechanisms may provide a plausible approach to suppressing the CYP3A5 pathway.

2.14 UBR5, DYRK2, and Hsp90β

By using siRNA-based screening and mass spectrometry analysis, Ong and colleagues found that UBR5 and dual-specificity tyrosine-phosphorylation–regulated kinase 2 (DYRK2) interact with and negatively regulate hPXR stability and, subsequently, *CYP3A4* promoter activity [104]. DYRK2 phosphorylates hPXR and facilitates hPXR ubiquitination by UBR5 [104]. In addition to ubiquitination, PXR stabilization was also regulated by the chaperone protein heat-shock protein 90β (Hsp90β) [105]. RIF-activated casein kinase 2 (CK2) was shown to phosphorylate Hsp90β at serine 225 and serine 254. The phosphorylation of Hsp90β increased its interaction with PXR and promoted PXR stabilization, consequently increasing the expression of MDR1—an essential mediator of multidrug resistance [105].

2.15 CDK2

The phosphorylation status of PXR fine-tunes its activities in response to various stimuli [37, 106]. By screening a library of known bioactive compounds for small-molecule hPXR activators, Lin and colleagues identified two CDK inhibitors, kenpaullone and roscovitine, that strongly activated the hPXR signaling pathway but only weakly bound to hPXR. Consistent with this observation, this group showed that the activation of CDK2 led to the abrogation of hPXR transcriptional activity. Furthermore, CDK2 was shown to directly phosphorylate hPXR in an *in vitro* kinase assay. By using phosphomimetic hPXR constructs, Ser350 was demonstrated to be a putative CDK phosphorylation site [107]. Interestingly, however, the repressive effect of CDK on PXR was counteracted by the protein phosphatase type 2C isoform beta long (PP2Cβl) [108]. These findings suggest a link between cell cycle regulation and PXR signaling and highlight the importance of considering the cell cycle status when analyzing PXR activity and CYP expression.

2.16 p300 and SIRT1

Recently and for the first time, acetylation of PXR was shown to regulate PXR transcriptional activity. PXR was show to be acetylated in its unstimulated state, and deacetylated in response to RIF [109]. In a genome-wide profiling of PXR regulated genes in response to RIF, Smith *et al.* discovered that p300 was recruited with PXR to putative regulatory elements upon RIF stimulation [110]; furthermore, Pasquel and colleagues uncovered the relevance of PXR-p300 association and showed that PXR is directly acetylated by p300 on lysine 109, by using LC-MS/MS analysis *in vitro* and performing confirmatory studies in cells [109]. The authors went on to demonstrate that PXR deacetylation was mediated partly by SIRT1 resulting in activation of PXR's lipogenic functions in a ligand-independent manner [109]. A SUMO-acetyl "switch" model was recently suggested by Cui *et al.* for PXR transcriptional regulation wherein acetylation was a prerequisite for SUMOylation of PXR. PXR was shown to differentially associate with HDAC-SMRT corepressor complex, and was transcriptionally repressed following acetylation and SUMOylation. The repressive effect was independent of interaction with HDAC-SMRT complex, but depended directly on the SUMO-modification. Furthermore, acetylation was shown to regulate the subcellular localization of PXR [111]. These recent findings underscore the interconnectedness between SUMOylation and acetylation in post-translationally regulating PXR activity.

3. Regulation of CAR through protein-protein interaction and signaling crosstalk

3.1 Hsp90, PP2A, ERK1/2, and RACK1

As a well-characterized indirect activator of CAR, PB is able to induce nuclear accumulation and transcriptional activation of CAR in *CYP* genes by binding to the PBREM in their promoters [2, 112]. In normal mouse liver cells, CAR binds to Hsp90 and resides in the cytoplasm, and PB treatment results in the recruitment of protein phosphatase 2A (PP2A) to CAR-Hsp90 complex, leading to the dephosphorylation and nuclear translocation of CAR [113].

Coimmunoprecipitation using an antibody that specifically recognizes phospho-ERK confirmed the interaction between active extracellular signal–regulated kinase (ERK) 1/2 and T38D CAR phosphomimetic mutant. Kabayashi *et al.* described that CAR complexes with CCRP and Hsp90 to accumulate in the cytoplasm [114]. T38D CAR was shown to retain primarily in the cytoplasm; however, T38A CAR mimicking the dephosphorylation of Thr38 on CAR redistributes into the nucleus and has greater basal and induced transcriptional activity [115, 116]. Presumably, the phosphorylation of thr38 on CAR is essential for its interaction with CCRP and Hsp90, thus regulates the cytoplasmic localization of CAR. On the other hand, the C-terminal xenobiotic response signal (XRS) peptide of CAR is responsible for its direct interaction with ERK1/2 and essential for its nuclear localization [115, 117]. As shown by Mutoh *et al.*, PB competitively binds to EGFR and compromises the downstream activation of Src kinase, thereby enabling the interaction between the dephosphorylated receptor for activated C kinase 1 (RACK1) and Thr38-phosphorylated CAR and the dephosphorylation of phosphorylated Thr-38 by PP2A [118]. Collectively, these observations suggested that the binding partners of CAR could modulate CAR activity by regulating the subcellular localization of CAR through modifying its phosphorylation status.

3.2 FoxO1

As discussed in section *2.12*, FoxO1, a member of the family of Forkhead box transcription factors [119], broadly participates in cellular processes modulated by various growth factors [120–122]. FoxO1 could be phosphorylated by Akt through insulin stimulation, resulting in its transcriptional repression and nuclear exportation to downregulate the targeted glucogenetic genes [123, 124]. By means of yeast two-hybrid screening and a GST pull-down assay, FoxO1 was identified as a direct binding partner of mouse CAR, which enhanced *CYP2B6* transcriptionin the presence of 1,4-bis[2-(3,5-dichloropyridoxy)]benzene (TCPOBOP) [125, 126]. Conversely, mCAR functions as a suppressor of FoxO1 transactivity on gluconeogenic genes such as *G6Pase* and *PEPCK1* [93]. A study in mouse liver verified that, in correlation with increased cyclin D1, the level of p21, a cell cycle inhibitor, was downregulated in response to CAR activation by preventing FoxO1 from binding to its target promoters [127]. As CAR is an inhibitor of gluconeogenesis, lipogenesis, and fatty acid synthesis genes in the mouse via different mechanisms [7, 128, 129], its interaction with FoxO1 provides more insight into how energy homeostasis is regulated by transcriptional factors in response to stimuli.

3.3 PGC-1α

PGC-1α was initially identified as a coregulator of PPARγ in brown fat cells [130]. In addition to its roles in adipocyte differentiation, several studies have revealed that PGC-1α can also augment the expression of key enzymes on glucose uptake and gluconeogenesis in skeletal muscle and liver cells [131–133].

Shiraki *et al.* showed that PGC-1α enabled the ligand-independent transcriptional activation of CAR, which could be further enhanced by RXRα [134]. A GST pull-down assay demonstrated that the LXXLL motif and serine/arginine-rich domain (RS domain) in PGC-1α are direct binding regions for the CAR LBD. Notably, the RS domain is also required for CAR accumulation in nuclear speckles [134]. CAR can also induce the ubiquitination and degradation of PGC-1α through the recruitment of Cullin1 E3 ligase [135]. Interestingly, CAR crosstalks with HNF-4α in a functionally inhibitory manner by competitively binding to PGC-1α [136]. In addition, PGC-1α and FoxO1 are both glucagon sensors, and they could couple with each other to promote the expression of glycogenesis genes in human and mouse liver cells, which is blocked by insulin [137]. On the other hand, the activation of CAR improves glucose tolerance and enhances insulin sensitivity, thereby improving a diabetic condition [138]. Taken together, these results indicate that understanding the crosstalk between energy expenditure and drug metabolism might provide more avenues to explore in the search for therapies of metabolic diseases.

3.4 SRCs

The SRC family of p160 coactivators consists of SRC-1, SRC-2/GRIP1/TIF2, and SRC-3/pCIP/ACTR/AIB1/RAC-3/TRAM-1 [139]. With their highly conserved LXXLL motifs, SRCs are capable of interacting with nuclear receptors in a ligand-dependent manner to enhance transcriptional activation [140]. Studies showed that the recruitment of SRC by CAR to trigger transactivation may be ligand-independent; however, the levels of SRC1 recruitment are proportional to the liganded state of the CAR:RXR heterodimer [141, 142]. In rat hepatocytes and HepG2 cells, SRC-1 coactivates mCAR-induced cytochrome P450 2B1 (*Cyp2b1*) expression, which can be further enhanced by transcription factor Sp1 [143].

By using GST pull-down, glucocorticoid receptor–interacting protein 1 (GRIP1) was identified as a direct partner of mCAR, and the interaction could be strengthened in the presence of RXR and TCPOBOP [144]. In addition to activating CAR-regulated genes, interaction with GRIP1 could also promote CAR nuclear accumulation in hepatocyte-based assays [144]. Furthermore, although the three SRCs possess overlapping roles in the activation and nuclear translocation of mCAR [145], SRC-3 has been identified as the preferred coactivator for mCAR functions, as demonstrated by GST pull-down and cell-based reporter assays [146]. This finding was supported by an *ex vivo* model in which the proliferation and CAR-mediated induction of drug-metabolizing enzymes were repressed in cells isolated from SRC3−/− mice, even in the presence of an agonist [146].

3.5 SMRT, NCoR, DAX-1, and SHP

Among the cofactors that regulate nuclear receptor transcriptional activity is a group of corepressors, including SMRT and NCoR, that inhibit transcriptional activity [147, 148] by

coordinating with mSin3A/B and histone deacetylases [149–151]. A mammalian two-hybrid assay uncovered an androstanol-dependent interaction between mCAR and SMRT that could be effectively enhanced in the presence of RXR [152]. By using yeast two-hybrid and GST pull-down assays, NCoR was also shown to be a binding partner of mouse and human CAR. In addition, a series of mCAR and hCAR agonists and antagonists were shown to affect CAR transcription, which was concomitant with the binding affinity of CAR for coactivators or corepressors [153, 154].

DAX-1, an atypical DBD-deficient orphan nuclear receptor which has been characterized as a repressor for many nuclear receptors [155], was shown to require NCoR for its inhibitory roles [156]. Cell reporter and mammalian two-hybrid assays indicated that the activity of human CAR was abrogated in response DAX-1 expression. Coimmunoprecipitation and alpha-screen assays further confirmed direct interaction between CAR and DAX-1, and this interaction was enhanced by CITCO, an hCAR agonist [157, 158]. Another nuclear receptor SHP was initially identified as an interacting partner of mCAR by two-hybrid screening [159]. Similar to DAX-1, SHP also lacks the zinc finger DBD. GST pull-down and gel mobility shift assays demonstrated that SHP acts as a suppressor of *CYP2B* gene transcription without affecting DNA binding or GRIP1 recruitment of the CAR/RXR complex [160].

3.6 GADD45B

Growth arrest and DNA damage–inducible 45b (GADD45B) is a negative regulator of cell growth and apoptosis [161, 162] and its expression could be induced by TCPOBOP in a CAR-dependent manner in mouse liver [29]. GST-pull down and coimmunoprecipitation experiments have confirmed a direct interaction between CAR and GADD45B and MAPK kinase 7 (MKK7) [163]. Upon pre-stimulation with TCPOBOP and co-stimulation with actinomycin D and TNFα, CAR potentiates GADD45B to inhibit MKK7 from phosphorylating and activating Jun N-terminal kinase 1 (JNK1). This mechanism describes CAR's function in suppressing mouse hepatocyte death [163]. These studies also provided a better understanding of the CAR-mediated tumorigenicity induced by agonists [163, 164].

4. Conclusion

We have discussed many of the interactions and pathways that control PXR and CAR activity, as summarized in Tables 1 and 2. The protein-protein interactions of these receptors regulate their localization in cells, recruitment of cofactors, stability, and binding to the promoters of their target genes. As summarized in Table 3, it is not surprising there is an overlap in the binding partners of PXR and CAR, which explains their similar regulation of a subset of genes. As we learn more about the roles of PXR and CAR in xenobiotic and endobiotic responses and their regulation thereof, it remains to be determined whether this knowledge can be harnessed in treating human diseases.

5. Expert opinion

The nuclear receptors PXR and CAR are key mediators of the xenobiotic response in the liver that is regulated by an array of ligands [3, 4, 15] and this discovery has biased the study

of PXR and CAR towards their xenobiotic functions. It is now clear that these nuclear receptors are versatile in their activity, as demonstrated by their involvement in energy homeostasis, through their effects on lipogenesis and gluconeogenesis, and in the immune response, through their effects on the NF-κB and TLR signaling pathways [75, 77, 137]. Many studies have been conducted in an attempt to understand the ligand-dependent regulation of PXR and CAR; however, it has become increasingly apparent that the regulation of these xenobiotic receptors is multifaceted. In addition to direct regulation by ligand binding, indirect regulatory mechanisms, including transcriptional, posttranscriptional, and posttranslational regulation, affect the levels or activities of these receptors [165]. Although there is no experimental evidence to support that endogenous PXR and CAR are phosphorylated *in vivo*, partly due to the low expression levels of the receptors in cell models frequently used, exogenous expression of the mutant proteins have teased out important residues required for their activity [107]. There are strong evidences that support that PXR and CAR are modulated to an extent by phosphorylation. It is also becoming evident that acetylation and SUMOylation play important roles in PXR activity [109, 111]. Furthermore, PXR and CAR activity is modulated by (1) interaction with other proteins, (2) crosstalking pathways that feed into PXR and CAR signaling, and (3) the subcellular localization of the receptors [32, 75, 77], all of which affect the overall responsiveness of the receptors to stimuli. The regulation of PXR and CAR is finely tuned, because the binding of ligand confers a conformational change on the receptors. The conformation assumed by the receptors dictates their interaction with cofactors and, hence, the transcriptional regulation of target genes. As most cofactors are involved in regulating multiple transcription factors, those that interact with PXR may recruit PXR to targets of other transcription factors and *vice versa*, thus giving rise to the PXR crosstalk in multiple pathways.

Our recently acquired understanding of PXR and CAR crosstalk with other signaling pathways has broadened the scope of xenobiotic receptor studies. This also means that more attention should be paid when designing agonists or antagonists of PXR and CAR because of the likelihood of adversely altering other interconnected pathways. Caution should also be exercised when interpreting data obtained from experiments involving PXR and CAR activation or inhibition: The observed effects may not necessarily be PXR or CAR dependent, and for this reason, more stringent controls should be included in experimental setups. With the recent observation of CYP3A5-mediated drug resistance in pancreatic cancer [102], one might wonder how *CYP3A4* and *CYP3A5* are differentially regulated by PXR and how such differential regulation might affect hepatic systemic drug metabolism and extra-hepatic tumor cell–autonomous drug metabolism and drug resistance. In addition, the contribution of HNF4α to regulating *CYP3A4* and *CYP3A5* expression suggests that a potentially more efficacious drug to prevent resistance would be one that simultaneously inhibited both HNF4α and PXR. Thus, many questions remain unanswered, and this is an area that warrants further investigation.

The upregulation of drug-metabolizing enzymes and transporters, mediated by PXR and CAR, affects the pharmacokinetics of drugs and other co-administered drugs, potentially resulting in drug-drug interactions. If the metabolites or the interactions of metabolites are toxic, this could result in liver injury [166]. Additionally, the immunosuppressive effects of

PXR and CAR activation have long being known [167, 168]; therefore, the activation of these xenobiotic receptors may affect the outcome of different therapies through the regulation of drug metabolism and the immune response in patients.

Lastly, in view of the recent discoveries regarding the complex network of factors that regulate PXR and CAR, it is clear that the full potential of these receptors as therapeutic targets has yet to be fully explored. We have discussed many of the metabolic circuits and proteins controlling PXR and CAR, and alterations in these pathways could have substantial physiologic consequences. The pharmacologic manipulation of these complex networks of factors may present novel therapeutic opportunities or improve the current drug therapies for numerous metabolic diseases, neoplasms, and immune disorders.

PXR and CAR were first discovered and recognized as master xenobiotic receptors, but it is clear that they have much broader cellular functions. Through protein-protein interactions and signaling crosstalk, the function of not only PXR and CAR but also their interacting or crosstalking partners can be modulated, and this needs to be fully investigated.

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Article highlights

1. PXR and CAR are well-recognized xenobiotic receptors.
2. PXR and CAR interact with other proteins.
3. PXR and CAR crosstalk with other signaling pathways.
4. Protein-protein interaction and signal crosstalk mutually affect the function of all partners involved.
5. Investigations of protein-protein interaction and signaling crosstalk reveal the broader cellular functions of PXR and CAR.

Table 1

Representative regulators of PXR in protein-protein interactions and signaling crosstalk.

Proteins interacting with PXR	Effect on PXR	References	Notes
PKA	Enhances or represses PXR transcriptional activity	[36, 37]	The effect of PKA on PXR is species specific
PKC	Phosphorylates and represses PXR activity	[41]	
P70 S6K	Represses PXR activity	[44]	
PRMT1	Required for maximum PXR transcriptional activity	[50]	
SRC1, RIP140, SUG1, GRIP1, PBP	Enhances PXR activity	[36, 55, 56]	
p53	Inhibits PXR transcriptional activity	[78]	
HNF4α	Synergizes with PXR to upregulate PXR target genes	[99, 102]	
DYRK2, UBR5	DYRK2 phosphorylates PXR and facilitates PXR ubiquitination by UBR5	[104]	
CDK2	Phosphorylates and represses PXR transcriptional activity	[107]	
p300	Acetylates and potentiates RIF-induced PXR transcriptional activity	[109]	
SIRT1	Deacetylates and enhances ligand-independent PXR transcriptional activity	[109]	

Table 2

Representative regulators of CAR in protein-protein interactions and signaling crosstalk.

Proteins interacting with CAR	Effect on CAR	References	Notes
PP2A	Dephosphorylation and nuclear translocation of CAR	[113]	
ERK1/2	Phosphorylation and CAR cytoplasmic retention	[115, 116]	
RACK1	Recruits PP2A to CAR to enhance dephosphorylation and nuclear transportation	[118]	
SRC2 and SRC3	Promote nuclear accumulation	[144, 146]	Interaction with SRC2/3 was shown with mCAR
NCoR and DAX-1	Repress transcriptional activity	[153, 154, 157, 158]	
GADD45B	CAR potentiates the antiapoptotic activity of GADD45B	[163]	

Table 3

Representative regulators of both PXR and CAR in protein-protein interactions and signaling crosstalk.

Proteins interacting with PXR and CAR	Effect on receptors	References
SMRT	Represses receptor transcriptional activity	[32, 152]
SREBP1	Represses transcriptional activity by inhibiting coactivator recruitment	[60]
PGC-1α	Enhances receptor activity	[64, 136]
TLR	Downregulates PXR- and CAR-mediated drug metabolizing enzymes	[76, 77]
IL-6 and NF-κB	Downregulate PXR- and CAR-mediated drug metabolizing enzymes	[87, 89]
FoxO1	Upregulates receptor activity	[93, 126]
SHP	Enhances receptor activity	[81, 159]
Hsp90	Stabilizes PXR and CAR	[105, 113]