Published in final edited form as:

Biochim Biophys Acta. 2016 September ; 1859(9): 1130–1140. doi:10.1016/j.bbagr.2016.02.006.

Mechanisms of Xenobiotic Receptor Activation: Direct vs. Indirect

Bryan Mackowiak and Hongbing Wang

Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 Penn Street, Baltimore, MD 21201

Abstract

The so-called xenobiotic receptors (XRs) have functionally evolved into cellular sensors for both endogenous and exogenous stimuli by regulating the transcription of genes encoding drug-metabolizing enzymes and transporters, as well as those involving energy homeostasis, cell proliferation, and/or immune responses. Unlike prototypical steroid hormone receptors, XRs are activated through both direct ligand-binding and ligand-independent (indirect) mechanisms by a plethora of structurally unrelated chemicals. This review covers research literature that discusses direct vs. indirect activation of XRs. A particular focus is centered on the signaling control of the constitutive androstane receptor (CAR), the pregnane X receptor (PXR) and the aryl hydrocarbon receptor (AhR). We expect that this review will shed light on both the common and distinct mechanisms associated with activation of these three XRs.

Keywords

AhR; CAR; PXR; xenobiotic receptor; activation mechanism

1. Introduction

Nuclear receptors (NRs) are important cellular proteins that govern the expression of numerous genes involved in a wide range of cellular processes including cell growth, differentiation, metabolism, and stress responses. Prototypical NRs exert their effects by acting as transcription factors, which sense both intracellular and extracellular signals and respond by inducing the transcription of their target genes [1]. Although the cellular impact of each NR is different, all NRs share a number of characteristic structures, functional domains, and sequence similarities. In general, NRs feature a variable N-terminal region with an activation function 1 (AF-1) domain, a highly conserved DNA-binding domain

Corresponding author: Hongbing Wang, Ph.D, Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 Penn Street, Baltimore, MD 21201, Phone: (410)-706-1280; Fax: (410)-706-5017, hwang@rx.umaryland.edu.

Declaration of Interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(DBD), a hinge region, and a ligand-binding domain (LBD) that contains the activation function 2 (AF-2) domain. The AF-1 domain mediates ligand-independent activation of the NR while AF-2 activation is ligand-dependent [2]. The hinge region connects the DBD to the LBD and upon ligand binding, helices in this region undergo a conformational change allowing coactivators to bind, ultimately leading to nuclear localization and activation [2, 3]. These features differentiate NRs from many other regulatory proteins and allow them to be dynamic sensors that respond to various stimuli and regulate cellular responses.

NRs can be roughly divided into two main classes based on their ligand specificity – endocrine NRs and orphan NRs [4]. Endocrine NRs bind with nanomolar-affinity to specific endogenous ligands such as hormones and steroids that are present at low concentrations physiologically. Examples of endocrine NRs include, but are not limited to, the thyroid hormone receptor, the retinoic acid receptor, the androgen receptor, and the estrogen receptor [2, 4]. On the other hand, orphan NRs usually have no identified high-affinity endogenous ligands and are instead activated by abundant and low-affinity endogenous metabolites or xenobiotics. However, it is important to note that some NRs previously designated as orphan NRs have been “adopted” after discovering an endogenous ligand, such as the farnesoid x receptor, which was “adopted” after bile acids were identified as high-affinity ligands [5]. A number of orphan NRs promiscuously bind to a wide range of both endogenous compounds and xenobiotics, often at micromolar concentrations, and are instrumental in mounting cellular responses to toxic compounds and their metabolites. This subset of orphan NRs, termed xenobiotic receptors (XRs), have become increasingly important in coordinating toxic or protective responses when humans are exposed to accumulated endotoxins or high concentrations of environmental chemicals [6]. XRs coordinate the gene transcription of numerous phase I and II drug-metabolizing enzymes and transporters in the liver and intestine, where XRs are also enriched [7]. To date, extensive investigations have been centered on three XRs; namely, the constitutive androstane receptor (CAR, NR1I3), the pregnane x receptor (PXR, NR1I2), and the aryl hydrocarbon receptor (AhR), due majorly to their predominance in regulating hepatic responses to drugs and environmental chemicals. Although AhR, a member of the Per-ARNT-Sim (PAS) family of proteins, is not typically classified as a nuclear receptor, it has similar functionality and follows the same overall paradigm as other XRs in a pharmacological or toxicological perspective [8]. While all of these XRs are capable of altering cellular metabolism and homeostasis individually, significant cross-talk exists between these receptors leading to multifaceted regulation of xenobiotic detoxification. For example, previous studies have shown that PXR activation in human primary hepatocytes increases the conversion of AhR antagonist omeprazole-sulfide to a prototypical AhR activator, omeprazole [9]. In addition, all three XRs are known to regulate the important drug-metabolizing enzyme UDP glucuronosyltransferase 1A1 and the transporter breast cancer resistance protein (ABCG2) at the transcriptional level [10-15]. Also, shared ligands such as non-coplanar polychlorinated biphenyls and flavonoids derived from Ginkgo biloba extract activate CAR, PXR, and AhR, which can lead to complicated effects in the liver [16, 17]. As these XRs exhibit complex interplay that markedly alters cellular metabolism and homeostasis, understanding the types of compounds that activate these XRs and the underlying mechanical bases of XR activation are essential.

Traditionally, ligand-binding has been thought of as an essential component of XR activation and has been studied with methods such as mammalian two-hybrid assays, luciferase reporter assays, and more recently, fluorescence resonance energy transfer (FRET) assays [18]. However, recent evidence has shown that many compounds activate XRs in lieu of direct ligand binding; rather, they activate XRs via ligand-independent (indirect) mechanisms that have yet to be fully elucidated. This paradigm of XR activation mediated through both direct and indirect mechanisms raises new challenges in our understanding of how XR signaling is controlled when exposed to various cellular stresses and has profound implications on how XR modulators will be identified in the future. As such, this review will highlight the recent research advances regarding mechanisms of direct and indirect activation for XRs with the focus on CAR, PXR, and AhR.

2. Constitutive androstane receptor

Screening of a human liver library with a degenerate oligonucleotide based on the sequence of the conserved DNA binding domain of NRs led to the cloning of CAR, originally named MB67, in 1994 [19]. CAR heterodimerizes with the retinoid x receptor (RXR) and transactivates genes that contain the retinoic acid response element “constitutively” in the absence of a ligand, leading to its early name as the constitutive activated receptor [20]. The mouse and rat orthologs of CAR were cloned soon thereafter, exhibiting the same heterodimerization and constitutive activation features as human CAR (hCAR) [20, 21]. Under normal physiological conditions, CAR is sequestered in the cytoplasm in a complex with heat-shock protein (HSP) 90 and CAR cytoplasmic retention protein (CCRP); and HSP70 has recently been shown to stabilize this complex in the inactive state [22-24]. Interestingly, although CAR is retained in the cytoplasm in the inactive state and translocates to the nucleus upon activation in physiologically-relevant primary hepatocytes, CAR is localized to the nucleus and constitutively active in immortalized cell lines [25, 26].

As a xenobiotic receptor, CAR governs the inductive expression of many phase I and phase II drug-metabolizing enzymes and transporter proteins, coordinating a defensive network against xenobiotic challenges in the liver [27, 28]. Although the well-established xeno-sensing role of CAR continues to be important in predicting potential drug-drug interactions (DDIs) and pharmacokinetic profiles of drugs, emerging evidence reveals that CAR also affects physiological and pathophysiological conditions including obesity, diabetes, and tumor development by modulating hepatic energy homeostasis, insulin signaling, and cell proliferation [29-32]. For instance, in contrast to the up-regulation of genes encoding drug-metabolizing enzymes and transporters, activation of CAR by the selective mouse CAR (mCAR) activator 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) significantly represses a cluster of genes associated with gluconeogenesis and lipogenesis, and attenuates high-fat diet (HFD)-induced obesity and diabetes in wild-type but not CAR-knockout mice [29-31]. Assuming such beneficial effects also occur in humans, CAR may function as a novel therapeutic target for metabolic disorders in addition to its known role as a xenobiotic sensor. Therefore, understanding of the mechanistic basis of CAR activation is pivotal, and these recent developments have stimulated much interest in identifying selective and potent activators of human CAR.

2.1. Activation of CAR

The pharmacological importance of CAR was first appreciated when CAR activation was linked to the induction of CYP2b10 expression by phenobarbital (PB) in mouse liver [33]. Subsequent studies revealed that numerous structurally unrelated PB-like compounds induce CYP2B genes in different species through the activation of CAR [25, 27, 34]. The initial step of CAR activation involves cellular translocation of the receptor from the cytoplasm to the nucleus, where it interacts with its heterodimer partner RXR and other transcriptional proteins to stimulate the expression of target genes [25, 35]. Further analysis of the 5' upstream regions of CAR target genes revealed key promoter elements often containing direct repeats of the hexamer AGGTCA separated by 3-5 nucleotides which directly interact with the CAR/RXR heterodimer [36]. These findings led to the establishment of specific cell-based luciferase reporter assays, by which pharmacological modulation of CAR activity could be monitored efficiently. Initial ligands identified for CAR include two endogenous testosterone metabolites, 5 α -androst-16-en-3 α -ol (androst-enol) and 5 α -androst-3 α -ol (androst-anol) [37]. Both compounds repressed the constitutive activity of mCAR *in vitro* and disrupted its interactions with coregulatory proteins such as the nuclear receptor coactivator 1 (SRC-1) [37]. Notably, although this finding leads to the current name of CAR as the constitutive androstane receptor, these steroid metabolites are not likely to be “real” endogenous ligands of CAR *in vivo* because the concentrations needed to antagonize CAR *in vitro* are several magnitudes higher than their physiological levels. Subsequently, TCPOBOP was identified as a potent and selective agonist of mCAR with the ability to reverse the antagonism conferred by androstanes while promoting CAR interaction with coactivator SRC-1 [38, 39]. Interestingly, although CAR demonstrates promiscuity in ligand binding, it also exhibits divergent activation profiles across species. For instance, TCPOBOP activates mouse but not human CAR while androstanol represses mouse but not human CAR [34, 38]. The later discovery of a potent and selective hCAR agonist, 6-(4-chlorophenyl)imidazo[2,1- β][1,3]-thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime (CITCO), has been instrumental in studying the effects of CAR in human systems and conferring physiological relevance to many studies carried out in mice only [40].

CAR displays unique activation mechanisms compared with typical nuclear receptors, requiring both nuclear translocation and nuclear activation. In HepG2 cells, transfected CAR spontaneously accumulates in the nucleus in the absence of an inducer and exhibits constitutive activation of its target genes [20, 25]. This would suggest that nuclear translocation alone is sufficient to confer CAR activation. However, several lines of evidence support nuclear activation as a distinct step in CAR-mediated gene regulation in primary hepatocytes and intact liver *in vivo*. For example, pretreatment of primary hepatocytes with the protein phosphatase inhibitor okadaic acid (OA) inhibits PB-induced CAR nuclear translocation but does not repress CAR-mediated activation of reporter genes in HepG2 cells since CAR is constitutively localized in the nuclei of HepG2 cells [25, 41]. Additionally, pretreatment of primary mouse hepatocytes with KN-62, a calcium/calmodulin-dependent kinase inhibitor, repressed PB- and TCPOBOP-associated CYP2b10 induction and reporter gene activation without affecting CAR nuclear accumulation [42]. Nevertheless, although these results indicate nuclear translocation is only one of the two required steps for target gene activation, nuclear localized CAR prefers interaction with coactivators instead of

corepressors even in the absence of agonist binding in contrast to other nuclear receptors [43, 44]. Many compounds, like PB, have no CAR binding activity and indirectly activate CAR, most likely by facilitating nuclear translocation of the receptor instead. These ligand-dependent and -independent activation mechanisms of CAR will be discussed in the following sections.

2.1.1. Direct Activation of CAR—Although the basic structure of CAR is similar to other NRs, the structure-activity relationship of CAR is quite distinct and has been widely studied to understand the factors behind its constitutive activity. In general, the ligand-binding pocket of a NR is enclosed by 12 α -helices, where helix 12 (H12) containing the AF-2 domain is the master regulator of activation. Early mutagenesis studies showed that the AF-2 motif of CAR mediates ligand-independent coactivator binding and confers constitutive activity [45]. Compared to other NRs, CAR has a shortened loop between helices 11 and 12 in addition to a shortened H12 helix which allows the negatively charged carboxy-terminus to interact with the positive K195 (K205 for mCAR) residue, favoring the active conformation of H12 [43, 44]. The crystal structures of TCPOBOP- and CITCO-bound mCAR:RXR and hCAR:RXR heterodimers have given much insight into how direct activators bind to CAR and impart activity [44, 46]. The direct activator TCPOBOP is often referred to as a “superagonist” of mCAR, as it drastically increases mCAR transcriptional activity above constitutive levels, unlike CITCO for hCAR [46]. Most likely, this is due to the interactions of TCPOBOP with L353 on the AF-2 domain and L346 and T350 on the linker helix of mCAR, directly stabilizing H12 in the active conformation [44]. In contrast, the structure of hCAR bound to CITCO is remarkably similar to the unbound constitutively active form, suggesting that the mechanism of hCAR activation by CITCO may result majorly in a conformational change that promotes nuclear localization [46]. This is consistent with observations that CITCO cannot induce hCAR activity over its basal level in immortalized cell lines, but strongly induces translocation-dependent hCAR activation in human primary hepatocytes [47].

Acquiring crystal structures of the CAR LBD bound to activators has facilitated computational screening for possible CAR activators based on both structural and pharmacophore models. This method is normally used to narrow down a compound library to likely activators, which can then be further screened in a less high-throughput manner [48]. Many different binding assays have been designed to find direct ligands of CAR over the years, and most of them feature the CAR LBD and its ability to interact with coactivators or heterodimerize with RXR. For instance, two-hybrid mammalian cell assays have been extensively used to characterize CAR binding to coactivators in the presence of different compounds to determine possible ligands [49]. FRET assays are also frequently employed in this capacity, representing another method to assess CAR binding and activation [50]. Once a potential activator is found to bind to the CAR LBD and induce association with coactivators, it is usually subjected to testing in luciferase reporter assays, normally in HepG2, Huh7, COS-1, or CV-1 cells [18]. This type of assay determines whether a compound can induce CAR transcriptional activity, which is the hallmark of CAR activation. However, the basal constitutive activity and nuclear localization of CAR in immortalized cell lines provide challenges to this approach. As CAR is constitutively active, even the most

potent hCAR inducers can only increase CAR transcriptional activity moderately over the high basal level in cellular systems. In addition, the constitutive nuclear localization of CAR in immortalized cells prevents the use of nuclear translocation assays when probing compounds as potential activators. Accordingly, many studies have sought to modify or supplement the basic luciferase assay hoping to more effectively identify CAR modulators *in vitro*. Among others, genetic variations within the *CAR* gene may alter the expression and function of the encoded protein. To date, more than 25 alternatively spliced transcripts of hCAR have been identified [51, 52]. Although their functional significance *in vivo* remains unknown, several splice variants exhibit altered enhancer binding and coregulatory recruitment, as well as differential chemical responses [51-53]. The splicing variant hCAR3, which contains an in-frame insertion of five amino acids (APYLT) in the LBD, was reported to exhibit significantly reduced basal, but potent ligand-induced activities in cell-based reporter assays [53]. Further delineation of the five amino acid insertion found that retaining the alanine alone (hCAR1+A) is sufficient to convert the constitutively activated hCAR into its xenobiotic-sensitive surrogate *in vitro* [54]. Importantly, hCAR1+A displays chemical-mediated activation superior to that of hCAR3 [54]. A similar approach was taken by Kanno and Inouye by inserting three consecutive alanine residues between helices 11 and 12, which lowered the high basal activity of CAR in immortalized cells [55]. However, it is important to note that the addition of any residue to a protein, especially in the ligand-binding pocket, may alter the chemical binding specificity of the protein. Indeed, the splice variant hCAR2 containing an additional four amino acids (SPTV) reshapes the ligand-binding pocket of CAR and recognizes the common plasticizer, di(2-ethylhexyl) phthalate as a specific and highly potent agonist for hCAR2 without affecting the activity of the reference hCAR1 [56, 57].

Although androstanol and androstenol were among the first known inverse agonists of mCAR, discovery of potent inverse agonists/antagonists of hCAR has lagged behind. Clotrimazole was one of the first inverse agonists discovered for hCAR [34]; however, studies since have shown complex outcomes regarding the effects of clotrimazole on CAR activity. In CV-1 cells, the maximal deactivation of CAR achieved by clotrimazole was approximately 50%. In contrast, in HEK293 and COS1 cells, clotrimazole failed to repress the constitutively activated hCAR1, but potently activated hCAR3 and the hCAR1+A construct [54, 58]. A typical peripheral benzodiazepine receptor ligand, PK11195, was later established as a potent hCAR antagonist that decreases the high basal activation of wild-type CAR in HepG2 cells by directly competing with agonists such as CITCO for binding to the LBD [47]. Interestingly, cell-based luciferase reporter assays showed that PK11195-mediated repression of CAR activity can be efficiently recovered by CITCO (a direct activator) but not by PB (an indirect activator) [47]. Utilizing this model, Lynch et al. recently established a quantitative high-throughput screening (qHTS) assay for identification of hCAR modulators. By screening approximately 2800 compounds from the NIH Chemical Genomics Center Pharmaceutical Collection, 115 activators of hCAR were identified, which include both novel and known hCAR activators and CYP2B6 inducers [59].

One of the concerns surrounding the identification of selective CAR activators lies in the fact that CAR and PXR share many xenobiotic ligands and have significant overlap in the regulation of their target genes [7, 34]. Many drugs previously identified as PXR agonists

are also activators of CAR, such as the antimalarial artemisinin [60]. Notably, the CAR inverse agonists PK11195 and clotrimazole are also potent activators of human PXR, making the identification of selective hCAR activators extremely challenging [34, 47]. Most recently, Cherian et al. reported CINPA1 as a more selective deactivator of hCAR that does not activate PXR [61]. In HepG2 cells, CINPA1 and PK11195 exhibit comparable deactivation of CAR, but CINPA1 does not activate PXR. More importantly, CINPA1 effectively repressed CITCO-induced CYP2B6 expression in human primary hepatocyte cultures [61]. This new compound could be used as a novel molecular tool for elucidating hCAR activators.

Overall, ligand-dependent modulation of CAR activity represents the fundamental mechanisms of direct chemical-protein interactions. Due to the relatively low sequence homology between LBD of human CAR and its rodent counterparts, direct modulators of CAR (agonists and antagonists) such as CITCO and TCPOBOP often exhibit more species selectivity than indirect activators, such as PB. Identification of a human-specific CAR modulator may eventually be of clinical importance. In the meantime, it is important to point out that the physiological condition and microenvironment of cells are crucial for the investigation of CAR activation. Many compounds that have shown robust induction of CAR transcriptional activity in immortalized cell lines fail to induce CAR and PXR target genes in human primary hepatocytes. This can be exemplified by the fact that buprenorphine was identified as an effective activator for both PXR and CAR in cell-based luciferase assays, but failed to either translocate CAR to the nucleus or induce CAR and PXR target genes in human primary hepatocytes [62]. Further investigation revealed that buprenorphine experienced a dramatically different rate of elimination between primary hepatocytes and HepG2 cells, leading to the observed discrepancy and highlighting the need for cautious interpretation of data obtained from *in vitro* assays.

2.1.2. Indirect activation of CAR—Shortly after PB was discovered to be a CAR activator, Kawamoto et al. demonstrated that activation of CAR by PB was majorly related to nuclear translocation that could be blocked by the protein phosphatase inhibitor OA, suggesting that PB-mediated CAR nuclear translocation was associated with a signaling cascade that requires phosphatase-mediated protein dephosphorylation [25]. It was later confirmed by scintillation-proximity binding assays that PB does not directly bind to CAR even though it effectively activates CAR in multiple species [34]. Hosseinpour et al., showed that when Ser202 of CAR was mutated to an aspartate mimicking the status of protein phosphorylation, CAR was sequestered in the cytoplasm of mouse primary hepatocytes and unable to translocate to the nucleus in response to PB treatment, while mutation of Ser202 to an alanine did not affect PB-mediated mCAR nuclear translocation [63]. Furthermore, Western-blotting analysis revealed that an antibody specific for phospho-Ser202 only recognized cytoplasmic CAR protein but not nuclear-localized CAR protein in liver cells, suggesting that dephosphorylation of Ser202 is likely an early, essential step of PB-mediated CAR activation.

CAR resides in the cytoplasm of primary hepatocytes and non-induced liver in contrast to the nuclear localization seen in immortalized cell lines. In primary hepatocytes, cytoplasmic chaperones associated with CAR have been proposed as important determinants for CAR's

cytoplasmic retention before activation and nuclear translocation following PB treatment [64]. The initial simplified CAR protein complex was reported by Yoshinari et. al in non-induced mouse liver, where CAR forms a complex in the cytoplasm with heat-shock protein (HSP) 90 [65]. Pretreatment with geldanamycin, a compound known to interrupt the chaperoning function of HSP90, profoundly repressed CYP2b10 expression and mCAR nuclear translocation induced by PB-type activators. It appears that the CAR:HSP90 complex also transiently recruits protein phosphatase 2A (PP2A) in response to PB treatment, further validating that chemically-stimulated CAR nuclear translocation might be a OA-sensitive, protein phosphatase-mediated process [65]. Interestingly, although TCPOBOP does not bind hCAR when expressed in mouse liver, hCAR translocates into the nuclei of mice livers in response to TCPOBOP treatment, suggesting CAR nuclear translocation is not initiated by ligand binding [66]. Further work by Negishi and colleagues identified CCRP (a member of the HSP40 family) as another protein that could bind to the mCAR:HSP90 complex important in the cellular localization of CAR [22]. When co-expressed with CCRP in HepG2 cells, mCAR protein levels dramatically increased in the cytosol, providing evidence that CCRP may stabilize the CAR:HSP90 complex and affect CAR nuclear translocation. Additional studies revealed that CCRP is subjected to ubiquitination and proteasomal degradation in cells when exposed to PB-type CAR activators [24]. In HepG2 cells overexpressing both CCRP and CAR, MG132 (a proteasome inhibitor) treatment further increased the retention of CAR in cytoplasm [24]. However, whether CAR can be directly ubiquitinated is debatable [67]. Negishi and colleagues also identified HSP70, another chaperone protein, as a novel component of the cytosolic CAR protein complex [24]. When HepG2 cells expressing CAR and CCRP were exposed to thermal stress, the increase in HSP70 protein levels was correlated with increased cytoplasmic CAR levels, mimicking the effects of MG132. Collectively, these findings represent important steps towards a better understanding of how CAR is released from the cytoplasmic protein complex and translocated to the nucleus – the crucial step for CAR activation.

Although PB has long been known as a CYP2B inducer and represents a class of compounds that activate CAR without binding to the receptor, the hunt for the direct target of PB has been a lengthy journey. Early observations by Bauer et al. indicated that treatment with epidermal growth factor (EGF) could repress the induction of CYP2B1 by PB in primary rat hepatocytes [68]. Subsequently, Joannard et al. revealed that activation of the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways could play a role in PB-mediated gene transcription [69]. These findings turned out to be important clues for elucidating the indirect activation mechanism of CAR (Figure 1). Negishi and colleagues went on to show that hepatocyte growth factor treatment increased ERK1/2 phosphorylation, leading to the inhibition of TCPOBOP-induced CAR nuclear translocation and transcription of CAR target genes, while inhibition of mitogen-activated protein kinase kinase (MEK) upstream of ERK by U0126 enhanced the induction of CYP2b10 [70]. Subsequently, the Thr38 residue of hCAR was found to be phosphorylated by protein kinase C (PKC), dephosphorylation of Thr38 was found to be required for CAR nuclear translocation, and most importantly, PB treatment resulted in dephosphorylation of the Thr38 of hCAR and the corresponding Thr48 residue of mCAR [71]. Additional studies

indicated that the ERK1/2:CAR interaction was increased in response to EGF treatment, while inhibition and knockdown of MEK led to a decrease in ERK1/2:CAR interaction [72]. Most recently, Mutoh et al., defined the epidermal growth factor receptor (EGFR) as the initial binding protein for PB to induce CAR activation in the liver. This binding potentially inhibited EGF-mediated signaling and led to the dephosphorylation of the downstream receptor for activated C kinase 1 (RACK1) at Tyr52, which promoted dephosphorylation of the Thr38 residue of hCAR by PP2A, leading to the nuclear translocation and activation of CAR [73]. Subsequent studies with the flavonoids chrysin, baicalein, and galangin, which are known to activate CAR, found that these compounds do not bind to CAR and instead activate CAR through inhibition of EGF-mediated signaling, further validating inhibition of the EGF signaling pathway as a common mechanism for indirect CAR activation [74].

Unlike classical NRs that are often activated or deactivated in a ligand-dependent manner, CAR is activated by both direct and indirect activators through overlapping yet distinct mechanisms. This dual-mechanism paradigm for CAR activation presents challenges in studying CAR's signaling and function, heightened by the fact that traditional ligand binding assays are essentially useless in finding indirect activators, which make up a large portion of currently available activators of CAR. The constitutive nuclear localization and activation of CAR in immortalized cell lines also renders extra difficulties in using cell-based luciferase reporter assays to identify activators. It is worth noting that even with all these challenges, our understanding of both the biological function of CAR and the mechanistic bases of CAR activation has expanded considerably in the past decade.

2. Pregnane X receptor

The pharmacological importance of PXR, also referred to as steroid X receptor (SXR) and pregnane-activated receptor (PAR), was almost immediately recognized after its cloning in 1998 when its primary gene target was found to be CYP3A4, the most abundant human hepatic P450 [75-77]. As a promiscuous mediator for metabolism-based DDIs, PXR significantly induces the expression of numerous drug-metabolizing enzymes and transporters important in xenobiotic disposition, which can result in clinically important DDIs [28]. To date, a large number of compounds, including drugs and environmental chemicals, have been established as PXR activators due in part to the unique structure of the PXR LBD, which contains a bulky and flexible ligand-binding pocket characterized by a 5-strand β -sheet in contrast to the 3-strand β -sheet exhibited by the majority of NRs [78-80]. Upon ligand binding and activation, PXR recruits many of the same coactivators/repressors as CAR to carry out its transcriptional activities [6]. In fact, PXR is the closest sister receptor of CAR in the whole NR superfamily tree, sharing many small molecule activators and participating in cross-talk over common target genes [81].

2.1. Activation of PXR

PXR is activated by a broad range of chemicals with no obvious common structural features, including endobiotics such as steroid hormone metabolites, vitamins, and bile acids, and xenobiotics such as herbals, macrolide antibiotics, antifungals, etc. [81, 82]. Interestingly, although PXR is a promiscuous receptor, it exhibits divergent ligand activation profiles

across species. Clearly, the low homology in the PXR LBD regions contributes to the observed species differences in PXR activation and target gene induction [83]. For instance, the anti-glucocorticoid pregnenolone 16 α -carbonitrile (PCN) and DTBA activate rat PXR but not human PXR, while rifampin and SR12813 activate human but not rat and mouse PXR [84]. Remarkably, rabbit PXR was activated by both rifampin and DTBA, reflecting its evolutionary conservation [84, 85]. It was speculated that differences in diet and xenobiotic exposure among species were responsible for evolutionary changes in amino acids lining the ligand-binding pockets of different PXRs [86].

Currently, there is controversy regarding the cellular localization of PXR. Like CAR, human PXR (hPXR) is consistently localized in the nucleus in immortalized cell lines [87]. Nevertheless, such auto-accumulation in the nucleus does not exhibit spontaneous activation without chemical stimulation. In contrast, multiple studies have shown that mouse PXR (mPXR) is primarily localized in the cytoplasm of non-induced mouse liver and translocates to the nucleus following treatment with the prototypical mPXR agonist PCN [88]. In addition, mPXR can form a complex in the cytoplasm with HSP90 and CCRP similar to CAR, although its PCN-dependent translocation could not be reversed by overexpression of CCRP [88]. Studies have also demonstrated that the nuclear localization signal of hPXR is essential to its translocation from the cytoplasm to the nucleus and transcription of target genes in immortalized cells [89]. More studies *in vivo* or in physiologically-relevant cell culture systems are needed to determine the importance of nuclear translocation in hPXR activation. Regardless, PXR is not constitutively active like CAR and therefore, nuclear localization alone is not sufficient for PXR to induce transcription of its target genes. Although all PXR activators identified to date have either been proven or assumed to be directly binding to PXR, a number of studies have shown that signaling pathways can influence PXR activation status.

2.2. Direct Activation of PXR

Ligand-dependent activation of PXR has long been a focus of investigation, due to the extensive ligand promiscuity and robust target gene induction. Without ligand binding, PXR is constantly silenced by recruitment of corepressors instead of coactivators when residing in the nucleus. Agonist binding of PXR results in release of preoccupied corepressors, such as silencing mediator of retinoid and thyroid receptors (SMRT) and the nuclear receptor corepressor 1 (NcoR1), and recruitment of coactivators such as SRC-1 and glucocorticoid receptor interacting protein 1 (GRIP1) (Figure 2) [81]. Similar to CAR, PXR also dimerizes with RXR and binds to two AGGTCA hexamers formed as DR 3-5, or ER6 motifs [84]. Accumulating evidence reveals that the ligand promiscuity of PXR stems from the unique structure of its LBD. The crystal structures of the hPXR LBD complexed with prototypical activators, including macrolide antibiotic rifampicin, St. John's Wort component hyperforin, and cholesterol-lowering drug SR12813, have provided important structural information that has dramatically increased our understanding of the structure-activity relationship of PXR [78, 90, 91]. The PXR LBD contains a rather large (1280-1600Å), flexible spherical ligand-binding pocket, and several unique structural features allow it to fit different sizes and types of ligands [78, 91]. The ligand-binding pocket of PXR is mostly made up of hydrophobic residues, but contains a few important polar residues which participate in hydrogen-bonding

interactions with ligands. Importantly, these features of the PXR ligand-binding pocket allow a single compound to dock in more than one orientation and contact different polar residues to exert its activity [83]. However, when the PXR LBD and a peptide of coactivator SRC-1 were co-crystallized with SR12813, the ligand was bound in only one conformation, showing the inherent limitations of crystallographic analysis [92].

Extensive efforts have been made to understand the structural basis that makes a compound a PXR activator. Although structural models based on the LBD of PXR have been developed, they have had limited success in predicting PXR activators. Diverse predictive models have also been developed for ligand-based computational methods, including machine learning techniques, pharmacophore modeling, and quantitative structure-activity relationship analysis, which have all been used to virtually screen compound libraries with varying rates of success [80]. As with CAR, many of these computational models are used in combination with binding and luciferase assays to validate potential PXR activators [18, 93]. One HTS assay developed for PXR used time-resolved FRET between fluorescent-tagged PXR LBD and coactivator SRC-1 to identify PXR activators, similar to those used in CAR activator screening [94]. Stable HepG2 cell lines expressing a reporter plasmid have also been developed for HTS and successfully used to identify PXR activators, but again, these immortalized cell lines do not exhibit metabolic capacity and therefore, will not necessarily predict physiologically-relevant PXR activators, showing the need for screening in human primary hepatocytes [95].

2.3. Indirect Activation of PXR

Although classical understanding of PXR activation necessitates that a ligand must be bound to impart transcriptional activity, emerging evidence indicates that the status of PXR activation can be influenced by different cellular signaling pathways. Ding and Staudinger observed that activation of protein kinase A (PKA) signaling by forskolin potentiates PXR-mediated induction of CYP3A gene expression in mouse hepatocytes [96]. On the other hand, increase of PKC activity by cytokines dramatically repressed PXR activity in both luciferase reporter and target gene expression assays [97]. These initial findings suggest that PXR activity is altered by signaling pathways that influence the phosphorylation status of PXR and/or its associated protein partners. Subsequently, utilization of computational analysis and site-directed mutagenesis approaches predicted 18 consensus phosphorylation sites of PXR that were further characterized *in vitro* [98]. A number of phosphomimetic mutants altered PXR transcriptional activity by affecting response element binding or coactivator binding, including a phosphomimetic mutation at Ser350 of PXR which inhibited its heterodimerization with RXR [99]. Interestingly, mutation of Thr248 to aspartate converts PXR to a constitutively active format that is irresponsive to ligand stimulation while mutations at Thr408 mimicking both phosphorylation (T408D) and lack thereof (T408A) abolished the ability of rifampicin to translocate PXR to the nucleus of CV-1 cells [98, 100]. Additionally, Sugatani et. al showed that the dephosphorylation of Thr290 by PP1 was essential to xenobiotic-induced nuclear translocation of PXR while phosphorylation of Thr290 by Ca^{2+} /calmodulin-dependent protein kinase II led to repression of PXR nuclear translocation, suggesting that phosphorylation status may alter the activity of PXR by affecting its subcellular localization as well [98, 101]. PXR has also been shown

to interact with a number of other signaling kinases and phosphatases, including PKA, PKC, cyclin-dependent kinases (Cdk), and the 70 kDa ribosomal protein S6 kinase (S6K), which all generate different effects on PXR activation [96, 97, 102-104]. For instance, PKC signaling represses PXR activity, potentially by strengthening PXR interaction with corepressor NcoR1, while PKA signaling enhances the recruitment of SRC-1 to PXR, potentiating target gene transcription [96, 97]. Notably, the protein phosphatase inhibitor OA can completely abolish PXR activity in both reporter assays and cultured hepatocytes, providing further evidence that protein phosphatases play role in PXR activation [97].

Indirect activation of PXR can also be exemplified by the Cdk signaling pathway. Chen and colleagues found that the small-molecule inhibitors of Cdk2, kenpaullone and roscovitine, induced PXR-mediated gene expression in HepG2 luciferase assays, while activation of the Cdk2 pathway led to repression of PXR-mediated CYP3A4 activation [102]. Kinase assays showed that Cdk2 phosphorylates the Ser350 residue of PXR *in vitro*. Notably, kenpaullone binds to PXR with moderate affinity compared to its robust activation of PXR activity, suggesting that direct ligand-binding may not be essential for PXR activity, particularly for chemicals that predominantly influence signaling pathways [102]. Further studies found that the flavonoids luteolin and apigenin could bind to PXR only at concentrations 10 μ M; however, these compounds significantly enhanced PXR-mediated CYP3A4 gene expression at concentrations lower than 10 μ M, indicating that signaling pathways may be involved in flavonoid-mediated PXR activation [105]. Mechanistic analysis revealed that Cdk5 phosphorylated PXR *in vitro* and expression of Cdk5 and its regulatory subunit repressed PXR activation by rifampicin in HepG2 cells, while this effect of Cdk5 on PXR was efficiently reversed by both the knockdown of Cdk5 as well as the addition of flavonoids that are known inhibitors of Cdk5 [105].

Collectively, accumulating evidence demonstrates that many factors can alter PXR's cellular localization and association with other proteins in addition to direct ligand-binding. Post-translational modification of PXR by signaling molecules clearly plays a crucial role in modulation of its biological function and represents a currently understudied area. Further studies are needed to fully characterize the mechanism of PXR indirect activation in more physiologically-relevant cell systems.

3. Aryl hydrocarbon receptor

Compared to the relatively short history of CAR and PXR, AhR has been a subject for research over the past 40 years and has proven to be crucial in diverse cellular processes. Early studies leading to the identification of AhR focused mainly on the aryl hydrocarbon hydroxylase activity of polychlorinated dibenzo-*p*-dioxins, like 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), highlighted by the fact that AhR was once named the TCDD receptor [106, 107]. To date, numerous ligands of AhR have been identified with environmental toxins constituting the vast majority of the potent activators [108]. The xenobiotic activators of AhR are mainly derived from two classes of compounds – polycyclic aromatic hydrocarbons (PAHs) such as 3-methylcholanthrene (3-MC), and halogenated aromatic hydrocarbons (HAHs) exemplified by TCDD which has extremely high affinity for AhR in comparison to most known ligands [109]. On the other hand,

searching for endogenous activators of AhR has only resulted in a short list of candidates. Among others, the tryptophan photo-derivative 6-formylindolo[3,2-b]carbazole (FICZ) was established as an endogenous ligand of AhR with the highest binding affinity reported to date [110].

The most well-established functions of AhR involve its roles in mediating metabolism of toxic compounds and tumor-promotion [111]. Upon activation, AhR enhances the expression of its target genes such as CYP1A1, CYP1A2, and CYP1B1, a number of UGT1A family members, and drug transporters such as breast cancer resistance protein (ABCG2) [13, 112]. For instance, known AhR activator benzo[a]pyrene induces the expression of CYP1A1, which in turn increases the biotransformation of benzo[a]pyrene, a procarcinogen, to benzo[a]pyren-diolepoxide, the ultimate mutagen [113]. In addition to modulating drug metabolism and disposition, accumulating evidence revealed that AhR also mediates the transcription of many genes participating immune responses [114]. Moreover, AhR was recently linked to obesity and energy metabolism [115]. Lu et al., reported that transgenic activation of AhR in the liver alleviated mice from HFD-induced obesity and insulin resistance likely through AhR-dependent activation of fibroblast growth factor 21 [116]. Thus, similar to CAR and PXR, AhR is also broadly involved in both xenobiotic metabolism and clearance, as well as endobiotic immune response and energy homeostasis. Regulation of gene expression at the transcriptional level by AhR also plays an important role influencing drug safety as well as disease development.

3.1. Activation of AhR

Although AhR does not belong to the NR superfamily, it functionally resembles both CAR and PXR, mediating the cellular response to xenobiotics by upregulating drug-metabolizing enzymes and transporters. AhR is a ligand-activated transcription factor of the PAS family, which facilitates the toxicological response to many environmental chemicals, clinically used drugs such as the proton-pump inhibitor omeprazole, and endogenous compounds like bilirubin [111, 112]. Unlike CAR and PXR, inactive AhR is predominantly expressed in the cytoplasm in nearly all tissues and immortalized cell lines, which may be due to the difference in cytoplasmic binding partners between the XRs [117]. AhR is sequestered in the cytoplasm by a protein complex containing HSP90, hepatitis B virus protein X-associated protein 2 (XAP2), and p23 in the absence of stimulation, and can be translocated to the nucleus in a ligand-dependent fashion which constitutes the first essential step in AhR activation [118, 119]. Interestingly, AhR translocates to the nucleus while still bound to HSP90, and this AhR:HSP90 complex is not affected by ligand-binding [120]. Once inside the nucleus, AhR breaks away from HSP90 and heterodimerizes with the aryl hydrocarbon nuclear translocator (ARNT) protein, enabling the AhR:ARNT complex to act as a high-affinity DNA binding chimera and activate target genes (Figure 3) [121].

The AhR protein is comprised of two multi-functional structural domains, the basic helix-loop-helix (bHLH) domain and the Per-ARNT-Sim (PAS) domain which can be further divided into the PAS-A and PAS-B domains based on particular functions. The bHLH domain at the amino-terminus of the protein contains the NLS and nuclear export signals (NES) that are essential to AhR translocation, functions in ARNT heterodimerization and

DNA-binding, and interacts with HSP90. The PAS-A domain of AhR aids in ARNT heterodimerization while the PAS-B domain mediates ligand binding and interacts with HSP90 [111, 112]. Interestingly, an AhR mutant with a deleted PAS domain fully localizes to the nucleus independent of ligand activation, indicating that the PAS domain may contain functional modules that help retain AhR in the cytoplasm and providing early evidence that AhR is activated by both ligand- and signal-dependent pathways [122].

3.1.1. Direct Activation of AhR—AhR can bind a wide array of chemicals, with the most potent xenobiotic activators identified thus far being the planar, hydrophobic PAHs and HAHs [108]. TCDD is the prototypical AhR activator and is by far the most potent xenobiotic ligand of AhR [109]. To this end, screening assays that measure AhR transcriptional activity have been developed in yeast expressing AhR/ARNT and HepG2 cells using luciferase reporter plasmids to identify new AhR activators [18, 123]. Recently, a stable AhR reporter HepG2 cell line has been developed and validated for HTS, similar to those generated for CAR and PXR [124]. Another method for determining whether a compound is a direct activator is a competition assay using TCDD to determine AhR ligand-binding. However, it must be noted that TCDD has an extremely high affinity for AhR and therefore, weak ligands are not able to compete with TCDD for binding [109]. Low-affinity ligands are hypothesized to interact with different residues in the AhR ligand binding pocket than high-affinity ligands like TCDD, which could explain the inability of weak activators to compete with TCDD for binding [125]. All of these methods can provide valuable information about potential activators, but these compounds must be further validated in physiologically-relevant systems even though the activation mechanism of AhR in immortalized cell lines is similar to that *in vivo*.

Although AhR has been studied for much longer than PXR or CAR, it has lagged behind in computational ligand identification due to the lack of a crystal structure of the AhR LBD to date. The first partial crystal structure of AhR, which contained the PAS-A domain of mouse AhR, was recently solved and has provided critical insight into the PAS-mediated heterodimerization of AhR with ARNT, which is important in DNA binding and target gene recognition [126]. However, the ligand-binding properties of AhR LBD are still largely unknown, and thus, most computational models of AhR rely on homology modeling. A number of homology models of AhR have been developed based on the crystal structure of hypoxia-inducible factor (HIF) 2 α [127-129]. Although functionally related, HIF2 α and AhR only share 25% sequence homology and therefore, the predictive abilities of these models are relatively limited even though some compound screenings have been successfully completed. A more robust computational method of virtual compound screening was developed using 3D-QSAR (quantitative structure activity relationship) models for AhR, which do not require structural information and instead rely on the vast amount of known AhR ligands to predict new activators [130]. Although this is a good method for screening vast libraries, the AhR LBD crystal structure should dramatically increase screening accuracy and provide information on the conformational changes required for direct AhR activation.

3.1.2. Indirect Activation of AhR—Although anecdotal evidence of indirect activation of AhR by signaling cascades has been around for years, only recently have mechanisms for AhR activation independent of direct ligand-binding come to the forefront of this field. One of the first observations that led to the discovery of an indirect activation mechanism for AhR was that the known activator omeprazole does not directly bind to AhR and instead mediates its effects through indirect mechanisms [131, 132]. The exact mechanism of AhR activation by omeprazole was further investigated in the rat hepatoma H4IIE cell line, leading to the important discovery that both genistein, a tyrosine kinase inhibitor (TKI), and daidzein, a casein kinase II inhibitor, were able to inhibit the indirect activation of AhR by omeprazole, but not the direct activation by TCDD [133]. Additionally, insulin pretreatment also abolished AhR activation by omeprazole while only mildly affecting direct activation by TCDD [133]. These results suggest that different signaling pathways are involved in the translocation and activation of AhR by direct and indirect activators.

Cyclic AMP (cAMP) is an important second messenger in all cells, acting as an energy sensor that mediates numerous endocrine signaling cascades. Recently, cAMP has been shown to mediate subcellular localization of AhR, as increased levels of cAMP efficiently translocate AhR to the nucleus. However, cAMP- and TCDD-mediated mechanisms of AhR activation are distinct from each other and induce different conformational changes of the AhR protein structure, where cAMP-activated AhR is not a structurally-favorable binding partner for ARNT [134]. Instead, AhR may adopt novel structural changes in response to cAMP, favoring ligand-independent protein-protein interactions and gene activation over the traditional mechanism.

Another possible pathway that could have an impact on AhR activation is the protein tyrosine kinase pathway, since omeprazole activation of AhR was potently inhibited with a typical TKI [133]. This pathway was further characterized by using specific inhibitors and a dominant negative form of *c-src* kinase to interrogate its role in AhR activation [135]. Both direct and indirect activation of AhR by TCDD and omeprazole were ameliorated when *c-src* signaling was inhibited, which implies that *c-src* is an essential mediator of overall AhR function. In addition, mutation of the Tyr322 residue on AhR abolishes the ability of omeprazole to activate AhR, providing further evidence that this phenomenon is mediated by a tyrosine kinase-dependent pathway [136]. However, the TKI Sunitinib has recently been shown to indirectly activate AhR independently of ligand-binding, showing that the activation of AhR through the protein tyrosine kinase signaling cascade is probably complex and mediated by specific members of the protein tyrosine kinase family [137].

4. Conclusion

It is now evident that activation of the XRs CAR, PXR, and AhR can occur through two distinct mechanisms: the direct activation mechanism, which involves ligand binding to induce a conformational change to the active form of the XR; and the indirect activation mechanism, which involves in the alteration of cellular signaling to change the phosphorylation status of XRs without direct ligand interaction with the receptors. The indirect activation mechanisms of CAR and AhR have been widely studied and accepted as an important route of activation, while this phenomenon is quite controversial for PXR even

though emerging evidence suggests that indirect activation of PXR is possible. It is imperative that the indirect activation mechanism of PXR is elucidated, as it could play a major role in predicting unknown DDIs. There is also a dire need for more physiologically-relevant screening methods for all of these receptors which can accurately identify activators of these XRs. One of the most important features of a physiologically-relevant screening system is the inclusion of phase I and II metabolic capacities, as compounds that activate reporter assays in immortalized cells might be extensively metabolized, while those that are not considered activators may be metabolically activated. Unfortunately, using human primary hepatocytes for compound screening is cost-prohibitive and are better used in the validation of potential activators. New cell models like HepaRG cells that are metabolically competent and express relevant transporters may be an optimal system to bridge the gap between immortalized cell lines and primary hepatocytes, perhaps through the creation of stable HepaRG cell lines containing reporter plasmids for each XR [138].

Overall, there is a significant need to better understand the signaling mechanisms that govern the activation status of CAR, PXR, and AhR because it could have a profound effect on everything from DDIs and toxicity response to energy metabolism and cell growth. The ability of these receptors to be activated in the absence of a ligand also suggests that they might not be “orphan” receptors after all, and instead could have endogenous physiologically-relevant ligands that can activate them in the absence of a direct activator. Hopefully we will gain a more thorough understanding of all aspects of these XRs over the next few years, but in our opinion, the most consequential discoveries will be made outside of the “traditional” activation mechanisms and gene regulation of these XRs.

Acknowledgments

This work was supported by NIH grants R01 DK061652 and R01 GM107058.

ABBREVIATIONS

AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon nuclear translocator
CAR	constitutive androstane receptor
CCRP	cytoplasmic retention protein
CITCO	6-(4-chlorophenyl)imidazo [2,1-beta][1,3]thiazole-5-carbaldehyde- <i>O</i> -(3,4-dichlorobenzyl)oxime
DBD	DNA-binding domain
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FRET	fluorescence resonance energy transfer
GRIP1	glucocorticoid receptor interacting protein 1

HSP	heat-shock protein
LBD	ligand-binding domain
MEK	mitogen-activated protein kinase kinase
NcoR1	nuclear receptor co-repressor 1
NR	nuclear receptor
OA	okadaic acid
PB	phenobarbital
PKA	protein kinase A
PKC	protein kinase C
PP	protein phosphatase
PXR	pregnane x receptor
RXR	retinoid x receptor
SMRT	silencing mediator of retinoid and thyroid receptors
SRC-1	nuclear receptor coactivator 1
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)] benzene
TKI	tyrosine-kinase inhibitor
XR	xenobiotic receptors

References

- [1]. Forman BM, Evans RM. Nuclear hormone receptors activate direct, inverted, and everted repeats. *Ann N Y Acad Sci.* 1995; 761:29–37. [PubMed: 7625727]
- [2]. Nagy L, Schwabe JWR. Mechanism of the nuclear receptor molecular switch. *Trends in Biochemical Sciences.* 2004; 29:317–324. [PubMed: 15276186]
- [3]. McKenna NJ, Lanz RB, O'Malley BW. Nuclear Receptor Coregulators: Cellular and Molecular Biology. *Endocrine Reviews.* 1999; 20:321–344. [PubMed: 10368774]
- [4]. Sonoda J, Pei L, Evans RM. Nuclear receptors: Decoding metabolic disease. *FEBS Letters.* 2008; 582:2–9. [PubMed: 18023286]
- [5]. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM. Bile acids: natural ligands for an orphan nuclear receptor. *Science.* 1999; 284:1365–1368. [PubMed: 10334993]
- [6]. Wang H, LeCluyse E. Role of Orphan Nuclear Receptors in the Regulation of Drug-Metabolising Enzymes. *Clinical Pharmacokinetics.* 2003; 42:1331–1357. [PubMed: 14674787]
- [7]. Tolson AH, Wang H. Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Advanced Drug Delivery Reviews.* 2010; 62:1238–1249. [PubMed: 20727377]
- [8]. Burbach KM, Poland A, Bradfield CA. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc Natl Acad Sci U S A.* 1992; 89:8185–8189. [PubMed: 1325649]

- [9]. Gerbal-Chaloin S, Pichard-Garcia L, Fabre J-M, Sa-Cunha A, Poellinger L, Maurel P, Daujat-Chavanieu M. Role of CYP3A4 in the regulation of the aryl hydrocarbon receptor by omeprazole sulphide. *Cellular Signalling*. 2006; 18:740–750. [PubMed: 16109480]
- [10]. Sugatani J, Kojima H, Ueda A, Kakizaki S, Yoshinari K, Gong Q-H, Owens IS, Negishi M, Sueyoshi T. The phenobarbital response enhancer module in the human bilirubin UDP-glucuronosyltransferase UGT1A1 gene and regulation by the nuclear receptor CAR. *Hepatology*. 2001; 33:1232–1238. [PubMed: 11343253]
- [11]. Xie W, Yeuh M-F, Radominska-Pandya A, Saini SPS, Negishi Y, Bottroff BS, Cabrera GY, Tukey RH, Evans RM. Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. *Proceedings of the National Academy of Sciences*. 2003; 100:4150–4155.
- [12]. Yueh M-F, Huang Y-H, Hiller A, Chen S, Nguyen N, Tukey RH. Involvement of the Xenobiotic Response Element (XRE) in Ah Receptor-mediated Induction of Human UDP-glucuronosyltransferase 1A1. *Journal of Biological Chemistry*. 2003; 278:15001–15006. [PubMed: 12566446]
- [13]. Tompkins LM, Li H, Li L, Lynch C, Xie Y, Nakanishi T, Ross DD, Wang H. A novel xenobiotic responsive element regulated by aryl hydrocarbon receptor is involved in the induction of BCRP/ABCG2 in LS174T cells. *Biochem Pharmacol*. 2010; 80:1754–1761. [PubMed: 20804740]
- [14]. Wang X, Sykes DB, Miller DS. Constitutive Androstane Receptor-Mediated Up-Regulation of ATP-Driven Xenobiotic Efflux Transporters at the Blood-Brain Barrier. *Molecular Pharmacology*. 2010; 78:376–383. [PubMed: 20547735]
- [15]. Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y, Fardel O. Differential Regulation of Sinusoidal and Canalicular Hepatic Drug Transporter Expression by Xenobiotics Activating Drug-Sensing Receptors in Primary Human Hepatocytes. *Drug Metabolism and Disposition*. 2006; 34:1756–1763. [PubMed: 16837569]
- [16]. Li L, Stanton JD, Tolson AH, Luo Y, Wang H. Bioactive Terpenoids and Flavonoids from Ginkgo Biloba Extract Induce the Expression of Hepatic Drug-Metabolizing Enzymes Through Pregnane X Receptor, Constitutive Androstane Receptor, and Aryl hydrocarbon Receptor-Mediated Pathways. *Pharmaceutical Research*. 2008; 26:872–882. [PubMed: 19034627]
- [17]. Al-Salman F, Plant N. Non-coplanar polychlorinated biphenyls (PCBs) are direct agonists for the human pregnane-X receptor and constitutive androstane receptor, and activate target gene expression in a tissue-specific manner. *Toxicology and Applied Pharmacology*. 2012; 263:7–13. [PubMed: 22664347]
- [18]. Raucy JL, Lasker JM. Cell-based systems to assess nuclear receptor activation and their use in drug development. *Drug Metabolism Reviews*. 2013; 45:101–109. [PubMed: 23330544]
- [19]. Baes M, Gulick T, Choi HS, Martinoli MG, Simha D, Moore DD. A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Molecular and Cellular Biology*. 1994; 14:1544–1552. [PubMed: 8114692]
- [20]. Choi H-S, Chung M, Tzamelis I, Simha D, Lee Y-K, Seol W, Moore DD. Differential Transactivation by Two Isoforms of the Orphan Nuclear Hormone Receptor CAR. *Journal of Biological Chemistry*. 1997; 272:23565–23571. [PubMed: 9295294]
- [21]. Yoshinari K, Sueyoshi T, Moore R, Negishi M. Nuclear Receptor CAR as a Regulatory Factor for the Sexually Dimorphic Induction of CYP2B1 Gene by Phenobarbital in Rat Livers. *Molecular Pharmacology*. 2001; 59:278–284. [PubMed: 11160864]
- [22]. Kobayashi K, Sueyoshi T, Inoue K, Moore R, Negishi M. Cytoplasmic Accumulation of the Nuclear Receptor CAR by a Tetratricopeptide Repeat Protein in HepG2 Cells. *Molecular Pharmacology*. 2003; 64:1069–1075. [PubMed: 14573755]
- [23]. Yoshinari K, Kobayashi K, Moore R, Kawamoto T, Negishi M. Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital. *FEBS Letters*. 2003; 548:17–20. [PubMed: 12885400]
- [24]. Timsit YE, Negishi M. Coordinated regulation of nuclear receptor CAR by CCRP/DNAJC7, HSP70 and the ubiquitin-proteasome system. *PLoS One*. 2014; 9:e96092. [PubMed: 24789201]

- [25]. Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, Negishi M. Phenobarbital-Responsive Nuclear Translocation of the Receptor CAR in Induction of the CYP2B Gene. *Molecular and Cellular Biology*. 1999; 19:6318–6322. [PubMed: 10454578]
- [26]. Kanno Y, Suzuki M, Nakahama T, Inouye Y. Characterization of nuclear localization signals and cytoplasmic retention region in the nuclear receptor CAR. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2005; 1745:215–222. [PubMed: 16055207]
- [27]. Qatanani M, Moore DD. CAR, the continuously advancing receptor, in drug metabolism and disease. *Current Drug Metabolism*. 2005; 6:329–339. [PubMed: 16101572]
- [28]. Stanley L, Horsburgh B, Ross J, Scheer N, Roland Wolf C. PXR and CAR: Nuclear Receptors which Play a Pivotal Role in Drug Disposition and Chemical Toxicity. *Drug Metabolism Reviews*. 2006; 38:515–597. [PubMed: 16877263]
- [29]. Dong B, Saha PK, Huang W, Chen W, Abu-Elheiga LA, Wakil SJ, Stevens RD, Ilkayeva O, Newgard CB, Chan L, Moore DD. Activation of nuclear receptor CAR ameliorates diabetes and fatty liver disease. *Proceedings of the National Academy of Sciences*. 2009; 106:18831–18836.
- [30]. Gao J, He J, Zhai Y, Wada T, Xie W. The Constitutive Androstane Receptor Is an Anti-obesity Nuclear Receptor That Improves Insulin Sensitivity. *Journal of Biological Chemistry*. 2009; 284:25984–25992. [PubMed: 19617349]
- [31]. Masuyama H, Hiramatsu Y. Treatment with a constitutive androstane receptor ligand ameliorates the signs of preeclampsia in high-fat diet-induced obese pregnant mice. *Molecular and Cellular Endocrinology*. 2012; 348:120–127. [PubMed: 21839802]
- [32]. Huang W, Zhang J, Washington M, Liu J, Parant JM, Lozano G, Moore DD. Xenobiotic Stress Induces Hepatomegaly and Liver Tumors via the Nuclear Receptor Constitutive Androstane Receptor. *Molecular Endocrinology*. 2005; 19:1646–1653. [PubMed: 15831521]
- [33]. Honkakoski P, Zelko I, Sueyoshi T, Negishi M. The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol*. 1998; 18:5652–5658. [PubMed: 9742082]
- [34]. Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL, Kliewer SA. Orphan Nuclear Receptors Constitutive Androstane Receptor and Pregnane X Receptor Share Xenobiotic and Steroid Ligands. *Journal of Biological Chemistry*. 2000; 275:15122–15127. [PubMed: 10748001]
- [35]. Li H, Chen T, Cottrell J, Wang H. Nuclear Translocation of Adenoviral-Enhanced Yellow Fluorescent Protein-Tagged-Human Constitutive Androstane Receptor (hCAR): A Novel Tool for Screening hCAR Activators in Human Primary Hepatocytes. *Drug Metabolism and Disposition*. 2009; 37:1098–1106. [PubMed: 19196842]
- [36]. Mäkinen J, Frank C, Jyrkkärinne J, Gynther J, Carlberg C, Honkakoski P. Modulation of Mouse and Human Phenobarbital-Responsive Enhancer Module by Nuclear Receptors. *Molecular Pharmacology*. 2002; 62:366–378. [PubMed: 12130690]
- [37]. Forman BM, Tzamei I, Choi H-S, Chen J, Simha D, Seol W, Evans RM, Moore DD. Androstane metabolites bind to and deactivate the nuclear receptor CAR-[beta]. *Nature*. 1998; 395:612–615. [PubMed: 9783588]
- [38]. Tzamei I, Pissios P, Schuetz EG, Moore DD. The Xenobiotic Compound 1,4-Bis[2-(3,5-Dichloropyridyloxy)]Benzene Is an Agonist Ligand for the Nuclear Receptor CAR. *Molecular and Cellular Biology*. 2000; 20:2951–2958. [PubMed: 10757780]
- [39]. Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, Negishi M. The Repressed Nuclear Receptor CAR Responds to Phenobarbital in Activating the Human CYP2B6 Gene. *Journal of Biological Chemistry*. 1999; 274:6043–6046. [PubMed: 10037683]
- [40]. Maglich JM, Parks DJ, Moore LB, Collins JL, Goodwin B, Billin AN, Stoltz CA, Kliewer SA, Lambert MH, Willson TM, Moore JT. Identification of a Novel Human Constitutive Androstane Receptor (CAR) Agonist and Its Use in the Identification of CAR Target Genes. *Journal of Biological Chemistry*. 2003; 278:17277–17283. [PubMed: 12611900]
- [41]. Swales K, Kakizaki S, Yamamoto Y, Inoue K, Kobayashi K, Negishi M. Novel CAR-mediated Mechanism for Synergistic Activation of Two Distinct Elements within the Human Cytochrome P450 2B6 Gene in HepG2 Cells. *Journal of Biological Chemistry*. 2005; 280:3458–3466. [PubMed: 15563456]

- [42]. Yamamoto Y, Kawamoto T, Negishi M. The role of the nuclear receptor CAR as a coordinate regulator of hepatic gene expression in defense against chemical toxicity. *Arch Biochem Biophys*. 2003; 409:207–211. [PubMed: 12464260]
- [43]. Dussault I, Lin M, Hollister K, Fan M, Termini J, Sherman MA, Forman BM. A Structural Model of the Constitutive Androstane Receptor Defines Novel Interactions That Mediate Ligand-Independent Activity. *Molecular and Cellular Biology*. 2002; 22:5270–5280. [PubMed: 12101224]
- [44]. Suino K, Peng L, Reynolds R, Li Y, Cha J-Y, Repa JJ, Kliewer SA, Xu HE. The Nuclear Xenobiotic Receptor CAR: Structural Determinants of Constitutive Activation and Heterodimerization. *Molecular Cell*. 2004; 16:893–905. [PubMed: 15610733]
- [45]. Andersin T, Väisänen S, Carlberg C. The Critical Role of Carboxy-Terminal Amino Acids in Ligand-Dependent and -Independent Transactivation of the Constitutive Androstane Receptor. *Molecular Endocrinology*. 2003; 17:234–246. [PubMed: 12554751]
- [46]. Xu RX, Lambert MH, Wisely BB, Warren EN, Weinert EE, Waitt GM, Williams JD, Collins JL, Moore LB, Willson TM, Moore JT. A Structural Basis for Constitutive Activity in the Human CAR/RXR α Heterodimer. *Molecular Cell*. 2004; 16:919–928. [PubMed: 15610735]
- [47]. Li L, Chen T, Stanton JD, Sueyoshi T, Negishi M, Wang H. The Peripheral Benzodiazepine Receptor Ligand 1-(2-Chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide Is a Novel Antagonist of Human Constitutive Androstane Receptor. *Molecular Pharmacology*. 2008; 74:443–453. [PubMed: 18492798]
- [48]. Lynch C, Pan Y, Li L, Ferguson S, Xia M, Swaan P, Wang H. Identification of Novel Activators of Constitutive Androstane Receptor from FDA-Approved Drugs by Integrated Computational and Biological Approaches. *Pharmaceutical Research*. 2013; 30:489–501. [PubMed: 23090669]
- [49]. MÄKINEN J, REINISALO M, NIEMI K, VIITALA P, JYRKÄÄRINNE J, CHUNG H, PELKONEN O, HONKAKOSKI P. Dual action of oestrogens on the mouse constitutive androstane receptor. *Biochemical Journal*. 2003; 376:465–472. [PubMed: 12948398]
- [50]. Carazo A, Pavék P. The Use of the LanthaScreen TR-FRET CAR Coactivator Assay in the Characterization of Constitutive Androstane Receptor (CAR) Inverse Agonists. *Sensors (Basel)*. 2015; 15:9265–9276. [PubMed: 25905697]
- [51]. Arnold KA, Eichelbaum M, Burk O. Alternative splicing affects the function and tissue-specific expression of the human constitutive androstane receptor. *Nuclear Receptor*. 2004; 2:1–16. [PubMed: 15043764]
- [52]. Lamba JK, Lamba V, Yasuda K, Lin YS, Assem M, Thompson E, Strom S, Schuetz E. Expression of Constitutive Androstane Receptor Splice Variants in Human Tissues and Their Functional Consequences. *Journal of Pharmacology and Experimental Therapeutics*. 2004; 311:811–821. [PubMed: 15194709]
- [53]. Auerbach SS, Ramsden R, Stoner MA, Verlinde C, Hassett C, Omiecinski CJ. Alternatively spliced isoforms of the human constitutive androstane receptor. *Nucleic Acids Research*. 2003; 31:3194–3207. [PubMed: 12799447]
- [54]. Chen T, Tompkins LM, Li L, Li H, Kim G, Zheng Y, Wang H. A Single Amino Acid Controls the Functional Switch of Human Constitutive Androstane Receptor (CAR) 1 to the Xenobiotic-Sensitive Splicing Variant CAR3. *Journal of Pharmacology and Experimental Therapeutics*. 2010; 332:106–115. [PubMed: 19820207]
- [55]. Kanno Y, Inouye Y. A consecutive three alanine residue insertion mutant of human CAR: a novel CAR ligand screening system in HepG2 cells. *The Journal Of Toxicological Sciences*. 2010; 35:515–525. [PubMed: 20686338]
- [56]. DeKeyser JG, Stagliano MC, Auerbach SS, Prabhu KS, Jones AD, Omiecinski CJ. Di(2-ethylhexyl) phthalate Is a Highly Potent Agonist for the Human Constitutive Androstane Receptor Splice Variant CAR2. *Molecular Pharmacology*. 2009; 75:1005–1013. [PubMed: 19211671]
- [57]. DeKeyser JG, Laurenzana EM, Peterson EC, Chen T, Omiecinski CJ. Selective Phthalate Activation of Naturally Occurring Human Constitutive Androstane Receptor Splice Variants and the Pregnane X Receptor. *Toxicological Sciences*. 2011; 120:381–391. [PubMed: 21227907]

- [58]. Omiecinski CJ, Coslo DM, Chen T, Laurenzana EM, Peffer RC. Multi-species Analyses of Direct Activators of the Constitutive Androstane Receptor. *Toxicological Sciences*. 2011; 123:550–562. [PubMed: 21778469]
- [59]. Lynch C, Zhao J, Huang R, Xiao J, Li L, Heyward S, Xia M, Wang H. Quantitative High-Throughput Identification of Drugs as Modulators of Human Constitutive Androstane Receptor. *Scientific Reports*. 2015; 5:10405. [PubMed: 25993555]
- [60]. Burk O, Arnold KA, Nussler AK, Schaeffeler E, Efimova E, Avery BA, Avery MA, Fromm MF, Eichelbaum M. Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. *Mol Pharmacol*. 2005; 67:1954–1965. [PubMed: 15761118]
- [61]. Cherian MT, Lin W, Wu J, Chen T. CINPA1 Is an Inhibitor of Constitutive Androstane Receptor That Does Not Activate Pregnane X Receptor. *Molecular Pharmacology*. 2015; 87:878–889. [PubMed: 25762023]
- [62]. Li L, Hassan HE, Tolson AH, Ferguson SS, Eddington ND, Wang H. Differential Activation of Pregnane X Receptor and Constitutive Androstane Receptor by Buprenorphine in Primary Human Hepatocytes and HepG2 Cells. *Journal of Pharmacology and Experimental Therapeutics*. 2010; 335:562–571. [PubMed: 20829393]
- [63]. Hosseinpour F, Moore R, Negishi M, Sueyoshi T. Serine 202 Regulates the Nuclear Translocation of Constitutive Active/Androstane Receptor. *Molecular Pharmacology*. 2006; 69:1095–1102. [PubMed: 16377764]
- [64]. Zelko I, Negishi M. Phenobarbital-Elicited Activation of Nuclear Receptor CAR in Induction of Cytochrome P450 Genes. *Biochemical and Biophysical Research Communications*. 2000; 277:1–6. [PubMed: 11027630]
- [65]. Yoshinari K, Kobayashi K, Moore R, Kawamoto T, Negishi M. Identification of the nuclear receptor CAR:HSP90 complex in mouse liver and recruitment of protein phosphatase 2A in response to phenobarbital. *FEBS Letters*. 2003; 548:17–20. [PubMed: 12885400]
- [66]. Zelko I, Sueyoshi T, Kawamoto T, Moore R, Negishi M. The peptide near the C terminus regulates receptor CAR nuclear translocation induced by xenochemicals in mouse liver. *Mol Cell Biol*. 2001; 21:2838–2846. [PubMed: 11283262]
- [67]. Chen T, Laurenzana Elizabeth M, Coslo Denise M, Chen F, Omiecinski Curtis J. Proteasomal interaction as a critical activity modulator of the human constitutive androstane receptor. *Biochemical Journal*. 2014; 458:95–107. [PubMed: 24224465]
- [68]. Bauer D, Wolfram N, Kahl GF, Hirsch-Ernst KI. Transcriptional Regulation of CYP2B1 Induction in Primary Rat Hepatocyte Cultures: Repression by Epidermal Growth Factor Is Mediated via a Distal Enhancer Region. *Molecular Pharmacology*. 2004; 65:172–180. [PubMed: 14722249]
- [69]. Joannard F, Rissel M, Gilot D, Anderson A, Orfila-Lefevre L, Guillouzo A, Atfi A, Lagadic-Gossmann D. Role for mitogen-activated protein kinases in phenobarbital-induced expression of cytochrome P450 2B in primary cultures of rat hepatocytes. *Toxicology Letters*. 2006; 161:61–72. [PubMed: 16154717]
- [70]. Koike C, Moore R, Negishi M. Extracellular Signal-Regulated Kinase Is an Endogenous Signal Retaining the Nuclear Constitutive Active/Androstane Receptor (CAR) in the Cytoplasm of Mouse Primary Hepatocytes. *Molecular Pharmacology*. 2007; 71:1217–1221. [PubMed: 17314319]
- [71]. Mutoh S, Osabe M, Inoue K, Moore R, Pedersen L, Perera L, Reboloso Y, Sueyoshi T, Negishi M. Dephosphorylation of Threonine 38 Is Required for Nuclear Translocation and Activation of Human Xenobiotic Receptor CAR (NR1I3). *Journal of Biological Chemistry*. 2009; 284:34785–34792. [PubMed: 19858220]
- [72]. Osabe M, Negishi M. Active ERK1/2 Protein Interacts with the Phosphorylated Nuclear Constitutive Active/Androstane Receptor (CAR; NR1I3), Repressing Dephosphorylation and Sequestering CAR in the Cytoplasm. *Journal of Biological Chemistry*. 2011; 286:35763–35769. [PubMed: 21873423]
- [73]. Mutoh S, Sobhany M, Moore R, Perera L, Pedersen L, Sueyoshi T, Negishi M. Phenobarbital Indirectly Activates the Constitutive Active Androstane Receptor (CAR) by Inhibition of

Epidermal Growth Factor Receptor Signaling. *Science Signaling*. 2013; 6:ra31–ra31. [PubMed: 23652203]

- [74]. Carazo Fernández A, Smutny T, Hyrsová L, Berka K, Pavek P. Chrysin, baicalein and galangin are indirect activators of the human constitutive androstane receptor (CAR). *Toxicology Letters*. 2015; 233:68–77. [PubMed: 25625231]
- [75]. Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterström RH, Perlmann T, Lehmann JM. An Orphan Nuclear Receptor Activated by Pregnanes Defines a Novel Steroid Signaling Pathway. *Cell*. 92:73–82. [PubMed: 9489701]
- [76]. Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci U S A*. 1998; 95:12208–12213. [PubMed: 9770465]
- [77]. Blumberg B, Sabbagh W Jr, Juguilon H, Bolado J Jr, van Meter CM, Ong ES, Evans RM. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev*. 1998; 12:3195–3205. [PubMed: 9784494]
- [78]. Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA, Redinbo MR. The Human Nuclear Xenobiotic Receptor PXR: Structural Determinants of Directed Promiscuity. *Science*. 2001; 292:2329–2333. [PubMed: 11408620]
- [79]. Ngan C-H, Beglov D, Rudnitskaya AN, Kozakov D, Waxman DJ, Vajda S. The Structural Basis of Pregnane X Receptor Binding Promiscuity. *Biochemistry*. 2009; 48:11572–11581. [PubMed: 19856963]
- [80]. Ekins S, Kortagere S, Iyer M, Reschly EJ, Lill MA, Redinbo MR, Krasowski MD. Challenges predicting ligand-receptor interactions of promiscuous proteins: the nuclear receptor PXR. *PLoS Comput Biol*. 2009; 5:e1000594. [PubMed: 20011107]
- [81]. di Masi A, Marinis ED, Ascenzi P, Marino M. Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. *Molecular Aspects of Medicine*. 2009; 30:297–343. [PubMed: 19427329]
- [82]. Chang TKH, Waxman D. Synthetic Drugs and Natural Products as Modulators of Constitutive Androstane Receptor (Car) and Pregnane X Receptor (PXR). *Drug Metabolism Reviews*. 2006; 38:51–73. [PubMed: 16684648]
- [83]. Ostberg T, Bertilsson G, Jendeberg L, Berkenstam A, Uppenberg J. Identification of residues in the PXR ligand binding domain critical for species specific and constitutive activation. *Eur J Biochem*. 2002; 269:4896–4904. [PubMed: 12354121]
- [84]. Orans J, Teotico DG, Redinbo MR. The Nuclear Xenobiotic Receptor Pregnane X Receptor: Recent Insights and New Challenges. *Molecular Endocrinology*. 2005; 19:2891–2900. [PubMed: 15961506]
- [85]. Savas Ü, Wester MR, Griffin KJ, Johnson EF. Rabbit Pregnane X Receptor Is Activated by Rifampicin. *Drug Metabolism and Disposition*. 2000; 28:529–537. [PubMed: 10772631]
- [86]. Krasowski MD, Ni A, Hagey LR, Ekins S. Evolution of promiscuous nuclear hormone receptors: LXR, FXR, VDR, PXR, and CAR. *Molecular and Cellular Endocrinology*. 2011; 334:39–48. [PubMed: 20615451]
- [87]. Saradhi M, Sengupta A, Mukhopadhyay G, Tyagi RK. Pregnane and Xenobiotic Receptor (PXR/SXR) resides predominantly in the nuclear compartment of the interphase cell and associates with the condensed chromosomes during mitosis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2005; 1746:85–94. [PubMed: 16297466]
- [88]. Squires EJ, Sueyoshi T, Negishi M. Cytoplasmic Localization of Pregnane X Receptor and Ligand-dependent Nuclear Translocation in Mouse Liver. *Journal of Biological Chemistry*. 2004; 279:49307–49314. [PubMed: 15347657]
- [89]. Kawana K, Ikuta T, Kobayashi Y, Gotoh O, Takeda K, Kawajiri K. Molecular Mechanism of Nuclear Translocation of an Orphan Nuclear Receptor, SXR. *Molecular Pharmacology*. 2003; 63:524–531. [PubMed: 12606758]
- [90]. Watkins RE, Maglich JM, Moore LB, Wisely GB, Noble SM, Davis-Searles PR, Lambert MH, Kliewer SA, Redinbo MR. 2.1 Å Crystal Structure of Human PXR in Complex with the St. John's Wort Compound Hyperforin†,‡. *Biochemistry*. 2003; 42:1430–1438. [PubMed: 12578355]

- [91]. Chrencik JE, Orans J, Moore LB, Xue Y, Peng L, Collins JL, Wisely GB, Lambert MH, Klierer SA, Redinbo MR. Structural Disorder in the Complex of Human Pregnane X Receptor and the Macrolide Antibiotic Rifampicin. *Molecular Endocrinology*. 2005; 19:1125–1134. [PubMed: 15705662]
- [92]. Watkins RE, Davis-Searles PR, Lambert MH, Redinbo MR. Coactivator Binding Promotes the Specific Interaction Between Ligand and the Pregnane X Receptor. *Journal of Molecular Biology*. 2003; 331:815–828. [PubMed: 12909012]
- [93]. Xiao L, Nickbarg E, Wang W, Thomas A, Ziebell M, Prosise WW, Lesburg CA, Taremi SS, Gerlach VL, Le HV, Cheng KC. Evaluation of in vitro PXR-based assays and in silico modeling approaches for understanding the binding of a structurally diverse set of drugs to PXR. *Biochemical Pharmacology*. 2011; 81:669–679. [PubMed: 21145880]
- [94]. Shukla SJ, Nguyen D-T, MacArthur R, Simeonov A, Frazee WJ, Hallis TM, Marks BD, Singh U, Eliason HC, Printen J, Austin CP, Inglese J, Auld DS. Identification of Pregnane X Receptor Ligands Using Time-Resolved Fluorescence Resonance Energy Transfer and Quantitative High-Throughput Screening. *Assay and Drug Development Technologies*. 2009; 7:143–169. [PubMed: 19505231]
- [95]. Ratajewski M, Grzelak I, Wi niewska K, Ryba K, Gorzkiewicz M, Walczak-Drzewiecka A, Hoffmann M, Dastyh J. Screening of a chemical library reveals novel PXR-activating pharmacologic compounds. *Toxicology Letters*. 2015; 232:193–202. [PubMed: 25455453]
- [96]. Ding X, Staudinger JL. Induction of Drug Metabolism by Forskolin: The Role of the Pregnane X Receptor and the Protein Kinase A Signal Transduction Pathway. *Journal of Pharmacology and Experimental Therapeutics*. 2005; 312:849–856. [PubMed: 15459237]
- [97]. Ding X, Staudinger JL. Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. *Biochemical Pharmacology*. 2005; 69:867–873. [PubMed: 15710363]
- [98]. Lichti-Kaiser K, Brobst D, Xu C, Staudinger JL. A Systematic Analysis of Predicted Phosphorylation Sites within the Human Pregnane X Receptor Protein. *Journal of Pharmacology and Experimental Therapeutics*. 2009; 331:65–76. [PubMed: 19617467]
- [99]. Wang Y-M, Chai SC, Lin W, Chai X, Elias A, Wu J, Ong SS, Pondugula SR, Beard JA, Schuetz EG, Zeng S, Xie W, Chen T. Serine 350 of human pregnane X receptor is crucial for its heterodimerization with retinoid X receptor alpha and transactivation of target genes in vitro and in vivo. *Biochemical Pharmacology*. 2015; 96:357–368. [PubMed: 26119819]
- [100]. Dorcakova A, Novotna A, Vrzal R, Pavek P, Dvorak Z. The role of residues T248, Y249 and T422 in the function of human pregnane X receptor. *Archives of Toxicology*. 2013; 87:291–301. [PubMed: 22976785]
- [101]. Sugatani J, Hattori Y, Noguchi Y, Yamaguchi M, Yamazaki Y, Ikari A. Threonine-290 Regulates Nuclear Translocation of the Human Pregnane X Receptor through Its Phosphorylation/Dephosphorylation by Ca²⁺/Calmodulin-Dependent Protein Kinase II and Protein Phosphatase 1. *Drug Metabolism and Disposition*. 2014; 42:1708–1718. [PubMed: 25074870]
- [102]. Lin W, Wu J, Dong H, Bouck D, Zeng F-Y, Chen T. Cyclin-dependent Kinase 2 Negatively Regulates Human Pregnane X Receptor-mediated CYP3A4 Gene Expression in HepG2 Liver Carcinoma Cells. *Journal of Biological Chemistry*. 2008; 283:30650–30657. [PubMed: 18784074]
- [103]. Pondugula SR, Brimer-Cline C, Wu J, Schuetz EG, Tyagi RK, Chen T. A Phosphomimetic Mutation at Threonine-57 Abolishes Transactivation Activity and Alters Nuclear Localization Pattern of Human Pregnane X Receptor. *Drug Metabolism and Disposition*. 2009; 37:719–730. [PubMed: 19171678]
- [104]. Elias A, High AA, Mishra A, Ong SS, Wu J, Peng J, Chen T. Identification and characterization of phosphorylation sites within the pregnane X receptor protein. *Biochemical Pharmacology*. 2014; 87:360–370. [PubMed: 24184507]
- [105]. Dong H, Lin W, Wu J, Chen T. Flavonoids activate pregnane × receptor-mediated CYP3A4 gene expression by inhibiting cyclin-dependent kinases in HepG2 liver carcinoma cells. *BMC Biochemistry*. 2010; 11:1–9. [PubMed: 20044937]
- [106]. Poland A, Glover E, Kende AS. Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. Evidence that the binding species is receptor for

induction of aryl hydrocarbon hydroxylase. The Journal Of Biological Chemistry. 1976; 251:4936–4946. [PubMed: 956169]

- [107]. Carlstedt-Duke J, Elfström G, Snochowski M, Högberg B, Gustafsson J.-å. Detection of the 2,3,7,8-tetrapachlorodibenzo-p-dioxin (TCDD) receptor in rat liver by isoelectric focusing in polyacrylamide gels. Toxicology Letters. 1978; 2:365–373.
- [108]. Denison MS, Nagy SR. ACTIVATION OF THE ARYL HYDROCARBON RECEPTOR BY STRUCTURALLY DIVERSE EXOGENOUS AND ENDOGENOUS CHEMICALS. Annual Review of Pharmacology & Toxicology. 2003; 43:309.
- [109]. Denison MS, Pandini A, Nagy SR, Baldwin EP, Bonati L. Ligand binding and activation of the Ah receptor. Chemico-Biological Interactions. 2002; 141:3–24. [PubMed: 12213382]
- [110]. Rannug A, Rannug U, Rosenkranz HS, Winqvist L, Westerholm R, Agurell E, Grafstrom AK. Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances. J Biol Chem. 1987; 262:15422–15427. [PubMed: 2824460]
- [111]. Schmidt JV, Bradfield CA. AH RECEPTOR SIGNALING PATHWAYS. Annual Review of Cell & Developmental Biology. 1996; 12:55.
- [112]. Abel J, Haarmann-Stemmann T. An introduction to the molecular basics of aryl hydrocarbon receptor biology. Biological Chemistry. 2010; 391:1235–1248. [PubMed: 20868221]
- [113]. Tarantini A, Maitre A, Lefebvre E, Marques M, Rajhi A, Douki T. Polycyclic aromatic hydrocarbons in binary mixtures modulate the efficiency of benzo[a]pyrene to form DNA adducts in human cells. Toxicology. 2011; 279:36–44. [PubMed: 20849910]
- [114]. Esser C, Rannug A, Stockinger B. The aryl hydrocarbon receptor in immunity. Trends in Immunology. 2009; 30:447–454. [PubMed: 19699679]
- [115]. Lindén J, Lensu S, Tuomisto J, Pohjanvirta R. Dioxins, the aryl hydrocarbon receptor and the central regulation of energy balance. Frontiers in Neuroendocrinology. 2010; 31:452–478. [PubMed: 20624415]
- [116]. Lu P, Yan J, Liu K, Garbacz WG, Wang P, Xu M, Ma X, Xie W. Activation of aryl hydrocarbon receptor dissociates fatty liver from insulin resistance by inducing fibroblast growth factor 21. Hepatology (Baltimore, Md.). 2015; 61:1908–1919.
- [117]. Rowlands JC, Gustafsson JA. Aryl hydrocarbon receptor-mediated signal transduction. Critical Reviews In Toxicology. 1997; 27:109–134. [PubMed: 9099515]
- [118]. Ikuta T, Tachibana T, Watanabe J, Yoshida M, Yoneda Y, Kawajiri K. Nucleocytoplasmic Shuttling of the Aryl Hydrocarbon Receptor1. Journal of Biochemistry. 2000; 127:503–509. [PubMed: 10731723]
- [119]. Kazlauskas A, Sundström S, Poellinger L, Pongratz I. The hsp90 chaperone complex regulates intracellular localization of the dioxin receptor. Molecular And Cellular Biology. 2001; 21:2594–2607. [PubMed: 11259606]
- [120]. Tsuji N, Fukuda K, Nagata Y, Okada H, Haga A, Hatakeyama S, Yoshida S, Okamoto T, Hosaka M, Sekine K, Ohtaka K, Yamamoto S, Otaka M, Grave E, Itoh H. The activation mechanism of the aryl hydrocarbon receptor (AhR) by molecular chaperone HSP90. FEBS Open Bio. 2014; 4:796–803.
- [121]. Reisz-Porszasz S, Probst MR, Fukunaga BN, Hankinson O. Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT). Molecular and Cellular Biology. 1994; 14:6075–6086. [PubMed: 8065341]
- [122]. Ikuta T, Eguchi H, Tachibana T, Yoneda Y, Kawajiri K. Nuclear Localization and Export Signals of the Human Aryl Hydrocarbon Receptor. Journal of Biological Chemistry. 1998; 273:2895–2904. [PubMed: 9446600]
- [123]. Miller CA. Expression of the Human Aryl Hydrocarbon Receptor Complex in Yeast: ACTIVATION OF TRANSCRIPTION BY INDOLE COMPOUNDS. Journal of Biological Chemistry. 1997; 272:32824–32829. [PubMed: 9407059]
- [124]. Satsu H, Yoshida K, Mikubo A, Ogiwara H, Inakuma T, Shimizu M. Establishment of a stable aryl hydrocarbon receptor-responsive HepG2 cell line. Cytotechnology. 2015; 67:621–632. [PubMed: 24667997]

- [125]. Backlund M, Ingelman-Sundberg M. Different Structural Requirements of the Ligand Binding Domain of the Aryl Hydrocarbon Receptor for High- and Low-Affinity Ligand Binding and Receptor Activation. *Molecular Pharmacology*. 2004; 65:416–425. [PubMed: 14742684]
- [126]. Wu D, Potluri N, Kim Y, Rastinejad F. Structure and Dimerization Properties of the Aryl Hydrocarbon Receptor PAS-A Domain. *Molecular and Cellular Biology*. 2013; 33:4346–4356. [PubMed: 24001774]
- [127]. Goryo K, Suzuki A, Carpio CAD, Siizaki K, Kuriyama E, Mikami Y, Kinoshita K, Yasumoto K.-i, Rannug A, Miyamoto A, Fujii-Kuriyama Y, Sogawa K. Identification of amino acid residues in the Ah receptor involved in ligand binding. *Biochemical and Biophysical Research Communications*. 2007; 354:396–402. [PubMed: 17227672]
- [128]. Pandini A, Denison MS, Song Y, Soshilov AA, Bonati L. Structural and Functional Characterization of the Aryl Hydrocarbon Receptor Ligand Binding Domain by Homology Modeling and Mutational Analysis†. *Biochemistry*. 2007; 46:696–708. [PubMed: 17223691]
- [129]. Bisson WH, Koch DC, O'Donnell EF, Khalil SM, Kerkvliet NI, Tanguay RL, Abagyan R, Kolluri SK. Modeling of the Aryl Hydrocarbon Receptor (AhR) Ligand Binding Domain and Its Utility in Virtual Ligand Screening to Predict New AhR Ligands. *Journal of Medicinal Chemistry*. 2009; 52:5635–5641. [PubMed: 19719119]
- [130]. Lo Piparo E, Koehler K, Chana A, Benfenati E. Virtual Screening for Aryl Hydrocarbon Receptor Binding Prediction. *Journal of Medicinal Chemistry*. 2006; 49:5702–5709. [PubMed: 16970396]
- [131]. Lesca P, Peryt B, Larrieu G, Alvinerie M, Galtier P, Daujat M, Maurel P, Hoogenboom L. Evidence for the Ligand-Independent Activation of the AH Receptor. *Biochemical and Biophysical Research Communications*. 1995; 209:474–482. [PubMed: 7733914]
- [132]. Daujat M, Peryt B, Lesca P, Fourtanier G, Domergue J, Maurel P. Omeprazole, an inducer of human CYP1A1 and 1A2, is not a ligand for the Ah receptor. *Biochemical And Biophysical Research Communications*. 1992; 188:820–825. [PubMed: 1280125]
- [133]. Backlund M, Johansson I, Mkrtchian S, Ingelman-Sundberg M. Signal Transduction-mediated Activation of the Aryl Hydrocarbon Receptor in Rat Hepatoma H4IIE Cells. *Journal of Biological Chemistry*. 1997; 272:31755–31763. [PubMed: 9395520]
- [134]. Oesch-Bartlomowicz B, Huelster A, Wiss O, Antoniou-Lipfert P, Dietrich C, Arand M, Weiss C, Bockamp E, Oesch F. Aryl hydrocarbon receptor activation by cAMP vs. dioxin: Divergent signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:9218–9223. [PubMed: 15972329]
- [135]. Backlund M, Ingelman-Sundberg M. Regulation of aryl hydrocarbon receptor signal transduction by protein tyrosine kinases. *Cellular Signalling*. 2005; 17:39–48. [PubMed: 15451023]
- [136]. Powis M, Celius T, Matthews J. Differential ligand-dependent activation and a role for Y322 in aryl hydrocarbon receptor-mediated regulation of gene expression. *Biochemical and Biophysical Research Communications*. 2011; 410:859–865. [PubMed: 21703235]
- [137]. Maayah Z, El Gendy MM, El-Kadi A, Korashy H. Sunitinib, a tyrosine kinase inhibitor, induces cytochrome P450 1A1 gene in human breast cancer MCF7 cells through ligand-independent aryl hydrocarbon receptor activation. *Archives of Toxicology*. 2013; 87:847–856. [PubMed: 23288144]
- [138]. Aninat C, Piton A, Glaise D, Le Charpentier T, Langouët S, Morel F, Guguen-Guillouzo C, Guillouzo A. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metabolism And Disposition: The Biological Fate Of Chemicals*. 2006; 34:75–83. [PubMed: 16204462]

HIGHLIGHTS

- CAR, PXR, and AhR coordinate xenobiotic biotransformation and detoxification.
- All three xenobiotic receptors also regulate energy homeostasis and cell proliferation.
- These receptors are activated through both ligand binding (direct) and indirect mechanisms.
- A particular focus is given to ligand-independent (indirect) activation of these receptors.

Figure 1. Schematic Illustration of Mechanisms of CAR Activation

Direct Activation – CAR is sequestered in a cytoplasmic complex containing HSP90 and CCRP. Upon ligand binding, these chaperones dissociate and CAR translocates to the nucleus where it heterodimerizes with RXR, recruits coactivators GRIP1 and SRC1, and binds to its response element to initiate gene transcription. **Indirect Activation** – When sequestered in the cytoplasm, the Thr38 residue of CAR is phosphorylated by PKC, and dephosphorylation of this residue for CAR activation can be inhibited by ERK1/2 upon activation of the EGFR signaling pathway. When a PB-like compound binds to the EGFR receptor and inhibits EGF-mediated signaling, RACK1 is dephosphorylated at Tyr52 and recruits PP2A to the CAR protein complex, where it dephosphorylates the Thr38 residue and induces CAR nuclear translocation and activation.

Figure 2. Schematic illustration of Mechanisms of PXR Activation

Direct Activation – PXR is retained in a cytoplasmic complex with HSP90 and CCRP, which dissociate upon ligand binding leading to PXR nuclear accumulation. Unbound PXR in the nucleus is complexed to corepressors NcoR and SMRT, which dissociate upon ligand stimulation, leading to the recruitment of coactivators SRC1 and GRIP1 and the transcription of target genes. **Indirect Activation** – This is a less well-developed mechanism of PXR activation, although the Ser350, Thr248, Thr290, and Thr408 phosphorylation sites of PXR are known to affect its activation. Compounds disturbing signaling pathways may influence the phosphorylation, nuclear translocation, and target gene express of PXR.

Figure 3. Schematic Illustration of Mechanisms of AhR Activation

Direct Activation – AhR is sequestered in the cytoplasm complexed with HSP90, XAP2, and p23. Ligand binding induces the dissociation of XAP2 and p23 from AhR and the AhR:HSP90 complex translocates to the nucleus. In the nucleus, HSP90 dissociates and AhR heterodimerizes with ARNT to induce transcription of target genes. **Indirect Activation** – Drugs such as omeprazole and sunitinib translocate and activate AhR by affecting the c-*src*-dependent and tyrosine kinases (TK)-mediated signaling pathways, respectively. cAMP activates AhR and induces a structural change that translocates AhR to the nucleus and favors ligand-independent protein-protein interaction but not AhR:ARNT heterodimerization.