Orphan Nuclear Receptor Constitutive Androstan Receptor and 
Pregnane X Receptor Share Xenobiotic and Steroid Ligands*

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Xenobiotics induce the transcription of cytochromes 
P450 (CYPs) 2B and 3A through the constitutive androstan 
receptor (CAR; NR1I3) and pregnane X receptor 
(PXR; NR12), respectively. In this report, we have sys 
tematically compared a series of xenobiotics and natu 
ral steroids for their effects on mouse and human CAR 
and PXR. Our results demonstrate dual regulation of 
PXR and CAR by a subset of compounds that affect CYP expression. Moreover, there are marked pharma 
cological differences between the mouse (m) and human 
(h) orthologs of both CAR and PXR. For example, the planar hydrocarbon 1,4-bis[2-(3,5-dichloropyridyl-oxy-
)]benzene activates mCAR and hPXR but has little or no activity on hCAR and mPXR. In contrast, the CAR de 
activator androstanol activates both mouse and human 
PXR. Similarly, the PXR activator clotrimazole is a po 
tent deactivator of hCAR. Using radioligand binding and fluorescence resonance energy transfer assays, we 
demonstrate that several of the compounds that regu 
late mouse and human CAR, including natural steroids, 
bind directly to the receptors. Our results suggest that 
CAR, like PXR, is a steroid receptor that is capable of 
recognizing structurally diverse compounds. Moreover, 
our findings underscore the complexity in the physio 
logic response to xenobiotics.

The cytochromes P450 (CYPs)1 comprise a superfAMILY of 
heme-containing monooxygenases that play a crucial role in the 
metabolism of natural compounds and xenobiotics, includ 
ing environmental pollutants and drugs. Transcription of sev 
eral of the CYP gene families is induced by xenobiotics. For 
example, the barbiturate phenobarbital (PB) and the antibiotic 
rifampicin are classic inducers of CYP3A40% amino acid 
identity in their ligand binding domains (LBDs). CAR is most 
abundantly expressed in liver and has strong constitutive ac 
tivity in cell-based reporter assays in the absence of any added 
ligand (4, 5). In HepG2 cells or other cell lines, exogenously 
expressed CAR can enter the nucleus and regulate the expres 
sion of target genes. This constitutive activity can be inhibited 
by superphysiological concentrations of the testosterone me 
tabolites androstanol and androstenol (6). These androstanes 
inhbit the interaction of CAR with the steroid receptor coacti 
vator 1 (SRC-1), suggesting that “deactivation” is mediated by 
direct binding to the orphan receptor. In contrast to transfected 
cell lines, CAR is not present in the nucleus of primary hepa 
tocytes but is instead sequestered in the cytoplasm. Treatment 
of primary hepatocytes with either PB or the planar hydrocar 
bon TCPOBOP results in the translocation of CAR into the 
nucleus, where it binds to its cognate DNA response elements 
as a heterodimer with the 9-cis-retinoic acid receptor (RXR) 
and activates the transcription of target genes, including 
CYP2B (7–9). CAR/RXR binding sites have been identified in 
the PB-responsive regions of the mouse, rat, and human 
CYP2B genes. The effects of PB on CYP2B expression are 
blocked by the phosphatase inhibitor okadaic acid (8), suggest 
ing that dephosphorylation of CAR, rather than direct ligand 
interaction, is involved in its translocation into the nucleus.

Transcription of CYP3A is induced by a remarkable diversity of 
xenobiotics, including many widely used drugs. Several lines of 
evidence indicate that PXR mediates the induction of CYP3A 
expression by these compounds. First, PXR is abundantly and 
selectively expressed in the liver and intestine, the same tis 
iues in which induction of CYP3A4 occurs (10–13). Moreover, 
PXR binds as a heterodimer with RXR to xenobiotic response 
elements in the CYP3A4 gene promoters and is activated in 
cell-based reporter assays by the range of xenobiotics that are 
known to induce CYP3A4 expression (10–13). Finally, PXR or 
thologs from different species are differentially activated by 
xenobiotics with a profile that correlates with the species 
specific induction of CYP3A4 gene expression (11, 14).

Although CAR and PXR have been implicated as primary regu 
ulators of CYP2B and CYP3A expression, respectively, 
there is evidence for cross-talk between these two nuclear 
receptor signaling pathways. First, the CYP2B and CYP3A genes are regulat 
ed in rodent and/or human hepatocytes by
several of the same xenobiotics, including PB, clotrimazole, and rifampicin (15, 16). Moreover, CAR was recently shown to transactivate through the CYP3A4 xenobiotic response element that serves as a PXR/RXR binding site (9). In order to clarify the relative functions of CAR and PXR, we have systematically compared the activities of various xenobiotics on the human (h) and mouse (m) orthologs of CAR and PXR using cell-based transactivation and in vitro radioligand binding assays. Our results demonstrate an unexpected overlap in the compounds that regulate these two orphan receptors.

**EXPERIMENTAL PROCEDURES**

Reagents— RU486 was purchased from Biomol (Plymouth Meeting, PA). SR12813 was synthesized in house. All other xenobiotics and steroids used in the transfection and binding assays were purchased from either Sigma or Steraloids, Inc. (Wilton, NH). [3H]Clotrimazole (specific activity 78 Ci/mmol) was prepared by Amersham Pharmacia Biotech (Cardiff, United Kingdom).

**Cotransfection Assays—** CV-1 cells were plated in 96-well plates at a density of 20,000 cells/well in Dulbecco’s modified Eagle’s medium high glucose medium supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT). Transfection mixtures contained 5 ng of receptor expression vector, 20 ng of reporter plasmid, 12 ng of β-actin SPAP, 0.05 ng of bgl-gal control, and 45 ng of carrier plasmid. Human and mouse PXR expression plasmids and the XREM-CYP3A4-LUC reporter, containing the enhancer and promoter of CYP3A4 driving luciferase expression, were as described previously (10, 11, 17). Transfections were performed with LipofectAMINE (Life Technologies, Inc.) essentially according to the manufacturer’s instructions. Drug dilutions were prepared in phenol red-free Dulbecco’s modified Eagle’s medium/F-12 medium with 15 mM HEPES supplemented with 10% charcoal-stripped, delipidated calf serum (Sigma). Cells were incubated for 24 h in the presence of drugs, after which the medium was sampled and assayed for alkaline phosphatase activity. Luciferase reporter activity was measured using the LucLite assay system (Packard Instrument Co., Meriden, CT).

**Overexpression and Purification of CAR and PXR LBDs—** The LBDs of mouse and human CAR were expressed in *Escherichia coli* strain BL21(DE3) as polyhistidine-tagged fusion proteins. Sequence encoding residues 113–358 of mCAR (5) or 103–348 of hCAR a modified polyhistidine tag (MKKGHHHHHHG) was fused in frame to BL21(DE3) as polyhistidine-tagged fusion proteins. Sequence encoding the second L motif of SRC-1 (amino acids 676–700) labeled with europium chelate. Data were collected with a Wallac 1450 Microbeta counter. XREM scintillation proximity binding assays were performed as described previously (14).

**RESULTS**

**Activity of Xenobiotics and Steroids on CAR and PXR—** Previous studies have revealed an overlap in the xenobiotics that regulate the expression of CYP2B and CYP3A (15, 16). We sought to determine whether there was a corresponding overlap in the activities of xenobiotics on CAR and PXR. Accordingly, the mouse and human CAR and PXR were tested in transfection assays in CV-1 cells with xenobiotics that are well established inducers of CYP2B and/or CYP3A gene expression. The xenobiotics evaluated include PB, TCPOBOP, the antibiotic rifampicin, the synthetic pregnane pregnenolone 16α-carbonitrile (PCN), the synthetic glucocorticoid dexamethasone, the antiprogestin RU486, the antimycotic clotrimazole, and the cholesterol-lowering drug SR12813. We also included the sex steroid metabolites 5β-pregnan-3,20-dione and 5α,17α-androstanol, which activate and deactivate PXR and CAR, respectively. As a reporter, we used the recently described XREM-CYP3A4-LUC construct containing the enhancer (nucleotides 7836 to −7208) and promoter (nucleotides −962 to +53) of CYP3A4 driving luciferase gene expression (17). This reporter construct is responsive to both CAR and PXR (see below).

All compounds were initially screened at a concentration of 10 μM, except for PB, which was tested at 0.5 μM. As described previously (14), rifampicin, RU486, clotrimazole, SR12813, PB, and 5β-pregnan-3,20-dione were efficacious activators of hPXR (Fig. IA). PCN and dexamethasone were relatively weak activators of hPXR as compared with the other compounds. PCN, dexamethasone, RU486, and clotrimazole all activated mPXR on the XREM-CYP3A4-luciferase reporter with comparable efficacy. Interestingly, SR12813 also induced expression from the XREM-CYP3A4-luciferase reporter. We previously showed that this compound had little activity on mPXR when the (DR3)₃-tk-CAT reporter was used (14). Thus, the effects of SR12813 are promoter-specific.

**APA assays performed with xenobiotics known to regulate CAR revealed several interesting results. First, the CAR deacti-**

ator androstanol activated both the hPXR and mPXR (Fig. 1A and Table I). Moreover, TCPOBOP was an efficacious activator of hPXR but had only weak effects on mPXR. Thus, two compounds with established CAR activity also had marked effects on PXR.

The same panel of compounds was next tested on CAR. As expected, androstanol was an efficacious activator of mCAR (Fig. 1B). Androstanol also deactivated hCAR, although the effect was not as pronounced as on mCAR. By contrast, the antimycotic clotrimazole was an efficacious activator of hCAR but not mCAR. Full dose-response analysis revealed that clotrimazole deactivated hCAR with an EC₅₀ value of ∼700 nM (Fig. 1C and Table I). The PXR activators rifampicin, PCN, dexamethasone, RU486, and SR12813 had little or no effect on CAR activity (Fig. 1B). PB caused a weak but reproducible

**2 B. Goodwin and C. Liddle, unpublished data.**
deactivation of both hCAR and mCAR at high concentrations. TCPOBOP was a potent (EC$_{50}$ $\approx$ 100 nM) and efficacious activator of mCAR but had virtually no activity on hCAR (Fig. 1, B and D, and Table I). TCPOBOP was previously shown to activate mCAR, albeit weakly, on a reporter construct driven by a PB-responsive enhancer module from the CYP2B gene (8).

Interestingly, 5β-pregnane-3,20-dione activated hCAR 2-fold (Fig. 1, B and D) but had only weak effects on mCAR. These data suggest that natural ligands may exist that increase CAR activity above its high constitutive activity.

**Compounds Induce Conformational Changes in the CAR LBD**—Androstanol has been shown previously to inhibit the interaction between mCAR and a polypeptide derived from SRC-1 (6). We next tested whether the xenobiotics and steroids that modulated CAR activity in the transfection assay also affected CAR/SRC-1 interactions. A FRET ligand-sensing assay (18) was developed for hCAR. The LBD of hCAR was expressed in E. coli, purified to homogeneity, biotinylated, and labeled with the steptavidin-conjugated fluorophore allophycocyanin. Labeled hCAR LBD was incubated with a peptide that included the second LXXLL motif of SRC-1 (amino acids 676–700) labeled with europium chelate. As expected, hCAR LBD interacted efficiently with SRC-1 in the absence of compound, and androstanol disrupted this interaction (Fig. 2A). Clotrimazole was a potent inhibitor of the hCAR/SRC-1 interaction (IC$_{50}$; 100 nM) (Fig. 2A). We note that clotrimazole was 10-fold more potent in the FRET assay (Fig. 2A) than in transfection assays (Fig. 1C). This discrepancy may be due to cell permeability or the metabolism of clotrimazole in cells. Consistent with its ability to activate hCAR in transfection assays, 5β-pregnane-3,20-dione caused a marked increase in the binding of SRC-1 to hCAR (Fig. 2A). Thus, compounds can be identified that induce conformational changes in the hCAR LBD and either enhance or disrupt its basal interaction with SRC-1.

A similar FRET assay was developed for mCAR. In contrast to the hCAR assay, no basal interaction was observed between mCAR and the SRC-1 peptide. We also failed to detect any basal interaction between mCAR and three other peptides containing PXR (A) or mouse or human CAR (B) and the XREM-CYP3A4-luciferase reporter. Cells were treated with 10 μM amounts of each compound, except for phenobarbital, which was tested at 0.5 mM. Cell extracts were subsequently assayed for luciferase activity. Data represent the mean of assays performed in triplicate ± S.E. and are plotted as fold activation relative to transfected cells treated with vehicle alone. Cotransfection of hCAR or mCAR expression plasmids with the CYP3A4-XREM-luciferase plasmid increased reporter levels 3.3- and 5.2-fold, respectively, relative to transfection with reporter plasmid alone. Full dose-response curves are shown for deactivators (C) or activators (D) of human or mouse CAR.

**TABLE I**

| Compounds                | EC$_{50}$ (μM) for activation | hPXR | mPXR | hCAR | mCAR |
|--------------------------|-------------------------------|------|------|------|------|
| Rifampicin               | 0.10                          |      |      |      |      |
| PCN                      | >10                           | 0.20 |      |      |      |
| Dexamethasone            | >10                           | >10  | >10  | >10  | >10  |
| RU486                    | 5.5                           |      |      | 1.0  | 1.0  |
| Clotrimazole             | 1.6                           | 1.0  | 0.69 |      |      |
| SR12813                  | 0.12                          | 4.1  |      | 0.10 |      |
| TCPOBOP                  | 3.9                           |      |      | 0.10 |      |
| Androstanol              | >10                           | >10  | >10  | >10  | >10  |
| 5β-Pregnan-3,20-dione    | 3.1                           | 1.1  | 0.67 |      |      |

**FIG. 1.** Effects of xenobiotics on CAR and PXR activity. CV-1 cells were transfected with expression plasmids for mouse or human CAR and PXR Share Ligands

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**FIGURE 1**

*Effects of xenobiotics on CAR and PXR activity. CV-1 cells were transfected with expression plasmids for mouse or human CAR and PXR, and treated with compounds at various concentrations. The expression of luciferase activity was measured and normalized relative to the vehicle control. The graphs show the fold activation of luciferase activity for each compound tested.***
containing LXXLL motifs derived from either SRC-1 or the coactivator CBP (data not shown). The reason for this difference between the mouse and human CAR is unclear, but it may be due in part to an amino acid change (Gln → Gly) in the AF-2 helix. The lack of a basal interaction between these coactivator peptides and mCAR precluded us from testing whether androstanol disrupted coactivator-mCAR interactions. However, TCPOBOP resulted in a marked increase in the interaction of SRC-1 with mCAR (Fig. 2B). These data provide strong evidence that TCPOBOP activates mCAR by binding directly to the receptor's LBD. We note that PB had no effect on SRC-1 interactions with either mouse or human CAR at concentrations up to 1 mM (data not shown), suggesting that PB and TCPOBOP may activate CAR through distinct mechanisms.

Xenobiotics and Steroids Bind hCAR—The potency of clotrimazole in hCAR cell-based reporter and in vitro FRET assays suggested that [3H]clotrimazole might be used to develop a radioligand binding assay for this orphan receptor. To test this idea, an SPA (19) was developed using the purified, biotinylated hCAR LBD and streptavidin-coated polyvinyltoluene SPA beads. [3H]Clotrimazole bound specifically to hCAR with a Ki of 100 nM (Fig. 3A). Dexamethasone, which has no effect on hCAR in transfection assays, failed to compete with [3H]clotrimazole for binding to hCAR (Fig. 3A). No specific binding of [3H]clotrimazole to the SPA beads was detected in the absence of hCAR (data not shown). These data suggest that clotrimazole mediates its effects on hCAR activity through direct interactions with the receptor LBD.

The availability of radioligand binding assays for both hCAR and hPXR allowed us to directly compare the binding of compounds to these receptors. In agreement with the transfection data, the sex steroid metabolites androstanol and 5β-pregnane-3,20-dione competed efficiently with [3H]clotrimazole for binding to hCAR. TCPOBOP, dexamethasone, RU486, rifampicin, PCN, and SR12813, which had little or no activity on hCAR in the transfection assay, did not compete in the binding assay (Fig. 3B). PB, which deactivated hCAR in the transfection studies, did not compete at 1 mM concentration with [3H]clotrimazole for binding to hCAR, although it was a weak competitor of [3H]SR12813 for binding to hPXR. These data suggest that PB does not mediate its effects on hCAR through direct interactions with the receptor, but rather through cellular metabolism of PB to a compound that can bind CAR or via an indirect mechanism. The latter hypothesis is supported by the finding that PB effects on CYP2B gene expression are blocked by the phosphatase inhibitor okadaic acid (8). As expected, RU486, clotrimazole, SR12813, and 5β-pregnane-3,20-dione competed efficiently with [3H]SR12813 for binding to hPXR (Fig. 3B). The hCAR ligands androstanol and TCPOBOP also bound to hPXR (Fig. 3B). We conclude that many of the structurally diverse compounds that modulate CAR and PXR activity do so through direct interactions with the LBDs of these orphan receptors.

**PXR Is the Dominant Regulator of CYP3A4 In Vitro**—Since both clotrimazole and androstanol are ligands that activate...
hPXR and deactivate hCAR, we used these compounds to test which receptor would be dominant on the XREM-CYP3A4-luciferase reporter. CV-1 cells were transfected with a fixed amount of hCAR expression plasmid (5 ng) and increasing amounts of the hPXR expression plasmid (0.1–5 ng), and the cells were treated with 10 μM clotrimazole or androstanol, and cell extracts subsequently assayed for luciferase activity. Data represent the mean of assays (n = 8) ± S.E.

Fig. 4. PXR is dominant over CAR on the CYP3A4 gene promoter. CV-1 cells were transfected with the indicated amounts of hCAR and/or hPXR expression plasmids and the XREM-CYP3A4-luciferase reporter. Transfected cells were treated with 10 μM clotrimazole or androstanol, and cell extracts subsequently assayed for luciferase activity.

DISCUSSION

The orphan nuclear receptors CAR and PXR have recently been implicated in the induction of CYP expression (1–3). However, the relative contributions of these orphans in the regulation of particular CYPs have remained unclear. In this report, we have systematically compared the effects of a panel of xenobiotics with established activities on CYP expression on the mouse and human orthologs of CAR and PXR. Our results demonstrate a surprising degree of overlap in the compounds that are capable of binding and regulating the activities of these orphan receptors. In some cases, xenobiotics have opposing effects on these orphans; for example, clotrimazole activates hPXR but deactivates hCAR. Since CAR and PXR can share many target genes, the net effect of a xenobiotic on gene regulation and purification, and Ian Fellows of the Isotope Chemistry Group in Stevenage for assistance in the preparation of [3H]clotrimazole.

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