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DIFFERENTIAL REGULATION OF HEPATIC *CYP2B6* AND *CYP3A4* GENES BY CONSTITUTIVE ANDROSTANE RECEPTOR BUT NOT PREGNANE X RECEPTOR

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d) Abbreviations: AF2, activation function 2; CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl:imidazo[2,1-b]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; DR3 direct repeat separated by three base pairs; DR4, direct repeat separated by four base pairs; ER6, everted repeat separated by six base pairs; EYFP, enhanced yellow fluorescent protein; hCAR, human constitutive androstane receptor; hPXR, human pregnane X receptor; hRXRα, human 9-*cis* retinoic acid receptor-α; mCAR, mouse constitutive androstane receptor; mPXR, mouse

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pregnane X receptor; NR, nuclear receptor binding site; PBREM, phenobarbital responsive enhancer module; PXRE, pregnane X receptor response element; PCN, pregnenolone 16α-carbonitrile; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; XREM, xenobiotic responsive enhancer module

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ABSTRACT

Accumulated evidence suggests that cross-talk between the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) results in shared transcriptional activation of *CYP2B* and *CYP3A* genes. Although most data implies symmetrical cross-regulation of these genes by rodent PXR and CAR, the actual selectivities of the corresponding human receptors are unknown. The objective of this study was to evaluate the symmetry of human (h) PXR and hCAR cross-talk by comparing the selectivites of these receptors for CYP2B6 and CYP3A4. Human hepatocyte studies revealed non-selective induction of both CYP2B6 and CYP3A4 by hPXR activation, but marked preferential induction of CYP2B6 by selective hCAR activation. Gel shift assays demonstrated that hPXR exhibited strong and relatively equal binding to all functional response elements in both *CYP2B6* and *CYP3A4* genes, while hCAR displayed significantly weak binding to the CYP3A4 proximal ER6 motif. In cell-based transfection assays, hCAR displayed greater activation of CYP2B6 reporter gene expression compared with CYP3A4 with constructs containing both proximal and distal regulatory elements. Furthermore, in agreement with binding observations, transfection assays using promoter constructs containing repeats of CYP2B6 DR4 and CYP3A4 ER6 motifs revealed an even greater difference in reporter activation by hCAR. In contrast, hPXR activation resulted in less discernible differences between CYP2B6 and CYP3A4 reporter gene expression. These results suggest asymmetrical cross-regulation of CYP2B6 and CYP3A4 by hCAR but not hPXR in that hCAR exhibits preferential induction of CYP2B6 relative to CYP3A4 due to its weak binding and functional activation of the CYP3A4 ER6.

Introduction

The pregnane X receptor (PXR; NR1I2) and the constitutive androstane receptor (CAR; NR1I3) are two closely related members of the nuclear receptor family that mediate enhanced expression of genes involved in the detoxification and elimination of xenobiotics and endobiotics (Handschin and Meyer, 2003; Honkakoski et al., 2003). In response to structurally diverse compounds, these receptors increase transcriptional activity by binding to target enhancer sequences in the promoters of an overlapping set of xenobiotic metabolism and transport genes, including those encoding cytochromes P450 (CYPs), glucoronyltransferases, sulfotransferases, glutathione transferases, and drug transporters (Maglich et al., 2002; Rosenfeld et al., 2003; Sugatani et al., 2001; Ueda et al., 2002). The complex interplay between PXR and CAR regulation has been proposed to create a biological safety net to ensure comprehensive protection against drug, hormone, bilirubin, and bile acid toxicity (Huang et al., 2003; Maglich et al., 2004; Xie et al., 2003; Zhang et al., 2004).

PXR and CAR were established originally as the predominant regulators of xenobiotic-induced CYP3A and CYP2B hepatic expression, respectively (Blumberg et al., 1998; Honkakoski et al., 1998; Kliewer et al., 1998). However, recent lines of evidence suggest that cross-talk between PXR and CAR results in reciprocal activation of rodent and human *CYP2B* and *CYP3A* genes (Moore et al., 2000; Smirlis et al., 2001; Wei et al., 2002; Xie et al., 2000b). For example, Cyp2b10 is induced by the PXR ligand dexamethasone in CAR-null mice and Cyp3a11 is induced by the CAR activator PB in PXR-null mice (Wei et al., 2002; Xie et al., 2000a; Xie et al., 2000b). Furthermore, *CYP2B* and *CYP3A* genes can be induced simultaneously by the same compounds such as PCN in rats and RIF in humans (Burger et al., 1992; Faucette et al., 2004; Goodwin et al., 2001; Kocarek et al., 1994). Because only a limited

number of chemicals activate both PXR and CAR, cross-talk between these receptors results primarily from their binding of different response elements within the CYP2B and CYP3A promoters. In addition to binding to the rat DR3 or human ER6 motifs in the CYP3A PXRE, PXR binds to the DR4-type NR1 and NR2 sequences in the rodent and human CYP2B PBREM (Blumberg et al., 1998; Goodwin et al., 2001; Kliewer et al., 1998; Lehmann et al., 1998). Conversely, CAR binds CYP3A proximal response elements in addition to CYP2B NR1 and NR2 sequences (Honkakoski et al., 1998; Sueyoshi et al., 1999; Tzameli et al., 2000). hPXR and hCAR also share the ability to bind and activate the DR4-type NR3 motif in the CYP2B6 XREM and the DR3 (dNR1) and ER6 (dNR2) motifs in the CYP3A4 XREM (Goodwin et al., 2002; Goodwin et al., 1999; Wang et al., 2003). Previous studies have demonstrated that these distal enhancer modules function cooperatively with the more proximal elements to elicit maximum induction of the respective genes (Goodwin et al., 2002; Goodwin et al., 1999; Wang et al., 2003).

The current model of cross-talk between PXR and CAR is derived primarily from rodent data and commonly depicts symmetrical cross-regulation of CYP2B and CYP3A induction by PXR and CAR, respectively. However, because of documented species differences in nuclear receptor signaling pathways, it is speculative to extrapolate the extents of cross-regulation of *CYP2B* and *CYP3A* genes by rodent PXR and CAR to the corresponding human receptors. The selectivities of hPXR and hCAR for their cross-regulatory genes remain unclear due to the lack of comparisons of CYP2B6 and CYP3A4 promoter binding and functional activation between hPXR and hCAR as well as the previous unavailability of selective hCAR activators. These comparisons are now feasible due to the recent identification of CITCO and phenytoin as selective hCAR activators (Maglich et al., 2003; Wang et al., 2004). Consequently, these

compounds represent useful chemical-based tools to facilitate determination of the relative contributions of hPXR and hCAR to the respective cross-regulation of CYP2B6 and CYP3A4.

The objective of this study was to compare the target gene selectivities of hCAR and hPXR with respect to regulation of hepatic CYP2B6 and CYP3A4 induction. Studies in primary human hepatocytes revealed indiscriminant induction of CYP2B6 and CYP3A4 by hPXR activation, but preferential induction of CYP2B6 over CYP3A4 by hCAR activation. A mechanistic explanation for these observations was generated from gel shift and cell-based transfection reporter assays in that hCAR, but not hPXR, exhibits marked differential binding and activation of CYP2B6 and CYP3A4 response elements. Results presented here support the proposal of a new model to describe cross-regulation of *CYP2B6* and *CYP3A4* genes by hPXR and hCAR.

Materials and Methods

Materials. Rifampin and CITCO were purchased from Sigma-Aldrich (St. Louis, MO) and BIOMOL Research Laboratories (Plymouth Meeting, PA), respectively. Oligonucleotides for gel shift assays and TaqMan® real-time PCR were obtained from Sigma Genosys (The Woodlands, TX) and fluorogenic TaqMan® probes were synthesized by Applied Biosystems (Foster, CA). CellPhect Transfection Kit was purchased from Amersham Biosciences (Buckinghamshire, England), while Effectene® was acquired from Qiagen (Valencia, CA). The Dual-Luciferase Reporter Assay System was purchased through Promega (Madison, WI). Matrigel® was obtained from BD Biosciences (Bedford, MA). Other cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted. All reagents and chemicals were of the highest grade available from commercial sources.

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Plasmid Constructs. The pSG5-hPXR expression vector was provided generously by Dr. Steven Kliewer (Lehmann et al., 1998), and the CYP3A4-PXRE/XREM luciferase reporter construct (p3A4-362(7836/7208ins)) was provided generously by Dr. Bryan Goodwin, (GlaxoSmithKline, Research Triangle Park, NC). The latter construct consisted of the CYP3A4 native proximal promoter, including the PXRE containing an ER6 motif, as well as the distal XREM region bearing one DR3 motif (dNR1) and one ER6 motif (dNR2) (Goodwin et al., 1999). As reported earlier, the CYP2B6-PBREM/XREM construct consisted of 1.8 kb of the native promoter, including the 51 bp PBREM harboring two DR4 motifs (NR1 and NR2), and the 400 bp distal XREM region containing the DR4 motif termed NR3 (Wang et al., 2003). A mutant $hCAR_{1-338}$ expression plasmid that lacked the last ten C-terminal residues of wild-type hCAR was constructed using pEGFP-c1-$hCAR_{1-338}$ as template and cloning the amplified fragment between the BamHI and EcoRI sites of pCR3 vector (Zelko et al., 2001). Expression plasmids encoding full-length (1-348) hCAR (designated $hCAR_{wt}$), EYFP-$hCAR_{wt}$, and EYFP-$hCAR_{1-338}$ were described previously (Sueyoshi et al., 1999; Zelko et al., 2001). The CYP3A4 $(ER6)_2$ luciferase construct, generated using pGL3-promoter vector (Promega), was provided generously by Dr. Bingfang Yan (University of Rhode Island, Kingsport, RI). The CYP2B6 $(NR1)_2$ reporter plasmid was generated by inserting two repeats of the NR1 motif into the KpnI and XhoI sites of pGL3-promoter vector, respectively. Sequencing was performed to verify correct insert orientation of all generated plasmids. The pRL-TK vector was used as internal control (Promega).

Induction Studies in Primary Human Hepatocyte Cultures. Human hepatocytes were obtained commercially from CellzDirect, Inc. (Pittsboro, NC) or ADMET Technologies, Inc. (Research Triangle, NC). Alternatively, hepatic tissues were obtained through surgical

resections by qualified medical staff following donor consent and prior approval from the Institutional Review Board at the University of North Carolina at Chapel Hill. Hepatocytes were isolated from liver tissue resections by modification of a two-step collagenase digestion method described previously (LeCluyse et al., 2005).

Cells were seeded at 1.5 x 10^6 cells/well in six-well Biocoat® plates in DMEM supplemented with 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 4 μg/mL insulin, and 1 μM dexamethasone, and allowed to attach at 37°C in a humidified atmosphere of 5% CO_2. After 4 to 6 hr, cells were overlaid with Matrigel (0.25mg/mL) in serum-free Modified Chee's Medium supplemented with ITS^+ (6.25 μg/mL insulin, 6.25 μg/mL transferrin, and 6.25 ng/mL selenium) and 0.1 μM dexamethasone. After 36-48 hr in culture, hepatocytes were treated for 24 hr with vehicle (0.1% DMSO), 10 μM RIF, and 0.5 or 1 μM CITCO. For detailed concentration-response studies, cells were exposed for 24 hr to 0.1 to 150 μM RIF or 0.001 to 10 μM CITCO. RIF and CITCO stock solutions were prepared in DMSO and added to MCM at dilutions of 1/1000 to provide solvent concentrations of 0.1%.

Real-Time PCR. Total RNA was isolated from treated human hepatocytes using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the SuperScript First-Strand Synthesis System for PCR (Invitrogen). CYP2B6 and CYP3A4 primers and TaqMan® fluorescent probes were designed using Primer Express version 2.0 software (Applied Biosystems). Primers and probes for CYP2B6 and CYP3A4 mRNA detection are as follows, in the order of forward primer, probe, and reverse primer: CYP2B6 1299 to 1366 bp, 5'-AAGCGGATTTGTCTTGGTGAA-3', 6-FAM-CATCGCCCGTGCGGAATTGTTC-TAMRA, 5'-TGGAGGATGGTGGTGAAGAAG-3' and CYP3A4 59 to 179 bp, 5'-TCAGCCTGGTGCTCCTCTATCTAT-3', 6-FAM-TCCAGGGCCCACACCTCTGCCT-TAMRA, 5'-

AAGCCCTTATGGTAGGACAAAATATTT-3'. Intron and exon junctions were considered during primer and probe design to avoid potential genomic DNA contamination. CYP2B6 and CYP3A4 primers and probes were designed to exclude non-selective amplification of CYP2B7 and CYP3A5/7/43, respectively. CYP2B6 or CYP3A4 mRNA expression was normalized against that of human β-actin, which was detected using a predeveloped primer/probe mixture (Applied Biosystems). Multiplexed TaqMan® PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Fold induction values were calculated according to the equation $2^{\Delta\Delta Ct}$, where ΔCt represents the differences in cycle threshold numbers between the target gene and β-actin, and ΔΔCt represents the relative change in these differences between control and treatment groups.

Transient Transfection of Human Hepatoma Cells. HepG2 cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C, 5% CO_2. For reporter assays, cells were seeded into 24-well plates at 1.5 x 10^5 cells/well and transfected 24 hr later with CellPhect reagents according to supplier's recommendations (Amersham). With transfections involving a single nuclear receptor (hPXR or $hCAR_{wt}$), transfection mixes contained 100 ng firefly luciferase reporter construct, 10 ng pRL-TK, and 50 ng nuclear receptor expression vector in DMEM/F-12 supplemented with 10% charcoal/dextran-treated FBS. In the case of co-transfection with two receptors (hPXR + $hCAR_{wt}$, $hCAR_{wt}$ + $hCAR_{1-338}$, or hPXR + $hCAR_{1-338}$), mixes were the same as above except for a fixed amount of one receptor (25 ng) with increasing amounts of the other (25, 50, 75, 100, 175, 250 ng). pCR3 empty vector was transfected in varying amounts to ensure 275 ng of total DNA per transfection. Cells were washed with fresh DMEM/F-12 12 hr after transfection and treatment was initiated with vehicle

(0.1% DMSO) or RIF 10 μM. After 24 hr, cells were lysed and firefly and Renilla luciferase activities were measured from three to six independent transfections using the Dual-Luciferase Reporter Assay System (Promega). Transfection data was expressed as the fold induction of firefly to Renilla luciferase activities relative to empty vector or vehicle control.

For hCAR localization studies, 200 ng EYFP-hCAR$_{wt}$ or EYFP-hCAR$_{1-338}$ expression plasmid was transfected into HepG2 cells using Effectene® transfection reagent following the manufacturer's instructions. Twenty-four hr later, hCAR cellular distribution was visualized using a Zeiss Axiovert 100 TV fluorescent microscope (Carl Zeiss, Thornburg, NY).

Electrophoretic Mobility Shift Assays. Gel shift assays were performed as described previously (Wang et al., 2003). hPXR, hCAR, and hRXRα proteins were synthesized from pSG5-hPXR, pCR3-hCAR, and pGEM-hRXRα expression vectors by using the TNT Quick-Coupled *In Vitro* Transcription/Translation system (Promega). Oligonucleotide probes were labeled with [α-^{32}P]dATP and purified by Microspin G-25 columns (Amersham). All binding reactions consisted of 5 mM Tris-HCl (pH 7.5), 0.25 mM dithiothreitol, 10% glycerol, 0.05% Nonidet P-40, 25 mM NaCl, 1.5 μg of poly (dI-dC), 1 μl of each *in vitro* translated nuclear receptor protein, and 4×10^4 cpm of labeled probe. For competition experiments, binding reactions also contained unlabeled competitor in 25- or 50-fold excess of labeled probe. After incubation at room temperature for 10 min, reaction mixtures were resolved on non-denaturing 5% acrylamide gels in 7 mM Tris-acetic acid buffer (pH 7.5) containing 1 mM EDTA at 180 V for 1.5 hours. Shifted complexes were visualized by drying gels under vacuum for 1 hr and developing on X-ray film overnight at –70°C. Band densities were determined using NIH imaging software (ImageJ 1.34S).

Data Analysis. Results from reporter gene assays and real-time PCR are expressed as mean ± standard deviation of triplicate determinations unless otherwise indicated. Statistical comparisons were made where appropriate using an unpaired t-test. The criterion of significance was set at $p = 0.05$ and tests were performed using SigmaStat version 2.03 software (SPSS Inc, Chicago, IL).

Results

Comparison of CYP2B6 and CYP3A4 Induction by Selective hPXR or hCAR Activators. CYP2B6 and CYP3A4 induction profiles were compared between selective hPXR and hCAR activators using RIF and CITCO, respectively. Primary human hepatocytes from six donors were exposed to RIF (10 μM) or CITCO (0.5 or 1 μM) for 24 hr prior to RNA isolation. The selected concentrations of RIF and CITCO approximate those producing maximum induction of CYP3A4 and CYP2B6, respectively (Faucette et al., 2004; Maglich et al., 2003). Relative mRNA expression levels were quantitated by real-time PCR analysis.

RIF induced CYP3A4 by 5- to 13-fold among six human hepatocyte preparations (mean ± SD, 9.2 ± 3.2), whereas CITCO induced the gene by 1.4- to 2.7-fold (mean ± SD, 1.8 ± 0.5). Combining data from all hepatocyte donors, greater magnitudes of CYP3A4 induction were observed with RIF compared with CITCO ($p < 0.01$, Fig. 1A). In contrast, extents of RIF- and CITCO-mediated CYP2B6 induction were characterized by less discerning differences ($p > 0.05$, Fig. 1B). Among the six donors, CYP2B6 was induced from 3.7- to 8.9-fold by RIF (mean ± SD, 6.0 ± 2.0) and from 4.3- to 16.8-fold by CITCO (mean ± SD, 7.0 ± 4.8). Notably, CITCO exhibited marked preferential induction of CYP2B6 compared with CYP3A4 among all evaluated hepatocyte cultures, whereas RIF induced both genes efficaciously in a less discriminating manner (Fig. 1A vs. 1B).

To confirm selectivity differences between hPXR and hCAR activators with respect to CYP2B6 and CYP3A4 regulation, separate cultures of hepatocytes were exposed to extended concentration ranges of CITCO (0.001 to 10 μM) or RIF (0.1 to 150 μM). Although CITCO induced CYP2B6 in a concentration-dependent manner by a maximum of 10-fold, CYP3A4 was induced less than 2-fold over the entire concentration range (Fig. 2A). In contrast, parallel concentration-dependent induction of both CYP3A4 and CYP2B6 was observed with RIF treatment (Fig. 2B). Collectively, these observations suggest that selective hPXR activation results in promiscuous regulation of both CYP3A4 and CYP2B6, and that hCAR exhibits selective target gene activation, as demonstrated by preferred induction of CYP2B6 over CYP3A4.

Comparison of hCAR and hPXR Activation of CYP2B6 and CYP3A4 Reporter Genes Containing Proximal and Distal Response Elements. Because induction studies in primary human hepatocytes demonstrated the possibility of differential selectivities of hCAR and hPXR with respect to CYP3A4 and CYP2B6 regulation, additional evidence for this phenomenon was sought using cell-based transfection reporter assays. hCAR constitutive activation or RIF-mediated hPXR activation was compared between CYP3A4 and CYP2B6 reporter genes in HepG2 cells. Promoter sequences included in CYP3A4 and CYP2B6 reporter plasmids are shown in Figure 3A. Transfected reporter constructs contained both the proximal and distal regulatory elements of the CYP2B6 or CYP3A4 promoters that are required for optimal induction of the respective genes (Goodwin et al., 1999; Sueyoshi et al., 1999). For hCAR transfection, ligand-independent activation of reporter genes was examined because of the high basal activity of hCAR in immortalized cells resulting from its spontaneous nuclear accumulation (Kawamoto et al., 1999).

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Compared with empty vector, constitutive activation of hCAR increased CYP2B6-PBREM/XREM activities by 6.4-fold and CYP3A4-PXRE/XREM activities by only 3.3-fold ($p < 0.05$, Fig. 3B). In contrast, differences in hPXR activation by RIF were less pronounced between CYP2B6 and CYP3A4 reporters (15.8- vs. 23.7-fold relative to control) ($p > 0.05$, Fig. 3C). Although CYP3A4 reporter activities were slightly higher, differences between the extents of hPXR activation of CYP3A4 and CYP2B6 reporters were less prominent compared with hCAR activation. Results from cell-based transfection assays are in agreement with induction studies in human hepatocytes and provide additional evidence for greater CYP2B6 versus CYP3A4 selectivity of hCAR compared with hPXR.

Differential Binding of hPXR and hCAR to CYP2B6 and CYP3A4 Promoter Elements. It was hypothesized that preferred induction of CYP2B6 to CYP3A4 by hCAR, and lack of selective induction by hPXR, could be explained by differences in hPXR and hCAR binding to one or more motifs in the proximal and distal promoter regions of CYP2B6 and CYP3A4. To test this possibility, gel shift assays were performed to compare the binding affinities of hPXR- and hCAR-hRXRα heterodimers to the CYP2B6 NR1 (DR4) and NR3 (DR4) and to the CYP3A4 PXRE (ER6), dNR1 (DR3), and dNR2 (ER6) (Fig. 4A).

In agreement with previous studies (Goodwin et al., 1999; Goodwin et al., 2001; Wang et al., 2003), hPXR-hRXRα bound competently to the CYP2B6 NR1 and NR3 sequences and to the CYP3A4 ER6 and dNR1 sequences (Fig. 4B, left). hCAR-hRXRα complexed with these same sequences, as previously reported (Goodwin et al., 2002; Sueyoshi et al., 1999; Wang et al., 2003) (Fig. 4B, right). However, neither hPXR-RXRα nor hCAR-RXRα heterodimers significantly bound CYP3A4 dNR2. In contrast to hPXR, hCAR displayed a strikingly lower binding affinity for the CYP3A4 ER6 element compared with the other elements. Both hPXR

and hCAR bound the DR4-type NR1 and NR3 and the DR3-containing dNR1 elements with similar affinities or with less prominent differences than observed with the ER6 motif.

To evaluate whether differential or similar binding to DR4 or ER6 motifs could account for preferred induction of CYP2B6 over CYP3A4 by hCAR activation, or indiscriminant induction of these genes by hPXR activation, respectively, competition binding experiments were performed using labeled CYP2B6 NR3 probe with unlabeled CYP3A4 ER6 competitor or labeled ER6 probe with unlabeled NR3 competitor (Fig. 4C). For hPXR, binding to ^{32}P-labeled NR3 was decreased by 39% and 66% in the presence of 25- and 50-fold excess of ER6 competitor, respectively (relative densities of 1.0, 0.61 and 0.34 in the presence of 0, 25x, or 50x of cold competitor). Similarly, hPXR binding to ^{32}P-labeled ER6 was decreased by 29% and 58% by increasing amounts of NR3 competitor (relative densities of 1.0, 0.71, and 0.42 in the presence of 0, 25x, or 50x of cold competitor). In contrast, 25- and 50-fold excess ER6 decreased hCAR binding to NR3 by 7% and 28%, respectively (relative densities of 1.0, 0.93, and 0.72). Despite weak hCAR binding to the labeled ER6 probe in the absence of competitor, as observed in Fig. 4B, increasing amounts of unlabeled NR3 competitor further reduced this binding (Fig. 4C). These results indicate that hPXR binds CYP2B6 and CYP3A4 response elements with appreciable affinities, but that hCAR binds more strongly to the CYP2B6 DR4 motifs compared with the CYP3A4 proximal ER6. The comparatively lower affinity of hCAR for the CYP3A4 ER6 provides a mechanistic basis for selective hCAR-mediated induction of CYP2B6 relative to CYP3A4, while similar affinities of hPXR for the DR4 and ER6 motifs explain its non-selective induction of both genes.

Comparison of hPXR and hCAR Activation of CYP2B6 and CYP3A4 Reporter Genes Containing Repeats of Single Response Motifs. To determine the functional relevance of

differential binding to CYP2B6 and CYP3A4 promoter elements by hCAR but not hPXR, additional experiments focused on select elements from the CYP2B6 (DR4/NR1) and CYP3A4 (ER6) promoters. Thus, HepG2 cells were co-transfected with hPXR or hCAR expression plasmids and heterologous reporter constructs containing two repeats of the CYP2B6 NR1 or CYP3A4 ER6 motifs. Relative to empty vector, pGL3-CYP2B6 $(NR1)_2$ reporter gene expression was 12.7-fold higher in HepG2 cells transfected with hCAR, compared with 2.6-fold higher for pGL3-CYP3A4 $(ER6)_2$ ($p < 0.05$, Fig. 5A). In cells transfected with hPXR, RIF treatment was associated with similar degrees of CYP2B6 and CYP3A4 reporter gene activation (3.3- and 4.8-fold, respectively) ($p > 0.05$, Fig. 5B). Overall, these transfection results demonstrate the functional consequences of impaired binding of hCAR to the CYP3A4 ER6 motif. Furthermore, they suggest that lower levels of CYP3A4 induction by selective hCAR activators derive primarily from its weak ER6 binding.

Competition Between hPXR and hCAR for CYP2B6 and CYP3A4 Reporter Activation in HepG2-Based Transfection Assays. Co-transfection assays were performed in HepG2 cells to evaluate the competition between hPXR and hCAR for activation of a single element (CYP2B6 NR1 or CYP3A4 ER6). The first such experiment examined the ability of non-liganded hPXR to compete with hCAR for binding and constitutive activation of the pGL3-CYP2B6 $(NR1)_2$-luciferase construct (Fig. 6). A fixed amount of hCAR (25 ng) was transfected into HepG2 cells alone or with differing amounts of hPXR as binding competitor (25 to 250 ng). As expected, singly transfected hCAR, but not hPXR, exhibited constitutive activation of the CYP2B6 reporter gene relative to empty vector. Reporter activities decreased in a dose-dependent manner up to 75 ng hPXR and subsequently plateaued with higher transfected

amounts (Fig. 6). These results demonstrate that both hCAR and hPXR can compete effectively for binding and activation of the CYP2B6 DR4-type NR1 motif.

The second experiment examined the ability of hCAR to compete with RIF-activated hPXR for binding and activation of the pGL3-CYP3A4 $(ER6)_2$-luciferase reporter gene. However, $hCAR_{wt}$ was not appropriate to use in this study because of its inherently high constitutive activity. To avoid this potential confounding factor, we desired a mutant hCAR that displayed minimal or no constitutive activity, but similar binding to CYP2B6 and CYP3A4 response elements as $hCAR_{wt}$. Thus, a truncated hCAR mutant was used as competitor of hPXR instead of $hCAR_{wt}$. The hCAR mutant was generated by deleting the last ten C-terminal amino acid residues of the ligand binding domain comprising the ligand-independent AF2 region (Zelko et al., 2001).

The $hCAR_{1-338}$ deletion mutant exhibited predominant nuclear distribution in HepG2 cells, as did $hCAR_{wt}$ (Fig. 7A). Gel shift analysis revealed that mutant $hCAR_{1-338}$ was capable of binding to the CYP2B6 NR1 motif with similar affinity as $hCAR_{wt}$ (Fig. 7B), but only weakly to the CYP3A4 ER6 motif (data not shown). However, when co-transfected with the pGL3-CYP2B6 $(NR1)_2$ reporter in HepG2 cells, $hCAR_{1-338}$ failed to demonstrate constitutive activity compared with $hCAR_{wt}$, regardless of the amount of $hCAR_{1-338}$ transfected (10 ng to 250 ng) (Fig. 7C). These results validated that $hCAR_{1-338}$ shared the DNA binding characteristics and cellular localization of $hCAR_{wt}$ but not its constitutive activation.

In competition studies, HepG2 cells were transfected with the pGL3-CYP2B6 $(NR1)_2$-reporter plasmid and 25 ng $hCAR_{wt}$, with or without increasing amounts of mutant $hCAR_{1-338}$ (25 to 250 ng). As expected, constitutive activation of the CYP2B6 reporter by $hCAR_{wt}$ decreased in a dose-dependent manner with increasing amounts of co-transfected $hCAR_{1-338}$, reaching a

maximum decrease with 10-fold excess of the mutant receptor (Fig. 7C). Subsequently, HepG2 cells were co-transfected with hPXR alone or with increasing amounts of $hCAR_{1-338}$ and treated with solvent or RIF (Fig. 7D). In the absence of mutant hCAR, hPXR exhibited negligible constitutive activation, but 5-fold RIF-mediated activation of the pGL3-CYP3A4 $(ER6)_2$ reporter construct. This extent of hPXR activation did not decrease significantly when $hCAR_{1-338}$ was co-transfected over the range of 25 to 250 ng (Fig. 7D). Collectively, these transfection experiments indicate that hCAR cannot compete with hPXR for binding and activation of the CYP3A4 proximal ER6 motif, and support a lower affinity of hCAR for this element compared with hPXR.

Discussion

The objective of this study was to determine the degree of symmetry of CYP2B6 and CYP3A4 cross-regulation by hPXR and hCAR by comparing the abilities of these receptors to bind relevant promoter elements, to activate reporter genes driven by these promoter elements, and to induce the corresponding target genes in primary human hepatocytes. Results from this study indicate that cross-talk between hPXR and hCAR is asymmetrical for CYP3A4 but not CYP2B6. Whereas hPXR regulates both CYP3A4 and CYP2B6 with less selectivity, hCAR exhibits pronounced selectivity for CYP2B6 over CYP3A4. These results lead to the establishment of a functionally relevant model of cross-talk between hPXR and hCAR that challenges the existing paradigm based primarily on the corresponding rodent receptors (Fig. 8A vs. 8B).

Although CAR regulation of *CYP2B* and *CYP3A* genes has been well established in rodents (Honkakoski et al., 1998; Smirlis et al., 2001; Wei et al., 2002; Wei et al., 2000), the contribution of hCAR to CYP2B6 and CYP3A4 regulation has remained obscured in the absence of identified

ligands and/or activators. However, this study's comparison of the CYP2B6 and CYP3A4 transcriptional selectivity of hCAR was facilitated by the recent validation of CITCO as a selective hCAR activator (Maglich et al., 2003). In this investigation, selective hCAR activation by CITCO was associated with impaired induction of CYP3A4 in human hepatocytes relative to CYP2B6. Notably, this phenomenon was observed across an extended concentration range with 24 hr treatment. Although time course studies were not performed with CITCO, it is likely that preferential induction of CYP2B6 would occur with other treatment times because our previous study demonstrated similar temporal kinetics of CYP2B6 and CYP3A4 induction (Faucette et al., 2004). In addition, preferred induction of CYP2B6 over CYP3A4 has been observed with three other compounds identified by our laboratory as selective hCAR activators (data not shown), suggesting that this phenomenon is a shared class effect rather than a unique compound-specific effect of CITCO.

The selectivity of hCAR for CYP2B6 rather than CYP3A4 results presumably from its stronger binding to the DR4 motifs (NR1 and NR3) in the CYP2B6 promoter relative to the ER6 motif in the CYP3A4 proximal promoter. In gel shift assays conducted in this study, hCAR displayed appreciable binding to all known CYP2B6 and CYP3A4 regulatory elements except the ER6. Furthermore, the ER6 failed to compete with the CYP2B6 DR4-type NR3 element for binding to hCAR, although the NR3 competed effectively with the ER6. Other reports have confirmed the weaker ER6 binding potential of hCAR relative to other response elements. For example, Frank et al. (Frank et al., 2003) reported that hCAR-hRXR bound a perfect ER6 motif with 50% lower capacity than a perfect DR4 motif. In addition, Goodwin et al. (Goodwin et al., 2002) noted from competition binding studies that the affinity of hCAR-hRXR heterodimers was approximately 4- to 5-fold greater for the distal CYP3A4 DR3 motif (dNR1) than the proximal

ER6 motif. In this same study, it was observed that hCAR failed to activate a CYP3A4 reporter construct driven by ~400 bp of the native proximal promoter containing the PXRE (Goodwin et al., 2002).

The current study further illustrates the functional consequences of impaired ER6 binding by hCAR. Compared with the CYP2B6 NR1 motif, hCAR exhibited a diminished capacity to promote transcription of a heterologous reporter construct containing two copies of the ER6 motif. In addition, hCAR was unable to compete efficiently with hPXR for activation of this same construct. Although hCAR can bind to the DR3 motif in the CYP3A4 distal XREM with strong affinity (Fig. 4B; (Goodwin et al., 2002)), hCAR-mediated induction of CYP3A4 endogenous or reporter gene expression is significantly lower than for CYP2B6, suggesting that the proximal ER6 plays a critical role in determining the overall CYP3A4 transcriptional response. Previous studies have demonstrated that the ER6-containing PXRE interacts cooperatively with the DR3-containing XREM to achieve optimal CYP3A4 induction by both hPXR and hCAR (Goodwin et al., 2002; Goodwin et al., 1999). While maximum hCAR activation was achieved with a CYP3A4 reporter construct containing the native proximal promoter (including the PXRE) and the XREM, hCAR responsiveness was reduced by approximately 50% when the ER6 site within the PXRE was mutated and the XREM module was preserved (Goodwin et al., 2002). Dependence of the full hCAR-mediated CYP3A4 response on ER6 binding and activation is consistent with the current study findings of a greater difference in hCAR constitutive activation of CYP3A4 and CYP2B6 when CYP3A4 reporter constructs contained two repeats of the ER6 motif (4.9-fold) compared with both the proximal PXRE and XREM (2.3-fold).

In contrast to CYP3A4, patterns of CYP2B6 induction were similar or associated with less

discerning differences in response to selective hPXR and hCAR activators. In several human hepatocyte preparations, CYP2B6 was induced to similar extents by RIF and CITCO. Furthermore, hPXR and hCAR competed effectively for transcriptional activation of the CYP2B6 $(NR1)_2$-reporter gene in cell-based transfection assays. These findings are not surprising given that both receptors bind competently to the CYP2B6 DR4-type NR1 and NR3 elements, which differ from each other by only one base pair. In general, differences between hPXR and hCAR binding to these elements were not as pronounced as the difference noted for the CYP3A4 ER6 motif. Overall, these results reveal two important aspects of cross-regulation of CYP2B6 and CYP3A4 by hPXR and hCAR. First, the ability of hPXR to cross-regulate CYP2B6 is greater than the ability of hCAR to cross-regulate CYP3A4, and secondly, hPXR regulates these target genes in a non-differential fashion compared with the selective manner of hCAR.

The model proposed in this study to explain the degree of cross-regulation of CYP2B6 and CYP3A4 by hPXR and hCAR contrasts with the inferred model of cross-regulation of rodent *CYP2B* and *CYP3A* genes by the corresponding rodent receptors. Although illustrations of the rodent model of cross-talk portray equal cross-regulation of *CYP2B* and *CYP3A* genes by rodent PXR and CAR, respectively, it is unknown whether this representation is accurate because of the absence of studies designed to compare the cross-regulatory abilities of both receptors in parallel. Regardless of the actual selectivities of rodent PXR and CAR for these target genes, comparison of rodent data with the current study's data suggests that rodent CAR exhibits a stronger ability to regulate CYP3A relative to hCAR. In contrast to the weak CYP3A4 induction by CITCO observed in this study, previous studies have demonstrated that Cyp3a11 or CYP3A1 is induced moderately to strongly by the mCAR activators PB and TCPOBOP in CAR wild-type but not

deficient mice (Anakk et al., 2004; Wei et al., 2002; Wyde et al., 2003; Xie et al., 2000b). Furthermore, the magnitudes of PB- or TCPOBOP-mediated mCAR activation of a CYP3A1 heterologous reporter construct or CYP3A23 homologous reporter construct were similar to that of PCN-mediated mPXR or RIF-mediated hPXR activation in transfected primary rat hepatocytes (Smirlis et al., 2001; Xie et al., 2000b). Differences between rodent and human CAR in their capacities for CYP3A regulation may stem from dissimilarities in the proximal promoters of the rodent and human *CYP3A* genes; specifically, the rodent proximal PXRE contains a DR3 motif, while the human response element contains an ER6. Previous studies have demonstrated that mCAR can bind efficiently to the DR3 motifs in the rat CYP3A1 and CYP3A23 promoters (Tzameli et al., 2000; Xie et al., 2000b), which contrasts to the weak ER6 binding of hCAR. Overall, comparisons of cross-regulation of *CYP2B* and CYP3A genes between rodents and humans suggest that species differences in PXR and CAR regulation extend to target genes in addition to ligand binding and activation.

In conclusion, hPXR and hCAR exhibited differential target gene selectivities with respect to CYP2B6 and CYP3A4 gene induction, leading to a model of asymmetrical cross-regulation of these genes that differs from the presumed rodent model (Fig. 8A and B). The greater selectivity of hCAR activation resulted in preferred induction of CYP2B6 over CYP3A4, whereas the lower selectivity of hPXR activation conferred efficacious induction of both target genes. This study demonstrated that degrees of selectivities are related to similarities or differences in hPXR and hCAR binding to response elements in the CYP2B6 and CYP3A4 promoters. While hCAR binds the proximal CYP3A4 ER6 with weaker affinity than hPXR, both receptors bind similarly to the distal NR3 in the CYP2B6 XREM. Interestingly, the preference of hCAR for CYP2B6 over CYP3A4 may have clinical relevance with respect to drugs metabolized by both enzymes

and co-administered with inducers that are selective hCAR activators. Cyclophosphamide (CPA) is an example of such a drug. This antineoplastic undergoes N-dechloroethylation to an inactive toxic metabolite exclusively by CYP3A4, and 4-hydroxylation to a therapeutically active metabolite primarily by CYP2B6 (Roy et al., 1999; Schwartz et al., 2003). Assuming that hCAR selectivity for CYP2B6 also occurs *in vivo*, concurrent administration of CPA with a selective hCAR activator should facilitate enhanced production of its beneficial metabolite without simultaneously increasing formation of its toxic metabolite. These expected effects provide the opportunity to develop therapeutic regimens that optimize beneficial versus undesired toxic effects of CPA in cancer treatment.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. The effects of selective hPXR or hCAR activators on CYP3A4 (A) and CYP2B6 (B) gene expression in primary human hepatocytes from six donors. Cells were cultured for 24 hr in the presence of DMSO (0.1%), the selective hPXR activator 10 μM RIF, or the selective hCAR activator 0.5 μM CITCO (Hu116 and Hu117) or 1 μM CITCO (NLC-111, HCS-004, Hu141, Hu253). Total RNA was extracted, reverse transcribed, and subjected to real-time TaqMan® real-time PCR. CYP3A4 and CYP2B6 expression levels were normalized against those of β-actin. Fold induction of CYP3A4 and CYP2B6 relative to control was calculated as described under "Materials and Methods." Data represent the mean ± SD of triplicate determinations. ***p* < 0.01, where *p* indicates the level of statistical difference between fold induction of CYP3A4 by RIF and CITCO.

Figure 2. Concentration-dependent induction of CYP3A4 and CYP2B6 by CITCO (A) and RIF (B) CITCO in primary human hepatocytes from donor NLC-112 or NLC-111. Cells were treated for 24 hr with DMSO (0.1%), RIF (0.1-150 μM), or CITCO (0.001-10 μM). Total RNA was extracted, reverse transcribed, and subjected to real-time TaqMan® PCR. CYP3A4 (♦) and CYP2B6 ( ) expression levels were normalized against those of β-actin. Results are presented as fold induction relative to control and represent the mean ± SD of triplicate determinations.

Figure 3. Comparison of hCAR and hPXR activation of CYP2B6 and CYP3A4 reporter genes containing proximal and distal response elements. *A*, CYP2B6 and CYP3A4 promoter constructs were generated as described under "Materials and Methods." Sequences and orientations of hPXR and hCAR binding motifs are indicated. *B*, HepG2 cells were transfected with CYP2B6-PBREM/XREM or CYP3A4-PXRE/XREM reporter constructs (100 ng), pRL-TK (10 ng), and pCR3-hCAR or pCR3 empty vector (50 ng). Twelve hr later, cells were exposed to 0.1% DMSO

(vehicle control) for 24 hr prior to cell lysis. Luciferase activities were determined from six independent transfections using the Dual Luciferase Reporter Assay System and expressed as mean ± SD of fold activation. Hatched or solid bars represent results with empty vector or pCR3-hCAR, respectively. An unpaired t-test was used to evaluate statistical differences in fold activation of CYP2B6 and CYP3A4 reporter genes by hCAR (*$p < 0.05$). *C,* HepG2 cells were transfected and analyzed for CYP2B6 and CYP3A4 reporter activities as described in *B,* except that 50 ng of pSG5-hPXR was transfected. After 12 hr of transfection, cells were treated for 24 hr with 0.1% DMSO (hatched bars) or 10 μM RIF (solid bars).

Figure 4. Comparative binding of hPXR and hCAR to proximal and distal motifs in CYP2B6 and CYP3A4 promoters. *A,* Sequences and promoter positions of oligonucleotide probes used for gel shift assays. Bold uppercase lettering indicates putative nuclear receptor hexameric half-sites. *B,* Gel shift assays were performed as described under "Materials and Methods" using *in vitro* translated hPXR, hCAR, and hRXRα, and the CYP2B6 NR1 and NR3 or the CYP3A4 ER6, dNR1, and dNR2 motifs as labeled probes. *C,* The binding of hPXR- or hCAR-hRXRα heterodimers was assessed using CYP2B6 NR3 or CYP3A4 ER6 as labeled probes in the absence or presence of 25- or 50-fold excess of ER6 and NR3 cold competitors (CC), respectively. Arrows indicate the positions of the hPXR or hCAR binding complexes. Relative densities are included under each band, with no CC given an arbitrary value of 1.

Figure 5. Comparison of hCAR and hPXR activation of CYP2B6 and CYP3A4 reporter genes containing repeats of proximal response elements. *A,* HepG2 cells were transfected with CYP2B6 $(NR1)_2$ or CYP3A4 $(ER6)_2$ reporter constructs (100 ng), pRL-TK (10 ng), and pCR3 empty vector or pCR3-hCAR (50 ng). Twelve hr later, cells were treated with 0.1% DMSO (vehicle control) for 24 hr prior to cell lysis. Luciferase activities were measured using the Dual

Luciferase Reporter Assay System (Promega) from three independent transfections. Results are expressed as fold activation (mean ± SD) relative to empty vector. Hatched or solid bars represent transfection with empty vector or pCR3-hCAR, respectively. *$p < 0.05$; p is the level of statistical difference between fold activation of CYP2B6 and CYP3A4 reporter genes by hCAR. *B*, HepG2 cells were transfected and analyzed for CYP2B6 and CYP3A4 reporter activities as described in *A*, except that 50 ng of pSG5-hPXR was transfected. After 12 hr of transient transfection, cells were treated for 24 hr with 0.1% DMSO (hatched bars) or 10 μM RIF (solid bars).

Figure 6. Competition between hPXR and hCAR for activation of the CYP2B6 NR1 motif. HepG2 cells were transfected with CYP2B6 $(NR1)_2$ reporter construct (100 ng), pRL-TK (10 ng), and pCR3-$hCAR_{wt}$ (0 or 25 ng), in the absence or presence of increasing amounts of pSG5-hPXR (25 to 250 ng). The total amount of DNA transfected in each condition was normalized to 275 ng using pCR3 empty vector. Cells were treated for 24 hr with 0.1% DMSO (vehicle control) prior to determination of firefly and Renilla luciferase activities. Data depict mean ± SD of fold activation relative to empty vector, determined from three independent transfections. Pairs of numbers under each solid bar provide the respective amounts of transfected $hCAR_{wt}$ and hPXR.

Figure 7. Evaluation of cellular localization, DNA binding, and constitutive activity of mutant pCR3-$hCAR_{1-338}$ and its ability to compete with $hCAR_{wt}$ or hPXR. *A*, HepG2 cells were transfected with 200 ng of EYFP-tagged $hCAR_{wt}$ or $hCAR_{1-338}$ using Effectene®. Cellular localization of hCAR was observed and photographed using fluorescent microscopy. *B*, Gel shift was performed using CYP2B6 NR1 probe and *in vitro* translated $hCAR_{wt}$, $hCAR_{1-338}$, and hRXRα. *C*, HepG2 cells were transfected with pCR3-$hCAR_{wt}$ (0 or 25 ng), pCR3-$hCAR_{1-338}$ (0

to 250 ng), or combinations of both, in addition to CYP2B6 $(NR1)_2$ reporter construct (100 ng) and pRL-TK (10 ng). The total amount of DNA transfected in each condition was normalized to 275 ng using pCR3 empty vector. Cells were treated for 24 hr with 0.1% DMSO (vehicle control) prior to determination of dual luciferase activities. Data depict mean ± SD of fold activation relative to empty vector, determined from three independent transfections. Pairs of numbers under each solid bar provide the respective amounts of transfected $hCAR_{wt}$ and $hCAR_{1-338}$. *D*, HepG2 cells were transfected with CYP3A4 $(ER6)_2$ reporter construct (100 ng), pRL-TK (10 ng), and pSG5-hPXR (0 or 25 ng), in the absence or presence of increasing amounts of pCR3-$hCAR_{1-338}$ (25 to 250 ng). The total amount of DNA transfected in each condition was normalized to 275 ng using pCR3 empty vector. Cells were treated for 24 hr with 0.1% DMSO (hatched bars) or 10 μM RIF (solid bars) prior to determination of luciferase activities. Data represent the mean ± SD of three independent transfections.

Figure 8. Existing versus newly proposed models of cross-talk between PXR and CAR in the regulation of *CYP2B* and *CYP3A* genes. The model in *A* is based on interpretation of previous literature data, while the one in *B* is based on results generated from the current study. In *A*, +/- indicates that the rodent CYP3A and CYP2B promoters may or may not contain the distal XREM modules present in humans.

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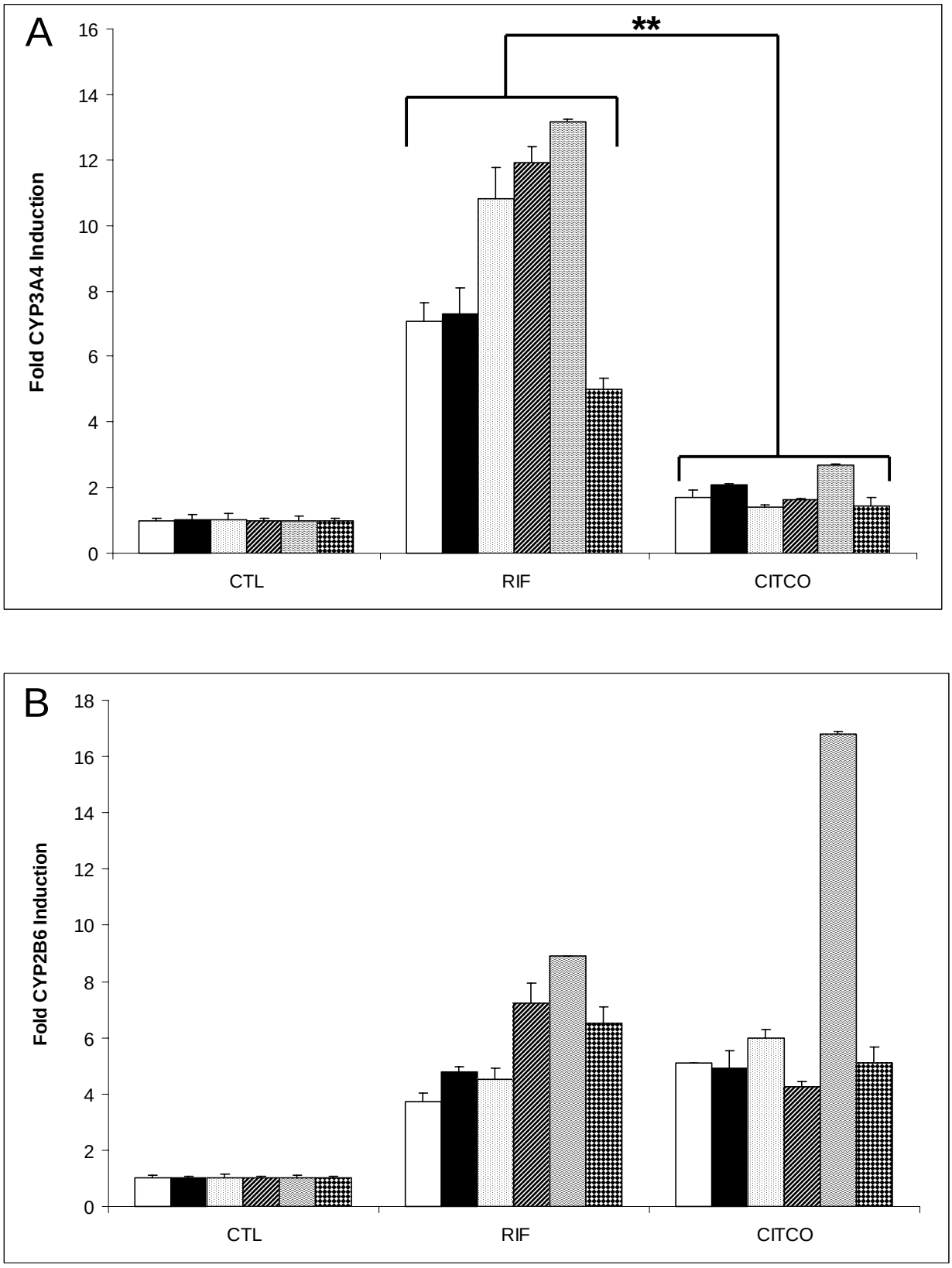

Figure 1

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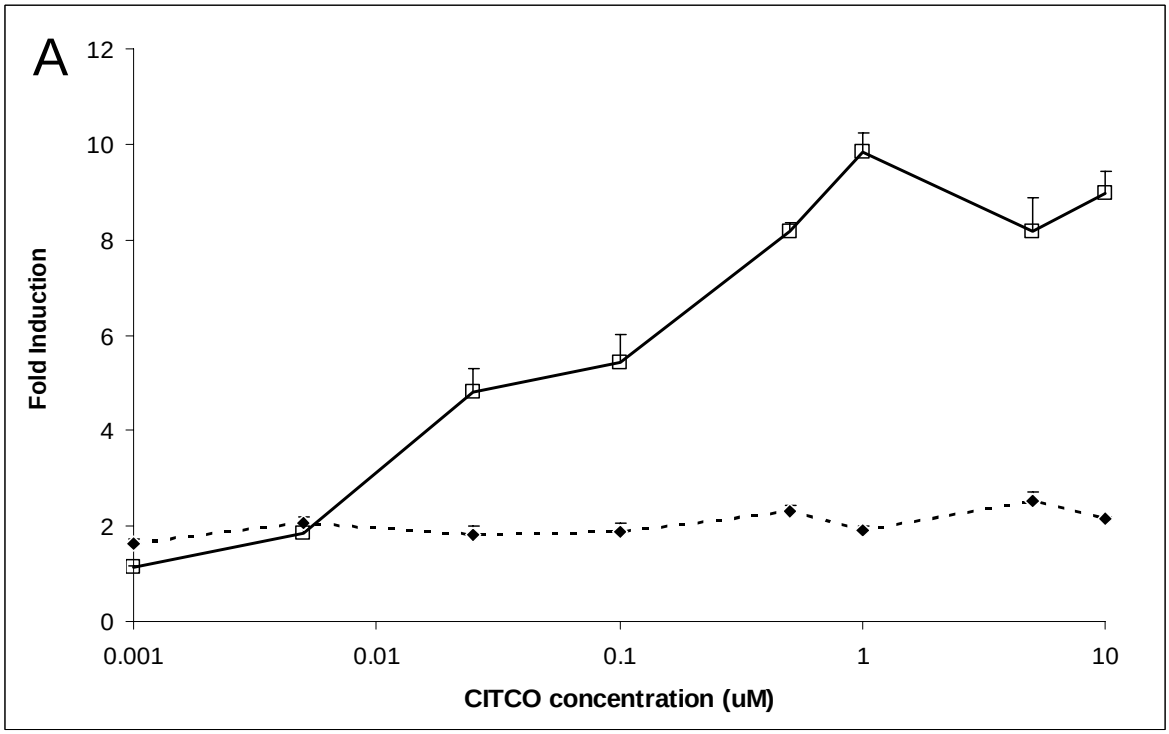

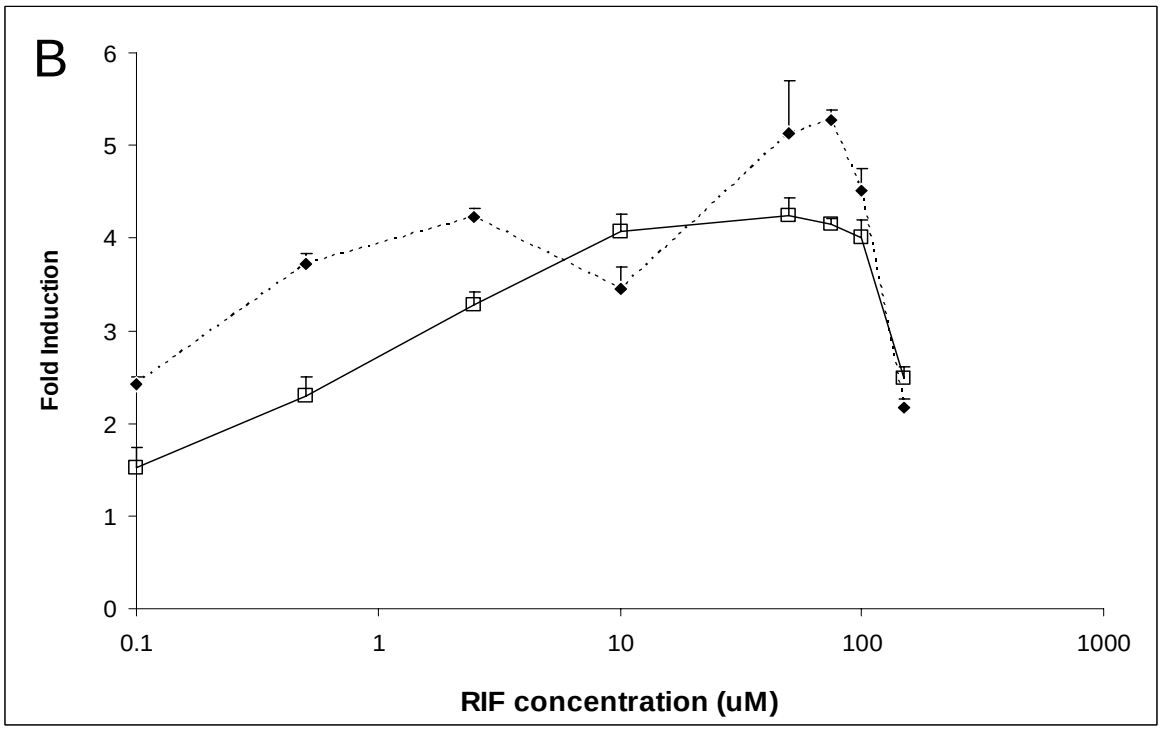

Figure 2

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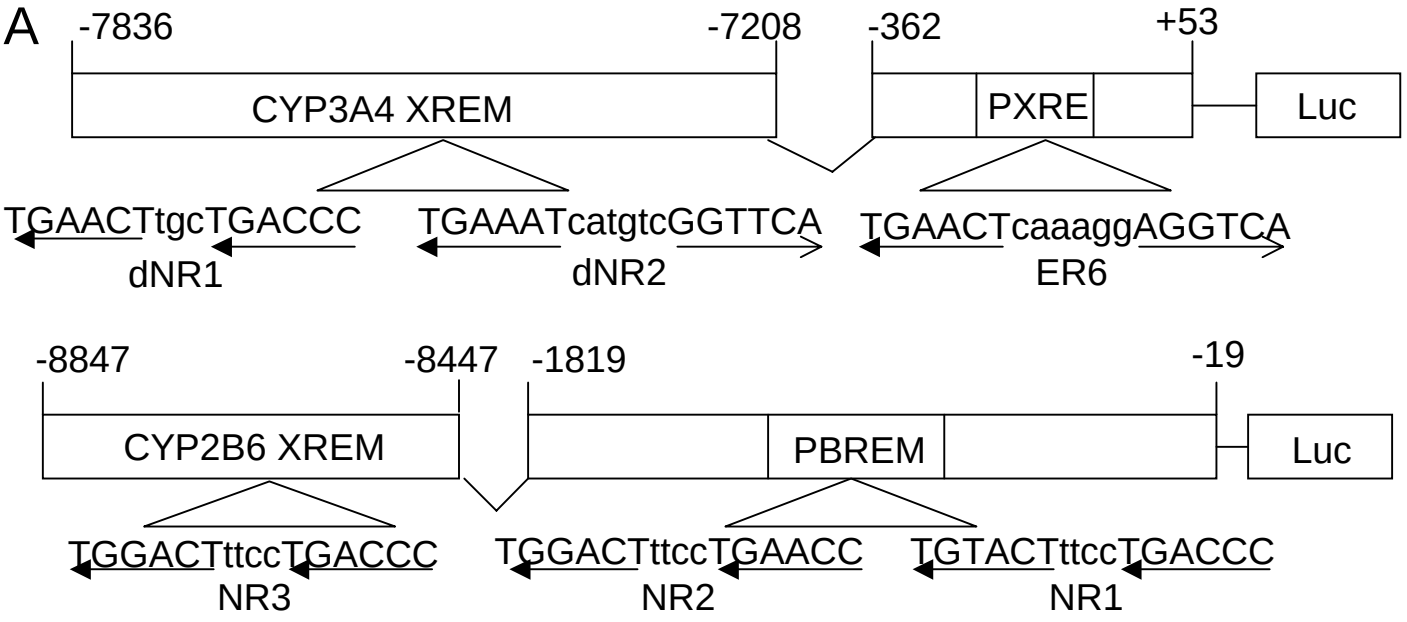

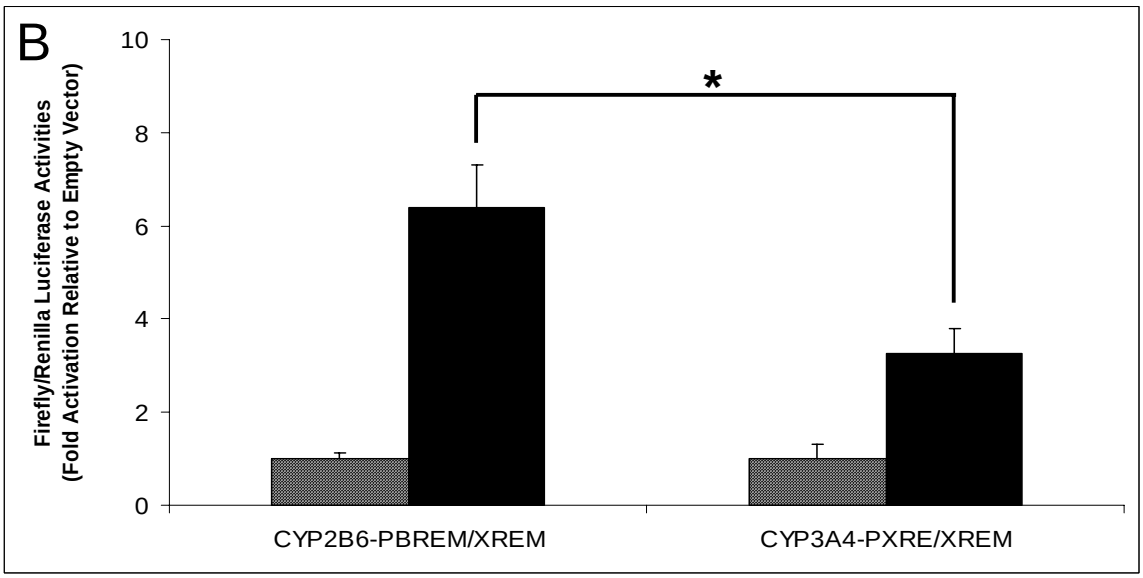

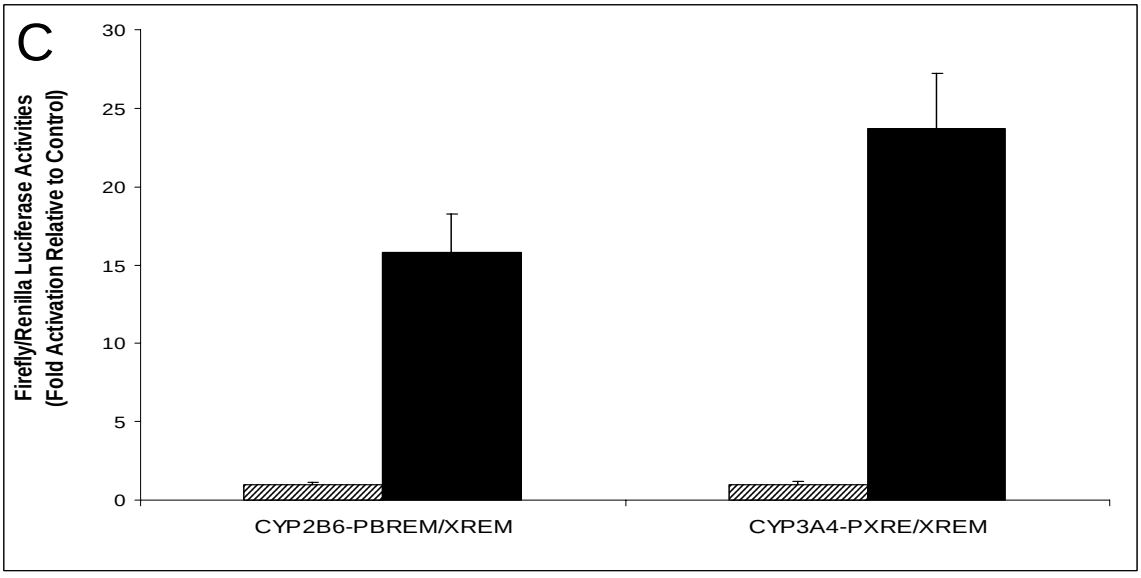

Figure 3

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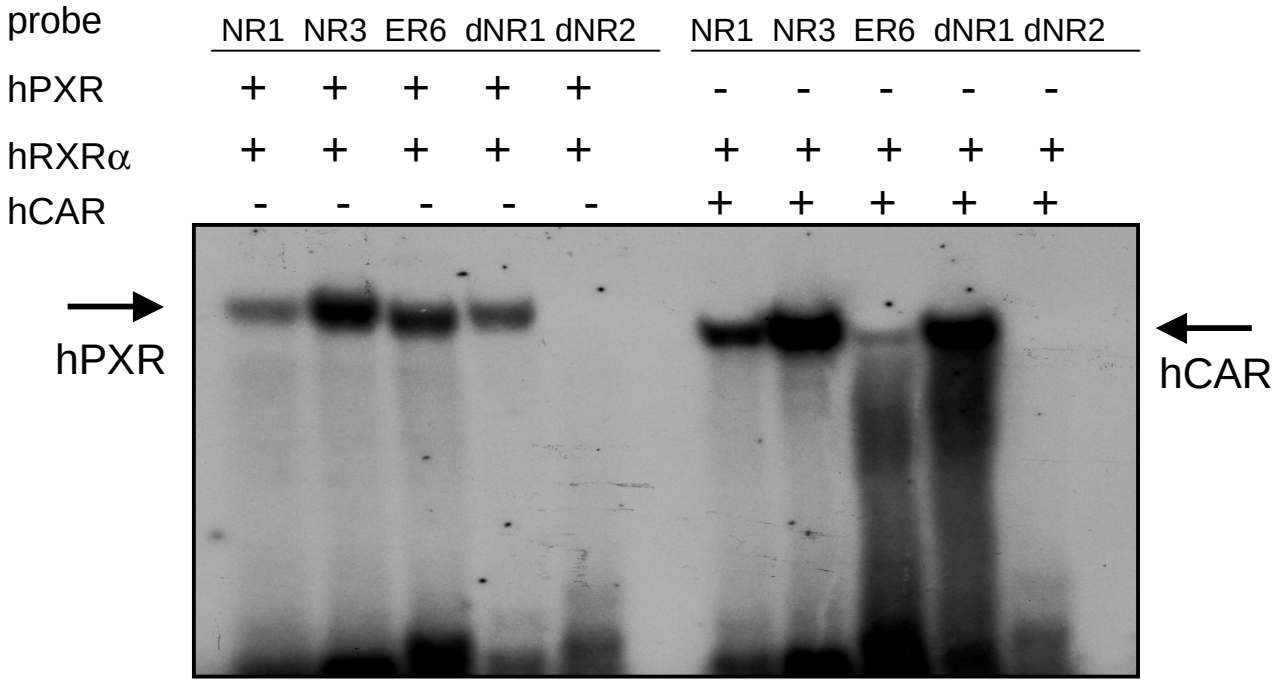
A

Probe	Sequence	Position
CYP2B6 NR1	ac **TGTACT** ttcc **TGACCC** tg	-1683 to -1702
CYP2B6 NR3	ca **TGGACT** ttcc **TGACCC** ca	-8554 to -8573
CYP3A4 ER6	ata **TGAACT** caaagg **AGGTCA** gtg	-172 to -149
CYP3A4 dNR1	gaa **TGAACT** tgc **TGACCC** tct	-7736 to -7716
CYP3A4 dNR2	cct **TGAAAT** catgtc **GGTTCA** agc	-7696 to -7669

B



C

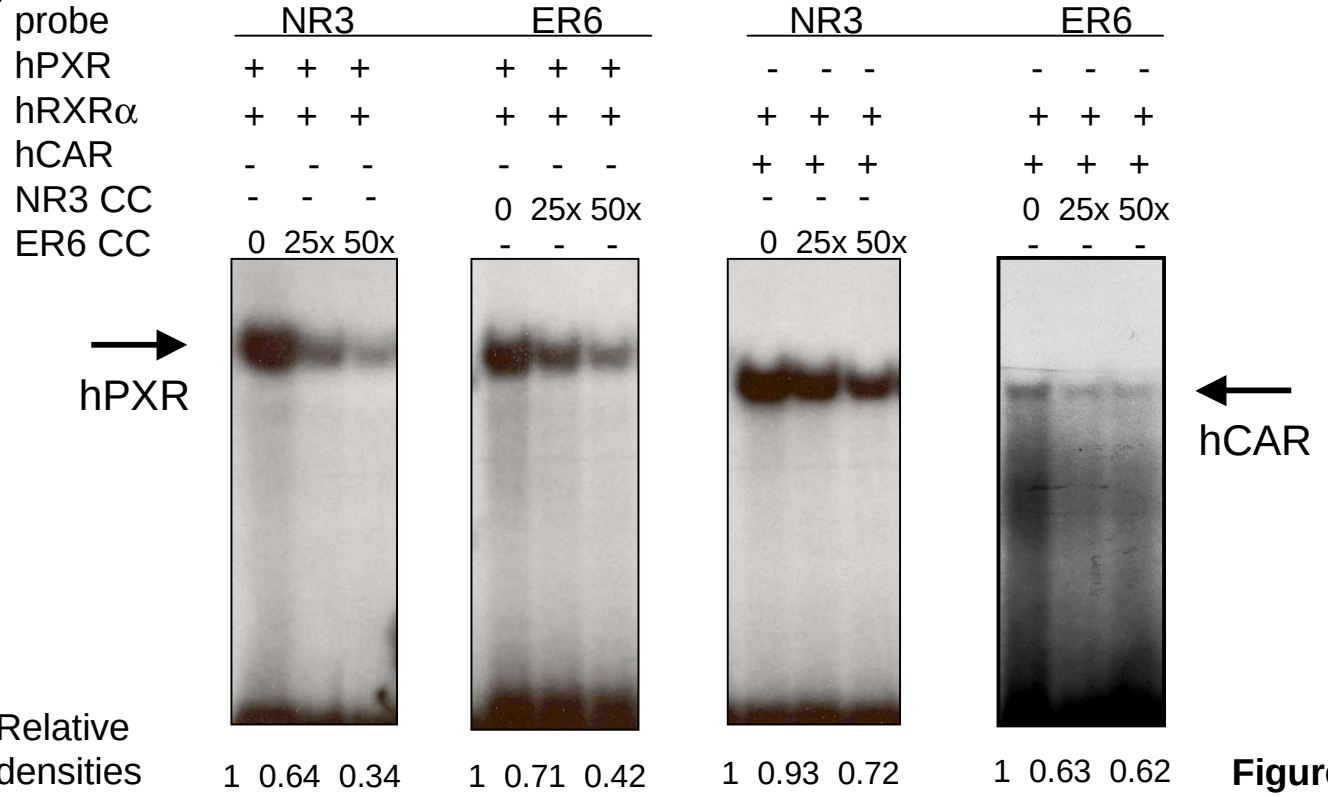


Figure 4

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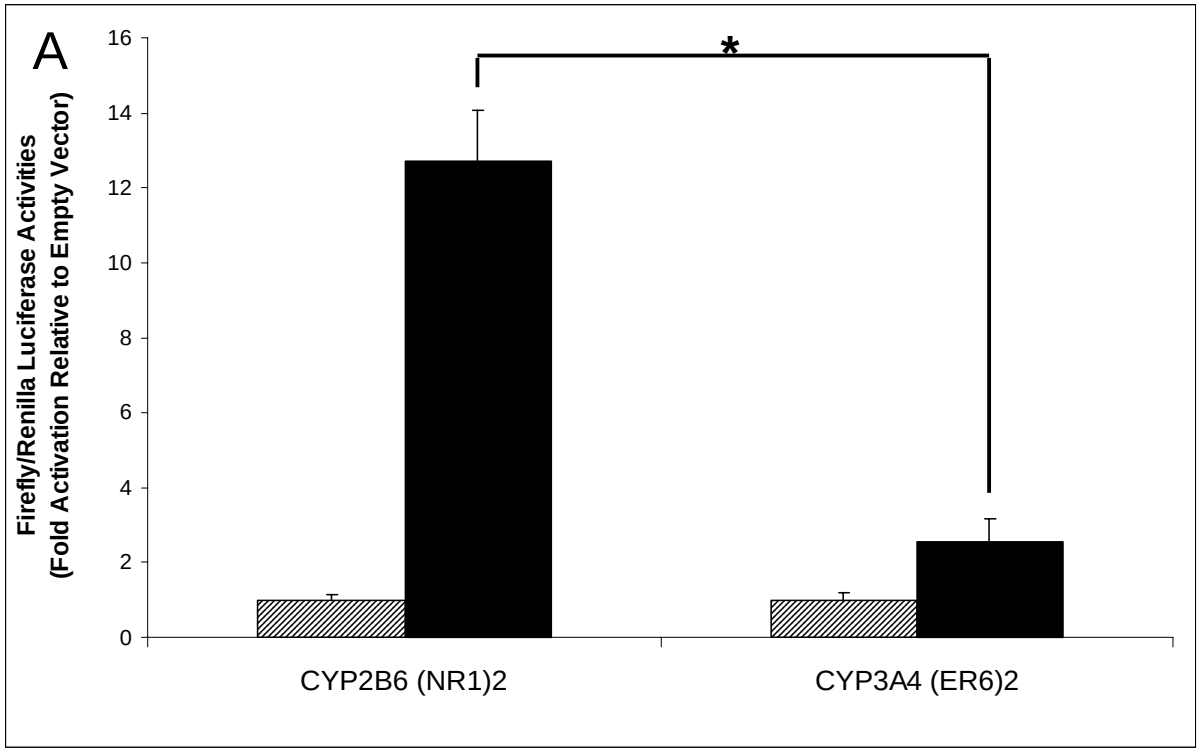

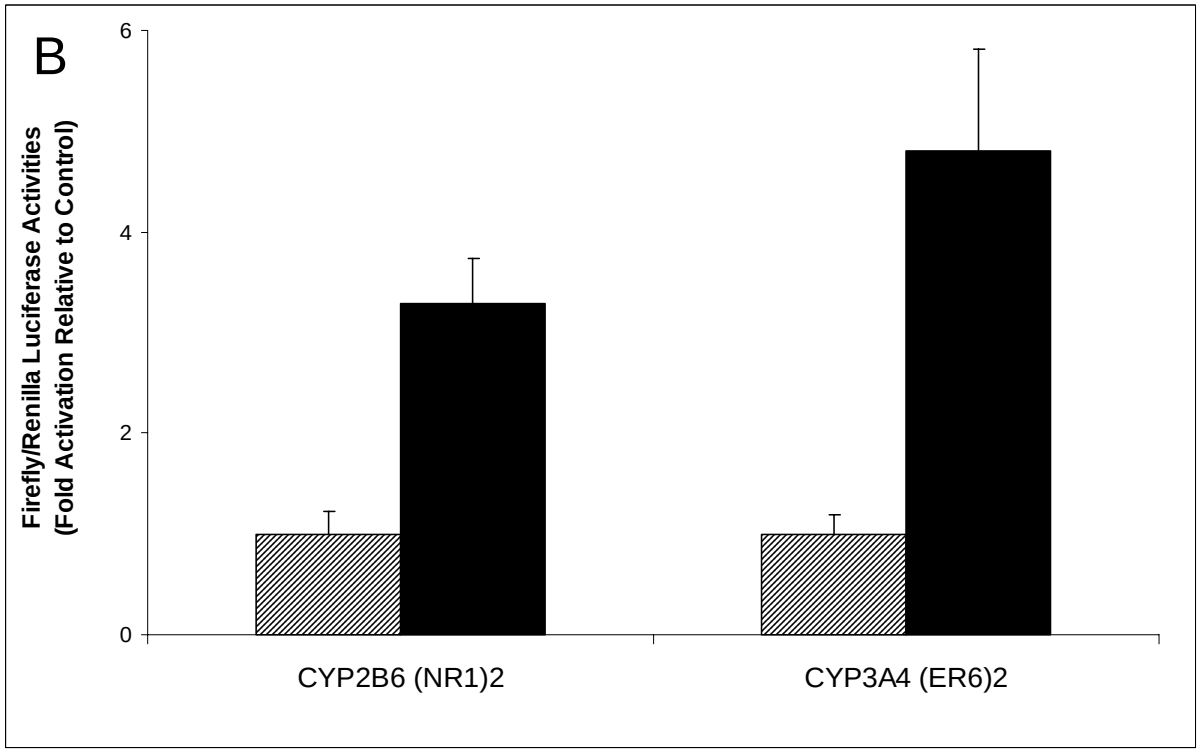

Figure 5

JPET Fast Forward. Published on March 2, 2006 as DOI: 10.1124/jpet.105.098160
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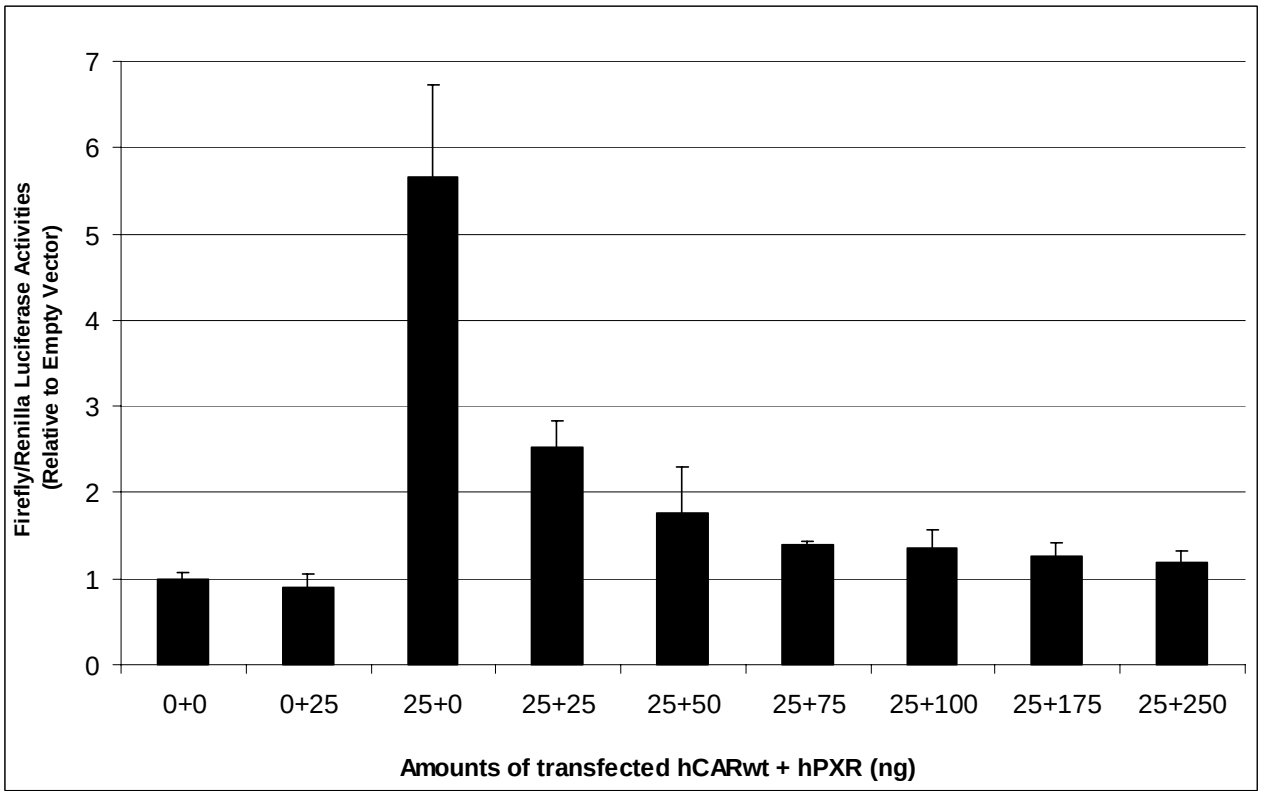

Figure 6

JPET Fast Forward. Published on March 2, 2006 as DOI: 10.1124/jpet.105.098160
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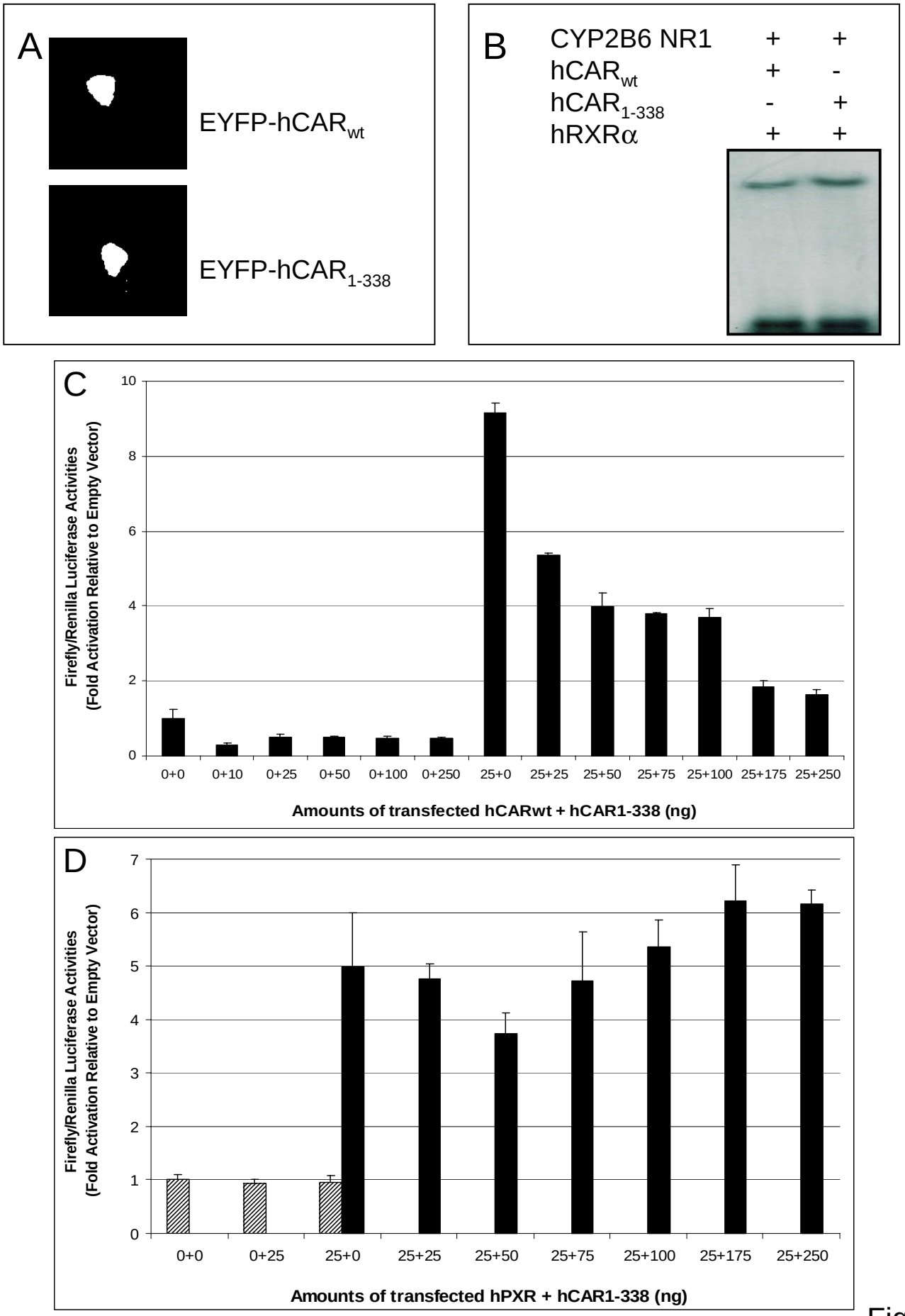

Fig. 7

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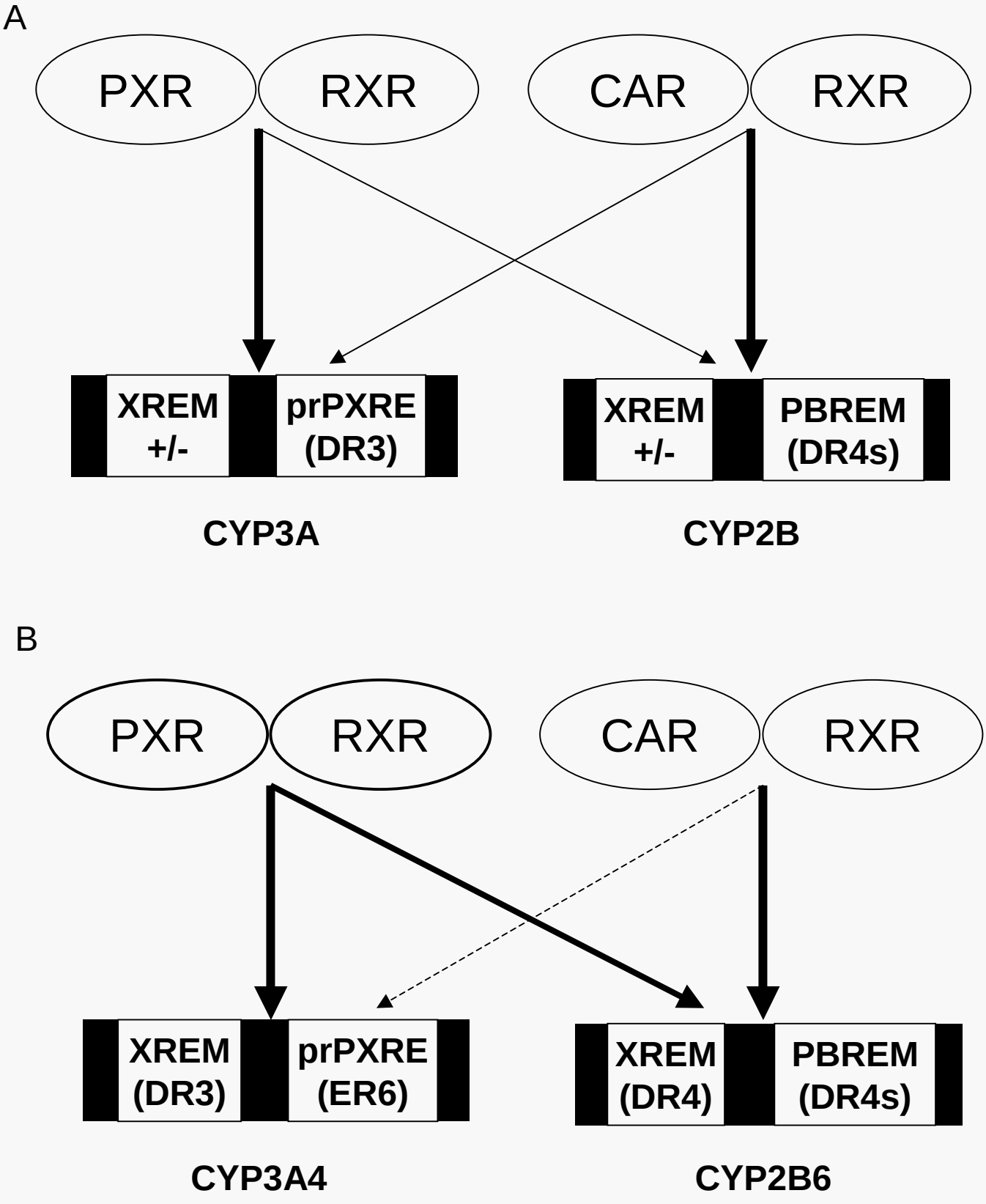

Figure 8