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Relative Activation of Human Pregnane X Receptor versus Constitutive Androstane Receptor Defines Distinct Classes of CYP2B6 and CYP3A4 Inducers

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Abstract

Both the human pregnane X receptor (hPXR) and constitutive androstane receptor (hCAR) are capable of regulating *CYP3A4* and *CYP2B6* gene expression. However, the majority of currently identified CYP3A4 and CYP2B6 inducers are confirmed activators of hPXR but not hCAR. To compare these receptors with respect to their chemical selectivities, 16 drugs known to induce CYP3A4 and/or CYP2B expression were evaluated for relative activation of hPXR versus hCAR. Because of the high basal but low chemical-induced activation of hCAR in immortalized cells, alternative methods were used to evaluate hCAR activation potential. Thirteen of the 16 compounds were classified as moderate to strong hPXR activators. In contrast, carbamazepine (CMZ), efavirenz (EFV), and nevirapine (NVP) were classified as negligible or weak hPXR activators at concentrations associated with efficacious CYP2B6 reporter or endogenous gene induction in primary human hepatocytes, suggesting potential activation of hCAR. Subsequent experiments demonstrated that these three drugs efficiently induced nuclear accumulation of in vivo-transfected enhanced yellow fluorescent protein-hCAR and significantly increased expression of a CYP2B6 reporter gene when hCAR was expressed in $CAR^{-/-}$ mice. In addition, using a recently identified, chemically responsive splice variant of hCAR (hCAR3), the hCAR activation profiles of the 16 compounds were evaluated. By combining results from the hPXR- and hCAR3-based reporter gene assays, these inducers were classified as hPXR, hCAR, or hPXR/hCAR dual activators. Our results demonstrate that CMZ, EFV, and NVP induce CYP2B6 and CYP3A4 preferentially through hCAR and that hCAR3 represents a sensitive tool for in vitro prediction of chemical-mediated human CAR activation.

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CYP3A4 and CYP2B6 are induced at the mRNA, protein, and activity levels by the same compounds, including rifampin, phenobarbital, clotrimazole, cyclophosphamide, calcium channel antagonists, HMG-CoA reductase inhibitors, and thiazolidinediones (Drocourt et al., 2001; Kocarek et al., 2002; Lindley et al., 2002; Sahi et al., 2003; Faucette et al., 2004). Coinduction of these enzymes is mediated through transcriptional activation of the corresponding genes by the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which are capable of binding to the same response elements in the promoter regions of the *CYP3A4* and *CYP2B6* genes (Goodwin et al., 1999, 2001; Sueyoshi et al., 1999; Wang et al., 2003). However, the majority of currently identified CYP3A4 and CYP2B6 inducers are confirmed activators of hPXR but not hCAR (Moore et al., 2000, 2002; Faucette et al., 2004). To date, only a limited number of compounds, including CITCO and the antiepileptic phenytoin (PHN), have been shown to induce CYP3A4 and/or CYP2B6 preferentially through hCAR instead of hPXR (Maglich et al., 2003; Wang et al., 2004). Besides a larger and more flexible ligand binding pocket of hPXR compared with that of hCAR (Watkins et al., 2001; Xu et al., 2004), the perceived predominance of hPXR activators may reflect the ease of their identification relative to hCAR activators.

Strong correlations have been observed between abilities of compounds to activate hPXR in cell-based reporter gene assays and induce CYP2B6 and/or CYP3A4 in human hepatocytes (Luo et al., 2002; Raucy et al., 2002; Vignati et al., 2004), In contrast, assessment of hCAR-mediated induction of CYP2B6 and CYP3A4 has been difficult due to the lack of an efficient in vitro system to screen for hCAR-mediated transcription. After transfection into immortalized cell lines, hCAR exhibits high constitutive activity and spontaneous nuclear localization, in contrast to its predominant cytosolic localization in primary hepatocytes and intact liver (Kawamoto et al., 1999; Wang et al., 2004). Because of difficulties in evaluation of hCAR activation, the contribution of this receptor to drug-drug interactions, relative to hPXR, has remained ambiguous. Recently, several groups have identified alternative splicing variants of wild-type hCAR with altered functional activity (Auerbach et al., 2003; Arnold et al., 2004; Jinno et al., 2004; Ikeda et al., 2005). One of these variants, hCAR3, exhibited significantly lower basal activity in immortalized cells than wild-type hCAR and was activated extensively by the known hCAR activator CITCO in a cell-based reporter gene assay (Auerbach et al., 2005), suggesting the possible utility of this variant as a novel tool for in vitro assessment of hCAR activation.

To compare the selectivities of hPXR and hCAR for coinducers of *CYP3A* and *CYP2B* genes, this study evaluated a series of 16 clinically used drugs for their relative activation of hPXR versus hCAR. Compared with the known hPXR activator rifampin (RIF), three of the 16 drugs (CMZ, EFV, and NVP) were associated with weak or negligible hPXR activation in cell-based transfection assays. In human hepatocytes, CMZ, EFV, and NVP induced CYP2B6 reporter gene expression, as well as CYP2B6 and CYP3A4 endogenous gene expression. Tail vein delivery of hCAR into $CAR^{-/-}$ mice demonstrated that these compounds induced nuclear translocation of hCAR and increased CYP2B6 reporter gene activities. In addition, xenobiotic-mediated in vitro hCAR3 activation was evaluated in HepG2 cell-based reporter gene assays with the 16 selected compounds. The splicing variant

hCAR3 was activated by both direct and indirect activators of wild-type hCAR. Combining results from hPXR- and hCAR3-based assays, the 16 inducers could be classified into three groups, including predominant hPXR activators [lovastatin (LOV), metyrapone (MET), mevastatin (MEV), nicardipine (NIC), nifedipine (NIF), omeprazole (OMP), and simvastatin (SIM)], predominant hCAR activators (CMZ, EFV, NVP), and dual activators of hPXR and hCAR [artemisinin (ART), chlorpromazine (CPZ), cyclophosphamide (CPA), reserpine (RES), RU486, and troglitazone (TGZ)]. CMZ, EFV, and NVP induce CYP2B6 and CYP3A4 primarily through hCAR, whereas other drugs act preferentially through hPXR or dually through hPXR and hCAR. Like hPXR, hCAR represents a potential mediator of drug interactions between inducers and substrates of CYP2B6 and CYP3A4. Furthermore, hCAR3 cell-based reporter gene assays may provide a powerful tool for screening of hCAR-mediated induction potential.

Materials and Methods

Chemicals and Biological Reagents

ART, CMZ, CPZ, CPA, LOV, MEV, RU486, NIC, NIF, OMP, PHN, phenobarbital (PB), RES, RIF, and TGZ were purchased from Sigma-Aldrich (St. Louis, MO). MET and CITCO were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA), whereas NVP and SIM were obtained from The United States Pharmacopoeia (Rockville, MD). EFV was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). Oligonucleotide primers and TaqMan fluorescent probes were synthesized by Sigma Genosys (The Woodlands, TX) and Applied Biosystems (Foster, CA), respectively. The CellPhect Transfection Kit was purchased from Amersham Biosciences (Buckinghamshire, UK), and the Dual-Luciferase Reporter Assay System was purchased through Promega (Madison, WI). Effectene transfection reagent was obtained from QIAGEN, Inc. (Valencia, CA). *Trans*IT *In Vivo* Gene Delivery System was acquired from Mirus (Madison, WI). Matrigel and ITS$^+$ were obtained from BD Biosciences (Bedford, MA). Charcoal-stripped/dextran-treated FBS was purchased from Hyclone (Logan, UT), whereas other cell culture reagents were purchased from Invitrogen (Carlsbad, CA) or Sigma-Aldrich.

Plasmid Constructs

The construction of the hCAR3 expression vector (CMV2-CAR3) has been described previously (Auerbach et al., 2005). The pSG5-hPXR expression vector and the CYP3A4-PXR-response element (PXRE)/XREM luciferase reporter construct [p3A4-362(7836/7208ins)] were obtained from Drs. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX) and Bryan Goodwin (GlaxoSmithKline, Research Triangle Park, NC), respectively. The CYP3A4-PXRE/XREM construct consisted of the CYP3A4 native proximal promoter, including the proximal PXRE containing an everted repeat separated by a 6-bp motif (ER6), 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 firefly luciferase construct consisted of 1.8 kb of the native promoter, including the 51-bp PBREM harboring two direct repeat separated by 4 bp

(DR4) motifs (NR1 and NR2) and the 400-bp distal XREM region containing the DR4 motif termed NR3 (Wang et al., 2003). Details of the CYP2B6 $(NR1)_2$ luciferase plasmid are included in a previous report (Faucette et al., 2006). pCR3-hCAR expression plasmid and fluorescently labeled hCAR (EYFP-hCAR) expression plasmid were constructed as described previously (Sueyoshi et al., 1999; Zelko et al., 2001). The pRL-TK and pRL-SV40 *Renilla* luciferase plasmids used to normalize firefly luciferase activities were from Promega.

Transfection Assays in Human Hepatoma Cells

HepG2 cells were cultured in 24-well plates in DMEM supplemented with 10% FBS before transfection and subsequently with charcoal-stripped/dextran-treated FBS. Cells were transfected with 50 ng of receptor expression vectors, 100 ng of luciferase reporter plasmids, and 30 ng of control plasmid (pRL-TK) using the CellPhect Transfection Kit as described previously (Faucette et al., 2006). After 12 h of transfection, cells were treated for 24 h with vehicle (0.1% DMSO, CTL), 10 μM RIF, 1 mM PB, 50 μM PHN, 1 μM CITCO, or indicated concentrations of test compounds, which included and/or bracketed plasma concentrations reported in humans. Cell lysates were assayed for firefly luciferase activities and normalized against the activities of cotransfected *Renilla* luciferase. Ratios of the two luciferase activities were expressed as fold activation relative to vehicle control or as percent activation achieved by 10 μM RIF. Reported data are representative of triplicate results obtained from three independent experiments.

Culture and Treatment of Primary Human Hepatocytes

Hepatocytes isolated from five human donors were supplied by Cell-zDirect, Inc. (Pittsboro, NC) or ADMET Technologies, Inc. (Research Triangle Park, NC). Cells were seeded at 1.5×10^6 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. After 4 to 6 h of attachment at 37°C in a humidified atmosphere of 5% CO_2, cells were overlaid with Matrigel (0.25 mg/ml) in serum-free modified Chee's medium supplemented with ITS^+ (insulin, transferrin, and selenium), linoleic acid, bovine serum albumin, and 0.1 μM dexamethasone. After 36 to 48 h in culture, hepatocytes were treated for 24 h with vehicle control (0.1% DMSO, CTL), 10 μM RIF, 1 μM CITCO, or indicated concentrations of test compounds.

Real-Time PCR

Total RNA was isolated from the treated human hepatocyte lysates (one to two wells per treatment) 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). Sequences of primers and probes are as follows, in the order of forward primer, probe, and reverse primer: CYP2B6, 5′-AAGCGGA-TTTGTCTTGGTGAA-3′, 6-FAM-CATCGCCCGTGCGGAATTGTTC-5-carboxytetramethylrhodamine, 5′-TGGAGGATGGTGGTGAAGA-AG-3′; and CYP3A4, 5′-TCAGCCTGGTGCTCCTCTATCTAT-3′, 6-FAM-

TCCAGGGCCCACACCTCTGCCT-5-carboxytetramethylrhod-amine, 5′-AAGCCCTTATGGTAGGACAAAATATTT-3′. CYP2B6 or CYP3A4 mRNA expression was normalized to that of human β-actin, which was detected using a predeveloped primer/probe mixture (Applied Biosystems). Multiplexed TaqMan PCR assays were performed 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. These values were expressed subsequently as the percentage induction attained by 10 μM RIF.

Transfection Assays in Primary Human Hepatocytes

As described above, hepatocytes from donor HCS-027 were plated in the same manner as hepatocytes used in induction studies. After cell attachment, medium was changed from plating medium (DMEM) to Williams' E medium supplemented with antibiotics, ITS^+, and 0.1 μM dexamethasone. Nonoverlaid cells were transfected with 250 ng of CYP2B6-PBREM/XREM reporter gene plasmid and 25 ng of pRL-TK plasmid using Effectene reagent and treated 24 h later with 10 μM RIF, 50 μM PHN, 25 μM CMZ, 10 μM EFV, and 50 μM NVP. Cell lysates were analyzed for firefly and *Renilla* luciferase activities as described above.

In Vivo Gene Transfection and Confocal Microscopy

$CAR^{-/-}$ mice were generated as described previously (Ueda et al., 2002). These mice were housed in a pathogen-free animal facility with standard 12-h light/dark cycles and provided autoclaved rodent chow and drinking water ad libitum. Animals weighing 18 to 25 g were used for hCAR translocation studies and CYP2B6 reporter gene assays. In the hCAR localization experiments, $CAR^{-/-}$ mice were injected through the tail vein with 10 μg of EYFP-hCAR expression plasmid using *Trans*IT *In Vivo* Gene Delivery System according to the manufacturer's protocol (Mirus). Treatment occurred at 2 and 5 h after gene delivery by i.p. injection of vehicle (DMSO, CTL), 50 mg/kg RIF, 100 mg/kg PB, 10 mg/kg CITCO, 25 mg/kg EFV, 50 mg/kg NVP, or 20 mg/kg CMZ at volumes of 0.1 ml/20 g body weight. Mouse livers were collected 7 h after the plasmid injection, embedded into Tissue-Tek OTC, and immediately frozen. Microscopic analysis of frozen liver sections was performed as described previously (Zelko et al., 2001). EYFP-hCAR was visualized in hepatocytes using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss GmbH, Jena, Germany) at an excitation wavelength of 514 nm and an emission wavelength of 530 nm. For each treatment group, approximately 150 mouse hepatocytes expressing EYFP-hCAR were counted per mouse liver and classified according to cytosolic, nuclear, or mixed (cytosolic and nuclear) hCAR localization. Livers from two mice were evaluated by confocal analysis for each treatment.

For the in vivo CYP2B6 reporter gene assays, $CAR^{-/-}$ mice were transfected with 5 μg of pCR3-hCAR expression vector, 4 μg of CYP2B6-PBREM/XREM reporter gene, and 1 μg of pRL-SV40 *Renilla* luciferase plasmid by tail vein injection. After 6 to 8 h, three to four mice per group were injected i.p. with vehicle (DMSO, CTL), 100 mg/kg PB, 25 mg/kg EFV, 50 mg/kg NVP, and 20 mg/kg CMZ at the same volumes given above. For CMZ, a repeat

dosage was administered approximately 12 h after the first injection. Mouse livers were harvested approximately 24 h after plasmid transfection and homogenized in 5 ml of passive lysis buffer one time (Promega). Homogenates were centrifuged at 14,000 rpm for 15 min, and 2 μl of the final supernatant was used for determination of firefly and *Renilla* luciferase activities.

Statistical Analysis

All results are expressed as mean ± S.D. of triplicate determinations unless otherwise specified. For most experiments, treatment groups were compared with the vehicle control or negative control group using one-way analysis of variance followed by Bonferroni's multiple comparisons test. For hPXR screening assays, -fold activation by test compounds was compared with a predefined level (30%) of fold activation attained by the positive control RIF using the same statistical approach. The criterion of significance was set at $p = 0.05$, and statistical tests were performed using SigmaStat version 2.03 software (SPSS Inc., Chicago, IL).

Results

Evaluation of hPXR Activation in Cell-Based Reporter Gene Assays

To identify negligible or weak hPXR activators and thus potential hCAR activators, 16 clinically used drugs with known or suspected CYP3A4 and/or CYP2B induction properties were screened in HepG2 cells transfected with hPXR expression plasmid and a CYP3A4-PXRE/XREM luciferase reporter construct. The test compounds consisted of the following sets of drugs: ART, CMZ, CPZ, CPA, EFV, LOV, MET, MEV, RU486, NVP, NIC, NIF, OMP, RES, SIM, and TGZ. Concentrations of test compounds were 1, 10, and 50 μM unless cytotoxicity or insolubility at 50 μM mandated reduction of the highest concentration to 25 μM. This study intends to compare extents of activation relative with rifampin, a positive control for hPXR assays, and to provide a qualitative measure of potency relative to a known control. The extents of hPXR activation by the test compounds were compared statistically with 30% of the extent observed with RIF. Although arbitrary, the 30% criterion was defined a priori to provide a benchmark for comparing relative hPXR activation by different compounds.

Figure 1 indicates drug-mediated increases in CYP3A4 reporter activities compared with vehicle control as a percentage of the increase achieved by the positive control RIF (10 μM). Compounds that achieved significantly greater than 30% of RIF-normalized hPXR activation of CYP3A4 reporter gene expression at one or more concentrations were classified as moderate to strong activators of hPXR, whereas drugs exhibiting less than 30% were classified as weak or nonactivators of hPXR. Consistent with previous reports, ART, CPA, LOV, MET, MEV, RU486, NIC, NIF, OMP, RES, SIM, and TGZ were classified as moderate to strong hPXR activators (fold activation > 30% of RIF response) (Drocourt et al., 2001; Kocarek et al., 2002; Lindley et al., 2002; Raucy, 2002; Burk et al., 2005). To our knowledge, this is the first demonstration of hPXR activation by CPZ. In contrast, CMZ, EFV, and NVP exhibited less than 30% of RIF-mediated hPXR activation to qualify as weak or negligible hPXR activators (Fig. 1).

Because CMZ, EFV, and NVP demonstrated weak hPXR activation in HepG2 cells cotransfected with the CYP3A4 PXRE-XREM reporter construct, these drugs also were assessed for hPXR activation of CYP2B6-PBREM/XREM reporter gene expression to ensure that results obtained with the CYP3A4 reporter gene did not represent promoter-specific effects. Figure 2 demonstrates that the three compounds were associated with less than 30% of hPXR-mediated CYP2B6 reporter activities achieved by 10 μM RIF (21-fold relative to control). Because results from hPXR-based reporter gene assays were inconsistent with the reported inductive effects of CMZ, EFV, and NVP, these drugs were subjected to additional studies to assess their hCAR activation potential.

Induction of *CYP2B6* and *CYP3A4* Gene Expression in Primary Human Hepatocytes

Previous experiments from this laboratory demonstrated efficacious induction of both CYP2B6 and CYP3A4 by the selective hPXR activator RIF but preferential induction of CYP2B6 over CYP3A4 by the selective hCAR activator CITCO (Faucette et al., 2006). To determine whether the suspected hCAR activators exhibited efficacious induction of CYP2B6 at greater magnitudes than CYP3A4, primary hepatocytes from five human donors were treated with the indicated concentrations of CMZ, EFV, and NVP in addition to the positive controls RIF (10 μM) and CITCO (1 μM). In each of five human hepatocyte preparations, the test compounds achieved maximum levels of CYP2B6 induction comparable with those of RIF and CITCO, ranging from 4.1- to 10.5-fold for CMZ, 3.7- to 7.9-fold for EFV, and 3.7- to 12.0-fold for NVP (Table 1).

To account for differences in CYP2B6 and CYP3A4 basal expression and thus magnitudes of fold induction, the extents of CYP2B6 and CYP3A4 induction by the suspected hCAR activators were compared by expressing their induction values as the percentage of induction observed with 10 μM RIF. This inducer was chosen over CITCO for normalizing test compound induction because of its ability to induce both gene efficaciously, compared with the selective induction of CYP2B6 observed with CITCO (Faucette et al., 2006). As demonstrated in Fig. 3A, CMZ, EFV, NVP, and CITCO exhibited efficacious induction of CYP2B6 relative to RIF in a representative culture of human hepatocytes (approximately 115–130% of RIF response for CMZ, EFV, and NVP and 160% for CITCO). These drugs also induced CYP3A4 mRNA in a concentration-dependent manner, albeit at lower levels compared with RIF (fold induction < 20% of 10 μM RIF) (Fig. 3B). Similar to CITCO, these compounds exhibited preferred induction of CYP2B6 over CYP3A4 in liver HCS014 (Fig. 3, A versus B) and other hepatocyte preparations (data not shown). The pattern of CYP2B6 versus CYP3A4 induction by these compounds is consistent with previous observations of preferential induction of CYP2B6 over CYP3A4 by hCAR (Faucette et al., 2006).

Induction of CYP2B6 Reporter Gene Expression in Primary Human Hepatocytes

Primary human hepatocytes maintain expression of hPXR and hCAR, the primary receptors implicated in CYP2B6 regulation. PHN, a selective hCAR activator, has been shown to activate CYP2B6 reporter gene expression in human hepatocytes despite its weak activation of hPXR (Wang et al., 2004). The abilities of CMZ, EFV, and NVP to activate CYP2B6-PBREM/XREM reporter gene expression were tested in hepatocytes from donor HCS027.

As shown in Fig. 4, these compounds were associated with 17-, 10-, and 7-fold increases in CYP2B6 reporter activities, respectively, which were comparable with the degree of increases observed with RIF (15-fold) and PHN (16-fold). Because of their weak hPXR activation in reporter gene assays, the effects of CMZ, EFV, NVP, and PHN were most probably mediated by hCAR endogenously expressed in human hepatocytes.

Human CAR Translocation Assay in CAR$^{-/-}$ Mice

Although CMZ, EFV, and NVP demonstrated characteristics consistent with hCAR activation, including weak hPXR activation, and preferred induction of CYP2B6 to CYP3A4, more direct evidence was required to establish these drugs as definitive hCAR activators. Compounds were not tested in cell-based reporter assays with wild-type hCAR because weak or negligible responses were expected given the high constitutive activity and spontaneous nuclear localization of the receptor in transformed cells (Kawamoto et al., 1999). Accordingly, an alternative approach was taken based on the requirement for hCAR translocation from the cytoplasm to the nucleus for its activation in intact liver in vivo (Kawamoto et al., 1999; Zelko et al., 2001). EYFP-tagged hCAR was tail vein-injected into CAR$^{-/-}$ mice using a previously described in vivo gene delivery technique (Zelko et al., 2001). Confocal microscopy analysis demonstrated examples of cytoplasmic, nuclear, and mixed distribution of EYFP-hCAR in liver sections from mice treated with vehicle, PB, CITCO, EFV, CMZ, or NVP (Fig. 5). Of the hCAR-expressing cells counted in the liver sections from two vehicle-treated mice, 77 to 88% exhibited cytoplasmic localization, 9 to 18% nuclear localization, and 3 to 5% mixed (cytoplasmic and nuclear) localization (Table 2). EYFP-hCAR expression was predominantly cytoplasmic in mice treated with the selective hPXR activator RIF (82–91% versus 6–16% nuclear versus 2–3% mixed). The known hCAR activators PB and CITCO demonstrated cytoplasmic distribution of hCAR in 27 to 33% and 30 to 37% of fluorescently labeled cells and nuclear distribution in 64 to 67% and 40 to 46%, respectively. CITCO-treated mice exhibited a greater extent of mixed localization compared with PB (17–30% versus 3–6%), as previously reported (Wang et al., 2004). In CMZ-, EFV-, and NVP-treated mice, the percentages of counted cells expressing EYFP-hCAR in the cytoplasm were 13 to 14%, 19%, and 21%, respectively. Treatment of mice with CMZ and EFV was associated with approximately 60% increases in nuclear localization of hCAR (75–81% and 76–78% of cells, respectively). In NVP-treated mice, 58 to 67% of cells displayed nuclear distribution of hCAR, and 12 to 21% displayed both nuclear and cytoplasmic. Overall, these results indicate that CMZ, EFV, and NVP are capable of inducing hCAR translocation from the cytoplasm to the nucleus as effectively as PB and CITCO (Table 2).

In Vivo Activation of CYP2B6 Reporter Gene Expression by hCAR in CAR$^{-/-}$ Mice

Because the suspected hCAR activators induced hCAR nuclear translocation, the first required step of hCAR activation, additional experiments were performed to determine whether these drugs were capable of inducing CYP2B6 reporter gene expression in the presence of hCAR. Accordingly, CAR-null mice were tail vein-injected with hCAR expression plasmid and CYP2B6-PBREM/XREM luciferase construct, followed by treatment with CMZ, EFV, NVP, or the positive control PB. As shown in Fig. 6, all treatments were associated with significant increases in CYP2B6 reporter activities relative

to vehicle control. PB and CMZ resulted in approximately 6-fold increases in CYP2B6 reporter activities, whereas EFV and NVP led to approximately 4-fold increases. These results suggested that the test compounds activated nuclear-localized hCAR in mouse liver, leading to increased CYP2B6 reporter gene expression.

Evaluation of hCAR3 Activation in Cell-Based Reporter Gene Assays

During the preparation of this manuscript, Auerbach et al. (2005) reported that a human CAR splice variant (hCAR3) demonstrated low basal activity, but CITCO-mediated activation, in COS-1 cells. In the current study, we have evaluated hCAR3 activation in HepG2 cells by using known hCAR activators such as CITCO, PB, and PHN, as well as the 16 test compounds. Consistent with previous reports, wild-type hCAR (CAR1) demonstrated constitutive activation of the transfected CYP2B6 $(NR1)_2$ reporter gene relative to empty vector, whereas hCAR3 exhibited approximately 80% lower basal activity compared with wild-type hCAR (Fig. 7). hCAR3 was activated by CITCO (1 μM), PB (1 mM), and PHN (50 μM) up to 18-, 18-, and 11-fold compared with empty vector control, respectively. In contrast, the selective hPXR activator RIF (10 μM) did not exhibit significant activation of hCAR3 (~1.5-fold relative to empty vector control). The extents of hCAR3 activation by the test compounds were compared statistically with the extent observed with the negative control RIF. For the 16 test compounds, a single concentration was selected for hCAR3 activation screening, which in most cases corresponded to the concentration associated with maximum hPXR activation (Fig. 1). Consistent with the in vivo hCAR translocation results, single concentrations of EFV, NVP, and CMZ demonstrated at least 3-fold activation of hCAR3 relative to empty vector (Fig. 7), and concentration-dependent hCAR3 activation was observed for all three compounds at a maximum of 7-fold for CMZ, 11-fold for EFV, and 5.5-fold for NVP (Fig. 8). In addition, ART, CPZ, CPA, RES, RU486, and TGZ demonstrated significant hCAR3 activation ranging from 2.4-fold for RU486 to 10-fold for ART (Fig. 7). Overall, these results indicate that hCAR3 activation is representative of hCAR chemical responsiveness observed in primary human hepatocytes and in vivo and, more importantly, that the hCAR3 cell-based reporter gene assay provides a novel and powerful tool for in vitro screening of hCAR activators.

Discussion

Both hPXR and hCAR are capable of regulating *CYP3A4* and *CYP2B6* gene expression in response to their xenobiotic activators. However, evidence to date indicates that the majority of CYP2B6 and CYP3A4 inducers are activators of hPXR rather than hCAR (Moore et al., 2000, 2002; Goodwin et al., 2001; Faucette et al., 2004). Exceptions include PB and 5β-pregnane-3,20-dione, mixed activators of both receptors, and PHN and CITCO, selective activators of hCAR (Moore et al., 2000; Maglich et al., 2003; Wang et al., 2004). Based on the relatively small numbers of hCAR activators identified, one could speculate that this receptor plays a secondary role to hPXR in regulating CYP2B6 and CYP3A4 expression. However, before accepting such a notion, a comprehensive investigation is required into the relative numbers of inducers capable of activating hPXR versus hCAR.

To gain insight into differences in chemical responsiveness between hPXR and hCAR, the current study evaluated 16 reported CYP3A4 and/or CYP2B inducers for their relative activation of hPXR and hCAR. Thirteen drugs were classified as moderate to strong hPXR activators. Three of the 16 compounds (CMZ, EFV, and NVP) were established as preferential hCAR activators. These findings were supported by a combination of direct and indirect experimental approaches. First, CMZ, EFV, and NVP induced CYP2B6 mRNA or reporter gene expression efficaciously in primary human hepatocytes at concentrations that produced weak activation of hPXR in cell-based reporter gene assays. Secondly, these compounds achieved greater magnitudes of CYP2B6 induction relative to CYP3A4, consistent with the pattern observed with the known hCAR activator CITCO (Faucette et al., 2006). Interestingly, clinical studies have suggested a pattern of greater CYP2B6 than CYP3A4 induction with EFV and NVP, based on clearance measurements of CYP2B6- and CYP3A4-selective pathways or discordance between the increase in CYP3A4 expression and systemic clearance (Mouly et al., 2002). Thirdly, CMZ, EFV, and NVP induced nuclear translocation of hCAR at levels comparable with or greater than PB and CITCO. Lastly, the three compounds activated CYP2B6 reporter expression in CAR-null mice transfected with hCAR. Overall, these results indicate greater numbers of hCAR activators than previously estimated and that reliance on hPXR-based transfection assays alone may result in incomplete prediction of efficacious inducers of CYP2B6 and CYP3A4.

Despite the need to screen for hCAR activators, there currently are difficulties associated with evaluating hCAR regulation of its target genes, particularly in a high-throughput format. In hepatic-derived immortalized cells, hCAR spontaneously translocates to the nucleus in the absence of ligand binding and/or activation (Kawamoto et al., 1999). Because of the ensuing high constitutive activity of the receptor, significant increases in reporter gene activities are difficult to discern in the presence of an activator. In contrast to mouse CAR, the constitutive activity of hCAR cannot be repressed by androstenol, androgens, progesterone, and protein kinase inhibitors, and subsequently reactivated by an activator (Zelko and Negishi, 2000). In addition, hCAR can be activated by both ligand binding (direct activator) and ligand-independent mechanisms (indirect activator) involving protein phosphorylation and/or dephosphorylation (Kawamoto et al., 1999; Zelko et al., 2001; Wang et al., 2004). This characteristic lowers the value of in vitro hCAR binding assays using radiolabeled competitive ligands to identify activators. Alternatively, primary cultures of human hepatocytes represent a potential in vitro system to evaluate hCAR function and activity. Advantages of primary cells include maintenance of physiologically relevant levels of transcription factors and coregulatory molecules and localization of hCAR in the cytoplasm as observed in vivo (Zelko et al., 2001). Previous studies in our group have demonstrated the value of performing transfection and localization studies in human hepatocytes to identify hCAR activators such as phenytoin (Wang et al., 2004). Unfortunately, the use of human hepatocytes is constrained by limited availability of fresh hepatic tissue, response variability among different donors, and unsuitability to high-throughput format.

Recently, several groups have identified and characterized hCAR splicing variants that are expressed in human liver at levels comprising 10 to 40% of the total hCAR transcript (Auerbach et al., 2003; Arnold et al., 2004; Jinno et al., 2004). One such variant, termed

hCAR3, contains a 15-bp insertion in intron 7 of wild-type hCAR. During the preparation of this manuscript, Auerbach et al. (2005) reported that hCAR3 exhibited a low basal activity, but strong ligand (CITCO)-dependent activation, in a COS-1 cell-based reporter gene assay. In the current study, we have evaluated the activation profile of hCAR3 more extensively by testing known hCAR activators as well as 16 other CYP3A4 and/or CYP2B inducers. Notably, all the known hCAR activators, including the direct activator CITCO and the indirect activators PB and PHN, strongly activated CYP2B6 reporter gene expression via hCAR3 in HepG2 cells, suggesting hCAR3 could be activated by both ligand-dependent and -independent mechanisms. Among the 16 test compounds, nine (CMZ, EFV, NVP, ART, CPZ, CPA, RES, RU486, and TGZ) demonstrated significant activation of hCAR3 relative to the negative control RIF. These observations are in agreement with our in vivo hCAR translocation and activation data for CMZ, EFV, and NVP, as well as a previous report demonstrating modest increases in wild-type hCAR activity by ART (Burk et al., 2005). Based on combined data from the hPXR and hCAR3 cell-based reporter gene assays, we classified known CYP3A4 and/or CYP2B inducers into three groups, including preferential hCAR activators (CMZ, EFV, and NVP), selective hPXR activators (RIF, LOV, MET, MEV, NIC, NIF, OMP, and SIM), and mixed hPXR and hCAR activators (ART, CPZ, CPA, RU486, RES, and TGZ). These results indicate multiple classes of CYP2B6 and CYP3A4 inducers based upon their relative activation of hPXR and hCAR.

The preferential hCAR activators identified in this study are similar in that their major metabolic pathways are catalyzed by CYP3A4 and/or CYP2B6 (epoxidation and 3-hydroxylation for CMZ; 8-hydroxylation for EFV; 2-, 3-, and 12-hydroxylation for NVP) (Erickson et al., 1999; Pearce et al., 2002; Ward et al., 2003). The dual hPXR/hCAR activators ART and CPA also are metabolized by CYP2B6 and/or CYP3A4 (Lindley et al., 2002; Simonsson et al., 2003). Due to induction of CYP3A4 and CYP2B6, these drugs are capable of enhancing their own metabolism (autoinduction), as evidenced by increased clearances and decreased half-lives with multiple dosing compared with single dosing (Bertilsson, 1978; Smith et al., 2001; Simonsson et al., 2003; de Jonge et al., 2005). In addition, these drugs are prescribed frequently in combination with other drugs, leading to drug interactions compromising the efficacy of coadministered therapeutics metabolized by CYP3A4 and CYP2B6 (Spina et al., 1996; Smith et al., 2001). From the current study, it can be concluded that hCAR, alone or in addition to hPXR, mediates the autoinductive effects of CMZ, EFV, NVP, ART, and CPA, as well as their ability to alter the pharmacokinetics and efficacy of other drugs.

In conclusion, this study demonstrates that CMZ, EFV, and NVP mediate their CYP2B6 and CYP3A4 inductive effects predominantly through hCAR. A proportion of identified hPXR activators, including ART, CPZ, CPA, RES, RU486, and TGZ, are capable of simultaneously activating hCAR, suggesting existence of greater numbers of hCAR activators than previously realized. The splicing variant hCAR3 can be activated by both direct ligand binding and indirect ligand-independent mechanisms in immortalized cell lines. In the absence of other suitable in vitro models and based on similar chemical sensitivities to wild-type hCAR, hCAR3 cell-based reporter gene assays may provide a powerful tool for screening hCAR activation in a relatively high-throughput format. Results from this study support routine screening of compounds for both hPXR and hCAR activation because both

receptors are capable of mediating drug-drug interactions involving CYP2B6 and CYP3A4 induction.

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ABBREVIATIONS

PXR	pregnane X receptor
CAR	constitutive androstane receptor
hPXR	human pregnane X receptor
hCAR	human constitutive androstane receptor
CITCO	6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenyzl)oxime
PHN	phenytoin
RIF	rifampin
CMZ	carbamazepine
EFV	efavirenz
NVP	nevirapine
LOV	lovastatin
MET	metyrapone
MEV	mevastatin
NIC	nicardipine
NIF	nifedipine
OMP	omeprazole
SIM	simvastatin
ART	artemisinin
CPZ	chlorpromazine
CPA	cyclophosphamide
RES	reserpine
RU486	mifepristone
TGZ	troglitazone

PB	phenobarbital
FBS	fetal bovine serum
PXRE	PXR-response element
XREM	xenobiotic-responsive enhancer module
NR	nuclear receptor binding site
EYFP	enhanced yellow fluorescent protein
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
CTL	control
PCR	polymerase chain reaction
ITS$^+$	insulin transferrin selenium
PBREM	phenobarbital-responsive enhancer module

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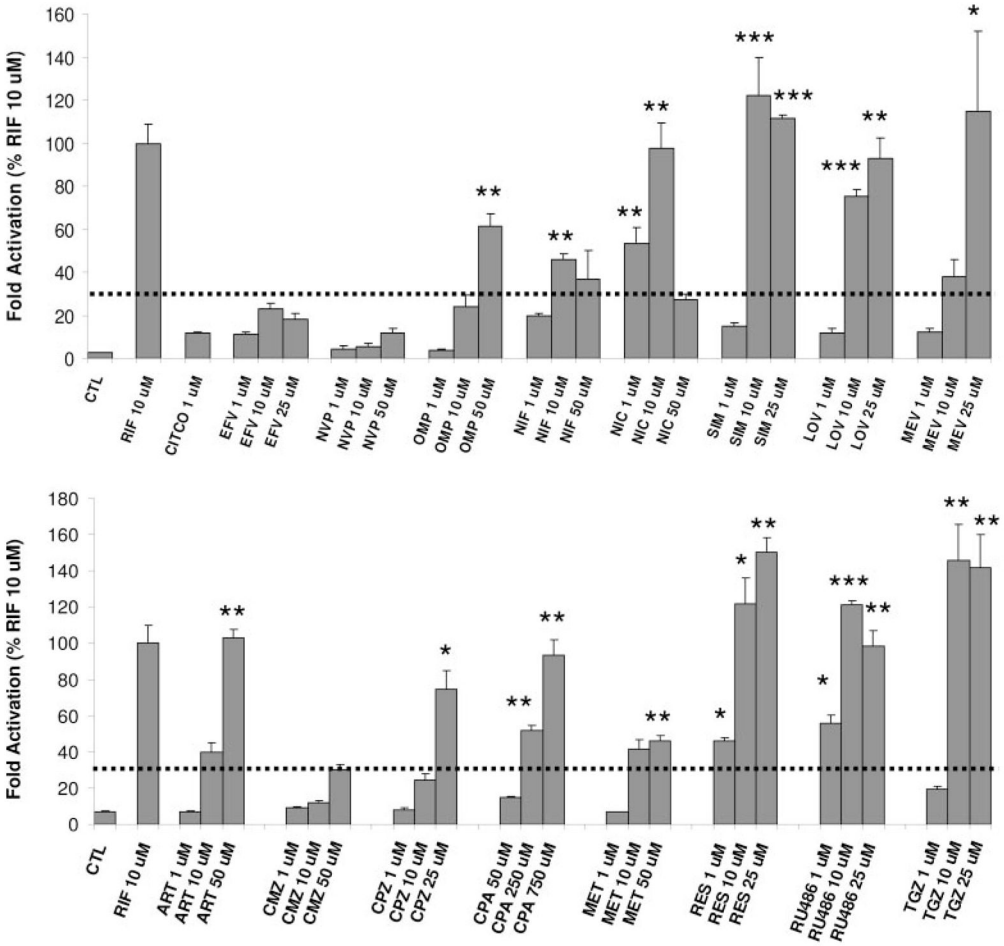

Fig. 1.

Effects of 16 compounds on hPXR-mediated CYP3A4 reporter gene activation. HepG2 cells were transfected with pSG5-hPXR expression plasmid (50 ng), CYP3A4-PXRE/XREM firefly luciferase reporter construct (100 ng), and pRL-TK *Renilla* luciferase reporter construct (30 ng) using CellPhect Transfection Kit. Following 12 h of exposure to transfection complexes, cells were treated with vehicle control (0.1% DMSO, CTL), 10 μM RIF as positive control, and the indicated concentrations of test compounds for 24 h. Normalized CYP3A4 reporter gene activities represent the mean ± S.D. of three independent transfections and are expressed as percent activation of 10 μM RIF. Dashed line, 30% of RIF-mediated hPXR activation; asterisks, level of statistical significance associated with test compound increases greater than 30% of the level attained by RIF (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

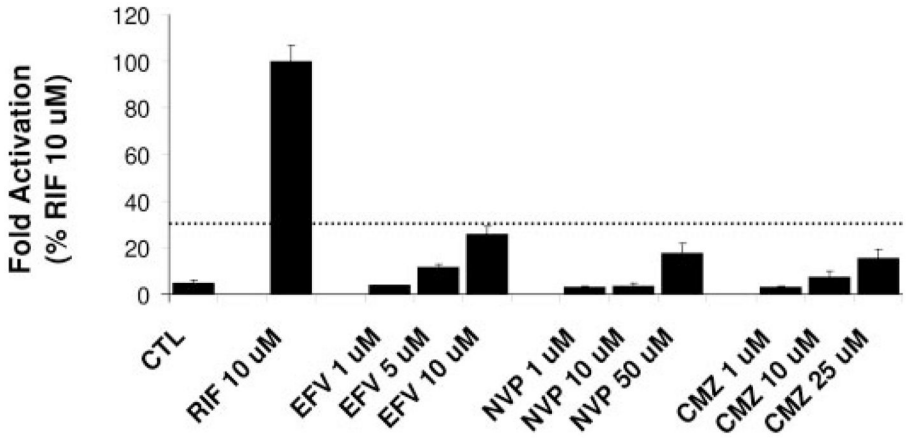

Fig. 2.

Effects of CMZ, EFV, and NVP on hPXR-mediated CYP2B6 reporter gene expression. HepG2 cells were transfected with pSG5-hPXR, pRL-TK, and CYP2B6-PBREM/XREM firefly luciferase reporter construct as described under *Materials and Methods*. After 12 h, cells were treated with vehicle control (0.1% DMSO, CTL), 10 μM RIF, and the indicated concentrations of CMZ, EFV, and NVP for 24 h. Luciferase activities were determined in triplicate and expressed as mean ± S.D. of percent activation achieved by RIF 10 μM. Dashed line, 30% of RIF-mediated hPXR activation. No compounds were associated with hPXR activation levels significantly greater than 30% RIF.

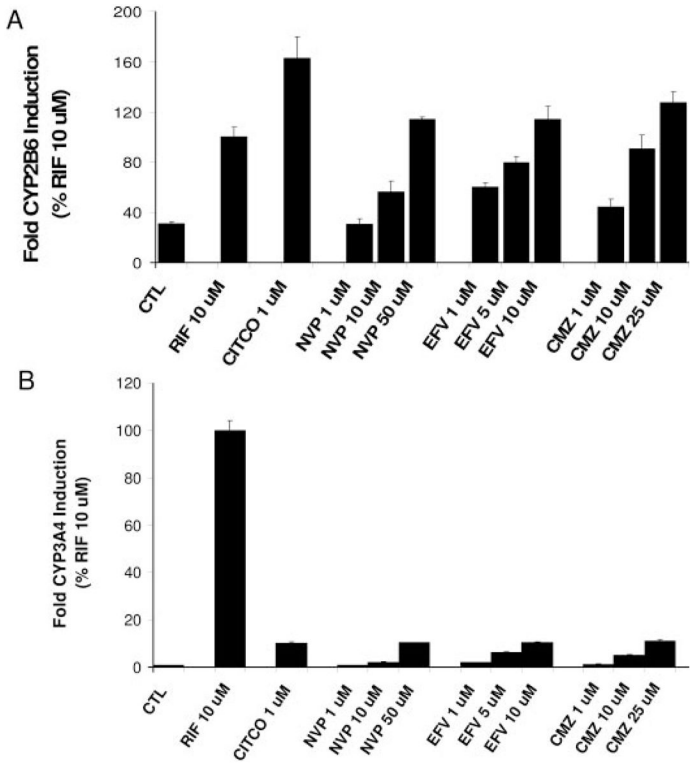

Fig. 3.

Evaluation of CYP2B6 (A) and CYP3A4 (B) induction by CMZ, EFV, and NVP in primary human hepatocytes. Human hepatocytes from donor HCS014 were exposed for 24 h to vehicle (0.1% DMSO, CTL), the selective hPXR activator RIF (10 μM), the selective hCAR activator CITCO (1 μM), and the indicated concentrations of test compounds. Total RNA was isolated from the treated human hepatocyte lysates (one to two wells per treatment) using the RNeasy Mini Kit (QIAGEN). CYP3A4 and CYP2B6 mRNA levels were determined in triplicate using real-time PCR and normalized to those of β-actin. Data are expressed as the mean ± S.D. of percent induction obtained with RIF 10 μM.

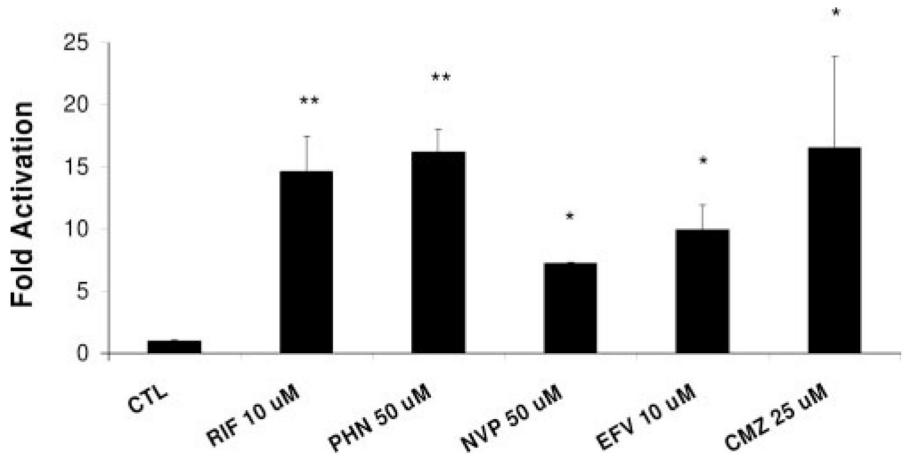

Fig. 4.

Activation of CYP2B6 reporter gene expression in primary human hepatocytes. Human hepatocytes from donor HCS027 were transfected with CYP2B6-PBREM/XREM luciferase reporter plasmid (250 μg) and pRL-TK control plasmid (25 μg) using Effectene reagent. Twenty-four hours later, hepatocytes were treated with vehicle control (0.1% DMSO, CTL), RIF (10 μM), PHN (50 μM), CMZ (25 μM), EFV (10 μM), and NVP (50 μM). Cells were lysed after a 24-h treatment period for determination of firefly and *Renilla* luciferase activities. Results represent fold activation relative to vehicle control (mean ± S.D. of triplicate transfections). *, $p < 0.05$; **, $p < 0.01$.

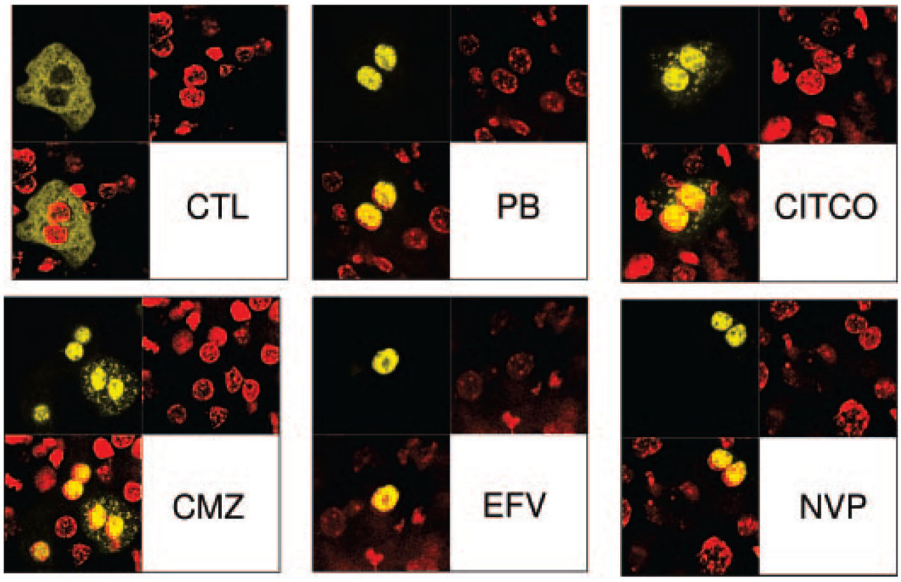

Fig. 5.

CMZ, EFV, and NVP promote cytoplasmic to nuclear translocation of hCAR. $CAR^{-/-}$ mice were transfected with 10 μg of expression plasmid encoding EYFP-tagged hCAR as described under *Materials and Methods* and treated 2 and 5 h later with vehicle (DMSO, CTL), 50 mg/kg RIF, 100 mg/kg PB, 10 mg/kg CITCO, 20 mg/kg CMZ, 25 mg/kg EFV, or 50 mg/kg NVP. Mice livers were harvested 7 h after tail vein delivery and slides of frozen liver sections were prepared for confocal microscopy. Approximately 150 counted cells were classified according to hCAR localization status. Representative images depict examples of cytoplasmic, nuclear, or mixed localization of hCAR. Three panels are shown for each treatment: upper left, EYFP-CAR (yellow) localization; upper right, nuclear staining (red); and lower left, merged image.

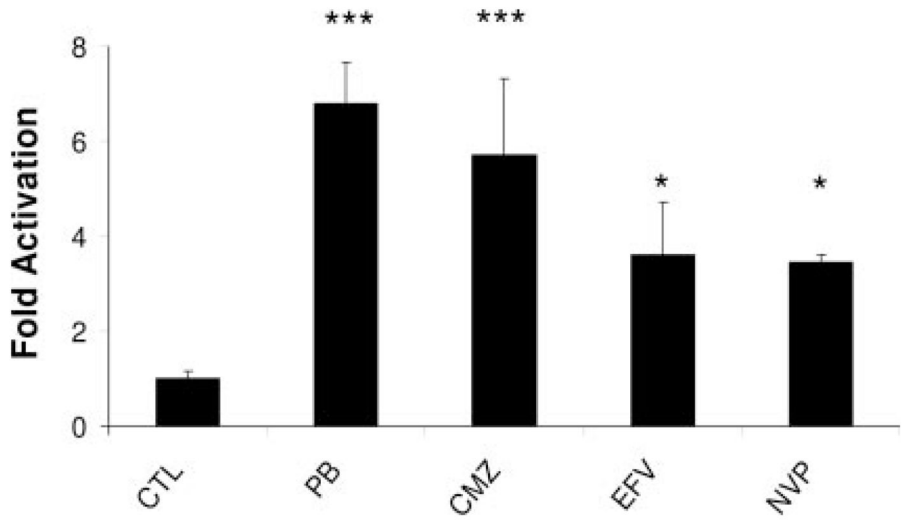

Fig. 6.

Effects of CMZ, EFV, and NVP on CYP2B6 reporter gene expression in hCAR-transfected $CAR^{-/-}$ mice. The *Trans*IT *In Vivo* Gene Delivery System was used for tail vein delivery of pCR3-hCAR (5 μg), CYP2B6-PBREM/XREM (4 μg), and pRL-SV40 (4 μg) into $CAR^{-/-}$ mice. Transfected mice were administered vehicle (DMSO, CTL), 100 mg/kg PB, 20 mg/kg CMZ, 25 mg/kg EFV, or 50 mg/kg NVP by i.p. injection at volumes of 0.1 ml/20 g body weight. Mouse livers were harvested approximately 24 h after delivery of plasmid DNA and processed as described under *Materials and Methods*. Luciferase activities were determined from three to four animals per treatment group. Data are presented as mean ± S.D. of fold activation over vehicle control. *, $p < 0.05$; ***, $p < 0.001$.

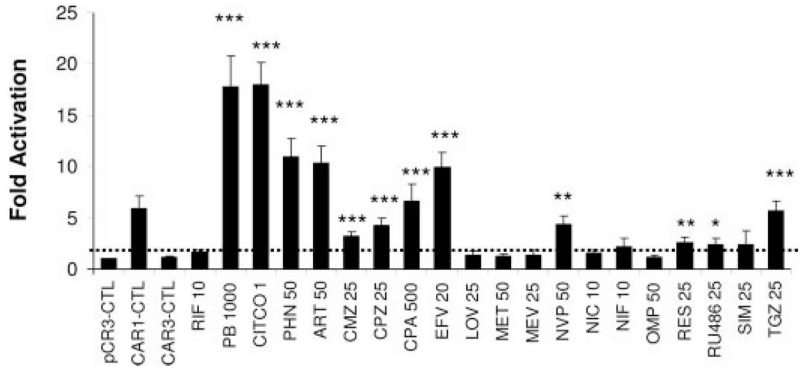

Fig. 7.

Evaluation of hCAR3 activation in HepG2 cell-based reporter gene assay. HepG2 cells were transfected with pCR3 empty vector, pCR3-hCAR (hCAR1), or CMV2-CAR3 expression plasmid (50 ng) along with CYP2B6 $(NR1)_2$ reporter gene plasmid (100 ng) and pRL-TK internal control plasmid (30 ng) as described under *Materials and Methods*. Subsequently, cells transfected with empty vector or wild-type hCAR were treated with vehicle (0.1% DMSO, CTL) (pCR3-CTL and CAR1-CTL, respectively) and hCAR3-transfected cells were treated with vehicle (CAR3-CTL), RIF (10 μM), PB (1 mM), CITCO (1 μM), and PHN (50 μM) in addition to the 16 test compounds at indicated concentrations (μM). Normalized CYP2B6 reporter gene activities represent the mean ± S.D. of three independent transfections. Results are presented as -fold activation relative to pCR3 empty vector. Dashed line, fold activation achieved by the negative control RIF; asterisks, level of statistical significance associated with test compound increases greater than RIF (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

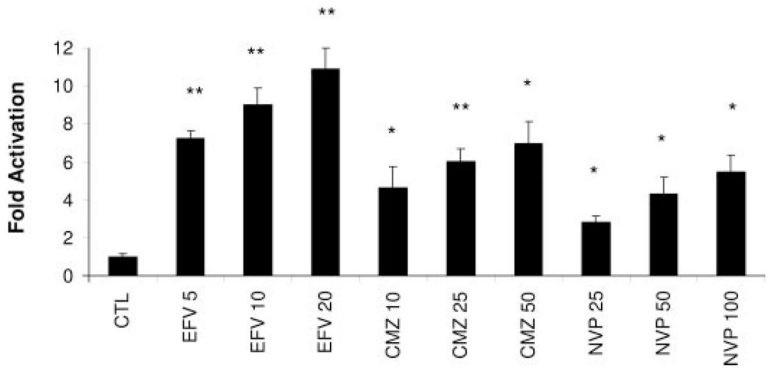

Fig. 8.

Concentration-dependent activation of hCAR3 by CMZ, EFV, and NVP. After transfection with CMV2-CAR3, CYP2B6 $(NR1)_2$, and pRL-TK plasmids as described under *Materials and Methods*, HepG2 cells were treated in triplicate with vehicle (0.1% DMSO, CTL) and the indicated concentrations of CMZ, EFV, and NVP. Results are expressed as fold activation (mean ± S.D.) relative to vehicle control. *, $p < 0.05$; **, $p < 0.01$.

TABLE 1

CYP2B6 induction in primary human hepatocytes

Human hepatocytes from five donors cultured in six-well Biocoat plates were treated for 24 h with DMSO (0.1%), 10 μM RIF, 1 μM CITCO, and the indicated concentrations of CMZ, EFV, and NVP. Total RNA was extracted from one to two wells per treatment, reverse-transcribed, and subjected to TaqMan real-time PCR. CYP2B6 mRNA levels were normalized to those of β-actin. Fold induction data relative to vehicle control represent the mean of triplicate PCR determinations.

Drug	Hu177	Hu190	Hu214	HCS009	HCS014
CMZ					
1	NA	1.6	1.3	2.0	1.4
10	6.0	4.3	4.6	4.9	2.9
25/50[a]	10.5	8.6	4.9	6.5	4.1
EFV					
1	4.4	3.4	2.1	4.2	1.9
5/10[b]	7.9	7.9	2.8	6.2	2.6
10/25[b]	5.5	7.4	3.3	5.8	3.7
NVP					
1	1.4	1.6	1.3	1.6	1.0
10	3.4	4.1	3.9	4.5	1.8
50	6.2	12.0	8.2	9.8	3.7
RIF					
10	NA	9.7	10.8	4.5	3.2
CITCO					
1	13.0	25.8	NA	10.8	5.3

NA, data not available.

[a] Highest tested concentration of CMZ was 25 μM in HCS009 and HCS014 and 50 μM in the remaining livers.

[b] Hepatocytes from donor HCS009 and HCS014 were treated with 1, 5, and 10 μM EFV, whereas those from other donors were treated with 1, 10, and 25 μM.

TABLE 2

Cellular localization of EYFP-hCAR in CAR-null mice

$CAR^{-/-}$ mice were tail vein-injected with 10 μ g of EYFP-hCAR and treated 2 and 5 h later by intraperitoneal injection of vehicle (DMSO, CTL), 50 mg/kg RIF, 100 mg/kg PB, 10 mg/kg CITCO, 20 mg/kg CMZ, 25 mg/kg EFV, or 50 mg/kg NVP at volumes of 0.1 ml per 20 g body weight. EYFP-hCAR expression was detected in mouse liver sections by confocal laser scanning microscope. For each treatment, over 150 hCAR-expressing cells from two mice livers (M1 and M2) were counted and classified as having cytosolic (C), nuclear (N), or mixed (C + N) hCAR localization. Numbers in parentheses represent the percentages of cells exhibiting the specified localization relative to the total number of counted cells. M1 = mouse 1, M2 = mouse 2.

Compounds	C		N		Mixed		Total	
	M1	M2	M1	M2	M1	M2	M1	M2
CTL	142 (88%)	152 (77%)	15 (9%)	35 (18%)	5 (3%)	10 (5%)	162 (100%)	197 (100%)
RIF (50 mg/kg)	155 (91%)	148 (82%)	10 (6%)	30 (16%)	5 (3%)	3 (2%)	170 (100%)	181 (100%)
PB (100 mg/kg)	59 (33%)	62 (27%)	113 (64%)	154 (67%)	6 (3%)	13 (6%)	178 (100%)	229 (100%)
CITCO (10 mg/kg)	67 (37%)	48 (30%)	84 (46%)	61 (40%)	32 (17%)	48 (30%)	183 (100%)	157 (100%)
CMZ (20 mg/kg)	21 (13%)	26 (14%)	118 (75%)	147 (81%)	18 (12%)	9 (5%)	157 (100%)	182 (100%)
EFV (25 mg/kg)	29 (19%)	41 (19%)	120 (78%)	163 (76%)	5 (3%)	11 (5%)	154 (100%)	215 (100%)
NVP (50 mg/kg)	46 (21%)	40 (21%)	129 (58%)	130 (67%)	47 (21%)	23 (12%)	222 (100%)	193 (100%)