Differential Activation of Pregnane X Receptor and Constitutive Androstane Receptor by Buprenorphine in Primary Human Hepatocytes and HepG2 Cells

Linhao Li, Hazem E. Hassan, Antonia H. Tolson, Stephen S. Ferguson, Natalie D. Eddington, and Hongbing Wang

Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, Maryland (L.L., H.E.H., A.H.T., N.D.E., H.W.); Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Helwan University, Helwan, Egypt (H.E.H.); and Life Technologies Corporation, Durham, North Carolina (S.S.F.)

Received July 21, 2010; accepted September 8, 2010

ABSTRACT

Buprenorphine is a partial μ -opioid receptor agonist used for the treatment of opioid dependence that has several advantages over methadone. The principal route of buprenorphine disposition has been well established; however, little is known regarding the potential for buprenorphine to influence the metabolism and clearance of other drugs by affecting the expression of drug-metabolizing enzymes (DMEs). Here, we investigate the effects of buprenorphine on the activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR), as well as the induction of DMEs, in both HepG2 cells and human primary hepatocytes (HPHs). In HepG2 cells, buprenorphine significantly increased human PXR-mediated CYP2B6 and CYP3A4 reporter activities. CYP2B6 reporter activity was also enhanced by buprenorphine in HepG2 cells cotransfected with a chemical-responsive human CAR variant. Real-time reverse transcription-polymerase chain reaction analysis re-

vealed that buprenorphine strongly induced CYP3A4 expression in both PXR- and CAR-transfected HepG2 cells. However, treatment with the same concentrations of buprenorphine in HPHs resulted in literally no induction of CYP3A4 or CYP2B6 expression. Further studies indicated that buprenorphine could neither translocate human CAR to the nucleus nor activate CYP2B6/CYP3A4 reporter activities in transfected HPHs. Subsequent experiments to determine whether the differential response was due to buprenorphine's metabolic stability revealed a dramatically differential rate of elimination for buprenorphine between HPHs and HepG2 cells. Taken together, these studies indicate that metabolic stability of buprenorphine defines the differential induction of DMEs observed in HepG2 and HPHs, and the results obtained from PXR and CAR reporter assays in immortalized cell line require cautious interpretation.

Introduction

Abuse of opioids is a rapidly escalating problem, with an estimated 980,000 people in the United States currently addicted to opiates and alarming increases reported in the number of opioid-related emergency department visits and

deaths (Mintzer et al., 2005). Although methadone (MD) continues to be the mainstay in the maintenance therapy of opioid dependence, buprenorphine (BUP), a partial μ -opioid receptor agonist, has been introduced as "a safe and effective" alternative that has several obvious advantages over MD (Milne et al., 2009; Orman and Keating, 2009). As a result, the use of BUP in this therapeutic scenario has increased dramatically in recent years. The potential for BUP-related drug-drug interactions (DDIs) are theoretically high, given that polypharmacy approaches are often applied to drug abusers, accompa-

This work was supported by the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grant DK061652].
Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.110.173187.

ABBREVIATIONS: MD, methadone; BUP, buprenorphine; DDI, drug-drug interaction; P450, cytochrome P450; UGT, UDP-glucuronosyl transferase; RIF, rifampicin; DME, drug-metabolizing enzyme; NR, nuclear receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RT-PCR, reverse transcription-polymerase chain reaction; HPH, human primary hepatocyte; DIP, diprenorphine; PK11195, 1-(2-chlorophenyl)-N-methylpropyl-3-isoquinoline-carboxamide; SFN, sulforaphane; PB, phenobarbital; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl) oxime; HPLC, high-performance liquid chromatography; h, human; PBREM, phenobarbital-responsive enhancer module; XREM, xenobiotic-responsive enhancer module; PXRE, pregnane X receptor response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s); DMSO, dimethyl sulfoxide; Ad/EYFP-hCAR, adenovirus expressing enhanced yellow fluorescence protein-tagged human CAR.

nied with medical complications such as cancer, HIV infection, and chronic pain (Köhler et al., 2000; Cone et al., 2003).

BUP is a natural morphine alkaloid of the opium poppy (*Papaver somniferum*) (Kress, 2009). Acting as a partial μ -opioid receptor agonist and a κ -opioid receptor antagonist, BUP's pharmacological features combine the benefits of both full opioid agonists and antagonists (Johnson et al., 2003). In the liver, BUP was extensively metabolized by the cytochrome P450 CYP3A4 and, to a lesser extent, CYP2C8. CYP3A4-mediated N-dealkylation and hydroxylation account for 70 to 90% of BUP metabolism, and the majority of these metabolites were further glucuronidated by UDP-glucuronosyl transferases (UGTs) and eliminated in the feces by efflux transporter-mediated biliary excretion (Cone et al., 1984; Iribarne et al., 1997). Over the past 10 years or so, metabolism- and transporter-related DDIs have received increasing attention, partly because of the frequent comedications necessitated by overlapping medical conditions, including opioid abusers under concomitant treatments for other diseases. To date, investigation of the interactions between opioid and other drugs has predominantly centered on how other drugs may affect the pharmacokinetics of opioids. For instance, studies demonstrated that rifampicin (RIF), the potent inducer of CYP3A4, CYP2B6, and P-glycoprotein, significantly accelerated the metabolism and clearance of MD, where sometimes double doses of MD were required to avoid withdrawal symptoms (Kharasch et al., 2004). Recent clinical studies also revealed that efavirenz, a non-nucleoside reverse-transcriptase inhibitor and CYP3A4 inducer, significantly decreased the BUP area under the concentration-time curve, whereas ritonavir, a protease inhibitor and potent inhibitor of CYP3A4, increased the area under the concentration-time curve of BUP in patients with HIV (McCance-Katz et al., 2006a,b). In addition, numerous other drugs are known to enhance the toxicity of opioids, which involves alteration in the pharmacokinetic properties of these opioids (Elkader and Sproule, 2005). On the other hand, there is an obvious paucity of data regarding the potential effects of opioids on the metabolic fate of other coadministered drugs.

In recent years, important advances have been made in our understanding of the mechanisms that regulate induction of DMEs and transporters. A family of ligand-activated transcription factors, known as nuclear receptors (NRs), has been identified as mediators of drug-induced expression of DMEs and transporters. Among them, the pregnane X receptor (PXR; NR1i2) and the constitutive androstane receptor (CAR; NR1i3) are promiscuously activated by a broad spectrum of chemicals with no obvious structural similarities, including endobiotics such as steroid hormones, fatty acids, and bile acids; and xenobiotics such as environmental chemicals and drugs (Kliwer et al., 2002; Hodgson and Rose, 2007). Together, these two NRs have been shown to transactivate an assortment of key DMEs and transporters, such as CYP2B6, CYP3A4, CYP2Cs, UGT1A1, and P-glycoprotein, the *MDR1* gene product (Xie et al., 2000; Wang and LeCluyse, 2003). A recent survey revealed that more than 64% of pharmaceutical companies in the United States have adopted cell-based PXR reporter assays routinely to assess the potential for DDIs as a result of DME inductions (Chu et al., 2009).

The primary objectives of this study were to characterize the ability of BUP to activate PXR and CAR and induce the

expression of hepatic DMEs. Cell-based reporter assays, transfection assays, cellular localization, and real-time RT-PCR assays were used to determine PXR and CAR activation and correlated P450 induction in HepG2 and human primary hepatocytes (HPHs). To our surprise, although BUP significantly activated PXR and CAR, as well as inducing CYP3A4 mRNA expression in HepG2 cells, there was no induction of CYP3A4 or CYP2B6 in HPHs treated with the same concentrations of BUP. Additional studies revealed that BUP was quickly eliminated in the physiologically relevant HPHs but not in HepG2 cells. These results warrant reasonable apprehension with respect to the interpretation and extrapolation of data obtained from NR reporter assays in immortalized cell lines.

Materials and Methods

Chemicals and Biological Reagents. BUP, RIF, MD, diprenorphine (DIP), PK11195, sulforaphane (SFN), and phenobarbital (PB) were purchased from Sigma-Aldrich (St. Louis, MO). 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl) oxime (CITCO) was obtained from Enzo Life Sciences Research Laboratories (Plymouth Meeting, PA). Morphine, codeine, and naloxone were supplied from Mallinckrodt, Inc. (St. Louis, MO). Meperidine, 6-desoxycodine, *N*-phenyl butyl normeperidine, 6-desoxymorphine, and normeperidine were kindly provided by Andrew Coop (University of Maryland School of Pharmacy, Baltimore, MD). Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The dual-luciferase reporter assay system was purchased through Promega (Madison, WI). CYP2B6 and CYP3A4 antibodies were obtained from Millipore Corporation (Billerica, MA). β -Actin antibody was obtained from Sigma-Aldrich. Matrigel, insulin, and insulin/transferrin/selenium were obtained from BD Biosciences Discovery Labware (Bedford, MA). Other cell culture reagents were purchased from Invitrogen (Carlsbad, CA) or Sigma-Aldrich. HPLC-grade acetonitrile was purchased from Thermo Fisher Scientific (Waltham, MA). Other cell culture reagents were purchased from Invitrogen or Sigma-Aldrich.

Plasmid Constructions. The pSG5-hPXR expression vector was obtained from Steven Kliwer (University of Texas Southwestern Medical Center, Dallas, TX). The pCR3-hCAR expression vector was generously provided by Masahiko Negishi (National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC). The CMV2-hCAR3 vector was from Curtis Omiecinski (Pennsylvania State University, University Park, PA). The pCR3-hCAR1+A expression vector and the CYP2B6 reporter construct, containing both phenobarbital-responsive enhancer module and the distal XREM (CYP2B6-2.2kb), were generated as described previously (Wang et al., 2003; Chen et al., 2010). The CYP3A4-PXRE/XREM reporter vector was obtained from Bryan Goodwin (GlaxoSmithKline, Research Triangle Park, NC). The pRL-TK *Renilla reniformis* luciferase plasmid used to normalize firefly luciferase activities was obtained from Promega.

Human Primary Hepatocyte Cultures and Treatments. Liver tissues were obtained by qualified medical staff after donor consent and prior approval from the Institutional Review Board at the University of Maryland at Baltimore. Hepatocytes were isolated from human liver specimens by a modification of the two-step collagenase digestion method as described previously (LeCluyse et al., 2005). Hepatocytes were seeded at 1.5×10^6 cells per well in six-well BioCoat plates in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 4 μ g/ml insulin, and 1 μ M dexamethasone. After 4 to 6 h of attachment at 37°C in a humidified atmosphere of 5% CO₂, cells were overlaid with Matrigel (0.25 mg/ml) in Williams' E medium supplemented with insulin, transferrin, and selenium; 0.1 μ M dexamethasone.

methasone; 100 U/ml penicillin; and 100 µg/ml streptomycin. The hepatocytes were maintained for 36 h before treatment with RIF (10 µM), CITCO (1 µM), PB (1 mM), BUP (10 and 50 µM), or DIP (10 µM) for another 24 or 72 h for detection of mRNA and protein expression, respectively.

Real-Time PCR Analysis. Total RNA was isolated from treated hepatocytes using the RNeasy Mini Kit (QIAGEN, Valencia, CA) and reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturers' instructions. CYP2B6 and CYP3A4 mRNA expression was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR assays were performed in 96-well optical plates on an ABI Prism 7000 sequence detection system with SYBR Green PCR Master Mix (Applied Biosystems). Primers for CYP2B6, CYP3A4, and GAPDH mRNA detection are as follows: CYP2B6, 1215 to 1319 bp, 5'-AGACGCCTTCAATCCTGACC-3' (forward) and 5'-CCTTCACCAAGACAAATCCGC-3' (reverse); CYP3A4, 213 to 462 bp, 5'-GTGGGGCTTTTATGATGGTCA-3' (forward) and 5'-GCCTCAGATTTCTCACCAACACA-3' (reverse); and GAPDH, 217 to 501 bp, 5'-CCCATCACCATTCTCCAGGAG-3' (forward) and 5'-GTTGTCATGGATGACCTTGGC-3' (reverse). Induction values (fold over control) were calculated according to the $2^{-\Delta\Delta C_t}$ method, where ΔC_t represents the differences in cycle threshold numbers between the target gene and GAPDH, and $\Delta\Delta C_t$ represents the relative change in these differences between the control and treatment groups.

Transient Transfection in HepG2 Cells and Human Primary Hepatocytes. HepG2 cells in 24-well plates were transfected with CYP3A4-PXRE/XREM or CYP2B6–2.2kb reporter construct in the presence of an hPXR, hCAR, hCAR3, or hCAR1+A expression vector using a FuGENE 6 transfection kit according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Twenty-four hours after transfection, cells were treated for another 24 h with solvent (0.1% DMSO) or test compounds RIF (10 µM), CITCO (1 µM), morphine (10 µM), MD (10 µM), oxycodone (10 µM), codeine (10 µM), meperidine (10 µM), BUP (1, 5, 10, 25, 50, and 100 µM), 6-desoxycodone (10 µM), naloxone (10 µM), DIP (10 µM), *N*-phenyl butyl normeperidine (10 µM), 6-desoxymorphine (10 µM), or normeperidine (10 µM). Cell lysates were subsequently assayed for firefly activities normalized against the activities of cotransfected *R. reniformis* luciferase using the Dual-Luciferase Kit (Promega). Data were represented as mean $\pm$ S.D. of three individual transfections. To analyze PXR- and CAR-mediated induction of CYP3A4 in hepatoma cells, HepG2 cells seeded in 12-well plates were transfected with a hCAR or hPXR expression vector; 24 h after transfection, cells were treated for another 24 h with RIF (10 µM), CITCO (1 µM), PK11195 (10 µM), SFN (10 µM), or BUP (10 and 50 µM) or were cotreated for 24 h with SFN plus RIF or BUP, and PK11195 plus CITCO or BUP, respectively. Total RNA was isolated and subjected to real-time RT-PCR analysis as described above. In separate experiments, HPHs seeded in 24-well BioCoat plates were transfected with CYP3A4-PXRE/XREM or CYP2B6–2.2kb construct in the presence of the pGL-TK vector using Effectene reagent (QIAGEN) as described previously (Wang et al., 2004). Transfected HPHs were treated with DMSO [0.1% (v/v)], RIF (10 µM), CITCO (1 µM), BUP (10 and 50 µM), or DIP (10 µM) for 24 h, respectively. Cell lysates were subjected to dual-luciferase analysis as described above.

Translocation of Adenovirus Expressing Enhanced Yellow Fluorescent Protein-Tagged hCAR in Human Primary Hepatocytes. The adenovirus expressing enhanced yellow fluorescent protein-tagged hCAR (Ad/EYFP-hCAR), which infects HPHs with high efficiency, was generated as described previously (Li et al., 2009a). Hepatocyte cultures in 24-well BioCoat plates were infected with 5 µl of Ad/EYFP-hCAR for 12 h before treatment with the vehicle control (0.1% DMSO), PB (1 mM), BUP (10 and 50 µM), or DIP (10 µM). After 24 h of incubation, cells were washed twice with phosphate-buffered saline and fixed for 30 min in 4% buffered paraformaldehyde. The cells were then stained with 4,6-diamidine-2-

phenylindole dihydrochloride for 30 min. Confocal microscopy analysis was performed with a Nikon C1-LU3 instrument based on an inverted Nikon Eclipse TE2000 microscope (Nikon, Melville, NY). The subcellular localization of hCAR was visualized and quantitatively characterized as a nuclear, cytosolic, or mixed (nuclear + cytosolic) expression by counting 100 Ad/EYFP-hCAR-expressing hepatocytes from each group.

Western Blot Analyses. The whole-cell homogenate proteins (20 µg) from treated HPHs were resolved on SDS-polyacrylamide gels and electrophoretically transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore Corporation). Membranes were subsequently incubated with specific antibodies against CYP2B6 or CYP3A4 (Millipore Corporation), diluted 1:4000 and 1:5000, respectively. β -Actin was used as an internal control. Blots were washed and incubated with horseradish peroxidase goat anti-rabbit IgG antibody, diluted 1:4000; they were developed using an ECL Western blotting detection reagent (GE Healthcare, Chalfont St. Giles, UK).

Metabolic Stability Studies. Metabolism studies were conducted in cryopreserved hepatocytes and HepG2 cells. Cell suspensions consisting of 0.5×10^6 viable cells per incubation were diluted in serum-free Dulbecco's modified Eagle's medium supplemented with ITS⁺ (BD Biosciences), 0.1 µM dexamethasone, and penicillin-streptomycin. Cells were incubated in triplicate under standard culture conditions with BUP and DIP at the final concentration of 5 µM. Time points used were 0, 30, 60, 90, 120, 150, and 180 min for BUP, and 0, 60, 120, and 180 min for DIP, respectively. The reactions were terminated using acetonitrile.

HPLC Quantitation of Buprenorphine and Diprenorphine. BUP and DIP samples were analyzed according to a validated HPLC method (Hassan et al., 2009). The assay was performed using a mobile phase of 5 mM sodium acetate buffer, pH 3.75, in acetonitrile [2:8 (v/v)] and a Waters 474 fluorescence detector (excitation at 210 nm, emission at 352 nm; Waters, Milford, MA). A flow rate of 1.2 ml/min at 25°C was used, and the injection volume was 150 µl. BUP and DIP were eluted at 3.83 and 4.57 min, respectively. The assay was linear ($r^2 \geq 0.987$) over the tested concentrations (0.05–4 µg/ml). The chromatographic HPLC system was composed of 1) a Waters 1525 Binary HPLC pump (Waters), 2) a 717 Waters autosampler, 3) a 3390A Hewlett Packard integrator/plotter (Hewlett Packard, Avondale, PA), and 4) Waters Symmetry C18 (4.6 $\times$ 250 mm) column (Waters). Accuracy and precision were determined by replicate injection of quality control samples. Both precision and accuracy were of satisfactory results below 11% CV.

Statistical Analysis. All data represent at least three independent experiments and are expressed as the mean $\pm$ S.D. Statistical comparisons were made using one-way analysis of variance followed by a post hoc Dunnett's test and Student's *t* test where appropriate. Statistical significance was set at $P < 0.05$.

Results

Effects of BUP on the Activation of hPXR in HepG2 Cells. To characterize the potential roles of opioids on the activation of xenobiotic receptors, we first screened 12 frequently prescribed opioids in HepG2 cells transfected with an hPXR expression vector in the presence of the CYP3A4-PXRE/XREM or CYP2B6–2.2kb luciferase reporter construct. The test opioids included morphine, MD, oxycodone, codeine, meperidine, BUP, 6-desoxycodone, naloxone, DIP, *N*-phenyl butyl normeperidine, 6-desoxymorphine, and normeperidine. All opioids were tested at 10 µM concentration; RIF (10 µM) and DMSO (0.1%, v/v) were the positive and vehicle controls, respectively. Figure 1 demonstrates opioid-mediated influence on CYP3A4 (Fig. 1A) and CYP2B6 (Fig. 1B) reporter activities in comparison with vehicle control as a percentage of the increase achieved by the hPXR positive control RIF (10

Fig. 1. Activation of hPXR by different opioids in cell-based reporter assays. HepG2 cells were transfected with an hPXR expression vector in the presence of CYP3A4-PXRE/XREM (A) or CYP2B6–2.2kb (B) reporter construct as described under *Materials and Methods*. Transfected cells were then treated with different opioids (morphine, MD, oxycodone, codeine, meperidine, BUP, 6-desoxycodone, naloxone, DIP, *N*-phenyl butyl normeperidine, 6-desoxynormeperidine, or normeperidine) at 10 μ M or with BUP at different concentrations (C) for 24 h. RIF (10 μ M) was used as a positive control for hPXR activation. Luciferase (Luc) activities were determined and expressed as a percentage of RIF activation or relative to vehicle control (0.1% DMSO). All data are expressed as mean $\pm$ S.D. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

μ M). The criteria adopted from previous publications (Sinz et al., 2006; Faucette et al., 2007; Chu et al., 2009) classify compounds that achieve more than 40% of RIF-normalized hPXR activation in reporter assays as strong activators of hPXR, whereas drugs exhibiting between 15 and 40% of RIF response are moderate activators; those exhibiting less than 15% of RIF response are recognized as nonactivators. Consistent with our previous report, MD was classified as a moderate-to-strong activator (Tolson et al., 2009). It is worth noting that BUP and DIP, with high structural similarity (see Fig. 7A), display strong activation of hPXR, reaching approximately 80 and 70% of RIF response, respectively, whereas all the other opioids exhibit negligible PXR activation.

Based on these initial screening data, additional hPXR reporter experiments were performed in HepG2 cells treated with BUP over a broader range of concentrations (1–100 μ M). Our preliminary experiments showed that BUP at a concentration of 100 μ M is associated with cytotoxicity in HepG2 cells (data not shown). Obvious concentration-dependent responses (1–50 μ M) for BUP-induced PXR activation of CYP2B6 and CYP3A4 reporter activities are shown in Fig. 1C.

Effects of BUP on the Activation of CAR in HepG2 Cells. Because PXR and CAR share a number of overlapping target genes and many xenobiotics as coactivators, we further investigated whether BUP could activate human CAR in HepG2 cells. Consistent with previous reports, cell-based reporter assays using the reference hCAR (hCAR1) displayed constitutively high basal activity and moderate response to the known hCAR activator CITCO in HepG2 cells (Li et al., 2009a). There was no activation of hCAR1 after the exposure of BUP in this system (Fig. 2A). On the other hand, using a hCAR ligand-responsive variant (hCAR3), BUP caused a marginal but concentration-dependent enhancement of CYP2B6 reporter activity, with the highest activation at 50 μ M (1.8-fold over vehicle control), whereas using the positive control CITCO resulted in 4.34-fold increase (Fig. 2B). More-

over, in separate reporter assays, we applied a recently developed hCAR1+A construct, which demonstrated robust response to chemical-mediated activation of hCAR in immortalized cell lines (Chen et al., 2010). As shown in Fig. 2C, BUP significantly enhanced the hCAR1+A-mediated expression of CYP2B6 reporter gene, with the highest response at 50 μ M (6.43-fold over vehicle control) challenging that of the positive control CITCO (6.92-fold over vehicle control).

BUP Increases hPXR- and hCAR-Mediated Induction of Endogenous CYP3A4 Expression in HepG2 Cells. To correlate the BUP-mediated activation of PXR and CAR with the actual induction of the *CYP3A4* gene in HepG2 cells, real-time PCR analysis was carried out in HepG2 cells transfected with an hPXR or hCAR expression vector as outlined under *Materials and Methods*. Without transfection, BUP induced CYP3A4 mRNA expression moderately but statistically significantly (Fig. 3A), mainly as a result of the limited endogenous expression of PXR and CAR in HepG2 cells. Consistent with the results from cell-based reporter assays, the induction of CYP3A4 was markedly increased by BUP in both hPXR- and hCAR-transfected HepG2 cells; moreover, this induction was significantly repressed by the cotreatment of SFN as a selective hPXR deactivator (Zhou et al., 2007) and PK11195 as a selective hCAR deactivator (Li et al., 2008), respectively (Fig. 3, B and C). Together, these results suggest that BUP could activate both PXR and CAR and induce CYP3A4 expression in HepG2 cells, accordingly.

Influence of BUP on CYP3A4 and CYP2B6 Expression in Human Primary Hepatocytes. Results obtained from HepG2 cell-based reporter assays and real-time PCR analysis indicated that BUP has agonistic effects on the activation of PXR and CAR. We further evaluated the effect of BUP on the expressions of *CYP3A4* and *CYP2B6*, two shared common target genes of PXR and CAR in HPHs. HPHs from four different donors were treated with CITCO, RIF, PB, and BUP as described under *Materials and Methods*. As expected, all prototypical PXR/CAR activators exhib-

Fig. 2. Effects of BUP on the activation of hCAR. HepG2 cells were transfected with an hCAR1 (A), hCAR3 (B), or hCAR1+A (C) expression vector in the presence of CYP2B6–2.2kb reporter construct as described under *Materials and Methods*. Transfected cells were subsequently treated with BUP at concentrations of 1 to 50 μ M for 24 h. CITCO (1 μ M) was used as a positive control for hCAR activation. Luciferase (Luc) activities were determined and expressed as fold activation relative to vehicle control (0.1% DMSO). All data are expressed as mean $\pm$ S.D. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

Fig. 3. PXR- and CAR-dependent induction of CYP3A4 by BUP in transfected HepG2 cells. B and C, HepG2 cells were transfected with an hPXR (B) or hCAR (C) expression vector as described under *Materials and Methods*. Twenty-four hours after transfection, cells were treated with RIF (10 μ M), CITCO (1 μ M), PK11195 (10 μ M), SFN (10 μ M), or BUP (10 and 50 μ M) or were cotreated with SFN and PK11195 as selective inhibitors of PXR and CAR, respectively. A, treatments in nontransfected HepG2 cells reflect the endogenous PXR and CAR responses. All cells were treated for 24 h before harvesting total RNA. Real-time RT-PCR was used to detect the mRNA expression of CYP3A4. Data are expressed as mean $\pm$ S.D. ($n = 3$). **, $P < 0.01$.

ited potent induction of *CYP2B6* and/or *CYP3A4* at mRNA and protein levels (Fig. 4). Nevertheless, the expression of *CYP2B6* and *CYP3A4* was literally not increased after the treatment of BUP at concentrations of 10 and 50 μ M, which significantly activated PXR and CAR and induced *CYP3A4* mRNA in HepG2 cells (Fig. 4). These unexpected results indicate that BUP may not be an efficient activator of either PXR or CAR in a more physiologically relevant cell system.

Expression of CYP2B6 and CYP3A4 Reporter Gene in Human Primary Hepatocytes. Primary hepatocyte cultures maintain the expression and function of major hepatic transcription factors including PXR and CAR, the major nuclear receptors implicated in the inductive expression of *CYP3A4* and *CYP2B6*. Previous reports showed that these endogenous nuclear receptors in human hepatocytes are sufficient to induce transfected *CYP2B6* and *CYP3A4* reporter

gene expression upon chemical stimulation (Wang et al., 2004; Faucette et al., 2007). The ability of BUP to activate *CYP2B6*/*CYP3A4* reporter gene expression was consequently evaluated in HPHs from two donors. As demonstrated in Fig. 5, selective activators of PXR (RIF, 10 μ M) and CAR (CITCO, 1 μ M) markedly increased the reporter gene expression of *CYP3A4* and *CYP2B6*, respectively. On the other hand, BUP at concentrations of 1, 10, and 50 μ M resulted in essentially no activation of either *CYP3A4* or *CYP2B6* reporter expression in HPHs prepared from both liver donors, which was in accordance with the observation that BUP failed to induce the actual *CYP2B6* and *CYP3A4* genes in HPHs.

Nuclear Translocation of hCAR in Human Primary Hepatocytes. In contrast to the constitutive nuclear expression in immortalized cells, human CAR is predominantly

Fig. 4. Induction of *CYP2B6* and *CYP3A4* expression in human primary hepatocytes. Human primary hepatocyte cultures from donors HL-017, HL-018, and HL-023 were treated for 24 h with CITCO (1 μ M), RIF (10 μ M), PB (1 mM), or BUP (10 and 50 μ M). A and B, total RNA was collected, reverse-transcribed, and subjected to real-time RT-PCR for detecting the *CYP3A4* (A) and *CYP2B6* (B) expression levels as outlined under *Materials and Methods*. C–E, in separate experiments, HPHs from HL-019 (C), HL-021 (D), and HL-023 (E) were treated with the same batch of chemicals as indicated for A and B for 72 h. After harvesting, whole-cell homogenates (20 μ g of each) were subjected to *CYP2B6* and *CYP3A4* immunoblotting analysis as described under *Materials and Methods*. RT-PCR data are expressed as mean $\pm$ S.D. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$.

sequestered in the cytoplasm of primary hepatocytes and translocated to the nucleus after exposure to prototypical CAR activators such as CITCO and PB (Kawamoto et al., 1999; Wang et al., 2004). We have recently generated an Ad-EYFP-hCAR. With high infection efficiency in HPHs, the Ad-EYFP-hCAR also displays the unique feature of hCAR distribution and activation in HPHs (Li et al., 2009a). Because our results thus far demonstrated that BUP-mediated activation of hCAR in HepG2 cells is associated with enhanced expression of *CYP3A4*, we continue to test whether

this opioid could translocate hCAR to the nucleus as the initial step of hCAR activation in HPHs. Cultured hepatocytes were infected with Ad-EYFP-hCAR and treated with vehicle control, positive control PB (1 mM), or BUP (10 and 50 μ M) as outlined under *Materials and Methods*. Confocal microscopic analysis revealed that without activation, Ad-EYFP-hCAR was localized primarily in the cytoplasm (90%), and the prototypical CAR activator PB efficiently translocated hCAR to the nucleus (92%), whereas BUP at 10 and 50 μ M showed no effect on hCAR nuclear accumulation with a

Fig. 5. *CYP2B6* and *CYP3A4* reporter gene expression in human primary hepatocytes. Human primary hepatocytes from two donors (HL-023 and HL-025) cultured in 24-well BioCoat plates were transfected with *CYP2B6*–2.2kb or *CYP3A4*–PXRE/XREM reporter construct and then treated with CITCO (1 μ M), RIF (10 μ M), PB (1 mM), or BUP (10 and 50 μ M) for 24 h as described under *Materials and Methods*. Luciferase (Luc) activities were determined and expressed as fold activation relative to control (0.1% DMSO). All data are expressed as mean $\pm$ S.D. ($n = 3$). **, $P < 0.01$.

Fig. 6. Localization of Ad-EYFP-hCAR in human primary hepatocytes. Human hepatocytes from donors (HL-018 and HL-020) in 24-well BioCoat plates were infected with Ad-EYFP-hCAR for 12 h and then treated with vehicle control (0.1% DMSO), PB (1 mM), and BUP (10 and 50 μ M) for 24 h. Cells were subsequently fixed for 30 min in 4% buffered paraformaldehyde and stained with 4,6-diamidino-2-phenylindole dihydrochloride for 30 min before being examined by a confocal microscopy. A, representative Ad-EYFP-hCAR localizations from vehicle control, PB-, and BUP-treated hepatocytes. B, percentage of Ad-EYFP-hCAR cellular localization in HPHs after treatment with vehicle control, PB, and BUP. One hundred Ad-EYFP-hCAR-expressing HPHs were counted from each treatment group.

cytoplasmic localization of 87 and 88%, respectively (Fig. 6, A and B). Overall, these results demonstrate that BUP is not an inducer of CYP2B6 and CYP3A4 because it fails to activate either PXR or CAR in HPHs.

Metabolic Stability of BUP and DIP in HepG2 and Human Hepatocytes. To further delineate the discrepancy observed between HepG2 and HPHs, the metabolic stability of BUP in these two cell systems were investigated. HepG2 cells and cryopreserved human hepatocytes suspension were used to compare the metabolic stability of BUP as described

under *Materials and Methods*. Because DIP also demonstrated potent activation of PXR in HepG2 cells (Fig. 1, A and B) and has high structural similarity with BUP (Fig. 7A), the metabolic stability of DIP was also investigated. As demonstrated in Fig. 7B, BUP was quickly metabolized in HPHs with a half-life ($t_{1/2}$) of approximately 2 h, whereas its concentration remains virtually unchanged up to the maximal incubation time (3 h) in HepG2 cells. It is noteworthy that DIP also displayed quick metabolism in HPHs versus HepG2 cells, where the estimated $t_{1/2}$ was approximately 50 min,

Fig. 7. Metabolic stability of BUP and DIP in HepG2 cells and human primary hepatocytes. A, the chemical structures of BUP and DIP are depicted. The metabolic stability of BUP and DIP were determined in suspensions of HepG2 cells and cryopreserved hepatocytes using HPLC analysis as described under *Materials and Methods*. B and C, the percentages of remaining BUP (B) and DIP (C) over time in HepG2 and HPHs are shown. All data points represent the average of three measurements $\pm$ S.D.

Fig. 8. DIP fails to activate PXR and CAR or to induce *CYP2B6* and *CYP3A4* expression in human primary hepatocytes. A and B, human hepatocytes from HL-023 (A) and HL-024 (B) were treated for 24 h with CITCO (1 μ M), RIF (10 μ M), PB (1 mM), or DIP (10 μ M). Real-time RT-PCR analysis of *CYP2B6* and *CYP3A4* expression was carried out as outlined under *Materials and Methods*. C, the cellular localization of Ad/EYFP-hCAR in transfected HPHs (HL-025) after the treatment with vehicle control (0.1% DMSO), PB (1 mM), or DIP (10 μ M). D and E, HPHs (HL-025) were transfected with *CYP2B6*-2.2kb (D) or *CYP3A4*-PXRE/XREM (E) reporter construct and then treated with CITCO (1 μ M), RIF (10 μ M), or DIP (1 and 10 μ M) for 24 h. Luciferase (Luc) activities were determined and expressed as fold activation relative to control (0.1% DMSO). All data are expressed as mean $\pm$ S.D. ($n = 3$). **, $P < 0.01$.

and less than 10% DIP was detected only in HPHs at the end of incubation (3 h) (Fig. 7C). Subsequent experiments in HPHs demonstrated that DIP, similarly to BUP, also failed to either activate PXR and CAR or induce the expression of *CYP2B6* and *CYP3A4* (Fig. 8). Together, these dramatic differences in the metabolic stability of BUP and DIP between HepG2 and HPHs may significantly contribute to the observed discrepancy in BUP- and DIP-mediated PXR and CAR activation and their target gene induction in these two cell systems.

Discussion

Prescription drug abuse and misuse has become a major public health concern worldwide. Opioids, among others, represent the highest prevalence of drug abuse and misuse given that they continue to be the mainstay for the treatment of chronic and severe pain, and they are extensively used for the management of heroin dependence (Pergolizzi et al., 2008). However, characterization of opioid interactions with other drugs to date has been heavily one-sided, focusing on how other drugs affect the metabolic and/or pharmacokinetic profiles of opioids. In contrast, the potential for opioids to influence the similar fates of coadministered drugs is largely unexplored. In the current study, we showed that BUP is the most potent PXR activator identified from 12 frequently prescribed opioids in the cell-based reporter assays. Subsequent

experiments in HepG2 cells confirmed that BUP can activate both PXR and CAR and can induce the expression of *CYP3A4* in a nuclear receptor-dependent manner. On the other hand, an unexpected profile of PXR/CAR activation and *CYP2B6*/*CYP3A4* induction was uncovered in cultured HPHs treated with BUP, where BUP treatments resulted in virtually no activation of PXR and CAR or induction of their target genes *CYP2B6* and *CYP3A4*.

Metabolism induction-related DDIs are one of the critical concerns in the overall safety profiles of clinical medication as well as drug development. Transcriptional up-regulation of drug-metabolizing genes by activation of nuclear receptors represents the principle mechanism by which induction-related DDIs occur. Owing to the ability to transactivate the expression of multiple DMEs and transporters, as well as the promiscuous selectivity of ligands and activators, PXR and CAR have been widely accepted as xenobiotic sensors, mediating the major inductive responses in hepatic metabolism and transport (Willson and Kliewer, 2002; Honkakoski et al., 2003). Cell-based PXR reporter assays in particular have been extensively used in the prediction of xenobiotic-mediated induction of PXR target genes such as *CYP3A4* and *CYP2B6* (Kliewer et al., 2002; Chu et al., 2009). Nevertheless, several significant drawbacks associated with the PXR reporter assay may affect the proper interpretation of data obtained from this broadly used in vitro system. For instance,

phenytoin, a commonly used antiepileptic drug, is a potent inducer of CYP2B6 and CYP3A4, but it does not activate PXR in cell-based reporter assays (Raucy, 2003; Wang et al., 2004). On the other hand, two flavonoids from *Ginkgo biloba* extract, quercetin and kaempferol, are efficient activators of PXR in cell-based reporter assays, yet they fail to induce the expression of either CYP3A4 or CYP2B6 in HPHs (Li et al., 2009b). Our current results illustrate that BUP is not a physiologically relevant activator of PXR and CAR or inducer of associated P450s in HPHs (Figs. 4–6), despite its demonstrated potent activation of PXR and induction of CYP3A4 in HepG2 cells (Figs. 1–3). These findings trigger the speculation that the obvious lack of metabolic capability in an immortalized cell line compared with an HPH may contribute to the differential induction.

Unlike immortalized hepatoma cell lines, HPHs retain physiologically relevant expression and function of the major DMEs, transporters, and hepatic-enriched transcription factors, and HPHs represent the most reliable in vitro model reflecting metabolic characteristics of the human liver (LeCluyse, 2001; Wang and LeCluyse, 2003). Many compounds underwent extensive metabolism in HPHs but not in HepG2 cells, which resulted in controversial bioactivities of these chemicals. Chrysin, a dietary flavonoid, has been shown to markedly induce the expression of UGT1A1 in HepG2 and Caco2 cells, and it was suggested to have clinical benefits in conditions such as hyperbilirubinemia by promoting the expression of UGT1A1 (Walle et al., 2000). Nonetheless, such inductive effect of chrysin was negligible in HPHs because of its rapid metabolism, indicating that the metabolic instability would most likely limit its UGT1A1 induction in vivo (Smith et al., 2005). On the other hand, Gerbal-Chaloin et al. (2006) reported that omeprazole sulfide, a pure antagonist of aryl hydrocarbon receptor, was efficiently converted to omeprazole as an agonist of aryl hydrocarbon receptor only in HPHs. It is well established that BUP was predominantly metabolized in the liver by CYP3A4, CYP2C8, and UGTs before eliminating in the bile (Cone et al., 1984; Orman and Keating, 2009). Thus the metabolic stability of BUP was compared between HPHs and HepG2 cells in the current study. Substantial differences in the rate of BUP elimination were observed between these two cell systems (Fig. 7), which strongly support the speculation that rapid clearance of BUP in HPHs contributes significantly to the noninduction of CYP2B6 and CYP3A4. In the meantime, these results imply that metabolites of BUP are not able to activate PXR and CAR or induce their target genes. In separate observations, the metabolic stability of DIP, an opioid antagonist structurally parallel to BUP, was also dramatically decreased in HPHs versus HepG2 cells. Moreover, a similarly differential activation of PXR and CAR, as well as induction of their P450 targets between HepG2 and HPHs, was noticed with the treatment of DIP (Fig. 8), suggesting that this phenomenon represents a shared class effect rather than a unique compound-specific role of BUP.

Compared with PXR, in vitro evaluation of CAR activation was more challenging because of the nature of constitutive activation of CAR in immortalized cell lines and the fact that CAR can be activated by both direct ligand binding and indirect mechanisms (Honkakoski et al., 2003; Qatanani and Moore, 2005). We have established several novel strategies to efficiently identify hCAR activators in vitro. A chimeric con-

struct generated by inserting alanine to amino acid position 270 of hCAR (hCAR1+A) converts the constitutively activated hCAR to a xenobiotic-sensitive receptor; this construct was significantly activated by a series of known hCAR activators (Chen et al., 2010). Using this unique system, we showed that BUP efficiently activated hCAR1+A to the extent that it is clearly superior to that of hCAR1 or hCAR3 in cell-based reporter assays. In addition, the Ad/EYFP-hCAR infection of HPHs was established as a valuable model to efficiently detect chemical-mediated nuclear translocation of hCAR as the initial step of CAR activation in HPHs (Li et al., 2009a). In agreement with the unstable feature of BUP and DIP in HPHs, both opioids were unable to translocate hCAR to the nucleus in transfected HPHs.

Taken together, we report here that BUP differentially transactivates PXR, CAR, and their target genes *CYP2B6* and *CYP3A4* in HepG2 and HPHs. The results demonstrate that although BUP functions as a potent activator of PXR and CAR as well as an inducer of CYP3A4 in the immortalized cell line, such effects were totally lost in a more physiologically relevant cell system, the HPHs. Further evidence reveals that the loss of inductive effects for BUP in HPHs is mainly due to the rapid metabolism and clearance of BUP. In addition, DIP displayed similar responses in HepG2 and HPHs, indicating that a class of structurally similar opioids and their derivatives may behave alike in this regard. Overall, these results raise sincere concerns in our use and interpretation of data obtained from cell-based nuclear receptor assays, in particular, the widely used PXR reporter assays.

Acknowledgments

We thank Masahiko Negishi (National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC), Steven Kliever (University of Texas Southwestern Medical Center, Dallas, TX), Curtis Omiecinski (Pennsylvania State University, University Park, PA), and Bryan Goodwin (GlaxoSmithKline, Research Triangle Park, NC) for kindly providing multiple PXR, CAR expression, and CYP3A4 reporter constructs; Andrew Coop (University of Maryland School of Pharmacy, Baltimore, MD) for generously providing assistance in obtaining opioids, and the University of Maryland Medical Center (Baltimore, MD) and Life Technologies (Durham, NC) for providing the human hepatocytes used in this study.

References

- Chen T, Tompkins LM, Li L, Li H, Kim G, Zheng Y, and Wang H (2010) A single amino acid controls the functional switch of human constitutive androstane receptor (CAR) 1 to the xenobiotic-sensitive splicing variant CAR3. *J Pharmacol Exp Ther* **332**:106–115.
- Chu V, Einolf HJ, Evers R, Kumar G, Moore D, Ripp S, Silva J, Sinha V, Sinz M, and Skerjanec A (2009) In vitro and in vivo induction of cytochrome P450: a survey of the current practices and recommendations: a Pharmaceutical Research and Manufacturers of America perspective. *Drug Metab Dispos* **37**:1339–1354.
- Cone EJ, Fant RV, Rohay JM, Caplan YH, Ballina M, Reder RF, Spyker D, and Haddox JD (2003) Oxycodone involvement in drug abuse deaths: a DAWN-based classification scheme applied to an oxycodone postmortem database containing over 1000 cases. *J Anal Toxicol* **27**:57–67.
- Cone EJ, Gorodetzky CW, Yousefnejad D, Buchwald WF, and Johnson RE (1984) The metabolism and excretion of buprenorphine in humans. *Drug Metab Dispos* **12**:577–581.
- Elkader A and Sproule B (2005) Buprenorphine: clinical pharmacokinetics in the treatment of opioid dependence. *Clin Pharmacokinet* **44**:661–680.
- Faucette SR, Zhang TC, Moore R, Sueyoshi T, Omiecinski CJ, LeCluyse EL, Negishi M, and Wang H (2007) Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *J Pharmacol Exp Ther* **320**:72–80.
- Gerbal-Chaloin S, Pichard-Garcia L, Fabre JM, Sa-Cunha A, Poellinger L, Maurel P, and Daujat-Chavanieu M (2006) Role of CYP3A4 in the regulation of the aryl hydrocarbon receptor by omeprazole sulphide. *Cell Signal* **18**:740–750.
- Hassan HE, Myers AL, Coop A, and Eddington ND (2009) Differential involvement of P-glycoprotein (ABCB1) in permeability, tissue distribution, and antinociceptive

- activity of methadone, buprenorphine, and diprenorphine: in vitro and in vivo evaluation. *J Pharm Sci* **98**:4928–4940.
- Hodgson E and Rose RL (2007) Human metabolic interactions of environmental chemicals. *J Biochem Mol Toxicol* **21**:182–186.
- Honkakoski P, Sueyoshi T, and Negishi M (2003) Drug-activated nuclear receptors CAR and PXR. *Ann Med* **35**:172–182.
- Iribarne C, Picart D, Dréano Y, Bail JP, and Berthou F (1997) Involvement of cytochrome P450 3A4 in N-dealkylation of buprenorphine in human liver microsomes. *Life Sci* **60**:1953–1964.
- Johnson RE, Strain EC, and Amass L (2003) Buprenorphine: how to use it right. *Drug Alcohol Depend* **70**:S59–S77.
- Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* **19**:6318–6322.
- Kharasch ED, Hoffer C, Whittington D, and Sheffels P (2004) Role of hepatic and intestinal cytochrome P450 3A and 2B6 in the metabolism, disposition, and mitotic effects of methadone. *Clin Pharmacol Ther* **76**:250–269.
- Kliwer SA, Goodwin B, and Willson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev* **23**:687–702.
- Köhler GI, Bode-Böger SM, Busse R, Hoopmann M, Welte T, and Böger RH (2000) Drug-drug interactions in medical patients: effects of in-hospital treatment and relation to multiple drug use. *Int J Clin Pharmacol Ther* **38**:504–513.
- Kress HG (2009) Clinical update on the pharmacology, efficacy and safety of transdermal buprenorphine. *Eur J Pain* **13**:219–230.
- LeCluyse EL (2001) Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci* **13**:343–368.
- LeCluyse EL, Alexandre E, Hamilton GA, Viollon-Abadie C, Coon DJ, Jolley S, and Richert L (2005) Isolation and culture of primary human hepatocytes. *Methods Mol Biol* **290**:207–229.
- Li H, Chen T, Cottrell J, and Wang H (2009a) Nuclear translocation of adenoviral-enhanced yellow fluorescent protein-tagged-human constitutive androstane receptor (hCAR): a novel tool for screening hCAR activators in human primary hepatocytes. *Drug Metab Dispos* **37**:1098–1106.
- Li L, Chen T, Stanton JD, Sueyoshi T, Negishi M, and Wang H (2008) The peripheral benzodiazepine receptor ligand 1-(2-chlorophenyl-methylpropyl)-3-isoquinoline-carboxamide is a novel antagonist of human constitutive androstane receptor. *Mol Pharmacol* **74**:443–453.
- Li L, Stanton JD, Tolson AH, Luo Y, and Wang H (2009b) Bioactive terpenoids and flavonoids from Ginkgo biloba extract induce the expression of hepatic drug-metabolizing enzymes through pregnane X receptor, constitutive androstane receptor, and aryl hydrocarbon receptor-mediated pathways. *Pharm Res* **26**:872–882.
- McCance-Katz EF, Moody DE, Morse GD, Friedland G, Pade P, Baker J, Alvanzo A, Smith P, Ogundele A, Jatlow P, et al. (2006a) Interactions between buprenorphine and antiretrovirals. I. The nonnucleoside reverse-transcriptase inhibitors efavirenz and delavirdine. *Clin Infect Dis* **43** (Suppl 4):S224–S234.
- McCance-Katz EF, Moody DE, Smith PF, Morse GD, Friedland G, Pade P, Baker J, Alvanzo A, Jatlow P, and Rainey PM (2006b) Interactions between buprenorphine and antiretrovirals. II. The protease inhibitors nelfinavir, lopinavir/ritonavir, and ritonavir. *Clin Infect Dis* **43** (Suppl 4):S235–S246.
- Milne M, Crouch BI, and Caravati EM (2009) Buprenorphine for opioid dependence. *J Pain Palliat Care Pharmacother* **23**:153–155.
- Mintzer MZ, Copersino ML, and Stitzer ML (2005) Opioid abuse and cognitive performance. *Drug Alcohol Depend* **78**:225–230.
- Orman JS and Keating GM (2009) Buprenorphine/naloxone: a review of its use in the treatment of opioid dependence. *Drugs* **69**:577–607.
- Pergolizzi J, Böger RH, Budd K, Dahan A, Erdine S, Hans G, Kress HG, Langford R, Likar R, Raffa RB, et al. (2008) Opioids and the management of chronic severe pain in the elderly: consensus statement of an International Expert Panel with focus on the six clinically most often used World Health Organization Step III opioids (buprenorphine, fentanyl, hydromorphone, methadone, morphine, oxycodone). *Pain Pract* **8**:287–313.
- Qatanani M and Moore DD (2005) CAR, the continuously advancing receptor, in drug metabolism and disease. *Curr Drug Metab* **6**:329–339.
- Raucy JL (2003) Regulation of CYP3A4 expression in human hepatocytes by pharmaceuticals and natural products. *Drug Metab Dispos* **31**:533–539.
- Sinz M, Kim S, Zhu Z, Chen T, Anthony M, Dickinson K, and Rodrigues AD (2006) Evaluation of 170 xenobiotics as transactivators of human pregnane X receptor (hPXR) and correlation to known CYP3A4 drug interactions. *Curr Drug Metab* **7**:375–388.
- Smith CM, Graham RA, Krol WL, Silver IS, Negishi M, Wang H, and Lecluyse EL (2005) Differential UGT1A1 induction by chrysin in primary human hepatocytes and HepG2 Cells. *J Pharmacol Exp Ther* **315**:1256–1264.
- Tolson AH, Li H, Eddington ND, and Wang H (2009) Methadone induces the expression of hepatic drug-metabolizing enzymes through the activation of pregnane X receptor and constitutive androstane receptor. *Drug Metab Dispos* **37**:1887–1894.
- Walle T, Otake Y, Galijatovic A, Ritter JK, and Walle UK (2000) Induction of UDP-glucuronosyltransferase UGT1A1 by the flavonoid chrysin in the human hepatoma cell line hep G2. *Drug Metab Dispos* **28**:1077–1082.
- Wang H, Faucette S, Moore R, Sueyoshi T, Negishi M, and LeCluyse E (2004) Human constitutive androstane receptor mediates induction of CYP2B6 gene expression by phenytoin. *J Biol Chem* **279**:29295–29301.
- Wang H, Faucette S, Sueyoshi T, Moore R, Ferguson S, Negishi M, and LeCluyse EL (2003) A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem* **278**:14146–14152.
- Wang H and LeCluyse EL (2003) Role of orphan nuclear receptors in the regulation of drug-metabolizing enzymes. *Clin Pharmacokinet* **42**:1331–1357.
- Willson TM and Klier SA (2002) PXR, CAR and drug metabolism. *Nat Rev Drug Discov* **1**:259–266.
- Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS, and Evans RM (2000) Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes Dev* **14**:3014–3023.
- Zhou C, Poulton EJ, Grün F, Bammler TK, Blumberg B, Thummel KE, and Eaton DL (2007) The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor. *Mol Pharmacol* **71**:220–229.

Address correspondence to: Dr. Hongbing Wang, Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20 Penn Street, Baltimore, MD 21201. E-mail: hwang@rx.umaryland.edu