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

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ABSTRACT

Buprenorphine is a partial \(\mu\)-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 revealed 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 lines 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 \(\mu\)-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, accompanying...
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 increased attention, partly because of the frequent comedication 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 ritasinavir, 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 toxicants, including endobiotics such as steroid hormones, fatty acids, and bile acids; and xenobiotics such as environmental chemicals, 6-desoxycodine, N-phenyl butyl normeperidine, 6-desoxymorphine, and normeperidine were kindly provided by Andrew Cooper (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 Kliewer (University of Texas Southwestern Medical Center, Dallas, TX). The pCR3-hCAR expression vector was generously provided by Masaaki Negishi (National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC). The CMV2-hCAR 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., 2005). The CYP3A4-PXR/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.

### 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 Mallinkrodt, Inc. (St. Louis, MO). Meperidine, 6-desoxycodine, N-phenyl butyl normeperidine, 6-desoxymorphine, and normeperidine were kindly provided by Andrew Cooper (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 or Sigma-Aldrich.

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#### 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 × 10⁶ 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 μg/ml streptomycin, 4 μg/ml insulin, and 1 μM dexamethasone. After 4 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 μM dexam...
methanone; 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.** Real-time PCR was 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, forward 5'-CCCATCACCATCTTCCAGGAG-3' (reverse) and 5'-GCCTCAGATTTCTCACCAACACA-3' (forward); and GAPDH, 213 to 462 bp, 5'-TGCGGGCTTTTATGATGGTCA-3' (forward) and 5'-GCTCTAGATTCTTCACACCAACA-3' (reverse); and GAPDH, 217 to 501 bp, 5'-CCCATACACATCCTCCAGGAG-3' (forward) and 5'-GGTCGATGATGATGCTTGGCA-3' (reverse). Induction values (fold over control) were calculated according to the 2^{-ΔΔCT} method, where ΔC is the differences in cycle threshold numbers between the target gene and GAPDH, and ΔΔC 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-desoxycodeine (10 μM), naloxone (10 μM), DIP (10 μM), N-phenyl butyl normeperidine (10 μM), 6-desoxyxymorphine (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 ± S.D. of three individual transfections.

To analyze XR- 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-diamidino-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 10 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 ≥ 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/plottter (Hewlett Packard, Avondale, PA), and 4) Waters Symmetry C18 (4.6 × 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 ± 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-desoxycodeine, 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 μM).
Buprenorphine (BUP) at a concentration of 100 μM was used to treat HepG2 cells over a broader range of concentrations (1–100 μM) and was compared to other opioids to determine their effects on PXR activation. The results showed that all the opioids, except for naloxone, diprenorphine, and norbinaltorphimine, induced hPXR activation in HepG2 cells. Naloxone at 10 μM was found to be a weak inhibitor of hPXR activation, while morphine, codeine, meperidine, BUP, 6-desoxycodone, naloxone, diprenorphine, and norbinaltorphimine exhibited moderate-to-strong activator effects. The effects of BUP were consistent with previous reports. MD was classified as a moderate activator of hPXR, whereas other opioids, such as RIF, PB, and BUP, were classified as strong activators, reaching approximately 80 and 70% of RIF response, respectively, as shown in Fig. 2A.

To correlate the BUP-mediated activation of PXR and CAR, we measured hPXR and hCAR activation by chemical-mediated activation of hCAR in immortalized cell lines. As shown in Fig. 2C, BUP significantly enhanced the hCAR1+mediated expression of CYP2B6 reporter gene, with the highest response at 50 μM (6.43-fold over vehicle control) challenging that of the positive control CITCO (6.92-fold over vehicle control).

Effects of BUP on the Activation of CAR in HepG2 Cells. BUP has been shown to activate CAR in HepG2 cells. Consistent with previous reports, cell-based reporter assays using the reference hCAR (hCAR1) demonstrated robust basal activation in HepG2 cells, with moderate response to chemical-mediated activation of hCAR in HepG2 cells (Fig. 2A). The results showed that BUP significantly enhanced the hCAR1-mediated expression of CYP2B6 reporter gene, with the highest response at 100 μM (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 was variable 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 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 effects 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 exhibited...
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 μM, 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 μM) and CAR (CITCO, 1 μM) markedly increased the reporter gene expression of CYP3A4 and CYP2B6, respectively. On the other hand, BUP at concentrations of 1, 10, and 50 μM 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
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 μM) 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 μM showed no effect on hCAR nuclear accumulation with a

![Fig. 4. Induction of CYP2B6 and CYP3A4 expression in human primary hepatocytes.](image)

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 μM), RIF (10 μM), PB (1 mM), or BUP (10 and 50 μM). 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 μg of each) were subjected to CYP2B6 and CYP3A4 immunoblotting analysis as described under Materials and Methods. RT-PCR data are expressed as mean ± S.D. (n = 3). *, P < 0.05; **, P < 0.01.

![Fig. 5. CYP2B6 and CYP3A4 reporter gene expression in human primary hepatocytes.](image)

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 μM), RIF (10 μM), PB (1 mM), or BUP (10 and 50 μM) 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 ± S.D. (n = 3). **, P < 0.01.
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,
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 (Kliwer 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 DMBS, 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. Chrysirin, 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 chrysirin 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.

When 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 construct 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.

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