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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.), and Life Technologies Corporation, 4301 Emperor Blvd, Durham, NC, USA 27703 (S. S. F.)
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Running title: Buprenorphine activates PXR in HepG2 not primary hepatocyte

Corresponding author: Hongbing Wang, Ph.D.

Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy,
20 Penn. Street, Baltimore MD 21201

Telephone: 410-706-1280; Fax 410-706-5017;

E-mail: hwang@rx.umaryland.edu

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Abbreviations: adenovirus expressing enhanced yellow florescence protein tagged human CAR (Ad/EYFP-hCAR), buprenorphine (BUP), constitutive androstane receptor (CAR), cytochrome P450 (CYP), 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl) oxime (CITCO), diprenorphine (DIP), drug-drug interaction (DDI), drug-
metabolizing enzyme (DME), methadone (MD), human primary hepatocytes (HPH), nuclear receptor (NR), 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinoline-carboxamide (PK11195), phenobarbital (PB), pregnane X receptor (PXR), phenobarbital- responsive enhancer module (PBREM), rifampicin (rifampin, RIF), sulforaphane (SFN), UDP-glucuronosyl transferase (UGT), xenobiotic-responsive enhancer module (XREM).
Abstract

Buprenorphine is a partial mu-opioid receptor agonist, used for the treatment of opioid dependence with 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); and 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 co-transfected with a chemical-responsive human CAR variant. Real-time RT-PCR analysis revealed that buprenorphine strongly induced CYP3A4 expression both in PXR and CAR transfected HepG2 cells. Unexpectedly, 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 hCAR to the nucleus nor activate CYP2B6/CYP3A4 reporter activities in transfected HPHs. Subsequent experiments to determine whether the differential response was due to its metabolic stability revealed a dramatically differential rate of elimination for buprenorphine between HPHs and HepG2 cells. Collectively, these studies indicate that metabolic stability of buprenorphine defines the differential induction of DMEs observed in HepG2 and HPHs, and 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 being currently addicted to opiates; and alarming increases have been 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 with several obvious advantages over MD (Milne et al., 2009; Orman and Keating, 2009). As such, the utilization 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 accompanied with medical complications such as cancer, HIV infection, and chronic pain (Kohler 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 mu-opioid receptor agonist and a kappa-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 cytochrome P450 (CYP) 3A4, and CYP2C8 to a lesser extent. CYP3A4-mediated N-dealkylation and hydroxylation account for 70-90% 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 ten years or so, metabolism- and transporter-related DDIs have received increasing attention partly due to the frequent co-medications 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 nonnucleoside reverse-transcriptase inhibitor and CYP3A4 inducer, significantly decreased the BUP area under the concentration-time curve (AUC), while ritonavir, a protease inhibitor and potent inhibitor of CYP3A4, increased the AUC of BUP in HIV patients (McCance-Katz et al., 2006a; McCance-Katz et al., 2006b). Additionally, numerous other drugs are also known to enhance the toxicity of opioids, which involve 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 co-administered 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 environment chemicals, and drugs (Kliewer 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 over 64% pharmaceutical companies in the US have adopted cell-based PXR reporter assays routinely to assess the potential for DDIs due to 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 to 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 HPHs. To our surprise, although BUP significantly activated PXR and CAR, as well as induced CYP3A4 mRNA expression in HepG2 cells, there was no induction of CYP3A4 or CYP2B6 in human primary hepatocytes (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 BIOMOL 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-desoxymorphone and normeperidine were kindly provided by Dr. 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 from Millipore Co. (Billerica, MA). β-Actin antibody were from Sigma-Aldrich. Matrigel, insulin, and insulin/transferrin/selenium were obtained from BD Biosciences (Bedford, MA). Other cell culture reagents were purchased from Invitrogen (Carlsbad, CA) or Sigma-Aldrich. HPLC grade acetonitrile was purchased from Fisher Scientific Co. (Pittsburgh, PA). Other cell culture reagents were purchased from Invitrogen or Sigma-Aldrich.

Plasmids Constructions. The pSG5-hPXR expression vector was obtained from Dr. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX). The pCR3-hCAR expression vector was generously provided by Dr. Masahiko Negishi (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC). The CMV2-hCAR3 vector was from Dr. Curtis Omiecinski (Pennsylvania State University, University Park, Pennsylvania). The pCR3-hCAR1+A expression vector and the CYP2B6 reporter construct, containing both PBREM and the distal XREM (CYP2B6-2.2 kb) 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 luciferase plasmid used to normalize firefly luciferase activities was 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/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 to 6 hr 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 dexamethasone, 100 U/ml penicillin, and 100 µg/ml streptomycin. The hepatocytes were maintained for 36 hr before the 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 hr for detection of mRNA and protein expression, respectively.

**Real-Time PCR Analysis.** Total RNA was isolated from treated hepatocytes using RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) following the manufacturers’ instruction. 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’-AGACGCTTCAATCCTGACC-3’ (forward) and 5’-CCTTCACCAAGACAAATCCGC-3’ (reverse); CYP3A4 213 to 462 bp, 5’-GTGGGGCTTTTATGATGGTCA-3’ (forward) and 5’-GCCTCAGATTTCACCAACACA-3’.
(reverse); and GAPDH 217 to 501 bp, 5'-CCCATCACCATCTTCCAGGAG-3' (forward) and 5'-GTTGTCATGGATGACCTTGGC-3' (reverse). Induction values (fold over control) were calculated according to the equation $2^{\Delta \Delta Ct}$, where $\Delta Ct$ represents the differences in cycle threshold numbers between the target gene and GAPDH and $\Delta \Delta Ct$ represents the relative change in these differences between 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 CYP2B6-2.2 kb reporter construct in the presence of hPXR, hCAR, hCAR3 or hCAR1+A expression vector using Fugene 6 Transfection Kit following the manufacturer’s instruction. Twenty four hours after transfection, cells were treated for another 24 hr with solvent (0.1% DMSO) or test compounds at the concentration of 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-desoxycodine (10 µM), naloxone (10 µM), DIP (10 µM), N-phenyl butyl normeperidine (10 µM), 6-desoxymorphine (10 µM), or normeperidine (10 µM). Subsequently, cell lysates were assayed for firefly activities normalized against the activities of cotransfected renilla luciferase using Dual-Luciferase Kit (Promega). Data were represented as mean ± SD 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 hCAR or hPXR expression vector, 24 hr after transfection cells were treated for another 24 hr with RIF (10 µM), CITCO (1 µM), PK11195 (10 µM), SFN (10 µM), BUP (10 and 50 µM), or cotreated for 24 hr 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 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 at 10 and 50 µM, or DIP (10 µM) for 24 hr, respectively. Cell lysates were subjected to Dual-luciferase analysis as described above.

**Translocation of Ad/EYFP-hCAR in Human Primary Hepatocytes.** The adenovirus expressing enhanced yellow fluorescent protein tagged hCAR (Ad/EYFP-hCAR), which infect 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 hr before treatment with the vehicle control (0.1% DMSO), PB (1mM), BUP (10 and 50 µM), or DIP (10 µM). After 24 hr 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 (DAPI) for 30 min. Confocal microscopy analysis was performed with a Nikon C1-LU3 instrument based on an inverted Nikon Eclipse TE2000 microscope. The subcellular localization of hCAR was visualized and quantitatively characterized as nuclear, cytosolic, and 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. Subsequently, membranes were incubated with specific antibodies against CYP2B6 or CYP3A4 (Millipore Co. Billerica, MA) 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, and developed using 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 × 10⁶ viable cells/incubation were diluted in serum-free DMEM supplemented with ITS⁺, 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 following 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 210 nm, emission 352 nm). 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 min 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) Waters 1525 Binary HPLC Pump (Waters-Millipore, Milford, MA, USA), (2) 717 Waters autosampler, (3) 3390A Hewlett Packard Integrator Plotter (Hewlett Packard, Avondale, PA), and (4) Waters Symmetry C-18 (4.6 × 250 mm) (Waters-Millipore, Milford, MA, USA).

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 ± SD. Statistical comparisons were made using One-way ANOVA followed by post hoc Dunnett’s test and Students’ t-test where appropriate. Statistical significance was set at *: $p < 0.05$ and **: $p < 0.01$. 


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 hPXR expression vector in the presence of the CYP3A4-PXRE/XREM or CYP2B6-2.2kb luciferase reporter construct. The test opioids include morphine, MD, oxycodone, codeine, meperidine, BUP, 6-desoxycodine, naloxone, DIP, N-phenyl butyl normeperidine, 6-desoxymorphine, and normeperidine. All opioids were tested at 10 µM concentration; and RIF (10 µM) and DMSO (0.1% v/v) were the positive and vehicle controls, respectively. Figure 1 demonstrates opioid-mediated influence on CYP3A4 (Figure 1A) and CYP2B6 (Figure 1B) reporter activities in comparison with vehicle control as a percentage of the increase achieved by the hPXR positive control RIF (10 µM). The criteria adopted from previous publications (Sinz et al., 2006; Faucette et al., 2007; Chu et al., 2009) defined that compounds achieved greater than 40% of RIF-normalized hPXR activation in reporter assays were classified as strong activators of hPXR, whereas drugs exhibiting between 15 and 40% of RIF response were moderate activators, and those exhibiting less than 15% of RIF response were recognized as nonactivators. Consistent with our previous report, MD was classified as a moderate-to-strong activator (Tolson et al., 2009). Notably, BUP and DIP, with high structural similarity (Figure 7A), displayed strong activation of hPXR, reaching approximately 80% and 70% of RIF response, respectively, while all the other opioids exhibited negligible PXR activation.

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

**Effects of BUP on the activation of CAR in HepG2 cells.** Since PXR and CAR share a number of overlapping target genes and many xenobiotics as co-activators, we further investigated whether BUP could activate human CAR in HepG2 cells. Consistent with previous reports, cell-based reporter assays utilizing 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 (Figure 2A). Alternatively, utilizing a hCAR ligand-responsive variant (hCAR3), BUP caused a marginal but concentration-dependent enhancement of CYP2B6 reporter activity with the highest activation at 50 µM (1.8-fold over vehicle control), while the positive control CITCO resulted in 4.34-fold increase (Figure 2B). Moreover, 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 Figure 2C, BUP significantly enhanced the hCAR1+A-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).

**BUP increases hPXR- and hCAR-mediated Induction of endogenous CYP3A4 Expression in HepG2 Cells.** In order to correlate the BUP-mediated activation of PXR and CAR with the actual induction of CYP3A4 gene in HepG2 cells, real-time PCR analysis was carried out in HepG2 cells transfected with hPXR or hCAR expression vector as outlined in Material and Methods. Without transfection, BUP induced CYP3A4 mRNA expression moderately but statistically significantly (Figure 3A) due mainly to 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 both in hPXR- and hCAR-transfected HepG2 cells and, moreover, this induction was significantly repressed by the co-treatment of
sulforaphane (SFN) as a selective hPXR deactivator (Zhou et al., 2007) and PK11195 as a selective hCAR deactivator (Li et al., 2008), respectively (Figure 3B and 3C). 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 in **materials and methods.** As expected, all prototypical PXR/CAR activators exhibited potent induction of CYP2B6 and/or CYP3A4 at mRNA and protein levels (Figure 4). Nevertheless, the expression of CYP2B6 and CYP3A4 was literally not increased after the treatment of BUP at 10 and 50 µM concentrations which significantly activated PXR and CAR and induced CYP3A4 mRNA in HepG2 cells (Figure 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). Consequently, the ability of BUP to activate CYP2B6/CYP3A4 reporter gene expression was evaluated in HPHs from two donors. As demonstrated in Figure 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 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 adenovirus construct expressing enhanced yellow fluorescence protein-tagged hCAR (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). Since 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 at 10 and 50 µM as outlined in Materials and Methods. Confocal microscopic analysis revealed that without activation, Ad-EYFP-hCAR was primarily localized in the cytoplasm (90%), and the prototypical CAR activator PB efficiently translocated hCAR to the nucleus (92%), while BUP at 10 and 50 µM showed no effect on hCAR nuclear accumulation with the cytoplasmic localization of 87% and 88%, respectively (Figure 6A and 6B). 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 in Materials and Methods. Since DIP also demonstrated potent activation of PXR in HepG2 cells (Figure 1A and 1B), and has high structural similarity with BUP (Figure 7A), the metabolic stability of DIP was also investigated. As demonstrated in Figure 7B, BUP was quickly metabolized in HPHs with a half-life ($t_{1/2}$) around 2 hr, while its concentration remains virtually unchanged up to the maximal incubation time (3 hr) in HepG2 cells. Notably, DIP also displayed quick metabolism in HPHs vs. HepG2 cells, where the estimated $t_{1/2}$ was approximately 50 min and only less than 10% DIP was detected in HPHs at the end of incubation (3 hr) (Figure 7C). Subsequent experiments in HPHs demonstrated that DIP similar to BUP also failed to either activate PXR and CAR or induce the expression of CYP2B6 and CYP3A4 (Figure 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 opioids continue to be the mainstay for the treatment of chronic and severe pain, and 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 (PK) profiles of opioids. In contrast, the potential for opioids to influence the similar fates of co-administered 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 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 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 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 (Figures 4-6) despite its demonstrated potent activation of PXR and induction of CYP3A4 in HepG2 cells (Figures 1-3). These findings trigger the speculation that the obvious lack of metabolic capability in an immortalized cell line in comparison to HPHs 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 represent the most reliable in vitro model reflecting metabolic characteristics of 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 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 due to the rapid metabolism, indicating that the metabolic instability would most likely limit its UGT1A1 induction in vivo (Smith et al., 2005). On the other hand, Cerbal-Chaloin et al., reported that omeprazole-sulphide, a pure antagonist of aryl hydrocarbon receptor (AhR), was efficiently converted to omeprazole as an agonist of AhR only in HPHs (Gerbal-Chaloin et al., 2006). It is well established that BUP was predominantly metabolized in the liver by CYP3A4, CYP2C8, and
UGTs before eliminating from 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 (Figure 7), which strongly support the speculation that rapid clearance of BUP in HPHs contributes significantly to the non-induction 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 either. In separate observations, the metabolic stability of DIP, an opioid antagonist structurally parallel to BUP, was also dramatically decreased in HPHs vs. 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 (Figure 8), suggesting that this phenomenon represents a shared class effect rather than a unique compound-specific role of BUP.

In comparison to PXR, in vitro evaluation of CAR activation was more challenging due to 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). Recently, our laboratory has established several novel strategies to efficiently identify hCAR activators in vitro. A chimeric construct by inserting alanine to the 270 amino acid position of hCAR (hCAR1+A) converts the constitutively activated hCAR to a xenobiotic-sensitive receptor; and 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 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 demonstrated that although BUP functions as a potent activator of PXR and CAR as well as inducer of CYP3A4 in the immortalized cell line, such effects were totally lost in a more physiologically relevant cell system, the HPHs. Further evidence revealed that loss of inductive effects for BUP in HPHs is majorly due to the rapid metabolism and clearance. Additionally, DIP displayed similar responses between HepG2 and HPHs, indicating that a class of structurally similar opioids and their derivative may behave alike in this regard. Overall, these results raise sincere concerns in our utilization and interpretation of data obtained from cell-based nuclear receptor assays, in particular, the widely used PXR-reporter assays.
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References


Hassan HE, Myers AL, Coop A and Eddington ND (2009) Differential involvement of P-glycoprotein (ABCB1) in permeability, tissue distribution, and antinociceptive activity of


Footnotes

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Address correspondence to: Dr. Hongbing Wang, Department of Pharmaceutical Sciences, School of Pharmacy, the University of Maryland at Baltimore, 20 Penn Street, Baltimore, MD 21201, USA, Telephone: 410-706-1280, Fax: 410-706-5017, email: hwang@rx.umd.edu
Figure Legends

Figure 1. Activation of hPXR by Different Opioids in Cell-based Reporter Assays. HepG2 cells were transfected with hPXR expression vector in the presence of CYP3A4-PXRE/XREM (A) or CYP2B6-2.2kb (B) reporter construct as described in Material and Methods. Transfected cells were then treated with different opioids (morphine, MD, oxycodone, codeine, meperidine, BUP, 6-desoxycodine, naloxone, DIP, N-phenyl butyl normeperidine, 6-desoxymorphine, or normeperidine) at 10 µM; or with BUP at different concentrations (C) for 24 hr. RIF (10 µM) was used as positive control for hPXR activation. Luciferase activities were determined and expressed as percentage of RIF activation or relative to vehicle control (0.1% DMSO). All data are expressed as mean ± SD (n=3) (*, P<0.05 and **, P<0.01).

Figure 2. Effects of BUP on the Activation of hCAR. HepG2 cells were transfected with hCAR1 (A), hCAR3 (B), or hCAR1+A (C) expression vector in the presence of CYP2B6-2.2kb reporter construct as described in Materials and Methods. Subsequently, transfected cells were treated with BUP at the concentrations (1 to 50 µM) for 24 hr. CITCO (1 µM) was used as a positive control for hCAR activation. Luciferase activities were determined and expressed as fold activation relative to vehicle control (0.1% DMSO). All data are expressed as mean ± SD (n=3) (*, P<0.05 and **, P<0.01).

Figure 3. PXR- and CAR-dependent Induction of CYP3A4 by BUP in transfected HepG2 cells. HepG2 cells were transfected with hPXR (B) or hCAR (C) expression vector as described in Materials and Methods. Twenty four hours after transfection, cells were treated with RIF (10 µM), CITCO (1 µM), PK11195 (10 µM), SFN (10 µM), BUP (10 and 50 µM), or cotreatment with SFN and PK as selective inhibitor of PXR and CAR, respectively. Treatments in non-transfected HepG2 cells (A) reflect the endogenous PXR and CAR responses. All cells were treated for 24
hr before harvesting total RNA. Real-time RT-PCR was employed to detect the mRNA expression of CYP3A4. Data are expressed as mean ± SD (n=3). (*, P<0.05 and **, P<0.01).

Figure 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 hr with CITCO (1 µM), RIF (10 µM), PB (1mM), or BUP (10 and 50 µM). 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 in Materials and Methods. In separate experiments, HPHs from HL-019 (C), HL-020 (D), and HL-023 (E) were treated with the same batch of chemicals as indicated in A and B for 72 hr. After harvesting, whole-cell homogenates (20 µg/each) were subjected to CYP2B6 and CYP3A4 immunoblotting analysis as described in Material and Methods. RT-PCR data are expressed as mean ± SD (n=3). (*, P<0.05 and **, P<0.01).

Figure 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, then treated with CITCO (1 µM), RIF (10 µM), PB (1mM), or BUP at 10 and 50 µM for 24 hr as described in Materials and Methods. Luciferase activities were determined and expressed as fold activation relative to control (0.1% DMSO). All data are expressed as mean ± SD (n=3) (*, P<0.05 and **, P<0.01).

Figure 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 hr then treated with vehicle control (0.1% DMSO), PB (1mM), and BUP (10
and 50 μM) for 24 hr. Subsequently, cells were fixed for 30 min in 4% buffered paraformaldehyde and stained with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) 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. A hundred Ad/EYFP-hCAR expressing HPHs were counted from each treatment group.

Figure 7. Metabolic stability of BUP and DIP in HepG2 cells and Human Primary Hepatocytes. The chemical structures of BUP and DIP are depicted in panel (A). The metabolic stability of BUP and DIP were determined in suspensions of HepG2 cells and cryopreserved hepatocytes using HPLC analysis as described in Materials and Methods. The remaining BUP (B) and DIP (C) over time in HepG2 and HPHs were demonstrated. All data points represent the average of three measurements ± SD.

Figure 8. DIP fails to activate PXR and CAR or to induce CYP2B6 and CYP3A4 expression in Human primary hepatocytes. Human hepatocytes from HL-023 (A) and HL-024 (B) were treated for 24 hr with CITCO (1 μM), RIF (10 μM), PB (1mM), or DIP (10 μM). Real-time RT-PCR analysis of CYP2B6 and CYP3A4 expression was carried out as outlined in Materials and Methods; (C) illustrates 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 μM). HPHs (HL-025) were transfected with CYP2B6-2.2kb (D) or CYP3A4-PXRE/XREM (E) reporter construct, then treated with CITCO (1 μM), RIF (10 μM), or DIP (1 and 10 μM) for 24 hr. Luciferase activities were determined and expressed as fold activation relative to control (0.1% DMSO). All data are expressed as mean ± SD (n=3) (*) P<0.05 and **, P<0.01.
Figure 2
Figure 3

(A) No Transfection

(B) hPXR Transfection

(C) hCAR Transfection

Legend:
- DMSO
- Rif
- CITCO
- PK11195
- SFN
- BUP
- 10 μM
- 25 μM
- 50 μM

Fold Induction for CYP3A4

** Indicates statistical significance.
Figure 4

A and B: Graphs showing the CYP3A4 and CYP2B6 fold induction in HL-017, HL-018, and HL-023 cell lines with various treatments including DMSO, CITCO, RIF, PB, BUP 10μM, and BUP 50μM.

C: Western blot analysis of CYP3A4, CYP2B6, and Actin in HL-019 cell line with treatment levels of 10 and 50 μM BUP.

D: Western blot analysis of CYP3A4, CYP2B6, and Actin in HL-021 cell line with treatment levels of 10 and 50 μM BUP.

E: Western blot analysis of CYP3A4, CYP2B6, and Actin in HL-023 cell line with treatment levels of 10 and 50 μM BUP.
Figure 5

A) HL 023 CYP2B6 (2.2k) Relative luc activity

B) HL 023 CYP3A4 (PXRE/XREM) Relative luc activity

C) HL 025 CYP2B6 (2.2k) Relative luc activity

D) HL 025 CYP3A4 (PXRE/XREM) Relative luc activity

** denotes statistical significance.
Figure 8