Differential Effect of Meclizine on the Activity of Human Pregnan X Receptor and
Constitutive Androstane Receptor

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ABBREVIATIONS: CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; DMSO, dimethyl sulfoxide; E_max, maximal effect; HBSS, Hanks’ Balanced Salt Solution; hCAR, human constitutive androstane receptor; HPRT, human hypoxanthine phosphoribosyltransferase 1; hPXR, human pregnane X receptor; PCN, pregnenolone 16α-carbonitrile; PCR, polymerase chain reaction; PK11195, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isouinoline-carboxamide; PXR, pregnane X receptor; rPXR, rat pregnane X receptor; SR12813, tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate; SRC-1, steroid receptor coactivator-1; TR-FRET, time-resolved fluorescence resonance energy transfer.

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ABSTRACT

Conflicting data exist as to whether meclizine is an activator of human pregnane X receptor (hPXR). Therefore, we conducted a detailed, systematic investigation to determine whether meclizine affects hPXR activity by performing a cell-based reporter gene assay, a time-resolved fluorescence resonance energy transfer competitive ligand-binding assay, a mammalian two-hybrid assay to assess coactivator recruitment, and a hPXR target gene expression assay. In PXR-transfected HepG2 cells, meclizine activated hPXR to a greater extent than rat PXR. It bound to hPXR ligand-binding domain and recruited steroid receptor coactivator-1 to the receptor. Consistent with its hPXR agonism, meclizine increased hPXR target gene expression (CYP3A4) in human hepatocytes. However, it did not increase but decreased testosterone 6β-hydroxylation, suggesting inhibition of CYP3A catalytic activity. Meclizine has also been reported to be an inverse agonist and antagonist of human constitutive androstane receptor (hCAR). Therefore, given that certain tissues (e.g. liver) express both hPXR and hCAR and that various genes are cross-regulated by them, we quantified the expression of a hCAR- and hPXR-regulated gene (CYP2B6) in cultured human hepatocytes treated with meclizine. This drug did not decrease constitutive CYP2B6 mRNA expression or attenuate hCAR agonist-mediated increase in CYP2B6 mRNA and CYP2B6-catalyzed bupropion hydroxylation levels. These observations reflect hPXR agonism and the lack of hCAR inverse agonism and antagonism by meclizine, which were assessed by hCAR reporter gene assay and mammalian two-hybrid assay. In conclusion, meclizine is a hPXR agonist and it does not act as a hCAR inverse agonist or antagonist in cultured human hepatocytes.
Introduction

Pregnane X receptor (PXR; NR1I2) (Lehmann et al., 1998) and constitutive androstane receptor (CAR; NR1I3) (Baes et al., 1994) are closely-related nuclear receptors expressed predominantly in human liver. These nuclear receptors regulate the transcription of a broad array of genes involved in bioactivation, detoxification, and transport of a structurally diverse group of endogenous and exogenous chemicals, and they are often implicated in drug-induced toxicity and drug-drug interaction (Kakizaki et al., 2008). There is also increasing evidence showing that these two receptors play a role in various physiological functions and pathophysiological processes, including glucose and lipid metabolism, thyroid hormone homeostasis, bile acid homeostasis, and inflammation (Kakizaki et al., 2008). Despite the structural differences in PXR and CAR, they coordinately regulate a distinct but overlapping set of target genes and biological functions (Maglich et al., 2002) and they share a set of ligands (Moore et al., 2000).

Meclizine, which is a piperazine-derived histamine H₁ antagonist (Fig. 1), is commonly used for the prevention and treatment of nausea, vomiting, vertigo, and motion sickness. Previously, it was reported to be an agonist of mouse CAR, but an inverse agonist of human CAR (hCAR) (Huang et al., 2004). In that study, meclizine decreased hCAR-mediated luciferase activity in a cell-based reporter gene assay, and attenuated coactivator recruitment in a mammalian two-hybrid assay. In addition to its apparent effect on hCAR function, this drug also appears to affect the action of human PXR (hPXR), although the published data are conflicting. Meclizine was reported to have no effect on hPXR reporter activity in one study (Huang et al., 2004), whereas it was classified as a weak activator in two other studies using high-throughput
cell-based screening assays (Sinz et al., 2006; Kim et al., 2010). Therefore, the effects of meclizine on hPXR function remain to be clarified.

In the present study, we conducted a detailed, systematic investigation to determine whether meclizine affects hPXR activity by performing: 1) a cell-based reporter gene assay, 2) a time-resolved fluorescence resonance energy transfer (TR-FRET) competitive ligand-binding assay, 3) a mammalian two-hybrid assay to assess coactivator recruitment, and 4) a hPXR target gene expression assay. Certain tissues (e.g. liver) may express both hPXR (Lehmann et al., 1998) and hCAR (Baes et al., 1994) and various target genes are cross-regulated by these receptors (Faucette et al., 2006). Therefore, activation of hPXR by meclizine may attenuate its apparent hCAR inverse agonistic effects, as reported previously (Huang et al., 2004). To test this hypothesis, we investigated whether meclizine would still be capable of suppressing hCAR target gene expression in primary cultures of human hepatocytes, which are known to express hCAR and hPXR (Martin et al., 2008). Given the pronounced species differences in the activation of PXR (Stanley et al., 2006), we also determined whether meclizine differentially affects rat PXR (rPXR) and hPXR function. Overall, our novel findings indicate species differences in hPXR and rPXR activation by meclizine and its mechanism of hPXR activation involves receptor agonism. Furthermore, this drug is not an effective inverse agonist or antagonist of hCAR in human hepatocytes.
Materials and Methods

Chemicals and Reagents. Meclizine dihydrochloride monohydrate (CAS number 31884-77-2) and hydroxybupropion were obtained from Toronto Research Chemicals, Inc. (North York, ON, Canada). Rifampicin, pregnenolone 16α-carbonitrile (PCN), phenobarbital, testosterone, bupropion hydrochloride, triprolidine, and 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline-carboxamide (PK11195) were purchased from Sigma-Aldrich (St Louis, MO). Tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate (SR12813) and 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) were obtained from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). 6β-Hydroxytestosterone was obtained from Steraloids, Inc. (Newport, RI). PureLink RNA Mini Kit, PicoGreen Double-Stranded DNA Quantitation Kit, and Lanthascreen TR-FRET PXR Competitive Binding Assay were purchased from Invitrogen (Carlsbad, CA). Charcoal-stripped, heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) was bought from Thermo Fisher Scientific, Inc. (Nepean, ON, Canada), and all other cell culture reagents were obtained from Invitrogen. FuGENE 6 transfection reagent was obtained from Roche Diagnostics (Laval, QC, Canada) and Dual-Luciferase Reporter Assay System was from Promega Corporation (Madison, WI). The suppliers of reagents for human hepatocytes isolation and culture (LeCluyse et al., 2005), and those for reverse transcription and real-time PCR analyses (Chang et al., 2006), were described previously.

Plasmids. pCMV6-XL4-hPXR, pCMV6-AC-rPXR, pCMV6-XL4-hCAR, pCMV6-XL4, pCMV6-AC, and pCMV6-XL4-hRXRα were purchased from OriGene Technologies, Inc. (Rockville, MD). Renilla luciferase pGL4.74[hRluc/TK] plasmid was obtained from Promega Corporation. A pGL3-basic-CYP3A4-XREM-luc reporter, originally named as p3A4-
362 (7836/7208ins) (Goodwin et al., 1999), and a pGL3-basic-CYP2B6-PBREM/XREM-luc reporter (Wang et al., 2003) were constructed as described previously.

The pVP16 and pM vectors were provided in the Matchmaker Mammalian Two-Hybrid Assay Kit (Clontech Laboratories, Inc., Mountain View, CA). PathDetect pFR-luc trans-reporter plasmid was purchased from Stratagene (La Jolla, CA). To construct the pVP16-hPXR-LBD plasmid, the ligand-binding domain (Met-107 to Ser-434) (Takeshita et al., 2002) of hPXR was amplified from pCMV6-XL4-hPXR and inserted into the pVP16 vector. The primers used to amplify the ligand-binding domain of hPXR were: 5′-GGA-GGA-ATT-CAT-GAA-GAA-GGA-GAT-GAT-CAT-GT-3′ (forward) and 5′-GGG-AGG-ATC-CTC-AGC-TAC-CTG-TGA-TGC-CG-3′ (reverse). To construct the pVP16-hCAR-LBD plasmid, the ligand-binding domain (Gln-105 to Ser-348) (Burk et al., 2005) of hCAR was amplified from pCMV6-XL4-hCAR and inserted into the pVP16 vector. The primers used to amplify hCAR-LBD were: 5′-GGA-GGA-ATT-CCA-ACT-GAG-TAA-GGA-GCA-AGA-A-3′ (forward) and 5′-GGG-AGG-ATC-CTC-AGT-TTG-GAG-TTG-ATC-TTA-AAT-3′ (reverse). To construct the pM-hSRC1-RID plasmid, the receptor-interacting domain of human SRC-1 (Asp-621 to Asn-765) (Chang et al., 1999) was amplified from pCMV6-XL4-NCOA1 (Origene Technologies, Inc.) and cloned into the pM vector. The primers used to amplify the nuclear receptor-interacting domain of hSRC-1 were: 5′-GGA-GGA-ATT-CGA-TGG-AGA-CAG-TAA-ATA-CTC-TC-3′ (forward) and 5′-GGG-AGG-ATC-CTC-AGT-TTG-GAG-TTG-ATC-TTA-AAT-3′ (reverse). All constructs were sequenced by the Nucleic Acid Protein Service Unit at the University of British Columbia (Vancouver, BC, Canada), and the identity of plasmids was confirmed by comparing their sequence with published sequence.
**HepG2 Cell Culture.** HepG2 human hepatocellular carcinoma cells were purchased from American Type Culture Collection (Manassas, VA), and grown in minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 10% v/v heat-inactivated fetal bovine serum. Cells were cultured in T-75 culture flasks and maintained at 37°C in a humidified incubator with 95% air and 5% CO₂.

**Transient Transfection and Reporter Gene Assays.** PXR-dependent reporter gene assay was conducted on HepG2 cells cultured in a supplemented culture medium containing 10% v/v heat-inactivated fetal bovine serum (Yeung et al., 2008). At 24 h after plating, HepG2 cells (100,000 cells/well) were transfected for 24 h with a master mix containing FuGENE 6 transfection reagent (0.6 µl/well), serum-free Opti-MEM (20 µl/well), pGL4.74[hRluc/TK] internal control plasmid (5 ng/well), pGL3-basic-CYP3A4-XREM-luc reporter (50 ng/well), and with pCMV6-XL4-hPXR (50 ng/well), pCMV6-AC-rPXR (50 ng/well), pCMV6-XL4 (50 ng/well; empty vector), or pCMV6-AC (50 ng/well; empty vector). Transfected HepG2 cells were treated for 24 h with 0.5 ml of fresh supplemented culture medium containing meclizine, rifampicin (positive control for hPXR assay) (Jones et al., 2000), PCN (positive control for rPXR assay) (Jones et al., 2000), or DMSO (0.1% v/v; vehicle), as detailed in each figure legend. Cells were lysed and the firefly luciferase and Renilla luciferase activities were determined using a Dual-Luciferase Reporter Assay System. Luciferase activity was expressed as a normalized ratio of firefly luciferase to Renilla luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with pCMV6-XL4 (empty vector) or pCMV6-AC (empty vector). Net luciferase activity was determined by subtracting the background value from the normalized luciferase activity. Fold increase was calculated by dividing the net luciferase activity of the treatment group by that of the vehicle-treated control group.
hCAR-dependent reporter gene activity was determined in a similar manner as that for PXR. HepG2 cells were suspended in a supplemented culture medium containing 10% v/v charcoal-stripped, heat-inactivated fetal bovine serum and seeded onto 24-well microplates at a density of 100,000 cells/well. At 24 h after plating, cells were transfected for 24 h with a master mix containing FuGENE 6 transfection reagent (0.6 μl/well), serum-free Opti-MEM (20 μl/well), pGL4.74[hRluc/TK] internal control plasmid (5 ng/well), pGL3-basic-CYP2B6-PBREM/XREM-luc reporter (50 ng/well), and with pCMV6-XL4-hCAR expression plasmid (50 ng/well) or pCMV6-XL4 empty vector (50 ng/well). Transfected HepG2 cells were treated for 24 h with 0.5 ml of fresh supplemented culture medium containing meclizine, PK11195 (hCAR inverse agonist/antagonist) (Li et al., 2008), CITCO (hCAR agonist) (Maglich et al., 2003), a combination of CITCO and meclizine, a combination of CITCO and PK11195, or DMSO (0.1% v/v; vehicle), as detailed in each figure legend. Luciferase activity was measured and normalized as described under PXR-dependent reporter gene assay. Data are expressed as a percentage of the normalized luciferase activity in the vehicle-treated control group or the CITCO-treated group, as indicated in each figure legend.

**Competitive Ligand-Binding Assay.** A LanthaScreen time-resolved fluorescence resonance energy transfer (TR-FRET) PXR Competitive Binding Assay was conducted according to the manufacturer’s protocol. First, 10 μl of a test chemical (diluted in TR-FRET PXR Assay Buffer; Invitrogen) was dispensed into triplicate wells of a white, non-treated 384-well assay plate (Corning Life Sciences, Lowell, MA). Second, 5 μl of Fluormone PXR Green (a fluorescein-labeled hPXR ligand; final concentration of 40 nM in assay buffer) was added into each well. Finally, 5 μl of a master mix containing hPXR ligand-binding domain (final concentration of 10 nM), terbium-labeled anti-glutathione-S-transferase (final concentration of
10 nM), and dithiothreitol (final concentration of 0.05 mM) was added into each well. The content was mixed briefly (10 s) and the plate was incubated in the dark at room temperature (22-24°C) for 1 h. TR-FRET was measured using a Synergy 4 Hybrid Multi-mode microplate reader in the filter mode (BioTek Instruments, Inc., Winooski, VT) and with the following settings: excitation wavelength of 340 nm (30 nm bandwidth), emission wavelengths of 520 nm (25 nm bandwidth; fluorescein emission) and 495 nm (10 nm bandwidth; terbium emission), delay time of 100 μs, and integration time of 200 μs. TR-FRET ratio was calculated by dividing the emission signal at 520 nm by the emission signal at 495 nm. Background TR-FRET ratio was determined from wells containing the same reagents as the vehicle-treated control group, but in the absence of hPXR ligand-binding domain. Net TR-FRET ratio was calculated by subtracting the background value from the TR-FRET ratio. Data are expressed as a percentage of the net TR-FRET ratio in the vehicle-treated control group.

**Mammalian Two-Hybrid Assay.** HepG2 cells were suspended in a supplemented culture medium containing 10% v/v charcoal-stripped, heat-inactivated fetal bovine serum and seeded onto 24-well microplates at a density of 100,000 cells/well. At 5 h after plating, PXR-dependent mammalian two-hybrid assay was conducted by transfecting cells with pM-hSRC1-RID plasmid (10 ng/well), pGL4.74[hRluc/TK] internal control plasmid (10 ng/well), pFR-luc reporter plasmid (100 ng/well), and pVP16-hPXR-LBD expression plasmid (40 ng/well) or pVP16 empty vector (40 ng/well), using FuGENE 6 transfection reagent (0.48 μl/well; diluted in 20 μl of serum-free Opti-MEM). At 24 h after transfection, cells were treated for 24 h with 0.5 ml of fresh supplemented culture medium containing meclizine, rifampicin (positive control) (Jones et al., 2000), or DMSO (0.1% v/v; vehicle), as detailed in the figure legend. Luciferase activity was measured and normalized as described under *Transient Transfection and Reporter
Gene Assays. Data are expressed as a percentage of the normalized luciferase activity in the rifampicin-treated group.

hCAR-dependent mammalian two-hybrid assay was modified from a previous method (DeKeyser et al., 2009). At 5 h after plating, HepG2 cells (cultured in a supplemented culture medium containing 10% v/v charcoal-stripped, heat-inactivated fetal bovine serum) were transfected with pM-hSRC1-RID plasmid (10 ng/well), pCMV6-XL4-hRXRα (10 ng/well) (DeKeyser et al., 2009), pGL4.74[hRluc/TK] internal control plasmid (10 ng/well), pFR-luc reporter plasmid (100 ng/well), and pVP16-hCAR-LBD expression plasmid (40 ng/well) or pVP16 empty vector (40 ng/well), using FuGENE 6 transfection reagent (0.51 µl/well; diluted in 20 µl of serum-free Opti-MEM). At 24 h after transfection, cells were treated for 24 h with 0.5 ml of fresh supplemented culture medium containing meclizine, PK11195 (hCAR inverse agonist/antagonist) (Li et al., 2008), CITCO (hCAR agonist) (Maglich et al., 2003), a combination of CITCO and meclizine, a combination of CITCO and PK11195, or DMSO (0.1% v/v; vehicle), as detailed in each figure legend. Luciferase activity was measured and normalized as described under Reporter Gene Assay. Data are expressed as a percentage of the normalized luciferase activity in the vehicle-treated control group or the CITCO-treated group, as indicated in each figure legend.

Isolation, Culture, and Treatment of Human Hepatocytes. Primary cultures of human hepatocytes were freshly isolated from resected hepatic tissue samples (LeCluyse et al., 2005). The demographics of the donors were described previously (Lau et al., 2010). Cell viability was at least 90%, as determined by trypan blue exclusion. Hepatocytes were suspended in Dulbecco’s Modified Eagle’s Medium supplemented with insulin (4 µg/ml), dexamethasone (1 µM), and fetal bovine serum (5% v/v), and were cultured at a density of 375,000 cells/well in 24-
well plates (pre-coated with a simple collagen Type I substratum) at 37°C in a humidified incubator with 95% air and 5% CO₂. Hepatocytes were allowed to attach for 2-4 h. Subsequently, culture medium containing unattached cells was aspirated, and fresh supplemented medium containing 1× ITS+ (Becton Dickinson, Bedford, MA) and Geltrex (0.35 mg/ml; Invitrogen) was added to each well. Hepatocytes were cultured for another 36-48 h prior to initiation of drug treatment, and culture medium was replaced daily. Human hepatocytes were treated with meclizine, rifampicin (hPXR agonist; positive control for CYP3A4 expression assay) (Faucette et al., 2006), CITCO (hCAR agonist; positive control for CYP2B6 expression assay) (Faucette et al., 2000), or DMSO (0.1% v/v; vehicle), as described in each figure legend. The chemical-containing culture medium was replaced every 24 h for a period of 72 h.

Total RNA Isolation and Reverse Transcription. Cultured human hepatocytes were harvested and cell lysates from 3 or 4 wells were pooled. Total cellular RNA was isolated using PureLink RNA Mini Kit and reverse-transcribed using Superscript II reverse transcriptase, according to the manufacturer’s protocol. Total cDNA was quantified using PicoGreen Double-Stranded DNA Quantitation Kit.

Polymerase Chain Reaction (PCR) Primers. Primer sequences are shown in Table 1. Primers were synthesized by Integrated DNA Technologies (Coralville, IA), and their specificity was verified by sequencing the purified amplicons at the University of British Columbia Nucleic Acid Protein Service Unit.

Real-time PCR Analysis. CYP3A4, CYP2B6, hCAR, hPXR, and human hypoxanthine phosphoribosyltransferase 1 (HPRT; housekeeping gene) cDNA were amplified using a real-time DNA thermal cycler (LightCycler, Roche Diagnostics). Each 20 μl PCR contained 1 ng total cDNA, 1 U Platinum Taq DNA polymerase in 1× PCR buffer [20 mM Tris-HCl (pH 8.4) and 50
mM KCl], MgCl₂ (4 mM for CYP3A4, CYP2B6, and hCAR; 2 mM for HPRT and hPXR), 0.2 mM dNTP, 0.25 mg/ml bovine serum albumin, 0.2 μM forward and reverse primers (except for HPRT in which the concentration was 0.5 μM), and 1:30,000 SYBR Green I solution. PCR cycling conditions are shown in Table 1. For all amplifications, the initial denaturation was performed at 95°C for 5 min. A calibration curve (cross point versus log cDNA copies) was constructed using known amounts of purified CYP3A4, CYP2B6, hCAR, hPXR, or HPRT cDNA. Each of these cDNAs was amplified from human liver QUICK-Clone cDNA (Clontech Laboratories, Inc.) and quantified by PicoGreen Double-Stranded DNA Quantitation Kit.

Testosterone 6β-Hydroxylation Assay. Cultured human hepatocytes were washed with Hanks’ Balanced Salt Solution (HBSS) and incubated for 14 min at 37°C with 0.5 ml of HBSS containing testosterone (final concentration of 200 μM) dissolved in DMSO (final concentration of 0.1% v/v in HBSS). The amount of 6β-hydroxytestosterone was quantified by a high-performance liquid chromatographic assay as described previously (Lau et al., 2010).

Bupropion Hydroxylation Assay. Cultured human hepatocytes were washed with HBSS and incubated for 20 min at 37°C with 0.5 ml of HBSS containing bupropion (final concentration of 500 μM) dissolved in DMSO (final concentration of 0.1% v/v in HBSS). The bupropion hydroxylation assay was performed using an ultra-performance liquid chromatography-tandem mass spectrometry assay as described previously (Lau and Chang, 2009), but with the following modifications: 1) the incubation mixture (200 μl) was spiked with triprolidine internal standard (0.1 μM in 200 μl methanol), evaporated to dryness under a stream of nitrogen gas, and the residue was then reconstituted with 60 μl of 33% methanol; and 2) hydroxybupropion and triprolidine were eluted at a flow rate of 0.2 ml/min using the following conditions: isocratic flow at 35% methanol containing 0.1% v/v formic acid (B) (0-3.5 min),
linear gradient from 35% to 100% B (3.5-7 min), isocratic flow at 100% B (7-8 min; 0.3 ml/min),
linear gradient from 100% to 35% B (8-8.05 min), and isocratic flow at 35% B (8.05-10.5 min).

**Statistical Analysis.** Data were analyzed by one-way or two-way analysis of variance, and when significant differences were detected, the Student Newman-Keuls multiple comparison test was performed (SigmaPlot 11.0, Systat Software, Inc., San Jose, CA). The level of statistical significance was set *a priori* at *P* < 0.05. EC$_{50}$ and maximal effect (E$_{max}$) were calculated by non-linear regression with the following equation (SigmaPlot 11.0): $y = E_0 + \frac{[E_{max} - E_0]}{[1+(x/EC_{50})^{Hill\ slope}]}$, where $y$ is PXR activation (fold increase over the vehicle-treated control group), $x$ is concentration (μM), and $E_0$ is minimum effect.
Results

**Meclizine and Rifampicin Activate hPXR to a Similar Extent, But Meclizine Activates rPXR to a Lesser Extent Than That of PCN.** To compare the effect of meclizine on hPXR activity with that of a typical hPXR agonist (rifampicin), a concentration-response experiment was conducted by treating hPXR-transfected HepG2 cells for 24 h with various concentrations of meclizine (1-30 µM) or rifampicin (0.01-30 µM). Fig. 2A shows that at 1-3 µM, meclizine had no effect on hPXR activity, whereas at 10, 20, and 30 µM, it increased hPXR activity in a log-linear manner by 5-, 8-, and 10-fold over the vehicle-treated control group, respectively. EC$_{50}$ and E$_{\text{max}}$ values of hPXR activation by meclizine could not be determined because maximal effect was not achieved at the highest concentration investigated in this study. Nevertheless, by comparing the concentration-response curves, it could be inferred that meclizine was less potent than rifampicin, and the extent of hPXR activation by meclizine (30 µM) was similar to that by rifampicin (30 µM). The experimentally-derived EC$_{50}$ and E$_{\text{max}}$ values of hPXR activation by rifampicin were 0.41 ± 0.03 µM and 13.5 ± 0.9-fold, respectively. These values are similar to those in the literature (Chang, 2009). As expected, 10 µM PCN (negative control) had no effect on hPXR activity (data not shown).

A concentration-response experiment was also conducted to compare the effect of meclizine (1-30 µM) on rPXR activity with that of a typical rPXR agonist, PCN (0.01-30 µM). As shown in Fig. 2B, meclizine had no effect at concentrations less than 20 µM, whereas 20 and 30 µM meclizine increased rPXR activity by 2.5- and 2.8-fold, respectively. By comparing the concentration-response curves, it is evident that meclizine activated rPXR to a far lesser extent than that by PCN. The EC$_{50}$ and E$_{\text{max}}$ values of rPXR activation by PCN were 1.0 ± 0.2 µM and 17.3 ± 1.9-fold, respectively. These values are similar to those reported previously (Chang,
2009). As expected, 10 µM rifampicin (negative control) had no effect on rPXR activity (data not shown). Overall, the extent of rPXR activation (Fig. 2B) by meclizine did not reflect that of hPXR activation (Fig. 2A). Therefore, subsequent experiments focused on hPXR.

**Meclizine Binds to hPXR Ligand-Binding Domain.** To determine whether meclizine activates hPXR by binding to the ligand-binding domain, a TR-FRET competitive ligand-binding assay was conducted. Preliminary experiment indicated that the drug did not quench fluorescence. As shown in Fig. 3, meclizine (30 µM) decreased TR-FRET emission ratio to 58% of that in the vehicle-treated control group. The extent of binding by meclizine was greater than that by 1000 µM phenobarbital (74% of control value), which is a known agonist of hPXR (Jones et al., 2000). By comparison, a maximal binding concentration (10 µM) of an efficacious hPXR ligand SR12813 (Jones et al., 2000) decreased the emission ratio to 8% of that in the control group. As expected, PCN (10 µM; negative control) had no effect.

**Meclizine Recruits Coactivator to hPXR.** Upon ligand binding, a PXR agonist induces a change in receptor conformation and recruits various coactivators, such as steroid receptor coactivator-1 (SRC-1) (Timsit and Negishi, 2007). Therefore, we investigated whether meclizine recruits SRC-1 by conducting a mammalian two-hybrid assay on HepG2 cells transfected with pM-hSRC1-RID plasmid, pGL4.74[hRluc/TK] internal control plasmid, pFR-luc reporter plasmid, and pVP16-hPXR-LBD expression plasmid or pVP16 empty vector. Transfected cells were treated with meclizine (30 µM), rifampicin (10 µM; positive control), or DMSO (0.1% v/v; vehicle). In HepG2 cells co-transfected with human SRC-1 and hPXR ligand-binding domain, meclizine increased the reporter activity to 32% of the level in the rifampicin-treated group (Fig. 4). Control analysis indicated that when cells were transfected with human SRC-1 alone, meclizine and rifampicin did not increase the reporter activity.
Meclizine Modulates hPXR Target Gene Expression in Cultured Human Hepatocytes. CYP3A4 is a typical target gene of hPXR (Lehmann et al., 1998). Given our data showing meclizine as an agonist of hPXR (Fig. 3 and Fig. 4), we determined the effect of meclizine on CYP3A4 gene expression in primary cultures of human hepatocytes. As shown in Fig. 5A, meclizine (10 µM) increased CYP3A4 mRNA expression by 5-fold in human hepatocyte sample Hu1108 and 10-fold in sample Hu1138. By comparison, rifampicin (10 µM; positive control) increased CYP3A4 mRNA level by 52-fold in Hu1108 and 29-fold in Hu1138.

To determine whether meclizine affects CYP3A4 function, we measured the enzymatic conversion of testosterone to 6β-hydroxytestosterone, which is a commonly used marker for CYP3A4 catalytic activity (Yuan et al., 2002). In contrast to the increase in CYP3A4 mRNA level, testosterone 6β-hydroxylation activity was not increased by 10 µM meclizine (Fig. 5B). Control analysis indicated that rifampicin (10 µM; positive control for CYP3A4 induction) increased testosterone 6β-hydroxylation by 51-fold in Hu1108 and 8-fold in Hu1138.

Concentration-response experiments were conducted to further characterize the differential effects of meclizine on CYP3A4 mRNA expression and catalytic activity. As shown in Fig. 6A, in sample Hu1108, meclizine, at concentrations < 10 µM, had minimal or no effect on CYP3A4 mRNA expression, whereas 10 and 60 µM meclizine increased it in a log-linear manner by 5- and 26-fold, respectively. Similarly, in sample Hu1138, meclizine, at concentrations < 3 µM, had minimal or no effect, whereas at 3, 10, 30, and 60 µM, it increased CYP3A4 mRNA level by 3-, 10-, 26-, and 20-fold, respectively (Fig. 6B). In contrast to the induction of CYP3A4 mRNA by meclizine, it did not increase testosterone 6β-hydroxylation activity (Fig. 6C and 6D). In fact, in Hu1108, 60 µM meclizine decreased the activity by 70%.
(Fig. 6C), whereas in Hu1138, 30 and 60 µM of the drug decreased it by 40 and 70% (Fig. 6D), respectively.

**Meclizine Does Not Decrease Constitutive Expression of a hCAR Target Gene in Cultured Human Hepatocytes.** Meclizine was previously reported to be an inverse agonist of hCAR (Huang et al., 2004), and our present data indicate that it is an agonist of hPXR. Therefore, we determined in human hepatocytes whether meclizine is capable of modulating the expression of a gene (e.g. CYP2B6) that is co-regulated by these two receptors (Sueyoshi et al., 1999; Goodwin et al., 2001). As shown in Fig. 7A and 7B, meclizine (10 µM) did not decrease CYP2B6 mRNA expression in both hepatocyte samples. A concentration-response experiment further indicated that meclizine (10 and 60 µM) marginally increased CYP2B6 mRNA expression (< 2-fold) in Hu1108 (Fig. 7C), whereas at 1, 3, 10, 30, and 60 µM, it increased CYP2B6 mRNA level by 2-, 3-, 7-, 12-, and 9-fold, respectively, in Hu1138 (Fig. 7D). As expected, CITCO (0.1 µM; positive control), which is a known hCAR agonist (Maglich et al., 2003), increased it by 12-fold in Hu1108 (Fig. 7A) and 21-fold in Hu1138 (Fig. 7B).

**Meclizine Does Not Attenuate Induction of hCAR Target Gene by CITCO in Cultured Human Hepatocytes.** Previously, meclizine was shown to suppress CITCO-mediated hCAR activation in a cell-based reporter gene assay conducted on hCAR-transfected HepG2 cells (Huang et al., 2004). Therefore, we determined whether meclizine blocks CITCO-mediated CYP2B6 induction in primary cultures of human hepatocytes. As shown in Fig. 8A and 8B, meclizine (10 µM) increased CYP2B6 mRNA expression by 2-fold in Hu1108 and 3-fold in Hu1043, whereas CITCO (0.1 µM) increased it by 12-fold in Hu1108 and 20-fold in Hu1043. Meclizine did not decrease the level of CYP2B6 induction by CITCO. Rather, it did not affect (Fig. 8A) or slightly increased (Fig. 8B) CYP2B6 mRNA expression in hepatocytes treated with
CITCO. To corroborate the gene expression findings, we also determined the enzymatic conversion of bupropion to hydroxybupropion, which is a selective marker for CYP2B6 catalytic activity (Faucette et al., 2000). The bupropion hydroxylation data (Fig. 8C and 8D) were similar to those obtained from the CYP2B6 mRNA assay (Fig. 8A and 8B).

**hCAR is Co-expressed with hPXR in Cultured Human Hepatocyte Samples Hu1108 and Hu1138.** Given that meclizine did not decrease the constitutive expression of CYP2B6 (e.g. Fig. 7A) and did not suppress CITCO-mediated CYP2B6 induction (e.g. Fig. 8A), we next assessed whether these observations were due to the absence of hCAR in our samples of human hepatocytes. As shown in Table 2, hCAR and hPXR mRNAs were detected in samples Hu1108 and Hu1138. In general, meclizine (0.03-60 µM) did not appear to decrease the expression of either receptor.

**Meclizine Does Not Act as an Inverse Agonist of hCAR.** To gain further insight into the lack of suppression of constitutive CYP2B6 expression by meclizine (Fig. 7A-7D), we conducted a hCAR reporter gene assay and a mammalian two-hybrid assay on transfected HepG2 cells treated with various concentrations of the drug. Meclizine, at 10-30 µM, did not decrease the constitutive activity of hCAR (Fig. 9A) or recruit SRC-1 to hCAR (Fig. 9B). In contrast, PK11195, which was reported to be a hCAR inverse agonist (Li et al., 2008), decreased hCAR reporter gene activity by 97% and SRC-1 recruitment by 89%. Control analysis indicated that the treatment groups did not affect the reporter activity when HepG2 cells were transfected with human SRC-1 alone (i.e., without hCAR ligand-binding domain).

**Meclizine Does Not Act as an Antagonist of hCAR.** To determine the reason for the lack of suppression of CITCO-mediated CYP2B6 induction in human hepatocytes (Fig. 8A-8D), we conducted a hCAR reporter gene assay and a mammalian two-hybrid assay on transfected...
HepG2 cells treated with CITCO or a combination of CITCO and meclizine. Meclizine (10 and 30 µM) did not decrease, but marginally increased, CITCO-mediated hCAR reporter gene activity (Fig. 10A). As shown in Fig. 10B, the drug also did not decrease CITCO-mediated SRC-1 recruitment to hCAR. In contrast, PK11195, which was reported to be a hCAR antagonist (Li et al., 2008), decreased CITCO-mediated reporter gene activity by 93% and CITCO-mediated SRC-1 recruitment by 79%. Control analysis indicated that all the treatment groups did not affect the reporter activity when HepG2 cells were transfected with human SRC-1 alone (i.e., without hCAR ligand-binding domain).
Discussion

A major conclusion in the present study is the agonism of hPXR by meclizine. This is evident by its ability to bind to hPXR ligand-binding domain and recruit SRC-1 coactivator, as analyzed by a TR-FRET competitive ligand-binding assay and a mammalian two-hybrid assay, respectively. Similar to rifampicin, which is a known hPXR agonist (Jones et al., 2000), meclizine increased luciferase reporter gene activity in hPXR-transfected HepG2 cells and hPXR target gene (CYP3A4 mRNA) expression in primary cultures of human hepatocytes. The extent of hPXR activation by 10-30 μM meclizine was 31-70% of that by 10 μM rifampicin in our reporter gene assay. This effect was greater than that obtained in other studies using high-throughput screening assays. In those studies, meclizine (17 μM) activated hPXR to 17% of that by 10 μM rifampicin (Sinz et al., 2006) and yielded an E_{max} value that was 25% of that by 10 μM rifampicin (Kim et al., 2010). Differences in the extent of PXR activation may reflect differences in experimental conditions or methodology, which are important factors to consider when comparing data obtained from reporter gene assays conducted in various laboratories (Stanley et al., 2006). Taken together, our findings indicate that meclizine is an agonist of hPXR.

An unexpected finding in our experiment with human hepatocytes is that meclizine, at 30 and 60 μM concentrations, decreased CYP3A-catalyzed testosterone 6β-hydroxylation, even though it increased CYP3A4 mRNA expression. A plausible explanation is inhibition of CYP3A4 catalytic activity in the hepatocytes, which were treated with meclizine for 72 h. In our experiments, cultured hepatocytes were washed prior to the addition of the substrate (testosterone). Therefore, meclizine is not likely to competitively inhibit CYP3A4 catalytic activity. Instead, irreversible inactivation of the enzyme may have occurred. In a recent study,
an antiemetic drug, aprepitant, increased CYP3A4 mRNA expression, but it also decreased CYP3A4 catalytic activity (Fahmi et al., 2010).

Species differences in PXR function have been reported and several chemicals have shown species-dependent activation of PXR (Stanley et al., 2006). As analyzed by a cell-based reporter gene assay in this study, meclizine was a much weaker activator of rPXR than hPXR. This can be explained by the structural differences (~76% amino acid identity) in the ligand-binding domains of rPXR and hPXR (Jones et al., 2000). In contrast, meclizine activates cynomolgus monkey PXR and hPXR to a similar extent (Kim et al., 2010), which reflects the greater degree of similarity (~96% amino acid identity) between the ligand-binding domains of monkey PXR and hPXR (Moore et al., 2002). Structural and site-directed mutagenesis studies suggest that the molecular basis for the species-specificity in rPXR and hPXR activation could be attributed to several key amino acids, such as Phe305 and Asp318 in rPXR and Leu308 in hPXR (Tirona et al., 2004), that are present near the ligand-binding cavity. Due to species differences in rPXR and hPXR activation by meclizine, our data indicate that rPXR is not an appropriate surrogate when studying the functional interaction between this drug and hPXR.

CYP2B6, which is a classical target gene of hCAR (Sueyoshi et al., 1999), is co-regulated by hPXR (Goodwin et al., 2001), and the expression of this gene is increased by known hPXR agonists, such as rifampicin and hyperforin (Faucette et al., 2004). As shown in the present study, meclizine did not decrease CYP2B6 expression in human hepatocytes. In fact, our findings indicate that the drug increased CYP2B6 expression in one of the human hepatocyte samples, which may reflect activation of hPXR by meclizine. As indicated by real-time PCR, both hCAR and hPXR were expressed in our samples. Consistent with the lack of decrease in CYP2B6 expression by meclizine, this drug did not act as an inverse agonist of hCAR, as
determined in a cell-based reporter gene assay and a mammalian two-hybrid assay. In a previous study, it was reported as an inverse agonist of hCAR, based on its ability to decrease hCAR activity in a cell-based reporter gene assay and coactivator recruitment in a mammalian two-hybrid assay (Huang et al., 2004). The reason for the differences in results is not known. Discrepancy has also been noted for clotrimazole, which was reported to increase (Kublbeck et al., 2008), decrease (Moore et al., 2000; Maglich et al., 2003), or had no effect (Toell et al., 2002) on hCAR activity in cell-based reporter gene assays. Overall, meclizine does not function as a hCAR inverse agonist in human hepatocytes.

In a previous study (Huang et al., 2004), meclizine was reported to act as an antagonist of hCAR, as suggested by its ability to block the increase in hCAR activation by CITCO in an *in vitro* cell-based reporter gene assay and attenuate the induction of mouse CAR target genes (e.g. *Cyp2b10*) by phenobarbital in humanized CAR mice. CITCO is an agonist of hCAR (Maglich et al., 2003), whereas phenobarbital activates mouse CAR and hCAR (Timsit and Negishi, 2007). However, in the present study, meclizine did not suppress CITCO-mediated increase in hCAR activity, as assessed in a reporter gene assay, or in SRC-1 recruitment, as determined in a mammalian two-hybrid assay. Consistent with these findings, the drug also did not suppress CITCO-mediated increase in CYP2B6 mRNA expression or CYP2B6-catalyzed bupropion hydroxylation in primary cultures of human hepatocytes. Therefore, our data indicate that meclizine is not effective as an antagonist of hCAR. The reason for the conflicting results obtained from a previous study (Huang et al., 2004) and the present study is not known. However, it may reflect, at least in part, the experimental model for hCAR target gene expression employed in the previous study (humanized CAR mice) and in the present study (primary cultures of human hepatocytes).
In conclusion, meclizine is a hPXR agonist and a stronger activator of hPXR than rPXR. In addition to the effect on hPXR activation, meclizine inhibits the catalytic activity of CYP3A4. This unexpected finding suggests that when studying the effects of a nuclear receptor, it is important to determine not only the target gene expression, but also the activity of the target gene product. In contrast to the previous findings (Huang et al., 2004), our data do not support the conclusion that meclizine is an inverse agonist and antagonist of hCAR, as assessed in the present study by a cell-based reporter gene assay, a mammalian two-hybrid assay, and hCAR target gene expression assays in primary cultures of human hepatocytes. Therefore, it is not appropriate to use meclizine as a pharmacological tool to study hCAR function.
JPET #175927

Authorship Contributions

Participated in research design: Lau and Chang.

Conducted experiments: Lau, Yang, Rajaraman, and Baucom.

Contributed new reagents or analytic tools: N/A

Performed data analysis: Lau.

Wrote or contributed to the writing of the manuscript: Lau and Chang.

Other: Chang acquired funding for the research.
References


Jordan #175927

Footnotes

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Figure Legends

**Fig. 1.** Chemical structure of meclizine.

**Fig. 2.** Meclizine and rifampicin activate hPXR to a similar extent, but meclizine activates rPXR to a lesser extent than that of PCN. HepG2 cells were transfected for 24 h with pGL3-basic-CYP3A4-XREM-luc, pGL4.74[hRluc/TK], and with (A) pCMV6-XL4-hPXR, pCMV6-XL4 (empty vector), (B) pCMV6-AC-rPXR, or pCMV6-AC (empty vector). Transfected cells were treated for 24 h with DMSO (0.1% v/v; vehicle), meclizine (1, 3, 10, 20, or 30 µM), rifampicin (0.01, 0.1, 0.3, 1, 3, 10, or 30 µM; positive control for hPXR), or PCN (0.01, 0.1, 0.3, 1, 3, 10, or 30 µM; positive control for rPXR). Firefly and Renilla luciferase activities were measured and normalized as described under Materials and Methods. Each independent experiment was performed in triplicate. Data are expressed as mean ± S.E.M. for four independent experiments. *Significantly different from the vehicle-treated control group (P < 0.05).

**Fig. 3.** Meclizine binds to hPXR ligand-binding domain. A TR-FRET competitive binding assay was performed as described under Materials and Methods. Human PXR ligand-binding domain (10 nM) was incubated with Fluormone PXR Green (a PXR ligand; 40 nM) in the presence of DMSO (1% v/v; vehicle), meclizine (30 µM), phenobarbital (1000 µM; positive control), SR12813 (10 µM; positive control), or PCN (10 µM; negative control). TR-FRET was measured using a fluorescence plate reader and the net TR-FRET ratio was calculated, as described under Materials and Methods. Each independent experiment was performed in triplicate. Data are expressed as a percentage of the mean value calculated for the vehicle-treated
control group and shown as mean ± S.E.M. of three or four independent experiments.

*Significantly different from the vehicle-treated control group (P < 0.05).

**Fig. 4.** Meclizine recruits coactivator to hPXR. HepG2 cells were transfected for 24 h with pM-hSRC1-RID, pGL4.74[hRluc/TK], pFR-luc, and pVP16-hPXR-LBD or pVP16 (empty vector). Transfected cells were treated for 24 h with DMSO (0.1% v/v; vehicle), meclizine (30 μM), or rifampicin (10 μM; positive control). Firefly and *Renilla* luciferase activities were measured and normalized as described under *Materials and Methods*. Each independent experiment was performed in triplicate. Data are expressed as a percentage of the normalized luciferase activity in the rifampicin-treated group and shown as mean ± S.E.M. for three independent experiments.

*Significantly different from the vehicle-treated control group in pVP16-transfected cells and pVP16-hPXR-LBD-transfected cells (P < 0.05). #Significantly different from the vehicle-treated control group in pVP16-transfected cells (P < 0.05).

**Fig. 5.** Meclizine modulates CYP3A4 expression to a lesser extent than that by rifampicin in primary cultures of human hepatocytes. Cultured hepatocytes (samples Hu1108 and Hu1138) were treated for 72 h with DMSO (0.1% v/v; vehicle), meclizine (10 μM), or rifampicin (10 μM; positive control). (A) Total RNA was isolated from pooled cell lysates (3 wells). CYP3A4 mRNA level was analyzed by real-time PCR and normalized to HPRT mRNA level, as described under *Materials and Methods*. Data are shown as mean of duplicate PCR analyses. (B) Testosterone 6β-hydroxylation level was determined by high-performance liquid chromatography, as described under *Materials and Methods*. Data are expressed as mean ± S.D.
of 3 wells. Testosterone 6β-hydroxylation in the DMSO-treated control group was 146 ± 13 pmol/375,000 cells and 502 ± 35 pmol/375,000 cells in Hu1108 and Hu1138, respectively.

**Fig. 6.** Meclizine increases CYP3A4 mRNA expression, but decreases testosterone 6β-hydroxylation, in primary cultures of human hepatocytes. Cultured hepatocytes were treated for 72 h with DMSO (0.1% v/v; vehicle) or meclizine (0.03, 0.1, 0.3, 1, 3, 10, or 60 µM for sample Hu1108; and 1, 3, 10, 30, or 60 µM for sample Hu1138). Total RNA was isolated from pooled cell lysates (3 wells). CYP3A4 mRNA level was analyzed by real-time PCR and normalized to HPRT mRNA level, as described under **Materials and Methods**. Data are shown as mean of duplicate PCR analyses for hepatocyte samples Hu1108 (A) and Hu1138 (B). Testosterone 6β-hydroxylation level was determined by high-performance liquid chromatography, as described under **Materials and Methods**. Data are expressed as mean ± S.D. of 3 wells for hepatocyte samples Hu1108 (C) and Hu1138 (D).

**Fig. 7.** Meclizine does not decrease hCAR target gene expression in primary cultures of human hepatocytes. Cultured hepatocytes were treated for 72 h with (A, B) DMSO (0.1% v/v; vehicle), meclizine (10 µM), CITCO (0.1 µM; positive control), or with (C, D) DMSO (0.1% v/v; vehicle) or meclizine (0.03, 0.1, 0.3, 1, 3, 10, or 60 µM for sample Hu1108; and 1, 3, 10, 30, or 60 µM for sample Hu1138). Total RNA was isolated from pooled cell lysates (3 wells). CYP2B6 mRNA level was analyzed by real-time PCR and normalized to HPRT mRNA level, as described under **Materials and Methods**. Data are shown as mean of duplicate PCR analyses for hepatocyte samples Hu1108 (A, C) and Hu1138 (B, D).
Fig. 8. Meclizine does not attenuate CITCO-mediated CYP2B6 induction in primary cultures of human hepatocytes. Cultured hepatocytes were treated for 72 h with DMSO (0.1% v/v; vehicle), meclizine (10 µM), CITCO (0.1 µM; positive control), or a combination of meclizine (10 µM) and CITCO (0.1 µM). Total RNA was isolated from pooled cell lysates (3 or 4 wells). CYP2B6 mRNA level was analyzed by real-time PCR and normalized to HPRT mRNA level, as described under Materials and Methods. Data are shown as mean of duplicate PCR analyses for hepatocyte samples Hu1108 (A) and Hu1043 (B). Bupropion hydroxylation level was determined by ultra-performance liquid chromatography-tandem mass spectrometry, as described under Materials and Methods. Data are expressed as mean ± S.D. of 3 or 4 wells for hepatocyte samples Hu1108 (C) and Hu1043 (D).

Fig. 9. Meclizine does not act as an inverse agonist of hCAR. (A) HepG2 cells were transfected for 24 h with pGL3-basic-CYP2B6-PBREM/XREM-luc, pGL4.74[hRluc/TK], and pCMV6-XL4-hCAR or pCMV6-XL4 (empty vector). (B) HepG2 cells were transfected for 5 h with pM-hSRC1-RID, pCMV6-XL4-hRXRα, pGL4.74[hRluc/TK], pFR-luc, and pVP16-hCAR-LBD or pVP16 (empty vector). Transfected cells were treated for 24 h with DMSO (0.1% v/v; vehicle), meclizine (10, 20, or 30 µM), or PK11195 (10 µM; positive control). Firefly and Renilla luciferase activities were measured and normalized as described under Materials and Methods. Each independent experiment was performed in triplicate. Data are expressed as a percentage of the normalized luciferase activity in the vehicle-treated control group and shown as mean ± S.E.M. for four or six independent experiments. *Significantly different from the vehicle-treated control group in pCMV6-XL4-hCAR-transfected cells (P < 0.05). †Significantly different from the vehicle-treated control group in pVP16-transfected cells (P < 0.05). #Significantly different...
from the vehicle-treated control group in pVP16-transfected cells and pVP16-hCAR-LBD-transfected cells ($P < 0.05$).

**Fig. 10.** Meclizine does not act as an antagonist of hCAR. (A) HepG2 cells were transfected for 24 h with pGL3-basic-CYP2B6-PBREM/XREM-luc, pGL4.74[$hRluc$/$TK$], and pCMV6-XL4-hCAR or pCMV6-XL4 (empty vector). (B) HepG2 cells were transfected for 5 h with pM-hSRC1-RID, pCMV6-XL4-hRXR$\alpha$, pGL4.74[$hRluc$/$TK$], pFR-luc, and pVP16-hCAR-LBD or pVP16 (empty vector). Transfected cells were treated for 24 h with DMSO (0.1% v/v; vehicle), CITCO (0.1 µM), a combination of CITCO (0.1 µM) and meclizine (10 or 30 µM), or a combination of CITCO (0.1 µM) and PK11195 (10 µM; positive control). Firefly and Renilla luciferase activities were measured and normalized as described under *Materials and Methods*. Each independent experiment was performed in triplicate. Data are expressed as a percentage of the normalized luciferase activity in the CITCO plus DMSO-treated group and shown as mean ± S.E.M. for four or six independent experiments. *Significantly different from the CITCO plus DMSO-treated group in pCMV6-XL4-hCAR-transfected cells ($P < 0.05$). #Significantly different from the CITCO plus DMSO-treated group in pVP16-transfected cells and pVP16-hCAR-LBD-transfected cells ($P < 0.05$).
### TABLE 1
Primer sequences and PCR cycling conditions

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<th>Gene</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
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<td>CCT-TAC-ACA-TAC-ACA-CCC-TTT-GGA-AGT (forward)$^a$</td>
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<td>60°C, 10 s</td>
<td>72°C, 15 s</td>
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<tr>
<td></td>
<td>AGC-TCA-ATG-CAT-GTA-CAG-AAT-CCC-CGG-TTA (reverse)$^a$</td>
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<td>CYP2B6</td>
<td>GCG-TGT-GGT-TCA-TTC-ACA-AA (forward)$^b$</td>
<td>94°C, 5 s</td>
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<tr>
<td></td>
<td>AAT-TTA-GCC-AGG-CGT-GGT-G (reverse)$^b$</td>
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<tr>
<td>hPXR</td>
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<td>95°C, 5 s</td>
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<td>72°C, 15 s</td>
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<td>HPRT</td>
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<td>GCG-ACC-TTG-ACC-ATC-TTT-G (reverse)$^c$</td>
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</table>

$^a$ Schuetz et al. (1996); $^b$ Chang et al. (2003); $^c$ Qiu et al., (2007).
TABLE 2

hCAR is co-expressed with hPXR in cultured human hepatocyte samples Hu1108 and Hu1138. Hepatocytes were treated for 72 h with DMSO (0.1% v/v; vehicle) or meclizine (0.03, 0.1, 0.3, 1, 3, 10, or 60 µM for sample Hu1108; and 1, 3, 10, 30, or 60 µM for sample Hu1138). Total RNA was isolated from pooled cell lysates (3 wells). hCAR and hPXR mRNA levels were analyzed by real-time PCR and normalized to HPRT mRNA level, as described under Materials and Methods. Data are shown as mean of duplicate PCR analyses.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chemical</th>
<th>Concentration</th>
<th>hCAR mRNA</th>
<th>hPXR mRNA</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Relative mRNA Expression</td>
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<tr>
<td>Hu1108</td>
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<td>0.1 µM</td>
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<td>0.3 µM</td>
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<td>1 µM</td>
<td>0.39</td>
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<td>3 µM</td>
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<td>10 µM</td>
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<tr>
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<td>0.18</td>
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<td>Meclizine</td>
<td>1 µM</td>
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<td>3 µM</td>
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<td>30 µM</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>60 µM</td>
<td>0.69</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Figure 3

Net TR-FRET Ratio (percentage of control)

DMSO  Meclizine  Phenobarbital  SR12813  PCN

* *
Figure 4

Normalized Luciferase Activity (percentage of rifampicin)

DMSO  Meclazine  Rifampicin

DMSO  Meclazine  Rifampicin

pVP16  pVP16-hPXR-LBD

pM-hSRC1-RID

*  *

#
Figure 5

A. CYP3A4 mRNA

B. Testosterone 6β-OH

Relative mRNA Expression (fold increase over control)

Testosterone 6β-Hydroxylation (fold increase over control)

DMSO, Meclizine, Rifampicin
Figure 6

A. CYP3A4 mRNA (Hu1108)

B. CYP3A4 mRNA (Hu1138)

C. Testosterone 6β-OH (Hu1108)

D. Testosterone 6β-OH (Hu1138)