Species-Dependent and Receptor-Selective Action of Bilobalide on the Function of Constitutive Androstane Receptor and Pregnan X Receptor

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ABSTRACT:
Bilobalide is a naturally occurring sesquiterpene trilactone with therapeutic potential in the management of ischemia and neurodegenerative diseases such as Alzheimer’s disease. In the present study, we investigated the effect of bilobalide on the activity of rat constitutive androstane receptor (rCAR) and rat pregnane X receptor (rPXR) and compared that with human CAR (hCAR) and human PXR (hPXR). Bilobalide activated rCAR in a luciferase reporter gene assay and increased rCAR target gene expression in cultured rat hepatocytes, as determined by the CYP2B1 mRNA and CYP2B enzyme activity (benzyloxyresorufin O-dealkylation) assays. This increase in hepatocyte CYP2B1 expression by bilobalide was not accompanied by a corresponding increase in rCAR mRNA level. In contrast to the activation of rCAR, the activity of rPXR, hCAR, and hPXR was not influenced by this chemical in cell-based reporter gene assays. Consistent with these results, bilobalide did not alter rPXR, hCAR, or hPXR target gene expression in rat or human hepatocytes, as evaluated by the CYP3A23, CYP2B6, CYP3A4 mRNA assays and the CYP3A (testosterone 6-hydroxylation) and CYP2B6 (buproprion hydroxylation) enzyme activity assays. Bilobalide was not an antagonist of rPXR, hCAR, or hPXR, as suggested by the finding that it did not attenuate rPXR activation by pregnenolone 16α-carbonitrile, hCAR activation by 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime, or hPXR activation by rifampicin in reporter gene assays. In conclusion, bilobalide is an activator of rCAR, whereas it is not a ligand of rPXR, hCAR, or hPXR. Likewise, it is an inducer of rat CYP2B1, but not of rat CYP3A23, human CYP2B6, or human CYP3A4.

Introduction
Bilobalide is a naturally occurring sesquiterpene trilactone (Fig. 1) that is present in Ginkgo biloba (van Beek and Montoro, 2009). As a single chemical entity, bilobalide possesses several pharmacological activities, including antagonizing GABA_A receptor (Kiewert et al., 2007), decreasing β-amyloid protein levels (Shi et al., 2011), and preventing ischemia-induced edema formation (Mdzinarishvili et al., 2007), as shown in various in vitro and in vivo experimental models. These promising actions of bilobalide have led to considerable interest in developing it as a potential pharmacotherapy for conditions such as cerebral ischemia (Mdzinarishvili et al., 2007) and neurodegenerative diseases, including Alzheimer’s disease (Shi et al., 2011).

Constitutive androstane receptor (CAR; NR1I3) and pregnane X receptor (PXR; NR1I2) regulate the transcription of a large number of genes, including those that encode drug-metabolizing enzymes and drug transporters (Köhle and Bock, 2009). These two nuclear receptors have overlapping, but distinct, sets of ligands. Various chemicals, such as phenobarbital, activate both CAR and PXR (Chang and Waxman, 2006). However, there are also chemicals that activate either CAR or PXR. For example, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) activates mouse CAR (mCAR) but not mouse PXR (mPXR), whereas pregnenolone 16α-carbonitrile (PCN) and dexamethasone activate mPXR but not mCAR (Moore et al., 2000). In addition to these differences in ligand selectivity, pronounced species differences exist in chemical activation of CAR and PXR. For example, TCPOBOP activates mCAR but not human CAR (hCAR) (Moore et al., 2000), whereas rifampicin activates human PXR (hPXR) but not...
rat PXR (rPXR), and PCN activates rPXR but not hPXR (Jones et al., 2000).

The expression of CYP2B is regulated predominantly by CAR (Fauchette et al., 2006). Previous ex vivo studies indicate that bilobalide increased rat hepatic CYP2B expression (Deng et al., 2008a; Taki et al., 2009). Likewise, in a cell culture study on rat hepatocytes, bilobalide increased the levels of CYP2B1 mRNA and enzyme activity (Chang et al., 2006). By comparison, it is not clear whether bilobalide induces human CYP2B6 because it had minimal effect (less than 2-fold at 50 μM concentration) on CYP2B6 mRNA expression, as reported in a cell culture study on human hepatocytes (Li et al., 2009). In that same study, 50 μM bilobalide had no effect on hCAR activity in a cell-based reporter gene assay (Li et al., 2009). Taken together, these ex vivo and in vitro findings lead us to hypothesize that bilobalide is an activator of rat CAR (rCAR) and to the possibility that bilobalide activates CAR in a species-dependent manner.

The expression of CYP3A is regulated predominantly by PXR (Fauchette et al., 2006). In a previous ex vivo study, bilobalide increased the activity of rat hepatic microsomal CYP3A (Suzuki et al., 2004). However, data on the effect of this chemical on rat CYP3A mRNA, protein, and enzyme activity are internally inconsistent in each of the other two ex vivo studies (Deng et al., 2008a; Taki et al., 2009). In a cell culture study on rat hepatocytes, bilobalide (3–15 μM) did not increase CYP3A mRNA or enzyme activity (Chang et al., 2006), whereas in another study, the chemical at lesser concentrations of 2 to 50 ng/ml (equivalent to 0.006–0.153 μM) was reported to apparently increase CYP3A protein expression in rat hepatocytes (Deng et al., 2008b). In cultured human hepatocytes, bilobalide (50 μM) had no effect on CYP3A4 mRNA level in one study (Li et al., 2009), whereas the same chemical at lesser concentrations (0.006–0.153 μM) was shown to increase CYP3A protein expression in another study (Deng et al., 2008b). Therefore, conflicting ex vivo and in vitro experimental observations exist on the effect of bilobalide on the expression of rat and human CYP3A. It is now recognized that another approach to predict drug or chemical induction of CYP3A is to characterize the effect of the drug or chemical on PXR activity (Chu et al., 2009).

The primary goal of the present study is to investigate the effect of bilobalide on the activity of rCAR and rPXR and compare that with hCAR and hPXR. The experiments, which were performed in a hepatoma cell line and primary cultures of hepatocytes, involved reporter gene assays and CAR/PXR target gene expression assays. The results are discussed in the context of the species-dependent and receptor-selective effect of bilobalide.

Materials and Methods

Chemicals and Reagents. (+)-Bilobalide (Chemical Abstracts Service no. 33570-04-6) was obtained from LKT Laboratories (St. Paul, MN). Rifampicin, PCN, sodium phenobarbital, dexamethasone, TCPOBOP, 1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isooquinoline-carboxamide (PK11195), testosterone, buproprion hydrochloride, triprolidine hydrochloride, resorufin, 7-benzoyloxyresorufin, dicumarol, dextran, and Trition X-100 (Union Carbide Corporation, Houston, TX) were purchased from Sigma-Aldrich (St. Louis, MO). Hydroxybupropion and ketoconazole were obtained from Toronto Research Chemicals, Inc. (North York, ON, Canada). 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carboxaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) was obtained from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). 6p-Hydroxytestosterone and 5α-androstane-3,17α-diol (androstanol) were obtained from Steraloids, Inc. (Newport, RI). PureLink RNA Mini Kit, PicoGreen Double-Stranded DNA Quantitation Kit, Lipofectamine LTX and PLUS reagent, Opti-MEM, and all other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Fugene 6 transfection reagent (Fugent, LLC, Madison, WI), Cytotoxicity Detection Kit, β-glucuronidase, and asialylfetuin were obtained from Roche Diagnostics (Laval, QC, Canada), and Dual-Luciferase Reporter Assay System was from Promega (Madison, WI). The suppliers of reagents for isolation and culturing of rat (Chang et al., 2006) and human (Lau et al., 2010) hepatocytes, and those for reverse transcription and real-time polymerase chain reaction (PCR) analyses (Chang et al., 2006) were described previously.

Plasmids. pCMV6-AC-rCAR, pCMV6-XL4-hCAR, pCMV6-AC-rPXR, pCMV6-XL4-hPXR, pCMV6-AC, and pCMV6-XL4 were purchased from OriGene Technologies, Inc. (Rockville, MD). Renilla reniformis luciferase pGL4.74[hrLuc/TK] plasmid was obtained from Promega. pGL3-basic-CYP2B1-luc reporter was generously provided by Dr. Thomas A. Kocarek (Wayne State University, Detroit, MI) (Kocarek and Mercer-Haines, 2002). pGL3-basic-CYP2B6-PBREM/XREM-luc reporter (Lau et al., 2011b) and pGL3-basic-CYP3A4-XREM-luc reporter (Lau et al., 2010) were constructed as described previously. 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 sequences with published sequences.

HepG2 Cell Culture. HepG2 human hepatocellular carcinoma cells were purchased from American Type Culture Collection (Manassas, VA) and cultured as described previously (Lau et al., 2010).

Isolation, Culture, and Treatment of Rat Hepatocytes. Primary cultures of rat hepatocytes were isolated by a two-step collagenase perfusion method as described previously (Chang et al., 2006). Isolated rat hepatocytes were suspended in Williams’ Medium E supplemented with 10% v/v heat-inactivated fetal bovine serum, 1 μM insulin, 100 nM dexamethasone, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Hepatocytes were cultured at a density of 300,000 (for reporter gene assays) or 560,000 (for gene expression assays) cells/well in 12-well plates, which were precoated with Matrigel at approximately 1 h before use. The plates were allowed to attach for 4 h in a 37°C humidified incubator with 95% air and 5% CO2. Subsequently, the culture medium was aspirated and replaced with supplemented Williams’ Medium E (without 10% v/v heat-inactivated fetal bovine serum) containing Matrigel (20 μg/ml culture medium) (Discovery Labware, Inc., Franklin Lakes, NJ) for the formation of a sandwich culture. In transient transfection experiments, Matrigel was overlaid only after the transfection step. In gene expression experiments, at 24 h after plating, hepatocytes were treated for 48 h with bilobalide, phenobarbital (rCAR activator; positive control for CYP2B1 expression assay) (Yoshinari et al., 2001), dexamethasone (rPXR activator; positive control for CYP3A23 expression assay) (Lu and Li, 2001), or di-methyl sulfoxide (DMSO) (0.1% v/v; vehicle), as described in each figure legend. The chemical-containing culture medium was replaced daily.

Isolation, Culture, and Treatment of Human Hepatocytes. Donor demographics, isolation of human hepatocytes, and hepatocyte culture conditions were described previously (Lau et al., 2010). Human hepatocytes were treated for 72 h with bilobalide, CITCO (hCAR agonist; positive control for CYP2B6 expression assay; Fauchette et al., 2006), rifampicin (hPXR agonist; positive control for CYP3A4 expression assay; Fauchette et al., 2006), or DMSO (0.1% v/v; vehicle), as described in each figure legend. The chemical-containing culture medium was replaced daily.

Lactate Dehydrogenase Assay. Cultured rat hepatocytes (560,000 cells/well) and HepG2 cells (100,000 cells/well) were seeded onto 12- and 24-well plates, respectively. On the third day after plating, hepatocytes and HepG2 cells were treated for 24 h with bilobalide (1–100 μM), dextran (1% v/v; vehicle), or DMSO (0.1% v/v; vehicle) as positive controls. Enzyme activity was measured by a lactate dehydrogenase assay (Cayman Chemical, MI) according to the manufacturer’s instructions. Results are expressed as percent of control and are the mean ± S.E.M. of three separate experiments, each performed in triplicate.
were transfected for 24 h with a master mix containing FuGENE 6 transfection reagent on HepG2 cells. At 24 h after plating, HepG2 cells (100,000 cells/well) were transfected with a master mix containing Lipofectamine LTX reagent (5 µl/well), PLUS reagent (1 µl/well), Opti-MEM (600 µl/well), pGL4.74[hRluc/TK] internal control plasmid (100 ng/well), a reporter plasmid (800 ng/well), and either a receptor expression plasmid (100 ng/well) or the corresponding empty vector (pCMV6-AC or pCMV6-XL4; 100 ng/well). pGL3-basic-CYP2B1-luc reporter and pGL3-basic-CYP3A4-XREM-luc reporter were the reporter plasmids. pCMV6-AC-rCAR, pCMV6-XL4-hCAR, pCMV6-AC-rPXR, and pCMV6-XL4-hPXR were the receptor expression plasmids. At 5 h after transfection, the culture medium containing the transfection mixture was replaced with supplemented Williams’ Medium E (without 10% v/v heat-inactivated fetal bovine serum) containing Matrigel (20 µl/ml culture medium).

Transfected hepatocytes and HepG2 cells were treated for 24 h with 0.5 ml of fresh culture medium containing bilobalide, phenobarbital (a rCAR activator) (Yoshinari et al., 2001), CITCO (a hCAR agonist) (Maglich et al., 2000), TCPOBOP (a mouse CAR agonist) (Trameli et al., 2000), PCN (a rPXR activator) (Jones et al., 2000), rifampicin (a hPXR agonist) (Jones et al., 2000), a combination of the chemicals, or DMSO (0.1% v/v; vehicle), as detailed in each figure legend. Cells were lysed, and the firefly luciferase and R. reniformis luciferase activities were determined using a Dual-Luciferase Reporter Assay System. Luciferase activity was expressed as a normalized ratio of firefly luciferase to R. reniformis luciferase activity. Background luciferase activity was determined in cells transfected with pCMV6-XL4 (empty vector) or pCMV6-AC (empty vector). Data are expressed as a percentage of the luciferase activity in the corresponding positive control-treated group.

**Isolation of Total RNA and Reverse Transcription.** Cultured rat and human hepatocytes were harvested, and cell lysates from 2 to 4 wells were pooled. Isolation of total cellular RNA and reverse transcription were performed as described previously (Chang et al., 2006).

**PCR Primers.** The primer sequences for CYP2B1, CYP3A23, rCAR, rPXR, rat cyclophilin, CYP2B6, CYP3A4, hCAR, hPXR, and hHPRT cDNAs were used for reverse-transcribed RNA thermal cycling (LightCycler; Roche Diagnostics). Each 20-µl PCR mixture contained 1 ng of total cDNA (except that 5 ng was used for rCAR and 10 ng was used for rPXR), 1 U Platinum Taq DNA polymerase (Invitrogen) in a PCR buffer [20 mM Tris-HCl (pH 8.4) and 50 mM KCl], MgCl₂ (2 mM for hHPRT and hPXR; 3 mM for CYP2B1, CYP3A23, rPXR, and rat cyclophilin; 4 mM for CYP2B6, CYP3A4, and hCAR; and 5 mM for rCAR), 0.2 mM deoxyribonucleotide triphosphate, 0.25 mg/ml bovine serum albumin, 0.2 µM forward and reverse primers (except for hHPRT in which the concentration was 0.5 µM), and 1:300 SYBR Green I solution (Invitrogen). For all amplifications, the initial denaturation was performed at 95°C for 5 min. The cycling conditions for the amplification of CYP2B1 cDNA were 95°C for 1 s, 56°C for 6 s, and 72°C for 12 s. The cycling conditions for the amplification of CYP3A23 and rPXR cDNAs were 95°C for 1 s, 60°C for 6 s, and 72°C for 14 s. The cycling conditions for the amplification of rCAR cDNA were 95°C for 1 s, 60°C for 6 s, and 72°C for 12 s. The cycling conditions for the amplification of CYP2B6 cDNA were 94°C for 4 s, 65°C for 5 s, and 72°C for 15 s. The cycling conditions for the amplification of CYP3A4 and hHPRT cDNAs were 94°C for 5 s, 65°C for 10 s, and 72°C for 15 s. The cycling conditions for the amplification of hCAR cDNA were 95°C for 5 s, 65°C for 10 s, and 72°C for 25 s. The cycling

<table>
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<td>AGG-ATT-GCC-TTC-TTC-TCG-CC</td>
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<td>TGG-GTG-TCA-CCA-AAG-AGA-AG</td>
<td>Bauer et al., 2004</td>
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<td>AAT-GCA-TCC-COA-GAG-GTC-TG</td>
<td>Morris and Davila, 1996</td>
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<td>GGG-TGT-GTT-CTA-TTC-AAC-AA</td>
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<td>NM_000194</td>
<td>GAA-GAG-CTA-CTG-TCA-AGC-AG</td>
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**TABLE 1**

Primer sequences of the genes analyzed in the present study.
conditions for the amplification of hPXR cDNA were 95°C for 5 s, 65°C for 10 s, and 72°C for 15 s. A calibration curve (cross-point versus log cDNA copies) was constructed using known amounts of purified cDNA of each gene. Each of these purified cDNAs was amplified from human liver QUICK-Clone cDNA (Clontech Laboratories, Mountain View, CA) and quantified by Pi- coGreen Double-Stranded DNA Quantitation Kit. CYP2B1, CYP3A23, rCAR, and rPXR mRNA levels were normalized to rat cyclophilin mRNA level, whereas CYP2B6, CYP3A4, hCAR, and hPXR mRNA levels were normalized to hHPRT mRNA level.

Benzylxoyresorfin O-Dealkylation Assay. At the end of the treatment period, cultured rat hepatocytes were washed with phosphate-buffered saline (pH 7.4) and incubated for 60 min at 37°C with 0.5 ml of culture medium containing 7-benzyloxyresorufin (final concentration of 15 μM) and dicumarol (final concentration of 10 μM) dissolved in DMSO (final concentration of 0.1% v/v in culture medium). The benzylxoyresorfin O-dealkylation (BROD) assay was performed as described previously (Chang et al., 2006). The fluorescence intensity was measured at an excitation wavelength of 530 nm and an emission wavelength of 580 nm using a Synergy MX fluorescence microplate reader (BioTek, Winooski, VT). A standard curve was constructed with 10 to 150 pmol of resorufin.

Bupropion Hydroxylation Assay. At the end of the treatment period, cultured human hepatocytes were washed with Hanks’ balanced salt solution (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 ultrasensitive liquid chromatography-tandem mass spectrometry assay as described previously (Lau et al., 2011b).

Testosterone 6β-Hydroxylation Assay. At the end of the treatment period, cultured rat hepatocytes were washed with Krebs-Henseleit buffer (containing 12.5 mM HEPES; pH 7.4) and incubated for 10 min at 37°C with 0.5 ml of the buffer containing testosterone (final concentration of 200 μM) dissolved in DMSO (final concentration of 0.1% v/v in buffer). Subsequently, the catalytic reaction was stopped by transferring 400 μl of the incubation mixture into a microcentrifuge tube containing 200 μl of ice-cold acetonitrile. The contents were centrifuged at 10,000g for 3 min. At the end of the treatment period, cultured human hepatocytes were washed with 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), as described previously (Lau et al., 2010). In both rat and human hepatocytes experiments, the amount of 6β-hydroxysterosterone was quantified by a high-performance liquid chromatographic assay as described previously (Lau et al., 2010).

Statistical Analysis. Data were analyzed by one- or two-way analysis of variance as appropriate, 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.

Results

LDH Release in Cultured Hepatocytes and HepG2 Cells Treated with Bilobalide. In the first experiment, we determined the range of nontoxic concentrations of bilobalide by measuring LDH release, which is a marker of cytotoxicity (Jauregui et al., 1981). Bilobalide at 1 to 100 μM did not increase LDH release in cultured rat hepatocytes or HepG2 cells (Fig. 2). Control analysis indicated that dextran (1% v/v; negative control) and Triton X-100 (0.1% v/v; positive control) produced the expected results. Based on these data, subsequent experiments were conducted at bilobalide concentrations of up to 100 μM.

Bilobalide Activates rCAR but Not hCAR. To compare the effects of bilobalide on rCAR and hCAR activities with those of phenobarbital (rCAR activator; Yoshinari et al., 2001) and CPTCCO (hCAR activator; Maglich et al., 2003), reporter gene assays were conducted on primary cultures of rat hepatocytes and human HepG2 cells. As shown in cultured rat hepatocytes (Fig. 3A), bilobalide (100 μM) increased rCAR activity to a similar extent as that by phenobarbital (100 μM), which activated rCAR by 9-fold over the vehicle-treated control group. In contrast, CPTCO (1 μM; negative control for rCAR) did not increase rCAR activity (Fig. 3A). To determine the range of rCAR-activating concentrations of bilobalide, we conducted a detailed concentration-response experiment in rat hepatocytes. Bilobalide at concentrations of 0.1, 0.3, 1, 3, and 10 μM had no statistically significant effect on rCAR activity, as analyzed by one-way analysis of variance, whereas at 30, 60, and 100 μM, it increased the activity to 189, 176, and 115% of the level in the phenobarbital-treated group, respectively (Fig. 3B).

In contrast to rCAR, hCAR activity was not increased by bilobalide (100 μM) in cultured rat hepatocytes (Fig. 3C). By comparison, CPTCO (10 μM), which is a hCAR agonist (Maglich et al., 2003), increased hCAR activity by 4-fold over the vehicle-treated control group, whereas TCPBOB (1 μM; a negative control for hCAR) had no effect (Fig. 3C). As shown in Fig. 3D, the same pattern of response was obtained in cultured HepG2 cells treated with bilobalide (0.1–100 μM), CPTCO (10 μM), or TCPBOB (0.25 μM).

Bilobalide Increases rCAR but Not hCAR Target Gene Expression in Cultured Hepatocytes. To corroborate the activation of rCAR by bilobalide in the reporter gene assays (Fig. 3, A and B), we determined the effect of the chemical on the expression of a rCAR target gene (CYP2B1; Yoshinari et al., 2001) in primary cultures of rat hepatocytes. As shown in Fig. 4A, 1 μM bilobalide had no effect on CYP2B1 mRNA expression, whereas at 3, 10, and 30 μM, it increased the mRNA expression to 6, 18, and 37% of the levels in the phenobarbital-treated group, respectively. By comparison, phenobarbital (100 μM), which is a rat CYP2B2 inducer (Yoshinari et al., 2001), increased the expression by 48-fold over the vehicle-treated control group. Likewise, 1, 3, 10, and 30 μM bilobalide increased CYP2B-mediated BROD activity to 30, 44, 64, and 83% of the levels in the phenobarbital-treated group, respectively (Fig. 4B). As a positive control, phenobarbital (100 μM) increased BROD activity by 12-fold over the vehicle-treated control group.

We also determined the effect of bilobalide on the expression of a hCAR target gene (e.g., CYP2B6; Fauckett et al., 2006) in primary
cultures of human hepatocytes. As shown in Fig. 4C, bilobalide (10–60 μM) had no effect on CYP2B6 mRNA expression. In contrast, CITCO (0.1 μM), which is a hCAR agonist (Maglich et al., 2003) and a CYP2B6 inducer (Faucette et al., 2006), increased the expression by 20- and 37-fold over the vehicle-treated control group in samples Hu1043 and Hu1108, respectively (Fig. 4C). Consistent with the mRNA findings, bilobalide did not increase CYP2B6-catalyzed bupropion hydroxylation in human hepatocytes, whereas...
Bilobalide Does Not Activate rPXR or hPXR. To compare the effect of bilobalide on the activity of rPXR and hPXR, a cell-based reporter gene assay was conducted on primary cultures of rat hepatocytes and HepG2 cells treated with varying concentrations of bilobalide. This chemical (100 μM) did not increase rPXR activity in cultured rat hepatocytes (Fig. 5A). By comparison, PCN (10 μM), which is a rPXR agonist (Jones et al., 2000), increased it by 9-fold in hepatocytes, whereas rifampicin (10 μM), which is a hPXR agonist (Jones et al., 2000), had no effect on rPXR activity (Fig. 5A). Likewise, bilobalide (0.1–100 μM) also did not increase rPXR activity in HepG2 cells, whereas PCN (10 μM) increased it by 19-fold and rifampicin (10 μM) had no effect (Fig. 5B). hPXR was also not activated by bilobalide in cultured rat hepatocytes (100 μM; Fig. 5C) or HepG2 cells (0.1–100 μM; Fig. 5D). As expected, rifampicin (10 μM) increased hPXR activity in hepatocytes (11-fold) and HepG2 cells (12-fold), whereas PCN (10 μM) had no effect (Fig. 5, C and D).

Bilobalide Does Not Increase rPXR or hPXR Target Gene Expression in Cultured Hepatocytes. To evaluate the effect of bilobalide on the expression of a PXR target gene in primary cultures of rat or human hepatocytes, the mRNA and enzyme activity assays were performed. As shown in Table 2, bilobalide (1–50 μM) did not affect the mRNA level of each of these receptors in cultured rat or human hepatocytes.

Discussion

The present study provides the first demonstration that bilobalide is an activator of rCAR. Compared with other rCAR activators reported in the literature, bilobalide is one of the most efficacious. As assessed by a reporter gene assay conducted on rat hepatocytes, the extent of rCAR activation by bilobalide (100 μM) was similar to that by phenobarbital (100 μM), which is the prototypic rCAR activator (Yoshinari et al., 2001). Although it activates rCAR, our detailed dose-response experiment indicated that this chemical at concentra-
tions of up to 100 μM did not affect the activity of hCAR, as shown by a cell-based reporter gene assay. This result is in accord with that demonstrated in previous studies, indicating that bilobalide at concentrations of 50 μM (Li et al., 2009) and ~70 μM (Lau et al., 2011a) had no effect on hCAR activity. Consistent with the data from the reporter gene assays, bilobalide induced a rCAR target gene (CYP2B1) but not a hCAR target gene (CYP2B6). Collectively, our findings highlight the species-dependent activation of CAR by bilobalide. The amino acid sequence identity of rCAR and hCAR is 77% (Yoshinari et al., 2001). Due to the lack of structural studies on rCAR, the molecular determinants of species differences in chemical activation of rCAR and hCAR are currently not known. It is noteworthy that structure-function studies indicate that Phe-243 in hCAR is important for the species-dependent effect of 17α-ethynyl-3,17β-estradiol, which represses hCAR but activates mCAR (Jyrkkärinne et al., 2005). Given that rCAR has a high degree (91%) of amino acid sequence identity with mCAR (Yoshinari et al., 2001), it would be of interest to investigate the role of this amino acid in the species-dependent activation of rCAR by bilobalide.

Various chemicals demonstrate differential effects on the function of CAR and PXR. For example, PCN, dexamethasone, and mifepristone (also referred to as RU486) activate mPXR but not mCAR, whereas TCP0BOP activates mCAR but not mPXR (Moore et al., 2000). Another novel finding in our study is that bilobalide exhibits receptor selectivity for rCAR but not rPXR. To date, it is among a few chemicals shown to activate rCAR but not rPXR. Another chemical with the same receptor selectivity is squalestatin 1, which is an endogenous isoprenoid (Kocarek and Mercer-Haines, 2002). In fact, there are more chemicals that activate PXR than CAR (Chang and Waxman, 2006). This may relate to the notion that the ligand-binding pocket of PXR has a greater flexibility and a larger volume than that of CAR (Timsit and Negishi, 2007).

Pronounced species differences exist in chemical activation of rPXR and hPXR (Jones et al., 2000). The ligand-binding domains of these two receptors share approximately 76% amino acid identity (Jones et al., 2000). Site-directed mutagenesis studies have identified several amino acids, such as Phe-305 and Asp-318 in rPXR and Leu-308 in hPXR, that account for the species differences in PXR activation (Tirona et al., 2004; Song et al., 2005). Previously, it was reported that bilobalide at concentrations of ~35 μM (Lau et al., 2010) and 50 μM (Li et al., 2009) had no effect on hPXR activity. In the current study, we confirmed the lack of hPXR activation by bilobalide over the concentration range of 0.1 to 100 μM and showed for the first time that it did not activate rPXR. Therefore, bilobalide, which is a sesquiterpene trilactone, is not expected to produce PXR-mediated actions in rats or humans. It is noteworthy that a naturally occurring sesquiterpene lactone known as artemisinin was shown to activate hPXR (Burk et al., 2005).

To date, several antagonists of CAR and PXR have been identified. For example, PK11195 is a hCAR antagonist (Li et al., 2008), whereas ketoconazole is an antagonist of rPXR and hPXR (Huang et al., 2007). In the present study, our data indicate that bilobalide is not an antagonist of hCAR, rPXR, or hPXR. This conclusion is based on the findings that it did not attenuate the magnitude of the luciferase activity in receptor plasmid-transfected cells treated with an agonist of hCAR (CITCO), rPXR (PCN), or hPXR (rifampicin). However, it should be noted that under the same assay conditions, the positive controls (PK11195 and ketoconazole) produced the expected results. As shown in the reporter gene assays to assess receptor activation (Figs. 3, C and D, and 5, A–D) and the corresponding target gene expression assays (Figs. 4, C and D, and 6, A–D), this naturally occurring sesquiterpene trilactone was not an activator of hCAR, rPXR, or hPXR. Collectively, these data suggest that bilobalide is not a ligand of these receptors.

Bilobalide increased CYP2B1 gene expression in cultured rat hepatocytes. In principle, this effect may be the consequence of increased transcriptional activation of rCAR and/or increased receptor expression. However, according to our real-time PCR analyses, bilobalide
CYP3A4 for 10 days (Deng et al., 2008a). Taken together, the data from the ex vivo rat studies raise the possibility that a metabolite(s) of bilobalide is formed in vivo rat studies are inconclusive. In a cell culture study, bilobalide at submicromolar concentrations (2–50 ng/ml; equivalent to 0.006–0.153 μM) was reported to increase CYP3A expression in rat and human hepatocytes (Deng et al., 2008b). However, this was not observed in two other studies that were conducted with micromolar concentrations of this chemical in rat (3–15 μM Chang et al., 2006) or human hepatocytes (50 μM Li et al., 2009). In the present study, bilobalide also did not affect the mRNA or enzyme activity levels of CYP3A in rat (1–30 μM) and human hepatocytes (1–60 μM). The reason for the conflicting data is not clear. However, it is not likely that bilobalide is an inducer of CYP3A. As shown in cell-based reporter gene assays, it did not activate rPXR (present study) or hPXR (Li et al., 2009 and present study). As mentioned above, the expression of CYP3A is regulated predominantly by PXR (Faucette et al., 2006). Collectively, the results from the ex vivo and in vitro rat studies raise the possibility that a metabolite(s) of bilobalide is formed.

### TABLE 2

**CAR and PXR mRNA expression in cultured rat and human hepatocytes treated with bilobalide**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
<th>Rat Hepatocytes</th>
<th>Human Hepatocytes</th>
<th>Fold increase over control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rCAR</td>
<td>hCAR</td>
<td>rPXR</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.1% v/v</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>N.A.</td>
</tr>
<tr>
<td>Bilobalide</td>
<td>1 μM</td>
<td>1.3 ± 0.5</td>
<td>1.1 ± 0.1</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>3 μM</td>
<td>1.3 ± 0.4</td>
<td>1.0 ± 0.3</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>10 μM</td>
<td>1.1 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>25 μM</td>
<td>N.A.</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>1.0 ± 0.3</td>
<td>0.8 ± 0.5</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N.A.</td>
<td>N.A.</td>
<td>1.4 ± 0.0</td>
</tr>
</tbody>
</table>

N.A., not available.
in vivo in rats but not in cultured hepatocytes, and that it is the metabolite(s) that is responsible for the previously reported (e.g., Suzuki et al., 2004) induction of hepatic CYP3A in rats treated with bilobalide. Future studies will be needed to address this possibility.

In conclusion, bilobalide has a species-dependent and receptor-selective effect on CAR and PXR. It activates rCAR, but it does not activate or antagonize hCAR, rPXR, or hPXR. Therefore, in humans, bilobalide is not expected to alter the disposition of drugs that are metabolized or transported by CAR/PXR-regulated drug-metabolizing enzymes or transporters. Thus, our study provides insight into the safety of bilobalide in humans. Due to the contrasting findings in rat and human receptors and enzymes, our study also serves as a reminder of the importance of evaluating the actions of drugs and other chemicals in the relevant species of interest.

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Authorship Contributions

Participated in research design: Lau and Chang.
Conducted experiments: Lau, Yang, Rajaraman, and Baucom.
Performed data analysis: Lau.
Wrote or contributed to the writing of the manuscript: Lau and Chang.

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