Human Pregnane X Receptor Agonism by *Ginkgo biloba* Extract: Assessment of the Role of Individual Ginkgolides

Aik Jiang Lau, Guixiang Yang, Ganesh Rajaraman¹, Christie C. Baucom, and Thomas K. H. Chang

Running Title: Human PXR Agonism by Ginkgo biloba

Address correspondence to: Thomas K. H. Chang, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, BC, V6T 1Z3, Canada. E-mail address: tchang@interchange.ubc.ca.

Number of text pages: 38
Number of tables: 2
Number of figures: 8
Number of references (maximum of 40): 40
Number of words in Abstract (maximum of 250): 239
Number of words in Introduction (maximum of 750): 645
Number of words in Discussion (maximum of 1500): 1477

ABBREVIATIONS: DMSO, dimethyl sulfoxide; HBSS, Hanks’ Balanced Salt Solution; HPRT, human hypoxanthine phosphoribosyltransferase 1; hPXR, human pregnane X receptor; PCN, pregnenolone 16α-carbonitrile; PCR, polymerase chain reaction; PXR, pregnane X receptor; rPXR, rat pregnane X receptor; SR12813, tetraethyl 2-(3,5-di-t-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate; SRC-1, steroid receptor coactivator-1; TO901317, N-(2,2,2-trifluoro-ethyl)-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl] benzenesulfonamide; TR-FRET, time-resolved fluorescence resonance energy transfer.

Recommended Section Assignment: Cellular and Molecular
JPET #172338

ABSTRACT

*Ginkgo biloba* extract activates pregnane X receptor (PXR), but how this occurs is not known. Therefore, we investigated the mechanism of PXR activation by the extract and the role of five individual terpene trilactones in the activation. In a cell-based reporter gene assay, *G. biloba* extract activated human PXR (hPXR), and at a concentration present in the extract, ginkgolide A, but not ginkgolide B, ginkgolide C, ginkgolide J, or bilobalide, was partially responsible for the increase in hPXR activity of the extract. Similarly, in cultured human hepatocytes, only ginkgolide A contributed to the increase in hPXR target gene expression (CYP3A4 mRNA and CYP3A-mediated testosterone 6β-hydroxylation). The extract, but none of the terpene trilactones, bound to hPXR ligand-binding domain, as analyzed by time-resolved fluorescence resonance energy transfer competitive binding assay. Only the extract and ginkgolide A recruited SRC-1, as determined by a mammalian two-hybrid assay. When compared to hPXR, rat PXR (rPXR) was activated to a lesser extent by *G. biloba* extract. Similar to hPXR, only ginkgolide A contributed to rPXR activation by the extract. In contrast to the effect of *G. biloba* extract on PXR function, it did not affect hPXR expression. Overall, the main conclusions are that *G. biloba* extract is a hPXR agonist and among the five terpene trilactones investigated, only ginkgolide A contributes to the actions of the extract. Our findings provide insights into the biological and chemical mechanisms of hPXR activation by *G. biloba* extract.
Introduction

Pregnane X receptor (PXR; NR1I2) is a ligand-activated nuclear hormone receptor that is expressed predominantly in the liver (Lehmann et al., 1998). It was initially identified as a “xenobiotic sensor” that plays a central role in regulating the expression of genes involved in the detoxification, bioactivation, and transport of endogenous and exogenous chemicals (Zhou et al., 2009). However, it is now known that PXR also impacts on the expression of genes that regulate various physiological and pathological processes, including lipid metabolism, bile acid homeostasis, energy homeostasis, and inflammation (Zhou et al., 2009). These potential roles of PXR in disease pathogenesis suggest PXR as a novel target for drug discovery.

PXR transcriptional activity may be modulated by numerous ligands, including drugs, environmental toxicants, phytochemicals, and herbal medicines (Chang and Waxman, 2006). The structural diversity of PXR ligands is due in part to the large and flexible ligand-binding domain of the receptor. Extensive research has now shown that ligand activation of PXR occurs by a series of complex signaling pathways (Timsit and Negishi, 2007). Upon ligand binding, the ligand-receptor complex dissociates from co-chaperone partners (cytoplasmic CAR retention protein and heat shock protein 90) and translocates from the cytoplasm to the nucleus. The activated PXR then forms a heterodimer with retinoid X receptor α, recruits coactivators [e.g. steroid receptor coactivator-1 (SRC-1); also known as NCoA-1], releases corepressors, binds to DNA responsive elements in the promoter or enhancer regions of PXR target genes, and leads to an increase in gene transcription.

*Ginkgo biloba* is one of the top ten most frequently used natural products in the United States (Barnes et al., 2008). Its reported biological/pharmacological activities include free radical scavenging activity, inhibition of platelet-activating factor, stimulation of endothelium-
derived relaxing factor, and suppression of peripheral benzodiazepine receptor expression in the cerebral cortex (Smith and Luo, 2004). It is often used to improve cognitive function in neurodegenerative conditions, such as Alzheimer’s disease (Weinmann et al., 2010). Recently, *G. biloba* extract was identified as a PXR activator (Yeung et al., 2008; Li et al., 2009), and it increased the expression of target genes in LS180 human colon adenocarcinoma cells (*CYP3A4*, *CYP3A5*, and *ABCB1*) and cultured human hepatocytes (*CYP3A4*) (Deng et al., 2008; Yeung et al., 2008; Li et al., 2009). However, the mechanism by which it activates PXR is not known. *G. biloba* extract contains approximately 6% w/w terpene trilactones, such as ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide (Fig. 1), 24% w/w flavonol glycosides, and other chemical classes, such as alkylphenols, organic acids, proanthocyanidins, catechins, biflavones, and non-flavonol glycosides (van Beek and Montoro, 2009). Previous reports have shown that ginkgolide A, ginkgolide B, and ginkgolide C activated human PXR (hPXR) in an *in vitro* cell-based reporter gene assay (Satsu et al., 2008; Li et al., 2009). However, the concentrations used in those studies are greater than the levels present in *G. biloba* extract. Therefore, it is not known whether any of the individual ginkgolides is responsible for hPXR activation by the extract.

The primary objectives of the current study were to: 1) determine whether *G. biloba* extract binds to hPXR and recruits SRC-1 coactivator to the receptor; and 2) investigate the role of five individual terpene trilactones (ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide) in the effects of *G. biloba* extract on hPXR activity. Given the pronounced species differences in ligand activation of PXR (Jones et al., 2000), a secondary objective was to compare the effect of *G. biloba* extract and terpene trilactones on rat PXR (rPXR) and hPXR activities. In addition to function, the expression of PXR may also be altered by a drug (Pascussi
et al., 2000). Therefore, we also assessed the effect of *G. biloba* extract on hPXR mRNA levels in cultured human hepatocytes. Our novel findings provide insights into the biological and chemical mechanisms of hPXR activation by *G. biloba* extract.


Materials and Methods

G. biloba extract, chemicals, and reagents. G. biloba extract (in dry powder form) was provided by Indena S.A. (Milan, Italy). The extract contained 1.1% w/w ginkgolide A, 0.3% w/w ginkgolide B, 1.4% w/w ginkgolide C, 0.6% w/w ginkgolide J, and 2.8% w/w bilobalide, as determined by gas chromatography (Indena S.A., Milan, Italy); it also contained 6.3% w/w kaempferol glycosides, 10.6% w/w quercetin glycosides, and 4.1% w/w isorhamnetin glycosides, as analyzed by liquid chromatography-mass spectrometry (ChromaDex, Inc., Irvine, CA). Ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide were obtained from LKT Laboratories, Inc. (St. Paul, MN), and ginkgolide J was from ChromaDex. Tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate (SR12813) was purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). Rifampicin, pregnenolone 16α-carbonitrile (PCN), N-(2,2,2-trifluoro-ethyl)-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl] benzenesulfonamide (TO901317), phenobarbital, testosterone, dextran, Triton® X-100 (Union Carbide), dimethyl sulfoxide (DMSO), trypan blue, and Tris-HCl were purchased from Sigma-Aldrich (St Louis, MO). 6β-hydroxytestosterone was purchased from Steraloids, Inc. (Newport, RI). Minimum essential medium, L-glutamine, penicillin G-streptomycin, heat-inactivated fetal bovine serum, trypsin-EDTA, phosphate-buffered saline (pH 7.4), EDTA, Opti-MEM®, Hank’s Balanced Salt Solution (HBSS), PureLink® RNA Mini Kit, PicoGreen® Double-Stranded DNA Quantification Kit, and LanthaScreen® TR-FRET PXR Competitive Binding Assay were purchased from Invitrogen (Carlsbad, CA). Charcoal-stripped, heat-inactivated fetal bovine serum (Hyclone) was bought from Fisher Scientific, Inc. (Nepean, ON, Canada). FuGENE® 6 transfection reagent (Fugent) and Cytotoxicity Detection Kit (LDH)® were 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 isolation of total RNA, reverse transcription, and real-time polymerase chain reaction (PCR) analyses (Chang et al., 2006a) were described previously.

**Plasmids.** pCMV6-XL4-hPXR expression plasmid, pCMV6-XL4 (empty vector), pCMV6-AC-rPXR expression plasmid, and pCMV6-AC (empty vector) 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), was constructed as described previously (Goodwin et al., 1999). The pVP16 and pM empty 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 (a division of Agilent Technologies, Inc., Santa Clara, CA). To construct the pVP16-hPXR-LBD plasmid, the ligand-binding domain (Lys 107 to Ser 443) of hPXR (Synold et al., 2001) was amplified from pCMV6-XL4-hPXR and inserted into the pVP16 vector at the EcoRI and BamHI sites. The primers used to amplify the ligand-binding domain of hPXR were: 5′-GGA-GGA-ATT-CAA-GAA-GGA-GAT-GAT-CAT-GTC-C-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 at the EcoRI and BamHI sites. The primers used to amplify the nuclear receptor-interacting domain of hSRC-1 were: 5′-GGA-GGA-ATT-CCA-GGA-GAT-GAT-CAT-GTC-C-3′ (forward) and 5′-GGG-AGG-ATC-CTG-GTG-TAG-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. Culture medium was changed every two or three days, and cells were sub-cultured weekly.

**Lactate Dehydrogenase (LDH) Assay.** The LDH assay was performed essentially as described previously (Rajaraman et al., 2006). Human PXR-transfected cells were treated with culture medium (vehicle control), *G. biloba* extract (200, 400, 600, or 800 µg/ml), dextran (1% v/v; negative control), or Triton® X-100 (1% v/v; positive control). At the end of the 24 h treatment period, culture medium was collected and cells were lysed. To determine intracellular LDH release, 5 µl aliquot of culture medium or 1 µl aliquot of cell lysate was transferred into the wells of a 96-well microplate containing 95 µl or 99 µl of phosphate-buffered saline (pH 7.4), respectively. A 100 µl of LDH reaction mixture (Cytotoxicity Detection Kit) was added into each well, and the plate was incubated for 30 min at room temperature. The absorbance was measured at 492 nm using a Multiskan Ascent® microplate reader (Thermo Fisher Scientific Inc., Waltham, MA). The amount of LDH release into the culture medium was expressed as a percentage of the total cellular LDH content (sum of LDH content in culture medium and cell lysate).
Reporter Gene Assay. PXR-dependent reporter activity was determined as described previously (Yeung et al., 2008). HepG2 cells were seeded onto 24-well microplates at a density of 100,000 cells per well and in a volume of 0.5 ml culture medium. At 24 h after plating, cells were transfected for 24 h with 20 μl of a transfection master mix containing FuGENE® 6 transfection reagent (0.6 μl per well), serum-free Opti-MEM® (19.2 μl per well), pGL4.74[hRluc/TK] internal control vector (5 ng per well), pGL3-basic-CYP3A4-XREM-luc reporter construct (50 ng per well), pCMV6-XL4-hPXR expression plasmid (50 ng per well), pCMV6-AC-rPXR expression plasmid (50 ng per well), pCMV6-XL4 (50 ng per well; empty vector), or pCMV6-AC (50 ng per well; empty vector). Transfected HepG2 cells were treated for 24 h with 0.5 ml of fresh supplemented culture medium containing G. biloba extract, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, bilobalide, a mixture of the five terpene trilactones, rifampicin (positive control for hPXR assay) (Jones et al., 2000), PCN (positive control for rPXR assay) (Jones et al., 2000), or vehicle (culture medium for G. biloba extract or 0.1% v/v DMSO for individual chemicals), as detailed in each figure legend. At the end of the 24 h treatment period, transfected HepG2 cells were lysed for the determination of firefly luciferase and Renilla luciferase activities using a Dual-Luciferase® Reporter Assay System (Promega Corporation). Luminescence was measured using a GloMax™ 96 microplate luminometer (Promega Corporation). 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 the empty vector, and the value was subtracted from the normalized luciferase activity. Fold-activation was calculated by dividing the net luciferase activity of the treatment group by that of the vehicle-treated control group.
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. *G. biloba* extract and each of the test chemicals were diluted to two times of their final concentration using TR-FRET PXR Assay Buffer (Invitrogen), and 10 μl of the extract or chemical was dispensed into triplicate wells of a white, non-treated 384-well assay plate (catalog #3674, Corning, Inc., Lowell, MA). A 5 μl aliquot of diluted Fluormone PXR Green (a fluorescein-labeled PXR ligand; final concentration of 40 nM in assay buffer) was then added into each well. A master mix containing PXR 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) in assay buffer was prepared. A 5 μl aliquot of the PXR master mix was added into each well, and the content was mixed for 10 s. The plate was protected from light and incubated 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, except for the absence of PXR ligand-binding domain. Net TR-FRET ratio was calculated by subtracting the background TR-FRET ratio from the TR-FRET ratio. Data are expressed as a percentage of net TR-FRET ratio in the vehicle-treated control group. Preliminary experiments
showed that rifampicin quenched the fluorescence in this assay. Consequently, it was not used as a positive control.

**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, cells were transfected 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, culture medium containing the transfection mixture was removed and cells were treated for 24 h with 0.5 ml of fresh supplemented culture medium containing *G. biloba* extract (400 μg/ml), ginkgolide A (4.4 μg/ml), rifampicin (10 μM; positive control) (Jones et al., 2000), PCN (10 μM; negative control) (Jones et al., 2000), or vehicle (culture medium for *G. biloba* extract or 0.1% v/v DMSO for individual chemicals), as detailed in the 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 rifampicin-treated group.

**Isolation, Culture, and Treatment of Human Hepatocytes.** Hepatocytes were isolated from resected hepatic tissue samples (LeCluyse et al., 2005), which were obtained from three donors (see Table 1 for donor demographics). Cell viability was at least 90%, as determined by trypan blue exclusion. Human hepatocytes were suspended in Dulbecco’s modified Eagle’s medium supplemented with insulin (4 μg/ml), dexamethasone (1 μM), and 5% fetal bovine serum, and were cultured at a density of 375,000 cells per 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%
Hepatocytes were allowed to attach for 2-4 h. Subsequently, the culture medium containing unattached cells was aspirated, and fresh supplemented medium containing 1X ITS+ (Becton Dickinson, Bedford, MA) and Geltrex™ (0.35 mg/ml; Invitrogen, Carlsbad, CA) was added to each well. Plates were returned to the incubator and hepatocytes were cultured for another 36-48 h prior to the initiation of drug treatment. Medium was replaced daily. Human hepatocytes in culture were treated with *G. biloba* extract, culture medium (vehicle for *G. biloba* extract), ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, bilobalide, rifampicin (positive control for CYP3A4 expression) (Pascussi et al., 2000), or DMSO (0.1% v/v; vehicle for individual chemicals), as described in each figure legend. The extract- or chemical-containing culture medium was replaced every 24 h for a period of 72 h, which had been shown to yield maximum CYP3A4 induction (Faucette et al., 2004).

**Total RNA Isolation and Reverse Transcription.** Cultured human hepatocytes were harvested and total cellular RNA was isolated using PureLink® RNA Mini Kit, according to the manufacturer’s protocol. Total RNA was quantified by spectrophotometric method and transcribed using Superscript® II reverse transcriptase, and total cDNA was quantified using PicoGreen® dsDNA Quantitation Kit.

**PCR Primers.** The sequences of primers for amplification of CYP3A4 cDNA (GenBank accession number NM_017460) were 5’-CCT-TAC-ACA-TAC-ACA-CCC-TTT-GGA-AGT-3’ (forward) and 5’-AGC-TCA-ATG-CAT-GTA-CAG-AAT-CCC-CGG-TTA-3’ (reverse) (Schuetz et al., 1996), for amplification of human hypoxanthine phosphoribosyltransferase 1 (HPRT) cDNA (GenBank accession number NM_000194) were 5’-GAA-GAG-CTA-TTG-TAA-TGA-CCG-TTA-3’ (forward) and 5’-GCG-ACC-TTG-ACC-ATC-TTT-G-3’ (reverse) (Qiu et al., 2007), and for amplification of hPXR cDNA (GenBank accession number NM_003889) were 5’-CAA-
GCG-GAA-GAA-AAG-TGA-ACG-3’ (forward) and 5’-CAC-AGA-TCT-TTC-CGG-ACC-TG-3’ (reverse) (Chang et al., 2003). The primers were synthesized by Integrated DNA Technologies (Coralville, IA), and their specificity was verified by sequencing the purified PCR amplicons at the University of British Columbia Nucleic Acid Protein Service Unit.

**Real-Time PCR Analysis.** Real-time PCR analyses were performed using LightCycler® (Roche Diagnostics). CYP3A4 and hPXR cDNA were amplified using the PCR conditions described previously (Yeung et al., 2008), except that 2 mM of MgCl₂ was used in the amplification of hPXR cDNA and 1 ng of total cDNA was used in the amplification of CYP3A4 and hPXR cDNA. For HPRT cDNA (housekeeping gene) amplification, each 20 µl PCR reaction contained 1 ng total cDNA, 1 U Platinum Taq DNA polymerase in 1X PCR reaction buffer [20 mM Tris-HCl (pH 8.4) and 50 mM KCl], 2 mM MgCl₂, 0.2 mM deoxynucleoside triphosphate, 0.25 mg/ml bovine serum albumin, 0.5 µM forward and reverse primers, and 1:30,000 SYBR® Green I solution. The PCR conditions for HPRT cDNA were 95°C for 5 min (initial pre-incubation), followed by 40 cycles of 94°C for 5 s (denaturation), 60°C for 10 s (annealing), and 72°C for 15 s (extension). A calibration curve (cross point versus log cDNA copies) was constructed using known amount of purified CYP3A4, hPXR, or HPRT cDNA, which was amplified from human liver QUICK-Clone™ cDNA (Clontech Laboratories, Inc., Mountain View, CA) and quantified by PicoGreen® dsDNA Quantitation Kit.

**Testosterone 6β-Hydroxylation Assay.** Cultured human hepatocytes were washed with HBSS. Subsequently, the cells were 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 incubation mixture was then transferred into the wells of a 96-well plate and stored at -80°C until analysis. The testosterone 6β-hydroxylation assay was performed
by a high performance liquid chromatographic assay (Chang et al., 2006a). The amount of 6β-hydroxytestosterone metabolite in a 50 µl aliquot of the incubation mixture was analyzed by a HPLC system (Waters Corporation, Milford, MA) equipped with Waters model 1525 binary pump, Waters model 717 plus autosampler, and Waters model 2487 dual wavelength absorbance detector. Chromatography was performed at 25°C on a Waters SymmetryShield™ C18 column (150 x 4.6 mm i.d., 5 µm) linked to a Phenomenex® SecurityGuard™ cartridge (4.0 x 3.0 mm i.d., 5 µm). The mobile phases consisted of (A) water containing 0.5% v/v acetic acid and (B) methanol containing 0.5% v/v acetic acid. The 6β-hydroxytestosterone analyte was detected at a wavelength of 242 nm and eluted at a flow rate of 0.8 ml/min with the following conditions: linear gradient from 55% to 90% B (0-8 min), isocratic at 90% B (8-10 min), linear gradient from 90% to 55% B (10-11 min), and isocratic at 55% B (11-25 min). Data were acquired and processed using Waters Breeze software (version 3.20). A calibration curve was constructed with authentic 6β-hydroxytestosterone standard (75 to 12,500 pmol; diluted in HBSS) for each experiment.

Statistical Analysis. Data were analyzed by one-way or two-way ANOVA, and when significant differences were detected, the Student Newman-Keuls multiple comparison test was performed (SigmaPlot 11.0, Systat Software, Inc., Chicago, IL). The level of statistical significance was set a priori at $P < 0.05$. 

15
Results

Effect of *G. biloba* Extract on LDH release in Cultures of hPXR-Transfected HepG2 Cells. To determine whether *G. biloba* extract affected cell viability in transfected HepG2 cells, we conducted a LDH assay on hPXR-transfected HepG2 cells treated with 200-800 µg/ml of the extract. As shown in Fig. 2, *G. biloba* extract did not increase LDH release in hPXR-transfected HepG2 cells, as compared to the LDH release by the vehicle-treated control group. Control analysis indicated that dextran (1% v/v; negative control) did not increase LDH release, whereas Triton® X-100 (1% v/v; positive control) resulted in complete LDH release. LDH assay was not performed using extract concentrations greater than 800 µg/ml due to solubility problems. Based on these LDH data, subsequent experiments were conducted using *G. biloba* extract at concentrations up to a maximum of 800 µg/ml.

Comparative Effect of *G. biloba* Extract, PCN, and Rifampicin on rPXR and hPXR Activation in Cultured HepG2 Cells. The effects of *G. biloba* extract, PCN (positive control for rPXR), and rifampicin (positive control for hPXR) on rPXR- and hPXR-dependent reporter gene activities were compared in cultures of transfected HepG2 cells. As shown in Fig. 3, *G. biloba* extract (600 µg/ml) increased rPXR activity by 22-fold over culture medium-treated control group, and the fold-increase was less than that by PCN (10 μM; 35-fold). In contrast, the extract activated hPXR (38-fold) to a greater extent than that by rifampicin (10 μM; 24-fold over the DMSO-treated control group). There was a species difference in PXR activation by *G. biloba* extract, PCN, and rifampicin, as determined by two-way ANOVA. As expected, rifampicin and PCN had no effect on rPXR or hPXR activity, respectively.

Concentration-Dependent Effect of *G. biloba* Extract on rPXR and hPXR Activation in Cultured HepG2 Cells. A concentration-response experiment was conducted by treating
rPXR- or hPXR-transfected HepG2 cells for 24 h with six concentrations (30, 100, 200, 400, 600, or 800 µg/ml) of *G. biloba* extract. Fig. 4A shows that *G. biloba* extract, at 30-200 µg/ml, had no effect on rPXR-dependent reporter gene activity, but it increased the activity in a log-linear manner at 400-800 µg/ml. At 400, 600, and 800 µg/ml, the extract increased rPXR activity by 13-, 16-, and 20-fold, respectively. Comparatively, *G. biloba* extract, at 30-100 µg/ml, had no effect on hPXR activity, but it increased the activity in a log-linear manner at 200-800 µg/ml. At 200, 400, 600, and 800 µg/ml concentrations, the extract increased hPXR activity by 13-, 27-, 34-, and 46-fold, respectively. Overall, *G. biloba* extract activated hPXR to a greater extent than rPXR.

**Role of Terpene Trilactones in rPXR and hPXR Activation by *G. biloba* Extract in Cultured HepG2 Cells.** In this study, we used a *G. biloba* extract with known levels of terpene trilactones, namely 1.1% w/w ginkgolide A, 0.3% w/w ginkgolide B, 1.4% w/w ginkgolide C, 0.6% w/w ginkgolide J, and 2.8% w/w bilobalide. To determine whether these terpene trilactones were responsible for the increase in rPXR- and hPXR-dependent reporter gene activities by the extract, transfected HepG2 cells were treated with an individual terpene trilactone or a mixture of terpene trilactones at levels present in a PXR-activating concentration of the extract. A concentration of 400 µg/ml extract was selected because it was a log-linear concentration that activated both rPXR and hPXR. When tested at levels present in 400 µg/ml of *G. biloba* extract, ginkgolide A (4.4 µg/ml) activated rPXR by 6-fold and hPXR by 4-fold, whereas ginkgolide B (1.2 µg/ml), ginkgolide C (5.6 µg/ml), ginkgolide J (2.4 µg/ml), and bilobalide (11.2 µg/ml) had no effect on both receptors (Fig. 4B). By comparison, the extract activated rPXR by 12-fold and hPXR by 19-fold in the same experiment. Therefore, ginkgolide A accounted for 47% of rPXR activation and 22% of hPXR activation. A mixture of the five
individual terpene trilactones (at the above concentrations) activated rPXR and hPXR to a similar extent as that by ginkgolide A alone. Given the extent of rPXR activation by *G. biloba* extract did not reflect that of hPXR activation (Fig. 4A) and the qualitatively similar contribution of the individual terpene trilactones to rPXR and hPXR activities (Fig. 4B), subsequent experiments focused only on hPXR.

**Binding of *G. biloba* Extract to hPXR Ligand-Binding Domain and Role of Terpene Trilactones.** A TR-FRET competitive ligand binding assay was conducted to determine whether *G. biloba* extract binds to the ligand-binding domain of hPXR. Preliminary experiment showed that extract concentration at \( \leq 30 \mu g/ml \) did not quench fluorescence. Therefore, a concentration of 30 \( \mu g/ml \) was selected for subsequent experiments. As shown in Fig. 5, *G. biloba* extract decreased TR-FRET emission ratio to 33\% of vehicle control (assay buffer), whereas a maximal binding concentration (10 \( \mu M \)) of efficacious PXR ligands SR12813 (Jones et al., 2000) and TO901317 (Mitro et al., 2007) decreased the emission ratio to 8\% and 5\% of vehicle control (DMSO), respectively. By comparison, the extent of binding by the extract was similar to that by 1000 \( \mu M \) phenobarbital (58\% of vehicle control), which is another known agonist of hPXR (Jones et al., 2000). As expected, PCN (10 \( \mu M \); negative control) had no effect. To determine whether terpene trilactones were responsible for the hPXR binding by *G. biloba* extract, the binding assay was conducted with each of the five individual terpene trilactones at concentrations equivalent to those present in 30 \( \mu g/ml \) of *G. biloba* extract. Ginkgolide A (0.33 \( \mu g/ml \)), ginkgolide B (0.09 \( \mu g/ml \)), ginkgolide C (0.42 \( \mu g/ml \)), ginkgolide J (0.18 \( \mu g/ml \)), and bilobalide (0.84 \( \mu g/ml \)) did not decrease TR-FRET emission ratio, as compared to the vehicle-treated control group.
Effect of *G. biloba* Extract and Ginkgolide A on Recruitment of SRC-1 Coactivator to hPXR. Transactivation of hPXR can lead to recruitment of various coactivators, such as steroid receptor coactivator-1 (SRC-1) (Timsit and Negishi, 2007). To investigate whether activation of hPXR by *G. biloba* extract and ginkgolide A resulted in recruitment of SRC-1, we conducted a mammalian two-hybrid assay in HepG2 cells transfected with pVP16-hPXR-LBD expression plasmid, pM-hSRC1-RID plasmid, pFR-luc reporter plasmid, and the internal control pGL4.74[hRluc/TK] plasmid. Transfected cells were treated with *G. biloba* extract (400 µg/ml), ginkgolide A (4.4 µg/ml), or the corresponding vehicle. As shown in Fig. 6, in HepG2 cells co-transfected with both SRC-1 and hPXR ligand-binding domain, *G. biloba* extract increased the reporter activity to 25% of that by the rifampicin-treated group. At a level present in a 400 µg/ml concentration of the extract, ginkgolide A increased the reporter activity to 22% of the level in the rifampicin-treated group. As expected, PCN (negative control) had no effect. Control analysis indicated that when the cells were transfected with SRC-1 alone, none of these treatment groups increased the reporter activity over the vehicle-treated control group.

Concentration-Dependent Effect of *G. biloba* Extract on PXR Target Gene Expression in Cultured Human Hepatocytes. The next experiment was to characterize the effect of *G. biloba* extract on the transcription of a hPXR target gene (*CYP3A4*) in human hepatocytes in culture. The extract increased CYP3A4 mRNA expression in a log-linear manner at concentrations 25-200 µg/ml. At less than 25 µg/ml, the extract had minimal or no effect, whereas at 200 µg/ml, it increased the mRNA level to 11-fold in sample Hu1043 (Fig. 7A) and 17-fold in sample Hu1108 (Fig. 7B). Compared to the extract, rifampicin (10 µM; positive control) increased CYP3A4 mRNA expression by 44-fold in Hu1043 and 42-fold in Hu1108 (data not shown). To corroborate the gene expression findings, testosterone 6β-hydroxylation...
was determined in the same wells as those used for CYP3A4 mRNA analyses. At concentrations less than 50 µg/ml, the extract had minimal or no effect on testosterone 6β-hydroxylation, but at 50-200 µg/ml, it increased the catalytic activity in a log-linear manner by 4- to 11-fold in Hu1043 (Fig. 7C) and 3- to 13-fold in Hu1108 (Fig. 7D). In the same experiment, rifampicin increased testosterone 6β-hydroxylation by 51-fold in both Hu1043 and Hu1108 (data not shown).

**Role of Terpene Trilactones in the Induction of a PXR Target Gene by *G. biloba* Extract in Cultured Human Hepatocytes.** To evaluate the role of terpene trilactones in CYP3A4 induction by *G. biloba* extract, cultured human hepatocytes were treated with each of the five individual terpene trilactones at levels present in a CYP3A4-inducing concentration (100 µg/ml) of the extract. Ginkgolide A (1.1 µg/ml) increased CYP3A4 mRNA levels by 5-fold in Hu1108 (Fig. 8A) and 3-fold in Hu1138 (Fig. 8B), whereas ginkgolide B (0.3 µg/ml), ginkgolide C (1.4 µg/ml), ginkgolide J (0.6 µg/ml), and bilobalide (2.8 µg/ml) had minimal or no effect on CYP3A4 mRNA expression in Hu1108 and Hu1138. Comparatively, ginkgolide A increased CYP3A4 mRNA level to a lesser extent than the increase (10-fold in Hu1108 and 4-fold in Hu1138) by *G. biloba* extract. Consistent with the mRNA data, ginkgolide A (1.1 µg/ml) increased CYP3A-catalyzed testosterone 6β-hydroxylation activity by 5-fold in Hu1108 (Fig. 8C) and 2-fold in Hu1138 (Fig. 8D), which was less than the increase (8-fold in Hu1108 and 3-fold in Hu1138) by *G. biloba* extract (100 µg/ml). In contrast, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide did not affect CYP3A enzyme activity in both samples of human hepatocytes.

**Effect of *G. biloba* Extract on hPXR Expression in Cultured Human Hepatocytes.** To assess whether *G. biloba* extract increases hPXR expression, hPXR mRNA levels were
measured in cultured human hepatocytes treated with *G. biloba* extract (25-200 µg/ml) or culture medium (vehicle control). As shown in Table 2, the extract did not increase hPXR mRNA expression in human hepatocyte sample Hu1108 or Hu1138.
Discussion

A major conclusion of the present study is that *G. biloba* extract functions as a human PXR agonist. This conclusion is supported by the experimental evidence indicating that *G. biloba* extract: 1) bound to hPXR ligand-binding domain, as analyzed by a TR-FRET competitive ligand binding assay; and 2) recruited a coactivator, SRC-1, to the receptor, as demonstrated by a mammalian two-hybrid assay. Consistent with the finding that *G. biloba* extract is an agonist of hPXR, we showed herein that the extract activated hPXR in a cell-based reporter gene assay and increased target gene expression, as quantified by CYP3A4 mRNA and testosterone 6β-hydroxylation assays in cultured human hepatocytes. However, it is less clear whether *G. biloba* extract elicits hPXR-mediated drug interactions in humans. The reasons include the lack of characterization of the individual chemical constituents in the extract used in the limited number of pharmacokinetic studies (Abad et al., 2010) and the variability reported in the clearance of marker substrate in a small number of subjects (Uchida et al., 2006). Other than *G. biloba* extract, St John’s wort is another herbal medicine that is a hPXR agonist. It binds to the receptor, recruits SRC-1, and increases target gene expression (*CYP3A4*) in human hepatocytes (Moore et al., 2000a; Wentworth et al., 2000). However, it is not possible to compare the capacity (*B*<sub>max</sub>) or affinity (*K*<sub>d</sub>) of hPXR binding by St John’s wort and *G. biloba* extract because kinetic experiments have yet to be conducted to delineate their receptor-binding constants.

Previous studies reported hPXR activation by ginkgolide A, ginkgolide B, and ginkgolide C (Satsu et al., 2008; Li et al., 2009). However, the concentrations used in these studies were greater than those present in commercial preparations of *G. biloba* (Kressmann et al., 2002). Therefore, in the present study, we determined whether individual ginkgolides and bilobalide are
responsible for PXR-activation by the extract. Our findings lead us to conclude that ginkgolide A contributes to the increase in rPXR- and hPXR-mediated luciferase reporter activity, SRC-1 recruitment, and hPXR target gene (CYP3A4) expression by G. biloba extract. This conclusion is based on data obtained from experiments conducted using a concentration of ginkgolide A equivalent to the level present in a PXR-activating or CYP3A4-inducing concentration of the extract. This approach has also led to the identification of ginkgolide A as a partial contributor to induction of CYP3A23 (Chang et al., 2006a) and potentiation of acetaminophen hepatotoxicity (Rajaraman et al., 2006) by the extract in cultured rat hepatocytes. Although ginkgolide A contributed to the increase in rPXR and hPXR-mediated luciferase reporter activity, SRC-1 recruitment, and CYP3A4 expression by G. biloba extract, it was not responsible for hPXR binding. A possible explanation for this lack of contribution to hPXR binding could be that ginkgolide A binds to hPXR only at concentrations far greater than the levels present in the extract. In fact, ginkgolide A does not bind to hPXR at concentrations less than 1000 μM. Another explanation is that ginkgolide A, at concentrations relevant to the extract, may activate hPXR by a mechanism other than binding to the ligand-binding domain of the receptor. Although it has not been shown for PXR, it is known that phenobarbital activates another member of the superfamily of human nuclear receptors, constitutive androstane receptor, without binding to the ligand-binding domain of the receptor (Moore et al., 2000b). Therefore, it is of interest to elucidate the detailed molecular mechanism by which ginkgolide A activates hPXR.

Among the remaining terpene trilactones investigated in the present study, bilobalide has been shown to be responsible for CYP2B1 induction by G. biloba extract in cultured rat hepatocytes (Chang et al., 2006a). However, based on the studies published to date, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide do not play a role in the other biological activities
of the extract; for example, these chemicals do not contribute to the \textit{in vitro} inhibition of CYP1 (Chang et al., 2006b) and CYP2B6 (Lau and Chang, 2009) catalytic activities, induction of \textit{CYP1A1} and \textit{CYP1B1} gene expression (Rajaraman et al., 2009), or activation of aryl hydrocarbon receptor (Rajaraman et al., 2009) by \textit{G. biloba} extract. As shown in the present study, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide were also not responsible for hPXR-mediated luciferase reporter activity, hPXR binding, or induction of hPXR target gene (\textit{CYP3A4}) by the extract. These findings together with those obtained from ginkgolide A indicate that the individual ginkgolides, although structurally similar (Fig. 1), selectively contribute to the biological activities of \textit{G. biloba} extract. Given that herbal extracts contain numerous chemicals, it is possible that they may interact synergistically to give a greater effect than a single chemical. However, when compared to ginkgolide A alone, a mixture of ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide did not further increase hPXR-mediated luciferase reporter activity. Overall, it is not known whether other chemical constituents present in the extract contribute to hPXR activation. Flavonols, such as quercetin and kaempferol, are present in \textit{G. biloba} extracts. However, they exist mainly as monoglycosides, diglycosides, and other glycosides (van Beek and Montoro, 2009), and the levels of the respective aglycones would be expected to be relatively modest given they are subject to extensive metabolism by phase II conjugation enzymes (Cermak and Wolfram, 2006). Moreover, quercetin and kaempferol aglycones have been classified as weak activators of hPXR with estimated EC\textsubscript{50} values of 300 and 380 \textmu M, respectively, according to computational molecular-modeling studies (Ekins and Erickson, 2002). These compounds do not activate hPXR at low micromolar concentrations (\textless 25 \textmu M), as assessed in \textit{in vitro} cell-based reporter gene assays (Moore et al., 2000a).
PXR shows considerable structural diversity among different species, and there is only 76% amino acid sequence homology between the ligand-binding domain of rat and human PXR (Jones et al., 2000). Data from our luciferase reporter gene assay indicate that G. biloba extract activated rPXR to a lesser extent than hPXR, whereas ginkgolide A, at a concentration present in the extract, activated rPXR to a slightly greater extent than hPXR. This activation of rPXR provides a molecular basis for the increase in CYP3A expression by the extract in cultured rat hepatocytes (Rajaraman et al., 2006; Chang et al., 2006a; Deng et al., 2008) and rat liver (Shinozuka et al., 2002). Structural studies suggest that the species-specific PXR activation could be attributed to several different amino acid residues in the ligand binding cavity of PXR. Using site-directed mutagenesis assays, Phe305 in rPXR and Leu308 in hPXR, which are amino acids present in the flexible loop adjacent to the ligand binding cavity, have been identified as critical for the species-specific activation by several PXR ligands (Tirona et al., 2004).

The expression of hPXR is subjected to regulation by certain drugs (Pascussi et al., 2000). However, as shown in this study, G. biloba extract did not affect hPXR expression in cultured human hepatocytes. An increase in hPXR target gene (CYP3A4) expression may be a consequence of increased receptor expression or receptor activation. For example, submicromolar (physiological) concentrations of dexamethasone upregulate PXR expression through a glucocorticoid receptor-mediated mechanism (Pascussi et al., 2001), whereas micromolar (pharmacological) concentrations of this drug activate PXR (Pascussi et al., 2000; Pascussi et al., 2001). However, as shown by our data, the effect of G. biloba extract on hPXR target gene (CYP3A4) expression in cultured human hepatocytes was not due to an increase in hPXR expression, but likely the result of hPXR activation.
In conclusion, *G. biloba* extract is a hPXR agonist. It bound to hPXR and recruited SRC-1 coactivator to the receptor. Ginkgolide A, but not ginkgolide B, ginkgolide C, ginkgolide J, or bilobalide, contributed to hPXR activation by the extract, as assessed by an *in vitro* cell-based reporter gene assay, a mammalian two-hybrid assay, and target gene expression assays. However, the effect of ginkgolide A did not involve binding to the ligand-binding domain of the receptor. While the extract had a greater effect on hPXR than on rPXR activity, ginkgolide A was also a contributor to the rPXR-activating effect of the extract. Although the extract affected hPXR function, it did not modulate hPXR expression, as assessed in cultured human hepatocytes. Overall, our major findings provide insights into the biological and chemical mechanisms of hPXR activation by *G. biloba* extract. Recently, it has been suggested that PXR activation may be a new therapeutic strategy for Alzheimer’s disease by increasing expression of P-glycoprotein (ABCB1) and clearance of brain β-amyloid (Hartz et al., 2010). Given that *G. biloba* extract is a hPXR agonist and is capable of increasing P-glycoprotein (Yeung et al., 2008; Li et al., 2009), future studies are warranted to elucidate whether PXR activation may be a molecular mechanism underlying the purported use of the extract for neurodegenerative conditions such as Alzheimer’s disease. In this context, the discovery that low micromolar concentrations of ginkgolide A activate hPXR may lead to an interest in drug development efforts.
Acknowledgements

The authors thank Indena S. A. (Milan, Italy) for the generous provision of *G. biloba* extract.
References


Chang TKH and Waxman DJ (2006) Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR). Drug Metab Rev 38:51-73.


Footnote

This research was supported by the Canadian Institutes of Health Research [Grant MOP-84581] and a major equipment grant from the Dawson Endowment Fund in Pharmaceutical Sciences. T.K.H.C. received a Senior Scholar Award from the Michael Smith Foundation for Health Research.

1Present address: NoAb BioDiscoveries, Inc., Mississauga, ON, Canada.

2Lau et al., manuscript in preparation.
Figure Legends

Fig. 1. Chemical structures of ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide (van Beek and Montoro, 2009).

Fig. 2. Effect of *G. biloba* extract on LDH release in human PXR-transfected HepG2 cells. Cultured cells were transfected for 24 h with pGL3-basic-CYP3A4-XREM-luc, pGL4.74[hRluc/TK], and pCMV6-XL4-hPXR. The transfected cells were treated for 24 h with culture medium (vehicle), *G. biloba* extract (200, 400, 600, or 800 µg/ml), dextran (1% v/v; negative control), or Triton® X-100 (1% v/v; positive control). LDH levels in the culture medium and cell lysates were determined as described under Materials and Methods. Data are expressed as mean ± S.E.M. for three independent experiments. *Significantly different from the vehicle-treated control group (*P* < 0.05).

Fig. 3. Comparative effect of *G. biloba* extract, PCN, and rifampicin on rPXR and hPXR activation in cultured HepG2 cells. Cells were transfected for 24 h with pGL3-basic-CYP3A4-XREM-luc, pGL4.74[hRluc/TK], and with pCMV6-AC-rPXR, pCMV6-AC (empty vector), pCMV6-XL4-hPXR, or pCMV6-XL4 (empty vector). The transfected cells were treated for 24 h with culture medium (vehicle control for *G. biloba* extract), *G. biloba* extract (600 µg/ml), DMSO (0.1% v/v; vehicle control for chemicals), PCN (10 µM; positive control for rPXR), or rifampicin (10 µM; positive control for hPXR). Firefly and *Renilla* luciferase activities were measured and normalized as described under Materials and Methods. Data are expressed as mean ± S.E.M. for four independent experiments. *Significantly different from the vehicle-
treated control group and shows a species difference in PXR activation within each treatment group ($P < 0.05$).

**Fig. 4.** Concentration-dependent effect of *G. biloba* extract on rPXR and hPXR activation and role of terpene trilactones in the activation of these receptors in cultured HepG2 cells. Cells were transfected for 24 h with pGL3-basic-CYP3A4-XREM-luc, pGL4.74[hRluc/TK], and with pCMV6-XL4-rPXR, pCMV6-AC (empty vector), pCMV6-XL4-hPXR, or pCMV6-XL4 (empty vector). The transfected cells were treated for 24 h with (A) culture medium (vehicle control), *G. biloba* extract (30, 100, 200, 400, 600, or 800 µg/ml), (B) DMSO (0.1% v/v; vehicle control for chemicals), ginkgolide A (GA; 4.4 µg/ml), ginkgolide B (GB; 1.2 µg/ml), ginkgolide C (GC; 5.6 µg/ml), ginkgolide J (GJ; 2.4 µg/ml), bilobalide (BB; 11.2 µg/ml), a mixture of the five terpene trilactones (GA + GB + GC + GJ + BB) at the above concentrations, culture medium (vehicle control for *G. biloba* extract), or *G. biloba* extract (400 µg/ml). The concentration of terpene trilactones represented the levels present in a 400 µg/ml concentration of *G. biloba* extract. Firefly and *Renilla* luciferase activities were measured and normalized as described under Materials and Methods. Data are expressed as mean ± S.E.M. for four independent experiments. *Significantly different from the vehicle-treated control group and shows a species difference in PXR activation within each concentration or treatment group ($P < 0.05$). #Significantly different from the vehicle-treated control group ($P < 0.05$).

**Fig. 5.** Binding of *G. biloba* extract to human PXR ligand-binding domain and role of terpene trilactones. 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 assay buffer (vehicle for *G. biloba* extract), *G. biloba* extract (30 µg/ml), DMSO (1% v/v; vehicle for individual chemicals), ginkgolide A (0.33 µg/ml), ginkgolide B (0.09 µg/ml), ginkgolide C (0.42 µg/ml), ginkgolide J (0.18 µg/ml), bilobalide (0.84 µg/ml), SR12813 (10 µM; positive control), TO901317 (10 µM; positive control), phenobarbital (1000 µM; positive control), or PCN (10 µM; negative control). The concentrations of terpene trilactones represented the levels present in 30 µg/ml of *G. biloba* extract. Net TR-FRET ratio (520 nm/495 nm) was determined using a fluorescence plate reader. Data are expressed as a percentage of vehicle-treated control group and shown as mean ± S.E.M. (n = 3). *Significantly different from the vehicle-treated control group (*P* < 0.05).

**Fig. 6.** Effect of *G. biloba* extract and ginkgolide A on the recruitment of SRC-1 coactivator to human PXR in cultured HepG2 cells. Cells were transfected for 24 h with pM-hSRC1-RID, pGL4.74[hrLuc/TK], pFR-luc, and pVP16 empty vector or pVP16-hPXR-LBD expression plasmid. The transfected cells were treated for 24 h with culture medium (vehicle for *G. biloba* extract), *G. biloba* extract (400 µg/ml), DMSO (0.1% v/v; vehicle for individual chemicals), ginkgolide A (4.4 µg/ml, a concentration that is present in 400 µg/ml of extract), rifampicin (10 µM; positive control), or PCN (10 µM; negative control). Firefly and *Renilla* luciferase activities were measured and normalized as described under Materials and Methods. 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 or five 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. 7. Concentration-dependent effect of *G. biloba* extract on CYP3A4 expression in primary cultures of human hepatocytes. Cultured hepatocytes were treated for 72 h with culture medium (vehicle) or various concentrations of *G. biloba* extract (25, 50, 100, or 200 µg/ml for sample Hu1043, and 1, 2.5, 10, 25, 50, 100, or 200 µg/ml for sample Hu1108). Total RNA was isolated from pooled cell lysates (three or four wells) and CYP3A4 mRNA level was analyzed by real-time PCR, as described under Materials and Methods. CYP3A4 mRNA level was normalized to HPRT mRNA level. Data are shown as mean of duplicate PCR analyses for hepatocyte samples Hu1043 (A) and Hu1108 (B). Testosterone 6β-hydroxylation level was determined by HPLC analyses. Data are expressed as mean ± S.D. of three or four wells for hepatocyte samples Hu1043 (C) and Hu1108 (D).

Fig. 8. Role of terpene trilactones in the induction of CYP3A4 by *G. biloba* extract in primary cultures of human hepatocytes. Cultured hepatocytes were treated for 72 h with DMSO (0.1% v/v; vehicle for individual chemicals), ginkgolide A (1.1 µg/ml), ginkgolide B (0.3 µg/ml), ginkgolide C (1.4 µg/ml), ginkgolide J (0.6 µg/ml), bilobalide (2.8 µg/ml), culture medium (vehicle control for *G. biloba* extract), or *G. biloba* extract (100 µg/ml). The concentration of terpene trilactones represented the levels present in 100 µg/ml of *G. biloba* extract. Total RNA was isolated from pooled cell lysates (three wells) and CYP3A4 mRNA level was analyzed by real-time PCR, as described under Materials and Methods. CYP3A4 mRNA level was normalized to HPRT mRNA level. Data are shown as mean of duplicate PCR analyses for hepatocyte samples Hu1108 (A) and Hu1138 (B). Testosterone 6β-hydroxylation activity was
determined by HPLC analyses. Data are expressed as mean ± S.D. of three wells for hepatocyte samples Hu1108 (A) and Hu1138 (B).
### TABLE 1

Demographics of human liver donors

<table>
<thead>
<tr>
<th>Donor Identification</th>
<th>Sex</th>
<th>Age (Years)</th>
<th>Race</th>
<th>Smoker</th>
<th>Medication History</th>
<th>Medical History</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hu1043</td>
<td>Male</td>
<td>73</td>
<td>Caucasian</td>
<td>No</td>
<td>Amlodipine, Simvastatin, Omeprazole, Atenolol, Vitamin B6, Chemotherapy</td>
<td>Hypertension, cardiac, gastrointestinal disorders, urinary disorders, rectal cancer</td>
</tr>
<tr>
<td>Hu1108</td>
<td>Female</td>
<td>51</td>
<td>Caucasian</td>
<td>Yes</td>
<td>Vitamin D, Vicodin (Acetaminophen and Hydrocodone), Alprazolam</td>
<td>None, documented</td>
</tr>
<tr>
<td>Hu1138</td>
<td>Female</td>
<td>53</td>
<td>Caucasian</td>
<td>No</td>
<td>Not reported</td>
<td>Colon cancer</td>
</tr>
</tbody>
</table>
TABLE 2

Effect of *G. biloba* extract on PXR mRNA expression in primary cultures of human hepatocytes

Cultured hepatocytes isolated from two donors (Hu1108 and Hu1138) were treated for 72 h with *G. biloba* extract or culture medium (vehicle). Total RNA was isolated from pooled cell lysates (three wells per treatment group). PXR and HPRT mRNA levels were analyzed by real-time PCR, as described under Materials and Methods. PXR mRNA level was normalized to HPRT mRNA level. Data are shown as mean of duplicate PCR analyses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Final Concentration (µg/ml)</th>
<th>Relative PXR mRNA Expression (fold-increase over control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hu1108</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture medium</td>
<td>N/A</td>
<td>1.0</td>
</tr>
<tr>
<td><em>G. biloba</em> extract</td>
<td>25</td>
<td>0.9</td>
</tr>
<tr>
<td><em>G. biloba</em> extract</td>
<td>50</td>
<td>1.0</td>
</tr>
<tr>
<td><em>G. biloba</em> extract</td>
<td>100</td>
<td>0.8</td>
</tr>
<tr>
<td><em>G. biloba</em> extract</td>
<td>200</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Hu1138</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture medium</td>
<td>N/A</td>
<td>1.0</td>
</tr>
<tr>
<td><em>G. biloba</em> extract</td>
<td>100</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Figure 1

Ginkgolides

Bilobalide

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginkgolide A</td>
<td>OH</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Ginkgolide B</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Ginkgolide C</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Ginkgolide J</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
</tr>
</tbody>
</table>
Figure 2

LDH Release
(percentage of total LDH content)

- Culture medium
- G. biloba (200 µg/ml)
- G. biloba (400 µg/ml)
- G. biloba (600 µg/ml)
- G. biloba (800 µg/ml)
- Dextran
- Triton X-100

*
Figure 5

Net TR-FRET Ratio (percentage of control)

Buffer G. biloba
DMSO Ginkgolide A
Ginkgolide B
Ginkgolide C
Ginkgolide J
Bilobalide
SR12813
TO901317
Phenobarbital
PCN
Figure 8

A. CYP3A4 mRNA (Hu1108)

B. CYP3A4 mRNA (Hu1138)

C. Testosterone 6β-OH (Hu1108)

D. Testosterone 6β-OH (Hu1138)