Selective Agonism of Human Pregnan X Receptor by Individual Ginkgolides

Aik Jiang Lau, Guixiang Yang, Chun Wei Yap, and Thomas K. H. Chang

Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, British Columbia, Canada (A.J.L., G.Y., T.K.H.C.); Department of Pharmacy, Faculty of Science, National University of Singapore, Singapore (C.W.Y.)
DMD #45013

Running Title: Ginkgolides, PXR, GR, and CAR

Corresponding author: Dr. Thomas K. H. Chang, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, British Columbia, V6T 1Z3, Canada. Tel.: 1-604-822-7795; Fax: 1-604-822-3035; E-mail: thomas.chang@ubc.ca.

Number of text pages: 36
Number of tables: 2
Number of figures: 7
Number of references: 41
Number of words in Abstract: 250
Number of words in Introduction: 627
Number of words in Discussion: 1204

ABBREVIATIONS: CAR, constitutive androstane receptor; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; DEHP, di-(2-ethylhexyl)phthalate; DMSO, dimethyl sulfoxide; GR, glucocorticoid receptor; hCAR, human constitutive androstane receptor; hGR, human glucocorticoid receptor; hHPRT, human hypoxanthine phosphoribosyltransferase 1; hPXR, human pregnane X receptor; MM/GBVI, molecular mechanics generalized born/volume integral; PCN, pregnenolone 16α-carbonitrile; PCR, polymerase chain reaction; PXR, pregnane X receptor; SR12813, tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate; SRC-1, steroid receptor coactivator-1; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene; TR-FRET, time-resolved fluorescence resonance energy transfer.
ABSTRACT:

Ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J are structurally-related terpene trilactones present in *Ginkgo biloba* extract. Pregnane X receptor (PXR), glucocorticoid receptor (GR), and constitutive androstane receptor (CAR) regulate the expression of genes involved in diverse biological functions. In the present study, we investigated the effects of individual ginkgolides as single chemical entities on the function of human PXR (hPXR), human GR (hGR), and human CAR (hCAR). In cell-based reporter gene assays, none of the ginkgolides activated hGR or hCAR (wild-type and its SV23, SV24, and SV25 splice variants). Concentration-response experiments showed that ginkgolide A and ginkgolide B activated hPXR and rat PXR to a greater extent than ginkgolide C, whereas ginkgolide J had no effect. As determined by a time-resolved fluorescence resonance energy transfer competitive binding assay, ginkgolide A and ginkgolide B, but not ginkgolide C or ginkgolide J, were shown to bind to the ligand-binding domain of hPXR, consistent with molecular docking data. When compared with SR12813 (a known agonist of hPXR), ginkgolide A and ginkgolide B were considerably less potent in binding to hPXR. These two ginkgolides recruited steroid receptor coactivator-1 to hPXR and increased hPXR target gene (*CYP3A4*) expression, as assessed by a mammalian two-hybrid assay and real-time polymerase chain reaction, respectively. In conclusion, the individual ginkgolides regulate the function of nuclear receptors in a receptor-selective and chemical-dependent manner. This study identifies ginkgolide A and ginkgolide B as naturally-occurring agonists of hPXR and provides mechanistic insight into the structure-activity relationship in ligand-activation of hPXR.
Introduction

Ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J are a group of structurally-related terpene trilactones (Fig. 1). They represent a subset of the bioactive chemical constituents in *Ginkgo biloba* extract (van Beek and Montoro, 2009). As shown in studies with these ginkgolides as single chemical entities, differences exist in their biological properties, despite being highly similar in their chemical structures. For example, ginkgolide B and ginkgolide C are more efficacious than ginkgolide A and ginkgolide J in antagonizing glycine-gated chloride channel (Ivic et al., 2003). Ginkgolide B (Xiao et al., 2010) and ginkgolide J (Vitolo et al., 2009) also inhibit β-amyloid-induced cell death in rodent hippocampal neurons. Other actions of ginkgolide B include antagonism of platelet-activating factor receptor and anti-apoptotic, anti-inflammatory, antioxidant, and anti-proliferative activities (Xia and Fang, 2007).

As part of the goal to develop ginkgolides as drug candidates, efforts have been made to synthesize various analogues of ginkgolides and elucidate their structure-activity relationships (Jensen et al., 2010).

Pregnane X receptor (PXR; NR1I2), constitutive androstane receptor (CAR; NR1I3; originally known as MB67), and glucocorticoid receptor (GR; NR3C1) are members of the superfamily of nuclear receptors (Germain et al., 2006). These three receptors play important roles in maintaining homeostasis and essential physiological functions. For example, PXR and CAR are involved in glucose homeostasis, bile acid homeostasis, lipid metabolism, and the biotransformation and transport of drugs and endogenous chemicals (Kakizaki et al., 2008), whereas GR plays a role in growth, anti-inflammatory and immune response (Nicolaides et al., 2010). Previous studies have shown that GR regulates the expression of PXR and CAR (Pascussi et al., 2001), which in turn may modulate the actions of these two receptors. Due to
the interactions between PXR, CAR, and GR, various genes, such as CYP3A4, are regulated directly or indirectly by these receptors.

In cell-based reporter gene assays, ginkgolide A, ginkgolide B, and ginkgolide C (to a small extent), but not ginkgolide J, were shown to activate human PXR (hPXR), as assessed with a reporter plasmid containing the ABCB1 promoter (Satsu et al., 2008). Ginkgolide A and ginkgolide B have also been shown to activate hPXR in a study that used a CYP2B6 reporter containing the phenobarbital-responsive enhancer module and the distal xenobiotic-responsive enhancer module (Li et al., 2009). Our previous studies showed that ginkgolide A, but not ginkgolide B, ginkgolide C, or ginkgolide J, contributed to hPXR activation by G. biloba extract (Lau et al., 2010), whereas none of these chemicals was responsible for the activation of the wild-type form of human CAR (hCAR-WT) or one of its splice variants (hCAR-SV23) by the extract (Lau et al., 2011). However, it is not known how the ginkgolides activate hPXR and whether the ginkgolides differentially affect the function of hPXR, human GR (hGR), and human CAR (hCAR). Given that this class of ginkgolides is structurally similar, they are potentially useful chemical tools to study the structural determinants of nuclear receptor function.

In the present study, we compared the effects of individual ginkgolides (i.e. ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J) as single chemical entities on the function of hPXR, hGR, and hCAR. Recently, several splice variants of hCAR (hCAR-SV23, hCAR-SV24, and hCAR-SV25) have been shown to be differentially activated by drugs and other chemicals (Lau et al., 2011). Therefore, we determined whether the ginkgolides affect the activity of these hCAR splice variants. Given the pronounced species differences in PXR activation (Jones et al., 2000), we conducted detailed dose-response experiments to compare the effect of the ginkgolides on the activity of hPXR and rat PXR (rPXR). Additional mechanistic experiments
and molecular docking analysis were conducted to gain insight on how the ginkgolides activate hPXR. Our results are discussed in the context of receptor-selective and chemical-dependent regulation of nuclear receptors by individual ginkgolides.
Materials and Methods

Chemicals and Reagents. Ginkgolide A (lot #2398804; purity of 99.58%), ginkgolide B (lot #220962; purity of > 95%), and ginkgolide C (lot #23922902; purity of > 99.9%) were purchased from LKT Laboratories, Inc. (St. Paul, MN), and ginkgolide J (lot #00007186-112; purity of 98.2%) was from ChromaDex (Irvine, CA). Rifampicin, pregnenolone 16α-carbonitrile (PCN), sodium phenobarbital, di-(2-ethylhexyl)phthalate (DEHP), 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO). 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) and tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate (SR12813) were from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA), and 5α-androstan-3α-ol (androstanol) was from Steraloids (Newport, RI). Opti-MEM, PureLink RNA Mini Kit, and LanthaScreen Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) PXR Competitive Binding Assay were purchased from Invitrogen, Inc. (Burlington, ON, Canada). FuGENE 6 transfection reagent and Cytotoxicity Detection Kit 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 cell culture, reverse transcription, and real-time polymerase chain reaction (PCR) analyses were described previously (Lau et al., 2010).

Plasmids. pCMV6-XL4-hPXR, pCMV6-AC-rPXR, pCMV6-XL5-hGR, pCMV6-XL4-hCAR-WT, pCMV6-neo-hCAR-SV23, pCMV6-XL4-hCAR-SV24, pCMV6-XL5-hCAR-SV25, pCMV6-XL4, pCMV6-AC, pCMV6-XL5, and pCMV6-neo were purchased from OriGene Technologies (Rockville, MD). pGRE-luc reporter and pFR-luc reporter (contains five tandem repeats of yeast GAL4 binding sites) were bought from Agilent Technologies (Santa Clara, CA).
**Renilla reniformis** luciferase pGL4.74[hRluc/TK] plasmid was obtained from Promega Corporation (Madison, WI). pGL3-basic-CYP3A4-XREM-luc reporter (Goodwin et al., 1999) and 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). pVP16-hPXR-LBD and pM-hSRC1-RID were constructed as described previously (Lau et al., 2010). To construct the pM-hPXR-LBD plasmid, the ligand-binding domain of hPXR (Met-107 to Ser-434) was amplified from pCMV6-XL4-hPXR and inserted into the pM vector (contains the yeast GAL4 DNA-binding domain). Contrary to what was stated in a previous article (Synold et al., 2001), hPXR (NP_003880) has 434 amino acids, as indicated in the NCBI Reference Sequence Database. Methionine is the amino acid at position 107, whereas lysine is at position 108. The constructed plasmids were sequenced by the Nucleic Acid Protein Service Unit at the University of British Columbia (Vancouver, BC, Canada), and the identity of the plasmids was confirmed by comparing their sequence with published sequence.

**Cell Culture.** LS180 human colon adenocarcinoma cells and HepG2 human hepatocellular carcinoma cells were purchased from American Type Culture Collection (Manassas, VA). LS180 cells (Yeung et al., 2008) and HepG2 cells (Lau et al., 2010) were cultured as described previously.

**Treatment of LS180 Cells.** Cultured LS180 cells were seeded onto 6-well plates at a cell density of 200,000 cells per well. At 72 h after plating, cells were treated with DMSO (0.1% v/v; vehicle), ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, or rifampicin (positive control) (Kolars et al., 1992), at the concentrations indicated in the figure legend. The chemical-containing culture medium was replaced every 24 h for a period of 72 h.
Isolation of Total RNA and Reverse Transcription. Cultured LS180 cells were harvested at the end of the chemical treatment period. Total cellular RNA was isolated using PureLink RNA Mini Kit (Invitrogen, Inc.), according to the manufacturer’s protocol, and reversed transcribed using Superscript II reverse transcriptase. Quantification of total RNA and total cDNA were performed as described previously (Chang et al., 2006).

PCR Primers. The sequences of primers for amplification of CYP3A4 cDNA 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) and for amplification of human hypoxanthine phosphoribosyltransferase 1 (hHPRT) cDNA were 5′-GAA-GAG-CTA-TTG-TAA-TGA-CC-3′ (forward) and 5′-GCG-ACC-TTG-ACC-ATC-TTT-G-3′ (reverse) (Qiu et al., 2007). The primers were synthesized by Integrated DNA Technologies (Coralville, IA), and their specificity was verified by sequencing the purified amplicons (University of British Columbia Nucleic Acid Protein Service Unit, Vancouver, BC, Canada).

Real-Time PCR Analysis. Amplification of cDNA was performed using LightCycler (Roche Diagnostics). CYP3A4 and hHPRT cDNAs were amplified using PCR conditions described previously (Lau et al., 2010). A calibration curve (cross point versus log cDNA copies) was constructed using known amounts of purified CYP3A4 or hHPRT cDNA, which was amplified from human liver QUICK-Clone cDNA (Clontech Laboratories, Inc., Mountain View, CA) and quantified as described previously (Chang et al., 2006).

Transient Transfection and Reporter Gene Assays. Cultured HepG2 cells were seeded onto 24-well microplate at a density of 100,000 cells per well (in 0.5 ml culture medium). At 24 h after plating, cells were transfected 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 plasmid (5 ng per well), pGL3-basic-CYP3A4-XREM-luc reporter construct (50 ng per well), and pCMV6-XL4-hPXR expression plasmid (50 ng per well), pCMV6-AC-rPXR expression plasmid (50 ng per well), pCMV6-XL4 empty vector (50 ng per well), or pCMV6-AC empty vector (50 ng per well) for 24 h. Subsequently, transfected HepG2 cells were treated with 0.5 ml of fresh culture medium containing DMSO (0.1% v/v; vehicle), ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, rifampicin (positive control for hPXR and negative control for rPXR) (Moore et al., 2000), or PCN (10 µM; negative control for hPXR and positive control for rPXR) (Moore et al., 2000) for 24 h at the concentrations indicated in the figure legend. At the end of the treatment period, transfected HepG2 cells were lysed for the determination of firefly luciferase and *R. reniformis* 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 *R. reniformis* luciferase activity. Background luciferase activity was determined in HepG2 cells transfected with an empty vector (pCMV6-XL4 or pCMV6-AC), and the value was subtracted from each of 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. Each independent experiment was performed in triplicate, and a total of four independent experiments were conducted.

hGR-dependent reporter gene assay was determined in HepG2 cells in the same manner as that described for the hPXR-dependent reporter gene assays, except that: a) pCMV6-XL5-hGR (50 ng per well) was the receptor expression plasmid; b) pCMV6-XL5 (50 ng per well) was the empty vector; c) pGRE-luc (50 ng per well) was the reporter plasmid; d) dexamethasone (0.1
µM) was used as positive control for hGR (Pascussi et al., 2001); and e) rifampicin (10 µM) was used as a negative control for hGR (Herr et al., 2000).

hCAR-WT-, hCAR-SV23-, hCAR-SV24-, and hCAR-SV25-dependent reporter gene assays were performed in HepG2 cells, as described previously (Lau et al., 2011). Transfected cells were treated with DMSO (0.1% v/v; vehicle), ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, CITCO (positive control for hCAR-WT and hCAR-SV24) (Maglich et al., 2003; Faucette et al., 2007), DEHP (positive control for hCAR-SV23) (DeKeyser et al., 2009), or TCPOBOP (negative control for hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25) (Lau et al., 2011) for 24 h at the concentration indicated in the figure legend. Androstanol (10 µM; inverse agonist of hCAR-WT) (Moore et al., 2000) was added to all the treatment groups in the hCAR-WT-dependent reporter gene assay.

In the pM-hPXR-LBD assay, cultured HepG2 cells were transfected with pM-hPXR-LBD (Met-107 to Ser-434; 100 ng per well) or pM empty vector (100 ng per well) along with pFR-luc reporter plasmid (100 ng per well) and pGL4.74[hRluc/TK] internal control plasmid (5 ng per well), using FuGENE 6 transfection reagent (3 µl per µg of DNA) diluted in 20 µl of Opti-MEM. Transfected HepG2 cells were treated with 0.5 ml of fresh culture medium containing DMSO (0.1% v/v; vehicle), ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, rifampicin (positive control for hPXR) (Moore et al., 2000), SR12813 (positive control for hPXR) (Jones et al., 2000), sodium phenobarbital, or PCN (negative control for hPXR) (Moore et al., 2000) for 24 h at the concentration indicated in the figure legend.

**Competitive Ligand-Binding Assay.** A LanthaScreen TR-FRET PXR Competitive Binding Assay (Invitrogen, Inc.) was conducted according to the manufacturer’s protocol as detailed previously (Lau et al., 2010).
Mammalian Two-Hybrid Assay. HepG2 cells were seeded onto 24-well microplates at a density of 75,000 cells per well. At 24 h after plating, cells were transfected with pM-hSRC1-RID plasmid (10 ng per well), pFR-luc reporter plasmid (100 ng per well), and pVP16 (40 ng per well; empty vector control) or pVP16-hPXR-LBD expression plasmid (40 ng per well), using FuGENE 6 transfection reagent as described previously (Lau et al., 2010). At 24 h after transfection, culture medium containing the transfection mixture was removed and cells were treated with 0.5 ml of fresh culture medium containing DMSO (0.1% v/v; vehicle), ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, rifampicin (positive control) (Moore et al., 2000), SR12813 (positive control) (Jones et al., 2000), or PCN (negative control) (Moore et al., 2000) for 24 h at the concentration indicated in the figure legend. Phenobarbital was included for comparative purposes. Luciferase activity was measured and normalized as described under Transient Transfection and Reporter Gene Assays.

Lactate Dehydrogenase (LDH) Assay. LS180 and HepG2 cells (100,000 cells per well) were seeded onto 24-well plates. At 48 h after plating, cultured cells were treated with ginkgolide A (10-100 μM), ginkgolide B (10-100 μM), ginkgolide C (10-100 μM), ginkgolide J (10-100 μM), dextran (1% v/v; negative control), Triton X-100 (0.1% v/v; positive control), DMSO (0.1% v/v; vehicle for ginkgolides), or culture medium (vehicle for dextran and Triton X-100) for 24 h (HepG2) or 72 h (LS180). At the end of the treatment period, culture medium was collected and cells were lysed with 2% v/v Triton X-100 in phosphate-buffered saline (pH 7.4) containing 20 mM EDTA (0.5 ml per well). The LDH assay was performed using the Cytotoxicity Detection Kit, as described previously (Lau et al., 2010). To determine intracellular LDH release, 5 μl aliquot of culture medium or cell lysate was transferred into the wells of a 96-well microplate containing 95 μl of phosphate-buffered saline (pH 7.4). 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).

**Molecular Docking.** There are nine X-ray crystallography structures of hPXR-ligand complexes in the RCSB Protein Data Bank. Three criteria, (1) resolution ≤ 2.5 Å, (2) R value ≤ resolution/10, and (3) difference between R-value and R-free ≤ 0.05, were used to identify structures that were of sufficiently good quality for docking studies. The R-value is a measure of the degree of fit between the structure and the crystallographic data. R-free is calculated using the same method as R-value, except that it is based on data that have not been used to derive the structure. The difference between R-value and R-free shows the degree of over-fitting for the structure (Kleywegt and Jones, 1997). There was only one structure (PDB ID: 1M13) that met these criteria, and its quality was confirmed in a study comparing five different hPXR structures (Ekins et al., 2009).

Molecular Operating Environment (MOE) 2010.10 (Chemical Computing Group, Inc., Montreal, QC, Canada) was used for docking the individual ginkgolides, rifampicin, SR12813, and PCN to the 1M13 structure. 1M13 was first processed by removing bound ligand and water molecules. Hydrogen atoms were then added and ionization states were assigned using the Protonate3D function in MOE. The active site was defined using residues known to line the site. These residues consist of 17 hydrophobic residues (Leu-206, Leu-209, Val-211, Leu-240, Met-243, Phe-251, Phe-281, Phe-288, Trp-299, Leu-308, Met-323, Leu-324, Leu-411, Ile-414, Phe-420, Met-425, and Phe-429), five polar residues (Ser-208, Ser-247, Cys-284, Gln-285, and Tyr-306), and four charged residues (Glu-321, His-327, His-407, and Arg-410) (Liu et al., 2011).

The chemical structures of ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, rifampicin, SR12813, and PCN were drawn and minimized using MOE. These were saved to the
DMD database format and partial charges of the molecules were calculated. The docking function in MOE was used to dock the seven molecules to hPXR. The default settings were used, except that the force field refinement scheme was used to refine the docked molecules and the positions of the side chains of the 26 residues surrounding the active site. The default settings produced up to 30 possible poses for each molecule. The pose with the best molecular mechanics generalized born/volume integral (MM/GBVI) binding free energy was selected for analysis of interaction with hPXR if it fulfilled the following criteria (Gao et al., 2007): (1) the t-butyl group of the ginkgolides fits into the Trp-299 site; and (2) a H-bond acceptor of the ginkgolides interacts with either the His-407 or Gln-285 side chain. Given the similarity in the structures of the four ginkgolides (Fig. 1), each was assumed to bind to the receptor in a similar pose as the other ginkgolides.

**Statistical Analysis.** Data were analyzed by one-way or two-way ANOVA 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 $P < 0.05$. The half-maximal effective concentration ($EC_{50}$) and maximal response ($E_{\text{max}}$) of the concentration-response curves were calculated by non-linear regression with the following equation (SigmaPlot 11.0): 

$$y = E_0 + \frac{\left(E_{\text{max}} - E_0\right)}{1+(x/EC_{50})^{-\text{Hill slope}}},$$

where $y$ is effect (fold-increase over the vehicle-treated control group or percentage of the values in the vehicle-treated control group), $x$ is concentration ($\mu$M), and $E_0$ is minimum effect.
Results

Effect of Individual Ginkgolides on hPXR Target Gene Expression in Cultured LS180 Cells. Our initial experiment was to verify the previously reported effect of ginkgolide A, ginkgolide B, and ginkgolide C on the transcription of a hPXR target gene (CYP3A4) in LS180 cells (Satsu et al., 2008), which was reported to be an useful model for studying CYP3A4 induction (Gupta et al., 2008). In agreement with a previous study (Satsu et al., 2008), ginkgolide A (60 µM) and ginkgolide B (60 µM) increased CYP3A4 mRNA expression, whereas ginkgolide C (60 µM) had no effect (data not shown). In the same experiment, our novel finding indicates a lack of an effect by ginkgolide J (60 µM). Rifampicin (10 µM), which is a positive control for the induction of CYP3A4 (Kolars et al., 1992), increased CYP3A4 mRNA level by 15-fold.

Comparative Effect of Individual Ginkgolides on the Activity of hPXR, hGR, and hCAR in Transfected HepG2 Cells. hPXR (Lehmann et al., 1998), hGR (Pascussi et al., 2001), and hCAR (Goodwin et al., 2002) regulate CYP3A4 expression. Therefore, we compared the effect of the four structurally-related ginkgolides on hPXR, hGR, and hCAR activities. As shown in Fig. 2A, ginkgolide A (100 µM), ginkgolide B (100 µM), and ginkgolide C (100 µM) increased hPXR activity by 11-, 15-, and 4-fold, respectively, whereas ginkgolide J (100 µM) had no effect. Control analysis indicated that rifampicin (10 µM; positive control) (Moore et al., 2000) activated hPXR by 14-fold and PCN (10 µM; negative control) (Moore et al., 2000) had no effect. Unlike hPXR, hGR (Fig. 2B) and hCAR-WT (Fig. 2C) activities were not increased by any of the four individual ginkgolides. Dexamethasone (0.1 µM; positive control) (Pascussi et al., 2001) increased hGR activity by 10-fold (Fig. 2B) and CITCO (10 µM; positive control) (Maglich et al., 2003) increased hCAR-WT activity by 4-fold (Fig. 2C), whereas rifampicin (10
µM; negative control) (Herr et al., 2000) had no effect on hGR (Fig. 2B) and TCPOBOP (0.25 µM; negative control) (Moore et al., 2000) had no effect on hCAR-WT (Fig. 2C).

Given that hCAR splice variants are differentially activated by drugs and other chemicals (Lau et al., 2011), we determined the effect of the ginkgolides on the activity of hCAR-SV23, hCAR-SV24, and hCAR-SV25 using reporter gene assays conducted on HepG2 cells. As shown in Fig. 2D-F, none of the ginkgolides affected the activity of hCAR-SV23, hCAR-SV24, or hCAR-SV25. In contrast, DEHP (10 µM; positive control for hCAR-SV23) (DeKeyser et al., 2009) and CITCO (10 µM; positive control for hCAR-SV24) (Faucette et al., 2007) activated hCAR-SV23 (Fig. 2D) and hCAR-SV24 (Fig. 2E) by 17- and 4-fold, respectively. In agreement with published data (Lau et al., 2011), CITCO (10 µM) did not activate hCAR-SV25 (Fig. 2F), and TCPOBOP (0.25 µM; negative control) did not affect each of the three hCAR splice variants (Fig. 2D-F). Given that ginkgolides activated hPXR, but not hCAR or hGR, subsequent experiments focused on PXR.

**Concentration-Response Relationship in hPXR and rPXR Activation by Individual Ginkgolides in Transfected HepG2 Cells.** To further characterize in detail the differential effect of the four individual ginkgolides on hPXR activation, a concentration-response experiment was conducted by treating hPXR-transfected HepG2 cells with DMSO (0.1% v/v; vehicle) or varying concentrations (0.1, 1, 3, 10, 30, 60, or 100 µM) of ginkgolide A, ginkgolide B, ginkgolide C, or ginkgolide J. As shown in Fig. 3A, ginkgolide A, at 0.1-3 µM, had no effect on hPXR activity, whereas at 10, 30, and 60 µM, it increased the activity in a log-linear manner by 5-, 9-, and 11-fold, respectively. The maximum effect was achieved with 60 µM ginkgolide A. The EC50 and Emax values of hPXR activation by ginkgolide A were 16 ± 1 µM and 12 ± 1-fold, respectively. Ginkgolide B, at 0.1-1 µM, had no effect on hPXR activity, whereas at 3, 10,
30, 60, and 100 µM, it increased the activity in a log-linear manner by 3-, 5-, 11-, 14, and 15-fold, respectively. Ginkgolide C, at 0.1-30 µM, had no effect on hPXR activity, whereas at 60 and 100 µM, it increased the activity by 3- and 4-fold, respectively. In contrast to the other ginkgolides, ginkgolide J at each of the concentrations (0.1 to 100 µM) investigated had no effect. By comparison, rifampicin (positive control) (Moore et al., 2000), at 1, 3, 10, and 30 µM, increased hPXR activity by 11-, 14-, 11-, and 12-fold, respectively. Its EC_{50} value was 0.4 ± 0.1 µM and its E_{max} value was 12 ± 2-fold, which were consistent with literature values (Chang, 2009).

To determine whether the ginkgolides activate rPXR, a concentration-response experiment was conducted on HepG2 cells transfected with rPXR. As shown in Fig. 3B, ginkgolide A, at 0.1-3 µM, had no effect on rPXR activity, whereas at 10, 30, and 60 µM, it increased the activity in a log-linear manner by 4-, 8-, and 9-fold, respectively. The maximum effect was achieved with 60 µM ginkgolide A. The EC_{50} and E_{max} values of rPXR activation by ginkgolide A were 12 ± 1 µM and 9 ± 1-fold, respectively. Ginkgolide B, at 0.1-3 µM, had no effect on rPXR activity, whereas at 10, 30, 60, and 100 µM, it increased the activity in a log-linear manner by 3-, 6-, 7-, and 8-fold, respectively. Ginkgolide C, at 0.1-10 µM, had no effect on rPXR activity, whereas at 30, 60, and 100 µM, it increased the activity by 2-, 2-, and 3-fold, respectively. In contrast to the other ginkgolides, ginkgolide J at all concentrations (0.1 to 100 µM) had no effect. By comparison, PCN (positive control) (Moore et al., 2000), at 1, 3, 10, and 30 µM, increased rPXR activity by 9-, 15-, 16-, and 17-fold, respectively. Its EC_{50} value was 1.0 ± 0.2 µM and its E_{max} value was 17 ± 2-fold, which were consistent with literature values (Chang, 2009). In general, the individual ginkgolides showed a similar pattern of activation in hPXR and rPXR. Subsequent experiments focused on hPXR activation.
**Effect of Individual Ginkgolides on Transactivation of the Ligand-Binding Domain of hPXR.** The mechanism of hPXR activation by the ginkgolides is not known. Therefore, to determine whether the ginkgolides transactivate the ligand-binding domain of hPXR, a reporter gene assay was conducted on HepG2 cells transfected with pM-hPXR-LBD (Met-107 to Ser-434). As shown in Fig. 4, ginkgolide A (100 µM) and ginkgolide B (100 µM) increased the luciferase activity by 5- and 8-fold, respectively, whereas ginkgolide C (100 µM) and ginkgolide J (100 µM) had no effect. Analyses with positive controls indicated that phenobarbital (1000 µM) (Jones et al., 2000), SR12813 (10 µM) (Jones et al., 2000), and rifampicin (10 µM) (Moore et al., 2000) increased the reporter activity by 9-, 10-, and 11-fold, respectively. As expected, PCN (10 µM; negative control) (Moore et al., 2000) had no effect. None of the treatment groups affected the activity in cells transfected with the pM empty vector.

**TR-FRET Competitive Ligand Binding of Individual Ginkgolides to the Ligand-Binding Domain of hPXR.** To corroborate the ligand-binding domain transactivation data shown in Fig 4, we conducted a TR-FRET competitive ligand binding assay. A preliminary experiment showed that rifampicin, but not the ginkgolides, phenobarbital, PCN, or SR12813, quenched fluorescence in the ligand binding assay (data not shown). As shown in Fig. 5A, ginkgolide A (1000 µM) and ginkgolide B (1000 µM) decreased the TR-FRET emission ratio by 60 and 50%, respectively, whereas ginkgolide C (1000 µM) and ginkgolide J (1000 µM) had no effect. By comparison, phenobarbital (1000 µM) and SR12813 (10 µM), which are known PXR ligands (Jones et al., 2000), decreased the emission ratio by 42% and 92%, respectively. PCN (10 µM), which is a negative control (Moore et al., 2000), yielded the expected result. We also conducted a detailed concentration-response experiment to further characterize the binding effect of ginkgolide A and ginkgolide B. As shown in Fig. 5B, ginkgolide A, at 3-300 µM, had no
effect on the emission ratio, but at 1000 µM, it decreased the ratio by 54%. Ginkgolide B, at 3-30 µM, had no effect on the emission ratio, whereas at 100, 300, and 1000 µM, it decreased the ratio by 33%, 37%, and 48%, respectively. Comparatively, SR12813, at 0.01 nM to 100 µM, decreased the TR-FRET emission ratio by 27% to 97%.

**Effect of Individual Ginkgolides on SRC-1 Coactivator Recruitment to hPXR in Cultured HepG2 Cells.** To determine whether there is ligand selectivity in the effect of the individual ginkgolides on SRC-1 recruitment, a mammalian two-hybrid assay conducted in HepG2 cells transfected with pFR-luc, pM-hSRC1-RID, and either pVP16-hPXR-LBD or pVP16 (empty vector). In HepG2 cells co-transfected with both pM-hSRC1-RID and pVP16-hPXR-LBD, ginkgolide A (100 µM) and ginkgolide B (100 µM) increased the luciferase reporter activity by 10- and 13-fold, respectively (Fig. 6). In contrast, ginkgolide C and ginkgolide J had no effect. As expected, phenobarbital (1000 µM; positive control) (Jones et al., 2000), SR12813 (10 µM; positive control) (Jones et al., 2000), and rifampicin (10 µM; positive control) (Moore et al., 2000) increased the reporter activity, whereas PCN (10 µM; negative control) (Moore et al., 2000) had no effect. Control analysis also indicated a lack of increase in the reporter activity by each of the treatment groups in cells not transfected with the pVP16-hPXR-LBD expression plasmid.

**Effect of Individual Ginkgolides on LDH Release in LS180 and HepG2 Cells.** Ginkgolide J did not affect hPXR target gene expression or PXR activity in various cell-based assays (Fig. 2, 3, 4, and 6). However, it is not known whether cellular necrosis was associated with the concentrations of this ginkgolide used in those experiments. Therefore, we determined LDH release in LS180 and HepG2 cells treated with ginkgolide J. For comparative purposes, we also investigated the effect of ginkgolide A, ginkgolide B, and ginkgolide C on LDH release.
None of these chemicals at 10-100 µM increased LDH release in cultured LS180 or HepG2 cells (data not shown). Control analysis indicated that dextran (1% v/v; negative control) had no effect, whereas Triton X-100 (0.1% v/v; positive control) resulted in complete cell lysis.

**Molecular Docking of Ginkgolides to the Ligand-Binding Domain of hPXR.** To investigate how the ginkgolides interact with the ligand-binding domain of hPXR, we conducted a molecular docking study. Table 1 shows the MM/GBVI binding free energy for these chemicals and the various controls. Compared with rifampicin and SR12813 (positive controls), the ginkgolides had weaker binding free energy. Among the four individual ginkgolides, the rank order was ginkgolide A ~ ginkgolide B > ginkgolide C > ginkgolide J. PCN (negative control) did not interact with Gln-285 or His-407. Table 2 shows the amino acid residues of hPXR that were involved in the binding of the various ginkgolides and Fig. 7 shows the various interactions between the residues and the ginkgolides. Hydrogen bonds linking to Gln-285 and His-407 were shorter for ginkgolide A and ginkgolide B than those for ginkgolide C and ginkgolide J.
Discussion

A major finding in the present study is that ginkgolide A, ginkgolide B, and ginkgolide C activate hPXR, but not hGR, hCAR-WT, hCAR-SV23, hCAR-SV24, or hCAR-SV25. This receptor selectivity is similar to that of rifampicin, which also activates hPXR (Lehmann et al., 1998), but not hGR [(Herr et al., 2000) and present study] or hCAR-WT (Moore et al., 2000). The receptor selectivity of ginkgolides may be related to differences in the size and flexibility of the ligand-binding domain of these receptors. hPXR (~1200 Å³) (Timsit and Negishi, 2007) has a larger and more flexible ligand-binding domain, when compared with hGR (~600 Å³ in each subunit) (Veleiro et al., 2010) and hCAR-WT (~675 Å³) (Timsit and Negishi, 2007). This allows hPXR to accommodate a diverse group of ligands of different shapes and sizes. Our detailed concentration-response experiments indicate that ginkgolide A and ginkgolide B activated hPXR to a similar extent as rifampicin, whereas ginkgolide C was a weak activator and ginkgolide J had no effect. However, ginkgolide A, ginkgolide B, and ginkgolide C were less potent than rifampicin. Among the naturally-occurring chemicals, hyperforin, forskolin, and cryptotanshinone are potent hPXR activators with EC₅₀ in the submicromolar range (Chang, 2009). By comparison, ginkgolide A was less potent (EC₅₀ = 16.2 μM), but similarly efficacious (Eₘₐₓ = 11.5 fold) as hyperforin (EC₅₀ = 0.02-0.2 μM; Eₘₐₓ = 6-12 fold) (Chang, 2009). Overall, we have identified specific ginkgolides as selective, naturally-occurring ligands of hPXR, but not of hGR or hCAR-WT.

Despite the small structural differences among the four ginkgolides investigated, only ginkgolide A and ginkgolide B demonstrated agonism of hPXR. This conclusion is based on the findings that they transactivated the ligand-binding domain of hPXR in a cell-based reporter gene assay, bound to the ligand-binding domain of hPXR in a competitive TR-FRET binding.
assay, and recruited SRC-1 coactivator to the receptor in a mammalian two-hybrid assay. Although ginkgolide C increased hPXR-mediated luciferase reporter activity (Satsu et al., 2008 and present study), it did not transactivate or bind to the ligand-binding domain of hPXR, as assessed by the assays employed in the present study. The functional differences among the four ginkgolides may be explained by their structural differences. Ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J differ structurally in their substitutions at the C₁ and C₇ positions (Fig. 1). Whereas the OH-group at the C₁ position does not affect the activation of hPXR because ginkgolide A and ginkgolide B activate hPXR to similar extent (Satsu et al., 2008 and present study), the presence of an OH-group at the C₇ position appears to be unfavorable for hPXR activation. The latter is illustrated by our finding that hPXR activation is completely abolished when a H-group at the C₇ position of ginkgolide A is changed to an OH-group in ginkgolide J. Likewise, with an additional OH-group at the C₁ position, a change from a H-group at the C₇ position of ginkgolide B to an OH-group in ginkgolide C also substantially decreases hPXR activation. Overall, the type of substituents at the C₇ position of these diterpenes appears to be a determinant in hPXR activation. Although the ligand-binding domain of hPXR can accommodate ligands with diverse structures (Timsit and Negishi, 2007), our present study with the individual ginkgolides indicates that a slight modification in chemical structure can lead to drastic changes in the functionality of this receptor. Interestingly, in contrast to the structure-activity relationship in hPXR agonism, the OH group at the C₁ position of ginkgolides is necessary for glycine receptor antagonism (Ivic et al., 2003). Overall, our novel findings provide mechanistic information on hPXR activation by individual ginkgolides and show that subtle differences in chemical structure can confer pronounced differences in the functionality of hPXR.
Molecular docking analysis indicates that the binding energy was greatest for ginkgolide A and ginkgolide B, followed by ginkgolide C and ginkgolide J. This rank order in the binding energies of these chemicals is similar to that in cell-based reporter gene assays and the TR-FRET binding assay. The docking analysis also suggests that ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J are capable of interacting with the same amino acid residues in the ligand-binding domain of hPXR. The ginkgolides have a molecular weight of > 400 daltons. They have a 6-ring “cage-like” backbone that allows hydrophobic interactions with various hydrophobic amino acid residues in the ligand-binding domain of hPXR. They also have three lactone groups and two to four OH groups that can interact with polar amino acid residues. These features of ginkgolides fit the proposed pharmacophore of hPXR ligands, which includes a molecular weight > 300 daltons, an ability to form hydrophobic interactions (e.g. aromatic π-π interactions), and the presence of hydrogen bond acceptors (one or two) and hydrogen bond donors (Xiao et al., 2011). Although molecular docking analysis predict that ginkgolide J, which is bioactive (Vitolo et al., 2009), binds very weakly to the ligand-binding domain of the receptor, it does not activate hPXR, as shown in the various assays conducted in the present study. However, it is possible that ginkgolide J is capable of acting as an antagonist of hPXR, but future studies are needed to address this issue.

Pronounced species differences in the activation of hPXR and rPXR have been reported. For example, rifampicin activates hPXR but not rPXR, whereas PCN activates rPXR but not hPXR (Jones et al., 2000). Our novel findings indicate that the pattern of activation of hPXR and rPXR by the four ginkgolides were qualitatively similar, although ginkgolide B activated hPXR to a somewhat greater extent than rPXR. In general, these findings indicate that species differences in the structure of the ligand-binding domains of hPXR and rPXR are not a critical
factor in the activation of these receptors by the individual ginkgolides. The ligand-binding
domains of hPXR and rPXR share only approximately ~76% amino acids identity (Jones et al.,
2000), and site-directed mutagenesis studies identified that Leu-308 in hPXR accounts for the
species differences in hPXR and rPXR activation by rifampicin, paclitaxel, hyperforin,
nifedipine, and PCN (Tirona et al., 2004). However, according to our molecular docking data,
Leu-308 is not involved in the binding of the individual ginkgolides to the ligand-binding
domain of hPXR. Therefore, this may explain the observed lack of pronounced species
differences in hPXR and rPXR activation by these chemicals. Molecular modeling also
predicted that several other amino acid residues in hPXR (Table 2) interact with the individual
ginkgolides. Given that these ginkgolides did not show pronounced species-dependent activation
of hPXR and rPXR in the reporter gene assays, it is proposed that those amino acid residues are
not critical determinants of species-dependent activation of these receptors.

In conclusion, the individual ginkgolides regulate the function of nuclear receptors in a
receptor-selective and chemical-dependent manner. None of the ginkgolides activates hGR or
hCAR, whereas ginkgolide A, ginkgolide B, and ginkgolide C, but not ginkgolide J, activate
hPXR and rPXR. Among the ginkgolides investigated, only ginkgolide A and ginkgolide B
exhibit hPXR agonism. Overall, our study provides mechanistic insight into the structure-
activity relationship in hPXR activation by ginkgolides. This class of naturally-occurring
chemicals may have the potential for PXR-based drug development because this receptor has
been proposed as a therapeutic target for various disease conditions (Kakizaki et al., 2011).
Authorship Contributions

Participated in research design: Lau and Chang.

Conducted experiments: Lau, Yang, and Yap

Contributed new reagents or analytic tools: N/A

Performed data analysis: Lau and Yap.

Wrote or contributed to the writing of the manuscript: Lau, Yap, and Chang.
DMD #45013

References


Footnotes

This research was supported by the Canadian Institutes of Health Research [Grant MOP-84581], Singapore Ministry of Education Academic Research Fund Tier 1 [R-148-000-136-112], and a major equipment grant from the Dawson Endowment Fund in Pharmaceutical Sciences at the University of British Columbia. T.K.H.C. received a Senior Scholar Award from the Michael Smith Foundation for Health Research.

Address correspondence to: Dr. Thomas K. H. Chang, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, British Columbia, V6T 1Z3, Canada. E-mail: thomas.chang@ubc.ca.
Figure Legends

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

FIG. 2. Comparative effects of individual ginkgolides on the activity of hPXR, hGR, hCAR-WT, hCAR-SV23, hCAR-SV24, and hCAR-SV25 in cultured HepG2 cells. Cells were transfected with: (A) pGL3-basic-CYP3A4-XREM-luc, pGL4.74[hRluc/TK], and pCMV6-XL4-hPXR or pCMV6-XL4 (empty vector); (B) pGRE-luc, pGL4.74[hRluc/TK], and pCMV6-XL5-hGR or pCMV6-XL5 (empty vector); (C) pGL3-basic-CYP2B6-PBREM/XREM-luc, pGL4.74[hRluc/TK], and pCMV6-XL4-hCAR-WT or pCMV6-XL4 (empty vector); (D) pGL3-basic-CYP2B6-PBREM/XREM-luc, pGL4.74[hRluc/TK], and pCMV6-neo-hCAR-SV23 or pCMV6-neo (empty vector); (E) pGL3-basic-CYP2B6-PBREM/XREM-luc, pGL4.74[hRluc/TK], and pCMV6-XL4-hCAR-SV24 or pCMV6-XL4 (empty vector); or (F) pGL3-basic-CYP2B6-PBREM/XREM-luc, pGL4.74[hRluc/TK], and pCMV6-XL5-hCAR-SV25 or pCMV6-XL5 (empty vector). Transfected cells were treated with DMSO (0.1% v/v; vehicle), ginkgolide A (100 µM), ginkgolide B (100 µM), ginkgolide C (100 µM), ginkgolide J (100 µM), rifampicin (10 µM), PCN (10 µM), dexamethasone (0.1 µM), CITCO (10 µM), DEHP (10 µM), or TCPOBOP (0.25 µM) for 24 h. In the hCAR-WT assay, androstanol (10 µM; inverse agonist of hCAR-WT) was added to each treatment group. Firefly luciferase and R. reniformis luciferase activities were measured and normalized. Data are shown as mean ± SEM for three or four independent experiments. *Significantly different from the vehicle-treated control group (P < 0.05). Androstanol decreased hCAR-WT activity in the vehicle-treated control group by 59 ± 6%.
FIG. 3. Concentration-response curves for the effect of individual ginkgolides on hPXR and rPXR activities in transfected HepG2 cells. Cells were transfected with pGL3-basic-CYP3A4-XREM-luc, pGL4.74[hRluc/TK], and (A) either pCMV6-XL4-hPXR or pCMV6-XL4 (empty vector), or (B) either pCMV6-AC-rPXR or pCMV6-AC (empty vector). Transfected cells were treated with DMSO (0.1% v/v; vehicle), ginkgolide A (0.1-100 µM), ginkgolide B (0.1-100 µM), ginkgolide C (0.1-100 µM), ginkgolide J (0.1-100 µM), rifampicin (0.01-30 µM), or PCN (0.01-30 µM) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized. Data are shown as mean ± SEM for four independent experiments. In the hPXR experiment, ginkgolide A (10-100 µM), ginkgolide B (3-100 µM), ginkgolide C (60-100 µM), and rifampicin (1-30 µM) were significantly different from the vehicle-treated control group (*P* < 0.05). In the rPXR experiment, ginkgolide A (10-100 µM), ginkgolide B (10-100 µM), ginkgolide C (30-100 µM), and PCN (1-30 µM) were significantly different from the vehicle-treated control group (*P* < 0.05).

FIG. 4. Effect of individual ginkgolides on transactivation of the ligand-binding domain of hPXR in cultured HepG2 cells. Cells were transfected with pGL4.74[hRluc/TK], pFR-luc, and either pM-hPXR-LBD (Met-107 to Ser-434) or pM empty vector. Transfected cells were treated with DMSO (0.1% v/v; vehicle), ginkgolide A (100 µM), ginkgolide B (100 µM), ginkgolide C (100 µM), ginkgolide J (100 µM), PCN (10 µM), phenobarbital (1000 µM), SR12813 (10 µM), or rifampicin (10 µM) for 24 h. Firefly luciferase and *R. reniformis* luciferase activities were measured and normalized. Data are expressed as fold-increase over the vehicle-treated control group in cells transfected with the same expression plasmid (pM-hPXR-LBD or pM), and shown
as mean ± SEM for three independent experiments. *Significantly different from the same treatment group transfected with pM empty vector and the vehicle-treated control cells transfected with pM-hPXR-LBD (P < 0.05).

FIG. 5. (A) Comparative effect of individual ginkgolides on binding to the ligand-binding domain of hPXR. A TR-FRET competitive binding assay was performed. hPXR-LBD (10 nM) was incubated with Fluormone PXR Green (a PXR ligand; 40 nM) in the presence of DMSO (1% v/v; vehicle), ginkgolide A (1000 µM), ginkgolide B (1000 µM), ginkgolide C (1000 µM), ginkgolide J (1000 µM), PCN (10 µM), phenobarbital (1000 µM), or SR12813 (10 µM). (B) Concentration-response curves for binding to the ligand-binding domain of hPXR by SR12813, ginkgolide A, and ginkgolide B. hPXR-LBD (10 nM) was incubated with Fluormone PXR Green in the presence of DMSO (1% v/v; vehicle), ginkgolide A (3-1000 µM), ginkgolide B (3-1000 µM), or SR12813 (0.01 nM to 100 µM). Net TR-FRET ratio (520 nm / 495 nm) was determined using a fluorescence plate reader. Data are expressed as a percentage of the net TR-FRET ratio in the vehicle-treated control group, and shown as mean ± SEM of three or four independent experiments. *Significantly different from the vehicle-treated control group (P < 0.05).

FIG. 6. Effect of individual ginkgolides on the recruitment of SRC-1 coactivator to hPXR in cultured HepG2 cells. Cells were transfected with pM-hSRC1-RID, pGL4.74[hRluc/TK], pFR-luc, and either pVP16-hPXR-LBD or pVP16 (empty vector). Transfected cells were treated with DMSO (0.1% v/v; vehicle), ginkgolide A (100 µM), ginkgolide B (100 µM), ginkgolide C (100 µM), ginkgolide J (100 µM), PCN (10 µM), phenobarbital (1000 µM), SR12813 (10 µM), or...
rifampicin (10 μM) for 24 h. Firefly luciferase and \textit{R. reniformis} luciferase activities were measured and normalized. Data are expressed as fold-increase over the vehicle-treated control group in cells transfected with the same expression plasmid (pVP16-hPXR-LBD or pVP16), and shown as mean ± SEM for four independent experiments. *Significantly different from the same treatment group transfected with pVP16 and the vehicle-treated control cells transfected with pVP16-hPXR-LBD (\(P < 0.05\)).

FIG. 7. Molecular docking of individual ginkgolides to the ligand-binding domain of hPXR. (A) Ginkgolide A, (B) ginkgolide B, (C) ginkgolide C, and (D) ginkgolide J were docked to the ligand-binding domain of hPXR. Shown are key amino acids involved in the hydrophobic interactions and hydrogen bonding between each of the ginkgolides and the ligand-binding domain of hPXR.
TABLE 1

**MM/GBVI binding free energy of ginkgolides and other chemicals in the binding of hPXR**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>MM/GBVI binding free energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>-31.83</td>
</tr>
<tr>
<td>SR12813</td>
<td>-23.21</td>
</tr>
<tr>
<td>Ginkgolide A</td>
<td>-21.79</td>
</tr>
<tr>
<td>Ginkgolide B</td>
<td>-21.72</td>
</tr>
<tr>
<td>Ginkgolide C</td>
<td>-19.84</td>
</tr>
<tr>
<td>Ginkgolide J</td>
<td>-18.56</td>
</tr>
<tr>
<td>PCN</td>
<td>N.A.(^{a})</td>
</tr>
</tbody>
</table>

\(^{a}\) No pose with Gln-285 or His-407 interactions.
### TABLE 2

*Amino acid residues involved in the binding of individual ginkgolides to hPXR*

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Hydrophobic residues</th>
<th>Polar residues</th>
<th>Charged residues</th>
</tr>
</thead>
</table>
**Figure 4**

Comparison of normalized luciferase activity in different conditions. The x-axis represents various compounds, including DMSO, Ginkgolide A, Ginkgolide B, Ginkgolide C, Phenobarbital, SR12813, Rifampicin, Ginkgolide A, Ginkgolide B, Ginkgolide C, Phenobarbital, SR12813, and Rifampicin, while the y-axis shows normalized luciferase activity (fold increase over control). The y-axis scale ranges from 0 to 15. The data points are represented with error bars, indicating the variability in the measurements. The chart includes a horizontal line at the baseline (pM-empty vector) for reference.

Significant differences are indicated by an asterisk (*) above the bars for each compound.

**Legend:**
- **PM-hPXR-LBD** indicates the experimental condition involving the PXR-LBD part of the protein.
(A) Ginkgolide A

(B) Ginkgolide B

(C) Ginkgolide C

(D) Ginkgolide J