Identification of Ginkgo biloba as a Novel Activator of Pregnane X Receptor

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ABBREVIATIONS: PXR, pregnane X receptor; AUC, area under the plasma concentration-time curve; ABCB1, P-glycoprotein; LDH, lactate dehydrogenase; hPXR, human PXR; mPXR, mouse PXR; PCN, pregnenolone 16β-carbonitrile; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; CAR, constitutive androstane receptor.

Pregnane X receptor (PXR; gene designation NR1I2) is a ligand-activated transcription factor that belongs to the superfamily of nuclear receptors (Kliewer et al., 1998). It plays an important role in the regulation of numerous genes involved not only in drug metabolism and transport but also in various other biological processes. Ginkgo biloba is a herbal medicine commonly used to manage memory impairment. Treatment of primary cultures of rat hepatocytes with G. biloba extract increases the mRNA expression of CYP3A23, which is a target gene for rat PXR. The present study was conducted to test the hypothesis that G. biloba extract activates PXR. Treatment of mouse PXR (mPXR) or human PXR (hPXR)-transfected HepG2 cells with G. biloba extract at 200 μg/ml increased mPXR and hPXR activation by 3.2- and 9.5-fold, respectively. Dose-response analysis showed a log-linear increase in hPXR activation by the extract over the range of 200 to 800 μg/ml. To determine whether G. biloba extract induces hPXR target gene expression, cultured LS180 human colon adenocarcinoma cells were treated for 72 h with the extract. G. biloba extract at 200, 400, and 800 μg/ml increased CYP3A4 mRNA expression by 1.7-, 2.4-, and 2.5-fold, respectively. The same concentrations of the extract increased CYP3A5 (1.3–3.6-fold) and P-glycoprotein (ABCB1) (2.7–6.4-fold) mRNA expression. At concentrations (5 and 10 μM) that did not down-regulate PXR gene expression and were not cytotoxic, L-sulforaphane (an hPXR antagonist) decreased CYP3A4, CYP3A5, and ABCB1 gene expression in cells treated with G. biloba extract. In summary, G. biloba extract activated mPXR and hPXR in a cell-based reporter gene assay and induced CYP3A4, CYP3A5, and ABCB1 gene expression in hPXR-expressing LS180 cells.
area under the plasma concentration-time curve (AUC) of an orally administered CYP3A substrate, nicardipine, and this was accompanied by decreased hypotensive action of this calcium channel blocker (Shinozuka et al., 2002). The extract also increases rat hepatic CYP3A expression (Shinozuka et al., 2002) and its associated microsomal testosterone 6β-hydroxylation activity (Umegaki et al., 2002). As demonstrated in primary cultures of rat hepatocytes, G. biloba extract increases CYP3A2, CYP3A18, and CYP3A23 mRNA levels (Chang et al., 2006). The in vivo administration of G. biloba extract has also been shown to increase CYP3A-mediated testosterone 6β-hydroxylation activity in mouse hepatic microsomes (Umegaki et al., 2007). Given that PXR controls the transcriptional regulation of CYP3A genes (Kliwer et al., 1998), our hypothesis is that G. biloba extract activates PXR.

In the present study, we determined the effect of G. biloba extract on rodent and human PXR activity and extended the findings by investigating the effect of the extract on the expression of various PXR target genes (CYP3A4, CYP3A5, and ABCB1) in PXR-expressing rodent and human cells. The extracts used in the current study are similar to those present in a commonly studied G. biloba extract known as EgB 761 (van Beek, 2002). The extract was isolated from leaves and supplied in a powder form) obtained as three individual lots from Indena S.A. (Milan, Italy). The total amount of terpene tri lactones [i.e., ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide; see van Beek (2002) for the chemical structures] in lots A to C was 6.2, 6.2, and 6.8% (w/w), respectively, as quantified by gas chromatography (ChromaDex, Irvine, CA). The total amount of flavonoids [i.e., quercetin, kaempferol, and isorhamnetin as the aglycone and glycosides; see van Beek (2002) for the chemical structures] in lots A to C was 21.0, 24.4, and 24.4% (w/w), respectively, as quantified by liquid chromatography-mass spectrometry (ChromaDex). The levels of terpene tri lactones and flavonol glycosides contained in the extract used in the current study are similar to those present in a commonly studied G. biloba extract known as EgB 761 (van Beek, 2002). HepG2 human hepatoma cells and LS180 human colon adenocarcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). FuGENE 6 transfection reagent was purchased from Roche Diagnostics (Mannheim, Germany). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). Minimum essential medium, OPTI-MEM, heat-inactivated fetal bovine serum, l-glutamine, penicillin G, streptomycin, and trypsin-EDTA were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Triton X-100, dextran, and l-sulfoparaffone were purchased from Sigma-Aldrich (St. Louis, MO). The suppliers for the chemicals and reagents used in the isolation of total RNA, reverse transcription, real-time polymerase chain reaction, and the lactate dehydrogenase (LDH) assay are detailed elsewhere (Chang et al., 2006; Rajaraman et al., 2006). Human CYP3A4, CYP3A5, ABCB1, and PXR gene-specific primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Plasmid Constructs. hpXR and mpXR full-length coding cDNAs were cloned into BamHI and XhoI sites of pCR3. XREM-CYP3A4-Luc, where XREM is the xenobiotic-responsive enhancer module originally named as p3A4-362(7836/7208ins) (Goodwin et al., 1999), was a kind gift from Bryan Goodwin (GlaxoSmithKline, Research Triangle Park, NC). The plh-TK Renilla luciferase plasmid was purchased from Promega.

Cell Culture. HepG2 human hepatoma cells and LS180 human colon adenocarcinoma cells were cultured in T-75 flasks at 37°C in a humidified, 5% CO2 incubator. Cells were grown in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Culture medium was changed once every 2 to 3 days, and cells were subcultured once weekly.

Transient Transfection. HepG2 cells were plated in 24-well microplates at a density of 100,000 cells/well. At 1 day after plating, cells were transfected for 24 h with XREM-CYP3A4-Luc (50 ng/well) and pCR3-mPXR (100 ng/well), pCR3-hpXR (100 ng/well), or pCR3 (100 ng/well; empty vector control) using FuGENE 6 (0.6 μl diluted in 20 μl of OPTI-MEM/well), according to the manufacturer’s instructions. Cells were also transfected with plh-TK (50 ng/well), which was used as an internal control to normalize the firefly luciferase activity in each sample.

Treatment of Transfected HepG2 Cells and Luciferase Reporter Assay. Transfected cells were treated for 24 h with G. biloba extract (30, 100, 200, 400, or 800 μg/ml) or culture medium (vehicle control for G. biloba). As controls, cells were treated with rifampin (10 μM), pregnenolone 16β-carbonitrile (PCN; 10 μM), or dimethyl sulfoxide (DMSO) (0.1% [v/v]; vehicle control for rifampin and PCN). Firefly luciferase and Renilla luciferase levels were determined using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions. Luminescence was measured using a Glomax 96 luminescence microplate reader (Promega).

Treatment of LS180 Cells. Cultured LS180 cells were plated at a density of 1 × 106 cells in T-25 flasks. Drug treatment was initiated 3 days later. LS180 cells were treated once every 24 h for a total of 72 h with varying concentrations of G. biloba extract or l-sulfoparaffone, as indicated in each figure legend. Control cells were treated with DMSO (0.1% [v/v]; vehicle for l-sulfoparaffone) or culture medium (vehicle for G. biloba extract).

Isolation of Total RNA, Reverse Transcription, and Real-Time Polymerase Chain Reaction. At the end of the treatment period, total RNA was isolated from LS180 cells using TRIzol and reverse transcribed using SuperScript II reverse transcriptase, as described previously (Chang et al., 2006). Total RNA concentration and total cDNA concentration were quantified by the RiboGreen RNA Quantitation Kit and PicoGreen dsDNA Quantitation Kit, respectively (Chang et al., 2006). Polymerase chain reaction (PCR) was performed in a real-time DNA thermal cycler (LightCycler; Roche Diagnostics). Each 20-μl PCR reaction contained 1 U of Platinum Taq DNA polymerase in 1X PCR II buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 3 mM MgCl2 (except that 4 mM was used in the amplification of CYP3A4 cDNA), 0.25 ng/ml bovine serum albumin, 0.2 mM dNTP, 0.2 μM forward and reverse primers specific for CYP3A4, CYP3A5, and ABCB1, 1:30,000 SYBR Green I, and 10 ng of total cDNA. The sequences of the forward and reverse primers to amplify human CYP3A4 cDNA were 5’-CCT-TAC-ATA-TAC-GAT-CAG-AAT-CCC-CGG-TTA-3’, respectively (Schuetz et al., 1996). The sequences of the forward and reverse primers to amplify human CYP3A5 cDNA were 5’-CCT-TAC-ATA-TAC-CCC-TTT-GGA-AC-3’ and 5’-GGT-GAA-GAA-GTC-CTT-GCG-CCT-C-3’, respectively (Yamaori et al., 2005). The sequences of the forward and reverse primers to amplify human ABCB1 cDNA were 5’-GGC-CTA-ATG-CCG-AAC-ATC-3’ and 5’-CAG-GTG-CTG-GCC-CTT-C-3’, respectively (Li et al., 2002). The sequences of the forward and reverse primers to amplify human PXR cDNA were 5’-CAAGCG-GAA-GAA-AAG-TAC-GAG-3’ and 5’-CAC-AGA-TCT-TTC-CCG-ACC-TG-3’, respectively (Chang et al., 2003). The cycling conditions for the real-time amplification (LightCycler; Roche Diagnostics) were 94°C for 5 s (denaturation), 60°C for 10 s (annealing), and 72°C for 15 s (extension) for CYP3A4; 94°C for 1 s (denaturation), 65°C for 10 s (annealing), and 72°C for 10 s (extension) for CYP3A5; 94°C for 1 s (denaturation), 61°C for 6 s (annealing), and 72°C for 10 s (extension) for ABCB1; and 95°C for 5 s (denaturation), 65°C for 10 s (annealing), and 72°C for 15 s (extension) for PXR. The initial denaturation was performed at 95°C for 5 min. Calibration curves were constructed by plotting the cross-point against known amounts of purified ampiclon, as determined by the PicoGreen dsDNA Quantitation Kit. Initial experiments were performed to verify the specificity of the primers by purifying and sequencing the ampiclons. Purification was carried out using the QiAquick Gel Extraction Kit, according to the manufacturer’s instructions (QIAGEN, Valencia, CA). Purified ampiclons were sequenced using the Applied Biosystems 377 DNA sequencer (Applied Biosystems, Inc., Foster City, CA) at the Nucleic Acid and Protein Service Unit at the University of British Columbia. The identity of the purified ampiclons was confirmed by the BLAST program (http://www.ncbi.nlm.nih.gov).

LDH Assay. Leakage of intracellular LDH into the culture medium was used to assess cellular toxicity in a method described previously (Rajaraman et al., 2006), but with the following modifications: 1) cells were plated at a density of 100,000 cells/well in 24-well plates; 2) 5 μl of the supernatant from each well containing cultured cells was transferred to a well in a 96-well microplate containing 95 μl of phosphate-buffered saline, pH 7.4, and 100 μl of the reaction mixture (provided in the Cytotoxicity Detection Kit; Roche...
The initial experiment was to determine whether G. biloba extract activates mouse and human PXR. There-}

\[ \text{Effect of } G. \text{ biloba Extract, PCN, and Rifampin on Mouse and Human PXR Activity.} \]

The LDH assay was performed to determine the viability of cultured LS180 cells treated with concentrations of G. biloba extract at 200, 400, and 800 μg/ml or culture medium (vehicle control). As shown in Fig. 3A, these concentrations of the extract increased CYP3A4 mRNA expression. The -fold increase (2.5 ± 0.1-fold) obtained at the highest concentration (800 μg/ml) of the extract was considerably less than the 49.4 ± 12.1-fold increase obtained with rifampin (10 μM). G. biloba extract at 200, 400, and 800 μg/ml also increased CYP3A5 mRNA levels (Fig. 3B) and ABCB1 mRNA levels (Fig. 3C), and the extent of induction by the extract at the highest concentrations tested was similar to or greater than that by rifampin.

\[ \text{Effect of } G. \text{ biloba Extract on Viability of Cultured LS180 Cells.} \]

The LDH assay was performed to determine the viability of cultured LS180 cells treated with concentrations of G. biloba extract that were effective in modulating PXR target gene expression (Fig. 3). The results indicated that treatment of cultured LS180 cells for 72 h with G. biloba extract (200, 400, or 800 μg/ml) did not increase LDH release (data not shown). The positive [% (v/v) Triton X-100] and negative [% (w/v) dextran] controls produced the expected outcomes (data not shown).

\[ \text{PXR Expression in Cultured LS180 Cells Treated with } G. \text{ biloba Extract.} \]

Next, we determined whether the increase in CYP3A4 (Fig. 3A), CYP3A5 (Fig. 3B), and ABCB1 (Fig. 3C) gene expression by
G. biloba extract was associated with an increase in PXR gene expression. Therefore, real-time PCR analysis was performed to measure PXR mRNA levels in LS180 cells treated with G. biloba extract (200, 400, or 800 μg/ml) or culture medium (vehicle control). In agreement with previous data (Thummel et al., 2001), PXR mRNA was expressed in control LS180 cells (data not shown). Our finding indicated that the levels in G. biloba-treated cells were not significantly different from those in vehicle-treated cells (data not shown).

Effect of a PXR Antagonist, L-Sulforaphane, on the Induction of CYP3A4, CYP3A5, and ABCB1 Gene Expression by G. biloba Extract. As demonstrated in a recent study, sulforaphane is an antagonist of human PXR, whereas it does not affect the activity of retinoid X receptor, constitutive androstane receptor (CAR), vitamin D receptor, or peroxisome proliferator-activated receptors α and γ (Zhou et al., 2007). Therefore, to investigate the role of endogenous PXR in the induction of CYP3A4, CYP3A5, and ABCB1 gene expression by G. biloba extract, cultured LS180 cells were treated for 72 h with G. biloba extract (400 μg/ml) and L-sulforaphane (5, 10, or 20 μM) or DMSO (0.1% v/v; vehicle control). Each of these concentrations of L-sulforaphane attenuated the increase in CYP3A4 (Fig. 4A), CYP3A5 (Fig. 4B), and ABCB1 (Fig. 4C) mRNA levels by G. biloba extract.

Assessment of Viability of Cultured LS180 Cells Treated with L-Sulforaphane and G. biloba Extract. We performed the LDH assay to determine whether treatment of LS180 cells with G. biloba extract and L-sulforaphane resulted in cytotoxicity and thereby led to the observed decrease in CYP3A4 (Fig. 4A), CYP3A5 (Fig. 4B), and ABCB1 (Fig. 4C) gene expression. As shown in Fig. 5, G. biloba extract (400 μg/ml) did not affect the extent of LDH release when compared with the vehicle-treated control group. Similarly, the combination of L-sulforaphane (5, 10, or 20 μM) and G. biloba extract (400 μg/ml) did not increase LDH release. Control analysis with 1% (v/v) Triton X-100 (positive control) and 1% (w/v) dextran (negative control) yielded the expected results.

Effect of L-Sulforaphane on PXR Expression in Cultured LS180 Cells Treated with G. biloba Extract. To rule out the possibility that decreased PXR expression is an explanation for the attenuating effect of L-sulforaphane on the induction of CYP3A4 (Fig. 4A), CYP3A5 (Fig. 4B), and ABCB1 (Fig. 4C) by G. biloba extract, real-time PCR analysis was conducted to determine PXR mRNA expression. As shown in Fig. 6, the administration of 5 or 10 μM L-sulforaphane did not affect the extent of PXR mRNA levels in LS180 cells treated with G. biloba extract (400 μg/ml). By comparison, PXR mRNA expression was decreased by 55% in cells cotreated with 20 μM L-sulforaphane and the extract. The extract alone did not affect PXR mRNA expression, when compared with the levels in vehicle-treated control cells (data not shown).

Discussion

Although many studies conducted to date have investigated the role of individual chemicals, particularly synthetic drugs, as agonists or
antagonists of PXR, considerably less is known as to which herbal medicine is capable of activating this receptor (Chang and Waxman, 2006). The present study provides the first demonstration that a novel action of *G. biloba* extract is the activation of mouse and human PXR, as assessed by an in vitro cell-based reporter gene assay. The activation of mouse PXR by *G. biloba* extract is consistent with the previous finding that the in vivo administration of this herbal medicine to mice increases the hepatic microsomal enzyme activity of CYP3A, which is a PXR target gene product (Umegaki et al., 2007). However, *G. biloba* extract was more effective in activating human PXR than in activating mouse PXR. When analyzed at the same concentration (200 μg/ml), *G. biloba* extract increased human PXR activation by 9.5-fold, whereas it increased mouse PXR activation by only 3.2-fold. It is important to assess not only rodent PXR activity, but also human PXR activity, because of the well-established species-dependent chemical activation of PXR. For example, as demonstrated previously (Jones et al., 2000) and confirmed in our control experiments, rifampin activates human PXR, but not mouse PXR, whereas PCN activates mouse PXR, but not human PXR. These differences in PXR activation reflect the relatively modest degree (77%) of sequence identity in the ligand binding domain of mouse and human PXR (Moore et al., 2002).

As shown in our dose-response experiments, a log-linear increase in human PXR activation was obtained with *G. biloba* extract at a concentration range of 200 to 800 μg/ml. A complete dose-response experiment was not performed because of solubility problems. As a consequence, it was not possible to calculate the EC50 (concentration required to achieve half of the maximal activity) or Emax (maximal activity) values for the activation of human PXR by *G. biloba* extract. However, the magnitude of the increase (32-fold) in human PXR activation by the 800 μg/ml concentration of the extract was comparable with the 31-fold increased by 10 μM rifampin. This concentration of rifampin was previously shown to be a near-maximal concentration in the in vitro activation of human PXR (El-Sankary et al., 2001).

Our data obtained from the reporter gene assay were corroborated by the finding that *G. biloba* extract increased the expression of PXR target genes, namely CYP3A4, CYP3A5, and ABCB1, as determined in LS180 human colon adenocarcinoma cells. LS180 cells were chosen because of their: 1) endogenous expression of PXR (Thummel et al., 2001); 2) constitutive and inducible expression of CYP3A4, CYP3A5, and ABCB1 (Schuetz et al., 1996; Gupta et al., 2008); and 3) lack of CAR expression (Gupta et al., 2008). The absence of CAR in the LS180 cell line renders it a useful model to study PXR-mediated gene transcription because the expression of some of the PXR target genes, including CYP3A4, CYP3A5, and ABCB1 (Stanley et al., 2006), is also controlled, at least in part, by CAR. As shown in the present study, PXR plays a role in the induction of CYP3A4, CYP3A5, and ABCB1 expression by *G. biloba* extract in LS180 cells. This conclusion is based on the results obtained from our gene expression experiment conducted with 1-tulforaphane, which is an antagonist of human PXR (Zhou et al., 2007). At 5 and 10 μM, which was not cytotoxic and did not alter PXR gene expression, 1-tulforaphane was effective in attenuating the increase in CYP3A4, CYP3A5, and ABCB1 gene expression in LS180 cells treated with *G. biloba* extract.

The human CYP3A subfamily consists of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (Plant, 2007). Among these enzymes, CYP3A4 and CYP3A5 are the most important in adult human tissues, such as liver and small intestine. Our findings in cultured LS180 cells that *G. biloba* extract increases CYP3A4 and CYP3A5 expression are consistent with those reported in a recent study conducted with primary cultures of human hepatocytes. In that study, treatment of cultured hepatocytes with an extract of *G. biloba* (reported to contain 24% flavonol glycosides and 6% terpene trilactones) was shown to increase the levels of CYP3A4 mRNA, CYP3A protein, and CYP3A-mediated testosterone 6β-hydroxylation activity (Deng et al., 2008). Collectively, results from the cell culture studies with LS180 cells and human hepatocytes indicate that *G. biloba* extract is an inducer of
cells, and that the effects were attenuated by a PXR antagonist. Given that PXR regulates the expression of a broad array of genes that are of fundamental importance in mammalian biology (Stanley et al., 2006; Nakamura et al., 2007; Moreau et al., 2008), results from the present study will provide an impetus to conduct studies in the future to delineate novel physiological, pharmacological, and toxicological actions of G. biloba.

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