DISTINCT ROLE OF BILOBALIDE AND GINKGOLIDE A IN THE MODULATION OF RAT CYP2B1 AND CYP3A23 GENE EXPRESSION BY GINKGO BILOBA EXTRACT IN CULTURED HEPATOCYTES

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ABSTRACT:

In the present study, primary cultures of rat hepatocytes were treated for 48 h with one of several extracts of Ginkgo biloba (10, 100, or 1000 μg/ml). Maximal increase in CYP2B1 and CYP3A23 mRNA levels was obtained at 100 μg/ml. This concentration of G. biloba extract also increased CYP3A2 and CYP3A18 mRNA expression in addition to CYP2B-mediated 7-benzylxyresorufin O-dealkylation (BROD) and CYP3A-mediated testosterone 6β-hydroxylation. In other experiments, cultured hepatocytes were treated for 48 h with bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, kaempferol, quercetin, isorhamnetin, or a flavonol diglycoside at a concentration that represented the level present in a 100 μg/ml concentration of an extract. Only bilobalide (2.8 μg/ml) increased CYP2B1 mRNA expression, and the -fold increase (7.9 ± 0.5; mean ± S.E.M.) was similar to that (8.3 ± 1.7) by the extract. By comparison, only ginkgolide A (1.1 μg/ml) increased CYP3A23 mRNA expression, but the extent (2.6 ± 0.5-fold) was less than the 5.3 ± 1.7-fold increase by the extract. A greater concentration (5 μg/ml) of ginkgolide A was required to elevate CYP3A2 and CYP3A18 mRNA expression. Over the range of 1 to 5 μg/ml, bilobalide increased CYP2B1 mRNA and BROD, but not CYP3A23 mRNA or testosterone 6β-hydroxylation, whereas ginkgolide A increased CYP3A23 mRNA and testosterone 6β-hydroxylation, but not CYP2B1 mRNA or BROD. Overall, our novel results indicate a distinct role of bilobalide and ginkgolide A in the modulation of CYP2B1 and CYP3A23 gene expression and enzyme activities by G. biloba extract in primary cultures of rat hepatocytes.

The use of herbal medicines has been increasing rapidly, especially in recent years (De Smet, 2002). Many consumers believe that herbal products, unlike conventional drugs, are safe and have no side effects (Barnes, 2003). A survey indicated that among the respondents who consumed herbal medicines, 31% ingested them concomitantly with conventional drugs (Abebe, 2002). Therefore, a concern is the potential risk of adverse effects due to herb-drug interactions (Huang et al., 2004). It was used by 21% of adults who consumed herbal medicines. Many individual chemical constituents are present in the variety of disorders; for example, memory impairment (De Smet, 2002). Many consumers believe that herbal medicines, 31% ingested them concomitantly with conventional drugs. Therefore, a concern is the potential risk of adverse effects due to herb-drug interactions (Huang et al., 2004).

According to a 2002 survey conducted in the United States, Ginkgo biloba was the third most popular herbal medicine (Barnes et al., 2004). It was used by 21% of adults who consumed herbal medicines. G. biloba extract is used by consumers for the management of a variety of disorders; for example, memory impairment (De Smet, 2002). Many individual chemical constituents are present in G. biloba extract, including terpene trilactones, such as diterpenes (e.g., ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J) and sesquiterpenes (e.g., bilobalide), and ginkgo flavonol glycosides (e.g., glycosides of kaempferol, quercetin, and isorhamnetin) (van Beek, 2002).

Previous rodent studies indicated that G. biloba extract, when administered as a single i.p. dose (25 or 30 mg/kg) (Brochet et al., 1999) or fed for 2 weeks via the diet (0.5% or 1% w/w) (Kubota et al., 2003). The effect of this drug, specifically, via a decrease in the maximum plasma concentration and area under the plasma concentration-time curve (Kubota et al., 2003). The effect of G. biloba on the pharmacodynamics and pharmacokinetics of these drugs may be related to induction of cytochrome P450 (P450) enzymes by this herbal medicine. It has been reported that the administration of a G. biloba extract (containing 24.9% flavonol glycosides and 10.6% terpene trilactones) to rats increased hepatic expression of several P450 enzymes, including CYP2B (Shinozuka et al., 2002; Umegaki et al., 2002). However, it is not known whether the flavonols or the terpene trilactones are responsible for the reported effects of G. biloba extract. The levels of the terpene trilactones in the extracts used in the previous studies (Shinozuka et al., 2002; Umegaki et al., 2002) were greater than those in many of the commercially available G. biloba products formulated to contain only 6% terpene trilactones (van Beek, 2002). Therefore, it is not known whether the results from those studies (Shinozuka et al., 2002; Umegaki et al., 2002) can be generalized to other extracts of G. biloba.

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ABBREVIATIONS: P450, cytochrome P450; BROD, 7-benzylxyresorufin O-dealkylation; DEX, dexamethasone; DMSO, dimethyl sulfoxide; PB, phenobarbital; PCR, polymerase chain reaction.

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The present study was conducted to 1) investigate the effect of multiple extracts of G. biloba containing known amounts of bilobalide, ginkgolides, and flavonol glycosides on CYP2B1, CYP3A23, CYP3A2, and CYP3A18 gene expression in primary cultures of rat hepatocytes, as determined by reverse transcription and real-time polymerase chain reaction (PCR); 2) assess the effect of G. biloba extract on CYP2B- and CYP3A-mediated enzyme activities in cultured hepatocytes, as analyzed by the 7-benzyloxyresorufin O-dealkylation (BROD) assay and the testosterone 6β-hydroxylation assay, respectively; and 3) determine whether terpene trilactones, such as bilobalide and ginkgolides A, B, C, and J, or flavonol aglycones and glycosides, such as those of kaempferol, quercetin, and isorhamnetin, are responsible for the effect of G. biloba extract on CYP2B1, CYP3A23, CYP3A2, and CYP3A18 expression. Our results indicate that bilobalide and ginkgolide A are novel inducers of CYP2B1 and CYP3A, respectively, and that these chemicals play a distinct role in the modulation of CYP2B1 and CYP3A3 mRNA expression and enzyme activities by G. biloba extract.

### Materials and Methods

#### Chemicals and Reagents.
G. biloba and rat hepatocyte P450 expression

<table>
<thead>
<tr>
<th>Ginkgo biloba Extract</th>
<th>Extract A</th>
<th>Extract B</th>
<th>Extract C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diterpene</strong></td>
<td></td>
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<tr>
<td>Ginkgolide A</td>
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</tr>
<tr>
<td>Ginkgolide B</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Ginkgolide C</td>
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<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Ginkgolide J</td>
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<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Total</td>
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<td>3.4</td>
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<tr>
<td><strong>Sesquiterpene</strong></td>
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<td>Bilobalide</td>
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<td>3.2</td>
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<tr>
<td>Total Terpene Trilactones</td>
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<td>6.2</td>
<td>6.6</td>
</tr>
<tr>
<td><strong>Flavonol and Its Glycosides</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Kaempferol (aglycone)</td>
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<td>n.d.</td>
</tr>
<tr>
<td>Kaempferol (diglycosides)</td>
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<td>n.d.</td>
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<td>n.d.</td>
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<tr>
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<td>n.d.</td>
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<td>Quercetin (diglycosides)</td>
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<td>n.d.</td>
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<td>n.d.</td>
</tr>
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<td>Quercetin (sum of aglycone and glycosides)</td>
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<td>10.9</td>
<td>11.6</td>
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<tr>
<td>Isorhamnetin (aglycone)</td>
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<tr>
<td>Isorhamnetin (3-O-rutinoside)</td>
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<tr>
<td>Isorhamnetin (other glycosides)</td>
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<td>n.d.</td>
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<tr>
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<td>2.9</td>
</tr>
<tr>
<td>Total Flavonol Glycosides</td>
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<td>24.4</td>
<td>24</td>
</tr>
</tbody>
</table>

N.D., not determined.

The levels of terpene trilactones in G. biloba extracts A and B were quantified by gas chromatography (Indena S.A.) and those of flavonols in these extracts were quantified by liquid chromatography-mass spectrometry (ChromaDex, Inc., Santa Ana, CA). The levels of terpene trilactones and flavonols in G. biloba extract C (GK501) were determined by high performance liquid chromatography (Pharmaton S.A.).
Media. Cells were mechanically dispersed using a blunt glass rod. The cell suspension was filtered through sterile Nytex nylon mesh (64-μm pore size; TETCO, Briarcliff Manor, NY) into a 50-ml Falcon tube. The suspension was centrifuged (50g) at 4°C for 5 min and the pellet was suspended in fresh ice-cold Hepatocyte Wash Media. A portion (20 ml) of the suspension was added to ice-cold Percoll solution (26.1 ml of Percoll and 3.9 ml of 10× Dulbecco’s phosphate-buffered saline) and mixed gently by inversion (five times). The mixture was centrifuged (50g) at 4°C for 10 min. The supernatant was decanted, and the pellet was washed again in Hepatocyte Wash Media and subsequently suspended in supplemented Williams’ medium E containing heat-inactivated fetal bovine serum (10% v/v). Supplemented Williams’ medium E contained insulin (1 μM), dexamethasone (100 nM), t-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cell viability was 80 to 90%, as assessed by trypan blue exclusion.

Hepatocyte Cultures. Culture dishes or microplates were coated with Matrigel at approximately 1 h before use (Schuetz et al., 1988). In the experiments investigating P450 gene expression, 170 μl of Matrigel was applied onto a 60-mm Permanox culture dish. Hepatocyte suspension was diluted to 106 cells/ml in serum-free, supplemented Williams’ medium E, and a 3-ml volume was plated onto each culture dish. In experiments investigating P450-mediated enzyme activities, 30 μl of Matrigel (diluted 1:1 with culture medium) was applied onto each well in a 24-well microplate. Each well was loaded with 3 × 105 cells in a volume of 500 μl containing serum-free, supplemented Williams’ medium E. Cells were allowed to attach for 4 h in a humidified 37°C incubator with 5% CO2 and 95% air. Subsequently, the medium was decanted to remove unattached cells and the hepatocytes were cultured in serum-free, supplemented Williams’ medium E. Culture medium was changed daily.

Treatment of Primary Cultures of Rat Hepatocytes. At 48 h after plating, hepatocytes were treated for 48 h with G. biloba extract (10, 100, or 1000 μg/ml) or culture medium (vehicle control). In other experiments, cultured hepatocytes were treated with bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, kaempferol, kaempferol-3-O-rutinoside, quercetin, quercetin-3-O-rutinoside, isorhamnetin, isorhamnetin-3-O-rutinoside, or DMSO (vehicle control) at the concentration indicated in each figure legend. For comparison, hepatocytes were treated with PB (100 μM, positive control) or DEX (10 μM, positive control) for 48 h with G. biloba extract (100 μM, positive control, and 0.1% DMSO, negative control). Control incubations contained culture medium (vehicle for the extract) or DMSO (0.1% final concentration, vehicle for the individual chemicals). At the end of the treatment period, culture medium was removed and the cells were washed with 1× phosphate-buffered saline. The BROD assay was initiated by the addition of a 150-μl mixture containing 7-benzoylresorufin (15 μM) and dicumarol (10 μM) dissolved in DMSO (0.1% final concentration) (Donato et al., 1993). The reaction proceeded for 60 min in a 37°C incubator. Subsequently, a 75-μl aliquot of the reaction mixture was transferred to another well containing a 25-μl mixture of β-glucuronidase (15 Fishman units) and arylsulfatase (120 Roy units) dissolved in 0.1 M sodium acetate (pH 4.5). The microplates were placed in a shaking 37°C incubator for 2 h. Following the addition of ethanol (200 μl), the microplates were centrifuged at 3000 rpm for 10 min. The fluorescence reading of a 200-μl sample of the supernatant was recorded at an excitation wavelength of 530 nm (5-nm bandwidth), an emission wavelength of 580 nm (5-nm bandwidth), and a gain of 50 in a Cytofluor 2350 fluorescence microplate reader (Millipore Corporation, Billerica, MA). A standard curve was constructed with 10 to 80 pmol of resorufin.

Testosterone 6β-Hydroxylation Assay. Hepatocytes cultured in 24-well microplates were treated as described above for the BROD assay, except that the positive control was DEX (10 μM). At the end of the treatment period, unadsorbed analytes remaining after the cells were remixed with the cell suspension was analyzed by a rat hepatocyte microplate (Millipore Corporation, Billerica, MA). A standard curve was constructed with 10 to 80 pmol of resorufin.

Real-Time PCR Analysis. Each 20-μl PCR reaction mixture contained 1 unit of Platinum Taq DNA polymerase in 1× PCR reaction buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 3 mM magnesium chloride (except that 5 mM was used to amplify CYP3A2 cDNA), 1 ng of total cDNA, 200 nM deoxynucleoside-5'-triphosphate mix, 0.2 μM concentration, each, of the forward and reverse primers, 0.25 mg/ml bovine serum albumin, and 2 μl of a 3.3× SYBR Green I solution. The conditions for the amplification of CYP2B1 cDNA were 95°C for 1 s (denaturation), 56°C for 6 s (annealing), and 72°C for 23 s (extension). The conditions for the amplification of CYP3A2 cDNA were 95°C for 1 s, 60°C for 6 s, and 72°C for 14 s. In all cases, the initial denaturation was performed at 95°C for 5 min. Under these conditions, no primer-dimer formation was detected. However, to prevent any potential contribution to the fluorescence signal due to primer-dimer formation, the real-time DNA thermal cycler (LightCycler; Roche Diagnostics, Mannheim, Germany) was programmed to record fluorescence readings after each cycle at a temperature several degrees lower than the melting temperature of the amplicon. Calibration curves were constructed as described previously (Cheung et al., 2004). The level of gene expression (copies of cDNA) was normalized to the amount of total cDNA used in the amplification, as quantified by the PicoGreen dsDNA Quantitation Kit (Singer et al., 1997). This approach to normalizing gene expression data was previously shown to be comparable to normalizing it to the amount of a “housekeeping” gene, such as cyclophilin (Yu et al., 2005). Results are expressed as fold increase over the vehicle-treated control group.

BROD Assay. Hepatocytes cultured in 24-well microplates were treated for 48 h with G. biloba extract (100 μg/ml), bilobalide (1–5 μg/ml), ginkgolide A (1.1–5 μg/ml), or PB (100 μM, positive control). Control incubations contained culture medium (vehicle for the extract) or DMSO (0.1% final concentration, vehicle for the individual chemicals). At the end of the treatment period, culture medium was removed and the cells were washed with 1× phosphate-buffered saline. The BROD assay was initiated by the addition of a 150-μl mixture containing 7-benzoylresorufin (15 μM) and dicumarol (10 μM) dissolved in DMSO (0.1% final concentration) (Donato et al., 1993). The reaction proceeded for 60 min in a 37°C incubator. Subsequently, a 75-μl aliquot of the reaction mixture was transferred to another well containing a 25-μl mixture of β-glucuronidase (15 Fishman units) and arylsulfatase (120 Roy units) dissolved in 0.1 M sodium acetate (pH 4.5). The microplates were placed in a shaking 37°C incubator for 2 h. Following the addition of ethanol (200 μl), the microplates were centrifuged at 3000 rpm for 10 min. The fluorescence reading of a 200-μl sample of the supernatant was recorded at an excitation wavelength of 530 nm (5-nm bandwidth), an emission wavelength of 580 nm (5-nm bandwidth), and a gain of 50 in a Cytofluor 2350 fluorescence microplate reader (Millipore Corporation, Billerica, MA). A standard curve was constructed with 10 to 80 pmol of resorufin.

Testosterone 6β-Hydroxylation Assay. Hepatocytes cultured in 24-well microplates were treated as described above for the BROD assay, except that the positive control was DEX (10 μM). At the end of the treatment period, unadsorbed analytes remaining after the cells were remixed with the cell suspension was analyzed by a rat hepatocyte microplate (Millipore Corporation, Billerica, MA). A standard curve was constructed with 10 to 80 pmol of resorufin.

Reverse Transcription and Quantification of Total cDNA Concentration. Reverse transcription was performed and total cDNA concentrations were quantified using the PicoGreen dsDNA Quantitation Kit (Singer et al., 1997) as detailed elsewhere (Chang et al., 2003).

Isolation of Total RNA and Quantification of Total RNA Concentration. Hepatocytes were harvested at the end of the treatment period. Total cellular RNA was isolated using the Trizol reagent, and total RNA concentration was determined using the Ribogreen RNA Quantitation Kit (Jones et al., 1998) as detailed elsewhere (Chang et al., 2003).

PCR Primers. The sequences of the forward and reverse primers used to amplify CYP2B1 cDNA (GenBank accession number J00719) were 5’-CT- GTGGGTCATGGAGAGCTG-3’ and 5’-TCACACCGGTACCAACCTT-3’, respectively (Li and Kupfer, 1998). The sequences of the forward and reverse primers used to amplify CYP3A2 cDNA (GenBank accession number NM153341) were 5’-TGATCTGGTACCTGATCTGA-3’ and 5’-GGCCAG-GAAATACAAAGCAAA-3’, respectively (Zhang et al., 1996). The sequences of the forward and reverse primers used to amplify CYP1A18 cDNA (gene accession number NM145782) were 5’-CAACTACGGTGATGGCGATGT-3’ and 5’-CAGTCTGGTACCTGATCTGA-3’, respectively (Maehne et al., 1997). The sequence of the forward and reverse primers used to amplify CYP3A23 cDNA (GenBank accession number X96721) were 5’-GGAAATTCGATGG-GAGTGGC-3’ and 5’-AGGTGGTCCTTCTTCTTGG-3’, respectively (Maehne et al., 1997). The specificity of the primers was confirmed by sequencing analysis of the purified amplicons as described previously (Yu et al., 2005).

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Ginkgo biloba AND RAT HEPATOCYTE P450 EXPRESSION

Fig. 1. Concentration-dependent effect of Ginkgo biloba extract on CYP2B1 and CYP3A23 mRNA expression. Primary cultures of rat hepatocyte cultures were treated for 48 h with various concentrations (10, 100, or 1000 μg/ml) of a G. biloba extract (i.e., extract A, Table 1) or culture medium (vehicle control). As a positive control, hepatocytes were treated for the same length of time with PB (100 μg/ml) or DEX (10 μM) dissolved in DMSO (0.1% final concentration). CYP2B1 (A) and CYP3A23 (B) mRNA levels were determined by reverse transcription and real-time PCR. Data are expressed as mean ± S.E.M. for hepatocyte cultures from three individual rats per treatment group. * significantly different from the corresponding vehicle-treated control group (p < 0.05).

Results

Concentration-Dependent Effect of G. biloba Extract on CYP2B1 and CYP3A23 Gene Expression. To determine the effect of G. biloba extract on CYP2B1 gene expression, primary cultures of rat hepatocytes were treated for 48 h with various concentrations (10, 100, or 1000 μg/ml) of a G. biloba extract containing known amounts of diterpenes, sesquiterpene, and flavonols (extract A, Table 1). Control hepatocyte cultures were treated with culture medium (vehicle control). As shown in Fig. 1A, G. biloba extract, at concentrations of 10 μg/ml and 100 μg/ml, increased CYP2B1 mRNA levels by 3.1 ± 0.6-fold (mean ± S.E.M.) and 8.4 ± 2.2-fold, respectively, whereas no increase was obtained with the 1000 μg/ml concentration of the extract. As a positive control, PB (100 μM for 48 h) increased CYP2B1 mRNA expression by 26 ± 2-fold, which was 3.1-fold greater than the effect produced by the 100 μg/ml concentration of G. biloba extract (Fig. 1A). By comparison, treatment of primary cultures of rat hepatocytes for 48 h with ginkgolide A (1.1 μg/ml) or ginkgolide B (0.3 μg/ml) increased CYP3A23 mRNA expression by 9-fold, which was similar to the increase (8.3 ± 1.7-fold) by the extract. In contrast, it did not increase CYP3A23 mRNA levels (Fig. 2B).

Role of Bilobalide in the Modulation of CYP2B1 and CYP3A23 Gene Expression by G. biloba Extract. G. biloba extract contains various individual chemicals, including bilobalide (Table 1). Therefore, to determine whether this compound was responsible for the induction of CYP2B1 and CYP3A23 by G. biloba extract, primary cultures of rat hepatocytes were treated for 48 h with bilobalide (2.8 μg/ml). This concentration of bilobalide was chosen to represent the level present in a CYP2B1-inducing concentration (100 μg/ml; Fig. 1A) of a G. biloba extract (i.e., extract A, Table 1). As shown in Fig. 2A, bilobalide increased CYP2B1 mRNA expression by 7.9 ± 0.5-fold, which was similar to the increase (8.3 ± 1.7-fold) by the extract. In contrast, it did not increase CYP3A23 mRNA levels (Fig. 2B).

Role of Ginkgolides A, B, C, and J in the Modulation of CYP2B1 and CYP3A23 Gene Expression by G. biloba Extract. G. biloba extracts also contain diterpenes, such as the ginkgolides (Table 1). Therefore, primary cultures of rat hepatocytes were treated for 48 h with ginkgolide A (1.1 μg/ml), ginkgolide B (0.3 μg/ml), ginkgolide C (1.4 μg/ml), ginkgolide J (0.6 μg/ml), or the vehicle (0.1% DMSO).

TABLE 2
CYP2B1 and CYP3A23 mRNA expression in primary cultures of rat hepatocytes treated with different brands and different lots of G. biloba extract

<table>
<thead>
<tr>
<th>G. biloba Extract</th>
<th>Fold Increase Relative to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP2B1 mRNA</td>
</tr>
<tr>
<td>Extract A</td>
<td></td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>3.1 ± 0.6*</td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>8.4 ± 2.2*</td>
</tr>
<tr>
<td>1000 μg/ml</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Extract B</td>
<td></td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>2.2 ± 0.3*</td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>4.0 ± 0.6*</td>
</tr>
<tr>
<td>1000 μg/ml</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td>Extract C</td>
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<tr>
<td>10 μg/ml</td>
<td>2.2 ± 0.3*</td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>4.2 ± 0.2*</td>
</tr>
<tr>
<td>1000 μg/ml</td>
<td>1.9 ± 0.6</td>
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</table>

* Significantly different from the control group (p < 0.05).

containing 0.5% acetic acid, increasing to 90% methanol containing 0.5% acetic acid over 8 min, and staying at 90% for 2 min before reequilibration at the initial condition. A standard curve was constructed with 30 to 5000 pmol of 6p-hydroxytestosterone.

Statistics. Data analyses were conducted using one-way analysis of variance and the Student Newman-Keuls multiple comparison test (SigmaStat software program; SPSS Inc., Chicago, IL). Statistical significance was set a priori at p < 0.05.
The concentrations of the individual ginkgolides were chosen to reflect the levels present in a CYP3A23-inducing concentration (100 μg/ml; Fig. 1B) of a G. biloba extract (i.e., extract A, Table 1). None of the ginkgolides influenced CYP2B1 mRNA expression (Fig. 2A), whereas only ginkgolide A increased (2.6 ± 0.5-fold) CYP3A23 mRNA levels (Fig. 2B), although the magnitude of the effect was less than the 5.3 ± 1.7-fold increase by the extract (Fig. 2B).

**Differential Effect of Bilobalide on CYP2B1 and CYP3A23**

**Gene Expression.** In a study that quantified the amount of bilobalide in 27 commercial products of *G. biloba*, the abundance of this compound ranged from 0.8% w/w to 4.6% w/w (Kressmann et al., 2002). In the present study, a concentration-response experiment was conducted whereby primary cultures of rat hepatocytes were treated for 48 h with bilobalide at 1, 2.8, or 5 μg/ml. As shown in Fig. 3A, bilobalide increased CYP2B1 mRNA expression in a concentration-dependent manner, but it had no effect on CYP3A23 mRNA levels.

**Differential Effect of Ginkgolide A on CYP3A23 and CYP2B1**

**Gene Expression.** The abundance of ginkgolide A ranged from 1.1% w/w to 3.8% w/w in a survey of 27 commercial products of *G. biloba* (Kressmann et al., 2002). Therefore, a concentration-response experiment was performed. Primary cultures of rat hepatocytes were treated for 48 h with ginkgolide A at 1.1, 2.5, or 5 μg/ml. As shown in Fig. 3B, ginkgolide A increased CYP3A23 mRNA levels linearly over this range of concentrations, but it did not affect CYP2B1 mRNA expression.

**Role of Flavonol Glycosides and Aglycones in the Modulation of**

**CYP2B1 and CYP3A23**

**Gene Expression by G. biloba Extract.** Flavonol glycosides are present in *G. biloba* extracts (van Beek, 2002). Therefore, primary cultures of rat hepatocytes were treated for 48 h with kaempferol-3-O-rutinoside (1.9 μg/ml), quercetin-3-O-rutinoside (4 μg/ml), isorhamnetin-3-O-rutinoside (0.6 μg/ml), or the vehicle (0.1% DMSO). The concentration of isorhamnetin-3-O-rutinoside was chosen to reflect the level present in a 100 μg/ml concentration of a *G. biloba* extract (i.e., extract A, Table 1). However, the levels of kaempferol-3-O-rutinoside and quercetin-3-O-rutinoside were not known in extract A. Therefore, we used a concentration that...
represented the level of all the kaempferol diglycosides or quercetin diglycosides present in the extract. As shown in Table 3, isorhamnetin-3-O-rutinoside, kaempferol-3-O-rutinoside, and quercetin-3-O-rutinoside did not affect CYP2B1 or CYP3A23 mRNA expression.

Flavonol glycosides are converted to the corresponding aglycones by the action of intestinal microflora (Walle, 2004). Therefore, primary cultures of rat hepatocytes were treated for 48 h with kaempferol (6.3 μg/ml), quercetin (10.6 μg/ml), isorhamnetin (4.1 μg/ml), or the vehicle (0.1% DMSO). These concentrations were selected to reflect the total levels of each of the flavonols (i.e., the sum of the aglycone and the glycosides). As shown in Table 3, kaempferol, quercetin, and isorhamnetin did not increase CYP2B1 or CYP3A23 mRNA expression.

**Effect of G. biloba Extract, Ginkgolides, Bilobalide, and Flavonoids on CYP3A2 and CYP3A18 Gene Expression.** To determine the effect of *G. biloba* extract and some of its individual chemical constituents on CYP3A2 and CYP3A18 gene expression, primary cultures of rat hepatocytes were treated for 48 h with a *G. biloba* extract (i.e., extract A, Table 1; 100 μg/ml), culture medium (vehicle for the extract), ginkgolide A (1.1 or 5 μg/ml), bilobalide (2.8 μg/ml), kaempferol-3-O-rutinoside (1.9 μg/ml), quercetin-3-O-rutinoside (4 μg/ml), isorhamnetin-3-O-rutinoside (0.6 μg/ml), kaempferol (6.3 μg/ml), quercetin (10.6 μg/ml), or isorhamnetin (4.1 μg/ml). Control cultures were treated with culture medium (vehicle for the extract) or DMSO (0.1%, vehicle for the individual chemicals). Data are expressed as the mean ± S.E.M. fold-increase in mRNA expression (relative to the vehicle-treated control group) for hepatocyte cultures from three individual rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold Increase Relative to Control</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CYP2B1 mRNA</td>
</tr>
<tr>
<td>Kaempferol-3-O-rutinoside</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Quercetin-3-O-rutinoside</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Isorhamnetin-3-O-rutinoside</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>Isorhamnetin</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td><em>Ginkgo biloba</em> extract</td>
<td>8.3 ± 1.7*</td>
</tr>
</tbody>
</table>

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

**TABLE 3**

Effect of flavonol glycosides and aglycones on CYP2B1 and CYP3A23 mRNA expression in primary cultures of rat hepatocytes

Primary cultures of rat hepatocytes were treated with a *G. biloba* extract (i.e., extract A, Table 1; 100 μg/ml), kaempferol-3-O-rutinoside (1.9 μg/ml), quercetin-3-O-rutinoside (4 μg/ml), isorhamnetin-3-O-rutinoside (0.6 μg/ml), kaempferol (6.3 μg/ml), quercetin (10.6 μg/ml), or isorhamnetin (4.1 μg/ml). Control cultures were treated with culture medium (vehicle for the extract) or DMSO (0.1%, vehicle for the individual chemicals). Data are expressed as the mean ± S.E.M. fold-increase in mRNA expression (relative to the vehicle-treated control group) for hepatocyte cultures from three individual rats.

**FIG. 4.** Effect of *G. biloba* extract and ginkgolide A on CYP3A2 and CYP3A18 mRNA expression. Primary cultures of rat hepatocyte cultures were treated for 48 h with *G. biloba* extract (i.e., extract A, Table 1; 100 μg/ml), culture medium (vehicle for the extract), ginkgolide A (1.1 or 5 μg/ml), DEX (10 μM, positive control), or DMSO (0.1%, vehicle for ginkgolide A and DEX). CYP3A2 (A) and CYP3A18 (B) mRNA levels were determined by reverse transcription and real-time PCR. Data are expressed as mean ± S.E.M. for hepatocytes from three individual rats per treatment group. * significantly different from the corresponding vehicle-treated control group (p < 0.05).

Activity, DEX (10 μM, positive control for CYP3A activity), or DMSO (0.1%, vehicle for the individual chemicals). *G. biloba* extract increased CYP2B-mediated BROD by 5 ± 1-fold (Fig. 5A), whereas PB increased it by 23 ± 5-fold (Fig. 5A). The extract also increased CYP3A-mediated testosterone 6β-hydroxylation, although the extent (2.3 ± 0.7-fold) was less than the 9.6 ± 2.3-fold by DEX (Fig. 5B). Concentration-response experiments showed that bilobalide increased BROD, but not testosterone 6β-hydroxylation (Fig. 6A). In contrast, ginkgolide A increased testosterone 6β-hydroxylation, but not BROD (Fig. 6B).

**Discussion**

The impetus for the present study was the finding that the administration of *G. biloba* extract to rats increased hepatic P450-mediated enzyme activities (Shinozuka et al., 2002; Umegaki et al., 2002). However, those findings may not be applicable to other extracts of *G. biloba*. Most of the commercially available *G. biloba* products contain approximately 6% (w/w) of terpene trilactones (Kressmann et al., 2002). The extract used in the previous studies (Shinozuka et al., 2002; Umegaki et al., 2002) contained a greater amount (10.6% w/w) of terpene trilactones. The present study was conducted with *G. biloba* extract.
extracts from different lots and different manufacturers. These extracts contained known amounts of terpene trilactones (6.2–6.6% w/w) and flavonol glycosides (21–24.4% w/w) (Table 1). Our results indicated that *G. biloba* extracts increased rat hepatic CYP2B1, CYP3A23, CYP3A2, and CYP3A18 gene expression.

CYP2B1 is a major PB-inducible P450 enzyme in rat liver (Waxman, 1999). In the present study, treatment of primary cultures of rat hepatocytes with *G. biloba* extract increased CYP2B1 gene expression level to approximately one-third of that by 100 \( \mu \text{M} \) PB, which is a maximal CYP2B1-inducing concentration (LeCluyse et al., 1999). It also increased CYP2B-mediated BROD to approximately one-quarter of the level by PB. At a concentration of 1000 \( \mu \text{g/ml} \), *G. biloba* extract did not increase CYP2B1 gene expression. A potential explanation could be that one or more chemicals present in the extract might have suppressed the CYP2B induction response. Another explanation might be general cellular toxicity, although this was not assessed in our hepatocyte cultures. However, the same concentration of the extract resulted in a maximal increase in CYP3A23 gene expression.

Several hepatic CYP3A enzymes, such as CYP3A23, CYP3A2, and CYP3A18, are expressed in male rats (Mahnke et al., 1997; Matsubara et al., 2004). The constitutive expression of hepatic CYP3A23 is low, but it is a major DEX-inducible P450 enzyme (Mahnke et al., 1997). Our results obtained with a *G. biloba* extract (i.e., extract A, Table 1) showed that it induced CYP3A23 gene expression to a level that was approximately one-third of that by 10 \( \mu \text{M} \) DEX, which is a maximal CYP3A23-inducing concentration (Lu and Li, 2001). However, minor differences existed in the magnitude of the increase among the extracts tested. Similar to CYP3A23, CYP3A2 and CYP3A18 are also inducible by DEX (Mahnke et al., 1997). As shown in the present study, when compared with a maximal CYP3A3-inducing concentration (10 \( \mu \text{M} \)) of DEX (Lu and Li, 2001), *G. biloba* extract was less effective in elevating CYP3A23 and CYP3A2 mRNA levels, but both were similarly effective in inducing CYP3A18. Consistent with the CYP3A gene expression data, treatment of cultured hepatocytes with *G. biloba* extract increased testosterone 6\( \beta \)-hydroxylation, which is mediated by CYP3A23, CYP3A2, CYP3A18, and other CYP3A enzymes (Matsubara et al., 2004). Our cell culture results are in agreement with these findings.
agreement with a previous study indicating that treatment of 5-week-old male Wistar rats with an extract of *G. biloba* (10, 100, or 1000 mg/kg via intragastric gavage once daily for 5 days) increased hepatic microsomal testosterone 6β-hydroxylation activity (Umegaki et al., 2002).

Bilobalide is the most abundant terpene trilactone in commercial preparations of *G. biloba* (Kressmann et al., 2002). According to the German Commission E monograph, the amount of bilobalide in a *G. biloba* product should be within the range of 2.6 to 3.2% w/w (Blumenthal, 1998). A novel finding from the present study is that bilobalide is responsible for the modulation of CYP2B1 gene expression and CYP2B-mediated BROD by *G. biloba* extract. Our results also indicated a concentration-dependent relationship on the effect of bilobalide (1–5 μg/ml) on CYP2B1 mRNA and enzyme activity. We tested this range of concentrations because according to a survey of commercial *G. biloba* products, the bilobalide concentrations were between 0.8 and 4.6 μg/ml (Kressmann et al., 2002). Interestingly, an earlier study reported that the oral administration of bilobalide (30 mg/kg once daily for 4 days) to mice increased hepatic microsomal 7-methoxycoumarin O-demethylation activity (Sasaki et al., 1997). However, it is not known which P450 enzyme(s) is responsible for this PB-inducible activity in mouse liver microsomes.

Another group of terpene trilactones present in *G. biloba* extracts are the diterpenes, which include ginkgolides A, B, C, and J (van Beek, 2002). According to the German Commission E monograph, the total amount of ginkgolides in a *G. biloba* product should be within the range of 2.8 to 3.4% w/w (Blumenthal, 1998), although there is no specified amount for the individual ginkgolides. As shown for the first time in the present study, ginkgolide A is partially responsible for the modulation of CYP3A23 gene expression by *G. biloba* extract. This compound, when determined at a concentration (1.1 μg/ml) that reflected the level present in a CYP3A-inducing concentration (100 μg/ml) of a *G. biloba* extract (i.e., extract A, Table 1), increased CYP3A23 mRNA expression to approximately one-half of that by the extract. Ginkgolide A increased CYP3A23 mRNA expression and CYP3A-catalyzed testosterone 6β-hydroxylation over the range of 1.1 to 5 μg/ml, which reflected the concentrations (1.1–3.8%) of ginkgolide A present in a survey of 27 commercial products of *G. biloba* (Kressmann et al., 2002). Interestingly, ginkgolide A, at the concentration (1.1 μg/ml) present in extract A (Table 1), did not increase CYP3A2 or CYP3A18 mRNA expression. A greater concentration (5 μg/ml) was required for the modulation of these two CYP3A genes by ginkgolide A.

A novel finding from the present study is the differential induction of CYP2B1 and CYP3A23 gene expression by bilobalide and ginkgolide A. The molecular basis for these effects is not known. The current evidence indicates that the constitutive androstane receptor mediates the induction of CYP2B1 (Muangmoonchai et al., 2001), whereas the pregnane X receptor is a regulator of CYP3A23 induction (Zhang et al., 1999). Therefore, a potential explanation for the difference in the two compounds on CYP2B1 and CYP3A23 expression is the preferential activation of constitutive androstane receptor and pregnane X receptor by bilobalide and ginkgolide A, respectively. Studies are planned to elucidate the molecular mechanism of CYP2B and CYP3A induction by these naturally occurring chemicals.

Another major group of constituents in *G. biloba* extracts are the flavonol glycosides, including those of quercetin, kaempferol, andisorhamnetin (van Beek, 2002). However, as demonstrated in the present study, the aglycone and the 3-O-rutinoside of each of these flavonols did not increase CYP2B1, CYP3A23, CYP3A2, or CYP3A18 gene expression. This finding is consistent with the result from a previous study showing a lack of an increase in hepatic microsomal CYP2B-mediated pentoxyresorufin O-dealkylation activity in rats administered quercetin via the diet for 2 weeks at a dosage of 1% w/w (Brouard et al., 1988).

It is not known whether *G. biloba* extract alters CYP2B-mediated drug clearance in humans, but conflicting data appear to exist for the effect of this herbal medicine on the clearance of drugs metabolized by human CYP3A enzymes. In one study, the oral ingestion of *G. biloba* extract (60 mg four times daily for 28 days) by healthy human volunteers did not alter the 1-h serum ratio of 1-hydroxymidazolam/midazolam (Gurley et al., 2002), which is used as an in vivo metabolic index for CYP3A activity (Thummel et al., 1994). However, in another study, the oral ingestion of *G. biloba* extract (120 mg twice daily) for 14 days resulted in a modest but statistically significant reduction in the area under the plasma alprazolam concentration-time curve (Markowitz et al., 2003). This result suggests a CYP3A-inductive effect by *G. biloba* because alprazolam is metabolized predominantly by CYP3A enzymes (Gorski et al., 1999). In the studies that investigated the effect of *G. biloba* extract on drug elimination (Gurley et al., 2002; Markowitz et al., 2003), the levels of bilobalide and ginkgolide A in the extracts were not reported. Studies are in progress to determine directly whether *G. biloba* extract and some of its individual chemical constituents are capable of modulating CYP2B6 and CYP3A4 expression and function in primary cultures of human hepatocytes.

In summary, the present study in primary cultures of rat hepatocytes shows 1) induction of CYP2B1, CYP3A23, CYP3A2, and CYP3A18 gene expression by *G. biloba*, as shown with multiple extracts containing known amounts of terpene trilactones and flavonol glycosides; 2) bilobalide and ginkgolide A are novel inducers of CYP2B1 and CYP3A, respectively, as demonstrated by real-time PCR and enzyme activity assays; and 3) bilobalide is responsible for the modulation of CYP2B1 expression by *G. biloba* extract, whereas ginkgolide A contributes partially to the modulation of CYP3A23 expression by this herbal medicine.

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**References**


