Inhibition of Human CYP2B6-Catalyzed Bupropion Hydroxylation by *Ginkgo biloba* Extract: Effect of Terpene Trilactones and Flavonols

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Received April 18, 2009; accepted May 28, 2009

**ABSTRACT:**

Cytochrome P450 2B6 (CYP2B6) is expressed predominantly in human liver. It catalyzes the oxidative biotransformation of various drugs, including bupropion, which is an antidepressant and a tobacco use cessation agent. Serious adverse effects of high dosages of bupropion have been reported, including the onset of seizure. As *Ginkgo biloba* extract may be consumed with bupropion or another CYP2B6 drug substrate, potential exists for an herb-drug interaction. Therefore, we investigated the effect of *G. biloba* extract and some of its chemical constituents (terpene trilactones and flavonols) on the in vitro catalytic activity of CYP2B6 as assessed by the bupropion hydroxylation assay with recombinant enzyme and hepatic microsomes. The amount of hydroxybupropion was quantified by a novel and validated ultra-performance liquid chromatography/mass spectrometry method. Enzyme kinetic analysis indicated that *G. biloba* extract competitively inhibited hepatic microsomal CYP2B6-catalyzed bupropion hydroxylation (apparent $K_i$ was $162 \pm 14 \mu$g/ml). Bilobalide and ginkgolides A, B, C, and J were not responsible for the inhibition of CYP2B6 catalytic activity by the extract. Whereas the 3-O-gluicoside and 3-O-rutinoside of quercetin, kaempferol, andisorhamnetin had no effect, the corresponding aglycones (10 and 50 μg/ml) decreased hepatic microsomal bupropion hydroxylation. The inhibition of CYP2B6 by kaempferol was competitive ($K_i$ was 10 ± 1 μg/ml). In summary, *G. biloba* extract and its flavonol aglycones are naturally occurring inhibitors of in vitro CYP2B6 catalytic activity and bupropion hydroxylation. Future studies are needed to investigate whether *G. biloba* extract interacts in vivo with bupropion or other clinically important CYP2B6 drug substrates.

Cytochrome P450 2B6 (CYP2B6) is expressed mainly in human liver, although this enzyme has also been detected in various extrahepatic tissues (Gervot et al., 1999). Considerable variability exists not only in hepatic expression of CYP2B6 mRNA (280-fold) (Chang et al., 2003) and protein (>288-fold) (Hesse et al., 2004) but also CYP2B6 enzyme activity (80-fold) (Faucette et al., 2000). The basis for the interindividual variability may relate to pharmacogenetics (Hofmann et al., 2008) and the fact that this enzyme is subject to induction by various drugs and other chemicals in a mechanism that involves transcription factors such as the constitutive androstane receptor (Sueyoshi et al., 1999), which also exhibits large interindividual differences (240-fold) in hepatic expression (Chang et al., 2003). The magnitude of CYP2B6 catalytic activity may also be altered as a result of enzyme inhibition by various synthetic drugs (Turpeinen et al., 2004; Walsky et al., 2006); naturally occurring compounds, including phenethyl isothiocyanate (Nakajima et al., 2001), e-viniferin (Piver et al., 2003), and citral (Kim et al., 2008); and herbal supplements, such as Woolhwangcheongsimwon (Kim et al., 2008), *Andrographis paniculata* extract (Pekthong et al., 2008), and curcuminoid extract (Volak et al., 2008). Important CYP2B6 drug substrates include the alkylating anticancer prodrug cyclophosphamide (Chang et al., 1993) and the tobacco use cessation agent bupropion (Faucette et al., 2000; Hesse et al., 2000). The biotransformation of bupropion to hydroxybupropion is catalyzed predominantly by CYP2B6 in human liver (Faucette et al., 2000; Hesse et al., 2000). As a result, hepatic microsomal bupropion hydroxylation is used as an enzyme-selective catalytic marker for CYP2B6 in human liver (Turpeinen et al., 2004; Walsky et al., 2006).

Bupropion inhibits dopamine and noradrenaline reuptake, and it acts as an antagonist of neuronal nicotinic acetylcholine receptor (Dwoskin et al., 2006). This drug was available initially as an antidepressant. It is now widely used as a non-nicotine drug for smoking cessation. However, the use of high dosages of bupropion is associated with serious adverse effects (e.g., seizure), particularly among susceptible individuals (Beyens et al., 2008). In humans, bupropion undergoes extensive hepatic biotransformation to form hydroxybupropion, threo-hydrobupropion, and erythro-hydrobupropion, which are pharmacologically active metabolites (Dwoskin et al., 2006). Given that inhibition of bupropion biotransformation leads to greater plasma drug concentrations and the potential for the onset of serious adverse effects, it is therefore important to identify factors (e.g., concomitant

**ABBREVIATIONS:** UPLC, ultraperformance liquid chromatography; MS, mass spectrometry; DMSO, dimethylsulfoxide; LLOQ, lower limit of quantification; QC, quality control.
administration of a drug or herb) that may interfere with the biotransformation and clearance of bupropion.

*Ginkgo biloba* is an herbal medicine that contains terpene trilactones (~6% w/w), such as bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J; and flavonoids (~24% w/w), such as the aglycone and glycosides of kaempferol, quercetin, and isorhamnetin. In a recent survey, 20% of the 4202 individuals included in the survey were reported to be taking one or more herbal medicines (Gardiner et al., 2006). Given that *G. biloba* may be ingested with bupropion or another CYP2B6 drug substrate, potential exists for an herb-drug interaction, which may lead to adverse drug effects, especially among susceptible individuals, such as those with a chronic illness or compromised hepatic or renal function. Currently, it is not known whether *G. biloba* extract interferes with the biotransformation of bupropion or other CYP2B6 substrates.

The current study was designed to (1) investigate in detail the effect of *G. biloba* extract on bupropion hydroxylation catalyzed by recombinant CYP2B6 and human hepatic microsomes, as analyzed by a new and validated ultraperformance liquid chromatography (UPLC)/tandem mass spectrometry (MS) method and evaluated by enzyme kinetic analysis; (2) ascertain the role of bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J in the observed effect of the extract; and (3) compare the effect of the aglycone, 3-O-glucoside (a monoglycoside), and 3-O-rutinoside (a diglycoside) of kaempferol, quercetin, and isorhamnetin on hepatic microsomal CYP2B6-mediated bupropion hydroxylation. Our results show that *G. biloba* extract and the aglycones of kaempferol, quercetin, and isorhamnetin are naturally occurring inhibitors of CYP2B6 catalytic activity and bupropion hydroxylation in vitro.

**Materials and Methods**

*G. biloba* Extract. *G. biloba* extract was provided as dry powder by Indena S.A. (Milan, Italy). Shown in Table 1 are levels of individual terpene trilactones and flavonols in each of the five *G. biloba* extract used in the current study.

**Chemicals.** Bilobalide was provided by Indena S.A. Ginkgolide A, ginkgolide B, and ginkgolide C were purchased from LKT Labs (St. Paul, MN), and ginkgolide J was from ChromaDex (Irvine, CA). Kaempferol, isorhamnetin, quercetin 3-O-glucoside, kaempferol 3-O-glucoside, isorhamnetin 3-O-glucoside, quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside, and isorhamnetin 3-O-rutinoside were bought from Indofine Chemicals (Hillsborough, NJ). Quercetin dihydrate, bupropion hydrochloride (~98% purity), triprolidine hydrochloride (~99% purity), NADPH, dimethylsulfoxide (DMSO), and formic acid (LC/MS grade) were obtained from Sigma-Aldrich (St. Louis, MO). Authentic hydroxybupropion metabolite standard (95.2% purity) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). All the other commercially available chemicals were of analytical or high-performance liquid chromatographic grade.

**Human Hepatic Microsomes and Recombinant CYP2B6 Enzyme.** Human hepatic microsomes (lot 41207 pooled from 19 individual donors and lot 18888 pooled from 24 individual donors), microsomes from baculovirus-infected insect cells (Supersomes) that coexpressed CYP2B6 and NADPH/cytochrome P450 reductase, and control insect cell microsomes were purchased from BD Gentest (Woburn, MA).

**Bupropion Hydroxylation Assay.** Unless specified otherwise, each standard 200-μl incubation mixture contained potassium phosphate buffer (50 mM, pH 7.4), EDTA (1 mM), magnesium chloride (3 mM), human hepatic microsomes (100 μg of protein) or recombinant CYP2B6 (5 pmol), NADPH (1 mM), and bupropion (30 μM in incubations containing recombinant CYP2B6 and 50 μM in incubations containing human hepatic microsomes). Each incubation mixture was prewarmed for 2 min at 37°C in a shaking water bath. Enzymic reaction was initiated by the addition of NADPH and terminated 15 min (recombinant CYP2B6) or 30 min (human hepatic microsomes) later by the addition of 100 μl of ice-cold acetonitrile containing triprolidine (1 μM final concentration; internal standard). Each sample was mixed on a vortex and then centrifuged at 8000g for 10 min. Supernatant was transferred to an autosampler vial or 96-well plate for metabolite analysis by UPLC/MS/MS. To construct calibration curves, authentic hydroxybupropion (0.2–50 μM) was freshly prepared and added to the complete incubation mixture but with heat-inactivated enzymes (inactivated at 65°C for 20 min) and processed as described above. Enzyme kinetic analysis of bupropion hydroxylation was performed at substrate concentrations from 10 to 500 μM. The values of $V_{\text{max}}$ and apparent $K_m$ were determined by nonlinear regression analysis of the metabolite formation-substrate concentration data using the equation for the one-component Michaelis-Menten model (Enzyme Kinetics Module, version 1.1; SPSS Inc., Chicago, IL).

**Enzyme Inhibition Experiments.** *G. biloba* extract, a terpenic trilactone, a flavonol, or the corresponding vehicle (assay buffer for *G. biloba* extract and DMSO for the individual compounds) was added to the standard incubation mixture as described in each figure legend. Unless specified otherwise, the final concentration of DMSO was 0.1% v/v. A previous study showed that DMSO concentrations of ≥0.2% did not affect the enzyme kinetics of CYP2B6-catalyzed bupropion hydroxylation (Vuppugalla et al., 2007). To characterize the enzyme kinetics of CYP2B6 inhibition, multiple concentrations of the inhibitor (as specified in the figure legends) and bupropion (25, 50, 100, and 200 μM) were used. The apparent $K_i$ values and the mode of inhibition were determined by nonlinear regression analysis of the metabolite formation data collected at various substrate and inhibitor concentrations using equations for competitive, noncompetitive, mixed, and uncompetitive inhibition (Enzyme Kinetics Module, version 1.1; SPSS Inc.). The Akaike information criterion was used as a measure of goodness of fit. The mode of inhibition was verified by visual inspection of Lineweaver-Burk plots and Dixon plots of the enzyme kinetic data.

**Quantification of Hydroxybupropion by UPLC/MS/MS.** The amount of hydroxybupropion was quantified by an UPLC/MS/MS method. UPLC was performed using a Waters (Milford, MA) ACQUITY UPLC system equipped with a binary solvent manager and an autosampler. Chromatography was performed on a Waters ACQUITY UPLC BEH C18 column (100 × 2.1 mm i.d., 1.7 μm). The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The elution conditions were optimized as follows: isocratic at 2% B (0.0–1.5 min), linear gradient from 2% to 98% B (1.5–1.6 min).

**TABLE 1**

*Content of terpene trilactones and flavonols in various lots of *G. biloba* extract*

<table>
<thead>
<tr>
<th>Component</th>
<th>Lot A</th>
<th>Lot B</th>
<th>Lot C</th>
<th>Lot D</th>
<th>Lot E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diterpene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilobalide</td>
<td>0.9</td>
<td>1.1</td>
<td>1.3</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Ginkgolide A</td>
<td>0.3</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Ginkgolide C</td>
<td>1.5</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Ginkgolide J</td>
<td>0.6</td>
<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total diterpenes</strong></td>
<td>3.3</td>
<td>3.4</td>
<td>3.8</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td><strong>Sesquiterpene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilobalide</td>
<td>2.9</td>
<td>2.8</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Total sesquiterpenes</td>
<td>6.2</td>
<td>6.2</td>
<td>6.8</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td><strong>Flavonol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin (sum of aglycone and glycosides)</td>
<td>10.9</td>
<td>10.6</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Kaempferol (sum of aglycone and glycosides)</td>
<td>11.2</td>
<td>6.3</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Isorhamnetin (sum of aglycone and glycosides)</td>
<td>2.3</td>
<td>4.1</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td><strong>Total flavonoids</strong></td>
<td>24.4</td>
<td>21</td>
<td>24.4</td>
<td>24.3</td>
<td>24.4</td>
</tr>
</tbody>
</table>

N.A., not available.
The amount of hydroxybupropion in each sample was prepared independently on three separate days and quantified using the calibration curve constructed on each day. Accuracy was assessed based on the percentage bias of the measured concentration relative to the nominal concentration. Precision (% CV) was calculated by dividing the S.D. by the group mean of each set of QC samples and multiplied by 100. Matrix interference was investigated by comparing the MS response of authentic hydroxybupropion (0.2 μM) in the complete incubation mixture containing heat-inactivated enzyme (human hepatic microsomes or recombinant CYP2B6) with that in the complete incubation mixture without enzyme.

**Statistical Analysis.** Data were analyzed by one-way analysis of variance, followed by the Student Newman-Keuls multiple comparison test where appropriate (GraphPad Prism 3.0; GraphPad Software Inc., San Diego, CA). The level of statistical significance was set a priori at p < 0.05.

**Results**

**Analytical Method Development and Validation.** A new UPLC/MS/MS method was developed for the quantification of hydroxybupropion. The blank sample (incubations containing heat-inactivated microsomes but without analyte) did not display peaks at the m/z transition corresponding to hydroxybupropion or triprolidine (data not shown), thereby showing specificity and lack of interference by the matrix. The peak area ratio of the analyte (0.2 μM) to the internal standard in incubations containing heat-inactivated hepatic microsomes (0.091 ± 0.007, mean ± S.E.M.; n = 4) or recombinant CYP2B6 enzyme (0.095 ± 0.004; n = 4) was not significantly different (p = 0.11) from that obtained in incubations without enzyme (0.074 ± 0.008; n = 4), indicating that the matrix did not interfere with the magnitude of the MS response. The LLOQ for hydroxybupropion was 0.27 pmol. The calibration curve was linear from 0.2 to 100 μM as assessed by the coefficient of determination (r² > 0.99) and visual inspection of the regression line and residuals. The measured concentration of each of the standards was within 15% of the nominal concentration. Determination of intraday (n = 6) and interday (n = 3) accuracy of low, mid, and high analyte concentrations showed a bias of <10.2%, whereas the intraday and interday precision was <6.9% for low, mid, and high analyte concentrations (Table 2).

**Optimization of the Bupropion Hydroxylation Assay.** Initial experiments were performed to optimize the conditions of the enzyme assay. The bupropion hydroxylation assay was linear with respect to the amount of enzyme (10–200 μg of human hepatic microsomal protein; 0.25–10 pmol of recombinant CYP2B6) and incubation time (up to 20 min for recombinant CYP2B6 and 40 min for human hepatic microsomes). All the subsequent bupropion hydroxylation assays were performed with 100 μg of microsomal protein (or 5 pmol of recombinant CYP2B6) and incubation time of 20 min (recombinant CYP2B6) or 30 min (human hepatic microsomes). Enzyme kinetic analysis indicated that the apparent K_{in} values were 34 ± 5 μM and 59 ± 1 μM for bupropion hydroxylation catalyzed by recombinant
CYP2B6 and human hepatic microsomes, respectively. These values were comparable to those reported previously (Faucette et al., 2000; Hesse et al., 2000; Walsky and Obach, 2004).

**Effect of G. biloba Extract on CYP2B6 Catalytic Activity.** To determine the effect of G. biloba extract on the catalytic activity of CYP2B6, the bupropion hydroxylation assay was performed for five different lots of the extract (300 μg/ml) obtained from the same manufacturer. Each lot of the extract decreased bupropion hydroxylation catalyzed by recombinant CYP2B6 (Fig. 2). The magnitude of the inhibitory effect was similar among the five lots of the extract. In all the subsequent experiments, lot A was used.

**Dose-Response Relationship in CYP2B6 Inhibition by G. biloba Extract.** G. biloba extract (10, 100, 200, 300, 400, 600, or 800 μg/ml) or assay buffer (vehicle) was incubated with recombinant CYP2B6 or human hepatic microsomes. As shown in Fig. 3, a similar dose-response relationship was obtained in the inhibition of bupropion hydroxylation catalyzed by recombinant CYP2B6 and human hepatic microsomes. A log-linear decrease in bupropion hydroxylation activity was evident at extract concentrations of 100 to 800 μg/ml.

**Enzyme Kinetic Analysis of Inhibition of CYP2B6-Mediated Hepatic Microsomal Bupropion Hydroxylation by G. biloba Extract.** The bupropion hydroxylation assay was performed with varying concentrations of G. biloba extract (0, 200, 300, or 400 μg/ml) and bupropion (25, 50, 100, or 200 μM). Shown in Fig. 4 is a Lineweaver-Burk plot of the inhibition of hepatic microsomal CYP2B6-mediated bupropion hydroxylation by G. biloba extract. As determined by nonlinear regression analysis of the enzyme kinetic data and judged by Akaike information criterion and graphical plots of the enzyme kinetic data (Lineweaver-Burk plot and Dixon plot), the mode of inhibition was best described as competitive. The apparent Ki was 162 ± 14 μg/ml.

**Role of Terpene Trilactones in the Inhibition of CYP2B6-Catalyzed Bupropion Hydroxylation Activity by G. biloba Extract.** The next experiment was performed to determine whether a terpene trilactone was responsible for the inhibition of hepatic microsomal CYP2B6 enzyme activity by G. biloba extract. Therefore, the bupropion hydroxylation assay was performed with human hepatic microsomes in the presence of ginkgolide A (5.4 μg/ml), ginkgolide B (1.8 μg/ml), ginkgolide C (9 μg/ml), ginkgolide J (3.6 μg/ml), bilobalide (17 μg/ml), or a mixture of these five terpene trilactones. The concentration of each of these individual chemicals was chosen to reflect the level present in a selected concentration (600 μg/ml) of the extract (lot A, Table 1). The results showed that at the concentrations indicated above, none of these chemicals, either individually or as a mixture, decreased hepatic microsomal bupropion hydroxylation when compared with the vehicle-treated control group (data not shown). A lack of an effect by the terpene trilactones was also obtained when the enzymatic incubations were performed with recombinant CYP2B6 enzyme (data not shown).

**Effect of Flavonol Glycosides and Aglycones on CYP2B6 Catalytic Activity.** The flavonols in G. biloba extract are present primarily as a mixture of monoglycosides, diglycosides, and more complex structures.
glycosides of kaempferol, quercetin, and isorhamnetin (van Beek and Montoro, 2009). In the present study, the identity and levels of the individual flavonol glycosides and aglycones in our G. biloba extracts are not known (Table 1). Therefore, it was not possible to determine which flavonol glycoside(s) or aglycone(s) was responsible for the inhibition of CYP2B6 catalytic activity by the extract. However, as a proof-of-principle experiment to determine whether a flavonol glycoside is capable of inhibiting CYP2B6 catalytic activity by the extract, the bupropion hydroxylation assay was conducted with recombinant CYP2B6 and a flavonol monoglycoside or diglycoside. The concentrations of the monoglycosides [i.e., quercetin 3-O-glucoside (87 μg/ml), kaempferol 3-O-glucoside (90 μg/ml), and isorhamnetin 3-O-glucoside (18 μg/ml)] and diglycosides [i.e., quercetin 3-O-rutinoside (87 μg/ml), kaempferol 3-O-rutinoside (90 μg/ml), and isorhamnetin 3-O-rutinoside (18 μg/ml)] were chosen to reflect the levels present in an 800-μg/ml concentration of G. biloba extract, with the assumption that the glucoside or rutinoside accounted for the entire amount of each flavonol for lot A (Table 1). However, even at these concentrations, none of these flavonol glycosides affected CYP2B6 catalytic activity (data not shown).

To determine whether flavonol aglycones are capable of inhibiting CYP2B6 catalytic activity, we performed a dose-response experiment to investigate the effect of quercetin, kaempferol, and isorhamnetin on hepatic microsomal CYP2B6-mediated bupropion hydroxylation. As shown in Fig. 5, each of these flavonol aglycones at concentrations up to 2 μg/ml had little or no effect on hepatic microsomal bupropion hydroxylation, whereas decreases were evident at concentrations of 10 and 50 μg/ml.

Enzyme Kinetic Analysis of Inhibition of Hepatic Microsomal CYP2B6-Mediated Bupropion Hydroxylation by Kaempferol.

The bupropion hydroxylation assay was performed in incubations containing kaempferol (0, 5, 10, or 20 μg/ml), bupropion (25, 50, 100, or 200 μM), and human hepatic microsomes. Figure 6 is a Line-eweaver-Burk plot showing competitive inhibition of hepatic microsomal bupropion hydroxylation by kaempferol. The apparent K_i was 10 ± 1 μM (35 ± 3 μM). Enzyme kinetic experiment was not performed with quercetin or isorhamnetin because of the limited solubility of these chemicals at higher concentrations (>50 μg/ml).

Discussion

Relatively little scientific information is available on the effect of herbal medicines and naturally occurring compounds on the catalytic activity of CYP2B6, which plays an important role in the biotransformation of specific drugs and other chemicals. In the present study, we identified G. biloba extract as a novel inhibitor of CYP2B6 catalytic activity as assessed with recombinant enzymes and human hepatic microsomes. The extract competitively inhibited hepatic microsomal CYP2B6-mediated bupropion hydroxylation with an apparent K_i of 162 ± 14 μg/ml (IC_50 estimated to be 284 ± 10 μg/ml when the assay was performed at a substrate concentration of 50 μM). The potency in the inhibition of CYP2B6 catalytic activity by G. biloba extract appears to be in the same order of magnitude as that reported for another herbal medicine known as Wool-whangmengsiwon (Kim et al., 2008), which is a suspension of 29 herbs (mainly Calculus bovis, Moschus, Borneol rhizomatum synthetica, Radix ginseng, and Rhizonia dioscoreae). The apparent K_i was not determined in that study, although the reported IC_50 was 110 μg/ml for the inhibition of hepatic microsomal bupropion hydroxylation (at a substrate concentration of 50 μM). Inhibition of CYP2B6 catalytic activity has also been shown for three other herbal supplements: 1) a herbal cold remedy containing a mixture of eight different herbal extracts (maltodextrin, lonicera, forsythia, Chinese vitex, ginger, schizonepeta, isatis root, and echinacea) and nine vitamins and minerals (identity not reported) (Foti et al., 2007); 2) A. paniculata extract, which contained androgrophiol and deoxyandrogrophiol (Pekhong et al., 2008); and 3) curcuminoid extract, which contained the principal constituents curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Volak et al., 2008). However, a direct comparison of their CYP2B6 inhibitory potency with that of G. biloba extract is not possible because either the apparent K_i (or IC_50) was not determined (Foti et al., 2007) or the IC_50 was expressed in terms of the molar concentration of a principal constituent in the extract (Pekhong et al., 2008; Volak et al., 2008).

Another objective of the current study was to determine whether any of the terpene trilactones present in G. biloba was responsible for the inhibition of CYP2B6 enzyme activity by the extract. Therefore, experiments were performed in which the enzymatic incubation contained bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, or ginkgolide J at a concentration that reflected the level present in a specific concentration of the extract. However, none of the terpene trilactones, either individually or as a mixture, decreased bupropion hydroxylation catalyzed by recombinant CYP2B6 or human hepatic microsomes. These terpene trilactones are also not responsible for the inhibition of the catalytic activity of human CYP1A1, CYP1A2, or...
CYP1B1 by *G. biloba* extract as reported previously (Chang et al., 2006). Data from experiments with human recombinant enzymes support the notion that ginkgolides are not inhibitory toward cytochrome P450 enzymes because ginkgolide A, ginkgolide B, and ginkgolide C at a concentration of 200 μM do not affect the catalytic activity of human recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6, or CYP3A4 (Zou et al., 2002). In contrast, bilobalide inhibits only CYP2D6 (IC$_{50}$ = 11 μM) (Zou et al., 2002).

Flavonol is another class of chemicals found in *G. biloba*; however, they are present mainly as glycosides rather than as aglycones (van Beek and Montoro, 2009). Flavonol glycosides are hydrolyzed to the corresponding aglycones by the β-glucosidases present in intestinal microflora, and the resulting aglycones are absorbed (Cermak and Wolffram, 2006). Kaempferol and quercetin aglycones appear to be more potent than the corresponding glycosides in various biological activities; for example, antioxidant activity (Bedir et al., 2002). In the present study, 3-O-glucoside (a monoglycoside) and 3-O-rutinoside (a diglycoside) of kaempferol, quercetin, and isorhamnetin did not affect CYP2B6 catalytic activity. Structural studies have shown that CYP2B6 substrates are nonplanar, neutral or weakly basic, and fairly hydrophobic with one or two hydrogen bond acceptors (Lewis, 2000). Therefore, a plausible explanation for our findings is that the bulky and hydrophilic glycoside groups of kaempferol, quercetin, and isorhamnetin may hinder their interactions with the CYP2B6 enzyme binding site.

Kaempferol and quercetin aglycones have been reported to inhibit the in vitro catalytic activity of various human cytochrome P450 enzymes, for example, CYP1A2 and CYP3A (von Moltk et al., 2004), but the effect on CYP2B6 catalytic activity has not been investigated. As shown in the present study, kaempferol, quercetin, and isorhamnetin are capable of inhibiting hepatic microsomal CYP2B6-mediated bupropion hydroxylation. Consistent with our data for quercetin, a previous screening experiment reported inhibition of human recombinant CYP2B6 catalytic activity by quercetin when studied at a single concentration of 30 μM (9 μg/ml) (Walsky et al., 2006). As shown in Table 3, the apparent $K_{i}$ value for the inhibition of hepatic microsomal CYP2B6 by kaempferol is comparable with that reported for most of the other naturally occurring compounds. The most potent naturally occurring CYP2B6 inhibitors reported to date appear to be phenethyl isothiocyanate and e-viniferin, with apparent $K_{i}$ values of 2 and 3 μM, respectively, as analyzed with 7-benzoxylresorufin as the substrate (Nakajima et al., 2001; Piver et al., 2003). By comparison, the most potent CYP2B6 inhibitor reported to date is the synthetic drug ticlopidine (apparent $K_{i}$ = 0.2 μM) (Turpeinen et al., 2004).

It is not known whether the in vitro inhibition of CYP2B6 catalytic activity by *G. biloba* extract is of in vivo significance. The reason is that the chemical constituent(s) directly responsible for the in vitro inhibitory effect of the extract remains to be conclusively identified.

The results from the present study have allowed us to conclude that bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, kaempferol 3-O-glucoside, quercetin 3-O-glucoside, isorhamnetin 3-O-glucoside, kaempferol 3′-O-rutinoside, quercetin 3′-O-rutinoside, and isorhamnetin 3′-O-rutinoside are not responsible for CYP2B6 inhibition by the extract. Although kaempferol, quercetin, and isorhamnetin are shown to decrease hepatic microsomal CYP2B6-mediated bupropion hydroxylation, their in vitro inhibitory concentrations are greater than the maximal plasma levels (0.02–0.1 μM) achieved in human subjects who ingest *G. biloba* extract (Wójcicki et al., 1995). Other than terpene trilactones and flavonol glycosides, which account for approximately 30% of the chemical constituents in *G. biloba* extract, chemicals present in the extract include alkylphenols, organic acids, proanthocyanidins, catechins, biflavones, and nonflavonol glycosides (van Beek and Montoro, 2009). Whether any of these compounds affects CYP2B6 catalytic activity remains to be investigated.

Flavonols are not only present in various herbal medicines but also in fruits, vegetables, and certain beverages (Manach et al., 2005). In the case of quercetin, it is also available as a supplement in health food stores and is taken at dosages ranging from 500 mg to several grams per day. Median maximal plasma concentrations of 0.18, 0.30, and 0.43 μM have been reported in healthy human volunteers who ingested quercetin daily for 2 weeks at dosages of 50, 100, and 150 mg/day, respectively (Egert et al., 2008). By comparison, ingestion of shallots (*Allium cepa* L. var. *aggregatum*) that contain quercetin at a level of 1.4 mg/kg b.w. (Wiczkowski et al., 2008) or onions that yield an equivalent of 100 mg of quercetin (Manach et al., 2005) has produced plasma quercetin levels of 1 to 4 μM and 8 μM, respectively. Although hepatic levels of the ingested quercetin are not known, this compound accumulates in liver, as shown in an animal model (Bieger et al., 2008). It remains to be determined whether dietary exposure to quercetin or other flavonols has any influence on CYP2B6-mediated drug biotransformation.

Inhibition of cytochrome P450 enzymes may occur by various mechanisms, including reversible inhibition (also known as alternate substrate inhibition) and mechanism-based inactivation (Wienkers and Heath, 2005). Reversible inhibitors and mechanism-based inactivators of CYP2B6 have been identified (Turpeinen et al., 2006). The present study was designed only to investigate reversible inhibition. Future studies should address the question of whether *G. biloba* and its flavonols are mechanism-based inactivators of CYP2B6 because...
data from those in vitro studies may help in predicting the potential of G. biloba and flavonoids to inhibit CYP2B6-mediated drug biotransformation in vivo. The use of in vitro data to predict in vivo inhibitory effects in a given individual is complicated by many factors, including genetic variation (Wienkers and Heath, 2005). For example, when compared with the wild-type CYP2B6, the CYP2B6*4 and CYP2B6*6 variants appear to be less prone to inhibition (Talakad et al., 2009).

In summary, our major findings indicate that 1) G. biloba extract competitively inhibited human hepatic microsomal CYP2B6-catalyzed bupropion hydroxylation, with an apparent K_i of 162 µg/ml; 2) bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J were not responsible for the inhibitory effect of the extract; and 3) whereas a monoglycoside and a diglycoside of kaempferol, quercitin, and isorhamnetin had no effect, the corresponding aglycones inhibited hepatic microsomal CYP2B6-mediated enzyme activity. The discovery of G. biloba and flavonoids as in vitro inhibitors of CYP2B6 provides an impetus for future investigations to expand our understanding of the pharmacological and toxicological consequences of CYP2B6 inhibition by these natural products. Interestingly, it has been suggested that CYP2B6 inhibitors may be beneficial in preventing tamoxifen-mediated endometrial cancer (Stiborová et al., 2002).

Acknowledgments. We thank Indena S. A. (Milan, Italy) for the G. biloba extract and bilobalide. We also thank András Szeitz for technical assistance with the UPLC/MS instrumentation. T.K.H.C. received a Senior Scholar Award from the Michael Smith Foundation for Health Research.

References


