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

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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 bupro- pion 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 ul- tformance liquid chromatography/mass spectrometry method. Enzyme kinetic analysis indicated that G. biloba extract competi- tively 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-glucoside and 3-O-rutinoside of quercetin, kaempferol, and isolhamnetin had no effect, the corresponding aglycones (10 and 50 $\mu$g/ml) decreased hepatic microsomal bupropion hydroxylation. The inhibi- tion of CYP2B6 by kaempferol was competitive (apparent $K_i$ was $10 \pm 1$ $\mu$g/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 sub- strates.

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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 flavonols (~24% w/w), such as the aglycone and glycosides of kaempferol, quercetin, and isorhamnetin on hepatic microsomal CYP2B6-mediated bupropion hydroxylation (≥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. Enzymatic 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_{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 terpene 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 (1.5–16
using weighted \((1/x^2)\) linear least-squares regression analysis of the peak area
analysis of each set of samples. The amount of hydroxybupropion in each
were prepared independently on three separate days and quantified using the
authentic hydroxybupropion in the incubation mixture. The 18 QC samples
were added to the complete incubation mixture containing heat-inactivated microsomes and quantified by
UPLC/MS/MS. Intraday \((n = 6)\) and interday \((n = 3)\) accuracy and precision were determined.

<table>
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<th>Average Measured Concentration</th>
<th>S.D.</th>
<th>Precision</th>
<th>Accuracy</th>
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**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 \((0.2 \mu M)\) to the internal standard in incubations containing heat-inactivated hepatic microsomes \((0.991 \pm 0.007, \text{mean} \pm \text{S.E.M.}; n = 4)\) or recombinant CYP2B6 enzyme \((0.095 \pm 0.004; n = 4)\) was not significantly different \((p > 0.11)\) from that obtained in incubations without enzyme \((0.074 \pm 0.008; n = 4)\), 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 \(\mu M\) as assessed by the coefficient of determination \((r^2 > 0.99)\) 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 \((10–200 \mu g\) 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 \(\mu g\) 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 \(K_m\) values were 34 ± 5 \(\mu M\) and 59 ± 1 \(\mu M\) for bupropion hydroxylation catalyzed by recombinant
CYP2B6 and human hepatic microsomes, respectively, whereas the \( V_{\text{max}} \) values were 17 ± 2 pmol/min/pmol CYP2B6 and 473 ± 57 pmol/min/mg protein when the assay was conducted with recombinant CYP2B6 and human hepatic microsomes, respectively. These values are comparable with those reported previously (Faucette et al., 1999; 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 on 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 bupropion and 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 catalyzed by recombinant CYP2B6 and 268 ± 48 pmol/min/mg protein in incubations containing human hepatic microsomes.

**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 \( K_i \) 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...
glycosides of kaempferol, quercetin, andisorhametin (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), andisorhametin 3-O-glucoside (18 μg/ml)] and diglycosides [i.e., quercetin 3-O-rutinoside (87 μg/ml), kaempferol 3-O-rutinoside (90 μg/ml), andisorhametin 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 flavonol for lot A (Table 1). However, even at those 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, andisorhametin 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-weaver-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 orisorhametin 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 μM (IC50 estimated to be 284 ± 10 μM 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 Woohwangcheongsimwon (Kim et al., 2008), which is a suspension of 29 herbs (mainly Calculus bovis, Moschus, Borneolum syntheticum, Radix ginseng, and Rhizoma dioscoreae). The apparent K_i was not determined in that study, although the reported IC50 was 110 μM 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 (maldextrin, linonica, 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 andrographolide and deoxyandrographolide (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 IC50) was not determined (Foti et al., 2007) or the IC50 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
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, gingkoleide A, gingkoleide B, gingkoleide 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-cata

talyzed bupropion hydroxylation, with an apparent $K_I$ of $162 \mu$g/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, quercetin, andisorhamnetin had no effect, the corresponding aglycones inhibited human 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 (Stiborova et al., 2002).

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References


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