Inhibition of Human CYP2B6-Catalyzed Bupropion Hydroxylation by

Ginkgo biloba Extract: Effect of Terpene Trilactones and Flavonols

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Running Title

Ginkgo biloba and CYP2B6 Catalytic Activity

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ABBREVIATIONS: DMSO, dimethylsulfoxide; $K_i$, inhibition constant; $K_m$, Michaelis-Menten constant; MS, mass spectrometry; UPLC, ultra performance liquid chromatography.
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 a 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-$O$-glucoside and 3-$O$-rutinoside of quercetin, kaempferol, and isorhamnetin had no effect, the corresponding aglycones (10 and 50 µg/ml) decreased hepatic microsomal bupropion hydroxylation. The inhibition 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 substrates.
Introduction

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 inter-individual 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 inter-individual 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), ε-viniferin (Piver et al., 2003), and citral (Kim et al., 2008), and herbal supplements, such as Woohwangcheongsimwon (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 cytochrome P450 2B6 (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, threohydrobupropion, and erythrohydrobupropion, 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 administration of a drug or herb) that may interfere with the biotransformation and clearance of bupropion.

*G. biloba* is a 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 (van Beek and Montoro, 2009). Various commercial preparations of *G. biloba* extract are available, and a common use of these products by consumers is to improve cognitive performance (e.g. in dementia) (Ramassamy et al., 2007). *G. biloba* is one of the most popular herbal medicines. In a recent survey, 20% of the 4202 prescription and non-vitamin dietary supplement users reported consumption of *G. biloba* (Gardiner et al., 2006). Given that *G. biloba* may be ingested with bupropion or another CYP2B6 drug substrate, potential exists for a 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 ultra performance 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 lots of _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 Laboratories, Inc. (St. Paul, MN), and ginkgolide J from ChromaDex, Inc. (Irvine, CA). Kaempferol, isorhamnetin, quercetin 3-<i>O</i>-glucoside, kaempferol 3-<i>O</i>-glucoside, isorhamnetin 3-<i>O</i>-glucoside, quercetin 3-<i>O</i>-rutinoside, kaempferol 3-<i>O</i>-rutinoside, and isorhamnetin 3-<i>O</i>-rutinoside were bought from INDOFINE Chemical Co., Inc. (Somerville, NJ). Quercetin dihydrate, bupropion hydrochloride (≥ 98% purity), tripolidine 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 other commercially available chemicals were of analytical or high performance liquid chromatographic-grade.

_Human Hepatic Microsomes and Recombinant CYP2B6 Enzyme._ Human hepatic microsomes (lot no. 41207 pooled from 19 individual donors and lot no. 18888 pooled from 24 individual donors), microsomes from baculovirus-infected insect cells (Supersomes™) that co-expressed 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 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 pre-warmed 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 ice-cold acetonitrile containing tripolidine (1 μM final concentration; internal standard). Each sample was mixed on a vortex and then centrifuged at 8000 × g 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 to 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, non-competitive, mixed, and uncompetitive inhibition (Enzyme Kinetics Module, Version 1.1). The Akaike’s 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 ACQUITY UPLC™ system (Waters Corp., Milford, MA) equipped with a binary solvent manager and an autosampler. Chromatography was performed on a Waters ACQUITY UPLC BEH C₁₈ column (100 x 2.1 mm I.D., 1.7 μm). The mobile phases were: (A) 0.1% formic acid in water, and (B) 0.1% formic acid in methanol. The elution conditions were optimized as follows: isocratic at 2% B (0.0-1.5 min), linear gradient from 2% to 98% B (1.3-1.6 min), isocratic at 98% B (1.6-4.0 min), linear gradient from 98% to 2% B (4.0-4.1 min), and isocratic at 2% B (4.1-6.0 min). The total run time was 6 min. Column temperature was set at 30°C. The flow rate was 0.2 ml/min and the injection volume was 2 μl. UPLC effluent was introduced directly (without splitting) into the mass spectrometer interface from 2.5 to 4.0 min.

Mass spectrometry (MS) was performed using a Micromass Quattro Premier triple-quadrupole mass spectrometer (Waters Corp., Milford, MA) with a Z-spray electrospray ion source. The mass scale of the instrument was periodically calibrated using a solution of sodium and cesium iodides. The mass spectrometer was operated in the positive electrospray ionization mode. Nitrogen gas was used as the desolvation gas and cone gas at a flow rate of 900 L/h and 5 L/h, respectively. The mass spectrometer tune parameters were optimized to give the highest product ion intensities, using full MS and daughter scans. The optimized parameters were as
follows: electrospray capillary, 3.5 kV; cone, 20 V; extractor, 3.0 V; radiofrequency lens, 0.5 V; source temperature, 100°C; desolvation temperature, 300°C; mass resolution (low mass 1, high mass 1, low mass 2, high mass 2), 15.0; ion energy 1, 0.5; ion energy 2, 3.0; entrance, 0; exit, 3; and multiplier, 680 V. Tandem MS/MS experiments were performed using nitrogen as the collision gas, and the pressure in the collision cell was 4.3 x 10⁻³ mbar. The optimal collision energy was determined to be 15 eV. Hydroxybupropion and triprolidine (internal standard) were analyzed in the multiple reaction monitoring scan mode using the transitions m/z 257.3 → 239.1 and m/z 280.5 → 208.8, respectively (Fig. 1). The dwell time was 0.10 s, and the inter-scan delay was 0.10 s. The transitions were verified by daughter and parent scans. Data were acquired and processed using MassLynx™ version 4.1 software with QuanLynx™ application manager (Waters Corp., Milford, MA). A calibration curve was acquired before the analysis of each set of samples. The amount of hydroxybupropion in each sample was determined based on the calibration curve, which was constructed using weighted (1/x²) linear least-squares regression analysis of the peak area ratio of hydroxybupropion to triprolidine.

**Validation of the UPLC-MS-MS Method.** To determine the lower limit of quantification (LLOQ), varying amount of hydroxybupropion was added to the standard incubation mixture for the bupropion hydroxylation assay, except that heat-inactivated microsomes (100 µg protein) were used. The LLOQ was established based on a signal-to-noise ratio of 5:1, a precision of ± 20%, and an accuracy of ± 20%. To determine intra-day and inter-day accuracy and precision of the assay, quality control (QC) samples were prepared in six replicates at low (0.5 µM), mid (20 µM), and high (80 µM) concentrations of authentic hydroxybupropion in the incubation mixture. The 18 QC samples were 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 standard deviation 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) to that in the complete incubation mixture without enzyme.

**Statistical Analysis.** Data were analyzed by one-way ANOVA and, where appropriate, was followed by the Student Newman Keuls multiple comparison test (GraphPad Prism 3.0, GraphPad Software, Inc., La Jolla, 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 demonstrating
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 intra-day (n = 6) and inter-day (n = 3)
accuracy of low, mid, and high analyte concentrations showed a bias of < 10.2%, whereas the
intra-day and inter-day precision were < 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 to 200 μg of human hepatic microsomal
protein; 0.25 to 10 pmol recombinant CYP2B6) and incubation time (up to 20 min for
recombinant CYP2B6 and 40 min for human hepatic microsomes). All subsequent bupropion
hydroxylation assays were performed with 100 μg microsomal protein (or 5 pmol recombinant
CYP2B6) and an 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 to those reported previously (Hesse et al., 2000; Faucette 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 \( \mu \)g/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 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 \( \mu \)g/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 activity was evident at extract concentrations of 100-800 \( \mu \)g/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 \( \mu \)g/ml) and bupropion (25, 50, 100, or 200 \( \mu \)M). 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 the
Akaike’s 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 \( \mu \)g/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 \( \mu \)g/ml), ginkgolide B (1.8 \( \mu \)g/ml), ginkgolide C (9 \( \mu \)g/ml), ginkgolide J (3.6 \( \mu \)g/ml), bilobalide (17 \( \mu \)g/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 \( \mu \)g/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 to the vehicle-treated control group (data not shown). A lack of an effect by the terpene trilactones was also obtained when the enzymatic incubations was 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, 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, 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 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, 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. Fig. 6 is a Lineweaver-Burk plot showing competitive inhibition of hepatic microsomal bupropion hydroxylation by kaempferol. The apparent $K_i$ was 10 ± 1 µg/ml (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 309 ± 13 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 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 $IC_{50}$ 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, ionicera, forsythia, Chinese vitex, ginger, schizonepeta, isatis root, and echinacea) and nine vitamins and minerals (identity not reported) (Foti et al., 2007); 2) *Andrographis paniculata* extract, which contained andrographolide and deoxyandrographolide (Pekthong 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 (Pekthong 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\textsubscript{50} = 11 µM) (Zou et al., 2002).

Flavonol is another class of chemicals that is 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, anti-oxidant 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 non-planar, 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 Moltke 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 to 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 ε-viniferin, with apparent $K_i$ values of 2 µM and 3 µM, respectively, as analyzed with 7-benzyloxyresorufin 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 (Wojcicki 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 non-flavonol 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 two weeks at dosages of 50, 100, and 150 mg per 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 body weight (Wiczkowski et al., 2008) or onions that yield an equivalent of 100 mg quercetin (Manach et al., 2005) have produced plasma quercetin levels of 1-4 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 flavonols 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 to 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, quercetin, and isorhamnetin had no effect, the corresponding aglycones inhibited hepatic microsomal CYP2B6-mediated enzyme activity. The discovery of *G. biloba* and flavonols 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


Footnotes

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Figure Legends

FIG. 1. Total ion chromatograms of hydroxybupropion and triprolidine (internal standard). Pooled human hepatic microsomes (100 μg protein) were incubated with bupropion (50 μM) for 30 min at 37°C in 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 3 mM magnesium chloride. The internal standard (1 μM triprolidine in ice-cold acetonitrile) was added to terminate the reaction. Shown are total ion chromatograms of hydroxybupropion and triprolidine as analyzed by UPLC-MS-MS in the multiple reaction monitoring scan mode using the transitions m/z 257.30 → 239.10 and m/z 280.50 → 208.80, respectively.

FIG. 2. Effect of different lots of Ginkgo biloba extracts on bupropion hydroxylation catalyzed by recombinant CYP2B6. The assay was conducted with recombinant CYP2B6 (5 pmol), bupropion (30 μM), and G. biloba extract (0 or 300 μg/ml). The mixture was incubated for 15 min at 37°C. The amount of hydroxybupropion was quantified by UPLC-MS-MS as described under Materials and Methods. Results are expressed as mean ± S.E.M. for five independent experiments. *Significantly different from the control group (p < 0.05).

FIG. 3. Dose-response relationship on the inhibition of CYP2B6 catalytic activity by G. biloba extract. Pooled human hepatic microsomes (100 μg protein) or recombinant CYP2B6 (5 pmol) was incubated with bupropion (30 μM for CYP2B6 and 50 μM for hepatic microsomes) and G. biloba extract (0, 10, 100, 200, 300, 400, 600, or 800 μg/ml) at 37°C. Enzymatic reaction was initiated with 1 mM NADPH and terminated after 15 min (recombinant CYP2B6) or 30 min (hepatic microsomes). The amount of hydroxybupropion was quantified by UPLC-MS-MS as described under Materials and Methods. Results are expressed as mean ± S.E.M. for four
in independent experiments. Bupropion hydroxylation in the control group was 8.8 ± 1.1 pmol/min/pmol CYP2B6 in incubations containing recombinant CYP2B6 and 268 ± 48 pmol/min/mg protein in incubations containing human hepatic microsomes.

FIG. 4. Lineweaver-Burk plot for the competitive inhibition of human hepatic microsomal CYP2B6-mediated bupropion hydroxylation by *G. biloba* extract. Pooled human hepatic microsomes (100 μg protein) were incubated with bupropion (25, 50, 100, or 200 μM) and *G. biloba* extract (0, 200, 300, or 400 μg/ml) for 30 min at 37°C. The amount of hydroxybupropion was quantified by UPLC-MS-MS as described under Materials and Methods. Results are expressed as mean ± S.E.M. for four independent experiments.

FIG. 5. Dose-response effect of flavonol aglycones on human hepatic microsomal bupropion hydroxylation. Pooled human hepatic microsomes (100 μg protein) were incubated with bupropion (50 μM) in the presence of quercetin, kaempferol, isorhamnetin (5, 10, or 20 μg/ml), or DMSO (0.1% v/v final concentration; vehicle control) for 30 min at 37°C. The amount of hydroxybupropion was quantified by UPLC-MS-MS as described under Materials and Methods. Results are expressed as mean ± S.E.M. for four independent experiments. Bupropion hydroxylation in the control group was 190 ± 8 pmol/min/mg protein.

FIG. 6. Lineweaver-Burk plot for the competitive inhibition of human hepatic microsomal CYP2B6-mediated bupropion hydroxylation by kaempferol. Pooled human hepatic microsomes (100 μg protein) were incubated with bupropion (25, 50, 100, or 200 μM) and kaempferol (5, 10, or 20 μg/ml) or DMSO (0.1% v/v final concentration; vehicle control) for 30 min at 37°C. The
amount of hydroxybupropion was quantified by UPLC-MS-MS as described under Materials and Methods. Results are expressed as mean ± S.E.M. for four independent experiments.
TABLE 1

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

The content of terpene trilactones and flavonols were determined by gas chromatography (Indena S.A., Milan, Italy) and liquid chromatography-mass spectrometry (ChromaDex, Inc., Santa Ana, CA), respectively.

<table>
<thead>
<tr>
<th>Content in G. biloba Extract (%) w/w</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>Ginkgolide A</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 B</td>
<td>0.3</td>
<td>0.3</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>0.6</td>
<td>0.5</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Total diterpenes</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 terpene trilactones</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>Isorhamnnetin (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>Total flavonols</td>
<td>24.4</td>
<td>21</td>
<td>24.4</td>
<td>24.3</td>
<td>24.4</td>
</tr>
</tbody>
</table>

Abbreviation: N/A, not available.
### TABLE 2

**Intra-day and inter-day accuracy and precision in the UPLC-MS-MS analysis of hydroxybupropion**

Low, mid, and high concentrations of authentic hydroxybupropion were added to the complete incubation mixture and quantified by UPLC-MS-MS. Intra-day ($n = 6$) and inter-day ($n = 3$) accuracy and precision were determined.

<table>
<thead>
<tr>
<th>QC Sample</th>
<th>Nominal Concentration (μM)</th>
<th>Average Measured Concentration (μM)</th>
<th>SD</th>
<th>Precision (% CV)</th>
<th>Accuracy (% bias)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-day</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.5</td>
<td>0.48</td>
<td>0.02</td>
<td>4.75</td>
<td>-3.04</td>
</tr>
<tr>
<td>Mid</td>
<td>20</td>
<td>19.56</td>
<td>0.59</td>
<td>3.01</td>
<td>-2.19</td>
</tr>
<tr>
<td>High</td>
<td>80</td>
<td>71.86</td>
<td>2.98</td>
<td>4.15</td>
<td>-10.18</td>
</tr>
<tr>
<td><strong>Inter-day</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.5</td>
<td>0.52</td>
<td>0.04</td>
<td>6.91</td>
<td>3.91</td>
</tr>
<tr>
<td>Mid</td>
<td>20</td>
<td>20.91</td>
<td>1.20</td>
<td>5.73</td>
<td>4.54</td>
</tr>
<tr>
<td>High</td>
<td>80</td>
<td>74.23</td>
<td>3.43</td>
<td>4.63</td>
<td>-7.21</td>
</tr>
</tbody>
</table>
**TABLE 3**

*Apparent $K_i$ values for the inhibition of CYP2B6 catalytic activity by naturally occurring compounds*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>Apparent $K_i$ (μM)</th>
<th>Mode of Inhibition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenethyl isothiocyanate</td>
<td>7-Benzylhydroxresorufin</td>
<td>2 $^c$</td>
<td>Non-competitive</td>
<td>Nakajima et al., 2001</td>
</tr>
<tr>
<td>ε-Viniferin</td>
<td>7-Benzylhydroxresorufin</td>
<td>3 $^b$</td>
<td>Mixed</td>
<td>Piver et al., 2003</td>
</tr>
<tr>
<td>Isoborneol</td>
<td>Bupropion, Efavirenz</td>
<td>6 $^a$, 26 $^a$</td>
<td>Competitive</td>
<td>Kim et al., 2008</td>
</tr>
<tr>
<td>Citral</td>
<td>Bupropion</td>
<td>7 $^a$</td>
<td>Competitive</td>
<td>Seo et al., 2008</td>
</tr>
<tr>
<td>Borneol</td>
<td>Bupropion, Efavirenz</td>
<td>10 $^a$, 22 $^a$</td>
<td>Competitive</td>
<td>Kim et al., 2008</td>
</tr>
<tr>
<td>Geraniol</td>
<td>Bupropion</td>
<td>10 $^a$</td>
<td>Competitive</td>
<td>Seo et al., 2008</td>
</tr>
<tr>
<td>Curcumin</td>
<td>7-Benzylhydroxresorufin</td>
<td>33 $^c$</td>
<td>Competitive</td>
<td>Appiah-Opong et al., 2007</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>Bupropion</td>
<td>35 $^a$</td>
<td>Competitive</td>
<td>present study</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>7-Benzylhydroxresorufin</td>
<td>100 $^b$</td>
<td>Mixed</td>
<td>Piver et al., 2003</td>
</tr>
</tbody>
</table>

$^a$ pooled human hepatic microsomes, $^b$ individual human hepatic microsomes, $^c$ recombinant CYP2B6 enzyme
**Fig. 1**

**Hydroxybupropion**

$m/z 257.3 \rightarrow 239.1$

**Internal Standard**

$m/z 280.5 \rightarrow 208.8$
Fig. 2

G. biloba Extract
Fig. 3

![Graph showing the effect of G. biloba Extract (μg/ml) on Bupropion Hydroxylation (percentage of control). The graph compares CYP2B6 and Hepatic Microsomes.](image-url)
Fig. 4

**G. biloba Extract**

- **0 µg/ml**
- **200 µg/ml**
- **300 µg/ml**
- **400 µg/ml**

![Graph showing the effect of different concentrations of G. biloba extract on bupropion hydroxylation.](image)
Fig. 5

Bupropion Hydroxylation (percentage of control)

- Quercetin
- Kaempferol
- Isorhamnetin

Concentration (μg/ml)
Fig. 6

Kampferol
- 0 µg/ml
- 5 µg/ml
- 10 µg/ml
- 20 µg/ml

1/Bupropion Hydroxylation (pmol/min/mg protein)$^{-1}$

1/[Bupropion] (µM)$^{-1}$