Effect of *Ginkgo biloba* extract on procarcinogen-bioactivating human CYP1 enzymes: Identification of isorhamnetin, kaempferol, and quercetin as potent inhibitors of CYP1B1

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Abstract

In the present study, we investigated the effect of *Ginkgo biloba* extracts and some of its individual constituents on the catalytic activity of human cytochrome P450 enzymes CYP1B1, CYP1A1, and CYP1A2. *G. biloba* extract of known abundance of terpene trilactones and flavonol glycosides inhibited 7-ethoxyresorufin O-dealkylation catalyzed by human recombinant CYP1B1, CYP1A1, and CYP1A2, and human liver microsomes, with apparent $K_i$ values of 2 ± 0.3, 5 ± 0.5, 16 ± 1.4, and 39 ± 1.2 μg/ml (mean ± SE), respectively. In each case, the mode of inhibition was of the mixed type. Bilobalide, ginkgolides A, B, C, and J, quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside, and isorhamnetin 3-O-rutinoside were not responsible for the inhibition of CYP1 enzymes by *G. biloba* extract, as determined by experiments with these individual chemicals at the levels present in the extract. In contrast, the aglycones of quercetin, kaempferol, and isorhamnetin inhibited CYP1B1, CYP1A1, and CYP1A2. Among the three flavonol aglycones, isorhamnetin was the most potent in inhibiting CYP1B1 (apparent $K_i = 3 \pm 0.1$ nM), whereas quercetin was the least potent in inhibiting CYP1A2 (apparent $K_i = 418 \pm 50$ nM). The mode of inhibition was competitive, noncompetitive, or mixed, depending on the enzyme and the flavonol. *G. biloba* extract also reduced benzo[a]pyrene hydroxylation, and the effect was greater with CYP1B1 than with CYP1A1 as the catalyst. Overall, our novel findings indicate that *G. biloba* extract and the flavonol aglycones isorhamnetin, kaempferol, and quercetin preferentially inhibit the in vitro catalytic activity of human CYP1B1.

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Keywords: Bilobalide; Cytochrome P450; CYP1A1; CYP1A2; CYP1B1; Ginkgo biloba; Ginkgolide A; Ginkgolide B; Ginkgolide C; Ginkgolide J; Isoflavonoids; Kaempferol; Quercetin

Introduction

The cytochrome P450 1 (CYP1) family consists of CYP1A1, CYP1A2, and CYP1B1 (Lewis, 2004). These enzymes are important in the bioactivation of procarcinogens, such as polycyclic aromatic hydrocarbons, heterocyclic amines, aromatic amines, and nitropolycyclic hydrocarbons (Guengerich et al., 2003; Shimada and Fujii-Kuriyama, 2004), and they also play a role in the biotransformation of drugs (Rendic and Di Carlo, 1997), including anticancer drugs (McFadyen et al., 2004). In contrast to many of the cytochrome P450 enzymes, including CYP1A1 and CYP1A2, CYP1B1 is expressed in hormonally sensitive tissues, such as breast, ovary, and prostate (Muskhelishvili et al., 2001). A particular intriguing finding is that CYP1B1 is overexpressed in cancerous tissues (Murray et al., 1997). Recently, CYP1B1 was proposed as a target for cancer chemoprevention (Guengerich et al., 2003). Therefore, it is important to identify inhibitors of CYP1B1 that could be used to block the initiation stage of chemical carcinogenesis. In vitro studies over the last number of years have shown that herbal extracts and naturally occurring chemicals are capable of inhibiting CYP1B1 catalytic activity. These include *Panax ginseng*, *Panax quinquefolius*, and individual ginsenosides (Chang et al., 2002); polyphenolic compounds, such as trans-resveratrol (Chang et al., 2000, 2001); hesperetin (Doostdar et al., 2000), and baicalein (Chan et al., 2002); and coumarins, such as imperatorin (Mammen et al., 2005).

*Ginkgo biloba* extract is often used by individuals to improve central nervous system function, for example, cognitive enhancement in dementia (MacLennan et al., 2002).
Many of the commercial preparations of *G. biloba* extract are formulated to contain 6% terpene trilactones (i.e., bilobalide and ginkgolides A, B, C, and J) and 24% flavonol glycosides (i.e., those of quercetin, kaempferol, and isorhamnetin) (van Beek, 2002). Previous studies reported that *G. biloba* extract decreased CYP1A2-mediated enzyme activity in rat liver microsomes (Kuo et al., 2004) and human recombinant CYP1A2 catalytic activity (Gaudineau et al., 2004), and that flavonols, such as quercetin, kaempferol, and isorhamnetin, were capable of inhibiting human recombinant CYP1A2-catalyzed enzyme activities (Zou et al., 2002; von Molka et al., 2004). However, it remains to be determined whether *G. biloba* extract and its terpene trilactones and flavonols inhibit the catalytic activity of CYP1B1 and CYP1A1. This is important because CYP1B1 and CYP1A1 are considerably more active than CYP1A2 in catalyzing the bioactivation of benzo[a]pyrene (Shimada et al., 1997) and other carcinogenic polycyclic aromatic hydrocarbons (Shimada et al., 1996). Information on the effect of *G. biloba* extract on CYP1B1 and CYP1A1 function may help to explain a previous experimental finding indicating that treatment of female Swiss albino mice with a *G. biloba* extract reduces the multiplicity of forestomach tumors induced by benzo[a]pyrene (Agha et al., 2001).

The present in vitro study was conducted to determine whether *G. biloba* extract inhibits the catalytic activity of human CYP1B1 and CYP1A1 and to compare the effects to those on CYP1A2, as analyzed by the 7-ethoxyresorufin *O*-dealkylation assay. The *G. biloba* extracts used in our study contained known amounts of terpene trilactones (i.e., ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide) and flavonol glycosides (i.e., those of quercetin, kaempferol, and isorhamnetin (Table 1)). Therefore, we determined whether any of these individual chemicals are responsible for the effect of *G. biloba* extract on CYP1 catalytic activity. Finally, we investigated the effect of *G. biloba* extract on benzo[a]pyrene hydroxylation catalyzed by CYP1B1 or CYP1A1. Overall, our novel results indicate preferential inhibition of CYP1B1 by *G. biloba* extract and the flavonol aglycones quercetin, kaempferol, and isorhamnetin.

### Materials and methods

**Ginkgo biloba extracts.** *G. biloba* extracts were provided by Indena S.A. (Tours, France; batch no. 1306A; i.e., Extract A, and batch no. 302831; i.e., Extract B) and Pharmaton S.A. (Bioggio, Switzerland; GK501, Tours, France; batch no. 1306A; i.e., Extract A, and batch no. 302831; i.e., Extract B). In our experiments, the extract in its powder form was dissolved in buffer, as described in each figure legend. Shown in Table 1 is the amount of terpene trilactones and flavonol glycosides present in each of the extracts. According to the manufacturers of the *G. biloba* extracts used in the present study, the sum of all the ginkgolic acids was < 5 ppm.

**Chemicals and authentic metabolite standards.** Bilobalide (batch no. 157/25/1; 97.9% pure) was supplied by Indena S.A. (Milan, Italy). Ginkgolide A (lot no. 220961, 95% pure), ginkgolide B (lot no. 220962, 95% pure), and ginkgolide C (lot no. 2396706, 99.9% pure) were purchased from LKT Laboratories Inc. (St. Paul, MN, USA). Ginkgolide J (lot no. 01-07186-101, 95.6% pure) was bought from ChromaDex, Inc. (Santa Ana, CA, USA). Kaempferol (lot no. 1040952, 99% pure), kaempferol-3-O-rutinoside (lot no. 0110214, 99.8% pure), quercetin-3-O-rutinoside (lot no. 02031209, 99.3% pure), isorhamnetin (lot no. 02053126, 99% pure), and isorhamnetin-3-O-rutinoside (lot no. 00082202, 99% pure) were purchased from Indofine Chemical Co. Inc. (Somerville, NJ, USA). The stated purity of each of these chemicals was based on the label claim by the manufacturer. Dimethylsulfoxide (DMSO), quercetin, NADPH, benzo[a]pyrene, and 7-ethoxyresorufin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Resorufin and 3-hydroxybenzo[a]pyrene were bought from Pierce Chemical Co. (Rockford, IL, USA) and RTC Corp. (Laramie, WY, USA), respectively.

### Table 1

<table>
<thead>
<tr>
<th>Amount of ginkgolides, bilobalide, and flavonols in the Ginkgo biloba extracts used in the present study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract A</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>Diterpene</td>
</tr>
<tr>
<td>Ginkgolide A</td>
</tr>
<tr>
<td>Ginkgolide B</td>
</tr>
<tr>
<td>Ginkgolide C</td>
</tr>
<tr>
<td>Ginkgolide J</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Sesquiterpene</td>
</tr>
<tr>
<td>Bilobalide</td>
</tr>
<tr>
<td>Total terpene trilactones</td>
</tr>
<tr>
<td>Flavonol and its glycosides</td>
</tr>
<tr>
<td>Kaempferol (aglycone)</td>
</tr>
<tr>
<td>Kaempferol (diglycosides)</td>
</tr>
<tr>
<td>Kaempferol (other glycosides)</td>
</tr>
<tr>
<td>Kaempferol (sum of aglycone and glycosides)</td>
</tr>
<tr>
<td>Quercetin (aglycone)</td>
</tr>
<tr>
<td>Quercetin (diglycosides)</td>
</tr>
<tr>
<td>Quercetin (other glycosides)</td>
</tr>
<tr>
<td>Quercetin (sum of aglycone and glycosides)</td>
</tr>
<tr>
<td>Isorhamnetin (aglycone)</td>
</tr>
<tr>
<td>Isorhamnetin (3-O-rutinoside)</td>
</tr>
<tr>
<td>Isorhamnetin (other glycosides)</td>
</tr>
<tr>
<td>Isorhamnetin (sum of aglycone and glycosides)</td>
</tr>
<tr>
<td>Total flavonol glycosides</td>
</tr>
</tbody>
</table>

The amount of terpenes in *Ginkgo biloba* Extracts A and B was quantified by gas chromatography (Indena S.A., Milan, Italy), and the amount of flavonols in these extracts was quantified by liquid chromatography–mass spectrometry (ChromaDex, Inc., Santa Ana, CA). The amount of terpenes and flavonoids in *Ginkgo biloba* extract C (GK501™) were determined by high performance liquid chromatography (Pharmaton S.A., Bioggio, Switzerland). Abbreviation: n.d., not determined.

99% pure were purchased from Indofine Chemical Co. Inc. (Somerville, NJ, USA). The stated purity of each of these chemicals was based on the label claim by the manufacturer. Dimethylsulfoxide (DMSO), quercetin, NADPH, benzo[a]pyrene, and 7-ethoxyresorufin were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Resorufin and 3-hydroxybenzo[a]pyrene were bought from Pierce Chemical Co. (Rockford, IL, USA) and RTC Corp. (Laramie, WY, USA), respectively.

**Human liver microsomes and recombinant CYP enzymes.** Pooled human liver microsomes (cat. no. 452161), microsomes from baculovirus-infected insect cells co-expressing human NADPH-cytochrome P450 reductase and CYP1A1 (cat. no. 456211), CYP1A2 (cat. no. 456203) or CYP1B1 (cat. no. 456220), and the corresponding control insect cell microsomes (cat. no. 456200) were purchased from BD GENTEST (Woburn, MA, USA).

7-Ethoxyresorufin O-dealkylation assay. The 7-ethoxyresorufin O-dealkylation assay was conducted using a continuous spectrofluorometric method (Burke and Mayer, 1974), as described previously (Chang et al., 2001). Unless indicated otherwise, each standard 2-ml incubation contained 100 mM potassium phosphate buffer (pH 7.4), 0.2 μM 7-ethoxyresorufin, cDNA-expressed human CYP enzyme (1 pmol CYP1A1, 5 pmol CYP1A2, or 2.5 pmol CYP1B1) or human liver microsomes (75 pmol total CYP), and 0.25 mM NADPH. Reaction was initiated by the addition of NADPH and allowed to proceed at 37 °C for up to 3 min. Initial experiments indicated that the assay was linear with respect to incubation time and amount of enzyme. None of the extracts or the individual
chemicals interfered with the fluorescence of the enzymatic product in the 7-ethoxyresorufin O-dealkylation assay.

**Benzo[a]pyrene hydroxylation assay.** The benzo[a]pyrene hydroxylation assay was performed based on a published method (Nebert and Gelboin, 1964). Unless otherwise indicated, each standard 1-ml incubation contained 100 mM potassium phosphate buffer (pH 7.4), human recombinant CYP1B1 (10 pmol) or CYP1A1 (10 pmol), benzo[a]pyrene (0.6 μM for CYP1B1 and 3 μM for CYP1A1), and 0.4 mM NADPH. The substrate concentration was chosen to reflect the apparent \( K_m \) values. Initial experiments indicated that the apparent \( K_m \) values were 0.6 μM and 3 μM for the benzo[a]pyrene hydroxylation catalyzed by CYP1B1 and CYP1A1, respectively. Reaction was performed at 37 °C for 5 min (CYP1A1) or 10 min (CYP1B1) and initiated by the addition of NADPH. Reaction was stopped and extracted with 4 ml of an ice-cold mixture of acetone and hexane (1:3, v/v). Subsequently, 2 ml of the organic phase was transferred to a clean test tube and extracted with 5 ml of 1 M NaOH. Mixture of acetone and hexane (1:3, v/v). Subsequently, 2 ml of the organic phase was transferred to a clean test tube and extracted with 5 ml of 1 M NaOH. Following a 15-min period, the fluorescence of each sample was recorded at an excitation wavelength of 396 nm (slit width of 5 nm) and an emission wavelength of 522 nm (slit width of 5 nm). Initial experiments indicated that the assay was linear with respect to incubation time and amount of enzyme. None of the extracts or the individual chemicals interfered with the fluorescence of the enzymatic product in the benzo[a]pyrene hydroxylation assay.

**Enzyme inhibition experiments.** To determine the effect of G. biloba extract and some of its individual constituents, such as ginkgolides A, B, C, or J, bilobalide, kaempferol, quercetin, isorhamnetin, or the corresponding flavonol glycoside, enzyme assays were performed as described in each figure legend. To characterize the enzyme kinetics of CYP1 inhibition, the 7-ethoxyresorufin O-dealkylation assay was conducted in the presence of multiple concentrations of the substrate and the inhibitor, as described in each figure legend.

**Enzyme kinetic analysis.** The apparent \( K_i \) values were determined by fitting the initial velocity-substrate concentration data at various inhibitor concentrations to the equations for competitive inhibition, noncompetitive inhibition, and mixed inhibition (Webb, 1963), using the Enzyme Kinetics Module software program, version 1.1 (SPSS Science, Chicago, IL, USA), as described elsewhere (Chang et al., 2002). The mode of inhibition was determined on the basis of the Akaike information criterion, as a measure of the goodness of fit (Enzyme Kinetics Module software program), and by visual inspection of Lineweaver–Burk plots.

**Statistics.** The significance of the difference between the group means was assessed by one-way analysis of variance and the Student Newman–Keuls multiple range test (SigmaStat™, SPSS Science, Chicago, IL, USA). The level of significance was set a priori at \( P < 0.05 \).

**Results**

**Concentration-dependent effect of Ginkgo biloba extract on human CYP1 catalytic activity**

To determine the effect of G. biloba extract on the catalytic activity of human recombinant CYP1B1, CYP1A1, and CYP1A2, the 7-ethoxyresorufin O-dealkylation assay was performed with varying concentrations (1, 3, 10, 30, or 100 μg/ml) of the extract (i.e., Extract A, Table 1). As shown in Fig. 1, G. biloba extract reduced CYP1B1, CYP1A1, and CYP1A2 catalytic activities in a concentration-dependent manner. The dose–response curves for the inhibition of CYP1A1 and CYP1A2 by G. biloba extracts were rather similar, whereas the one for the inhibition of CYP1B1 was shifted to the left, suggesting that CYP1B1 was more susceptible than CYP1A1 or CYP1A2 to the inhibitory effect of the extract.

Parallel experiments were performed with human liver microsomes as a means to assess the effect of G. biloba extract on CYP1 enzymes in tissue microsomes. It should be noted that in human liver microsomes, CYP1A1 and CYP1B1 proteins are undetectable (Chang et al., 2003), and the 7-ethoxyresorufin O-dealkylation activity is mainly a reflection of CYP1A2 (Burke et al., 1994). As shown in Fig. 1, the dose–response curve for the inhibition of 7-ethoxyresorufin O-dealkylation activity, as assessed in pooled human liver microsomes, was similar to that for the inhibition of recombinant CYP1A2. In a panel of 9 individual human liver microsomes, the extent of inhibition of 7-ethoxyresorufin O-dealkylation activity by G. biloba extract (100 μg/ml) was 62 ± 2% (mean ± SE).

**Enzyme kinetic analysis of human CYP1 inhibition by Ginkgo biloba extract**

To characterize the kinetics of CYP1 enzyme inhibition by G. biloba extract, the 7-ethoxyresorufin O-dealkylation assay was conducted with multiple concentrations of the extract (i.e., Extract A, Table 1) and multiple concentrations of the substrate. Lineweaver–Burk plots for the inhibition of CYP1B1, CYP1A1, CYP1A2, and human liver microsomes are shown in Figs. 2A–D. Based on nonlinear regression analysis of the enzyme kinetic data, the mode of inhibition of each of these enzymes was determined to be of the mixed type (Table 2). The value of the apparent \( K_i \) was the least for the inhibition of CYP1B1 and the greatest for CYP1A2 (Table 2).

**Comparative effect of multiple extracts of Ginkgo biloba on human CYP1 catalytic activity**

To compare the effect of various extracts of G. biloba on CYP1B1, CYP1A1, and CYP1A2 catalytic activity, the 7-ethoxyresorufin O-dealkylation assay was performed with extracts of known abundance of ginkgolides, bilobalide, and flavonols.
As shown in Fig. 3, a similar extent of enzyme inhibition was obtained with each of the three extracts of *G. biloba*. (i.e., Extract A, Extract B, and Extract C; Table 1). As shown in Fig. 3, a similar extent of enzyme inhibition was obtained with each of the three extracts of *G. biloba*.

Table 2
Kinetic analysis of the inhibition of human CYP1 enzymes by *Ginkgo biloba* extract

<table>
<thead>
<tr>
<th>Human enzyme</th>
<th>Apparent $K_i$ (µg/ml)</th>
<th>Mode of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1B1</td>
<td>2 ± 0.3</td>
<td>Mixed</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>5 ± 0.5*</td>
<td>Mixed</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>16 ± 1.4*</td>
<td>Mixed</td>
</tr>
<tr>
<td>Liver microsomes</td>
<td>39 ± 1.2*</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

The apparent $K_i$ and mode of inhibition were determined by nonlinear regression analysis of the enzyme kinetic data (Fig. 2), as described under Materials and methods. Results are expressed as mean ± SE enzyme activity for four determinations.

* Significantly different from CYP1B1, $P < 0.05$.

Role of flavonol glycosides in the inhibition of human CYP1 catalytic activity by *Ginkgo biloba* extract

As shown in Table 1, flavonol glycosides are present in *G. biloba* extracts. Therefore, enzyme assays were performed with quercetin-3-O-rutinoside, kaempferol-3-O-rutinoside, isorhamnetin-3-O-rutinoside, or the vehicle (0.1% DMSO). The concentration of isorhamnetin-3-O-rutinoside was chosen to reflect the level present in an 100 µg/ml of a *G. biloba* extract (i.e. Extract A, Table 1). In contrast, the concentrations of kaempferol-3-O-rutinoside and quercetin-3-O-rutinoside in Extract A were not known. Therefore, we conducted the enzyme assay with a concentration that represented the total diglycosides of quercetin or kaempferol in 100 µg/ml of a *G. biloba* extract (i.e., Extract A, Table 1). However, isorhamnetin-3-O-rutinoside, kaempferol-3-O-
rutinoside, and quercetin-3-\textit{O}-rutinoside did not reduce CYP1B1, CYP1A1, or CYP1A2 catalytic activity (data not shown).

**Effect of flavonol aglycones on human CYP1 catalytic activity**

In vivo, flavonol glycosides are converted to the corresponding aglycone in the gastrointestinal tract (Walle, 2004). Therefore, we investigated the effect of flavonol aglycones on the catalytic activity of CYP1 enzymes. In the present study, quercetin (Fig. 4A), kaempferol (Fig. 4B), and isorhamnetin (Fig. 4C) decreased CYP1B1, CYP1A1, and CYP1A2 catalytic activities in a concentration-dependent manner. Among these three flavonols, isorhamnetin was the most potent in inhibiting CYP1B1, whereas quercetin was the least potent in inhibiting CYP1A2 (Table 3). With respect to the enzymes, CYP1B1 was the most susceptible to inhibition, as suggested by the dose–response curves (Figs. 4A–C) and the apparent $K_i$ values (Table 3) for the inhibition of CYP1B1, CYP1A1, and CYP1A2 by each of the flavonol aglycones. The

### Table 3

<table>
<thead>
<tr>
<th>Enzyme kinetic analysis of the inhibition of human recombinant CYP1B1, CYP1A1, and CYP1A2 by isorhamnetin, kaempferol, and quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent $K_i$ Mode of inhibition</td>
</tr>
<tr>
<td>(ng/ml)</td>
</tr>
<tr>
<td>Isorhamnetin CYP1B1</td>
</tr>
<tr>
<td>CYP1A1</td>
</tr>
<tr>
<td>CYP1A2</td>
</tr>
<tr>
<td>Kaempferol CYP1B1</td>
</tr>
<tr>
<td>CYP1A1</td>
</tr>
<tr>
<td>CYP1A2</td>
</tr>
<tr>
<td>Quercetin CYP1B1</td>
</tr>
<tr>
<td>CYP1A1</td>
</tr>
<tr>
<td>CYP1A2</td>
</tr>
</tbody>
</table>

The 7-ethoxyresorufin $O$-dealkylation assay was conducted with varying concentrations of the substrate (0.025–0.2 μM for CYP1B1 and CYP1A1; 0.1–0.8 μM for CYP1A2) and isorhamnetin (0–0.9 ng/ml for CYP1B1, 0–40 ng/ml for CYP1A1, and 0–400 ng/ml for CYP1A2), kaempferol (0–6 ng/ml for CYP1B1, 0–120 ng/ml for CYP1A1 and CYP1A2), or quercetin (0–12 ng/ml for CYP1B1, 0–120 ng/ml for CYP1A1, and 0–1200 ng/ml for CYP1A2), Control incubations contained 0.1% methanol (vehicle). Results are expressed as mean ± SE for four determinations.

* Significantly different from CYP1B1, $P < 0.05$.

** Significantly different from CYP1B1 and CYP1A1, $P < 0.05$.

**Effect of flavonol aglycones on human CYP1 catalytic activity**

In vivo, flavonol glycosides are converted to the corresponding aglycone in the gastrointestinal tract (Walle, 2004). Therefore, we investigated the effect of flavonol aglycones on the catalytic activity of CYP1 enzymes. In the present study, quercetin (Fig. 4A), kaempferol (Fig. 4B), and isorhamnetin (Fig. 4C) decreased CYP1B1, CYP1A1, and CYP1A2 catalytic activities in a concentration-dependent manner. Among these three flavonols, isorhamnetin was the most potent in inhibiting CYP1B1, whereas quercetin was the least potent in inhibiting CYP1A2 (Table 3). With respect to the enzymes, CYP1B1 was the most susceptible to inhibition, as suggested by the dose–response curves (Figs. 4A–C) and the apparent $K_i$ values (Table 3) for the inhibition of CYP1B1, CYP1A1, and CYP1A2 by each of the flavonol aglycones. The

![Fig. 3. Effect of various extracts of G. biloba on the catalytic activity of human CYP1 enzymes. The 7-ethoxyresorufin $O$-dealkylation assay was conducted with human recombinant CYP1B1 (2.5 pmol), CYP1A1 (1 pmol), or CYP1A2 (5 pmol), and in the presence of a G. biloba extract (i.e., Extract A, Extract B, or Extract C; Table 1) or assay buffer (100 mM potassium phosphate, pH 7.4; vehicle control). Based on the results shown in Fig. 1, the final concentration of G. biloba extract was 10 A g/ml (for CYP1B1) or 60 A g/ml (for CYP1A1 and CYP1A2). Results are expressed as mean ± SE enzyme activity for four determinations. *Significantly different from the control group, $P < 0.05$.](image1)

![Fig. 4. Comparative effects of quercetin, kaempferol, and isorhamnetin on the catalytic activity of human recombinant CYP1B1, CYP1A1, and CYP1A2. The 7-ethoxyresorufin $O$-dealkylation assay (0.2 μM substrate) was conducted with human recombinant CYP1B1 (2.5 pmol), CYP1A1 (1 pmol), or CYP1A2 (5 pmol), and in the presence of quercetin, kaempferol, or isorhamnetin (at the concentrations indicated) or assay buffer (100 mM potassium phosphate, pH 7.4; vehicle control). Results are expressed as mean ± SE percentage of control enzyme activity for four determinations.](image2)
mode of inhibition was competitive, noncompetitive, or mixed, depending on the aglycone and the enzyme (Table 3).

Role of ginkgolides and bilobalide in the inhibition of human CYP1 catalytic activity by Ginkgo biloba extract

To determine whether the terpene trilactones present in the *G. biloba* is responsible for the inhibition of CYP1 enzymes by the extract, the enzyme assay was performed with ginkgolide A (1.1 μg/ml), ginkgolide B (0.3 μg/ml), ginkgolide C (1.4 μg/ml), ginkgolide J (0.6 μg/ml), or bilobalide (2.8 μg/ml). The concentrations of these individual compounds were chosen to represent the levels present in an inhibitory concentration (100 μg/ml, Table 1) of the *G. biloba* extract (i.e., Extract A). However, none of these terpene trilactones affected CYP1B1, CYP1A1, or CYP1A2 catalytic activity (data not shown).

Effect of Ginkgo biloba extract on benzo[a]pyrene hydroxylation catalyzed by human CYP1B1 or CYP1A1

CYP1B1 and CYP1A1 are major catalysts in the bioactivation of polycyclic aromatic hydrocarbons, such as benzo[a]pyrene (Shimada et al., 1997). Therefore, we investigated the effect of *G. biloba* extract on benzo[a]pyrene hydroxylation by human recombinant CYP1B1 or CYP1A1. As shown in Fig. 5, *G. biloba* extract at a concentration of 100 and 500 μg/ml reduced CYP1B1-catalyzed benzo[a]pyrene hydroxylation by 13 ± 3% (mean ± SE) and 68 ± 2%, respectively. By comparison, the same extract at a concentration of 100 μg/ml did not affect CYP1A1-catalyzed benzo[a]pyrene hydroxylation, whereas the 500 μg/ml concentration decreased it by 34 ± 2%.

Discussion

Previous studies reported that *G. biloba* extract inhibited rat hepatic microsomal CYP1A2 (Kuo et al., 2004) and human recombinant CYP1A2 enzymes (Gaudineau et al., 2004). The present in vitro study on human CYP1 enzymes confirmed the inhibition of CYP1A2 by *G. biloba* extract, as determined by experiments with human recombinant CYP1A2 enzymes and human liver microsomes, using the 7-ethoxyresorufin O-dealkylation assay. In human liver microsomes, 7-ethoxyresorufin O-dealkylation activity reflects CYP1A2 rather than CYP1A1 or CYP1B1, based on the finding that CYP1A2 is the only CYP1 enzyme detectable in human liver (Chang et al., 2003). A novel result from the present study is the inhibition of CYP1B1 and CYP1A1 by *G. biloba* extract. Among the CYP1 enzymes, CYP1B1 appeared to be the most susceptible to inhibition by *G. biloba* extract, as assessed by the apparent *K*ₐ values (2 ± 3.5 ± 0.5, 16 ± 1.4 μg/ml for CYP1B1, CYP1A1, and CYP1A2, respectively). By comparison, a study reported that an extract of St. John’s wort inhibited human recombinant CYP1A1-catalyzed 7,8-dihydrodiol-benzo[a]pyrene epoxidation with an apparent *K*ₐ value of 3.9 ± 0.2 μg/ml (Schwarz et al., 2003). In an earlier study, *P. ginseng* extract inhibited human recombinant CYP1B1, CYP1A1, and CYP1A2 with apparent *K*ₐ values of 98 ± 5, 906 ± 78 μg/ml, respectively, whereas *Panax quinquefolis* extract inhibited CYP1B1, CYP1A1, and CYP1A2 with apparent *K*ₐ values of 44 ± 3, 61 ± 4, and 20 ± 5 μg/ml, respectively (Chang et al., 2002). Therefore, *G. biloba* extract appears to be more potent than either *P. ginseng* extract or *P. quinquefolis* extract in inhibiting CYP1B1 and CYP1A1 enzymes. By comparison, *G. biloba* extract and *P. quinquefolis* extract inhibit CYP1A2 with similar potency, and both are more potent than *P. ginseng* extract.

A major group of chemicals present in *G. biloba* extract is the flavonol glycosides (van Beek, 2002). In the extracts used in the present study (Table 1), quercetin, kaempferol, and isorhamnetin were not present as the aglycone or the monoglycoside. Rather, they were in the form of diglycosides and other glycosides. The total amount of flavonol glycosides in our extracts was from 21% to 24.4%, which was within the range indicated in the German Commission E monograph (Blumenthal, 1998). As shown in the present study, none of the diglycosides investigated (i.e., quercetin 3-O-rutinoside, kaempferol-3-O-rutinoside, or isorhamnetin-3-O-rutinoside) was responsible for the in vitro inhibition of CYP1 enzymes by *G. biloba* extract. However, the current view is that flavonol glycosides are hydrolyzed enzymatically to the corresponding aglycones in the gastrointestinal tract, and it is the aglycones that are absorbed (Walle, 2004). Our novel results indicate that the flavonol aglycones isorhamnetin, kaempferol, and quercetin are relatively potent inhibitors of each of the CYP1 enzymes. When compared to kaempferol and quercetin, isorhamnetin is the most potent inhibitor. This may relate to the more lipophilic substitution (O-methyl group) at the C-3' position in the isorhamnetin molecule (Fig. 6). As a group, the three flavonols are among the most potent inhibitors of CYP1B1 identified to date (Table 4). Isorhamnetin, kaemp-
ferol, and quercetin are also some of the more potent inhibitors of CYP1A1 (Table 4). In contrast, these three flavonols are considerably less potent than α-naphthoflavone and galangin in the inhibition of CYP1A2 (Table 4). It has been suggested that quercetin forms aggregates in solution, resulting in nonspecific inhibition of CYP1A2 (Table 4). It has been suggested that quercetin on human CYP1 enzymes, as indicated by the differences in the apparent \( K_a \) values in the inhibition of CYP1B1 (23 ± 2 nM; mean ± SE), CYP1A1 (77 ± 5 nM), and CYP1A2 (418 ± 50 nM).

Another group of chemicals present in *G. biloba* extracts is the terpene triactones, such as the diterpenes (i.e., ginkgolides A, B, C, and J) and sesquiterpene (i.e., bilobalide). The total amount of the terpene triactones in our extracts was from 6.2% to 6.6% (Table 1), which was within the range recommended by the German Commission E monograph (Blumenthal, 1998). However, none of these terpene triactones was responsible for the inhibition of CYP1 enzymes by *G. biloba* extract. This conclusion is based on the finding that bilobalide and ginkgolides A, B, C, and J did not affect the catalytic activity of CYP1B1, CYP1A1, or CYP1A2, when determined at the levels present in an inhibitory concentration (100 μg/ml) of a *G. biloba* extract (i.e., Extract A, Table 1). Our results on CYP1A2 are consistent with previous findings (Zou et al., 2002) indicating that bilobalide (153 μM), ginkgolide A (200 μM), ginkgolide B (200 μM), and ginkgolide C (200 μM) do not affect the catalytic activity of human recombinant CYP1A2 when analyzed at concentrations substantially greater than those in the present study.

It remains to be determined which chemical(s) is responsible for the observed in vitro inhibition of CYP1 enzymes by *G. biloba* extract. In addition to the specific flavonol glycosides and terpene triactones listed in Table 1, other known constituents of *G. biloba* extract include biflavones (e.g., amentoflavone, bilobetin, ginkgetin, isoginkgetin, sequojaflavone, and sciodopitysin) and flavonoid glycosides of unknown abundance in the extracts used in the present study (e.g., those derived from the aglycones of myricetin, apigenin, tamarixetin, and luteolin) (Bedir et al., 2002; van Beek, 2002). A study reported that apigenin inhibited CYP1A2-mediated phenacetin \( O \)-deethylation activity in human liver microsomes, and the IC\(_{50}\) value was 0.3 μg/ml (von Moltke et al., 2004). However, the glycosides of apigenin are not expected to have substantive inhibitory activity. According to our previous structure–activity relationship study with flavonol aglycones and their mono- and diglycosides (Kuo et al., 2004), a substitution at the C-3 position (Fig. 6) with a glycoside substantially reduces the inhibitory effect, when compared to the corresponding aglycone. The order of potency is aglycone > monoglycoside > diglycoside. Ginkgolic acids are also present in *G. biloba* extract (van Beek, 2002). In a previous in vitro study, ginkgolic acid I and ginkgolic acid II were reported to inhibit human recombinant CYP1A2-catalyzed oxidation of 7-ethoxy-3-cyanocoumarin, with IC\(_{50}\) values of 4.81 μM and 4.88 μM, respectively (Zou et al., 2002). In the present study, we did not determine whether ginkgolic acids affect the catalytic activity of human CYP1B1, CYP1A1, or CYP1A2. The abundance of the individual ginkgolic acids in the extracts was not known, but the total ginkgolic acid level was <5 ppm, based on information provided by the manufacturer.

In the present study, *G. biloba* extract inhibited benzo[\( a \)]pyrene hydroxylation catalyzed by CYP1B1 or CYP1A1. This finding may have implications in chemical carcinogenesis because CYP1B1 and CYP1A1 are the principal catalysts in the bioactivation of carcinogenic polycyclic aromatic hydrocarbons (Shimada et al., 1996). In an earlier study, pretreatment of female Swiss albino mice with a *G. biloba* extract was reported to attenuate the multiplicity of benzo[\( a \)]pyrene-induced forestomach tumors (Agha et al., 2001). The mechanism for the effect of *G. biloba* extract on benzo[\( a \)]pyrene-induced carcinogenesis is not known. However, CYP1B1 (Murray et al., 1997) and CYP1A1 (Tatemichi et al., 1999) are expressed in stomach tumors. Our results suggest that a potential mechanism may involve inhibition of CYP1B1- and CYP1A1-catalyzed benzo[\( a \)]pyrene bioactivation by *G. biloba* extract. Studies are planned to address this issue.

The in vivo relevance of the in vitro inhibition of human CYP1 enzymes by a *G. biloba* extract is not known. In a human study, the peak plasma concentrations of isorhamnetin, kaempferol, and quercetin after ingestion of *G. biloba* extract were reported to be 7 ng/ml, 27 ng/ml, and 12 ng/ml, respectively (Wojcicki et al., 1995). By comparison, the apparent \( K_a \) values for the in vitro inhibition of CYP1 enzymes, particularly CYP1B1 and CYP1A1, by isorhamnetin, kaempferol, and quercetin are also in the low ng/ml concentrations.

(Table 3). However, there is no information on the levels of these compounds in tissues where CYP1B1, CYP1A1, and CYP1A2 are expressed. Previous studies have investigated the effect of G. biloba extract on CYP1A2-mediated drug elimination in human subjects (Gurley et al., 2002, 2005). However, those studies were designed to show whether the extract increases drug elimination as a consequence of enzyme induction. Therefore, it remains to be determined whether G. biloba extract inhibits CYP1 enzymes and impairs drug clearance in vivo.

In summary, the novel findings from the present in vitro study with human CYP1 enzymes are (1) G. biloba extract inhibited the catalytic activity of CYP1B1, CYP1A1, and CYP1A2; however, CYP1B1 was preferentially inhibited, based on a comparison of the apparent $K_i$ values; (2) bilobalide, ginkgolides A, B, C, and J, and the 3-O-rutinosides of quercetin, kaempferol, and isorhamnetin were not responsible for the inhibition of CYP1 enzymes by G. biloba extract; and (3) the aglycones of quercetin, kaempferol, and isorhamnetin were potent inhibitors of CYP1B1, with apparent $K_i$ values in the low nanomolar concentrations; (4) the mode of inhibition by these three flavonols was competitive, noncompetitive, or mixed, depending on the compound and the enzyme; (5) among the three CYP1 enzymes, CYP1B1 was the most susceptible to the inhibitory effect by the flavonol aglycones; and (6) G. biloba extract inhibited benzo[a]pyrene hydroxylation, and the effect was greater with CYP1B1 than with CYP1A1 as the catalyst.

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References


Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dispos. 2, 583–588.


