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Inhibition of human cytochromes P450 by components of *Ginkgo biloba*

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Abstract

The extraction, isolation and characterization of 29 natural products contained in *Ginkgo biloba* have been described, which we have now tested for their in-vitro capacity to inhibit the five major human cytochrome P450 (CYP) isoforms in human liver microsomes. Weak or negligible inhibitory activity was found for the terpene trilactones (ginkgolides A, B, C and J, and bilobalide), and the flavonol glycosides. However 50% inhibitory activity (IC50) was found at concentrations less than 10 μg mL^{-1} for the flavonol aglycones (kaempferol, quercetin, apigenin, myricetin, tamarixetin) with CYP1A2 and CYP3A. Quercetin, the biflavone amentoflavone, sesamin, as well as (*Z*,*Z*)-4,4′-(1,4-pentadiene-1,5-diyl)diphenol and 3-nonadec-8-enyl-benzene-1,2-diol, were also inhibitors of CYP2C9. The IC50 of amentoflavone for CYP2C9 was 0.019 μg mL^{-1} (0.035 μM). Thus, the principal components of *Ginkgo biloba* preparations in clinical use (terpene trilactones and flavonol glycosides) do not significantly inhibit these human CYPs in-vitro. However, flavonol aglycones, the biflavonol amentoflavone and several other non-glycosidic constituents are significant in-vitro inhibitors of CYP. The clinical importance of these potential inhibitors will depend on their amounts in ginkgo preparations sold to the public, and the extent to which their bioavailability allows them to reach the CYP enzymes in-situ.

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Introduction

Awareness of drug interactions with herbal medicines is increasing as they become more widely used as dietary supplements (Eisenberg et al 1998; Goldman 2001; de Smet 2002) and clinically important interactions are reported (Fugh-Berman 2000; Heck et al 2000; Izzo & Ernst 2001; Ernst 2002; Valli & Giardina 2002). *Ginkgo biloba* is used extensively in many countries to improve cognition and enhance cerebral and peripheral circulation (Schulz et al 2001; de Smet 2002; MacLennan et al 2002; Valli & Giardina 2002) in conditions which are likely to affect older patients, who are also likely to be taking conventional drugs and are therefore more likely to be subject to drug interactions. Nevertheless, the possibility of drug interactions with ginkgo has received only limited attention in spite of the report that at least one ginkgo-related haemorrhage occurred in a patient who was also taking warfarin (Matthews 1998), of which the pharmacologically active *S*-enantiomer is metabolized by human cytochrome P450-2C9 (CYP2C9) (Rettie et al 1992; Kaminsky & Zhang 1997). Thus, if some component of ginkgo inhibited this enzyme, it would be the functional equivalent of increasing the dose of warfarin, thereby enhancing its anticoagulant activity. Limited clinical studies done to date, however, have not supported this possibility (Engelsen et al 2002; Lai et al 2002). Most often the serious haemorrhages reported in individuals taking ginkgo (reviewed by Vaes & Chyka 2000) have been attributed to an inhibitor of normal platelet aggregation found in ginkgo.

Because a drug interaction with ginkgo might occur with any of a large number of different prescription medications, each possible interaction cannot be addressed individually. A more practical way to screen for potentially significant drug interactions is to use in-vitro models to identify components of ginkgo capable of inhibiting any of the major CYP enzymes, since the drugs metabolized by each of them are already largely known (Venkatakrishnan et al 2001, 2003; Bjornsson et al 2003; Zhou et al 2003). Such in-vitro models are known to have a number of drawbacks and limitations (von Moltke

et al 1998c; Bertz & Granneman 1997; Venkatakrishnan et al 2001, 2003; Bjornsson et al 2003). In particular, it is not clearly established how such specific quantitative values of inhibitory potency in-vitro (such as the 50% inhibitory concentration, IC50, or the inhibition constant, K_i) translate into valid predictions of clinical drug interactions. Nonetheless, in-vitro procedures are now extensively used in drug development and clinical pharmacology to generate estimates of absolute or relative inhibitory potency that can be used in planning of clinical drug interaction studies. In this study, therefore, the in-vitro model was used to determine whether any of 29 constituents of *Ginkgo biloba* can be considered as potentially important inhibitors of any of the five major CYP isoforms in human liver.

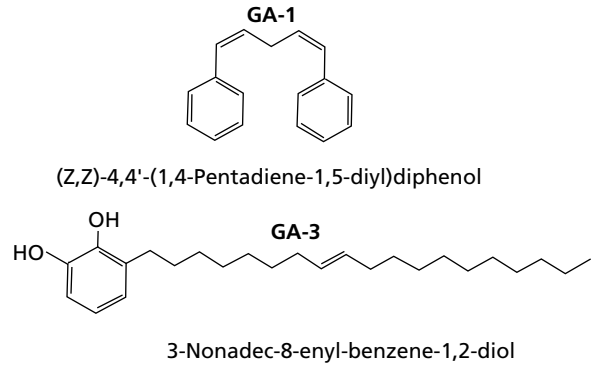

Figure 1 Structures of two ginkgo components not previously described in Bedir et al (2002).

Materials and Methods

Components of *Ginkgo biloba*

The extraction, isolation, purification, and characterization of 29 components of *Ginkgo biloba* used in this study have been described previously (Bedir et al 2002). Table 1 provides the names and references to their chemical structures and Figure 1 gives the structures of two additional compounds not described previously (GA-1 and GA-3). GA-1 was determined to be (*Z*,*Z*)-4,4′-(1,4-pentadiene-1,5-diyl) diphenol through comparison of its ^{1}H and ^{13}C NMR spectroscopic data with literature values (Weinges et al 1986), while GA-3 was elucidated as 3-nonadec-8-enyl-benzene-1,2-diol on the basis of spectroscopic evidence (1D- and 2D-NMR and MS).

Pure samples of these constituents were dissolved in methanol and stored at −18°C until used for in-vitro studies.

Table 1 Summary of ginkgo components

Name or description	Compound code from Bedir et al (2002)
Kaempferol	4
Quercetin	5
Apigenin	6
4′-O-methyl apigenin	7
Myricetin	8
Tamarixetin	9
Kaempferol glycoside	10
Kaempferol glycoside	11
Quercetin glycoside	12
Quercetin glycoside	13
Kaempferol glycoside	14
Kaempferol glycoside	15
Quercetin glycoside	16
Quercetin glycoside	17
Quercetin glycoside	18
Luteolin glycoside	19
Amentoflavone	20
Bilobetin	21
Ginkgetin	22
Sciadopitysin	23
Bilobalide	24
Ginkgolide A	25
Ginkgolide B	26
Ginkgolide C	27
Ginkgolide J	28
Sesamin	29
Syringaresinol glycoside	30
Pentadiene-1,5-diyl diphenol (GA-1)	*
3-Nonadec-8-enyl-benzene-1,2-diol (GA-3)	*

*Structure shown in Figure 1.

In-vitro studies of human CYP isoforms

Liver samples from individual human donors with no known liver disease were provided by the International Institute for the Advancement of Medicine (Exton, PA), the Liver Tissue Procurement and Distribution System, University of Minnesota (Minneapolis, MN) or the National Disease Research Interchange (Philadelphia, PA). Acquisition of discarded liver tissue from anonymous individuals for purposes of in-vitro metabolism studies was determined to be exempt from review by the Human Investigation Review Committee, the Institutional Review Board serving Tufts University School of Medicine and Tufts-New England Medical Center. All samples were of the CYP2D6 and CYP2C19 normal metabolizer phenotype based on previous studies in our laboratory.

Microsomes were prepared by ultracentrifugation as described in detail previously (von Moltke et al 1993). Microsomal pellets were suspended in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −80°C until use. Other chemical reagents and drugs were purchased from commercial sources or kindly provided by their manufacturers.

The inhibitory effects of ginkgo components on the activity of each of five human CYP isoforms were evaluated using the following index reactions as described by von Moltke et al (2002) (Table 2): CYP1A2, phenacetin (100 μM) to paracetamol (acetaminophen); CYP2C9, flurbiprofen (5 μM) to hydroxyflurbiprofen; CYP2C19,

Table 2 Summary of methods for studies of human cytochrome P-450 (CYP) inhibition in-vitro

CYP isoform	Substrate (concn)	Products	Method reference	Positive control
CYP1A2	Phenacetin (100 μM)	Paracetamol (acetaminophen)	von Moltke et al (1996a) Venkatakrishnan et al (1998a)	α-Naphthoflavone (1 μM)
CYP29C	Flurbiprofen (5 μM)	OH-Flurbiprofen	Giancarlo et al (2001)	Sulfaphenazole (2.5 μM)
CYP2C19	S-Mephenytoin (25 μM)	4′-OH-Mephenytoin	Venkatakrishnan et al (1998b)	Omeprazole (10 μM)
CYP2D6	Dextromethorphan (25 μM)	Dextrorphan	von Moltke et al (1998b)	Quinidine (2.5 μM)
CYP3A	Triazolam (250 μM)	α-OH-Triazolam 4-OH-Triazolam	von Moltke et al (1996b)	Ketoconazole (1.0 μM)

S-mephenytoin (25 μM) to 4′-OH-mephenytoin; CYP2D6, dextromethorphan (25 μM) to dextrorphan; CYP3A, triazolam (250 μM) to α-OH and 4-OH triazolam.

Index substrates (Table 2) in methanol solution were added separately to each incubation mixture, either alone or with each of the ginkgo test compounds. The solvent was evaporated to dryness at 40°C in-vacuo. Incubation mixtures in a volume of 250 μL were then prepared in these tubes to contain 50 mM phosphate buffer, 5 mM Mg^{2+}, 0.5 mM $NADP^+$ and an isocitrate/isocitric dehydrogenase regenerating system. Reactions at 37°C were initiated by addition of microsomal protein (0.25 mg mL^{-1}) and terminated by cooling the incubation mixture in ice and adding 100 μL of acetonitrile. Internal standard appropriate to the specific system (Table 2) was added, the incubation mixture was centrifuged and the supernatant solution transferred to an autosampling vial for HPLC analysis. The mobile phase consisted of a combination of acetonitrile and 50 mM phosphate buffer in proportions appropriate for isolating the product of each assay. The analytical column was stainless steel, 30 cm × 3.9 mm, containing reverse-phase C-18 microBondapak, or 15 cm × 3.9 mm, containing reverse-phase C-18 Novapak (Waters Associates, Milford, MA). Column effluent was monitored by ultraviolet absorbance at the appropriate wavelength, or by fluorescence deletion. Further procedural details for each assay are available in references cited in Table 2.

All biotransformation reactions were verified to be in the linear range with respect to incubation duration and protein concentration. All incubations were done in duplicate.

Analysis of data

Ginkgo components were initially evaluated for inhibitory activity at a high concentration (100 μg mL^{-1}). Components that produced less than 50% inhibition were considered weak inhibitors and not studied further. Each compound that produced more than a 50% inhibition at 100 μg mL^{-1} was further examined at various lower concentrations to determine the inhibitor concentration (IC50) reducing the reaction velocity to 50% of the control value. Reaction velocities with an inhibitor added were expressed as a ratio (R_V) of the control velocity in the absence of inhibitor. The relation of R_V to inhibitor concentration (C) was analysed by nonlinear regression to determine the concentration producing a 50% decrement in the control reaction velocity (von Moltke et al 1998a):

$$R_V = 100[1 - ((E_{max} \times C^A)/(C^A + IC^A))] \quad (1)$$

In equation 1, E_{max} is the projected maximum decrement in reaction velocity, A is an exponent, and IC is the concentration producing 50% of the maximum decrement. The true IC50 was calculated as:

$$IC50 = IC/(2E_{max} - 1)^{1/A} \quad (2)$$

With only rare exceptions, equation 1 was highly consistent with actual data points, based on r-square (goodness-of-fit) values in excess of 0.95. No additional statistical analyses were undertaken, since a direct quantitative comparison of IC50 values was not our objective.

Results

The terpene trilactones (Ginkgolides A, B, C, J; bilobalide) and the flavonol glycosides (Table 1) all had weak or negligible CYP inhibitory capacity, and were not studied further.

Significant inhibitory activity was observed for the flavonol aglycones (kaempferol, quercetin, apigenin, 4′-OH-methyl apigenin, myricetin, tamarixetin) (Table 3), which yielded IC50 values for CYP1A2 or CYP3A of less than 10 μg mL^{-1} (Figure 2). Quercetin was also a strong inhibitor of CYP2C9 (IC50 = 7.8 μg mL^{-1}, 25.8 μM), as was myricetin for CYP2D6 (IC50 = 9.6 μg mL^{-1}, 30.2 μM).

Of the four biflavones evaluated (amentoflavone, bilobetin, ginkgetin, sciadopitysin), only amentoflavone was found to be inhibitory; it was a highly potent inhibitor of CYP2C9 (IC50 = 0.019 μg mL^{-1}, 0.035 μM), and also inhibited CYP2C19, 2D6 and 3A (Table 3, Figure 3).

Sesamin was found to have inhibitory activity against CYP2C9 and CYP3A (Table 3) and CYP2C19. Both (*Z*,*Z*)-4,4′-(1,4-pentadiene-1,5-diyl)diphenol and 3-nonadec-8-enyl-benzene-1,2-diol (Figure 1) were found to be inhibitory for CYP2C9 and CYP3A.

Discussion

In-vitro models of human CYP inhibition, such as those used in these studies, are often used in the drug development process to estimate the risk of clinical drug

Table 3 IC50 values for ginkgo components as inhibitors of human cytochrome p450 (CYP) isoforms in-vitro

Compound*	IC50, (μg mL^{-1}) (mean value in μM shown in brackets)				
	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP3A
Quercetin (302)	3.0 ± 0.4 (9.9)	7.8 ± 1.2 (25.8)			4.1 ± 0.4 (13.6)
Tamarixetin (316)	2.9 ± 0.2 (9.2)				
Kaempferol (286)	1.5 ± 0.6 (5.2)				4.6 ± 0.6 (16.1)
Myricetin (318)				9.6 ± 0.7 (30.2)	6.4 ± .9 (18.9)
Apigenin (270)	0.3 ± 0.01 (1.1)				1.8 ± 0.1 (6.7)
4-O-Methyl-Apigenin (284)					4.3 ± 0.4 (15.1)
Amentoflavone (538)		0.019 ± 0.002 (0.035)	12.7 ± 4.3 (23.6)	13.1 ± 1.9 (24.3)	2.6 ± 0.5 (4.8)
Sesamin (354)		7.5 ± 1.3 (21.2)	2.2 ± 0.4 (6.2)		2.6 ± 0.2 (7.3)
GA-1 (220)		0.7 ± 2.2 (48.6)			16.8 ± 2.2 (76.4)
GA-3 (374)		2.1 ± 0.7 (5.6)		45.7 ± 4.6 (122)	28.0 ± 0.6 (74.9)

*Molecular weight shown in parentheses next to compound name. Data are means ± s.e., n = 4).

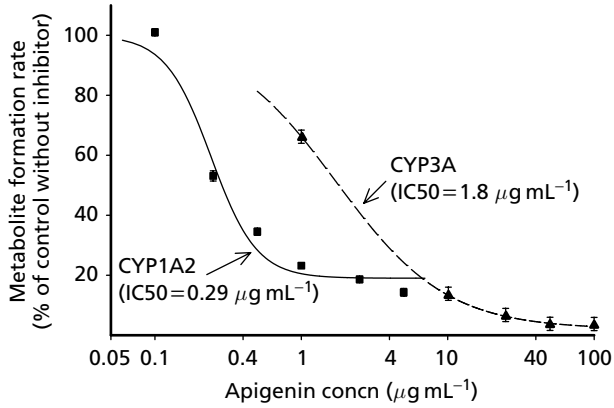

Figure 2 Effect of apigenin on the activity of human CYP1A2 (phenacetin O-deethylation) and CYP3A (triazolam hydroxylation) in human liver microsomes. Reaction velocities with apigenin added are expressed as a percent of the control velocity with no apigenin present. Each point is the mean ± s.e. for four separate liver preparations. Function lines and IC50 values are based on non-linear regression analysis of mean data points (equations 1 and 2). Table 3 provides IC50 values converted to molar units.

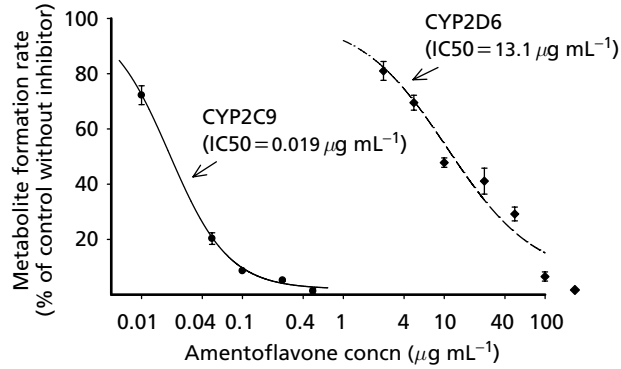

Figure 3 Effect of amentoflavone on the activity of human CYP2C9 (flurbiprofen hydroxylation) and CYP2D6 (dextromethorphan *o*-demethylation) in human liver microsomes. Reaction velocities with amentoflavone added are expressed as a percent of the control velocity with no amentoflavone present. Each point is the mean ± s.e. for four separate liver preparations. Function lines and IC50 values are based on non-linear regression analysis of mean data points (equations 1 and 2). Table 3 provides IC50 values converted to molar units.

interactions. Despite a number of well-recognized limitations, such models can be useful for anticipating pharmacokinetic interactions of possible clinical significance and help to plan more definitive human drug interaction studies (Bertz & Granneman 1997; von Moltke et al 1998c; Venkatakrishnan et al 2001; Bjornsson et al 2003). These models assume particular importance for assessing the risks of herbal medicines, for which drug interaction data are not mandated before product marketing. Thus, in-vitro screening allows a relatively inexpensive initial assessment of the risk of a drug interaction, which, along with clinical reports, helps to identify where further studies may be needed to detect clinically significant interactions between conventional drugs and herbal medicines.

Although clinical data on drug interactions with *Ginkgo biloba* are based almost exclusively on anecdotal case reports, for which cause-and-effect relationships cannot be established, some clinical investigations have been reported. Gurley et al (2002), using the cocktail approach (simultaneous administration of multiple index substrates), found that 18 days of exposure to a *Ginkgo biloba* preparation (240 mg daily) had no apparent effect on the activity of CYP3A, CYP1A2, CYP2D6 or CYP2E1. Similar findings were reported by Markowitz et al (2003) in a study of the effects of ginkgo on CYP2D6 and CYP3A. In contrast, Smith et al (2001) reported that exposure to ginkgo (120 mg daily) for 18 days resulted in reduced activity of CYP3A, based on pharmacokinetic changes in response to a

single dose of nifedipine. In addition, Sun et al (2002) found that ginkgo (240 mg daily) for 12 days slightly altered the in-vivo activity of CYP2C19 and CYP2E1. None of these four studies, however, evaluated the activity of human CYP2C9.

These in-vitro studies provide no evidence that terpene lactones or flavonol glycosides, the most abundant components of *Ginkgo biloba* preparations based on quantitative analyses (Kressman et al 2002; van Beek 2002; Yu et al 2003), are inhibitors of any of the CYP isoforms (He & Edeki 2002; Zou et al 2002). However, significant inhibitory activity of three isoforms, CYP1A2, 2C9 and 3A, was evident for several flavonol aglycones. Thus, the biflavone, amentoflavone, was a highly potent inhibitor of CYP2C9 ($IC50 = 0.019\ \mu g\,mL^{-1}$, $0.035\ \mu M$) using an assay based on the hydroxylation of flurbiprofen. Amentoflavone also was among the most potent inhibitors of CYP3A.

The clinical significance of these in-vitro demonstrations of inhibition by specific ginkgo components will depend on additional factors such as the amount of a specific inhibitor in commercial ginkgo preparations and the extent to which the inhibitor actually reaches the drug-metabolizing CYP in either intestinal mucosa or liver. It may be, for example, that flavonol aglycones undergo conjugation before reaching the liver or systemic circulation (Scalbert & Williamson 2000). Nonetheless, our findings suggest that certain constituents of *Ginkgo biloba* are sufficiently inhibitory for some human CYP isoforms to suggest the need for additional clinical studies to evaluate the possibility of clinically significant drug interactions. Of particular concern are amentoflavone and other ginkgo components that inhibit human CYP2C9, the isoform responsible for the clearance of such drugs as warfarin and phenytoin, known to have narrow therapeutic indexes and therefore subject to important clinical consequences even from small changes in plasma levels that might be caused by a drug interaction.

Conclusions

A number of flavonol aglycones, as well as the biflavonol amentoflavone, are inhibitors of several human CYP isoforms in-vitro. Inhibition of CYP2C9 is of potential concern, since this enzyme is responsible for clearance of some drugs (such as S-warfarin) that have narrow therapeutic ranges in clinical practice. The importance of the in-vitro findings requires evaluation in clinical studies.

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