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Characterization of cytochrome P450s mediating ipriflavone metabolism in human liver microsomes

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Abstract

Ipriflavone, a synthetic flavonoid for the prevention and treatment of osteoporosis, has been reported to be extensively metabolized in man to seven metabolites (M1–M7). This study was performed to characterize the human liver cytochrome P450s (CYP) responsible for the metabolism of ipriflavone. Hydroxylation at the β-ring to M3, *O*-dealkylation to M1 and oxidation at isopropyl group to M4 and M5 are major pathways for ipriflavone metabolism in three different human liver microsome preparations. The specific CYPs responsible for ipriflavone oxidation to the active metabolites, M1, M3, M4 and M5 were identified using a combination of correlation analysis, immuno-inhibition, chemical inhibition in human liver microsomes and metabolism by expressed recombinant CYP enzymes. The inhibitory potencies of ipriflavone and its five metabolites, M1–M5 on seven clinically important CYPs were investigated in human liver microsomes. Our results demonstrate that CYP3A4 plays the major role in *O*-dealkylation of ipriflavone to M1 and CYP1A2 plays a dominant role in the formation of M3, M4 and M5. Ipriflavone and/or its five metabolites were found to inhibit potently the metabolism of CYPs 1A2, 2C8, 2C9 and 2C19 substrates.

Keywords: *Ipriflavone, CYP3A4, CYP1A2, inhibition of P450s, inhibition of CYPs 1A2, 2C8, 2C9, 2C19*

Introduction

Ipriflavone (7-isopropoxy-3-phenyl-chromen-4-one) (Figure 1) is a synthetic flavonoid which is used in the prevention and treatment of osteoporosis (Reginster 1993; Head 1999). Ipriflavone is extensively metabolized in rats, dogs, rabbits and humans and undergoes an extensive first-pass metabolism (Ferenc and Istvan 1995; Kim et al. 2000; Kim and Lee 2002). Seven metabolites (M1–M7) were identified (Figure 1) in animals and humans (Yoshida et al. 1985; Ronelli et al. 1991; Ferenc and Istvan 1995; Rohatagi and

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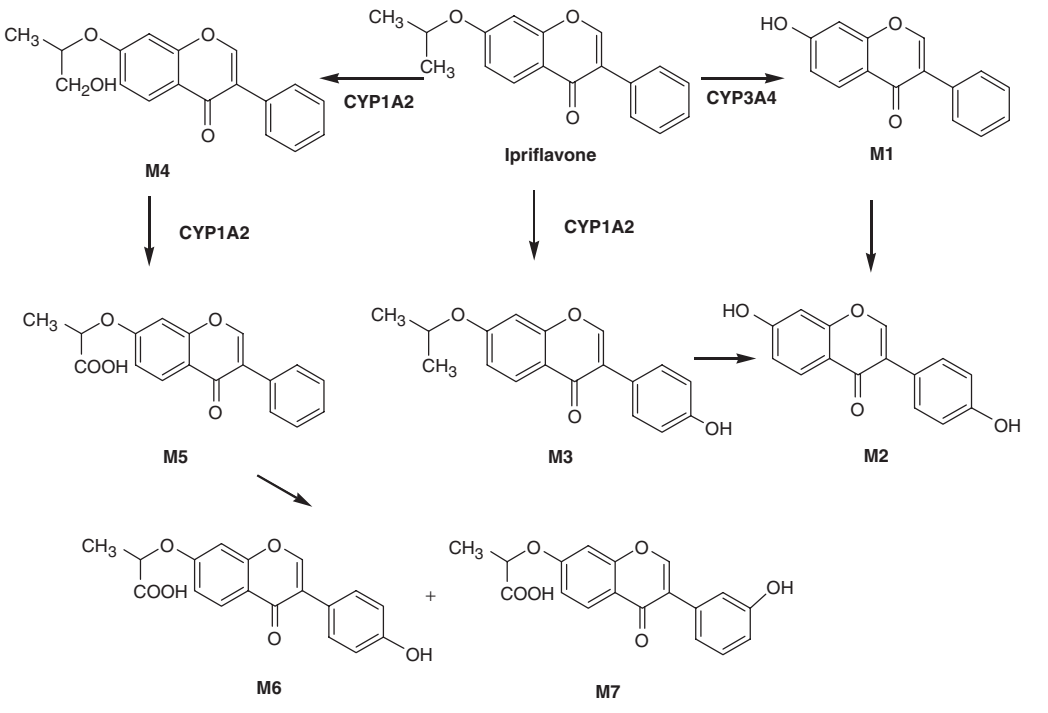

Figure 1. Metabolic pathways of ipriflavone.

Barrett 1997; Rohatagi et al. 1997). Ipriflavone is metabolized mainly in the liver by oxidation of the isopropyl group and/or hydroxylation of the β-ring followed by phase II glucuronidation or sulfation. Ipriflavone is biotransformed to M1, M3 and M4. Subsequently, M4 is biotransformed to M5, which is further metabolized to M6 and M7. Also, M1 and M3 are converted to M2 (Figure 1). Ipriflavone metabolites, M1, M2, M3 and M5 have been shown to possess pharmacological activity related to bone formation and bone resorption and may contribute to the overall efficacy of ipriflavone (Benvenuti et al. 1991; Cheng et al. 1994; Bassleer et al. 1996; Giossi et al. 1996).

The reduction of theophylline clearance by concomitant ipriflavone administration observed by Takahashi et al. (1992) is primarily due to the inhibition of CYPs responsible for theophylline *N*-demethylation by ipriflavone and/or its metabolites M1 and M5 (Monostory and Vereczkey 1995, 1996). Ipriflavone and M1 have been shown to be extensive inhibitors of phaenacetin *O*-deethylase (CYP1A2) and tolbutamide hydroxylase (CYP2C9) in human liver microsomes, whereas M5 had no effect on CYPs 1A2, 2A6, 2C9, 2C19, 2D6, 2E1 and 3A4 activities (Monostory et al. 1998). The total body clearance values of ipriflavone obtained after infusion of ipriflavone in rats pretreated with CYP inducers and inhibitors were compared with that in control rats, suggesting that CYP1A1/2, 2B1/2 and 2C11 might be responsible for ipriflavone metabolism in rats (Chung et al. 2006).

The information of specific CYP enzyme(s) involved in the biotransformation of a large number of therapeutic agents can be of considerable clinical impact in regard to potential drug interactions and inter-individual variation in drug metabolism. Consequently, the evaluation of human CYP enzyme(s) responsible for the metabolism of ipriflavone can be useful in understanding the disposition and the *in vivo* behavior of its active metabolites as well as ipriflavone and may have clinical implications.

The present study describes an *in vitro* investigation into the metabolism of ipriflavone to identify the human CYP enzymes responsible for the biotransformation of ipriflavone to the active metabolites and to investigate the inhibitory effects of ipriflavone and its five metabolites, M1–M5 on major human CYP enzymes in human liver microsomes.

Materials and methods

Chemicals

Ipriflavone, M1, M3, M4 and M5 (purity of 99.5%) were supplied by Samchundang Pharm. Co. (Seoul, Korea). 2-(3,4-Dimethoxy-phenyl)-5,7-dihydroxy-chromen-4-one (DPDC, internal standard) was synthesized by Dong-A Pharm. Co. (Yongin, Korea). Daidzein (M2), $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, furafylline, ketoconazole, sulfaphenazole and triethylenethiophosphoramide (thioTEPA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile and dichloromethane (HPLC grade) were obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and the other chemicals were of the highest quality available.

Human liver microsomes (coded H161, H003, H023, H042, H043, H056, H066, H070, H089, H093, H112, HK23 and HK34) and microsomes derived from baculovirus-infected insect cells transfected with human P450 cDNA over-expressing CYPs1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 were purchased from the Gentest Corporation (Woburn, MA, USA). Human selective antibodies for the immunoinhibition of human CYPs, i.e. anti-CYP1A2 (Cat. No. 458312, CYP1A2-specific), anti-CYP2D6, anti-CYP2E1 and anti-CYP3A4 (Cat. No. 458334, reactive for CYPs3A4 and 3A5), and antiserum of CYP2C were obtained from Gentest Co.

Metabolism of ipriflavone with human liver microsomes or cDNA-expressed CYP enzymes

Incubation mixtures contained 50 mM potassium phosphate buffer (pH 7.4), human liver microsomes (0.15 mg protein ml^{-1}) or eight cDNA expressing CYP enzymes (4 pmol), NADPH generating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate and 0.8 U ml^{-1} glucose-6-phosphate dehydrogenase), 3 mM magnesium chloride and ipriflavone or metabolite M4 (2 μM, added in methanol, final solvent concentration not exceeding 0.5%, v/v) in a final volume of 200 μl. Preliminary experiments showed that the formation of the three major metabolites, i.e. M1, M3 and M4, from ipriflavone was linear with respect to both incubation time over 45 min and liver microsomal protein concentration (0.1–0.3 mg ml^{-1}) at 37°C. Thus, a 20-min incubation time and a 0.15 mg ml^{-1} microsomal protein concentration were selected. The reactions were terminated by the addition of 50 μl of 500 mM hydrochloric acid, 10 μl of DPDC (internal standard, 500 ng ml^{-1}) and 1000 μl of dichloromethane. Following mixing with a vortex mixer and centrifugation, an aliquot (800 μl) of the organic layer was evaporated under a nitrogen stream. The residue was dissolved in 50 μl of 50% acetonitrile and an aliquot (10 μl) was analyzed by an LC-MS/MS method.

In kinetic experiments, seven concentrations of ipriflavone (1.0–100 μM) were incubated in duplicate with three different human liver microsome preparations (H043, H056 and H093) or cDNA-expressed CYP1A2, 2C9, 2C19 and 3A4 enzymes. Several enzyme kinetic equations were fitted to the untransformed data using enzyme kinetics software

(version 1.1, SPSS Science Inc., Richmond, CA, USA). The equation, $V = V_{max} \cdot S/(K_m + S)$ best described the kinetics of biotransformation of ipriflavone to M1, M3 and M4 in human liver microsomes and cDNA-expressed CYPs 1A2, 2C9, 2C19 and CYP3A4 enzymes. V is the velocity of the reaction at substrate concentration [S]; V_{max}, the maximum velocity; and K_m, the substrate concentration at which the reaction velocity is 50% of V_{max}. The intrinsic clearance (Cl_{int}) of *in vitro* incubation was calculated as V_{max}/K_m. Contributions of each cytochrome to ipriflavone oxidation were normalized for mean values of the relative abundance of individual cytochromes in the liver. The relative abundances of CYP1A2 (12.7%), CYP3A4 (28.8%), CYP2C9 (14.7%) and CYP2C19 (3.5%) and wide inter-individual variability in the expression of CYP3A4 and CYP1A2 have been reported (Shimada et al. 1994; Venkatakrishnan et al. 1998). The Cl_{int} attributable to CYPs 1A2, 2C9, 2C19 and 3A4 was multiplied by the relative abundance of each CYP in the liver. Percentages of the Cl_{int} of an individual CYP relative to the overall contribution of four CYPs (CYP1A2, -2C9, -2C19 and -3A4) were calculated.

For correlation analysis, comparative metabolic rates of ipriflavone in 12 different human liver microsomes were investigated by incubating 2 μM of ipriflavone with 0.15 mg ml^{-1} microsomal protein for 20 min. The formation rates of three metabolites, M1, M3 and M4 were correlated with specific CYP activities in human liver microsomes reported by Gentest using Pearson product moment correlation (SigmaStat software version 2.0, SPSS Science). For pairs with p values below 0.05, there is significant relationship between the two variables.

Inhibition experiments

Immunoinhibition studies were performed by incubating human liver microsomes with various amounts of human selective antibodies, anti-CYP1A2, anti-CYP2C, anti-CYP2D6, anti-CYP2E1 or anti-CYP3A4 for 15 min on ice before the addition of buffer, ipriflavone or M4 (2 μM) and an NADPH generating system. As a control, comparable incubations were done with microsomes and 25 mM Tris buffer.

To explore which CYP enzymes are involved in the metabolism of ipriflavone, the inhibitory effects were determined using selective CYP enzyme inhibitors. The concentrations of specific CYP enzyme inhibitors were chosen based on the published IC_{50} or K_i values for CYP enzyme-specific reactions: 10 μM furafylline for CYP1A2 (Sesardic et al. 1990), 100 μM coumarin for CYP2A6 (Yun et al. 1991), 5 μM thioTEPA for CYP2B6 (Rae et al. 2002), 0.1 μM montelukast for CYP2C8 (Walsky et al. 2005), 10 μM sulfaphenazole for CYP2C9 (Baldwin et al. 1995), 1 μM *S*-benzylnirvanol for CYP2C19 (Suzuki et al. 2002; Walsky and Obach 2003), 10 μM quinidine for CYP2D6 (Newton et al. 1995), 10 μM diethyldithiocarbamate for CYP2E1 (Newton et al. 1995) and 1 μM ketoconazole for CYP3A4 (Baldwin et al. 1995). Incubations were performed with CYP-selective inhibitor, pooled human liver microsomes (H161, 0.15 mg ml^{-1}) and ipriflavone or M4 (2 μM). Furafylline and diethyldithiocarbamate were pre-incubated for 10 min with microsomes and an NADPH generating system before addition of ipriflavone or M4 to initiate the reaction. Activities of all the inhibitors were compared with that of inhibitor-free controls.

LC-MS/MS analysis

The concentrations of ipriflavone and its five metabolites, M1, M2, M3, M4 and M5, in the microsomal incubates were analyzed by the modification of the previous LC-MS/MS

method (Ji et al. 2005). The chromatographic system consisted of a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan). The separation was performed on an XTerra C18 column (5 μm, 3.0 mm i.d. × 50 mm, Waters, USA) using a mixture of acetonitrile-ammonium formate (10 mM, pH 3.0) (50:50, v/v) at a flow rate of 0.5 ml min^{-1}. The column and autosampler tray temperature was 30°C and 4°C, respectively. The analytical run time was 11 min. The eluent was introduced directly into the positive ionization electrospray source of a tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Ltd, UK). The ion source and desolvation temperature were held at 120°C and 350°C, respectively. The optimum cone voltages were 32, 35, 25, 35 and 55 V for M1, M3, M4, M5 and DPDC (internal standard), respectively. Multiple reaction monitoring (MRM) mode was employed for the quantitation: m/z 238.9 → 165.3 for M1, m/z 297.6 → 254.9 for M3, m/z 297.3 → 239.1 for M4, m/z 311.1 → 238.8 for M5 and m/z 315 → 299 for DPDC. Peak areas for all components were automatically integrated using MassLynx version 3.5 software (Micromass UK Ltd.). Calibration curves were linear over the concentration range of 0.5–500 pmol of M1–M5. The inter-batch relative error values for M1–M5 ranged from −3.7% to 2.3% with coefficient of variation values of 2.2–5.7% at three QC levels, i.e. 1.0, 60 and 400 pmol.

Inhibitory potency of ipriflavone and its metabolites on CYP activities in human liver microsomes

The inhibitory potency of ipriflavone and its five metabolites, M1–M5, was determined with cytochrome P450 assays in the absence and presence of ipriflavone, M1, M2, M3, M4 or M5 (final concentrations of 1–100 μM with methanol concentration less than 0.5% v/v) using pooled human liver microsomes (H161). All experiments were performed in duplicate. Phenacetin *O*-deethylase, coumarin 7-hydroxylase, paclitaxel 6α-hydroxylase, diclofenac 4-hydroxylase, (*S*)-mephenytoin 4-hydroxylase, bufuralol 1′-hydroxylase and midazolam 1′-hydroxylase were determined as probe activities for CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, respectively, using cocktail incubation and tandem mass spectrometry with the modification of the method of Dierks et al. (2001). In brief, the incubation mixtures containing pooled human liver microsomes (0.25 mg ml^{-1}), CYP-selective substrates and ipriflavone or its metabolites (0.1–100 μM) were pre-incubated for 5 min at 37°C. The reaction was initiated by adding an NADPH generating system, and the reaction mixtures were incubated for 20 min at 37°C in a shaking water bath. The substrates were used at concentrations approximately equal to their respective K_m values: 50 μM phenacetin, 2.5 μM coumarin, 10 μM paclitaxel, 10 μM diclofenac, 100 μM [*S*]-mephenytoin, 5 μM bufuralol and 2.5 μM midazolam.

Results

Kinetics of ipriflavone oxidation in human liver microsomes

From the incubation of ipriflavone with human liver microsomes in the presence of NADPH-generating system, its four metabolites, i.e. M1, M3, M4 and M5 were identified but M2, M6 and M7 were not detected by LC-MS analysis. The incubation of M4 with human liver microsomes in the presence of NADPH-generating system resulted in the formation of M5.

The oxidation of ipriflavone to M1, M3 and M4 in three different human liver microsomes followed single-enzyme Michaelis–Menten kinetics (Figure 2). The formation rates of M5 were very low, and therefore kinetic parameters for M5 could not be determined. The apparent kinetic parameters for *O*-dealkylation of ipriflavone to M1 in three human liver microsomes were K_m of $3.0 \pm 0.8\,\mu M$ and V_{max} of 56.5 ± 37.0 pmol min^{-1} mg $protein^{-1}$ (Table I). The apparent kinetic parameters for isopropyl hydroxylation of ipriflavone to M4 in three human liver microsomes were K_m of $2.3 \pm 0.5\,\mu M$ and V_{max} of 105.2 ± 111.7 pmol min^{-1} mg $protein^{-1}$ (Table I). The apparent kinetic parameters for β-ring hydroxylation of ipriflavone to M3 in three human liver microsomes were K_m of $2.3 \pm 0.6\,\mu M$ and V_{max} of 51.0 ± 48.4 pmol min^{-1} mg $protein^{-1}$ (Table I). The metabolism of authentic standard M4 to M5 in three different human liver microsomes followed

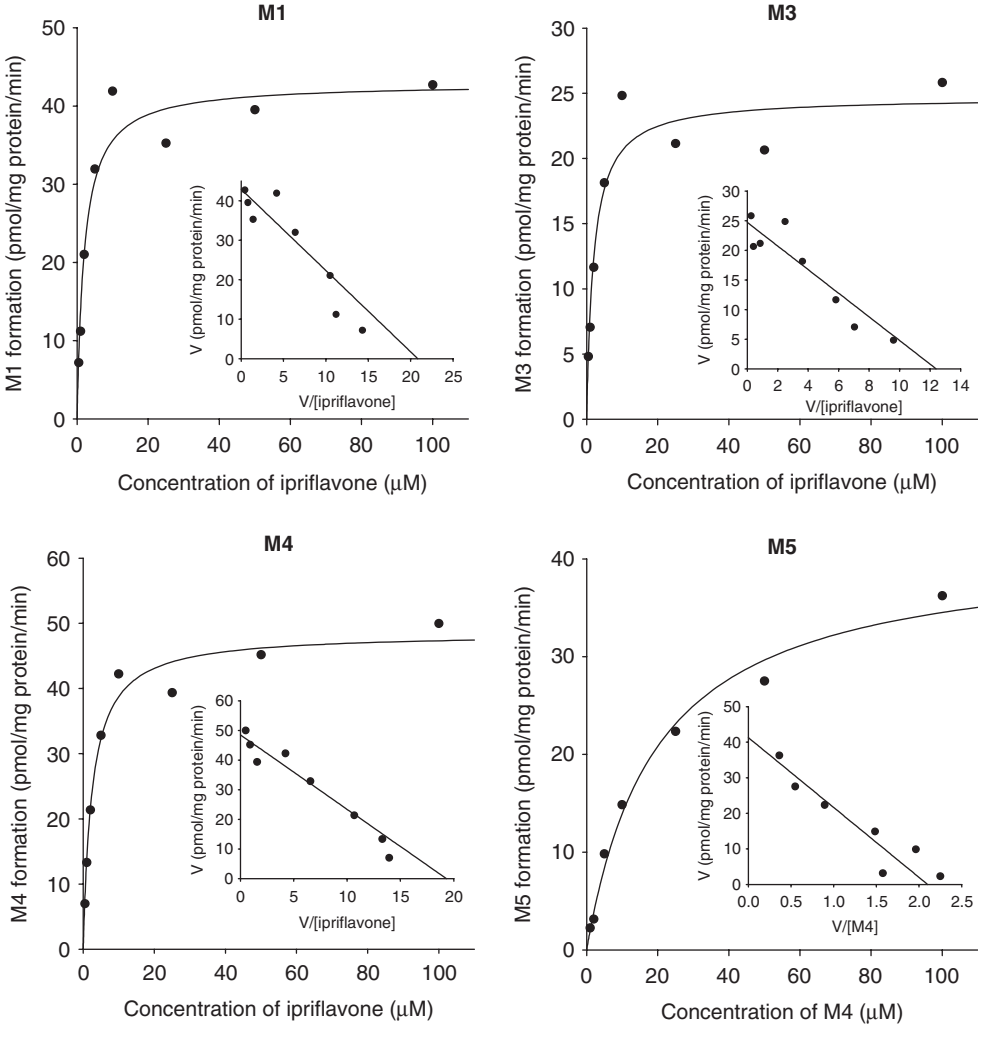

Figure 2. Representative Michaelis–Menten plots for the biotransformation of ipriflavone to M1, M3 and M4 and the formation of M5 from M4 in human liver microsome H093 with inset Eadie–Hostee plots demonstrating single enzyme kinetics. Each point represents the average of two determinations.

Table I. Kinetics of the formation of M1, M3 and M4 from ipriflavone and M4 oxidation to M5 in three human liver microsome preparations.

	Metabolite formation			
Human liver microsomes	M1	M3	M4	M5
H043				
K_m (μM)	3.2	2.0	1.8	14
V_{max} (pmol mg protein^{-1} min^{-1})	28.2	21.5	33.3	89.8
Cl_{int} (μl mg protein^{-1} min^{-1})	8.8	10.8	18.5	6.4
H056				
K_m (μM)	3.7	3.0	2.7	20.9
V_{max} (pmol mg protein^{-1} min^{-1})	98.4	106.9	233.9	200.5
Cl_{int} (μl mg protein^{-1} min^{-1})	26.6	35.6	86.6	9.6
H093				
K_m (μM)	2.1	2.0	2.5	19.7
V_{max} (pmol mg protein^{-1} min^{-1})	42.9	24.7	48.5	41.3
Cl_{int} (μl mg protein^{-1} min^{-1})	20.4	12.4	19.4	2.1
Mean ± SD				
K_m (μM)	3.0 ± 0.8	2.3 ± 0.6	2.3 ± 0.5	18.2 ± 3.7
V_{max} (pmol mg protein^{-1} min^{-1})	56.5 ± 37.0	51.0 ± 48.4	105.2 ± 111.7	110.5 ± 81.6
Cl_{int} (μl mg protein^{-1} min^{-1})	18.6 ± 9.0	19.6 ± 13.9	41.5 ± 39.1	6.0 ± 3.8

single-enzyme Michaelis–Menten kinetics (Figure 2). The apparent kinetic parameters for oxidation of M4 to M5 in three human liver microsomes were K_m of 18.2 ± 3.7 μM and V_{max} of 110.5 ± 81.6 pmol min^{-1} mg protein^{-1} (Table I). The order of the metabolic pathways for ipriflavone in human liver microsomes were hydroxylation to M4 (Cl_{int}; 41.5 ± 39.1 μl mg protein^{-1} min^{-1}) > hydroxylation to M3 (Cl_{int}; 19.6 ± 13.9 μl mg protein^{-1} min^{-1}) ≅ *O*-dealkylation to M1 (Cl_{int}; 18.6 ± 9.0 μl mg protein^{-1} min^{-1}) > oxidation of M4 to M5 (Cl_{int}; 6.0 ± 3.8 μl mg protein^{-1} min^{-1}).

Ipriflavone metabolism in human cDNA-expressed CYPs

A screen of ipriflavone oxidation at 2 μM concentration using microsomes containing human cDNA-expressed CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 or 3A4 identified the possible roles of CYPs 1A2, 2C9, 2C19, 2D6 and 3A4 for the oxidation of ipriflavone to M1, M3 and M4. CYP2A6, CYP2B6, CYP2C8 and CYP2E1 did not catalyze the biotransformation of ipriflavone to M1, M3 and M4 at a detectable rate (<0.006 pmol min^{-1} pmol CYP^{-1}). Screening of M4 oxidation to M5 in nine human cDNA-expressed CYP microsomes showed that only CYP 1A2 formed M5 from M4.

Table II shows the kinetic parameters for the formation of M1, M3 and M4 from ipriflavone and M4 oxidation to M5 in human cDNA-expressed CYPs 1A2, 2C9, 2C19 and 3A4. The formation of M1, M3, M4 and M5 was consistent with single-enzyme Michaelis–Menten kinetics. Contributions of these latter CYPs were determined after normalization for the predicted relative abundance of each CYP to ipriflavone oxidation. Normalization for the relative abundance of CYPs confirmed that CYP3A4 played a predominant role in *O*-dealkylation of M1 from ipriflavone. CYP1A2 played a major role in hydroxylation of ipriflavone to M3 and M4 as well as M4 oxidation to M5. Also, CYP2C9 partly contributed to ipriflavone oxidation to M1, M3 and M4.

Table II. Kinetic parameters for the formation of M1, M3 and M4 from ipriflavone and M4 oxidation to M5 in expressed human CYP enzymes. Since the role of each CYP has been determined relative to others, their Cl_{int} (V_{max}/K_m) values have been normalized for the relative hepatic abundance of each CYP.

Expressed human CYPs	M1	M3	M4	M5
CYP 3A4				
K_m (μM)	5.9	N.C.	N.C.	N.C.
V_{max} (pmol pmol CYP^{-1} min^{-1})	0.94	N.C.	N.C.	N.C.
Cl_{int} (μl pmol CYP^{-1} min^{-1})	0.16	N.C.	N.C.	N.C.
Cl_{int} (% of total)	62.3	N.C.	N.C.	N.C.
CYP 1A2				
K_m (μM)	3.8	4.0	3.9	23.8
V_{max} (pmol pmol CYP^{-1} min^{-1})	0.12	0.86	0.33	0.58
Cl_{int} (μl pmol CYP^{-1} min^{-1})	0.03	0.22	0.085	0.024
Cl_{int} (% of total)	5.4	77.9	59.7	100.0
CYP 2C9				
K_m (μM)	6.2	4.1	3.9	N.C.
V_{max} (pmol pmol CYP^{-1} min^{-1})	0.78	0.2	0.17	N.C.
Cl_{int} (μl pmol CYP^{-1} min^{-1})	0.13	0.049	0.044	N.C.
Cl_{int} (% of total)	25.1	20.1	35.6	N.C.
CYP 2C19				
K_m (μM)	2.7	3.3	2.3	N.C.
V_{max} (pmol pmol CYP^{-1} min^{-1})	0.40	0.067	0.055	N.C.
Cl_{int} (μl pmol CYP^{-1} min^{-1})	0.15	0.02	0.024	N.C.
Cl_{int} (% of total)	7.0	2.0	4.6	N.C.

N.C.: Not calculable.

Table III. Correlation of ipriflavone oxidation rates with other CYP activities in 12 human liver microsome preparations.

		Correlation coefficient (r)		
Enzyme activities	CYP	M1	M3	M4
Phenacetin *O*-deethylation	1A2	0.980*	0.984*	0.981*
Coumarin 7-hydroxylation	2A6	−0.115	−0.161	−0.112
Paclitaxel 6α-hydroxylation	2C8	−0.0578	−0.0983	−0.0879
Diclofenac 4′-hydroxylation	2C9	0.385	0.332	0.314
[*S*]-Mephenytoin 4′-hydroxylation	2C19	0.307	0.322	0.333
Bufuralol 1′-hydroxylation	2D6	−0.0264	0.00275	0.0286
Chlorzoxazone 6-hydroxylation	2E1	0.0987	0.144	0.109
Testosterone 6β-hydroxylation	3A4	−0.0749	−0.0130	−0.0537

*$p < 0.05$.

Correlation of rates of ipriflavone oxidation with other enzyme activities in human liver microsomes

The rates of ipriflavone oxidation at 2 μM concentration to M1, M3 and M4 in the 12 different human liver microsomes were 11.8–83.2, 4.0–49.0 and 8.2–132.7 pmol min^{-1} mg protein^{-1}, respectively. Table III shows correlations between the biotransformation rates of ipriflavone to M1, M3 or M4 in 12 human liver microsome preparations and their standard CYP isoform-specific activities. Significant correlations were observed between M1, M3 or M4 formation and CYP1A2-catalyzed phenacetin *O*-deethylation.

Immuno-inhibition of ipriflavone oxidation in human liver microsomes

To determine which CYP is primarily responsible for ipriflavone metabolism to M1, M3 and M4, immuno-inhibition studies with anti-CYP1A2, anti-CYP2C, anti-CYP2D6, anti-CYP2E1 or anti-CYP3A4 were performed in pooled human liver microsomes (H161) (Figure 3). The formation of M1 was inhibited by anti-CYP3A4, anti-CYP1A2 and anti-CYP2C, suggesting the involvement of CYPs 3A4, 2C and 1A2 in *O*-dealkylation of ipriflavone to M1 (Figure 3a). Anti-CYP1A2 potently inhibited the formation of M3 and M4 from ipriflavone as well as M4 oxidation to M5 (Figure 3b–d). Anti-CYP3A4, anti-CYP2C, anti-CYP2E1 and anti-CYP2D6 did not inhibit the formation of M3, M4 and M5. These results indicate that CYP1A2 may play a major role in the formation of M3, M4 and M5 from ipriflavone or M4.

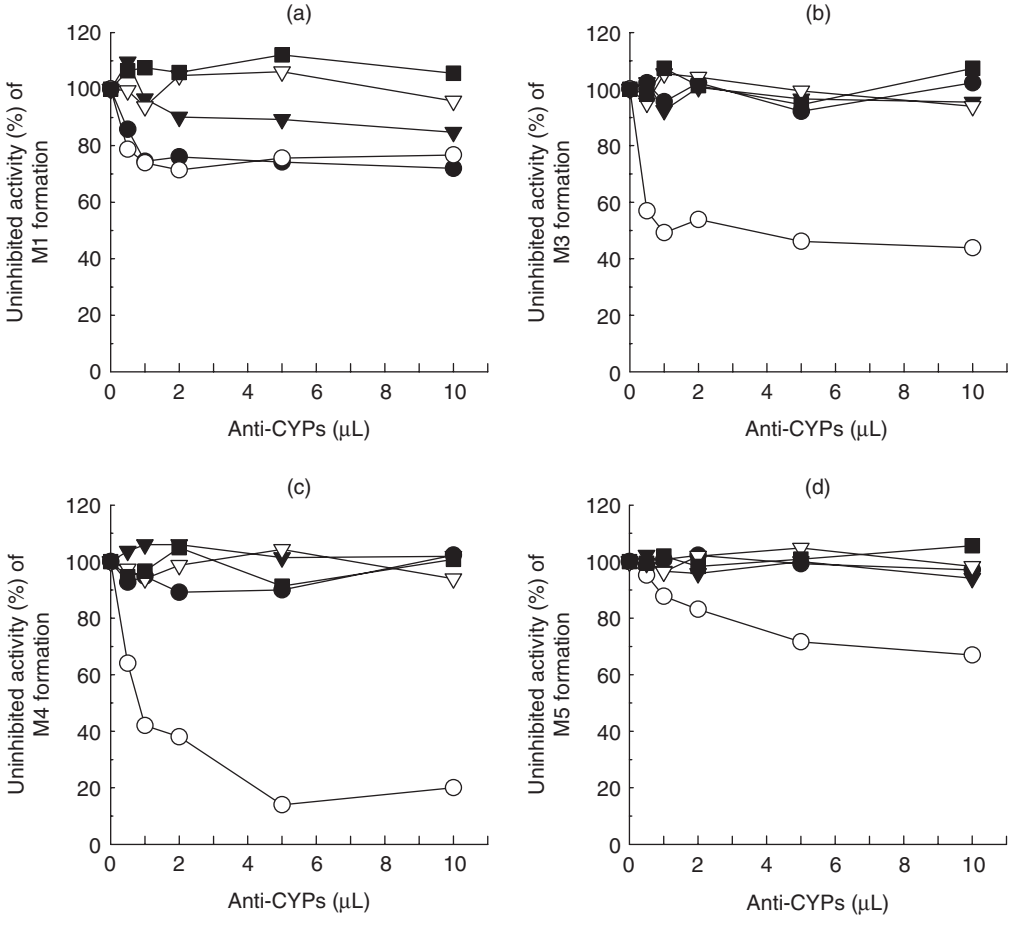

Figure 3. Effects of CYP antibodies on the biotransformation of ipriflavone to (a) M1, (b) M3 and (c) M4, and (d) the oxidation of M4 to M5. Human pooled microsomes (H161, 0.15 mg protein ml^{-1}) were pre-incubated with anti-CYP3A4 (●), anti-CYP1A2 (○), anti-CYP2D6 (■), anti-CYP2C (▼) or anti-CYP2E1 (▽). Data are derived from the average of two determinations. Rates of control reactions for the formation of M1, M3, M4 and M5 were 43.1, 26.2, 27.0 and 10.1 pmol min^{-1} mg $protein^{-1}$, respectively.

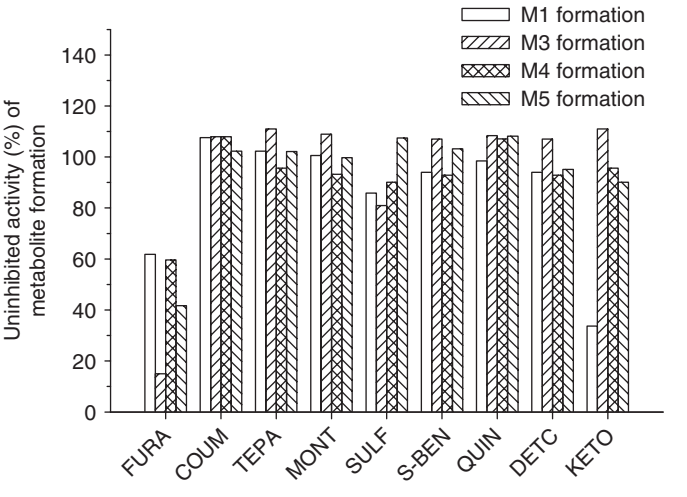

Figure 4. Effects of CYP isoform-selective inhibitors on the formation of M1, M3 and M4 from ipriflavone and M4 metabolism to M5. Pooled human liver microsomes (H161, 0.15 mg protein ml^{-1}) were incubated at 37°C for 20 min with 2 μM ipriflavone and NADPH generating system in the presence and absence of inhibitors. Each bar represents the average of two determinations. FURA, furafylline (10 μM); COUM, coumarin (100 μM); TEPA, triethylenethiophosphoramide (5 μM); MONT, montelukast (0.1 μM); SULF, sulfaphenazole (10 μM); S-BEN, *S*-benylnirvanol (1 μM); QUIN, quinidine (10 μM); DETC, diethyldithiocarbamate (10 μM); KETO, ketoconazole (1 μM). Rates of control reactions for the formation of M1, M3, M4 and M5 were 46.3, 24.8, 25.4 and 9.4 pmol min^{-1} mg $protein^{-1}$, respectively.

Chemical inhibition of ipriflavone metabolism in human liver microsomes

Figure 4 shows the effects of selective inhibitors of CYP450s on the metabolism of ipriflavone to M1, M3 and M4 as well as M5 formation from M4 in pooled human liver microsomes (H161). The rate of M1 formation was potently inhibited up to 70% and 40% by ketoconazole (1 μM, a CYP3A4 inhibitor) and furafylline (10 μM, a CYP1A2 inhibitor), respectively, whereas the effect of the other inhibitors tested on the formation of M1 was less than 15%. Furafylline (10 μM) extensively inhibited the formation of M3 (85%) and M4 (40%) from ipriflavone and M5 formation (60%) from M4. These results suggest that CYP3A4 might play a major role in *O*-dealkylation to M1 and CYP1A2 might play the major role in the formation of M1, M3, M4 and M5.

In vitro *inhibition of CYP enzymes by ipriflavone and its five metabolites*

Inhibition of CYP activity was evaluated at concentrations up to 100 μM of ipriflavone and its five metabolites, i.e. M1–M5, to investigate the effect of ipriflavone and its five metabolites on CYP-mediated drug interactions (Table IV). Ipriflavone was a potent inhibitor, with IC_{50} value of 7.3 μM against phenacetin O-deethylase (CYP1A2) activity and IC_{50} value of 10.8 μM against [*S*]-mephenytoin 4′-hydroxylase (CYP2C19) activity. Ipriflavone at a concentration of 100 μM did not affect coumarin 7-hydroxylation (CYP2A6), paclitaxel 6α-hydroxylation (CYP2C8), diclofenac 4-hydroxylation (CYP2C9), bufuralol 1′-hydroxylation (CYP2D6) and midazolam 1-hydroxylation (CYP3A4).

Table IV. Inhibitory potentials of ipriflavone and its metabolites M1–M5 on specific CYP activities in human liver microsomes.

Enzyme activities	CYP	IC_{50} (μM)					
		M1	M2	M3	M4	M5	Ipriflavone
Phenacetin *O*-deethylase	1A2	33.6	–	–	6.8	–	7.3
Coumarin 7-hydroxylase	2A6	13.4	–	65.2	–	–	–
Paclitaxel 6α-hydroxylase	2C8	9.9	10.2	–	31.8	2.5	–
Diclofenac 4-hydroxylase	2C9	11.2	19.6	–	21.9	6.0	–
[*S*]-Mephenytoin 4′-hydroxylase	2C19	4.5	–	1.7	1.5	–	10.8
Bufuralol 1′-hydroxylase	2D6	21.1	71.9	56.2	19.4	0.1	–
Midazolam 1-hydroxylase	3A4	86.6	–	–	63.7	–	–

-: unaffected at 100 μM.

M1 potently inhibited [*S*]-mephenytoin 4′-hydroxylation (CYP2C19), paclitaxel 6α-hydroxylation (CYP2C8), diclofenac 4-hydroxylation (CYP2C9) and coumarin 7-hydroxylation (CYP2A6) with IC_{50} values of 4.5, 9.9, 11.2 and 13.4 μM, respectively. M1 also inhibited bufuralol 1′-hydroxylation (CYP2D6), phenacetin *O*-deethylation (CYP1A2) and midazolam 1-hydroxylation (CYP3A4) with IC_{50} values of 21.1, 33.6 and 86.6 μM, respectively. M2, daidzein inhibited paclitaxel 6α-hydroxylation (CYP2C8), diclofenac 4-hydroxylation (CYP2C9) and bufuralol 1′-hydroxylation (CYP2D6) with IC_{50} values of 10.2, 19.6 and 71.9 μM, respectively. M3 potently inhibited [*S*]-mephenytoin 4′-hydroxylase (CYP2C19) activity with an IC_{50} value of 1.7 μM and weakly inhibited bufuralol 1′-hydroxylation (CYP2D6) and coumarin 7-hydroxylation (CYP2A6) with IC_{50} values of 56.2 and 65.2 μM, respectively. M4 was a potent inhibitor of [*S*]-mephenytoin 4′-hydroxylation (CYP2C19) and phenacetin O-deethylation (CYP1A2) with IC_{50} values of 1.5 and 6.8 μM, respectively. M4 inhibited bufuralol 1′-hydroxylation (CYP2D6), diclofenac 4-hydroxylation (CYP2C9), paclitaxel 6α-hydroxylation (CYP2C8) and midazolam 1-hydroxylation (CYP3A4) with an IC_{50} value of 19.4, 21.9, 31.8 and 63.7 μM, respectively. M5 potently inhibited paclitaxel 6α-hydroxylation (CYP2C8) and diclofenac 4-hydroxylation (CYP2C9) with an IC_{50} value of 2.5 and 6.0 μM, respectively.

Discussion

Ipriflavone has been reported to be metabolized to seven metabolites, i.e. M1–M7 in animals and human (Figure 1) but only M1, M3, M4 and M5 were identified from the incubation of ipriflavone with 12 different human liver microsome preparations in the current study. The formation rates of M5 from ipriflavone were very low and inconsistent in human liver microsomes, and therefore, M5 formation was determined from the incubation of M4 with human liver microsomes. Cl_{int} values for M1, M3, M4 and M5 formation in three human liver microsomes were 18.6 (±9.0), 19.6 (±13.9), 41.5 (±39.1) and 6.0 (±3.8) μl mg protein^{-1} min^{-1}, respectively (Table I), suggesting that isopropyl hydroxylation to M4, *O*-dealkylation to M1, β-ring hydroxylation to M3 and M4 oxidation to M5 are the major metabolic pathways for ipriflavone metabolism in human liver microsomes *in vitro*.

The specific CYP isoforms responsible for ipriflavone oxidation to M1, M3, M4 and M5 were identified using a combination of correlation analysis, immuno-inhibition, chemical

inhibition in human liver microsomes and metabolism by expressed recombinant CYP enzymes. From a screen of ipriflavone or M4 metabolism using microsomes containing cDNA-expressed CYPs, it was shown that the ipriflavone oxidations might be mediated by CYPs 3A4, 1A2, 2C9 and/or 2C19. The predominant role of CYP3A4 was evident in ipriflavone oxidation to M1 when the contributions of each CYP were normalized for their relative abundance in the liver (Table II). CYP1A2 played the major role in the formation of M3, M4 and M5. CYP2C9 played the role in the formation of M1, M3 and M4 from ipriflavone. Chemical inhibitor and CYP antibody studies also support the roles for CYPs 3A4, 1A2 and 2C9 in *O*-dealkylation of ipriflavone to M1 (Figures 3 and 4). Furafylline, a specific CYP1A2 inhibitor and anti-CYP1A2 antibody potently inhibited the formation of M3 and M4 from ipriflavone and M4 oxidation to M5. These results collectively suggest that CYP3A plays the major role in ipriflavone *O*-dealkylation to M1 and CYP1A2 plays the predominant role in the formation of M3, M4 and M5. CYP2C9 may partly contribute to the formation of M1, M3 and M4 from ipriflavone.

Expression of CYP3A4 has been observed to be highly variable among human liver samples (Wrighton *et al.* 1990; Shimada et al. 1994; Gibson et al. 2002). Wide inter-individual variability has also been reported in the expression of CYP1A2 in humans (Schweikl et al. 1993). Genetic polymorphisms in CYP2C9 have been shown to affect the metabolism, efficacy and toxicity of clinically important drugs (Goldstein 2001). CYPs 3A4, 1A2 and 2C9 are responsible for the metabolism of ipriflavone, and, therefore, inter-individual variability in plasma concentrations of ipriflavone may be expected owing to variable rates of metabolism. CYPs 3A4, 1A2 and 2C9 have been shown to be induced or inhibited by a variety of xenobiotics (Miners and Birkett 1998; Miners and McKinnon 2000; Wrighton and Thummel 2000; Luo et al. 2004). Therefore, any co-administered drugs that can inhibit or induce CYP3A4, CYP1A2 or CYP2C9 may alter the metabolism of ipriflavone and potentially lead to changes in the pharmacokinetics of ipriflavone in patients.

The effect of ipriflavone and its major metabolites, i.e. M1, M2, M3, M4 and M5, on the activities of clinically important human CYPs 1A2, 2A6, 2C8, 2C9, 2C19, 2D6 and 3A4 was also investigated in human liver microsomes (Table IV). Phenacetin *O*-deethylase (CYP1A2) activity was potently inhibited by ipriflavone and M4 with IC_{50} values of 7.3 and 6.8 μM, respectively. Diclofenac 4-hydroxylase, a marker of CYP2C9, activity was extensively inhibited by M5 and M1 with IC_{50} values of 6.0 and 11.2 μM, respectively. [*S*]-mephenytoin 4′-hydroxylase (CYP2C19) activity was strongly inhibited by M4, M3, M1 and ipriflavone with IC_{50} values of 1.5, 1.7, 4.5 and 10.8 μM, respectively. Paclitaxel 6α-hydroxylation (CYP2C8) was potently inhibited by M5, M1 and M2 with IC_{50} values of 2.5, 9.9 and 10.2 μM, respectively. These *in vitro* data suggest that ipriflavone should be used carefully with drugs metabolized by CYPs 1A2, 2C8, 2C9 and 2C19 to avoid possible drug–drug interactions. There is evidence that ipriflavone co-administration resulted in a decrease in theophylline clearance in patients as a result of CYP inhibition (Takahashi et al. 1992; Monostory and Vereczkey 1995, 1996). Coumarin 7-hydroxylation (CYP2A6), bufuralol 1′-hydroxylation (CYP2D6) and midazolam 1-hydroxylation (CYP3A4) activities were moderately or weakly inhibited, and, therefore, it is very unlikely that ipriflavone treatment will significantly alter the clearance of other compounds metabolized by CYPs 2A6, 2D6 and/or 3A4.

In conclusion, this study demonstrates that hydroxylation of the isopropyl moiety of ipriflavone to M4 and M5, β-ring hydroxylation to M3 and *O*-dealkylation to M1 are the major metabolic pathways for ipriflavone in human liver microsomes. CYP3A4 plays the major role in *O*-dealkylation to M1 and CYP1A2 plays the dominant role in the formation of M3, M4 and M5. CYP2C9 are responsible for ipriflavone oxidations. The possibility

of drug–drug interactions would therefore be predicted when prescribing ipriflavone concomitantly with known inducers or inhibitors of CYPs 3A4, 1A2 and 2C9. Ipriflavone and its five metabolites were observed to inhibit potently the metabolism of CYPs 1A2, 2C8, 2C9 and 2C19 probes, indicating that ipriflavone may have the significant effects on the pharmacokinetics of other drugs metabolized by CYPs 1A2, 2C8, 2C9 and/or 2C19.

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