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Ipriflavone as an inhibitor of human cytochrome P450 enzymes

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1 Reduction of theophylline metabolism and elimination were observed in a theophylline-treated patient during ipriflavone administration. After withdrawal of ipriflavone, the serum theophylline level decreased to an extent similar to that found before administration of ipriflavone. The effects of ipriflavone and its major metabolites 7-hydroxy-isoflavone and 7-(1-carboxy-ethoxy)-isoflavone on cytochrome P450 activities were studied *in vitro* in human liver microsomes from three donors.

2 Ipriflavone and 7-hydroxy-isoflavone competitively inhibited phenacetin O-deethylase and tolbutamide hydroxylase activity. The parent compound and its dealkylated metabolite were strong inhibitors exhibiting K_i values around 10–20 μM, while 7-(1-carboxy-ethoxy)-isoflavone had no effect on the cytochrome P450 activities investigated. 7-Hydroxy-isoflavone is the only one that influenced nifedipine oxidase activity. It competitively inhibited this activity with a K_i value of 129.5 μM.

3 The steady state concentrations of ipriflavone and 7-hydroxy-isoflavone in plasma of patients receiving 3×200 mg daily doses of ipriflavone for 48 weeks were found to be 0.33 ± 0.32 μM and 1.44 ± 0.77 μM, respectively.

4 The results indicate that the decrease in theophylline metabolism observed in a patient treated with ipriflavone may be due to a competitive interaction of ipriflavone or its metabolite, 7-hydroxy-isoflavone with CYP1A2. On the other hand, our *in vitro* findings predict some more interaction with CYP2C9.

Keywords: Ipriflavone; human cytochrome P450 enzymes; inhibition

Introduction

Cytochromes P450 (P450) play an important role in the biotransformation of lipophilic drugs to more polar compounds which are readily excreted. The metabolites of a drug can be inactive or less active than the parent compound, while some biotransformation products show enhanced pharmacological activity. The activity of P450 enzymes can determine a patient's response to a drug, thus any changes in the activity of P450 isoforms may influence the rate of activation or inactivation of drugs. The inhibition of drug-metabolizing enzymes as a side-effect of drug therapy is therefore of great clinical interest, because the resulting increased blood levels in patients can cause unexpected toxic side-effects or the lack of metabolic activation of a prodrug leads to the loss of a pharmacological effect.

Ipriflavone, a synthetic flavonoid developed for treatment of osteoporosis, may inhibit bone resorption and enhance the stimulating effect of oestrogen on calcitonin secretion (Yamazaki & Kinoshita, 1986). A 65-year-old patient with chronic obstructive pulmonary disease who showed an increase in serum theophylline level following the initiation of ipriflavone treatment has been studied (Takahashi *et al*., 1992). After withdrawal of ipriflavone, serum theophylline level decreased to a level similar to that found before administration of ipriflavone. An increase in serum theophylline level was observed again as a result of the resumption of ipriflavone treatment. The amount of theophylline and its metabolites in urine was also altered by ipriflavone treatment.

Our previous work (Monostory & Vereczkey, 1996) showed an inhibitory effect of ipriflavone and its major metabolites, 7-hydroxy-isoflavone and 7-(1-carboxy-ethoxy)-isoflavone, on theophylline metabolism in human liver microsomes. The compounds primarily decreased the N-demethylation to 1- or 3-methyl-xanthine, the major pathways of theophylline metabolism. The oxidation of theophylline to 1,3-dimethyluric acid was slightly affected by ipriflavone and its metabolites and the effect did not depend on the concentration of the inhibitors. Interaction of theophylline and ipriflavone was also observed at the cytochrome P450 level in male Wistar rats (Monostory & Vereczkey, 1995). CYP3A activities decreased in microsomes of rats treated with ipriflavone and an additional inhibition of CYP3A could be observed in microsomes of theophylline + ipriflavone treated animals as compared to the control group. It is known that several other flavonoids also inhibit hepatic mono-oxygenase activities (Beyeler *et al*., 1988; Merkel & Hoffmann, 1996).

These results suggest that the decrease in theophylline metabolism and increasing level of serum theophylline in a theophylline-treated patient during ipriflavone administration may be related to inhibition of cytochrome P450 enzymes of ipriflavone. Our present work deals with the effect of ipriflavone and its main metabolites (Figure 1) on specific reactions of the human liver cytochrome P450 enzymes, CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4.

Methods

Human liver microsomes

Human livers were obtained from kidney transplant donors. Permission from the Local Research Ethics Committee was obtained to use human tissues. Clinical histories of the donors are shown in Table 1. Human livers were perfused with Euro-Collin's solution (Fresenius AG, Bad Homburg v.d.H., Germany), excised and frozen immediately in liquid nitrogen. The tissues were homogenized in 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 154 mM KCl and micro-

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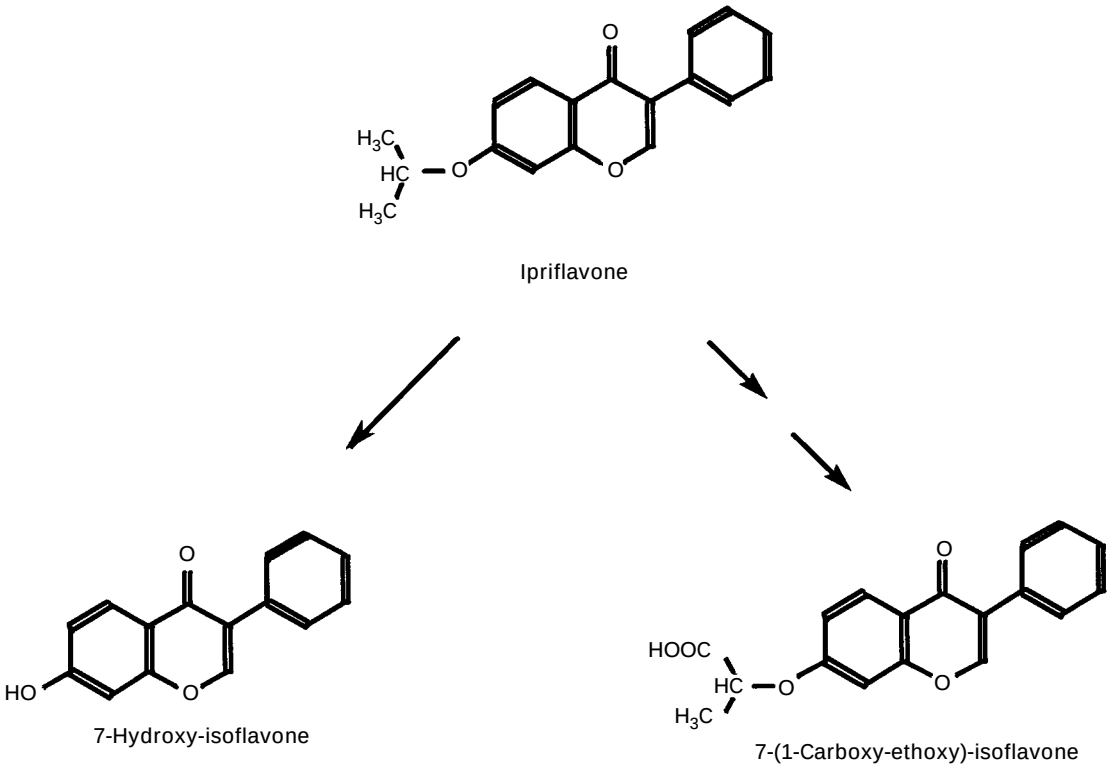

Figure 1 Chemical structure of ipriflavone and its main metabolites.

Table 1 Clinical histories of the liver donors

Donor code	*Sex*	*Age*	*Cause of death*	*Drug history*	*Anamnesis*
HHM-002	Male	24	Road traffic accident cranial fracture	Dopamine, dobutamine	Not known
HHM-003	Male	55	Home accident cranial fracture, cerebral contusion	Ampicylline, aminophylline, bromohexine, furosemide, dopamine, dexamethasone, lidocaine, ranitidine	High blood pressure
HHM-007	Male	38	Cranial fracture	Ampicylline, bromohexine, furosemide, dexamethasone, metronidazole brulamycin, sucralfate, ranitidine	Heavy drinker, obesity

somes were prepared as described (van der Hoeven & Coon, 1974). All procedures of preparation were performed at 0–4°C. The protein content of microsomes was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Inhibition studies

Published methods were followed to determine specific enzyme activities of the cytochromes P450. The incubation mixtures contained an NADPH-regenerating system (1 mM NADPH, 10 mM glucose 6-phosphate, 5 mM $MgCl_2$ and 2 unit ml^{-1} glucose 6-phosphate dehydrogenase) and various selective substrates for the cytochrome P450 isoforms (0.2 mM phenacetin for CYP1A2, 0.2 mM coumarin for CYP2A6, 1 mM tolbutamide for CYP2C9, 0.5 mM mephenytoin for CYP2C19, 2 mM dextromethorphan for CYP2D6, 0.5 mM chlorzoxazone for CYP2E1, 0.2 mM nifedipine for CYP3A). The amount of microsomal protein used in the enzymic reactions worked linearly in a 10–30 min incubation period. The metabolite extraction procedures and high performance liquid chromatographic (h.p.l.c.) or fluorometric analyses were performed according to published methods (Guengerich *et al.*, 1986; Kronbach *et al.*, 1987; Knodell *et al.*, 1987; Raunio *et al.*, 1988; Butler *et al.*, 1989a; Peter *et al.*, 1990; Sriwastava *et al.*, 1991). Ipriflavone or its metabolites was added in a final concentration of 17.8 μM before the addition of the substrates. During control incubations without selective substrates, it was proved that ipriflavone or its main metabolites investigated had no effect on the selective enzyme assays and did not cause

any detection problems. Inhibitory effects were expressed as the percentage of the rate of the control incubation with only the inhibitor vehicle present.

For those cytochrome P450 isoforms that were significantly inhibited by ipriflavone or any of its metabolites, inhibition constants (K_i values) were determined by use of different concentrations of the substrates and inhibitors in liver microsomes of the HHM-003 donor. K_i was calculated from Dixon plots of velocity^{-1} (expressed as (nmol product)$^{-1}$ mg protein min) versus inhibitor concentration (μM) at the three substrate concentrations. Each data point represents the mean of three determinations (s.d. was less than 5%). The apparent K_i was estimated from the intercept of three lines of Dixon plots and expressed as the mean $\pm$ s.d. of the intercepts.

Clinical investigations

During clinical trials, patients received 200 mg ipriflavone in tablet form 3 times daily and blood samples were taken from all patients at 11 time points. The time point at 48 weeks after the beginning of the ipriflavone treatment was chosen to characterize the steady state plasma levels of the compound and its metabolites. The plasma levels were determined with reversed phase h.p.l.c. applying u.v. detection (245 nm) and gradient elution. In order to follow the total conversion processes of ipriflavone, the plasma samples obtained from patients were digested with β-glucuronidase/arylsulphatase. For sample preparation solid phase extraction was developed and the h.p.l.c. separation was performed on a Symmetry C18 column (2 mm $\times$ 150 mm, 4 μm, Waters, U.S.A.), with 0.3 ml min^{-1} flow rate, the column temperature was 50°C. The samples were run with a gradient system using 7/1 (v/v) mixture of acetonitrile and water as solvent 'A' and 0.77/9 (v/v) mixture of acetonitrile and water (containing 0.2% phosphoric acid) as solvent 'B'. The method has been validated for ipriflavone and its metabolites: the lowest limit of quantification was 10 ng ml^{-1}; the linearity range was between 10 and 2000 ng ml^{-1}; the precision was 6.6% for ipriflavone, 8.7% for 7-hydroxy-isoflavone and 8.4% for 7-(1-carboxy-ethoxy)-isoflavone; the accuracy was 6.3% for ipriflavone, 3.0% for 7-hydroxy-isoflavone and 3.9% for 7-(1-carboxy-ethoxy)-isoflavone.

Chemicals

Ipriflavone and its metabolites, 7-hydroxy-isoflavone and 7-(1-carboxy-ethoxy)-isoflavone were provided by Chinoin Pharmaceutical and Chemical Works Co. Ltd. (Budapest, Hungary). D-Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and coumarin were purchased from Merck (Darmstadt, Germany). Dextromethorphan and bovine serum albumin were the products of Sigma Chemie GmbH (Deisenhofen, Germany). Phenacetin and chlorzoxazone were obtained from Aldrich Chem. Co. (Steinheim, Germany). Mephenytoin and nifedipine were from Ultrafine Chemicals (Manchester, U.K.). Tolbutamide was the product of Research Biochemicals International (Natick, MA, U.S.A.). The metabolites of the selective substrates of the cytochrome P450 isoforms (7-hydroxycoumarin, dextrorphan, 4-acetamidophenol, 6-hydroxychlorzoxazone, 4′-hydroxymephenytoin, oxidized nifedipine and 4-hydroxytolbutamide) were purchased from Ultrafine Chemicals (Manchester, U.K.), Research Biochemicals International (Natick, MA, U.S.A.) and Fluka Chemie AG (Buchs, Switzerland). Solvents for the h.p.l.c. mobile phases were ChemoLab (Budapest, Hungary) chromatography grade products. All other chemicals were obtained from Reanal (Budapest, Hungary).

Results

In vitro *effects of ipriflavone and its main metabolites*

Microsomal enzyme assays, with substrates selective for cytochrome P450 isoforms, were used to characterize the human enzymes that are responsible for the majority of oxidative metabolism of drugs. On the basis of phenotyping analysis of microsomes, none of the donors were poor metabolizers with respect to polymorphic cytochrome P450 isoforms that were tested in our studies (Table 2). For screening the inhibitory effects on cytochrome P450 isoforms of microsomal preparations, ipriflavone, 7-hydroxy-isoflavone or 7-(1-carboxy-ethoxy)-isoflavone were added to the incubation mixtures at a final concentration of 17.8 μM.

Results of studies on the inhibition of cytochrome P450 isoenzymes showed (Figure 2) that ipriflavone did not cause significant loss of coumarin 7-hydroxylation (CYP2A6),

Table 2 Cytochrome P450 enzyme activities of human liver microsomes

Enzyme activity (pmol mg^{-1} protein min^{-1})	*Donor code* HHM-002	HHM-003	HHM-007
Phenacetin O-deethylase (CYP1A2)	603.8	368.9	109.1
Coumarin 7-hydroxylase (CYP2A6)	559.0	725.3	1504.9
Tolbutamide hydroxylase (CYP2C9)	180.5	190.7	252.8
Mephenytoin 4′-hydroxylase (CYP2C19)	104.0	52.7	35.1
Dextromethrophan O-demethylase (CYP2D6)	328.4	550.3	607.6
Chlorzoxanzone 6-hydroxylase (CYP2E1)	504.0	1487.0	838.0
Nifedipine oxidase (CYP3A)	493.0	329.0	385.0

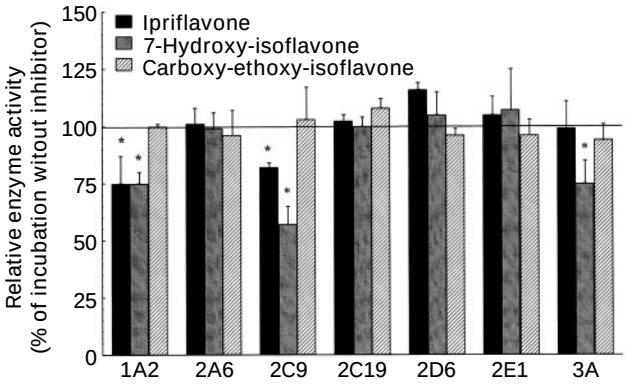

Figure 2 The effect of ipriflavone and its metabolites (7-hydroxy-isoflavone and 7-(1-carboxy-ethoxy)-isoflavone) on cytochrome P450 enzymes. Significant inhibition observed in specific reaction of P450 enzymes is marked by *. Probability value, $P<0.01$ calculated by Student's t test. Cytochrome P450 enzymes were determined by their activities towards selective substrates (CYP1A2: phenacetin O-deethylase; CYP2A6: coumarin 7-hydroxylase; CYP2C9: tolbutamide hydroxylase; CYP2C19: mephenytoin 4′-hydroxylase, CYP2D6: dextromethorphan O-demethylase; CYP2E1: chlorzoxazone 6-hydroxylase; CYP3A: nifedipine oxidase).

mephenytoin 4′-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1) and nifedipine oxidation (CYP3A), while decreases in phenacetin O-deethylation (CYP1A2) and tolbutamide hydroxylation (CYP2C9) were observed in the presence of ipriflavone. The phenacetin O-deethylation activity was inhibited by about 25%, while tolbutamide hydroxylase lost about 20% of its catalytic activity.

7-Hydroxy-isoflavone, one of the main metabolites of ipriflavone in man, is formed by the cleavage of the propoxy-group. This ipriflavone derivative caused a similar degree of inhibition of phenacetin O-deethylation (by 25%) as was observed for the parent compound. Tolbutamide hydroxylation was also altered; 7-hydroxy-isoflavone at a concentration of 17.8 μM decreased this activity by 40–50%. Furthermore, a decrease by about 25% was observed in nifedipine oxidation in the presence of 7-hydroxy-isoflavone, while ipriflavone did not show such an effect.

We also examined the inhibitory capacity of 7-(1-carboxy-ethoxy)-isoflavone, which is formed by oxidation of the ipriflavone side chain to a carboxy-derivative. The results demonstrated that it affected none of the reactions of the cytochrome P450 isoforms investigated.

Estimation of K_i *values*

Ipriflavone was found to be a selective inhibitor of phenacetin O-deethylation and tolbutamide hydroxylation with inhibition kinetic constants (K_i) of 11.9 ± 0.22 μM and 21.7 ± 1.33 μM, respectively (Figure 3). 7-Hydroxy-isoflavone displayed the same behaviour; it also was a potent inhibitor of phenacetin O-deethylase and tolbutamide hydroxylase with K_i values of 9.5 ± 1.74 μM and 13.4 ± 1.59 μM, respectively (Figure 4). The conclusion can be drawn from the type of intersection of the regression lines in the Dixon plots (Figures 3 and 4) that both the parent compound and its dealkylated derivative inhibited phenacetin O-deethylation and tolbutamide hydroxylation in a competitive manner. K_i values of ipriflavone and 7-hydroxy-isoflavone for both phenacetin O-deethylation and tolbutamide hydroxylation were quite close to each other, these two compounds had similarly strong inhibitory effects. In contrast, 7-hydroxy-isoflavone behaved as a competitive inhibitor towards nifedipine oxidation (Figure 5). Although its influence was somewhat weaker, the inhibition constant for nifedipine oxidation ($K_i = 129.5 \pm 12.49$ μM) was about 10 times higher

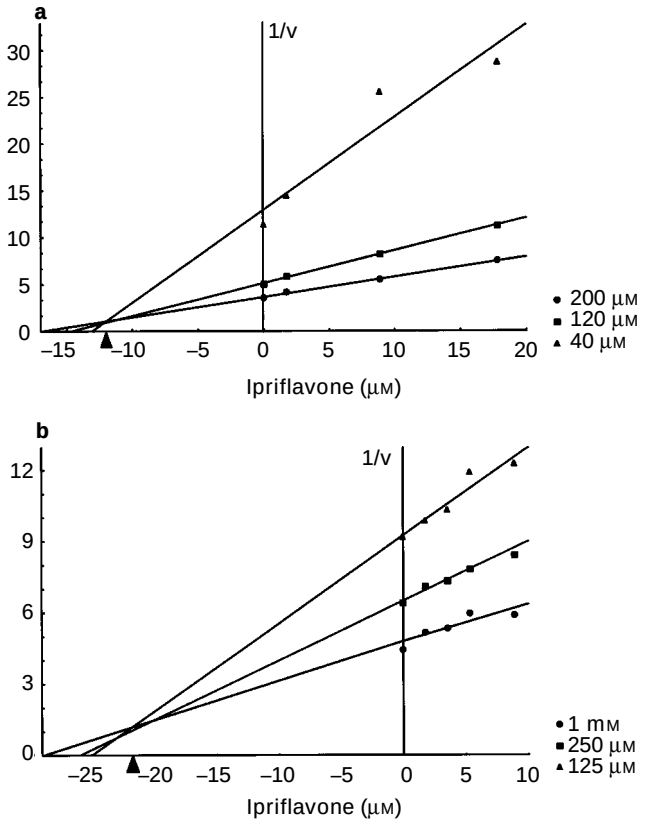

Figure 3 Dixon plots: the effects of ipriflavone on (a) phenacetin O-deethylation of CYP1A2 and (b) tolbutamide hydroxylation of CYP2C9. (a) $K_i = 11.9 \pm 0.22$ μM, (b) $K_i = 21.7 \pm 1.33$ μM.

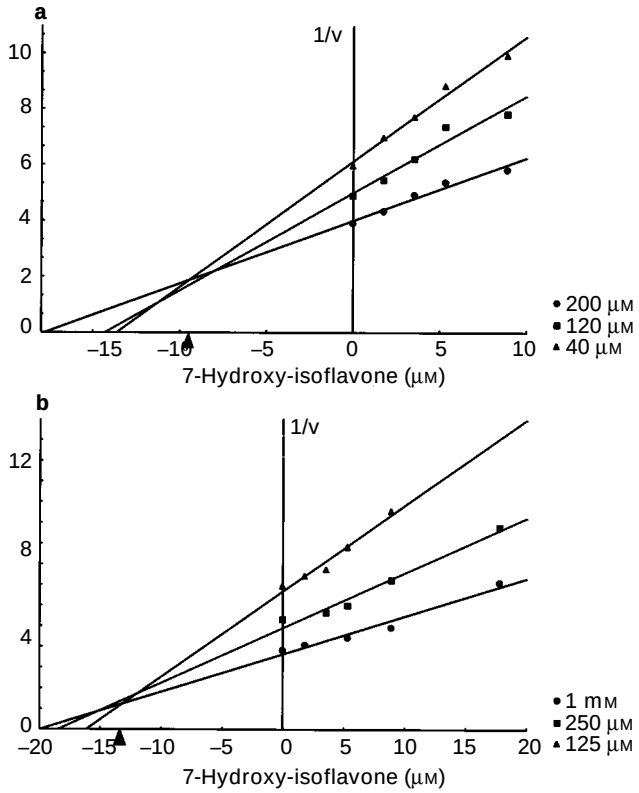

Figure 4 Dixon plots: the effects of 7-hydroxy-isoflavone on (a) phenacetin O-deethylation of CYP1A2 and (b) tolbutamide hydroxylation of CYP2C9. (a) $K_i = 9.5 \pm 1.74$ μM, (b) $K_i = 13.4 \pm 1.59$ μM.

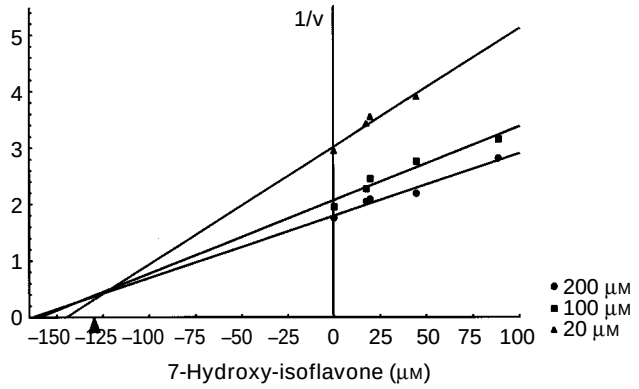

Figure 5 Dixon plot: the effect of 7-hydroxy-isoflavone on nifedipine oxidation of CYP3A. $K_i = 129.5 \pm 12.49$ μM.

than that for phenacetin O-deethylation or tolbutamide hydroxylation.

Clinical trial

The concentrations of ipriflavone and two of its metabolites were determined in plasma of patients receiving 3×200 mg daily doses of ipriflavone for 48 weeks. Ipriflavone could be measured in every sample, but it was only a minor component in the plasma. Its steady state level was 0.33 ± 0.32 μM (falling in the $0.06 - 1.78$ μM range). 7-(1-Carboxy-ethoxy)-isoflavone was the main metabolite, its level in the samples was 3.46 ± 1.70 μM (falling in the $0.30 - 6.33$ μM range). The dealkylated metabolite, 7-hydroxy-isoflavone showed the plasma level of 1.44 ± 0.77 μM (in the $0.14 - 2.86$ μM range).

Discussion

The intention of our study was to examine whether the delay of theophylline metabolism observed during concomitant treatment with ipriflavone in the patient mentioned above (Takahashi *et al*., 1992) is due to an interaction with any of the cytochrome P450 isoenzymes. The results obtained with liver microsomes of the three donors showed that ipriflavone, the parent compound and one of its major metabolites, 7-hydroxy-isoflavone had inhibitory effects on phenacetin O-deethylase (CYP1A2) and tolbutamide hydroxylase (CYP2C9), while the main metabolite, 7-(1-carboxy-ethoxy)-isoflavone did not alter the activities of the cytochromes P450 investigated. Ipriflavone and 7-hydroxy-isoflavone appeared to be quite strong inhibitors of CYP1A2 and CYP2C9 and the type of inhibition was competitive for both phenacetin O-deethylation and tolbutamide hydroxylation. Furthermore, we found that 7-hydroxy-isoflavone decreased nifedipine oxidation, but ipriflavone did not affect the activity of this cytochrome P450 isozyme. 7-Hydroxy-isoflavone had a somewhat lower inhibitory effect on nifedipine oxidation (CYP3A) than on phenacetin O-deethylation (CYP1A) or tolbutamide hydroxylation (CYP2C9).

Our findings, that ipriflavone and its dealkylated metabolite can inhibit several xenobiotic metabolizing cytochrome P450s *in vitro*, support the clinical observations on the inhibitory effect on theophylline metabolism *in vivo* (Takahashi *et al*., 1992). CYP1A enzymes mediated N-demethylations to 3-methylxanthine and 1-methylxanthine, the major primary metabolic pathways of theophylline in man (Robson *et al*., 1988; Sarkar *et al*., 1992). Thus inhibition of phenacetin O-deethylation (CYP1A2) by ipriflavone and 7-hydroxy-isoflavone observed in human liver microsomes corresponds to the effect on theophylline elimination and to the reduced formation of demethylated metabolites from theophylline found during the analysis of the patient's urine. It is worth mentioning that not only theophylline metabolism may be the subject of the interaction with ipriflavone. The inhibition of CYP1A2 may be expected to result in a reduction in the metabolism of caffeine, theobromine and some polycyclic aromatic xenobiotics, since CYP1A enzymes participate primarily in their biotransformation (Butler *et al*., 1989b).

CYP2C9 is not known to catalyze any step of theophylline metabolism; it cannot be responsible for the interaction between ipriflavone and theophylline. However, there are several drugs (tolbutamide, phenytoin, or S-warfarin) (Brian *et al*., 1989; Veronese *et al*., 1991; Rettie *et al*., 1992) that require CYP2C9 for their metabolism and thus concomitant treatment with ipriflavone may also result in drug-interaction. However, from the results of *in vitro* studies it is not easy to predict the clinical consequences of the supposed drug-interaction, since at the therapeutic dose, ipriflavone and 7-hydroxy-isoflavone had lower concentrations in human plasma than the inhibition constants found *in vitro*.

Nifedipine oxidation was affected by only 7-hydroxy-isoflavone with about a 10 times higher K_i than phenacetin O-deethylation or tolbutamide hydroxylation. However, it should be noted that the concentration of 7-hydroxy-isoflavone at the enzyme *in vivo* is unknown and possibly is not reflected by the given dose of parent compound or by plasma concentrations of the metabolite. These limitations in interpretation make the authors cautious in predicting any possible interaction of 7-hydroxy-isoflavone and drugs metabolized by CYP3A. It can be thought that the therapeutic use of ipriflavone may not bring about so high a 7-hydroxy-isoflavone concentration at the enzyme ($K_i = 129.5$ μM) that it will have consequences for the disposition of other drugs metabolized by CYP3A.

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