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Role of rat liver cytochrome P450 3A and 2D in metabolism of imrecoxib[1]

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Key words

imrecoxib; cytochrome P450; metabolism; liver microsomes

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Abstract

Aim: To investigate the *in vitro* metabolism of imrecoxib in rat liver microsomes and to identify the cytochrome P450 (CYP) forms involved in its metabolism. **Methods:** Liver microsomes of Wistar rats were prepared using an ultracentrifuge. The *in vitro* metabolism of imrecoxib was studied by incubation with rat liver microsomes. To characterize the CYP forms involved in the 4'-methyl hydroxylation of imrecoxib, the effects of typical CYP inducers (such as dexamethasone, isoniazid and β-naphthoflavone) and of CYP inhibitors (such as ketoconazole, quinine, α-naphthoflavone, methylpyrazole, and cimetidine) on the formation rate of 4'-hydroxymethyl imrecoxib were investigated. **Results:** Imrecoxib was metabolized to 3 metabolites by rat liver microsomes: 4'-hydroxymethyl imrecoxib (M4), 4'-hydroxymethyl-5-hydoxyl imrecoxib (M3), and 4'-hydroxymethyl-5-carbonyl imrecoxib (M5). Over the imrecoxib concentration range studied (5–600 μmol/L), the rate of 4'-methyl hydroxylation conformed to monophasic Michaelis-Menten kinetics. Dexamethasone significantly induced the formation of M4. Ketoconazole markedly lowered the metabolic rate of imrecoxib in a concentration-dependent manner. Moreover, a significant inhibitory effect of quinine on the formation of M4 was observed in microsomes obtained from control rats, isoniazid-induced rats, and β-naphthoflavone-induced rats. In contrast, α-naphthoflavone, cimetidine, and methylpyrazole had no inhibitory effects on this metabolic pathway. **Conclusion:** Imrecoxib is metabolized via 4'-methyl hydroxylation in rat liver microsomes. The reaction is mainly catalyzed by CYP 3A. CYP 2D also played a role in control rats, in isoniazid-induced rats and in β-naphthoflavone-induced rats.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the clinical treatment of pain, inflammation and fever[1,2]. These drugs (eg indomethacin, diclofenac and meloxicam) are thought to act by inhibiting cyclooxygenase (COX), an enzyme that limits the biosynthesis rate of prostaglandins from arachidonic acid[3–5]. Two forms of cyclooxygenase, designated COX-I and COX-II, exist[6,7]. COX-I is the major form, and is located in healthy tissues, catalyzes the formation of prostaglandins under normal physiological conditions, and plays a role in the maintenance of the gastrointestinal mucosa as well as platelet function[8]. In comparison, COX-II is an inducible enzyme that is predominantly expressed in association with inflammation[9]. It is believed that NSAID-induced gastrointestinal damage results from the inhibition of COX-I, whereas the therapeutic benefit results from the inhibition of COX-II expressed at the site of inflammation[10]. However, the division between the biological functions of COX-I and COX-II is not clear-cut. Moreover, treatment with COX-II selective inhibitors could theoretically lead to problems with thrombosis, and salt and water balance. Recently, much attention has been focused on the increased risk of cardiovascular events associated

with COX-II selective NSAIDs (such as celecoxib and rofecoxib) as compared with nonselective NSAIDs[11–13]. Therefore, NSAIDs that preferentially inhibit COX-II with moderate selectivity seem more promising. Imrecoxib, [4-(4-methane-sulfonyl-phenyl)-1-propyl-3-*p*-tolyl-1,5-dihydro-pyrrol-2 -one] (Figure 1), is a novel and moderately selective COX-II inhibitor[14–16]. The drug inhibits COX-I and COX-II with IC_{50} values of 115±28 nmol/L and 18±4 nmol/L, respectively. Imrecoxib exerts its anti-inflammatory effect by inhibiting COX-II mRNA expression[16]. In fact, imrecoxib is currently undergoing clinical trials in China for the treatment of acute and chronic inflammatory disease. We previously found that imrecoxib is extensively metabolized in rats, with less than 2% of the dose excreted unchanged in urine and feces (unpublished data). We characterized 7 metabolites of imrecoxib in rats: the 4'-hydroxymethyl (M4), 4'-carboxylic acid (M2), 4'-hydroxymethyl-5-hydroxy (M3), and 4'-hydroxymethyl-5-carbonyl (M5) metabolites, and glucuronide conjugates of M2, M3, and M4. The major route of metabolism appears to be 4'-methyl hydroxylation, with further oxidation of the corresponding carboxylic acid (Figure 1). The purpose of the present study was to study the in vitro metabolism of imrecoxib in rat liver microsomes and to identify the cytochrome P450 (CYP) forms involved in its metabolism.

Materials and methods

Chemicals Imrecoxib, the 4'-hydroxymethyl (M4) and 4'-carboxylic acid (M2) metabolites of imrecoxib, and BAP 910 (an analogue of imrecoxib, used as internal standard) were supplied by the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). 4'-Hydroxymethyl-5-hydroxyl (M3) and 4'-

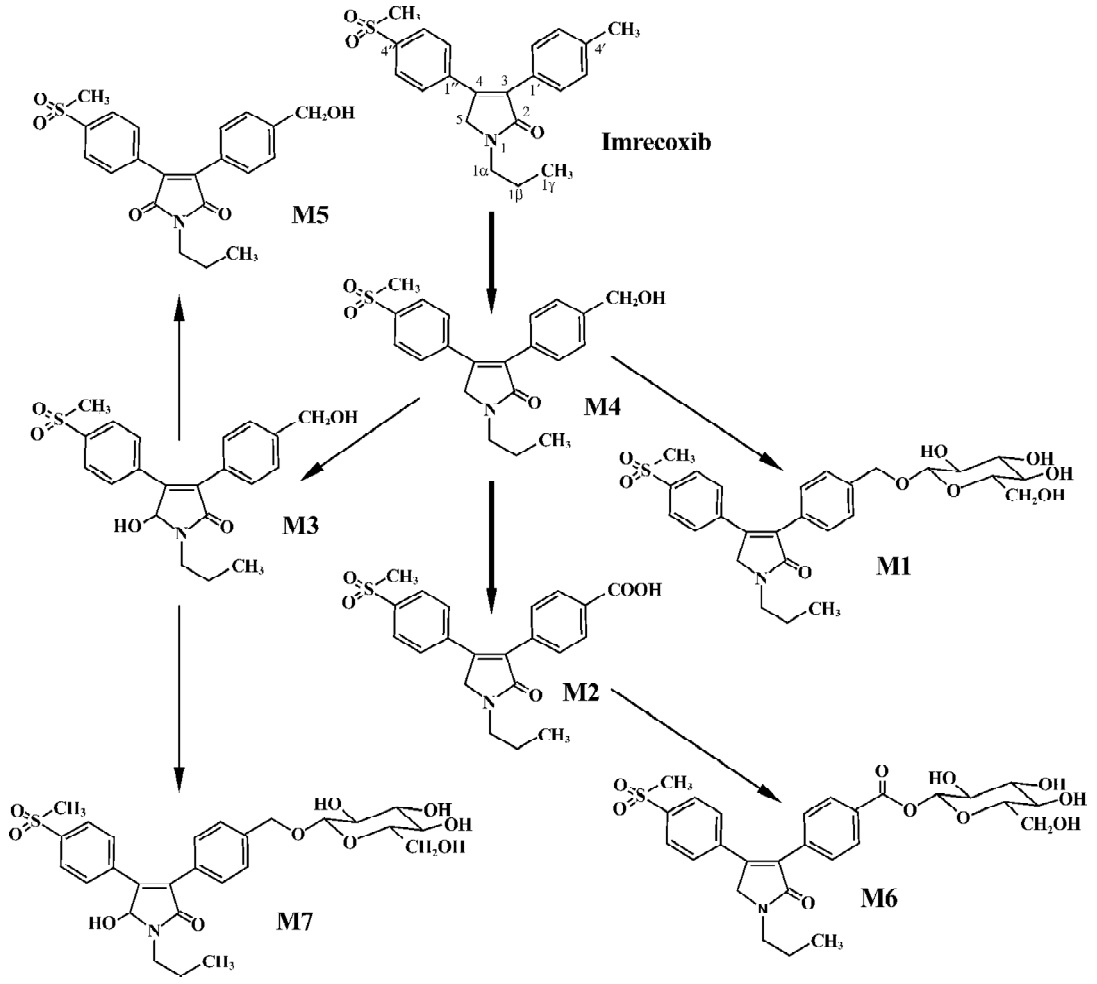

Figure 1. Proposed *in vivo* metabolic pathways of imrecoxib in rats.

hydroxy-methyl-5-carbonyl (M5) metabolites of imrecoxib, used as reference substances, were isolated from rat urine in our laboratory and identified by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. α-Naphthoflavone, β-naphthoflavone, methylpyrazole, quinine, codeine, and morphine were all purchased from Sigma-Aldrich (Deisenhofen, Germany). Cimetidine was obtained from Kaili Pharmaceutical Co (Jiangsu, China), ketoconazole from Dragon Pharmachemical Co (Zhejiang, China), dexamethasone from Tianjin Pharmaceutical Group Co (Tianjin, China), isoniazid from Jiangbei Pharmaceutical Co (Zhejiang, China), nifedipine from Zhongnuo Pharmaceutical Co (Shijiazhuang, China), diphenhydramine from Beijing Taiyang Pharmaceutical Co (Beijing, China), and gliclazide from Tianjin Zhongxin Pharmaceutical Co (Tianjin, China). Dehydronifedipine was synthesized in the Department of Pharmaceutical Chemistry, Shenyang Pharmaceutical University (Shenyang, China). β-Nicotinamine adenine dinucleotide phosphate (reduced form, NADPH) was obtained from Xinjingke Biotechnology Co (Beijing, China). *DL*-Dithiothreitol (DTT, ultrapure grade) and tris (hydroxymethyl) aminomethane (Tris, ultrapure grade) were obtained from Ameresco (Solon, Ohio, USA). Methanol and acetonitrile were of high performance liquid chromatography grade (Yuwang Co, Shandong, China). All other chemicals were of analytical grade.

Animal preparation Male Wistar rats (7*8 weeks old) weighing 200 to 250 g were supplied by the Laboratory Animal Center of Shenyang Pharmaceutical University (grade II, certificate No 042). Animals were fed a standard diet *ad libitum* and kept in a 12 h light/dark cycle. Groups of rats (n=6 per group) were treated daily (intraperitoneally) for 5 d with β-naphthoflavone (50 mg/kg, in corn oil, CYP 1A), isoniazid (100 mg/kg, in saline, CYP 2E), or dexamethasone (50 mg/kg, in corn oil, CYP 3A). Control animals (n=6 per group, 2 groups) were treated with an equivalent volume of corn oil or saline. The rats were fasted for 18 h before being killed, and were killed 24 h after the last injection. All experimental procedures were performed in accordance with the guidelines of the Experimental Animal Care and Use Committee of Shenyang Pharmaceutical University (Shenyang, China).

Preparation of rat liver microsomes Pooled liver microsomes from 6 rat livers in each group were prepared as previously described[17]. The microsomal protein concentrations were determined by using the method of Lowry *et al*[18]. Total cytochrome P450 contents were measured according to the method of Omura and Sato[19].

Incubation of imrecoxib with rat liver microsomes To determine the formation rate of the 4'-hydroxymethyl metabolite (M4), the basic incubation medium contained 0.1 mol/L Tris-HCl buffer (pH 7.4), 1.0 mmol/L NADPH, 10 mmol/L KCl, 10 mmol/L $MgCl_2$, 1.0 g/L microsomal protein and 5–600 μmol/L imrecoxib in a final volume of 200 μL. The mixture was incubated at 37 °C for various times (0, 5, 15, 30, or 60 min). The reactions were initiated by the addition of NADPH after 5 min preincubation and were terminated by the addition of 50 μL cold methanol. Then 20 μL internal standard (BAP 910, 20 μmol/L in methanol) and 200 μL of 20 μmol/L $NH_4H_2PO_4$ buffer (pH 3) were added to the reaction mixtures. The samples were extracted with 2 μL ethyl acetate and the supernatant was evaporated under a stream of nitrogen at 40 °C. The residue was dissolved in 100 μL of the mobile phase for LC/MS^n (liquid chromatography-ion trap mass spectrometry) assay. Controls were prepared in the same manner, except for the presence of NADPH. Blank samples were assayed without substrate to exclude analytical interference by the matrix.

Metabolite identification To identify the *in vitro* phase I metabolites of imrecoxib formed from rat liver microsomes, 200 μmol/L of imrecoxib (saturated concentration) was used as substrate. The final volume was 1 mL and the mixtures were incubated at 37 °C for 60 min. The reactions were initiated by addition of NADPH and were terminated with 1 mL of 20 μmol/L cold $NH_4H_2PO_4$ buffer (pH 3). Then the mixtures were applied to preconditioned 2.5 mL C_{18} cartridges (Tianjin Fuji Co, China). The columns were washed with 2 mL water and the metabolites were eluted with 1 mL methanol. The eluting solvents were evaporated and the residue was dissolved in 100 μL of the mobile phase for LC/MS^n analysis.

Enzyme kinetics Linear conditions for the formation of M4 were established with respect to protein content and incubation time. The rate of formation was linear over 60 min incubation and 0.5 to 1.5 g/L of microsomal protein. The Michaelis-Menten kinetics of imrecoxib 4'-methyl hydroxylation by rat liver microsomes was determined by using 14 substrate concentrations in the range of 5 to 600 μmol/L at 37 °C for 15 min. The rate of imrecoxib metabolism was analyzed by using the MULTI program, using a nonlinear least-squares method[20]. Data were also analyzed by linear transformation (Eadie-Hofstee plot) to confirm a single K_m model. The following Michaelis-Menten equation was used to analyze the relation between velocity and substrate concentration:

$$V=V_m \cdot S/(K_m+S),$$

where V, S, K_m, and V_m are the velocity of metabolite formation, the substrate concentration, the apparent Michaelis-Menten constant, and the maximum velocity of metabolism, respectively.

Inhibition study of imrecoxib metabolism Inhibitory effects on the 4'-methyl hydroxylation of imrecoxib in rat liver microsomes prepared from induced rats and control rats were determined at substrate concentrations of 50 μmol/L imrecoxib (~K_m) and at 3 inhibitor concentrations in the range of 2–50 μmol/L. Incubations were performed at 37 °C for 15 min. The selective P450 inhibitors α-naphthoflavone (CYP 1A), quinine (CYP 2D), methylpyrazole (CYP 2E), cimetidine (CYP 2C), and ketoconazole (CYP 3A) were used. Because all the inhibitors were dissolved in methanol, an equivalent volume of methanol (without inhibitors) was included in the control incubations to correct for any effects of the solvent on microsomal activity. The concentration of methanol was 0.5%.

LC/MS^n analysis A Finnigan LCQ liquid chromatography-ion trap mass spectrometer (San Jose, USA) was used to identify the *in vitro* metabolites of imrecoxib formed by rat liver microsomes. The instrument was operated in positive electrospray ionization mode. The source voltage was held at 4.5 kV. The capillary voltage was fixed at 13 V, and its temperature was set at 200 °C. Nitrogen was used as the sheath gas (0.75 L/min) and auxiliary gas (0.15 L/min). The MS^2 spectra were produced by collision-induced dissociation (CID) of the selected precursor ions with helium in the mass analyzer, and the relative collision energies were individually optimized for each compound. Liquid chromatography was performed with a Shimadzu LC-10 AD solvent delivery system (Kyoto, Japan). The samples were separated on a Diamonsil C_{18} column (200 mm×4.6 mm ID; 5 μm, Dikma Technologies, Beijing, China). A mobile phase consisting of methanol-ammonium acetate 10 mmol/L (60:40, v/v) was used at a flow rate of 0.5 mL/min. The injection volume was 20 μL. All data were analyzed by using Xcalibur software (version 1.2, Thermo Finnigan MAT).

The formation rate of M4 was determined by using the same LC/MS^n system, except that a mobile phase composed of acetonitrile-water-formic acid (75:25:0.5, v/v/v) was used and quantification was performed using selected reaction monitoring (SRM) of the transitions *m/z* 386 → *m/z* 356, 278 for M4 and *m/z* 374 → *m/z* 278 for BAP 910 (IS). Calibration standards were prepared by spiking 20 μL of appropriate standard solution of M4 into 200 μL of blank medium. The linear regressions of the peak area ratios versus concentrations were fitted over the concentration range of 0.10–50.0 μmol/L. The method was validated by determining quality control (QC) samples at 3 concentration levels on 3 consecutive days. The precisions were expressed as relative standard deviation (RSD) and the accuracy as relative error (RE%). The intra- and inter-day precision values were less than 8%. The accuracy for M4 was 7.5%, -5.3% and -4.8% at 0.1 μmol/L, 4.0 μmol/L and 50.0 μmol/L levels, respectively.

Codeine *O*-demethylase activity assay Codeine *O*-demethylation was carried out as a probe assay for CYP 2D[21,22]. The substrate concentration of codeine was 20 μmol/L. Incubations were performed at 37 °C for 20 min. After the reactions were finished, 40 μL internal standard (diphenhydramine, 20 μmol/L in methanol) and 200 μL of 0.1 mol/L Na_2CO_3 were added to the mixtures. The samples were extracted with 3 mL diethyl ether and the supernatant was evaporated under a stream of nitrogen at 40 °C. The residue was dissolved in 100 μL of the mobile phase. An aliquot of 20 μL of the solution was analyzed by using the same LC/MS^n system as described for LC/MS^n analysis except that the source voltage was held at 4.25 kV and its temperature was set at 180 °C; the SRM of the transitions of *m/z* 286 → *m/z* 201, 229 for morphine and *m/z* 256 → *m/z* 167 for diphenhydramine (IS) were used for quantification; and the mobile phase consisted of methanol-water-formic acid (60:40:0.5, v/v/v).

Nifedipine dehydrogenase activity assay Nifedipine dehydrogenation was used to probe the activity of CYP 3A[23]. Nifedipine (80 μmol/L) was incubated with microsomal protein at 37 °C for 10 min in a final volume of 200 μL. After the reactions were finished, 20 μL internal standard (gliclazide, 400 μmol/L in methanol) and 100 μL of 0.1 mol/L NaOH were added to the mixtures, respectively. The samples were extracted with 2 mL *n*-hexane-dichloromethane-iso-propyl alcohol (20:10:1, v/v/v). The dehydronifedipine formed was analyzed using the same LC/MS^n system as described earlier except that the SRM of the transitions of *m/z* 345 → *m/z* 284 for dehydronifedipine and *m/z* 324 → *m/z* 127, 168 for gliclazide (IS) were used for quantification; and the mobile phase consisted of acetonitrile-water-formic acid (85:15:0.5, v/v/v).

Data analysis All data are the means of 3 individual incubations. The significance of differences between means were evaluated by using ANOVA followed by the one-tailed Student's *t*-test.

Results

Metabolism of imrecoxib in rat liver microsomes Compared with the controls, 3 metabolites were found in rat liver microsomal incubates, in addition to the substrate imrecoxib (Figure 2). The structures of the metabolites were identified by investigation of their chromatographic behavior, and electrospray ionization MS and MS^2 spectra relative to reference substances. The retention times and main characteristic ions in mass spectra of imrecoxib and its metabolites are

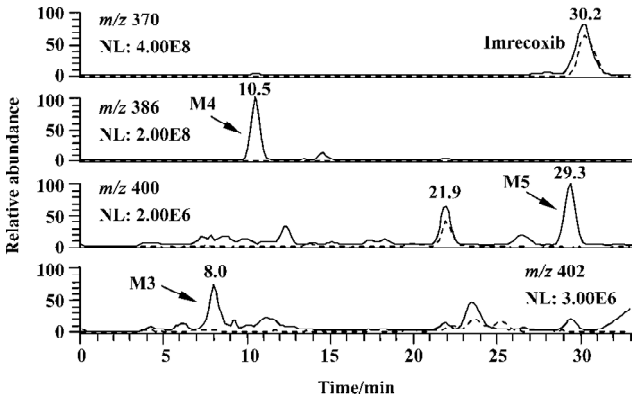

Figure 2. Representative chromatogram of metabolites of imrecoxib in dexamethasone-induced rat liver microsomes after incubation with 200 μmol/L imrecoxib for 60 min. Solid lines, with NADPH; dotted lines, without NADPH.

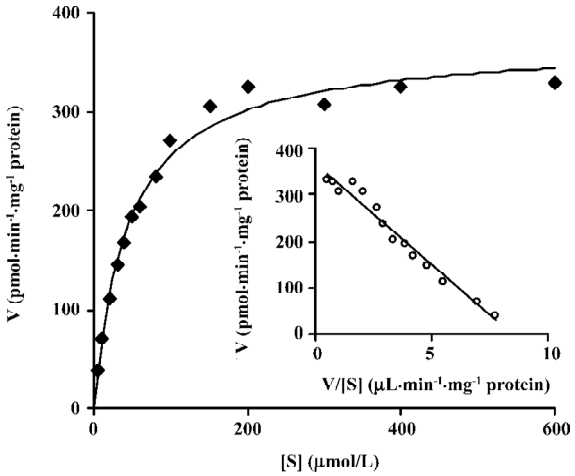

Figure 3. Representative plot of velocity versus imrecoxib concentration for the formation of 4'-hydroxymethyl imrecoxib in rat liver microsomes. Inset: Eadie-Hofstee plot.

summarized in Table 1.

The compound eluting at 30.2 min had the same pseudomolecular ion ($[M+H]^+$), full scan MS^2 spectrum, and chromatographic behavior as imrecoxib, therefore, it was identified as unchanged imrecoxib. By using the same method, the metabolites eluting at 8.0 min, 10.5 min, and 29.3 min were identified as the 4'-hydroxymethyl-5-hydroxyl metabolite (M3), the 4'-hydroxymethyl metabolite (M4), and the 4'-hydroxymethyl-5-carbonyl (M5) metabolite, respectively. No metabolites were detected in the absence of NADPH, indicating that metabolite formation is enzymatic and NADPH-dependent.

Enzyme kinetics Overall, the formation of M4 conformed to saturable kinetics and a representative Michaelis-Menten plot is shown in Figure 3. The Eadie-Hofstee plot (Figure 3, inset) for the formation of M4 from imrecoxib was indicative of monophasic behavior. Accordingly, a simple Michaelis-Menten kinetic analysis was used to estimate K_m and V_m (Table 2). The V_m of M4 in dexamethasone-induced microsomes increased significantly, to 7.5-fold higher than that in control microsomes. The same parameter in isoniazid-induced microsomes was 2-fold higher than that in control microsomes from rats treated with saline. The results suggest that CYP 3A and CYP 2E enzymes play important roles in the 4'-methyl hydroxylation of imrecoxib in rat liver microsomes.

Table 2. Michaelis-Menten parameters for the 4'-methyl hydroxylation of imrecoxib by rat liver microsomes.

Rat liver microsomes	K_m (μmol/L)	V_m (pmol·mim^{-1}·mg^{-1} protein)
Saline-treated	44.5±2.8	368.8±8.5
Corn oil-treated	42.5±4.1	380.7±10.0
β-Naphthoflavone-induced	37.4±4.4	358.8±14.8
Dexamethasone-induced	55.7±5.1	2871.1±103.9
Isoniazid-induced	46.2±6.0	743.6±35.6

Effects of inducers on the formation of M4 Imrecoxib (50 μmol/L or 200 μmol/L) was incubated at 37 °C for 15 min with induced and control rat liver microsomes (1.0 g/L) in the presence of NADPH. Among typical P450 inducers adminis-

Table 1. LC/MS^n data of imrecoxib and its metabolites in rat liver microsomes.

Metabolite code	Retention time (min)	$[M+H]^+$ (*m/z*)	MS^2 fragment ion (relative abundance, %) (*m/z*)
Imrecoxib	30.2	370	278, 236
M3	8.0	402	384 (100, -H_2O), 372 (17, -HCHO), 343 (39, -C_3H_9N), 271 (24)
M4	10.5	386	368 (100, -H_2O), 356 (40, -HCHO), 278 (10), 236 (3)
M5	29.3	400	382 (100, -H_2O), 370 (60, -HCHO)

tered intraperitoneally, dexamethasone caused the most induction of M4 formation, followed by isoniazid (Table 3). The formation rates of M4 in dexamethasone-induced microsomes were 3.4-fold and 6.1-fold higher than those in control microsomes from rats treated with corn oil, respectively, when substrate concentrations of 50 μmol/L and 200 μmol/L were used, respectively. The corresponding values in isoniazid-induced microsomes were 1.2-fold and 1.4-fold higher than those in control microsomes from rats treated with saline, respectively. However, β-naphthoflavone had no significant effect on the metabolism of imrecoxib. The results were in accordance with those regarding the kinetics of imrecoxib 4'-methyl hydroxylation (Table 2).

Effects of inhibitors on the formation of M4 The effects of inhibitors on the formation of M4 in rat liver microsomes are shown in Figures 4–6. The inhibitory activities are expressed as a ratio in comparison with the control activity without inhibitors. Ketoconazole, a selective inhibitor of CYP 3A, was shown to effectively decrease the formation rate of M4. When 2 μmol/L of ketoconazole was used, the hydroxylation activity was reduced to approximately 70% of the control activity in microsomes from control rats treated with saline or corn oil (Figure 4), whereas much lower enzyme activity (28% of the control activity) was observed in microsomes from dexamethasone-induced rats at the same ketoconazole concentration (Figure 5). In addition, a significant inhibition was observed in the presence of quinine (CYP 2D-selective) in control microsomes, but the other chemical inhibitors, α-naphthoflavone, cimetidine, and methylpyrazole, did not produce any significant effects on the 4'-methyl hydroxylation of imrecoxib (Figure 4). The effects of inhibitors on the formation of M4 by liver microsomes from isoniazid-induced rats and from β-naphthoflavone-induced rats were similar to those observed in control microsomes (Figure 6).

Table 3. Effects of pretreatment of rats with CYP inducers on the formation of the 4'-methyl hydroxyl metabolite of imrecoxib by liver microsomes. n=3. Mean±SD. $^{b}P<0.05$ *vs* saline-pretreated control microsomes. $^{f}P<0.01$ *vs* corn oil-pretreated control microsomes group).

Microsomes	Specific activity (pmol·mg^{-1}·min^{-1})	
	Imrecoxib (50 μmol/L)	Imrecoxib (200 μmol/L)
Saline-pretreated	336.4±28.6	422.8±50.8
Corn oil-pretreated	338.9±16.2	434.6±27.0
β-Naphthoflavone-induced	324.1±22.9^{c}	476.7±62.3^{c}
Dexamethasone-induced	1141.9±33.1^{f}	2664.4±329.4^{f}
Isoniazid-induced	414.8±26.1^{b}	602.1±89.2

Discussion

Three phase I metabolites of imrecoxib were observed in rat liver microsomal incubates using the LC/MS^{n} method with a saturated substrate concentration (200 μmol/L imrecoxib), a longer incubation time (60 min), and a larger final volume (1 mL). The identities of the metabolites were confirmed by chromatographic and mass spectra comparison with reference substances. These metabolites were identified as the

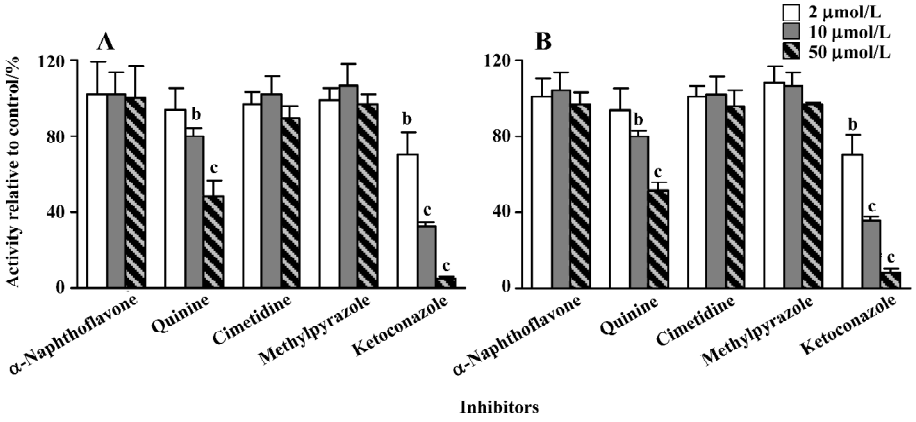

Figure 4. Effects of CYP inhibitors on 4'-methyl hydroxylation of imrecoxib in control liver microsomes. (A) From rats treated with saline; (B) from rats treated with corn oil. Imrecoxib (50 μmol/L) was incubated with rat liver microsomes at 37 °C for 15 min in the presence of NADPH. n=3. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ compared with the control samples without inhibitors. Control activities (without inhibitors) for 4'-methyl hydroxylation of imrecoxib in liver microsomes from saline-treated rats and from corn oil-treated rats were 172.4±18.9 pmol·mg^{-1}·min^{-1} and 160.8±12.4 pmol·mg^{-1}·min^{-1} respectively.

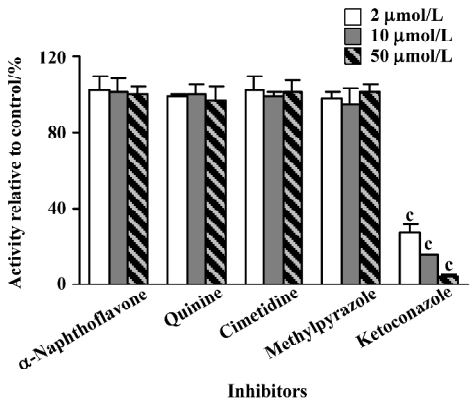

Figure 5. Effects of CYP inhibitors on 4'-methyl hydroxylation of imrecoxib in liver microsomes from dexamethasone-pretreated rats. Imrecoxib (50 μmol/L) was incubated with dexamethasone-induced microsomes at 37 °C for 15 min in the presence of NADPH. n=3. Mean±SD. ^cP<0.001 compared with the control samples without inhibitors. Control activity (without inhibitors) for 4'-methyl hydroxylation of imrecoxib in liver microsomes from dexamethasone-induced rats was 985.8±9.9 pmol·mg^{-1}·min^{-1}.

4'-hydroxymethyl-5-hydroxyl metabolite (M3), the 4'-hydroxymethyl metabolite (M4), and the 4'-hydroxymethyl-5-carbonyl (M5) metabolite. The formation of these metabolites was enzymatic and NADPH-dependent. However, when 50 μmol/L imrecoxib was incubated at 37 °C for 60 min in a final volume of 200 μL, imrecoxib metabolites other than M4 were not detected. Under these conditions, approximately 40% of imrecoxib was converted to M4 and the remainder was unchanged imrecoxib. It seems that 4'-methyl hydroxylation is the major metabolic pathway in NADPH-fortified rat liver microsomes. Although further oxidation of the 4'-hydroxymethyl metabolite to form the 4'-carboxylic acid metabolite (M2) was the predominant pathway in vivo, no carboxylic acid metabolite was found in the present study. This suggests that the generation of M2 *in vitro* may require the presence of cytosolic enzymes as in the metabolism of celecoxib[24]. This hypothesis is under investigation.

To identify the CYP isozymes involved in the 4'-methyl hydroxylation of imrecoxib, the effects of specific inducers and inhibitors of CYP on this reaction were examined. M4 was produced to the greatest extent by microsomes from dexamethasone-induced rats (Table 2 and 3). Dexamethasone is considered to be a specific inducer of CYP 3A[25,26]. However, ketoconazole, a well-known inhibitor of CYP 3A[22,26], strongly inhibited the reaction in a concentration-dependent manner (Figure 4–6). These results indicate that CYP 3A is the principal enzyme involved in the 4'-methyl hydroxylation of imrecoxib in rat liver microsomes.

In addition, 10 mmol/L of quinine (an inhibitor specific for CYP 2D[22,27]) significantly inhibited the rate of formation of M4 by approximately 20% compared with control activities in all types of microsomes except the microsomes obtained from dexamethasone-pretreated rats (Figure 4–6). When the concentration of quinine was increased to 50 μmol/L, the formation rate was decreased to a much lower level. With respect to the results mentioned above, participation of CYP 2D in the 4'-methyl hydroxylation of imrecoxib is also thought to be involved in control rats, and in β-naphthoflavone-induced and in isoniazid-induced rats.

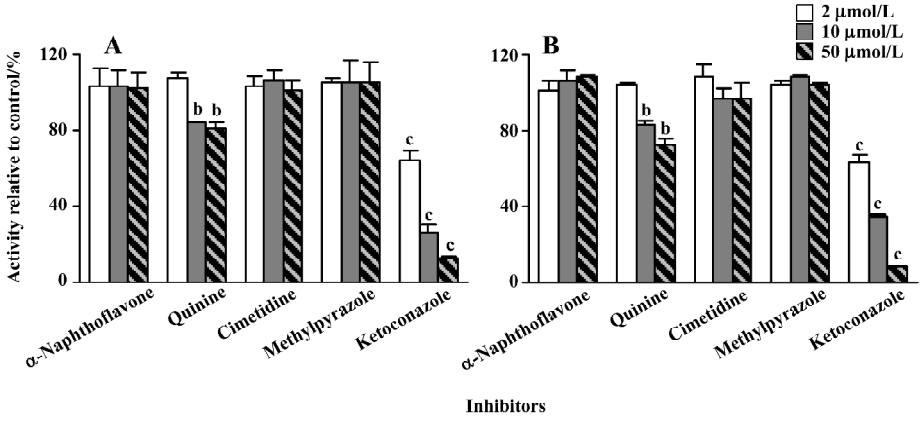

Figure 6. Effects of CYP inhibitors on 4'-methyl hydroxylation of imrecoxib. (A) In liver microsomes from β-naphthoflavone-pretreated rats; (B) in liver microsomes from isoniazid-pretreated rats. Concentrations of inhibitors were 2 μmol/L, 10 μmol/L, and 50 μmol/L. Imrecoxib (50 μmol/L) was incubated with rat liver microsomes at 37 °C for 15 min in the presence of NADPH. Data are mean±SD. n=3. ^bP <0.05, ^cP<0.01 *vs* control samples without inhibitors. Control activities (without inhibitors) for the 4'-methyl hydroxylation of imrecoxib in liver microsomes from β-naphthoflavone-induced rats and from isoniazid-induced rats were 174.4±16.5 pmol·mg·min^{-1} and 326.4±10.9 pmol·mg·min^{-1} per protein, respectively.

α-Naphthoflavone, which is often used as inhibitor of CYP 1A[22,26,28], did not decrease the rate of hydroxylation of imrecoxib. This result is in agreement with those of the induction experiments. β-naphthoflavone, an inducer of CYP 1A, did not significantly increase the formation of M4 (Tables 2 and 3). Cimetidine was used to confirm the participation of CYP 2C[27,28] in imrecoxib biotransformation; however, the formation of M4 was not inhibited by cimetidine in microsomes either from control rats or from induced rats. Thus, substantial participation of CYP 2C was unlikely in this reaction. Methylpyrazole, a selective inhibitor of CYP 2E[26], had no significant inhibitory effect on the 4'-methyl hydroxylation of imrecoxib. However, the reaction was elevated significantly in microsomes from isoniazid-pretreated rats compared with those from control rats treated with saline (Table 3). Isoniazid is an inducer of CYP 2E[25]. There appears to be a discrepancy between these two different phenomena. The results of inhibition studies clearly show that CYP 3A and 2D catalyze the 4'-methyl hydroxylation of imrecoxib, so the effects of isoniazid on CYP 3A and 2D were investigated in the present study. Codeine *O*-demethylation and nifedipine dehydrogenation were carried out as probe assays for CYP 2D and 3A, respectively, and their activities were determined as described in the materials and methods section. The activities of codeine *O*-demethylase in control microsomes from rats treated with saline and in isoniazid-induced microsomes were 45.8±5.9 pmol·min^{-1}·mg^{-1} protein and 59.4±7.5 pmol·min^{-1}·mg^{-1} protein, respectively. The activities of nifedipine dehydrogenase in control microsomes from rats treated with saline and in isoniazid-induced microsomes were 759.8±35.4 pmol·min^{-1}·mg^{-1} protein and 722.6±20.8 pmol·min^{-1}·mg^{-1} protein, respectively. There was no significant difference in the activities of CYP 3A between saline-treated control microsomes and isoniazid-induced microsomes, but the activity of CYP 2D in isoniazid-induced microsomes was significantly higher than that in saline-treated control microsomes. The results suggest that the faster formation rate of M4 observed in isoniazid-induced microsomes was caused by the higher activity of CYP 2D in isoniazid-induced microsomes. So the involvement of CYP 2E in the 4'-methyl hydroxylation of imrecoxib may be excluded.

Interestingly, despite CYP 3A and 2D being shown to catalyze the 4'-methyl hydroxylation of imrecoxib, the data obtained from rat liver microsomes were described by a single K_m model (Figure 3). This finding indicates that CYP 3A and 2D are likely to be characterized by similar apparent K_m values.

In conclusion, imrecoxib was metabolized to 3 metabolites by rat liver microsomes: 4'-hydroxymethyl imrecoxib (M4), 4'-hydroxymethyl-5-hydoxyl imrecoxib (M3), and 4'-hydroxymethyl-5-carbonyl imrecoxib (M5). This biotransformation study of imrecoxib in rat liver microsomes indicates that 4'-methyl hydroxylation represents the major metabolic pathway of imrecoxib. The reaction was mainly catalyzed by CYP 3A. CYP2D also played a role in control rats based on the results of inhibition studies. Other CYP (such as CYP 1A, 2C, and 2E) seem to not participate in the metabolic pathway.

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