

## Characterization of Benidipine and Its Enantiomers Metabolism by Human Liver Cytochrome P450 Enzymes

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**ABBREVIATIONS:**

P450: cytochrome P450, thio-TEPA, triethylenethiophosphoramidate; HLM: human liver microsome;  $CL_{int}$ , intrinsic clearance; LC/MS/MS, liquid chromatography/tandem mass spectrometry.

## Abstract

Benidipine is a dihydropyridine calcium antagonist, which has been used clinically as an antihypertensive and anti-anginal agent. It is used clinically as a racemate, containing the (-)-alpha and (+)-alpha isomers of benidipine. This study was performed to elucidate the metabolism of benidipine and its enantiomers in human liver microsomes and to characterize the cytochrome P450 (P450) enzymes that are involved in the metabolism of benidipine. Human liver microsomal incubation of benidipine in the presence of NADPH resulted in the formation of two metabolites, *N*-desbenzyl- and dehydro-benidipine. The intrinsic clearance ( $Cl_{int}$ ) of the formation of *N*-desbenzyl- and dehydro-benidipine metabolites from (-)- $\alpha$  isomer were similar to those from the (+)- $\alpha$  isomer ( $1.9 \pm 0.1$  versus  $2.3 \pm 2.3$   $\mu\text{l}/\text{min}/\text{pmol}$  P450 and  $0.5 \pm 0.2$  versus  $0.6 \pm 0.6$   $\mu\text{l}/\text{min}/\text{pmol}$  P450, respectively). Correlation analysis between the known P450 enzyme activities and the rate of the formation of benidipine metabolites in the 15 human liver microsomes showed that benidipine metabolism is correlated with CYP3A activity. The P450-isoform selective inhibition study in liver microsomes and the incubation study of cDNA-expressed enzymes also demonstrated that the *N*-debenzylation and dehydrogenation of benidipine are mainly mediated by CYP3A4 and CYP3A5. The total  $Cl_{int}$  values of CYP3A4-mediated metabolites formation from (-)- $\alpha$  isomer were similar to those from (+)- $\alpha$  isomer (17.7 versus 14.4  $\mu\text{l}/\text{min}/\text{pmol}$  P450, respectively). The total  $Cl_{int}$  values of CYP3A5-mediated metabolites formation from (-)- $\alpha$  isomer were also similar to those from (+)- $\alpha$  isomer (8.3 versus 11.0  $\mu\text{l}/\text{min}/\text{pmol}$  P450, respectively). These findings suggest that CYP3A4 and CYP3A5 isoforms are major enzymes contributing to the disposition of benidipine, but stereoselective disposition of

benidipine in vivo may be influenced not by stereoselective metabolism but by other factors.

## Introduction

Benidipine is a highly potent dihydropyridine calcium antagonist that is used clinically as a racemate. This agent shows slow onset and long-lasting antihypertensive and antianginal effects owing to the blockade of calcium ion entry to smooth muscle (Karasawa et al., 1988; Yamada et al., 1990). It is currently in clinical use for the treatment of hypertension as a single daily medication (2-8 mg). Like other dihydropyridine calcium antagonists (except nifedipine and mepirodipine) benidipine has asymmetric carbons both in the 1,4-dihydropyridine ring and in the side chain of the benzylpiperidine ring. This drug is a racemate consisting of two optical isomers ((+)-alpha- and (-)-alpha-isomer) out of the four possible optical isomers, since the other isomers are removed during the crystallization step on synthesis (Kobayashi and Kobayashi, 1998). The (+)-alpha isomer was 30- to 100-fold more active than the (-)-alpha isomer in terms of the antihypertensive effect after intravenous administration to the spontaneously hypertensive rat (Muto et al., 1988).

Pharmacokinetic studies in rat (Kobayashi and Kobayashi, 1998) and man (Kobayashi et al., 1997) have shown that benidipine is well absorbed but exhibits low absolute bioavailability due to marked first pass metabolism. Accordingly, various metabolites were identified in these species (Kobayashi et al., 1997; Kobayashi and Kobayashi, 1998). In our previous study involving human plasma, we found that the plasma concentrations of the (+)- $\alpha$ -benidipine were consistently higher than those of the (-)- $\alpha$ -

isomer after dosing of 8 mg racemic benidipine (Kang et al., 2005). The  $C_{\max}$  and  $AUC_{\text{inf}}$  values of (+)-alpha benidipine ( $1.47 \pm 0.75$  ng/ml and  $2.48 \pm 1.18$  ng h/ml, respectively) were higher than those of (-)-alpha benidipine ( $0.75 \pm 0.46$  ng/ml and  $1.34 \pm 0.48$  ng h/ml, respectively) by 1.96- and 1.85-fold, respectively ( $p < 0.001$ ). However, the mechanism of stereoselective disposition of benidipine in vivo has not yet been elucidated.

In the present study, we examined the metabolic pathways of benidipine using human liver microsomal fractions, and in particular identified the cytochrome P450 isoforms responsible for each metabolic pathway. Furthermore, to understand the mechanism of enantioselective pharmacokinetics of benidipine in vivo, we also investigated the stereoselectivity on benidipine metabolism in vitro.

## Methods

**Chemicals and Reagent.** Benidipine hydrochloride, its two enantiomers, dehydrobenidipine and *N*-desbenzylbenidipine, were kindly donated by Kyowa Hakko Kogyo Co. Ltd. (Shizuoka, Japan). Amlodipine, coumarin, diethyldithiocarbamate, furafylline, quinidine, ketoconazole, SKF-525A, sulfaphenazole, triethylenethiophosphoramidate (thio-TEPA),  $\beta$ -nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). The solvents were HPLC grade (Fisher Scientific CO., Pittsburgh, PA) and the other chemicals were of the highest quality available. Single-donor human liver microsomes (HLM) were obtained from tissue bank of PharmacoGenomics Research Center, Inje University (Busan, Korea).

Azamulin, *S*-benzylrivanol, pooled human liver microsomes, and 10 different human recombinant P450 isoforms were purchased from BD Gentest (Woburn, MA). All human P450 isoforms in Supersomes are coexpressed with human P450 reductase. The manufacturer supplied information regarding protein concentration and P450 content.

**Identification of benidipine metabolites in human liver microsomes.** The incubation mixtures, containing 0.25 mg of pooled human liver microsomes (H161, Gentest) and benidipine (100  $\mu$ M) were reconstituted in 100 mM phosphate buffer (pH 7.4) and prewarmed for 5 min at 37°C. The reaction was initiated by adding the NADPH-regenerating system (1.3 mM  $\beta$ -nicotinamide adenine dinucleotide phosphate, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl<sub>2</sub>, and 1.0 U/ml glucose-6-phosphate dehydrogenase) and further incubated (final volume of 250  $\mu$ l) for 30 min at 37°C in a shaking water bath. The control incubations were conducted with heat-denatured microsomal preparations (80°C for 10 min). The reaction was terminated by the addition of 100  $\mu$ l of acetonitrile on ice. The incubation mixtures were then centrifuged at 10,000g for 5 min at 4°C. Aliquots of the supernatant were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) for the identification of the metabolites.

**Metabolism of Benidipine and Its Enantiomers in Human Liver Microsomes or cDNA-Expressed P450 isoforms.** The optimal conditions for microsomal incubation were determined in the linear range for the formation of metabolites of benidipine. In all experiments, racemic benidipine and its enantiomers were dissolved, serially diluted with methanol to the required concentrations, and the final concentration of organic

solvent did not exceed 1% in final incubation mixtures. The incubation mixtures containing either 5  $\mu$ l of microsomes (5 mg protein/ml of stock, prepared from four different human liver microsomal preparations), or 25  $\mu$ l of cDNA-expressed P450 (diluted to 20 pmol/ml with 100mM phosphate buffer, pH 7.4) and various concentrations of benidipine or its enantiomers (0 to 200  $\mu$ M) were reconstituted in 100 mM phosphate buffer (pH 7.4) and prewarmed for 5 min at 37°C. The reaction was initiated by adding the NADPH-regenerating system and further incubated (final volume of 250  $\mu$ l) for 5 min at 37°C in a shaking water bath. The reaction was terminated by placing the incubation tubes on ice and by immediately adding 100  $\mu$ l of acetonitrile. After adding the internal standard (amlodipine, 10  $\mu$ M), the mixture was centrifuged at 10,000g for 5 min at 4°C, and aliquots of the supernatant were injected into an LC/MS/MS system.

**LC/MS/MS Analysis of Benidipine and Its Metabolites.** For the identification of benidipine and its metabolites, a tandem quadrupole mass spectrometer (QTrap 4000 LC/MS/MS, Applied Biosystems, Foster City, CA), coupled with an Agilent 1100 series HPLC system (Agilent, Wilmington, DE) was used. The separation was performed on a XTerra®MSC<sub>18</sub> column (2 mm i.d.  $\times$  30 mm, 2.5  $\mu$ m, Waters, USA) using the mobile phase that consisted of acetonitrile and water (40:60, v/v) at a flow rate of 0.2 mL/min. For identification of the metabolites, mass spectra were recorded by electrospray ionization with a positive mode. The turbo ion spray interface was operated at 5500 V and 500°C. The operating conditions were optimised by flow injection of an analyte and were determined as follows: nebulizing gas flow, 40 psi;

curtain gas flow, 10 psi; and collision energy, 50 eV. Quadruples Q1 and Q3 were set on unit resolution.

Multiple-reaction-monitoring (MRM) mode using specific precursor/product ion transition was employed for the quantification. Detection of the ions was performed by monitoring the transitions of  $m/z$  416  $\rightarrow$  315 for *N*-desbenzylbenidipine (collision energy 24 eV), 504  $\rightarrow$  91.2 for dehydrobenidipine (collision energy 95 eV), and 409  $\rightarrow$  238 for amlodipine (IS, collision energy 15 eV). Peak areas for all components were automatically integrated using Analyst software (version 1.4). The lower limits of quantification for two metabolites were 0.2 nM. The interassay precision for analyte was less than 15%.

**Chemical Inhibition Studies with Human Liver Microsomes.** The inhibitory effects of known P450 isoform-selective inhibitors on the formation of *N*-desbenzyl- and dehydro-benidipine were evaluated to determine the P450 isoform(s) involved in the metabolic pathway. The formation ratio of *N*-desbenzyl- and dehydro-benidipine from benidipine racemate or enantiomers were determined from the reaction mixtures incubated in the presence or absence of known P450 isoform-selective inhibitors (Kim et al., 2006; Lee et al., 2006). The P450 isoform-selective inhibitors used were furafylline (10  $\mu$ M) for CYP1A2, coumarin (100  $\mu$ M) for CYP2A6, thio-TEPA (5  $\mu$ M) for CYP2B6, montelukast (0.1  $\mu$ M) for CYP2C8, sulfaphenazole (10  $\mu$ M) for CYP2C9, *S*-benzylnirvanol (1  $\mu$ M) for CYP2C19, quinidine (10  $\mu$ M) for CYP2D6, diethyldithiocarbamate (10  $\mu$ M) for CYP2E1, ketoconazole (1  $\mu$ M) for CYP3A, and azamulin (5  $\mu$ M) for CYP3A. SKF-525A (100  $\mu$ M) was also used as non-selective P450s inhibitor. FMO was heat deactivated by incubating tubes (0.1 mg microsomal



protein and phosphate buffer) without NADPH generating system in a water-bath set at 45 °C for 5 min to elucidate whether FMO is responsible for benidipine metabolism or not. Except for the addition of P450 isoform-selective inhibitors, all other incubation conditions were similar to those described previously (Shin et al., 1999; Shin et al., 2002).

**Correlation Experiments.** Benidipine (5  $\mu$ M) were incubated with microsomes from 15 different livers to test the correlation of benidipine metabolism with the activity of individual P450s. The activities of each P450 isoform were determined using cocktail incubation and tandem mass spectrometry, as described previously (Kim et al., 2005). Isoform-specific reaction markers were used to determine the activity of each P450: phenacetin *O*-deethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), bupropion hydroxylation (CYP2B6), paclitaxel 6 $\alpha$ -hydroxylation (CYP2C8), tolbutamide 4-methylhydroxylation (CYP2C9), *S*-mephenytoin 4-hydroxylation (CYP2C19), dextromethorphan *O*-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), and midazolam 1'-hydroxylation (CYP3A). The correlation coefficients between the formation rates of benidipine metabolites and the activity of each P450 isoform in the different HLMs were calculated by parametric regression analysis (SAS version 8.01; SAS Institute Inc., Cary, NC). A *p*-value less than 0.05 was considered statistically significant.

**Data Analysis.** Results are expressed as means  $\pm$  S.D. of estimates obtained from four different liver microsomes in duplicate experiments. In the microsomal incubation

studies, the apparent kinetic parameters of benidipine biotransformation ( $K_m$  and  $V_{max}$ ) were determined by fitting an one-enzyme Michaelis-Menten equation ( $V = V_{max} \cdot S / (K_m + S)$ ) or a Hill equation ( $V = V_{max} \cdot S^n / (K_m + S^n)$ ). The calculated parameters were the maximum rate of formation ( $V_{max}$ ), the Michaelis constant (apparent  $K_m$ ), the intrinsic clearance ( $Cl_{int} = V_{max} / \text{apparent } K_m$ ), and Hill coefficient ( $n$ ). Calculations were performed using WinNonlin software (Pharsight, Mountain View, CA). The percentages of inhibition were calculated by the ratio of the amounts of metabolites formed with and without the specific inhibitor. In the incubation study using cDNA-expressed P450 isoforms, a Hill equation model was fitted to the unweighted data on the formation rate of both metabolites to estimate the enzyme kinetic parameters. The models that best fit were selected based on the dispersion of residuals and standard errors of the parameter estimates.

Contributions of each cytochrome to benidipine metabolism were normalized for mean values of the relative abundance of individual cytochromes in the liver (Rodrigues, 1999). Briefly, the reaction rates measured with individual cDNA-expressed P450 isoforms were normalized with respect to the nominal specific content of the corresponding P450 in native human liver microsomes. In this study, we adapted the data of immunologically determined P450 isoform liver contents reported by other researchers (Wrighton et al., 1990; Shimada et al., 1994); i.e., 4.0% for CYP2A6, 1.5% for CYP2D6, 23.0% for CYP3A4, and 5.8% for CYP3A5. In turn, the normalized rates for each cDNA-expressed P450 were summed, yielding a ‘total normalized rate’ ( $TNR = \sum f_i \cdot V_i$ ), and the normalized rate for each P450 isoform ( $= f_i \cdot V_i$ ) was expressed as a

percentage of the net reaction rate ( $= 100 \cdot f_i \cdot V_i / \sum f_i \cdot V_i$ , where  $f_i$  indicates the fraction of each P450 isoform content in the human liver).

## Results

**Identification of Benidipine Metabolites in Human Liver Microsomes.** Following the incubations of benidipine with human liver microsomes in the presence of a NADPH-generating system, the benidipine and its two metabolites (*N*-desbenzyl- and dehydro-benidipine) were profiled, characterized, and tentatively identified using LC/MS analysis (Fig. 1). Other metabolites that were observed in plasma, urine, and faeces after oral administration of benidipine to human were not found in human liver microsomal incubation sample. LC/MS/MS analysis of the unchanged benidipine and its two metabolites produced the informative and prominent product ions for structural elucidation. MS/MS spectrum of benidipine, having a protonated molecular ion  $[M+H]^+$  at  $m/z$  506, showed major fragment ions at  $m/z$  174 (the loss of benzylpiperidine group),  $m/z$  315 (carboxyl group cleavage), and  $m/z$  91 (the loss of benzyl group) (Fig. 2). Major metabolites *N*-desbenzyl- and dehydro-benidipine were identified by co-chromatography and the MS/MS spectral data of the authentic compounds (Fig. 2). The mass spectrum of *N*-desbenzylbenidipine has a protonated molecular ion peak  $[M+H]^+$  at  $m/z$  416, suggesting loss of benzyl molecule from benidipine ( $[M+H]^+$ ,  $m/z$  506). The MS/MS spectrum of *N*-desbenzylbenidipine also showed fragmentation pattern similar to parent compound (Fig. 2). Dehydrobenidipine gave an  $[M+H]^+$  molecular ion at  $m/z$  504, suggesting loss of hydrogen atoms from the parent compound. The MS/MS spectrum of dehydrobenidipine by fragmenting  $m/z$  504 through collision gave the characteristic daughter ions at  $m/z$  91, 174, 285, and 313, suggesting the possible oxidation of dihydropyridine ring (Fig. 2).

### **Identification of the P450 Isoforms Involved in the Metabolism of Benidipine.**

Human liver microsomal incubation of benidipine in the presence of NADPH resulted in the formation of dehydro- and *N*-desbenzyl-benidipine. Heat inactivation of the FMO activity did not inhibit benidipine metabolism, whereas, SKF-525A inhibit benidipine metabolism (Table 1), suggesting that cytochrome P450 is enzyme responsible for benidipine metabolism. The rates of formation of metabolite were proportional to incubation times up to 10 min and protein concentration up to 0.3 mg/ml at 5 min. A P450 isoform-selective inhibition study was performed to evaluate which P450 isoforms are involved in the metabolism of benidipine in human liver microsomes (Fig. 3). Among the ten inhibitors tested, ketoconazole, a well known CYP3A-selective inhibitor (Bourrie et al., 1996), inhibited *N*-desbenzyl- and dehydro-benidipine formation from benidipine. After treatment of 1  $\mu$ M ketoconazole, the level of *N*-desbenzyl- and dehydro-benidipine formation from benidipine enantiomers markedly decreased to 7-17% and 26-31% of that in the controls, respectively. It is difficult to explain why ketoconazole showed partial inhibition on dehydrobenidipine formation of benidipine with present data. However, CYP3A has multiple binding sites, therefore, sometimes shows partial inhibition (partial inhibition of erythromycin *N*-demethylation by testosterone in microsomes)(Wang et al., 1997). Therefore, further studies would be needed to clarify partial inhibition by ketoconazole. Benidipine metabolism was also inhibited by azamulin, another CYP3A inhibitor (Streel et al., 2002). Correlations between the rate of benidipine metabolism and the activities of P450s in HLMs are summarized in Table 2. The formation rates of dehydro- and *N*-desbenzyl-benidipine from benidipine were significantly correlated with the activity of CYP3A (Fig. 4).

Dehydro- and *N*-desbenzyl-benidipine formation from benidipine and its enantiomers (1 and 10  $\mu$ M) were also studied using the human cDNA-expressed P450 isoforms, P450s 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 (Fig. 5). CYP3A4 and CYP3A5 metabolized benidipine more efficiently than any other P450s, with little contribution of CYP2A6 and CYP2D6. To determine the relative contributions of four P450 isoforms to benidipine metabolism in human liver microsomes, we estimated the percentage of the net reaction rate from the abundance-adjusted simulation of each P450 isoform in human liver microsomes. This simulation also shows that CYP3A is the major P450 enzyme responsible for benidipine metabolism. The percentage of net reaction by CYP2A6 and CYP2D6 is lower than 3.6 and 2.3%, respectively, whereas that by CYP3A is higher than 90.0%. The levels of involvement of other recombinant P450 isoforms in the formation of benidipine metabolites were negligible.

**Enzyme Kinetic Analysis.** The formation of both *N*-desbenzyl and dehydro metabolites didn't show stereoselective metabolism on incubation of human liver microsomal preparations (Fig. 6). Under the experimental conditions used, the metabolism of benidipine by human liver microsomes was best fitted by a Hill equation (Table 3). The fitting of the data to the Michaelis-Menten expression of two-enzyme model did not significantly improve the regression, as compared with fitting of the data of the one-enzyme model. The sums of the formation  $Cl_{int}$  of both metabolites were 2.9 and 2.4  $\mu$ l/min/pmol P450 for (+)- $\alpha$ -benidipine and (-)- $\alpha$ -benidipine, respectively, indicating non-stereoselectivity in the metabolism of benidipine.

Next, we examined the enzyme kinetic parameters for the formation of *N*-desbenzyl- and dehydro-benidipine from benidipine enantiomers (0 – 200  $\mu$ M) on incubation with cDNA-expressed human CYP3A4 and CYP3A5 (Fig. 7). Under the experimental conditions used, the metabolism of benidipine by these P450 isoforms was also best described by a Hill equation (Table 4). CYP3A4 and CYP3A5 metabolized both benidipine enantiomers to *N*-desbenzyl- and dehydro-benidipine (Table 4). The CYP3A4-catalyzed *N*-debenzylation of benidipine enantiomers showed lower  $K_m$  and higher  $V_{max}$  than those of the dehydrogenation, resulting in a higher  $Cl_{int}$  of *N*-desbenzylbenidipine formation than dehydrobenidipine formation (12.3-16.1 versus 1.6-2.1  $\mu$ l/min/pmol CYP3A4, respectively). CYP3A5 also showed similar tendency as in the case of CYP3A4.

## Discussion

The metabolism studies of benidipine have been reported for various organisms such as rat and human (Kobayashi et al., 1988a; Kobayashi et al., 1988b; Kobayashi et al., 1997). As the result of previous research, a lot of metabolites were identified, and major metabolic pathways were *N*-debenzylation, dehydrogenation, ester hydrolysis, decarboxylation, and so on. In the present study, we determined that the major metabolites of human liver microsomal incubation were *N*-desbenzylbenidipine and an oxidized metabolite of the dihydropyridine ring (dehydrobenidipine) (Fig. 1). Benidipine was metabolized by human liver microsomes in the presence of an NADPH-generating system but was not metabolized in the absence of NADPH. This result suggests that benidipine metabolism in humans is cytochrome P450-dependent.

Owing to a common structural feature, *N*-debenzylation and oxidation of dihydropyridine ring pathways have been consistently reported as routes for other dihydropyridine calcium antagonists such as barnidipine (Teramura et al., 1997b) and efonidipine (Nakabeppu et al., 1995). Estimated intrinsic clearance, based on the  $V_{\max}/K_m$  for the two primary metabolic pathways, suggests *N*-debenzylation is more dominant metabolic pathway of benidipine than oxidation of the dihydropyridine ring. The  $Cl_{\text{int}}$  of both benidipine enantiomers indicated that *N*-desbenzyl metabolite formation contributes to 79% of benidipine metabolism and dehydro metabolite formation to 21% (Table 3).

It is therefore considered that benidipine is catalysed by the same mechanism as that proposed for other structural analogues (Guengerich and Bocker, 1988). Our present findings were further supported by the reports that P450s catalyse oxidation of the



dihydropyridine ring (Baarnhielm and Hansson, 1986), and that nifedipine and felodipine, typical 1,4-dihydropyridine type calcium antagonists, are oxidized by the CYP3A4 isoform (Bocker and Guengerich, 1986; Guengerich et al., 1991). The *N*-debenzylation pathway was specifically dependent on a particular substituent present in benidipine, and barnidipine, similar structural analogue, is also metabolized by the CYP3A enzyme (Teramura et al., 1997a).

We provide evidence that benidipine metabolism is catalysed by CYP3A subfamily. First, formation rates of *N*-desbenzyl- and dehydro-benidipine were inhibited (68-78%) by ketoconazole and azamulin, potent CYP3A-selective inhibitors (Fig. 3). Second, expressed human CYP3A4 and CYP3A5 metabolized benidipine to its two metabolites, whereas other P450 isoforms did not (Fig. 5). Third, midazolam 1'-hydroxylation activity (a marker of CYP3A activity) in 15 individual human liver microsomes exhibited high correlation with the formation rate of the metabolites ( $r = 0.66-0.80$ ) (Table 2). The significant correlation we observed between the activity CYP1A2 and benidipine metabolism in the panel of HLMs tested may not be due to the actual involvement of CYP1A2 in benidipine metabolism. Because our inhibition and recombinant experiments do not support a significant role of CYP1A2 in benidipine metabolism, the observed significant correlation between benidipine metabolism and CYP1A2 is probably derived from the significant correlation between the activity of CYP1A2 and CYP3A (Pearson  $r = 0.57$ ;  $p = 0.02$ ) in the bank of human livers tested. It is interesting that the two major metabolic pathways of benidipine are catalysed by the same CYP3A subfamily in spite of their different metabolic mechanisms, but may be explained by the fact that CYP3A has a broad substrate specificity (Spatzenegger and Jaeger, 1995; Patki et al., 2003). Taken together, these results suggest that CYP3A is the

major enzyme involved in the metabolism of the benidipine at concentrations in the usual experimental ranges.

In general, the stereoselective pharmacokinetics of dihydropyridine calcium antagonists such as felodipine, nimodipine, nitrendipine, and nilvadipine, in animals and man have been extensively reported (Niwa et al., 1988; Soons et al., 1993), and is reviewed (Tokuma and Noguchi, 1995; Inotsume and Nakano, 2002). These stereoselective pharmacokinetics can be explained by the rate-limiting step of a single cytochrome P450 enzyme and similar stereoselectivity for these structurally related drugs. (Niwa et al., 1989; Eriksson et al., 1991). At present, only limited pharmacokinetic data of the benidipine enantiomers is available. The preliminary data from a study in humans (Tokuma and Noguchi, 1995; Kang et al., 2005) suggest that the plasma concentrations of the (+)- $\alpha$ -enantiomer were higher than those of the (-)- $\alpha$ -enantiomer. Therefore, we evaluated the possibility of enantioselective metabolism of benidipine using human liver microsomes and recombinant P450 isoforms as examples of felodipine (Eriksson et al., 1991) and nivaldipine (Niwa et al., 1989). In this in vitro study, however, no significant differences were found for the estimated Michaelis-Menten parameters  $V_{max}$ ,  $K_m$ , or  $Cl_{int}$  (Table 3). Enzyme kinetic study using cDNA-expressed CYP3A4 and CYP3A5 didn't also show any stereoselective differences on benidipine metabolism (Table 4). Considering no enantioselective metabolism of benidipine in this in vitro study, the higher plasma levels of (+)- $\alpha$ -benidipine than (-)- $\alpha$ -benidipine could be due to enantioselectivity in plasma protein binding and/or to the enantioselectivity in the absorption via drug transporters such as p-glycoprotein. For example, the free fraction value of semotiadil (*R*-isomer), a calcium antagonist, is 0.7 times less than that of levosemotiadil (*S*-isomer) in human plasma (Rodriguez Rosas et al., 1997).

However, the protein binding of benidipine in human serum was almost constant at about 99% over a concentration range of 1-10,000 ng/mL (Kobayashi and Kobayashi, 1999). Therefore, the effect of enantioselective protein binding on enantioselective disposition of benidipine would not be important.

In conclusion, this study shows that the primary metabolic pathway of benidipine in human liver microsomes is *N*-debenzylation and oxidative dehydrogenation. The P450 isoform-specific chemical inhibition study, correlation study, and incubation study of cDNA-expressed P450 enzymes demonstrated that CYP3A4 and CYP3A5 were responsible for the formation of two metabolites from benidipine. In addition, benidipine didn't show stereoselectivity on metabolism. Therefore, detailed studies for elucidation of the mechanism of enantioselective disposition of benidipine in vivo are required.

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## Footnotes

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## Legends for Figures

**Fig. 1.** Metabolic pathway of benidipine by human liver microsomes.

**Fig. 2.** MS/MS spectra of benidipine and its two metabolites obtained by LC/MS/MS analysis of the human liver microsomal incubates of benidipine in the presence of NADPH-generating system.

**Fig. 3.** Stereoselective inhibition of known P450 isoform-selective inhibitors on *N*-debenzylation and dehydrogenation from racemic, (+)- $\alpha$ -, and (-)- $\alpha$ -benidipine in human liver microsomal incubations.

Pooled human liver microsomes (0.1 mg/ml, Gentest H161) were incubated with 5  $\mu$ M benidipine enantiomers or racemate in the absence or presence of various chemical inhibitors at 37°C for 5 min. The chemical inhibitors used were as follows: furafylline (FURA, 10  $\mu$ M) for CYP1A2; coumarin (COUM, 100  $\mu$ M) for CYP2A6; triethylenethiophosphoramidate (TEPA, 5  $\mu$ M) for CYP2B6; montelukast (MONT, 0.1  $\mu$ M) for CYP2C8; sulfaphenazole (SULF, 10  $\mu$ M) for CYP2C9; *S*-benzylrivanol (*S*-BEN, 1  $\mu$ M) for CYP2C19; quinidine (QUIN, 10  $\mu$ M) for CYP2D6; diethyldithiocarbamate (DETC, 10  $\mu$ M) for CYP2E1; ketoconazole (KETO, 1  $\mu$ M) for CYP3A, and azamulin (AZAM, 5  $\mu$ M) for CYP3A. Data shown are averages of remaining activity relative to the control metabolite formation rate estimated from duplicate experiments.

**Fig. 4.** Correlation analysis between the known P450 enzyme activities and the rate of formation of dehydro- (A) and *N*-desbenzyl-benidipine (B) from racemic benidipine in 15 human liver microsomes.

**Fig. 5.** Representative plot of the formation of *N*-desbenzyl- and dehydro-benidipine from racemic benidipine and two enantiomers by cDNA-expressed P450 isoforms.

Human cDNA-expressed P450 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 were incubated with 1  $\mu$ M (A) and 10  $\mu$ M (B) benidipine at 37°C for 5 min. Data shown are averages of duplicate experiments.

**Fig. 6.** Kinetics for the formation rate of *N*-desbenzyl-benidipine (A) and dehydro-benidipine (B) from racemic benidipine and two enantiomers in human liver microsomes.

An increasing concentration of benidipine (0 – 200  $\mu$ M) was incubated with HLMs (0.1 mg/ml) and an NADPH-generating system for 5 min at 37°C. Data points are the averages obtained from four different human liver microsomes.

**Fig. 7.** The formation rate of *N*-desbenzyl-benidipine and dehydro-benidipine from benidipine enantiomers in recombinant human CYP3A4 (A) and CYP3A5 (B).

An increasing concentration of benidipine (0 – 200  $\mu\text{M}$ ) was incubated with recombinant CYP3A and an NADPH-generating system for 5 min at 37°C. Each data point represents the average of triplicate incubations.

Table 1. The effect of heat treatment and SKF-525A on the oxidative metabolism of benidipine in human liver microsomes.

Treatment	Percentage of control activity <sup>a</sup>	
	<i>N</i> -Desbenzylbenidipine	Dehydro benidipine
Control	100	100
SKF-525A (100 $\mu$ M)	13	12
Heat treatment (5 min at 45°C)	91	94
Without NADPH	0	0

<sup>a</sup>Data are mean for three different human liver microsomes.

Table 2. Correlation of formation rates of dehydro- and *N*-desbenzyl-benidipine from benidipine (5  $\mu$ M) with the P450 maker activities in human liver microsomes ( $n = 15$ ).

Data were analyzed using the parametric correlation test (Pearson  $r$ ). The activity of each isoform was determined using the respective specific substrate probe reaction, as described previously (Kim et al., 2005).

Activity	P450 isoforms	Correlation coefficient ( $r$ )	
		Dehydro-benidipine	<i>N</i> -Desbenzyl-benidipine
Phenacetin <i>O</i> -deethylation	CYP1A2	0.55 ( $p=0.03$ )	0.60 ( $p=0.03$ )
Coumarin 7-hydroxylation	CYP2A6	0.09 ( $p=0.74$ )	0.24 ( $p=0.38$ )
Bupropion hydroxylation	CYP2B6	0.38 ( $p=0.16$ )	0.17 ( $p=0.54$ )
Paclitaxel 6 $\alpha$ -hydroxylation	CYP2C8	0.52 ( $p=0.06$ )	0.20 ( $p=0.47$ )
Tolbutamide 4-methylhydroxylation	CYP2C9	0.42 ( $p=0.12$ )	0.09 ( $p=0.74$ )
<i>S</i> -Mephenytoin 4-hydroxylation	CYP2C19	0.20 ( $p=0.47$ )	0.35 ( $p=0.20$ )
Dextromethorphan <i>O</i> -demethylation	CYP2D6	0.12 ( $p=0.68$ )	0.23 ( $p=0.41$ )
Chlorzoxazone 6-hydroxylation	CYP2E1	0.32 ( $p=0.25$ )	0.13 ( $p=0.66$ )
Midazolam 1'-hydroxylation	CYP3A	0.66 ( $p<0.01$ )	0.80 ( $p<0.01$ )

Table 3. Mean enzyme kinetic parameters of the formation of metabolites from benidipine and its enantiomers in human liver microsomes.

	(+)- $\alpha$ -Benidipine	Racemic benidipine	(-)- $\alpha$ -Benidipine
<i>N</i> -Debenzylation			
$V_{max}^a$	7.7 $\pm$ 3.4	9.8 $\pm$ 4.9	12.9 $\pm$ 10.1
$K_m$	10.1 $\pm$ 13.8	3.8 $\pm$ 0.9	6.9 $\pm$ 5.9
$Cl_{int}$	2.3 $\pm$ 2.3	3.0 $\pm$ 2.3	1.9 $\pm$ 0.1
<i>n</i>	0.9	0.8	0.9
Dehydrogenation			
$V_{max}^a$	8.7 $\pm$ 2.9	8.4 $\pm$ 4.7	10.7 $\pm$ 7.6
$K_m$	23.6 $\pm$ 12.9	17.3 $\pm$ 11.3	21.7 $\pm$ 7.2
$Cl_{int}$	0.6 $\pm$ 0.6	0.8 $\pm$ 0.9	0.5 $\pm$ 0.2
<i>n</i>	0.8	0.9	1.0

Values are mean  $\pm$  SD of estimates from four different human liver microsomes.

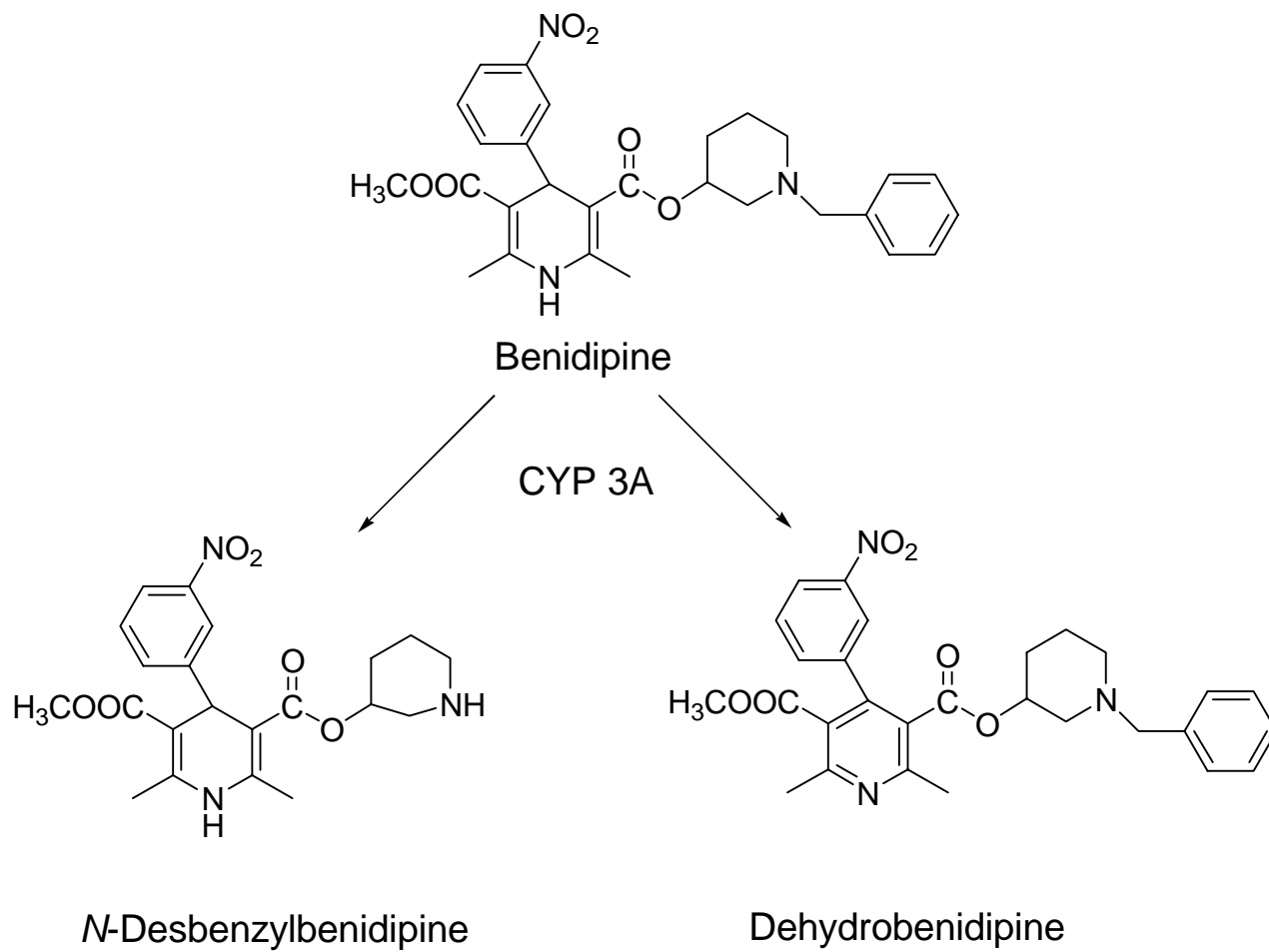
<sup>a</sup> $V_{max}$  is expressed as pmol/min/pmol P450,  $K_m$  as  $\mu$ M, and  $Cl_{int}$  as  $V_{max}/K_m$  ( $\mu$ l/min/pmol P450), *n* as Hill coefficient.

Table 4. Mean enzyme kinetic parameters of the formation of *N*-desbenzyl- and dehydro-benidipine from benidipine enantiomers from the cDNA-expressed P450s.

	CYP3A4		CYP3A5	
<i>N</i> -Debenzylation	(+)- $\alpha$ -Benidipine	(-)- $\alpha$ -Benidipine	(+)- $\alpha$ -Benidipine	(-)- $\alpha$ -Benidipine
$V_{max}^a$	62.7 $\pm$ 1.2	89.0 $\pm$ 6.3	25.7 $\pm$ 1.3	17.9 $\pm$ 1.4
$K_m$	5.5 $\pm$ 2.3	5.9 $\pm$ 2.2	2.5 $\pm$ 0.7	2.2 $\pm$ 0.1
$Cl_{int}$	12.3 $\pm$ 4.9	16.1 $\pm$ 5.0	10.4 $\pm$ 2.1	8.1 $\pm$ 0.1
<i>n</i>	1.2	1.2	1.6	0.9
Dehydrogenation	(+)- $\alpha$ -Benidipine	(-)- $\alpha$ -Benidipine	(+)- $\alpha$ -Benidipine	(-)- $\alpha$ -Benidipine
$V_{max}$	31.7 $\pm$ 2.5	33.6 $\pm$ 0.2	9.1 $\pm$ 0.1	8.9 $\pm$ 1.4
$K_m$	15.2 $\pm$ 0.5	20.8 $\pm$ 2.4	15.4 $\pm$ 0.9	38.2 $\pm$ 12.7
$Cl_{int}$	2.1 $\pm$ 0.1	1.6 $\pm$ 0.2	0.6 $\pm$ 0.1	0.2 $\pm$ 0.1
<i>n</i>	1.5	1.3	1.3	1.0

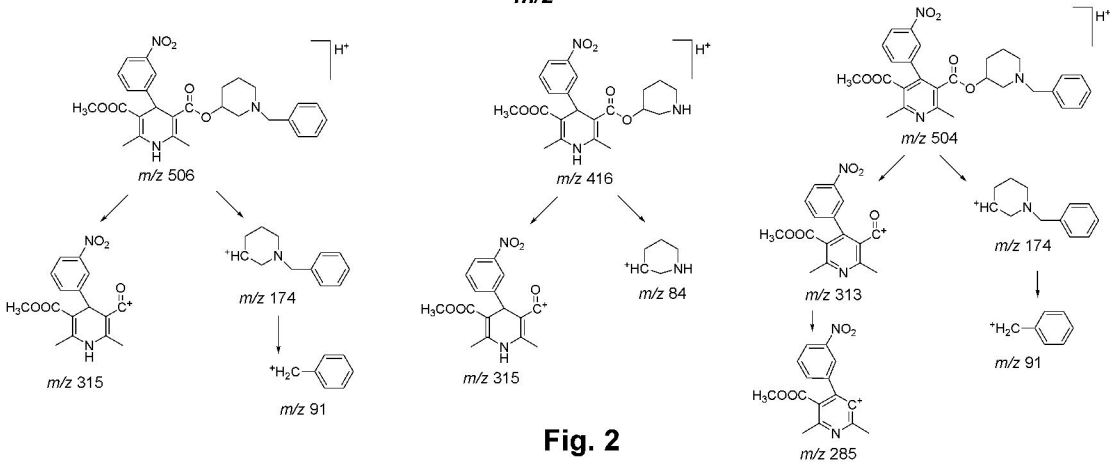
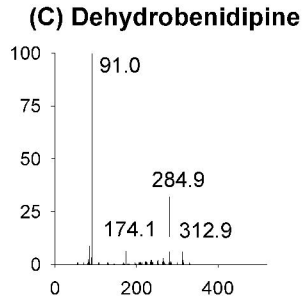
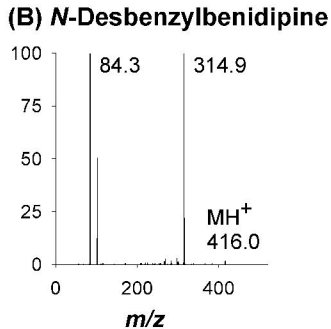
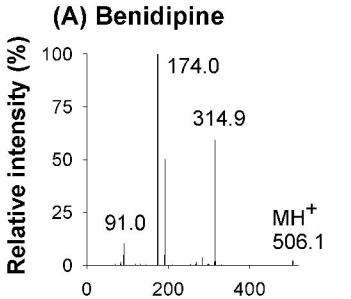
<sup>a</sup> $V_{max}$  is expressed as pmol/min/pmol P450,  $K_m$  as  $\mu$ M, and  $Cl_{int}$  as  $V_{max}/K_m$

( $\mu$ l/min/pmol of P450), *n* as Hill coefficient.

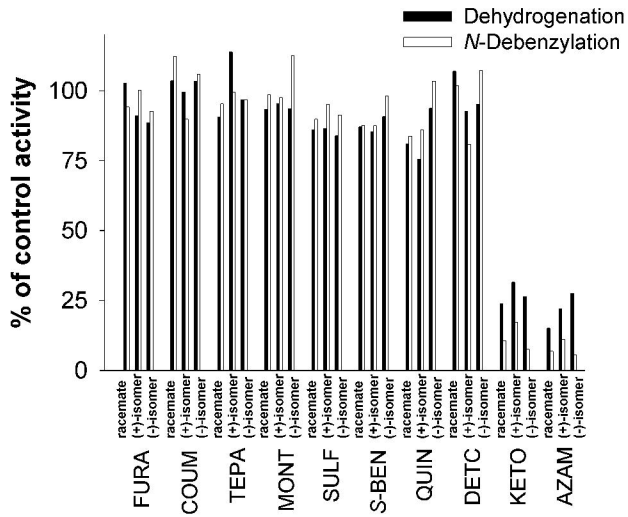


**Fig. 1**

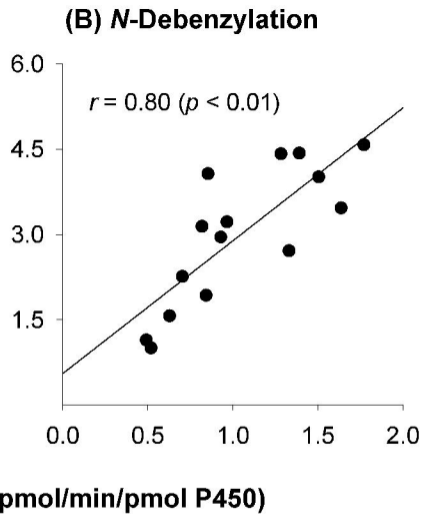
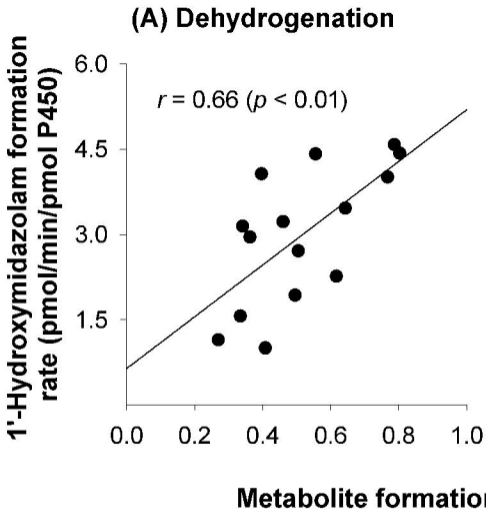




**Fig. 2**



**Fig. 3.**



**Fig. 4.**

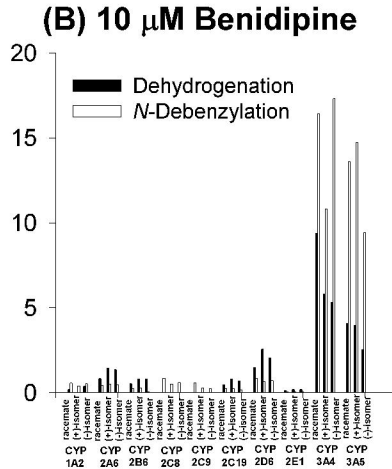
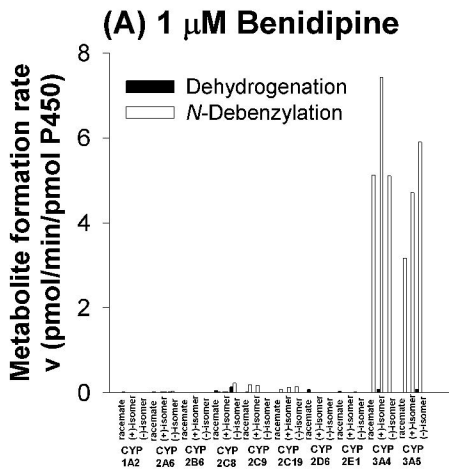
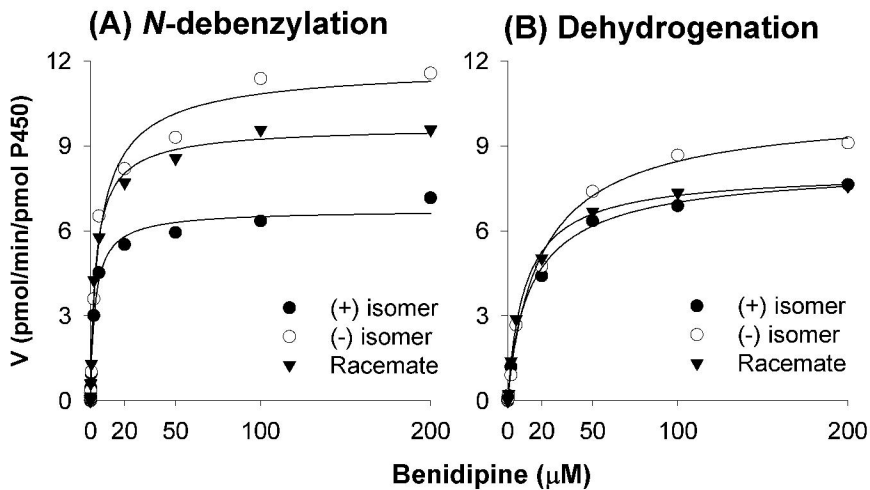
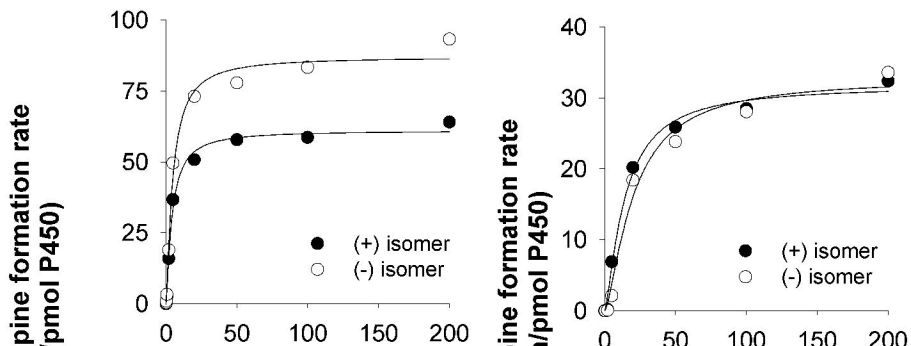


Fig. 5.



**Fig. 6.**

### (A) CYP3A4



### (B) CYP3A5

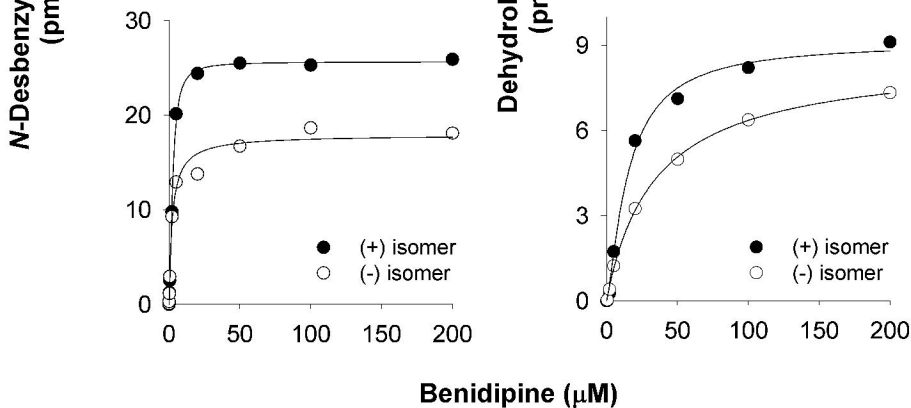


Fig. 7.