Inhibitory Effects of Nicardipine to Cytochrome P450 (CYP) in Human Liver Microsomes

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To anticipate drug–drug interactions by nicardipine *in vivo*, cytochrome P450 (CYP) forms responsible for the metabolism of nicardipine and inhibition of CYP-dependent drug metabolism by nicardipine were investigated. Microsomes of human B-lymphoblastoid cells expressing each human CYP form were used for the metabolism of nicardipine. Inhibitory effects of nicardipine on drug metabolism were studied using human liver microsomes. CYP2C8, CYP2D6 and CYP3A4 were identified as major CYP forms for the metabolism of nicardipine in human liver microsomes. Nicardipine strongly inhibited two-pathways of triazolam hydroxylation both catalyzed by CYP3A4. Comparison of three Ca^{2+} antagonists, nicardipine, nifedipine, and diltiazem revealed that only nicardipine showed such a strong inhibitory potency on the typical CYP2D6-catalyzed drug metabolism. Furthermore, nicardipine inhibited other reactions catalyzed by CYP1A, CYP2A6, CYP2C8, CYP2C9 and CYP2C19 with K_i values ranging from 1.1 to 29.4 μM. In conclusion, nicardipine was a relatively potent inhibitor of human CYP2D6, CYP3A4 and CYP2C (especially for CYP2C8 and CYP2C19) *in vitro*, suggesting that drug–drug interactions between nicardipine and other drugs metabolized mainly by these CYP forms appear to occur *in vivo*.

Key words Ca^{2+} blocker; diltiazem; drug–drug interaction; nifedipine

Nicardipine, a dihydropyridine derivative of calcium entry blockers, is widely used in the treatment of cardiovascular diseases. Clinical use of this drug has been approved for the treatment of hypertension.[6)] Many clinically used drugs are metabolized to pharmacologically inactive forms by one or more cytochrome P450s (CYPs).[7,8)] Recently, particular efforts have been paid to elucidating the pharmacokinetic mechanisms of drug–drug interactions. As one such mechanism, an administration of a certain drug alters the metabolism of other drugs *via* modulation of CYP activities. This interaction occurs by at least two distinct routes: induction and inhibition of CYP. In the latter case, a direct interaction of a drug with a certain CYP results in inhibition of its catalytic activity toward other drugs in a competitive or noncompetitive manner, or enhancement of its activity through allosteric activation. If a drug possessing potent inhibitory effects on CYP is administered simultaneously with another drug having a narrow therapeutic index, an exaggerated response and/or serious toxic effects may occur even at therapeutic doses as a result of increased plasma concentration of the parental drug. For instance, quinidine treatment inhibits CYP2D6 activity to cause orthostatic hypotension by increasing the plasma concentration of co-treated debrisoquine.[9)] In the same way, nicardipine was suspected as a modulator of other CYP substrates by information received from a patient. In the present study, we investigated the CYPs responsible for the metabolism of nicardipine in human liver microsomes. Then, inhibitory effects of nicardipine on reactions catalyzed by eight human CYP forms were examined to determine whether nicardipine would interact with other drugs in a CYP-mediated drug metabolism.

MATERIALS AND METHODS

Materials $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan), 7-ethoxycoumarin from Aldrich Chemical (Milwaukee, WI, U.S.A.), and nicardipine hydrochloride, nifedipine, diltiazem hydrocloride, coumarin, 7-hydroxycoumarin, *dl*-chlorpheniramine maleate and tolbutamide from Wako Pure Chemicals (Osaka, Japan). Sulfaphenazole was kindly provided by Ciba-Geigy Japan (Takarazuka, Japan), triazolam and triazolam metabolites from Pharmacia and Upjohn Japan (Tokyo, Japan), nicardipine metabolites from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan), midazolam and midazolam metabolites from Hoffmann-La Roche (Nutley, NJ, U.S.A.), and *S*-mephenytoin from Sumika Chemical Analysis Service (Tokyo, Japan), respectively. Pooled human liver microsomes were obtained from Daiichi Pure Chemicals (Tokyo, Japan). All other chemicals and solvents were of the highest grade commercially available.

Analytical Procedures Metabolites of nicardipine were determined as follows. A typical reaction mixture consisted of 100 mM potassium-phosphate buffer (pH 7.4), 50 μM EDTA, an NADPH-generating system (0.5 mM $NADP^+$, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate and 1 unit/ml glucose 6-phosphate dehydrogenase) and 0.2 mg protein of human liver microsomes in a final volume of 0.25 ml. After incubation at 37 °C for 10 min, ice-cold acetontrile (0.25 ml) was added to terminate the reaction. The mixture was cen-

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trifuged at 3000 rpm for 10 min and the resultant supernatant fraction was subjected to HPLC equipped with a L-7100 pump (Hitachi, Tokyo, Japan), a SPD-10A UV-visible absorbance detector (Shimadzu, Kyoto, Japan), a C-R6A integrator (Shimadzu, Kyoto, Japan) and an analytical column Nova-Pak Phenyl (3.9 mm×150 mm; Waters, Tokyo, Japan). The mobile phase consisting of 0.047 M ammonium acetate (pH 6.0)/acetonitrile (60 min of linear gradient from 81 : 19 to 54 : 46 v/v) was delivered at a flow rate of 0.8 ml/min. The elution of nicardipine and its metabolites was monitored at 238 nm. Coumarin 7-hydroxylase activity was measured as described by Pearce *et al*.[10] For this assay, 0.05 mg protein of microsomes was added to the reaction mixture and incubated at 37 °C for 5 min incubation. An F-2000 fluorescence detector (Hitachi, Tokyo, Japan) was employed for determination of a metabolite, 7-hydroxycoumarin. The elution of 7-hydroxycoumarin was monitored with excitation at 371 nm and emission at 454 nm. 7-Ethoxycoumarin *O*-deethylase activity was measured as described by Evans and Relling.[11] For this assay, microsomes (0.05 mg protein) were added and incubated for 30 min. HPLC equipped with a Capcell Pac C18 column (4.6 mm×250 mm, Shiseido, Tokyo, Japan) was employed for determination of a metabolite, 7-hydroxycoumarin. The elution of the parent compound and its *O*-deethylated metabolite was monitored with excitation at 360 nm and emission at 470 nm. *S*-Mephenytoin 4′-hydroxylase activity was determined as described by Yasumori *et al*.[12] The composition of an incubation mixture was the same as described above except that 0.1 mg protein of pooled microsomes was used. Incubations were performed at 37 °C for 60 min. Analysis of the metabolite was performed by HPLC equipped with a YMC-Pac Pro C18 column (4.6 mm×150 mm, YMC, Tokyo, Japan). The elution of *S*-mephenytoin and its 4′-hydroxylated metabolite was monitored at 204 nm. The method described by Cresteil *et al*.[13] was adopted for the assay of paclitaxel 6α-hydroxylase activity with minor modifications. Briefly, the final volume of the incubation mixture was 0.25 ml, and 0.1 mg protein of pooled microsomes was added to the reaction mixture. Incubations were carried out at 37 °C for 30 min. The same HPLC system employed for *S*-mephenytoin assay was used for the analysis of the 6α-hydroxy paclitaxel. The elution of paclitaxel and its 6α-hydroxylated metabolite was monitored at 230 nm. The assay of diclofenac 4′-hydroxylase activity was performed as described by Moncrieff[14] with minor modifications. Briefly, incubations were carried out at 37 °C for 15 min with 0.1 mg protein of pooled microsomes in a final incubation mixture of 0.25 ml. The metabolite was quantified by the same HPLC system as employed for the *S*-mephenytoin assay. The elution of diclofenac and 4′-hydroxylated metabolite was monitored at 280 nm. A typical incubation mixture for the assay of bufuralol and dextromethorphan metabolism[15] contained 100 mM potassium-phosphate (pH 7.4), 50 μM EDTA, substrate (bufuralol or dextromethorphan), the NADPH-generating system and 0.1 mg/ml of human liver microsomes in a final volume of 0.15 ml. To compare inhibitory potency on the metabolism of bufuralol with human liver microsomes, nicardipine, nifedipine or diltiazem was added to the incubation mixture. The reaction was stopped by addition of 60% perchloric acid. After centrifugation of the reaction mixture at 3000 rpm for 10 min, the supernatant was subjected to the HPLC systems equipped with a CCPE system pump (Tosoh, Tokyo, Japan), a C-R6A integrator (Shimadzu, Kyoto, Japan), an RF-530 Fluorescence HPLC monitor (Shimadzu, Kyoto, Japan) and an analytical column Capcell Pac C18 (4.6 mm×250 mm; Shiseido). The mobile phase consisting of 20 mM sodium perchlorate (pH 2.5)/acetonitrile (70 : 30, v/v) was delivered at a flow rate of 1.0 ml/min. The elution of the metabolites of bufuralol and dextromrthorphan was monitored fluorometrically with excitation at 252 nm and emission at 302 nm and with excitation at 270 nm and emission at 312 nm, respectively. Midazolam and triazolam hydroxylase activity was measured as described by Ring *et al*.[16] Briefly a typical incubation mixture for the assay of midazolam and triazolam metabolism contained 100 mM potassium-phosphate (pH 7.4), 50 μM EDTA, a substrate (midazolam or triazolam), the NADPH-generating system and 0.1 mg/ml of microsomal protein in a final volume of 0.25 ml. The sample was subjected to the same HPLC systems as described above, except that an analytical column TSK-GEL ODS-120T (4.6 mm×150 mm; Tosoh, Tokyo, Japan) was used. The mobile phase consisting of 100 mM sodium acetate (pH 7.8)/acetonitrile/methanol (53 : 29 : 18, v/v/v, for midazolam) or (61 : 24 : 15, v/v/v, for triazolam) was delivered at a flow rate of 1.0 ml/min. The elution of substrate and its metabolites was monitored at 240 nm.

Metabolism of Nicardipine by Human CYPs Expressed in Human B-Lymphoblastoid Cells Microsomes prepared from human B-lymphoblastoid cells expressing human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9-Arg, CYP2C9-Cys, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 with human NADPH-CYP reductase were obtained from Daiichi Pure Chemicals (Tokyo, Japan). As a negative control, the microsomes of cells transformed with an expression plasmid without the CYP cDNA were used. Incubations were carried out as described above, except that 1 unit of human P450 reductase and microsomal protein (0.15 mg) were added in a final volume of 0.15 ml.

RESULTS

Metabolism of Nicardipine The proposed *in vitro* metabolic pathways of nicardipine in human liver microsomes[17] are illustrated in Fig. 1. Incubation of the pooled human liver microsomes with nicardipine yielded debenzylated and oxidized metabolites. The rates of nicardipine debenzylation

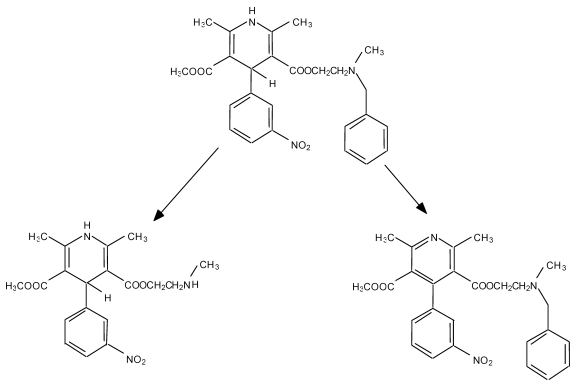

Fig. 1. Proposed Metabolic Pathways of Nicardipine in Human Liver Microsomes

and oxidation catalyzed by microsomes from B-lymphoblastoid cell lines expressing each CYP form were determined. Metabolism of nicardipine by the recombinant human CYPs indicated that the oxidation was mainly mediated by CYP2C8, 2D6 and 3A4 (Fig. 2). Although the debenzylation was catalyzed by essentially all CYPs examined, CYP2C8 and CYP3A4 were the major catalysts, at a low substrate condition (5 μM, Fig. 2). However, the extent of a difference in the debenzylase activity among all CYPs was relatively small at a high substrate concentration (50 μM, data not shown).

Effects of Nicardipine on the Catalytic Activities of CYP3A4 Since CYP3A4 appeared to be the major CYP form involved in the oxidation of nicardipine (Fig. 2), the inhibitory effects of nicardipine on the oxidation of triazolam, a clinically used drug metabolized by CYP3A4, was examined. Triazolam hydroxylation was competitively inhibited by co-incubation with nicardipine (Fig. 3). The K_i values for the α- and the 4-hydroxylation of triazolam are shown in Table 1.

Effects of Calcium Antagonists on the Catalytic Activities of CYP2D6 The inhibitory potencies of three Ca^{2+} antagonists, nicardipine, nifedipine or diltiazem, on bufuralol 1′-hydroxylase were compared (Fig. 4). As shown in Fig. 4, nicardipine inhibited the bufuralol 1′-hydroxylase in human liver microsomes more strongly than did nifedipine and diltiazem. CYP2D6-mediated dextromethorphan *O*-demethylation and mequitazine 3-hydroxylation (K_i, 2.1 μM) were also strongly inhibited by nicardipine. Nicardipine inhibited the CYP2D6-mediated metabolism competitively except for bufuralol 1′-hydroxylation (Fig. 5).

Effects of Nicardipine on the Catalytic Activities of Other CYPs The inhibitory potencies of nicardipine on the metabolism of prototype substrate for other CYPs were investigated. The K_i values of nicardipine for various reactions determined in human liver microsomes are summarized in Table 1. Nicardipine inhibited essentially all reactions examined in the present study, the inhibitory potency was extremely strong against CYP2C8, CYP2C19, CYP2D6 and CYP3A4.

DISCUSSION

One of the most frequent mechanisms of drug–drug inter-

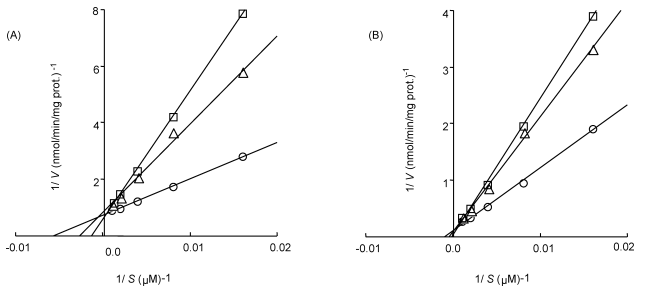

Fig. 3. Lineweaver–Burk Plots for the Effect of Nicardipine on Triazolam α-Hydroxylase (A) and 4-Hydroxylase (B) in Human Liver Microsomes

Nicardipine concentrations were 0 (○), 2.5 (△) and 5 μM (□), respectively. Each data point represents the mean value obtained from duplicate determinations.

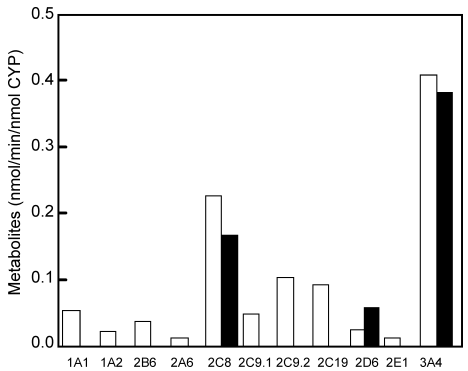

Fig. 2. Formation of Debenzylated (White Bars) and Oxidized (Black Bars) Metabolites in Microsomes from Human B-Lymphoblastoid Cells Expressing CYP Isoforms

Microsomes were preincubated in the presence of an NADPH-generating system for 5 min and then incubated with nicardipine at 5 μM. Each data point represents the mean of duplicate determinations.

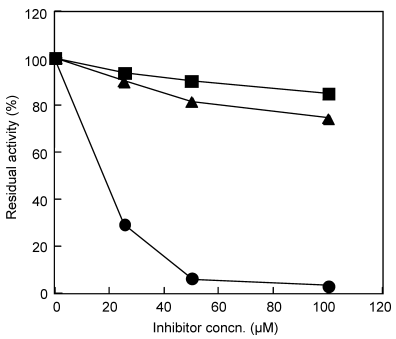

Fig. 4. Effects of Ca^{2+} Blockers on the Metabolism of Bufuralol in Human Liver Microsomes

Human liver microsomes were incubated with nicardipine (●), nifedipine (▲), or diltiazem (■). Residual activities were determined and expressed as percentage of the control value. Each data point represents the mean value obtained from duplicate determinations.

Table 1. Effect of Nicardipine on the Metabolism of Marker Substrate of CYPs in Human Liver Microsomes

Marker substrate	Reaction	K_m (μM)	V_{max} (nmol/min/mg prot.)	K_i (μM)
7-Ethoxycoumarin (1A)	*O*-Deethylation	33.3	33.3	27.0
Coumarin (2A6)	7-Hydroxylation	1.2	62.8	29.4
Paclitaxel (2C8)	6α-Hydroxylation	10.6	0.1	7.1
Diclofenac (2C9)	4′-Hydroxylation	10.1	17.9	17.3
S-Mephenitoin (2C19)	4′-Hydroxylation	31.3	0.4	1.1
Bufuralol (2D6)	1′-Hydroxylation	10.6	0.1	4.8
Dextromethorphan (2D6)	*O*-Demethylation	8.5	0.2	2.9
Midazolam (3A4)	1′-Hydroxylation	2.4	2.1	1.6
Triazolam (3A4)	α-Hydroxylation	175.4	1.4	2.1
	4-Hydroxylation	980.4	8.8	4.6

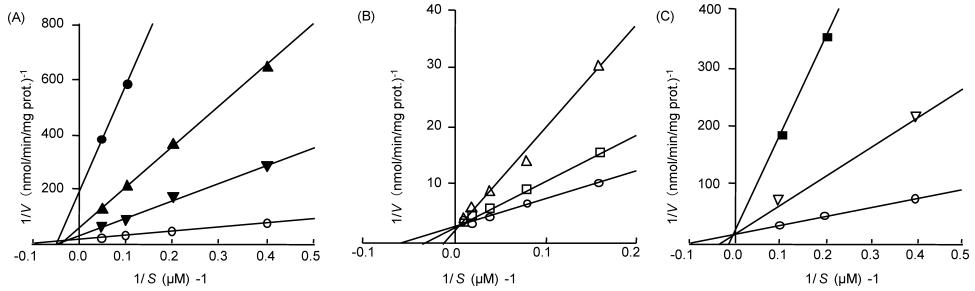

Fig. 5. Lineweaver–Burk Plots for the Effect of Nicardipine on Bufuralol 1′-Hydroxylase (A), Dextromethorphan *O*-Demethylase (B), and Mequitazine 3-Hydroxylase (C) in Human Liver Microsomes

Nicardipine concentrations were 0 (○), 2.5 (□), 5 (□), 10 (□), 12.5 (▼), 20 (■), 25 (▲), and 50 μM (●), respectively. Each data point represents the mean value obtained from duplicate determinations.

action is recognized to be a competition of several drugs on the same enzyme. Predominant binding of a drug with higher affinity to a certain enzyme results in a higher plasma concentration of a drug with lower affinity to the enzyme. Thus, to predict this type of interaction *in vivo*, the information of the *in vitro* K_i value of a reaction must be useful. Although the metabolism of nicardipine has been well characterized in experimental animals, the information on the metabolism of this drug in humans is limited. Co-administration of nicardipine at a dose of 5 mg/kg and triazolam at a dose of 0.1 mg/kg to monkeys resulted in a 1.9 times higher *AUC* value of triazolam than that after the administration of triazolam alone,[18] supporting the idea that nicardipine affected the pharmacodynamics of a certain drug metabolized by CYP3A4. In addition, inhibition on the metabolism of bufuralol and dextromethorphan was observed (Table 1). Thus, nicardipine may also interact with many clinically used drugs reported as a specific substrate for CYP2D6 such as anti-arrhythmic agents, β-blocking agents, H1 antagonists and antidepressants. Not only CYP3A4 and CYP2D6, but essentially all CYP forms investigated in the present study were also inhibited by nicardipine *in vitro* as reported by Katoh *et al*.[19] This may account for a reported *in vivo* pharmacokinetic interaction between nicardipine and other drugs such as propranolol.[20,21] Considering the mechanism of inhibition, a competitive inhibition of these CYPs was suggested in the present study (data not shown). In conclusion, nicardipine can be metabolized mainly by human CYP2C8, CYP2D6 and CYP3A4, whereas it could be a relatively potent competitive inhibitor of these CYPs. Interaction of nicardipine *in vivo* with a number of drugs metabolized by these CYPs should be worth evaluating in future studies.

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