Metabolism of a New 1,4-dihydropyridine Calcium Antagonist, Pranidipine, by cDNA-expressed Human Cytochrome P450

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Summary: To determine human cytochrome P450 isoform(s) (CYPs) involved in the metabolism of pranidipine, a new potent and long-acting 1,4-dihydropyridine calcium antagonist, the biotransformation of the compound was investigated *in vitro* using ten isoforms of human cytochrome P450 expressed in human AHH-1 TK +/− cell lines. Modulation of an ethyl acetate-extract of grapefruit juice on the activity of CYP3A4-mediated pranidipine metabolism was also investigated.

1. Pranidipine was dehydrogenated into pyridine metabolite by CYP3A4 with K_m and V_{max} values of 21.4 μM and 0.110 nmol/min/mg protein, respectively. CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6 and 2E1 were not involved in the pranidipine metabolism.

2. MOP-13031 constructed with dihydropyridine ring, a metabolite of pranidipine, was also oxidized into pyridine ring by the multi-isoforms of cytochrome P450 including CYP3A4, 2C19, 2D6 and 2E1. The K_m and V_{max} values on the oxidation of MOP-13031 to pyridine metabolite by CYP3A4 were 796 μM and 0.124 nmol/min/mg protein, respectively.

3. CYP3A4-mediated testosterone 6 β -hydroxylase activity was competitively inhibited by pranidipine with a K_i value of 5.44 μM but was not affected by MOP-13031 up to a concentration of 100 μM . ($\pm$) Miconazole inhibited the hydroxylation up to 94.7% at 10 μM .

4. CYP3A4-mediated pranidipine dehydrogenase activity was dose-dependently decreased by an addition of the ethyl acetate-extract of grapefruit juice.

Key words: Pranidipine, CYP3A4, Dehydrogenation, Grapefruit juice, cDNA-expressed microsomes

Introduction

Pranidipine, ($\pm$)-methyl 3-phenyl-2(E)-propenyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate, is a new potent and long-acting 1,4-dihydropyridine calcium antagonist¹⁻⁴⁾ that is being developed as a therapeutic drug for the treatment of essential hypertension and angina pectoris.⁵⁻⁹⁾

The absorption, distribution and excretion of pranidipine were previously reported in rats and dogs following oral or intravenous administration.¹⁰⁻¹²⁾ The absolute bioavailability of pranidipine following oral dosing at 1 mg/kg was 4.8% in rats and 6.8% in dogs,¹⁰⁾ and the AUC of unchanged pranidipine accounted for 6.3% of that of total radioactivity in plasma in dogs receiving oral dose of radiolabeled pranidipine at 10 mg/kg,¹¹⁾ showing extensive metabolism of the compound in the animals.

Several metabolic pathways of pranidipine have been identified in animals and humans, indicating the dehydrogenation of 1,4-dihydropyridine to pyridine ring, hydrolysis of the carbonyl ester, hydroxylation of the methyl group and glucuronide conjugation (Fig. 1).^{13,14)} In humans, it has been considered that the elimination of

pranidipine from the circulation was based on the metabolic clearance since no urinary excretion of unchanged pranidipine was observed in healthy subjects received an oral dose of the drug.¹⁴⁾ OPC-13463, which is a metabolite of pranidipine produced by the dehydrogenation of 1,4-dihydropyridine to pyridine ring and the hydrolysis of the carbonyl ester at the 3-position of pranidipine, is one of the main metabolites of pranidipine.¹³⁾

Identification of the human cytochrome P450(s) responsible for the metabolism of a drug is necessary for predicting any potential drug interaction or food interaction, particularly grapefruit juice, in therapeutic use. In this study, we examined the metabolism of pranidipine using ten human cDNA-expressed cytochrome P450 forms and identified CYP isoform responsible for the metabolism. We further demonstrated the modulating effect of an ethyl acetate-extract of grapefruit juice on the activities of CYP3A4-mediated pranidipine metabolism.

Materials and Methods

1. Chemicals and Reagents

Pranidipine, methyl 3-phenyl-2(E)-propenyl 2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate

Fig. 1 Presumed metabolic pathways of pranidipine

(OPC-13448), methyl 2,6-dimethyl-4-(3-nitrophenyl)-3-carboxy-5-pyridinecarboxylate (OPC-13463), ($\pm$)-methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3-carboxy-5-pyridine carboxylate (MOP-13031) and methyl 2-hydroxy-4-(3-nitrophenyl)-6-methyl-3-carboxy-5-pyridinecarboxylate (DM-1312) were provided by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Testosterone, 6β -hydroxytestosterone, ($\pm$)-miconazole, β -NADPH tetrasodium salt and β -NADH disodium salt were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents and solvents were of high analytical grade.

2. Human cDNA-expressed Cytochrome P450s

Microsomes derived from human AHH-1 TK +/− cells expressed human cytochrome P450s were purchased from Gentest Corp. (Woburn, MA, USA). The following microsome expressed cytochrome P450s were used in this study: microsome expressed CYP1A1 (34 pmol P450/mg protein, 7-ethoxyresorufin deethylase activity: 420 pmol/min/mg protein, lot 15), CYP1A2 (117 pmol/mg, 7-ethoxyresorufin deethylase activity: 170 pmol/min/mg protein, lot 37), CYP2A6 and P450 reductase (83 pmol/mg, coumarin 7-hydroxylase: 2430 pmol/min/mg, lot 24), CYP2B6 (120 pmol/mg, 7-ethoxy-4-trifluoromethyl-coumarin deethylase: 220 pmol/min/mg, lot 33), CYP2C8 (40 pmol/mg, 5-(and 6)-chloromethylfluorescein diethyl ether deethylase: 1 pmol/min/mg, lot 10), CYP2C9-cys and P450 reduc-

tase (37 pmol/mg, diclofenac 4'-hydroxylase: 226 pmol/min/mg, lot 22), CYP2C19 (16 pmol/mg, (s)-mephenytoin 4'-hydroxylase: 21 pmol/min/mg, lot 4), CYP2D6-val and P450 reductase (44 pmol/mg, (+)-bufuralol 1'-hydroxylase: 870 pmol/min/mg, lot 9), CYP2E1 and P450 reductase (150 pmol/mg, p-nitrophenol hydroxylase: 1430 pmol/min/mg, lot 25), and CYP3A4 and P450 reductase (56–65 pmol/mg, testosterone 6β -hydroxylase: 1000–2380 pmol/min/mg, lot 9, 15, 19, 25 and 42). The values listed were provided by the manufacturer.

3. Grapefruit Juice Extract

Grapefruit juice was all commercially available. Two reconstituted frozen concentrated (products of Florida, USA, by Tropicana and Dole Food Company Inc.) and one fresh regular-strength (Dole® straight select 100%, Indian River, Florida, USA) grapefruit juice was used in this study.

2 ml grapefruit juice was combined with 5 ml ethyl acetate and shaken for 10 min. After centrifuging at 3000 rpm for 10 min, 4 ml of the organic phase was evaporated to dryness under a stream of nitrogen. The samples extracted were dissolved in 50 μ l of methanol to prepare a stock solution for grapefruit juice extracts. The solution was diluted with methanol to obtain an adequate concentration of the extracts and used in subsequent experiments.

4. Assay Procedure for Pranidipine-, MOP-13031- or OPC-13463-metabolizing Activity

In the experiment to identify the CYP isoform(s) involved in the metabolism of pranidipine, MOP-13031 and OPC-13463, a 0.5 ml reaction mixture containing 2 mg/ml microsomal protein, 5 mM β -NADPH, 2.5 mM β -NADH, and 100 μ M pranidipine, MOP-13031 or OPC-13463 was incubated for 2 hr at 37°C. 100 mM Tris-HCl buffer, pH 7.4, was used for the CYP2A6 and 2C9 incubation mixtures, and 100 mM phosphate buffer, pH 7.4, was used for all other CYP reaction mixtures. The substrates were all dissolved with N, N-dimethylformamide (DMF) and added to the reaction mixtures. The final concentration of DMF was 1 % (v/v) in the reaction mixture.

The rate of formation of the dehydrogenated pranidipine (OPC-13448) was determined over the pranidipine concentration range of 50–200 μ M in a reaction mixture containing 0.4 mg/ml of microsomal protein expressing CYP3A4. A 0.5 ml reaction mixture, including cofactors, was incubated for 15 min at 37°C in an atmosphere of air. The reaction rate was linear with incubation time under this condition. The rate of formation of OPC-13463 was determined over the MOP-13031 concentration range of 100–500 μ M in a reaction mixture containing 0.5 mg/ml of microsomal protein expressing CYP3A4. Incubation was performed for 30 min since the reaction rate was linear up to the incubation time.

Reactions were quenched by adding 0.5 ml methanol. Reaction-terminated samples were centrifuged at 12,000 rpm for 10 min, and 50 μ M of the supernatant was analyzed by HPLC.

5. Effect of Grapefruit Juice-extract on CYP3A4-mediated Pranidipine Dehydrogenation

A reaction mixture (0.48 ml) containing 10 μ M pranidipine, 5 mM β -NADPH and 2.5 mM β -NADH was preincubated for 3 min in 100 mM phosphate buffer, pH 7.4, and then, the reaction was initiated by the addition of 20 μ l CYP3A4-expressing microsomal suspension (10 mg protein/ml, 56 pmol P450/mg protein). Incubation was performed in an atmosphere of air for 20 min. An ethyl acetate-extract of grapefruit juice was added to the reaction mixture before initiation of the reaction at final concentrations of 0.002–0.32 equivalence to original volume of grapefruit juice.¹⁵⁾ The final concentration of methanol used as a solvent for the extract was 1 % (v/v) in the mixture.

Samples after incubation were combined with 5 ml ethyl acetate and shaken for 10 min. After centrifuging at 3000 rpm for 10 min, 4 ml of the organic phase was evaporated to dryness under a stream of nitrogen. The samples extracted were dissolved in 100 μ l of methanol, and 30 μ l of the solution was analyzed by HPLC.

6. Effect of Pranidipine and MOP-13031 on CYP3A4-mediated Testosterone 6 β -hydroxylation

Testosterone 6 β -hydroxylation was assayed in microsomes expressing human cytochrome P450 as index of CYP3A4 activity. The effects of pranidipine, MOP-13031 and of the inhibitor probe, ($\pm$)-miconazole, for the isoform were examined.

Incubation system for the assay of testosterone 6 β -hydroxylation contained 120 μ M testosterone, 0.25 mg microsomal protein, 5 mM β -NADPH, 2.5 mM β -NADH and 100 mM phosphate buffer, pH 7.4, in a final volume of 0.5 ml. Pranidipine, MOP-13031 or ($\pm$)-miconazole was added to the above reaction mixture at concentrations of 1×10^{-6} – 1×10^{-4} M, 1×10^{-6} – 1×10^{-4} M and 1×10^{-7} – 1×10^{-5} M, respectively. The reaction mixture was incubated for 30 min at 37°C and the reaction was terminated by adding 0.5 ml methanol. 50 μ l supernatant fraction of the terminated sample obtained by centrifuging at 12,000 rpm was analyzed by HPLC to determine 6 β -hydroxytestosterone.

To estimate the apparent K_i value for pranidipine regarding the reaction, the rate of testosterone 6 β -hydroxylation was determined as described above in incubation systems containing final concentrations of 50–200 μ M for testosterone and 0–30 μ M for pranidipine.

7. HPLC Analysis

The HPLC apparatus included two Model 510 HPLC pumps (Waters), a Model WISP 712 auto sample processor (Waters), a Model 680 solvent programmer (Waters), a Model 484 or 486 tunable absorbance detector (Waters), and a Chromatopac C-R3A, C-R6A and C-R7A (Shimadzu). TSK gel ODS-80TS column (4.6 mm internal diameter $\times$ 150 mm; Tosoh) equipped with a TSK guardgel ODS-80TS guard column (3.2 mm i.d. $\times$ 15 mm; Tosoh) was used for the analysis.

The mobile phase used for pranidipine, MOP-13031 or OPC-13463 metabolism was a solution of 5 % CH₃CN in water containing 1 % acetic acid as solution A and a solution of 80% CH₃CN in water containing 1 % acetic acid as solution B, and a linear gradient from 10% to 80% solution B in 60 min following isocratic elution with 80% solution B in 10 min in a mixture of solution A and B was used for the determination of OPC-13448, MOP-13031, OPC-13463 and DM-1312. The flow rate was 1.0 ml/min, and UV detection was performed at 254 nm. The retention times for OPC-13463, DM-1312, MOP-13031, pranidipine, and OPC-13448 were 15.5, 17.8, 32.5, 60.3, and 62.5 min, respectively.

The mobile phase used for the determination of OPC-13448 in the study for the modulation of grapefruit juice-extract on the pranidipine metabolism was a solution of 10% CH₃CN in water containing 0.2% trifluoroacetic acid as solution A and a solution of 90% CH₃CN in water containing 0.2% trifluoroacetic acid as solution B, and a linear gradient from 50% to 80% solution B in a mixture

of solution A and B in 30 min was performed. The flow rate and UV detection were same as described previously. The retention times for OPC-13448 and pranidipine were 17 and 21 min, respectively.

The mobile phase used for testosterone metabolism was similar to that for pranidipine, MOP-13031 or OPC-13463 metabolism. The flow rate and UV detection were 1.0 ml/min and 254 nm, respectively. The retention times for 6 β -hydroxytestosterone and testosterone were about 25 and 36 min, respectively.

Calibration curves for OPC-13448, OPC-13463, DM-1312, MOP-13031 and 6 β -hydroxytestosterone were established by an absolute standard method with the respective standard solution at 2 μ g/ml (100 ng per assay). Detection limit for OPC-13448, OPC-13463, DM-1312 and MOP-13031 were all 1 ng per injection in the HPLC assay. OPC-13448 was also determined using an absolute standard method with a calibration range of 0.006–6 μ g/ml ($r=0.999$).

8. Calculations

Michaelis-Menten parameters were calculated from the linear regression line obtained by a Lineweaver-Burk reciprocal plot. The K_i value was calculated from the linear regression line obtained by a Dixon plot.

Results

1. Metabolism of Pranidipine, MOP-13031 and OPC-13463 by Various CYP Isoforms

The catalytic properties of each human CYP isoform for the metabolism of pranidipine are shown in Table I. The results showed that only CYP3A4 was involved in the metabolism of pranidipine. Dehydrogenated pyridine metabolite of pranidipine, OPC-13448, was produced by the enzyme catalysis, and the production activity was 70.9 nmol/2 hr/nmol P450. MOP-13031, OPC-13463 and DM-1312 were not produced by any of the CYP isoforms. Kinetic plots for pranidipine de-

hydrogenase activity catalyzed by CYP3A4 versus the concentration of pranidipine are shown in Fig. 2. The apparent K_m and V_{max} values, determined by Lineweaver-Burk representation, were 21.4 μ M and 0.110 nmol/min/mg protein, respectively.

The catalytic properties of each CYP isoform on the metabolism of MOP-13031 are shown in Table II. The results showed that CYP2C19, 2D6, 2E1 and 3A4 were involved in the metabolism of MOP-13031 and these isoforms produced dehydrogenated MOP-13031, OPC-13463. The production activity was 11.2, 3.18, 0.633 and 18.8 nmol/2 hr/nmol P450, respectively, showing

Fig. 2 Lineweaver-Burk plot of the initial velocity of pranidipine dehydrogenation by microsome-expressed CYP3A4.

Enzyme incubation and metabolite analysis were carried out three times. The linear regression line was $y=0.013x+0.607$ ($r=0.971$), yielding estimates 21.4 μ M for the K_m value and 0.110 nmol/min/mg protein for the V_{max} value.

Table I Catalytic properties of human cDNA-expressed cytochrome P 450 on pranidipine metabolism

CYP*	Formation rate (nmol/2 hr/nmol P 450)			
	OPC-13448	MOP-13031	OPC-13463	DM-1312
CYP1A1	<2.71	<1.37	<2.41	<4.71
CYP1A2	<0.786	<0.398	<0.701	<1.37
CYP2A6	<1.11	<0.561	<0.988	<1.93
CYP2B6	<0.767	<0.388	<0.683	<1.33
CYP2C8	<2.30	<1.17	<2.05	<4.00
CYP2C9	<2.49	<1.26	<2.22	<4.32
CYP2C19	<5.75	<2.91	<5.13	<10.0
CYP2D6	<2.09	<1.06	<1.86	<3.64
CYP2E1	<0.613	<0.311	<0.547	<1.07
CYP3A4	70.9	<0.717	<1.26	<2.46

* Human AHH-1 TK +/- cell microsomes expressing human cytochrome P 450.

Enzyme incubation and metabolite analysis were carried out in duplicate.

Inequality indicates below the detection limit.

Table II Catalytic properties of human cDA-expressed cytochrome P 450 on MOP-13031 metabolism

CYP*	Formation rate (nmol/2 hr/nmol P 450)	
	OPC-13463	DM-1312
CYP1A1	<2.41	<4.71
CYP1A2	<0.701	<1.37
CYP2A6	<0.988	<1.93
CYP2B6	<0.683	<1.33
CYP2C8	<2.05	<4.00
CYP2C9	<2.22	<4.32
CYP2C19	11.2	<10.0
CYP2D6	3.18	<3.64
CYP2E1	0.633	<1.07
CYP3A4	18.8	<24.6

* Human AHH-1 TK +/- cell microsomes expressing human cytochrome P 450.

Enzyme incubation and metabolite analysis were carried out in duplicate.

Inequality indicates below the detection limit.

Fig. 3 Lineweaver-Burk plot of the initial velocity of MOP-13031 dehydrogenation by microsome-expressed CYP3A4.

Enzyme incubation and metabolite analysis were carried out three times. The linear regression line was $y=0.214x+0.269$ ($\gamma=0.990$), yielding estimates 796 μM for the K_m value and 0.124 nmol/min/mg protein for the $V_{\max}$ value.

that CYP3A4 is the principal isoform involved in the catalytic reaction. DM-1312 was not produced by any of the CYP isoforms. Kinetic plots for MOP-13031 dehydrogenase activity catalyzed by CYP3A4 versus the concentration of MOP-13031 are shown in **Fig. 3**. The apparent K_m and $V_{\max}$ values were 796 μM and 0.124 nmol/min/mg protein, respectively.

Production of DM-1312 from OPC-13463 was not observed by any of the CYP isoforms.

Table III Effects of pranidipine, MOP-13031 and ($\pm$)-miconazole on CYP3A4-catalyzed testosterone 6 β -hydroxylase activity

Compound	Percentage of inhibition or (stimulation)			
	1×10^{-7} M	1×10^{-6} M	1×10^{-5} M	1×10^{-4} M
Pranidipine	NP ^a	10.4	67.4	94.7
MOP-13031	NP ^a	(9.5)	1.9	17.2
($\pm$)-miconazole	33.9	89.1	97.8	NP ^a

a) Not performed. Enzyme incubation and metabolite analysis were carried out in triplicate and the data expressed as mean value.

Fig. 4 Dixon plot for the inhibition by pranidipine of testosterone 6 β -hydroxylase activity catalyzed by microsome-expressed CYP3A4.

Enzyme incubation and metabolite analysis were carried out three times. The linear regression lines were $y=0.014x+0.093$ ($\gamma=0.962$) for 50 μM testosterone, $y=0.007x+0.065$ ($\gamma=0.972$) for 100 μM testosterone, and $y=0.004x+0.043$ ($\gamma=0.986$) for 200 μM testosterone, yielding an estimate of 5.44 μM for the K_i value.

2. Effects of Chemicals on Testosterone 6 β -hydroxylation

Effects of pranidipine, MOP-13031 and ($\pm$)-miconazole on CYP3A4-catalyzed testosterone 6 β -hydroxylation are shown in **Table III**. Pranidipine at concentrations of 1×10^{-4} , 1×10^{-5} and 1×10^{-6} M inhibited 94.7%, 67.4% and 10.4%, respectively, of the 6 β -hydroxytestosterone formation compared with the control value. No inhibition of CYP3A4-catalyzed testosterone 6 β -hydroxylation by MOP-13031 at 1×10^{-5} M was observed. ($\pm$)-miconazole at a concentration of 1×10^{-5} M completely inhibited the activity.

For pranidipine, the inhibitory kinetics were investigated using microsome-expressed CYP3A4 to determine the apparent K_i value. A Dixon plot illustrating the

inhibition of testosterone 6 β -hydroxylation by the compound is shown in **Fig. 4**. Pranidipine competitively inhibited the reaction with a K_i value of 5.44 μ M.

3. Effect of Grapefruit Juice Extract on CYP3A4-catalyzed Pranidipine Dehydrogenation

Effect of a grapefruit juice-extract on CYP3A4-catalyzed pranidipine dehydrogenation is shown in **Fig. 5**. Pranidipine dehydrogenase activity was dose-dependently decreased by the addition of grapefruit juice-extracts. A 50% inhibitory effect of the extracts on the activity was approximately 0.02 in volume equivalence of the original grapefruit juice. Two different brands and three commercially available grapefruit juice were examined in this study. The results showed that all three products inhibited CYP3A4-catalyzed pranidipine dehydrogenation and no difference was observed among three products on the inhibitory effect.

Discussion

To identify the CYP isoform(s) in the biotransformation of pranidipine, ten isoforms of cDNA-expressed human cytochrome P450 were examined. The results of this study indicated that the formation of dehydrogenated pranidipine is catalyzed only by CYP3A4, and that no other metabolites of pranidipine are produced by any of the CYP isoforms. MOP-13031, which has been identified as a metabolite of pranidipine¹³⁾ and is possessing a 1,4-dihydropyridine ring in the molecule, was dehydrogenated to a pyridine ring by the multi-isoforms of

cytochrome P450 including CYP3A4, 2C19, 2D6 and 2E1.

Nifedipine, a 1,4-dihydropyridine calcium antagonist that is widely used in clinical therapy, is the first substrate for CYP3A4 shown by Guengerich *et al.*,¹⁶⁾ and it is now commonly agreed that the oxidation of 1,4-dihydropyridine into pyridine is catalyzed by the enzyme.¹⁷⁻²⁰⁾ Pranidipine was also oxidized by the single isoform of CYP3A4 in this study as well as other 1,4-dihydropyridines. Cytochrome P450 has been demonstrated to catalyze not only the dehydrogenation of 1,4-dihydropyridine ring but also the oxidative cleavage of carboxylic acid esters of Hantzsch pyridine esters to the corresponding carboxylic acid,²¹⁻²³⁾ presumably following ring oxidation *in vivo*.²⁴⁾ A 2 hr incubation of pranidipine with CYP3A4 produced a dehydrogenated pranidipine metabolite but not methyl 2,6-dimethyl-4-(3-nitrophenyl)-3-carboxy-5-pyridinecarboxylate, OPC-13463. No information is available which CYP isoform(s) is involved in the oxidative cleavage of carboxylic acid ester in 1,4-dihydropyridine calcium antagonists. In pranidipine, CYP3A4 is probably not involved in the oxidative cleavage of the ester linkage but a further investigation is needed to clarify the responsible CYP isoform in the reaction.

MOP-13031 was dehydrogenated by not only CYP3A4 but also by other CYP isoforms. As mentioned above, only CYP3A4 has generally been responsible for the dehydrogenation of 1,4-dihydropyridine. There are few reports on the involvement of CYP isoform(s) except for CYP3A4 in the reaction. We thus believe this is the first study reporting on the involvement of CYP isoforms except for CYP3A4 in the catalytic activity on the dehydrogenation of 1,4-dihydropyridine. The carboxylic esters at the 3- or 5-position and substituents at the 4-position of 1,4-dihydropyridine ring did not appear to be important in causing dehydrogenation activity by CYP3A4.^{19,20)} MOP-13031 may thus be dehydrogenated by the multi-CYP isoforms since the compound has a carboxyl group at the 3-position of the dihydropyridine ring and the chemical structure is different compared to the specific dihydropyridine substrate for CYP3A4. However, the catalytic activity by CYP3A4 on the MOP-13031 dehydrogenation was higher than that by the other isoforms, indicating CYP3A4 to be a predominant isoform in the reaction.

The K_m for pranidipine or MOP-13031 dehydrogenation by CYP3A4 was 21.4 μ M or 796 μ M, respectively, and those for the metabolism of various 1,4-dihydropyridines by human liver preparation have been reported as follows: nifedipine (14.0 μ M),¹⁶⁾ ($\pm$)-felodipine (6.9 μ M),²⁵⁾ (R)- or (S)-felodipine (6.1 μ M or 12.2 μ M, respectively),²⁶⁾ (R)- or (S)-nilvadipine (14.5 μ M or 31.0 μ M, respectively),²⁷⁾ ($\pm$)-K8644 (32 μ M),¹⁷⁾ and (+)- or (-)-K8644 (17 μ M or 45 μ M, respectively).¹⁷⁾ From the above evidence, it seems that the affinity of pranidipine for CYP3A4 is generally similar to those of

Fig. 5 Effects of grapefruit juice-extracts on CYP3A4-catalyzed pranidipine dehydrogenase activity. Amounts corresponding to 0.002–0.08 equivalent volume of the original grapefruit juice were added to 500 μ l of the reaction mixture (1–40 μ l eq. juice/incubation). Incubations were carried out twice and the data expressed as the mean.

the other 1,4-dihydropyridines whereas that of MOP-13031 is the lowest among these compounds for the enzyme.

Pranidipine and ($\pm$)-miconazole inhibited the CYP3A4-catalyzed testosterone 6 β -hydroxylation with the K_m and V_{max} values of 76.9 μ M and 1.28 nmol/min/mg protein, respectively, estimated in our laboratory. MOP-13031 did not inhibit the catalytic reaction. It was considered that the K_m value for pranidipine dehydrogenation was lower than that for testosterone 6 β -hydroxylation, and so pranidipine competitively inhibited the hydroxylase activity with a relatively low K_i value of 5.44 μ M. To the contrary, the K_m for MOP-13031 dehydrogenation was higher than that for the hydroxylation, and so MOP-13031 probably did not inhibit the activity. ($\pm$)-miconazole is well known as a potent inhibitor for CYP3A4,²⁸⁾ and the inhibitory effect of the compound could be confirmed in this study.

OPC-13463, which is a pyridine metabolite contained a carboxyl group at the 3-position of the pyridine ring, is one of the main metabolites of pranidipine in humans and the plasma concentration of the metabolite is approximately 5-times higher than that of the unchanged pranidipine.¹³⁾ It is considered that there are two possible metabolic pathways for the production of OPC-13463 from pranidipine: one is via MOP-13031, and the other is via the dehydrogenated pranidipine (Fig. 1). In this study, it was clarified that CYP3A4 plays an important role in the biotransformation of both pranidipine to dehydrogenated pranidipine and MOP-13031 to OPC-13463. However, it seems that the metabolic clearance of MOP-13031 to OPC-13463 by the enzyme is extremely lower than that of pranidipine to dehydrogenated pranidipine because the intrinsic clearance of MOP-13031 metabolism calculated based on the V_{max}/K_m was lower than that of the pranidipine metabolism, accounting for 0.16 μ l/min/mg protein and 5.14 μ l/min/mg protein, respectively. Accordingly, it was considered that OPC-13463 is presumably produced via the metabolic route of dehydrogenated pranidipine, OPC-13448.

A food/drug interaction with grapefruit has been reported for 1,4-dihydropyridine calcium antagonists and an increase in the systemic bioavailability has been shown when these drugs are coadministered with grapefruit juice compared to being administered with water.²⁹⁾ The mechanism of the interaction is considered that grapefruit juice inhibits the first-pass metabolism of a drug metabolized by CYP3A (4) located in the wall of the gut rather than in the liver.³⁰⁾ Grapefruit juice contains flavonoids such as naringenin, quercetin and kaempferol being as glycosides. It has been considered that these flavonoids are potent active ingredients causing the interaction between grapefruit juice and drugs because the flavonoids, in particular correspond to the aglycones and inhibited the CYP3A activity in vitro.³¹⁻³⁴⁾ Nevertheless, the in vivo mechanism(s) causing the drug interaction by grapefruit juice is still un-

clear because naringin or quercetin did not cause the in vivo drug interaction,³⁵⁻³⁷⁾ and neither naringin nor naringenin were primarily responsible for the inhibitory effect in CYP3A activity in vitro.³⁸⁾ Recently, specific CYP3A4 inhibitors have been newly isolated from an ethyl acetate-extract of grapefruit juice and the chemical structures identified were furocoumarin derivatives.^{15,39)} These ingredients strongly inhibited CYP3A4-mediated testosterone 6 β -hydroxylase activity as well as ketoconazole, suggesting new candidates for causative ingredients in grapefruit juice causing drug interaction.³⁹⁾ From the above, we attempted to investigate the effect of an ethyl acetate-extract of grapefruit juice on CYP3A4-catalyzed pranidipine dehydrogenase activity. Our results showed that the pranidipine dehydrogenation by the enzyme was dose-dependently inhibited by the addition of the extract and no differences regarding the inhibitory effect were observed among three products from two different brands of grapefruit juice. These observations were similar to those by Fukuda *et al.*¹⁵⁾ It was thus considered that the pranidipine dehydrogenation by CYP3A4 was probably inhibited by the furocoumarin derivatives contained in an ethyl acetate-extract of grapefruit juice. To verify the details of the interaction between pranidipine and grapefruit juice, clinical investigation concerning the pharmacokinetics of pranidipine is now in progress.

In conclusion, the combined results obtained from the investigation in the present study of various CYP isoforms clearly demonstrated that the metabolic conversion of pranidipine to dehydrogenated pranidipine was caused mainly by a single isoform CYP3A4. Moreover, it was indicated that the pranidipine dehydrogenation by CYP3A4 was inhibited by an ethyl acetate-extract of grapefruit juice.

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