Inhibition of *In Vitro* Metabolism of Simvastatin by Itraconazole in Humans and Prediction of *In Vivo* Drug-Drug Interactions

Michi Ishigam,[1] Minoru Uchiyama,[1] Tomoko Kondo,[1] Haruo Iwabuchi,[1] Shin-ichi Inoue,[1] Wataru Takasaki,[1] Toshihiko Ikeda,[1] Toru Komai,[1] Kiyomi Ito,[2] and Yuichi Sugiyama[3,4]

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Purpose. To evaluate an interaction between simvastatin and itraconazole in *in vitro* studies and to attempt a quantitative prediction of *in vivo* interaction in humans.

Methods. The inhibitory effect of itraconazole on simvastatin metabolism was evaluated using human liver microsomes and the K_i values were calculated for the unbound drug in the reaction mixture. A physiologically-based pharmacokinetic model was used to predict the maximum *in vivo* drug-drug interaction.

Results. Itraconazole competitively inhibited the metabolism of simvastatin to M-1 and M-2 with K_i values in the nM range. The area under the curve (AUC) of simvastatin after concomitant dosing with itraconazole was predicted to increase ca. 84-101-fold compared with that without administration of itraconazole. Taking into consideration the fact that this method predicts the maximum interaction, this agrees well with the clinical observation of a 19-fold increase. A similar prediction, based on the K_i value without taking into account the drug adsorption to microsomes, led to an underevaluation of the interaction.

Conclusions. It was demonstrated that the competitive inhibition of CYP3A4-mediated simvastatin metabolism by itraconazole is the main cause of the drug interaction and that a K_i value corrected for drug adsorption to microsomes is the key factor in quantitatively predicting the maximum *in vivo* drug interactions.

KEY WORDS: drug-drug interaction; CYP3A4; HMG-CoA reductase inhibitor; simvastatin; pravastatin; itraconazole.

[1] Drug Metabolism and Pharmacokinetics Research Laboratories and Product Strategy Department, Sankyo Co., Ltd., Shinagawa-ku, Tokyo, Japan.

[2] School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo, Japan.

[3] Graduate School of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

[4] To whom correspondence should be addressed at Graduate School of Pharmaceutical Sciences, University of Tokyo, 3-1,7-Chome, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. (e-mail: sugiyama@mol.f.u-tokyo.ac.jp)

ABBREVIATIONS: AUC, area under the concentration-time curve; cMOAT, canalicular multispecific organic anion transporter; DMA, N,N-dimethylacetamide; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; KPB, potassium phosphate buffer; K_i, inhibition constant; K_m, Michaelis constant; NADP, β-nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; V_0, initial formation rate; V_{max}, maximum metabolic rate.

INTRODUCTION

Drug interactions mediated by the drug-metabolizing enzyme, cytochrome P450 (P450), are very common, and can lead to serious clinical toxicity. The most common P450-mediated interactions are those that are caused by inhibition and they account for approximately 70% of all reported cases (1). If, in clinical practice, a number of drugs metabolized by the same P450 isozyme are administered concomitantly, the metabolism of one drug may be strongly inhibited and its concentration in the blood can rise, resulting in the appearance of exaggerated pharmacological effects and adverse drug reactions (2–4).

The concomitant use of the HMG-CoA reductase inhibitor, simvastatin, and the antifungal agent, itraconazole, has led to rhabdomyolysis and this has been suspected of being caused by increased simvastatin blood levels (5). To confirm this interaction, a clinical trial was conducted on the effect of concomitant administration of itraconazole on the pharmacokinetics of simvastatin (6). The results clearly demonstrated that concomitant administration led to markedly elevated plasma concentrations of simvastatin. Since the administration of simvastatin alone has also been reported to produce rhabdomyolysis (7), it was presumed that the rhabdomyolysis observed after co-administration with itraconazole was due to the elevated plasma levels of simvastatin.

It is known that itraconazole exerts a powerful inhibitory action on CYP3A4, one of the principal P450 isozymes (8,9). There are also reports that simvastatin is metabolized by CYP3A4 (10,11), and so we considered that the plasma simvastatin concentration might have risen because itraconazole inhibited the CYP3A4-mediated metabolism of simvastatin. Similarly, in the case of lovastatin, which is known to be metabolized by CYP3A4 (12), concomitant administration with itraconazole has been reported to cause rhabdomyolysis (13,14). In contrast, pravastatin, which is a much more hydrophilic HMG-CoA reductase inhibitor than simvastatin or lovastatin, exhibited only a small or non-significant elevation in its plasma concentration following co-administration with itraconazole. In addition, there have been no reports of rhabdomyolysis after use of this particular combination of drugs and no proof so far of the CYP3A4-mediated metabolism of pravastatin rather than several other metabolic enzymes (15–17).

In recent years, the use of human liver microsomes and various recombinant human P450 isozyme has enabled *in vitro* experiments to be conducted to investigate the metabolic inhibition caused by concomitant administration of drugs. Also, it is now possible, by carrying out pharmacokinetic analyses, to predict the likelihood of *in vivo* drug interactions in patients based on data collected from *in vitro* systems (18–19). In this study, we have conducted *in vitro* experiments to clarify why, although all three drugs are HMG-CoA reductase inhibitors, only simvastatin and lovastatin, but not pravastatin, exhibit interactions when administered in combination with itraconazole. Furthermore, we have investigated the quantitative prediction of *in vivo* drug-drug interactions, when simvastatin and itraconazole were concomitantly administered, using *in vitro* metabolic and pharmacokinetic parameters.

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MATERIALS AND METHODS

Materials

Simvastatin, ^{14}C-simvastatin, pravastatin, ^{14}C-pravastatin, and itraconazole were synthesized at Sankyo Research Laboratories (Tokyo, Japan). Human liver microsomes, pooled from ten male or ten female subjects, were obtained from the International Institute for the Advancement of Medicine (IIAM, Exton, PA). Microsomal preparations of recombinant human CYP3A4 enzymes expressed in *Saccharomyces cerevisiae* AH22 were purchased from Sumika Chemical Analysis Service Ltd. (Osaka, Japan). Polyclonal antibodies for human CYP3A4 were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). All other reagents were of analytical or HPLC grade.

In Vitro Metabolism Studies

A final volume (0.2 ml) of a typical enzyme source mixture contained 0.04 mg liver microsomal protein, 2 μmol potassium phosphate buffer (pH7.4, KPB), 2 μmol $MgCl_2$, 0.5 μmol NADP, 5 μmol glucose-6-phosphate, and 0.4 units glucose-6-phosphate dehydrogenase. The enzyme source preincubated at 37°C for 3 min was added with ^{14}C-simvastatin (2–100 μM, dissolved in ethanol, final concentration 1%) or ^{14}C-pravastatin (20 μM, dissolved in ethanol, final concentration 1%), and the reaction was continued at 37°C for 10–30 min and then terminated by the addition of ethanol (0.4 ml). After centrifugation (19,000 g, 3 min) of the mixture, the metabolites in the supernatant were analyzed by thin-layer chromatography using silica-gel plates (0.25 mm thickness, 60 F_{254}, Merck KgaA, Darmstadt, Germany). Mobile phases of toluene : acetone : acetic acid (50 : 50 : 0.5, v/v/v) and chloroform : methanol : acetic acid (9 : 1 : 1, v/v/v) were used for simvastatin and pravastatin, respectively, to determine the unchanged forms and metabolites. The metabolites on the thin-layer plates were quantified by a BAS2000 Bio-Image Analyzer (Fuji Photo Film Co., Tokyo, Japan).

Preparation and Identification of Simvastatin Metabolites

The reaction mixture, after incubation of ^{14}C-simvastatin with the human liver microsomes on a large scale, was deproteinized by ethanol and concentrated for an initial purification using silica-gel chromatography. The radioactivity applied to the silica-gel was eluted with toluene : acetonitrile : methanol = 70 : 30 : 0.5–3 in a gradient manner. For further purification, collected fractions were evaporated, redissolved in 50% acetonitrile and subjected to HPLC, performed using a YMC-Pack ODS-A A-312 (150 mm × 6.0 mm I.D. S-5 μm, 120A, YMC Co., Ltd., Kyoto, Japan) column and 45% acetonitrile as the mobile phase, at a flow rate of 1 ml/min and a detection wavelength of 240nm. The final preparations, namely M-1 and M-2, were collected by monitoring their radioactivity in the HPLC system, evaporated to dryness and then subjected to structural analysis by mass spectrometry (MS) and NMR.

Identification of P450 Isozyme Involved in Simvastatin Metabolism

Incubation of simvastatin with human recombinant CYP3A4 was performed under almost the same conditions as described in "*In Vitro* Metabolism Studies" for human liver microsomes, except that the mixture contained 25 pmol CYP3A4 instead of human liver microsomes.

For immuno-inhibition studies, human microsomes (10 mg protein/ml, 10 μl) were first incubated with antiserum or normal rabbit serum (0–25 μl) at room temperature for 30 min. The mixture was added with the co-factor solution and ^{14}C-simvastatin solution (final concentration: 20 μM) containing the co-factor, and then a final volume of 0.5 ml was incubated in the same manner as "*In Vitro* Metabolism Studies." Finally, 1 ml ethanol was added to terminate the reaction, and the supernatant after centrifugation was subjected to thin-layer chromatography.

Kinetic Studies of Simvastatin Metabolism

Simvastatin was incubated at final concentrations of 2–100 μM with human liver microsomes to estimate the kinetic parameters. The production of simvastatin metabolites was determined, and fitted to Eq. (1) using a nonlinear regression program (WinNonlin, Scientific Consulting, Inc., Apex, NC) to estimate K_m, V_{max}, and CL_{ns}:

$$V_0 = V_{max} \cdot S/(K_m + S) + CL_{ns} \cdot S \quad (1)$$

where V_0 is the initial formation rate, V_{max} is the maximum metabolic rate, S is the substrate concentration, K_m is the Michaelis constant (calculated based on the concentration of simvastatin), and CL_{ns} is the intrinsic metabolic clearance for the nonsaturable component. The intrinsic metabolic clearance (CL_{int}) was evaluated using the following equation:

$$CL_{int} = V_{max}/K_m + CL_{ns} \quad (2)$$

Inhibition Studies of Simvastatin Metabolism by Itraconazole

To determine the K_i values for the inhibition of simvastatin metabolism by itraconazole, itraconazole (0.005–10 μM, dissolved in dimethylacetamide, DMA, final concentration 1%) and ^{14}C-simvastatin (20 μM) were added to the enzyme source mixture including human liver microsomes. The conditions of *in vitro* assay and determination of M-1 and M-2 produced from ^{14}C-simvastatin were the same as described in "*in vitro* metabolism studies." K_i values were then estimated by fitting nontransformed data to the following equation based on the nonlinear regression program, WinNonlin:

$$V_{0(+I)}/V_0 = \frac{CL_{ns} + V_{max}/(K_m \cdot (1 + I/K_i) + S)}{CL_{ns} + V_{max}/(K_m + S)} \quad (3)$$

where the subscript $_{(+I)}$ represents the value after alteration by the drug-drug interaction and S and I represent the concentration of substrate (simvastatin) and inhibitor (itraconazole), respectively.

To confirm the type of inhibition by itraconazole on simvastatin metabolism, ^{14}C-simvastatin (5–50 μM) was coincubated with itraconazole (0.2–0.5 μM) in human liver microsomes as described above.

Determination of Itraconazole Binding to Microsomal and Plasma Protein

The binding of itraconazole to microsomal and plasma protein was evaluated using a tube adsorption method. To

determine itraconazole in each fraction, HPLC was performed with a Deverosil ODS-UG-5 (150 mm × 4.6 mm I.D., Nomura Chemical Co., Inc., Aichi, Japan) column and water : acetonitrile (65 : 35, containing 0.28% triethylamine) as a mobile phase, at a flow rate of 1 ml/min. The peaks were monitored using a fluorescence detector (excitation at 260 nm and emission at 365 nm). The tube adsorption method utilizes the property of a lipophilic compound to adsorb to experimental materials such as tubes. To prepare itraconazole solutions as test samples at final concentrations of 0.05–10 μM (0.2 ml), itraconazole dissolved in DMA (final 1%) was added to 10 mM potassium phosphate buffer (pH 7.4, KPB), pooled human liver microsomes (ten male subjects, 0.2 mg protein/ml in KPB) and pooled human plasma (two male and three female subjects) in a polypropylene micro test tube (1.5 ml, Eppendorf). After 60 min, a 100 μl aliquot was transferred to the other tubes containing 200 μl methanol. After centrifugation (19,000 g, 3 min) of each sample, itraconazole in the supernatant fraction was analyzed by HPLC as the fraction not including a portion adsorbed to the tube wall (C_u, C_m and C_p for KPB, microsomes and plasma, respectively). To calculate the total drug concentration added initially (C_t, $C_{t(m)}$ and $C_{t(p)}$ for KPB, microsomes and plasma, respectively), the itraconazole solution prepared in the same tube was combined with methanol directly, then the supernatant was analyzed by HPLC. The drug concentration adsorbed to the tube wall (C_w) was calculated as follows:

$$C_w = C_t - C_u \quad (4)$$

The ratio of the unbound concentration to that adsorbed to the wall (a) was calculated as follows:

$$a = C_u/C_w \quad (5)$$

The concentration adsorbed to the tube wall ($C_{w(m)}$) when the drug was mixed with microsomes was calculated as follows:

$$C_{w(m)} = C_{t(m)} - C_m \quad (6)$$

The unbound drug concentration in microsome suspension ($C_{u(m)}$) was then calculated as follows:

$$C_{u(m)} = a_{mean} \cdot C_{w(m)} \quad (7)$$

where a_{mean} represents the mean value obtained at final itraconazole concentrations of 0.1–10 μM. The unbound fraction for microsomes ($f_{u(m)}$) was evaluated using the following equation:

$$f_{u(m)} = C_{u(m)}/C_m \quad (8)$$

The unbound fraction for plasma ($f_{u(p)}$) was calculated in the same manner as for microsomes.

Prediction of *In Vivo* Drug-Drug Interactions from *In Vitro* Data

The change in the area under the concentration-time curve (AUC) after a single oral administration of simvastatin was estimated on the basis of the "well-stirred" model. The change in AUC can be calculated by the following equation, if the protein binding ratio of the drug is not changed by inhibitor:

$$R = \frac{AUC_{(+I)}}{AUC} = \frac{CL_{int}}{CL_{int(+I)}} \quad (9)$$

where $_{(+I)}$ represents the value after alteration by the drug-drug interaction. In Eq. (9), it is assumed that the liver is the only organ involved in drug clearance.

The contribution of the metabolic process producing M-1 in CL_{int} ($f_{m,1}$) can be calculated by the following equation:

$$f_{m,1} = CL_{int,1}/(CL_{int,1} + CL_{int,2}) \quad (10)$$

$$CL_{int} = CL_{int,1} + CL_{int,2} \quad (11)$$

where $CL_{int,1}$ and $CL_{int,2}$ represent intrinsic clearances for the metabolic processes producing M-1 and M-2, respectively.

As the unbound drug concentration of simvastatin in plasma (0.5 nM) (20,21) is much less than K_m in clinical situations, $CL_{int,1(+1)}/CL_{int,1}$ and $CL_{int,2(+I)}/CL_{int,2}$ can be expressed as follows:

$$CL_{int,1(+I)}/CL_{int,1} = 1/(1 + I_u/K_{i,1}) \quad (12)$$

$$CL_{int,2(+I)}/CL_{int,2} = 1/(1 + I_u/K_{i,2}) \quad (13)$$

where $K_{i,1}$ and $K_{i,2}$ represent the inhibition constants for the metabolic processes producing M-1 and M-2, respectively.

Eq. (10) can be rearranged to give the following equation:

$$CL_{int,2} = (1/f_{m,1} - 1) \cdot CL_{int,1} \quad (14)$$

The change in the intrinsic clearance by the inhibitor can be expressed by the following equation:

$$CL_{int(+I)}/CL_{int} = (CL_{int,1(+I)} + CL_{int,2(+I)})/(CL_{int,1} + CL_{int,2}) \quad (15)$$

Combination of Eqs. (12), (13), (14) and (15) yields the following equation:

$$CL_{int(+I)}/CL_{int} = \frac{f_{m,1}}{1 + I_u/K_{i,1}} + \frac{1 - f_{m,1}}{1 + I_u/K_{i,2}} \quad (16)$$

In order to avoid a false negative prediction due to underestimation of I_u, the unbound drug concentration in plasma at the entrance to the liver ($C_{in,u}$), where the blood flow from the hepatic artery and the portal vein meet, was considered to be the maximum value of I_u and this was used in the prediction. $C_{in,u}$ was calculated from the following equation (ref. 18):

$$C_{in} \leqq I_{max} + \frac{k_a \cdot D \cdot F_a}{Q_h \cdot R_B} \quad (17)$$

$$C_{in,u} = C_{in} \cdot f_u \quad (18)$$

where I_{max} is the maximum plasma concentration in the circulation (the contribution from the systemic circulation), $k_a \cdot D \cdot F_a/(Q_h \cdot R_B)$ is contribution from the absorption, k_a is the absorption rate constant, D is the dose, F_a is the fraction absorbed from the gastrointestinal tract into the portal vein, Q_h is the blood flow in the hepatic vein, R_B is the blood to plasma concentration ratio and f_u is the unbound fraction of the drug.

Finally, comparing the values of $C_{in,u}$ ($= I_u$) and K_i obtained *in vitro* allows a prediction of the maximum degree of *in vivo* drug-drug interaction caused by the metabolic inhibition, according to Eq. (16).

RESULTS

Metabolism of Simvastatin and Pravastatin by Human Liver Microsomes and Recombinant Human CYP3A4

When ^{14}C-simvastatin was incubated with human liver microsomes or recombinant human CYP3A4, a number of metabolites were detected on the thin-layer chromatograms (Fig. 1a). The pattern of the metabolite formation by human liver microsomes was consistent with that obtained by recombinant human CYP3A4. In contrast, ^{14}C-pravastatin was not converted to any metabolites by either human liver microsomes or recombinant human CYP3A4 (Fig. 1b).

For structural analysis of the simvastatin metabolite, M-1, the purified metabolite was subjected to MS involving ionization by FAB and EI methods. The $(M + H)^+$ ion at m/z 435 was detected using FAB ionization mode. Therefore, the molecular weight of M-1 was proposed to be 434. Also, EI spectrum of M-1 was determined in order to obtain the structural information. The structure was found to be 6′-hydroxy simvastatin, which was also confirmed by ^{1}H- and ^{13}C-NMR of DQF-COSY, HMQC, and HNBC. The other main metabolite of simvastatin, M-2, was elucidated to be 3′,5′-dihydrodiol simvastatin by ESI-MS and EI-MS analysis. Molecular-related ions of $(M+H)^+$ and $(M+Na)^+$ peaks at m/z 453 and m/z 475, and their fragment ions m/z 435 and m/z 319 were detected in the ESI spectrum of M-2. Therefore, the molecular weight of M-2 was determined as 452. The structures of these simvastatin metabolites are shown in Fig. 2a, and it was suggested that CYP3A4 is involved in the hydroxylation of simvastatin to M-1 and M-2. In contrast, pravastatin was not a substrate for CYP3A4, judging from the *in vitro* metabolic studies (Fig. 2b).

Immuno-Inhibition of Simvastatin Metabolism Mediated by CYP3A4

As a result of the addition of inhibitory antibody against CYP3A4, the metabolism of simvastatin by human liver microsomes was inhibited depending on the concentration of the antibody (Fig. 3). When 25 μl of the antibody was added, the production of both M-1 and M-2 was inhibited by ca. 90% in male liver microsomes while the corresponding figure was ca. 75–80% in female liver microsomes. The isoform of P450 which was involved in the hydroxylation of simvastatin was identified to be CYP3A4 for the production of both M-1 and M-2.

Kinetics of the Formation of Simvastatin Metabolites in Human Liver Microsomes

The concentration-dependence of the formation of simvastatin metabolites by human liver microsomes is shown as Eadie-Hofstee plots (Fig. 4). In the formation of these metabolites, both saturated and unsaturated metabolic systems were identified by the plots. The calculated kinetic parameters, K_m, V_{max}, and CL_{ns} are shown in Table I. The K_m of M-1 was almost the same in both males and females, but the V_{max} was greater in females than in males by a factor of approximately 1.8. Moreover, the CL_{int} ($V_{max}/K_m + CL_{ns}$) of M-1 in females was 1.9 times higher than that in males. The K_m and V_{max} of M-2 in females were both higher than those

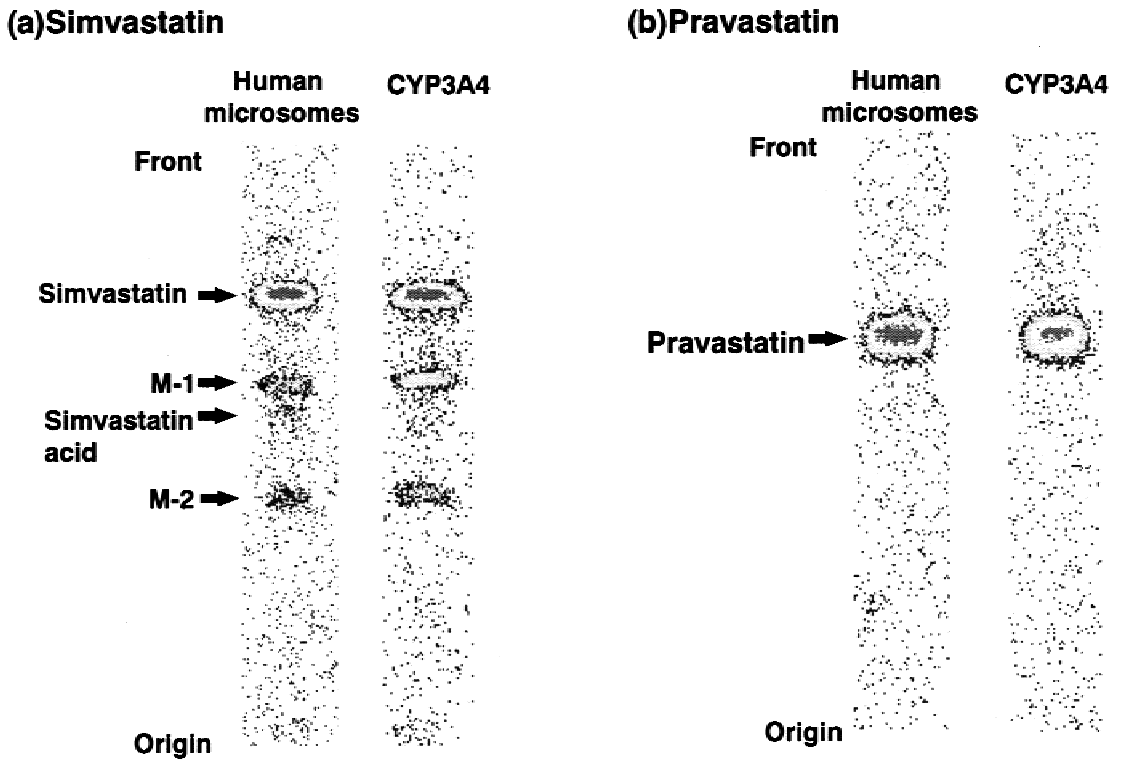

Fig. 1. Thin-layer chromatograms of simvastatin (a) and pravastatin (b) metabolites produced by human liver microsomes and recombinant human CYP3A4. The substrate (20 μM) was incubated at 37°C for 30 min with human liver microsomes (0.2 mg/ml) or recombinant human CYP3A4 (25 pmol/ml) in the presence of an NADPH generation system.

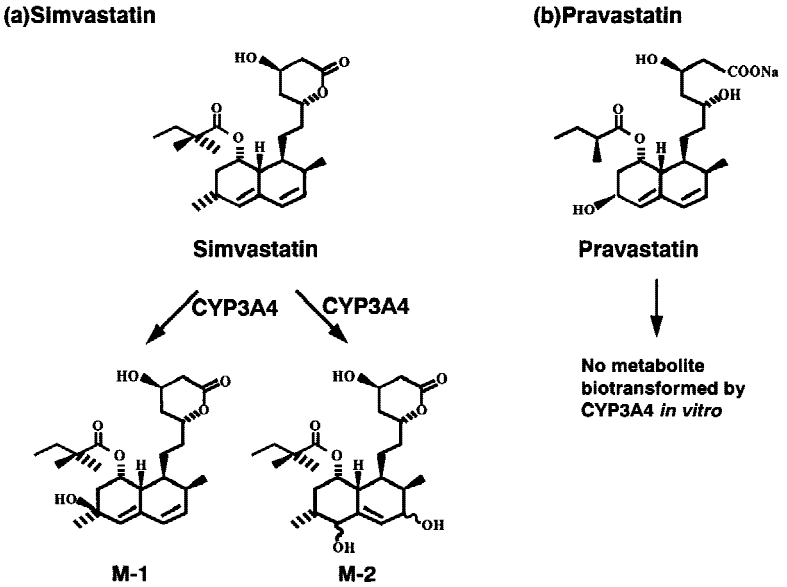

Fig. 2. Chemical structures of simvastatin, pravastatin and their *in vitro* metabolites.

in males by a factor of 3.2 and 2.9, respectively, but the CL_{int} of M-2 was almost equal in males and females. The CL_{int} of M-1 was higher than that of M-2 by a factor of 1.6 in males and by a factor of 3.4 in females. The total intrinsic clearance (M-1 + M-2) in females was approximately 1.5 times higher than that in males.

Binding of Itraconazole to Plasma and Microsomal Protein

The binding of itraconazole to plasma and microsomal protein was determined by the tube adsorption method and the unbound fractions are shown in Fig. 5 and Table II. The mean of unbound fraction of the drug in plasma, $f_{u(p)}$, and that in microsomes, $f_{u(m)}$, was 0.016 and 0.051, respectively.

Inhibition of the Formation of Simvastatin Metabolites by Itraconazole in Human Liver Microsomes

The metabolism of simvastatin by human liver microsomes was inhibited in a concentration-dependent manner by itraconazole (Fig. 6). The K_i values of itraconazole for the formation of M-1 and M-2 are shown in Table II. The K_i values calculated based on the concentration of itraconazole added were 0.075 (male) - 0.061 (female) μM for M-1 and

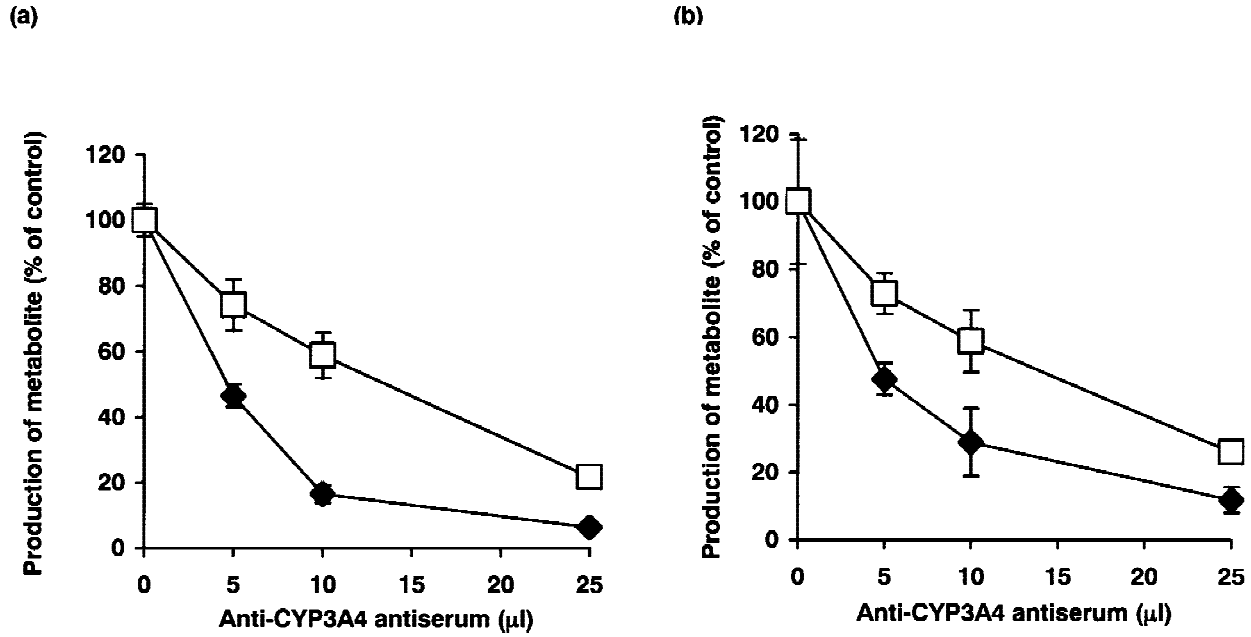

Fig. 3. Inhibition of the formation of simvastatin metabolites, M-1 (a) and M-2 (b), by anti-CYP3A4 antiserum in human liver microsomes. Pooled human liver microsomes (ten male subjects or ten female subjects, 0.1 mg protein/10 μl), preincubated at room temperature for 30 min with 5 to 25 μl of anti-CYP3A4 antiserum or control sera, were incubated with simvastatin (20 μM) and an NADPH generation system, at 37°C for 10 min. Control activities were obtained in the absence of antiserum. Results (mean ± S.E.) were based on triplicate determinations. ♦, human male microsomes; □, human female microsomes.

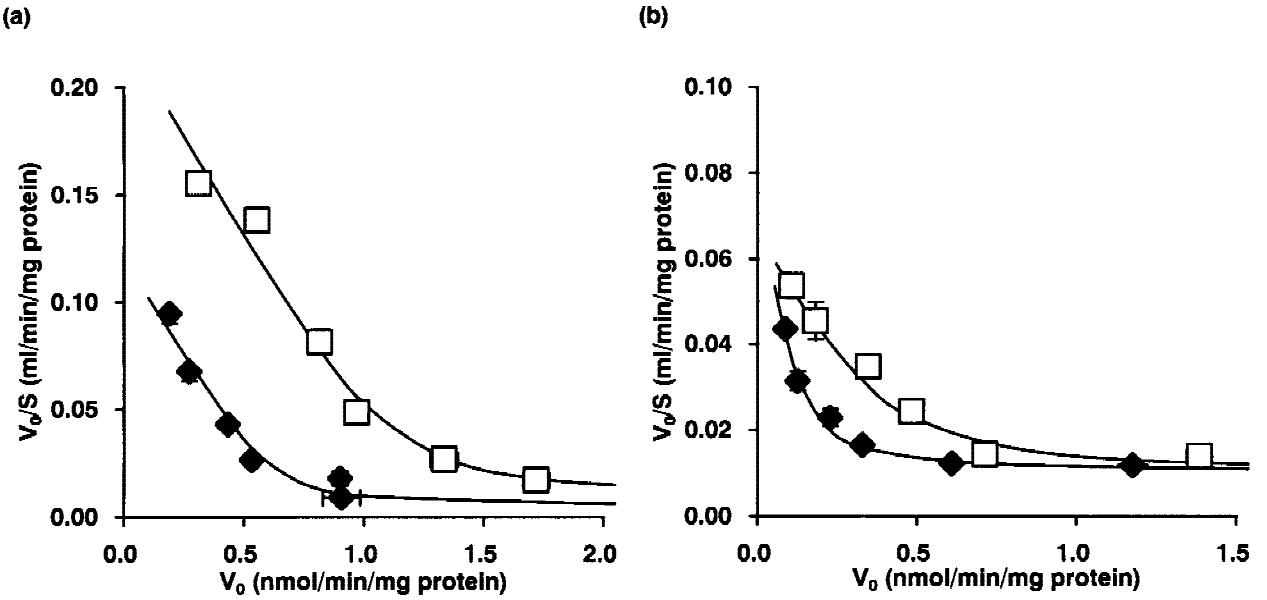

Fig. 4. Eadie-Hofstee plots for the formation of simvastatin metabolites, M-1 (a) and M-2 (b), in male and female human liver microsomes. Simvastatin was incubated at 37°C for 10 min with pooled human liver microsomes (ten male subjects or ten female subjects, 0.2 mg protein/ml). Each value represents the mean ± S.E. of triplicate determinations. ♦, human male microsomes; □, human female microsomes. Solid lines represent the theoretical curves calculated using the obtained values of K_m, V_{max} and CL_{ns}.

0.027 (male) - 0.093 (female) μM for M-2. There was no difference between males and females as far as the K_i values for the formation of M-1 were concerned, but in females the K_i value for the formation of M-2 was approximately 3.5 times higher than that in males. Considering the unbound fraction of itraconazole, $f_{u(m)}$, the K_i values were 0.0038 (male) - 0.0031 (female) μM for M-1 and 0.0014 (male) - 0.0047 (female) μM for M-2. All these K_i values were applied to the quantitative prediction of the *in vivo* drug interaction.

Dixon plots for the inhibition of simvastatin metabolism by itraconazole were examined using the data obtained from female liver microsomes, and a pattern of competitive inhibition was revealed for both M-1 (Fig. 7a) and M-2 (Fig. 7b).

Quantitative Prediction of the *In Vivo* Drug Interaction in Humans

I_{max} (the first term of Eq. (17)) was 0.721 μM (6), and $k_a \cdot D \cdot F_a/Q_h/R_B$ (the second term of Eq. (17)) was calculated to be 17.6 μM, and the sum of these values resulted in a C_{in} of 18.3 μM. The maximum unbound concentration in the portal vein (I_u) was calculated to be 0.290 μM (Table II).

The I_u/K_i and the contribution of M-1 clearance to the intrinsic metabolic clearance ($f_{m,1}$) were used to calculate the changing ratio of the intrinsic metabolic clearance of simvastatin in the case of the concomitant administration of itraconazole, when only the metabolic clearance through M-1 and M-2 is considered to be involved in the elimination of simvastatin. Correction of the unbound fraction, $f_{u(m)}$, of itraconazole showed a change in the intrinsic metabolic clearance ($CL_{int(+I)}/CL_{int}$) as 0.0099 (males) and 0.0119 (females). The maximum degree of elevation of simvastatin AUC was predicted to be 101 and 84 times higher in males and females, respectively, than in the case with no itraconazole (Table II).

DISCUSSION

The principal mechanism of simvastatin clearance in human was reported by Prueksaritanont *et al.* (11) to be *via* hydroxylation by CYP3A4 in the liver. We also confirmed that the principal metabolites of simvastatin in human liver, M-1 and M-2, were formed by CYP3A4. The intrinsic clearance of M-1 was higher than that of M-2 by a factor of 1.6–3.4 in human liver microsomes. It was suggested that the difference in V_{max} caused a difference in metabolic clearance, because the K_m values for M-1 and M-2 were almost equal. The Dixon plots also showed that the formation of simvastatin metabolites, M-1 and M-2, by human liver microsomes was competitively inhibited by itraconazole.

In a clinical report on the concomitant use of itraconazole and simvastatin, itraconazole caused a marked increase in the plasma AUC of simvastatin and simvastatin acid which is the active metabolite (6). However, the increase in the AUC of simvastatin and its acid form has not been accurately

Table I. Kinetics of the Formation of Simvastatin Metabolites in Human Liver Microsomes

	M-1				M-2				Total CL_{int} (M-1 + M-2) ml/min/mg
	K_m μM	V_{max} nmol/min/mg	CL_{ns} ml/min/mg	CL_{int} ml/min/mg	K_m μM	V_{max} nmol/min/mg	CL_{ns} ml/min/mg	CL_{int} ml/min/mg	
Male	5.25 ± 1.24	0.614 ± 0.085	0.004 ± 0.001	0.120	2.11 ± 0.87	0.135 ± 0.025	0.010 ± 0.001	0.074	0.194
Female	5.05 ± 0.55	1.10 ± 0.07	0.007 ± 0.001	0.224	6.81 ± 1.99	0.389 ± 0.083	0.009 ± 0.001	0.066	0.290

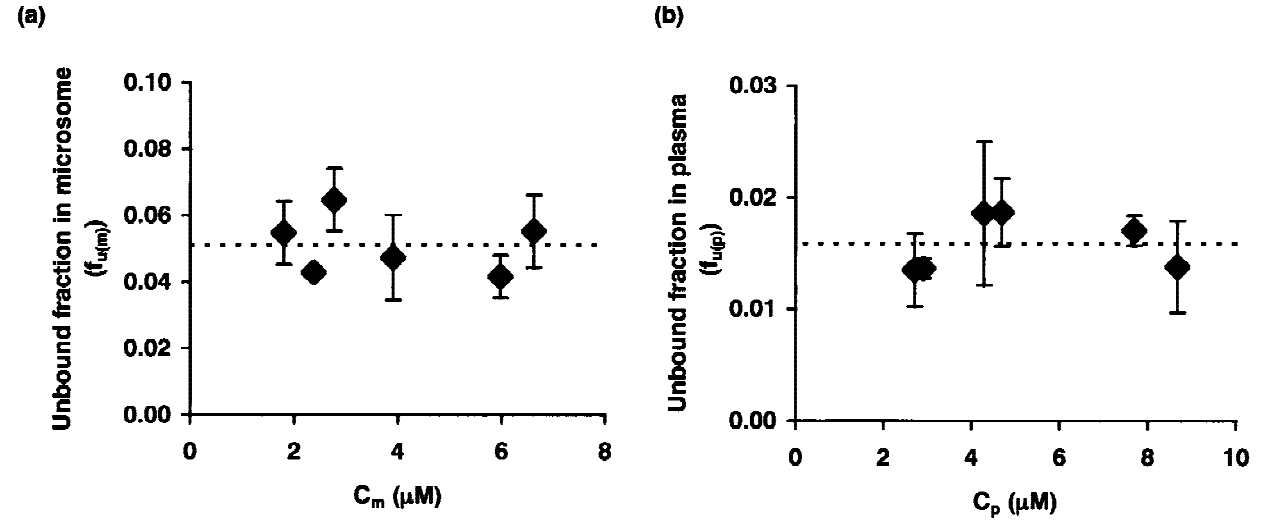

Fig. 5. Estimation of the unbound fraction of itraconazole in microsomes (a) and plasma (b). Itraconazole (0.05–10μM) was incubated at 37°C for 1 hr with KPB, pooled human liver microsomes (ten male subjects, 0.2 mg protein/ml in KPB) or pooled human plasma (two male and three female subjects) in a polypropylene micro test tube (1.5 ml, Eppendorf). C_m and C_p represent the drug concentration not adsorbed onto the tube wall in the presence of microsomes and plasma, respectively. Results (mean ± S.E.) were based on triplicate determinations. The dotted line represents the mean unbound fraction of the drug in plasma, $f_{u(p)}$, and microsomes, $f_{u(m)}$.

calculated, because the plasma levels of simvastatin are below the limit of quantitation when administered with a placebo. Determination of simvastatin acid is also difficult due to the need to subtract the simvastatin level from the total simvastatin acid (simvastatin and simvastatin acid) after hydrolysis. Only one paper (6) has so far been published to show an increase in their AUCs by a factor of 10 or more following the concomitant use of itraconazole. They also mentioned that the AUC of total simvastatin acid increased by a factor of 19.

The parameters obtained from the *in vitro* inhibition experiments and *in vivo* pharmacokinetic analysis enabled us to predict that the AUC of simvastatin co-administered with itraconazole in humans would be increased by a maximum of 84–101 times higher than that following a single dose of simvastatin. This value correlated well with the increase in AUC seen in clinical situations, considering that the maximum degree of *in vivo* interactions can be predicted by this method. When predictions of *in vivo* drug interactions are based on *in vitro* results, we need the values of I_u, which are the concentrations of the unbound form of the inhibitor at sites involving metabolic enzymes in the liver and which cannot be determined directly. In order to avoid a false negative prediction due to underestimation of I_u, the unbound drug concentration in plasma at the entrance to the liver, where the blood flow from the hepatic artery and the portal vein meet, was considered to be the maximum value of I_u (18). When the I_u of itraconazole was estimated by this method, the contribution of the absorption term (17.6 μM) was found to be 25 times that of the confluent fraction from the systemic blood circulation (0.721 μM). If the maximum unbound concentration in the systemic blood circulation is taken as the value of I_u, without considering the absorption term, this would lead to

Table II. Quantitative Prediction of *In Vivo* Drug Interaction in Human Based on *In Vitro* Data Obtained from Inhibition Studies of Simvastatin Metabolism by Itraconazole

Parameters of itraconazole				Parameters obtained from inhibition studies of simvastatin metabolism by intraconazole *in vitro*									
				M-1				M-2					
$f_{u(p)}$	$f_{u(m)}$	I_u[a] (μM)		K_i (μM)	I_u/K_i	$f_{m,1}$	$f_{m,1}$ /(1 + $I_u/K_{i,1}$)	K_i (μM)	I_u/K_i	1 − $f_{m,1}$	(1 − $f_{m,1}$) /(1 + $I_u/K_{i,2}$)	$CI_{int(+I)}$ /CL_{int}	Prediction of R (*in vivo* $AUC_{(+I)}/AUC$)
0.0158 ±0.0010	0.0510 ±0.0036	0.290	Male	0.0038	75.7	0.619	0.0081	0.0014	210	0.381	0.0018	0.0099	101.3
			Female	0.0031	93.1	0.772	0.0082	0.0047	61.0	0.228	0.0037	0.0119	84.2
0.0158 ±0.0010	1	0.290	Male	0.075	3.86	0.619	0.1274	0.027	10.72	0.381	0.0325	0.1599	6.26
			Female	0.061	4.75	0.772	0.1344	0.093	3.11	0.228	0.0554	0.1898	5.27

[a] $I_u = C_{in} \cdot f_u$, $C_{in} = I_{max} + ka \cdot D \cdot F_a/Q_h/R_B$, $I_{max} = 0.721$ μM and $D = 200$ mg were cited from reference 6, and $Q_h = 1610$ ml/min was from reference 18. $k_a = 0.1$ min^{-1}, $R_B = 1$, and $F_a = 1$ were values assumed.

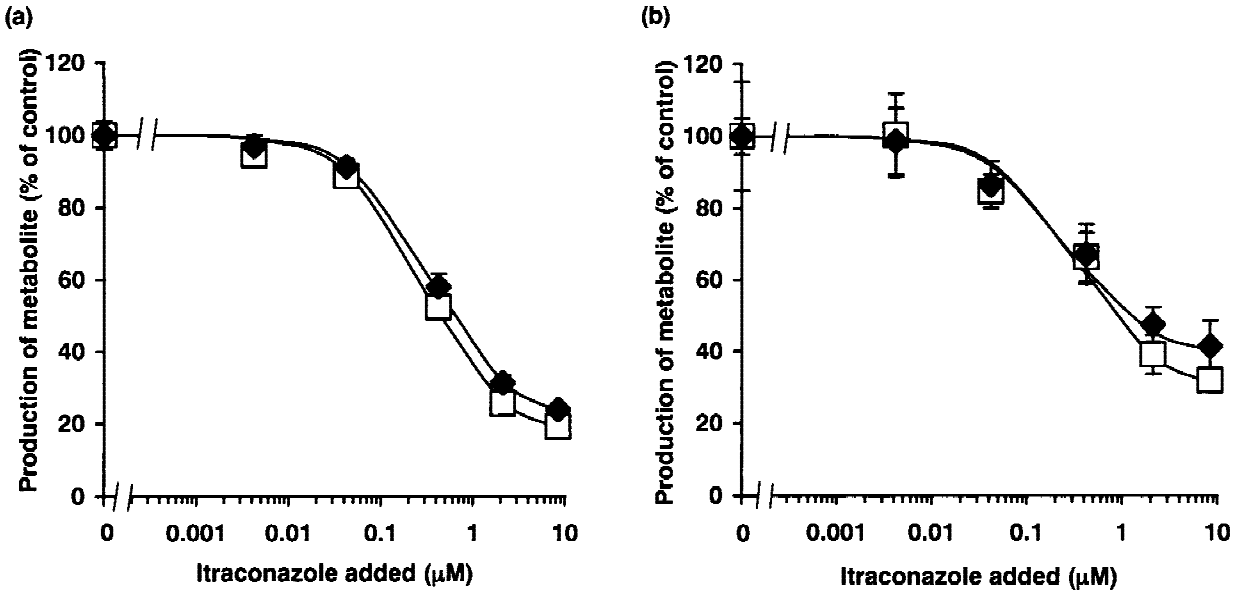

Fig. 6. Inhibition of the formation of simvastatin metabolites, M-1 (a) and M-2 (b), by itraconazole in human liver microsomes. Simvastatin (20 μM) was incubated at 37°C for 10 min with pooled human liver microsomes (ten male subjects or ten female subjects, 0.2 mg protein/ml) in the absence or presence of itraconazole (0.01–20 μM). Control activities were obtained in the absence of itraconazole. Results (mean ± S.E.) were based on triplicate determinations. ♦, human male microsomes; □, human female microsomes. Solid lines represent the theoretical curves calculated using the obtained values of K_m and K_i.

an underestimation of the inhibition. In fact, when the maximum unbound concentration in systemic circulating blood was used for the calculation of the I_u, the relative increase in the AUC of simvastatin was 5.3–6.3 times. Thus the obvious inhibition that has already been observed in clinical situations could not be predicted due to the underestimation of I_u.

In the *in vitro* experiment using microsomes, estimation of the unbound fraction of an inhibitor and a substrate in the reaction mixture is generally required to understand the real concentrations involved in metabolism. The concentration of the unbound drug in the microsomal reaction mixture is sometimes lower than that added because of adsorption to the

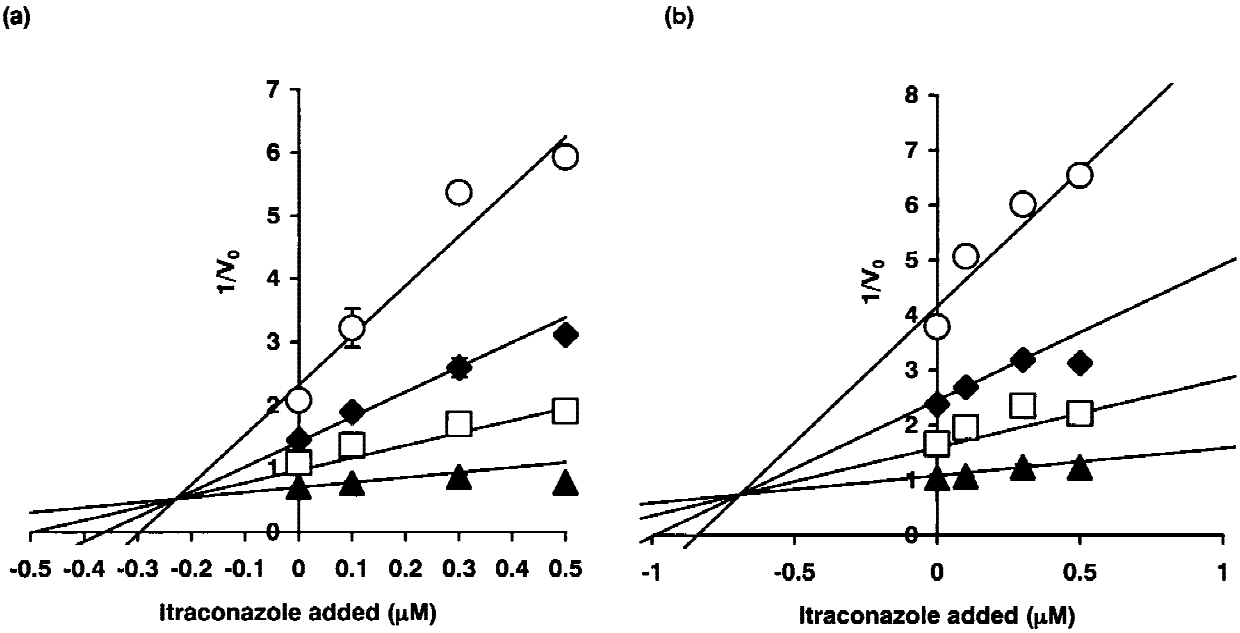

Fig. 7. Dixon plots for the inhibition of the formation of simvastatin metabolites, M-1 (a) and M-2 (b), by itraconazole in human liver microsomes. Simvastatin (5–50μM) was incubated at 37°C for 10 min with pooled human liver microsomes (ten female subjects, 0.2 mg protein/ml) in the absence or presence of itraconazole (0.1–0.5 μM). Results (mean ± S.E.) were based on triplicate determinations. ○, 5 μM of simvastatin; ♦, 10 μM; □, 20 μM; ▲, 50 μM; V_0: M-1 or M-2 formation rate (nmol/min/mg protein).

microsomes. As the binding ratio of a drug to microsomes usually depends on its lipophilicity, the unbound fraction of a lipophilic drug like itraconazole should be carefully considered when *in vitro* experiments are conducted. In addition, the value of K_i must be calculated based on the concentration of the unbound inhibitor. In such cases, if no attempt is made to correct for the unbound fraction of the inhibitor, the K_i value subsequently calculated will be underestimated (18,22,23). In the case of itraconazole, which binds extensively to microsomes, the value of K_i, when no correction for the unbound fraction was performed, was 0.061–0.075 μM for M-1 and 0.027–0.093 μM for M-2 which corresponded to 19 times higher than the corrected value. Moreover, the predicted degree of increase in the AUC of simvastatin, when no correction was performed for microsomal binding of itraconazole, was calculated to be 5.27 (female)—6.26 (male) times higher than that obtained by administering simvastatin alone. Then, there was a big difference between the AUC increase obtained by calculation and from clinical observations. The results of the present study support the importance of performing correction for the unbound fraction in the reaction mixture.

Itraconazole has been also reported to demonstrate the same type of drug interaction with lovastatin as with simvastatin (14). Although the increases in the AUCs of lovastatin and lovastatin acid were not calculated accurately, both were stated to be over 20-fold. The metabolism of lovastatin is mediated by CYP3A4, and is thought to be inhibited by itraconazole. Since hardly any lovastatin is excreted in human urine (24) and no unchanged drug is present in human bile (25), metabolism in the liver is considered to be the principal route of elimination. From the information about lovastatin described above, the *in vivo* drug interaction with itraconazole could be predicted in the same manner as for simvastatin, assuming that lovastatin is eliminated only by CYP3A-mediated metabolism and that the K_i value of itraconazole against lovastatin metabolism is equal to that against simvastatin metabolism determined in the present study. Our prediction suggested that the increase in lovastatin AUC could be 68–242 times higher than that when no itraconazole was administered. These values are in good agreement with the increase in AUC observed in clinical situations, and it was confirmed that the method of prediction used in the present study is a reasonable tool for *in vivo* drug interaction studies.

In the case of pravastatin, on the other hand, no observation of metabolism mediated by human liver microsomes and recombinant human CYP3A4 suggests that CYP3A4 does not play a part in the metabolism of pravastatin. For this reason, pravastatin is believed to be little affected by the inhibition of CYP3A4 metabolism by itraconazole. The process responsible for the elimination of pravastatin from the blood has been clearly demonstrated in rats, and uptake into hepatocytes mediated by the multispecific anion transporter (26–28) and the subsequent biliary excretion mediated by canalicular multispecific organic anion transporter (cMOAT) (29) were confirmed as main clearance pathways. It is reasonable to conclude that these clearance pathways of pravastatin are almost completely unaffected by the concomitant administration of itraconazole, because the inhibitor specificities of CYP3A4 and of organic anion transporters, such as cMOAT, are thought to be different from each other.

Simvastatin and lovastatin are lipophilic lactone-type prodrugs with high logP values, 4.40 and 3.91, respectively. In contrast, pravastatin, which is administered as an open-acid form and itself is an active drug, has a low logP of 0.47 and high solubility in water (30). Such results offer new evidence supporting the hypothesis that the difference in lipophilicity among HMG-CoA reductase inhibitors is related to their safety as far as drug-drug interactions during their clinical use are concerned.

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