ABSTRACT:

Taking into account the species and sex differences in drug interactions based on the inhibition of cytochrome P450 (P450)-mediated drug metabolism, we examined whether the interaction between simvastatin and itraconazole observed in humans could also occur in rats, the most commonly used animal species for pharmacokinetic studies. Itraconazole inhibited the in vitro metabolism of simvastatin in female rat liver microsomes, but not in male rat liver microsomes. Using anti-P450 antisera, the main P450 isozyme responsible for the metabolism of simvastatin was identified as CYP3A in female rats and CYP2C11 in male rats. Therefore, the sex difference in the inhibition of simvastatin metabolism by itraconazole seems to be caused by a difference in the P450 isozymes responsible for the metabolism of simvastatin in male and female rats and the different ability of itraconazole to inhibit CYP3A and CYP2C11. In addition, the effect of itraconazole on the pharmacokinetics of simvastatin in rats was also investigated. The area under the curve value of simvastatin increased approximately 1.6-fold by the concomitant use of itraconazole (50 mg/kg) in female rats, whereas in male rats, itraconazole had no effect. In conclusion, it was found that the results obtained in male rats did not reflect the results in humans as far as the inhibition of simvastatin metabolism by itraconazole was concerned. The P450 isozymes involved in the metabolism of drugs should be taken into consideration when rats are used as a model animal for humans in the investigation of drug interactions.

Drug interactions can be classified into two types: in one case the pharmacological effects or side-effects of drugs are altered by concomitantly administered drugs, and in the other case the effects and side-effects of the concomitantly administered drugs are altered by the original drugs. In both cases, the drug interactions have been evaluated based on changes in plasma drug levels in clinical situations. In the case of HMG-CoA reductase inhibitors, it has been reported in clinical situations that plasma levels of simvastatin and lovastatin, which are in their prodrug lactone forms, were increased more than 10-fold by the concomitantly administered antifungal agent, itraconazole (Neuvonen et al., 1998; Neuvonen and Jalava, 1996; Neuvonen et al., 1998). Itraconazole is known as a potent inhibitor of CYP3A4, one of the major cytochrome P450 (P450) isozymes involved in drug metabolism. For the prediction to be more accurate, it is necessary to choose an appropriate animal as a model. However, there is a possibility that the results obtained in experimental animals do not reflect the drug interactions in humans because species differences in pharmacokinetics are observed in clinical situations (Ishigami et al., 2001).

In the case of drugs at the development stage, in vivo drug interaction studies have been sometimes conducted with experimental animals because it is difficult to conduct such studies at this stage in humans (Damanhouri et al., 1988; Ikeda et al., 1988). It has become possible, to a certain extent, to predict the possibility of in vivo drug interactions in humans from results obtained in vitro using animal liver microsomes. For the scale-up of human metabolism from in vitro to in vivo, some information about the drug concentration in liver is required; however, the measurement is usually impossible. In experimental animals administered with the drug, the concentrations in liver can be easily measured, and the information can be referred to for prediction of in vivo drug interaction in human from in vitro metabolism. For the prediction to be more accurate, it is necessary to choose an appropriate animal as a model. However, there is a possibility that the results obtained in experimental animals do not reflect the drug interactions in humans because of species differences (Kato and Kamataki, 1982; Kamataki et al., 1983) in P450 isozymes.

Accordingly, taking into account the species and sex differences in the drug interactions based on the inhibition of P450 activities, we have investigated whether a drug interaction in humans actually occurs in rats, the most commonly used animal species in pharmacokinetic studies. In vitro and in vivo inhibition studies were conducted...
in male and female rats to investigate the effect of itraconazole, a specific inhibitor of CYP3A4 in humans, on simvastatin metabolism, and the results obtained were compared with the drug interaction observed in humans.

Materials and Methods

Chemicals and Reagents. 

\[ ^{14} \text{C}-\text{Labeled simvastatin (lactone form), simvastatin (lactone form), and simvastatin acid (Na}^+ \text{ salt)} \] used in the present study were synthesized at Sankyo Co., Ltd. (Tokyo, Japan). Rat liver microsomes were prepared from male and female Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) according to conventional methods. Anti-rat P450 antisera preparations (anti-rat CYP2C11 prepared from goat and anti-rat CYP3A2 prepared from rabbit) were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). All other chemicals and reagents used were commercially available and of guaranteed purity.

In Vitro Metabolism of Simvastatin. After preincubation of 0.2 ml of rat liver microsome (0.2 mg of protein/ml) containing an NADPH-generating system (2.5 mM NADP, 25 mM glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and 10 mM MgCl\(_2\)) at 37°C for 3 min, an ethanol solution of \[^{14}\text{C}\]simvastatin was added. After a 10-min incubation at 37°C, the reaction was stopped by adding 0.4 ml of ethanol and vortex mixing. The mixture was centrifuged at 10,000 rpm for 3 min, and the quantity of metabolites in the supernatant was analyzed by HPLC or TLC. The HPLC conditions were as follows: column, C\(_18\) ET250/4 Nucleosil 100-5, mobile phase (linear gradient), acetonitrile/0.05% phosphoric acid = 35:65 (0 min) → 75:25 (25 min); and flow rate, 1 ml/min. The HPLC eluate was collected at intervals of 30 s, and a certain volume of scintillation cocktail (Pico-Fluor, Packard Instrument Co., Meriden, CT) was added to each eluate. The radioactivity was determined by liquid scintillation counting (2250 CA, Packard). For quantification of the metabolites, 200 \(\mu\)l of acetonitrile was added to 100 \(\mu\)l of each plasma sample, and after centrifugation of the mixture the precipitate were re-extracted with a mixture of acetonitrile/water (3:1, v/v), and the supernatant obtained was combined with the supernatant collected above and evaporated to dryness under the stream of \(N_2\) gas. The residue was dissolved in acetonitrile/water (3:1, v/v), and the metabolites were separated by TLC and quantified by liquid scintillation counting. The AUC values (0–6 h or 0–8 h) were calculated by the trapezoidal method.

Results

Characteristics of Simvastatin Metabolism in Rat Liver Microsomes. During the incubation of simvastatin with female rat liver microsomes, metabolites M-1 (6'-hydroxy simvastatin), M-2 (3',5'-dihydridiol simvastatin), and simvastatin acid were formed as the main metabolites, which were identical to those observed in human liver microsomes, although their relative amounts differed. On the other hand, in male rat liver microsomes, metabolite M-3, which was not detected in human and female rat liver microsomes, and simvastatin acid were detected as main metabolites (Fig. 1).

The formation of M-1 and M-2, the main metabolites in female rat liver microsomes, was inhibited by about 80 and 50%, respectively, by the addition of 0.25 mg of IgG anti-CYP3A2 antisemur, while the formation of these metabolites was scarcely affected by the addition of anti-CYP2C11 antisemur (Fig. 2, a and b). These results suggest that the P450 isozyme responsible for the metabolism of simvastatin to M-1 and M-2 in female rats is CYP3A. On the other hand, the formation of M-3, the main metabolite in male rat liver microsomes, was inhibited by about 90% by the addition of 0.25 mg of IgG anti-CYP2C11 antisemur, but not by the addition of anti-CYP3A2 antisemur (Fig. 2c). These findings suggest that the P450 isozyme responsible for the metabolism of simvastatin to M-3 in male rats is CYP2C11.

Effect of Itraconazole on the Formation of Simvastatin Metabolites (M-1, M-2, and M-3) in Rat Liver Microsomes. The metabolism of simvastatin in female rat liver microsomes was inhibited

\[ \frac{1}{V_o} = \frac{1 + K_m}{S} + \frac{I + K_i}{K_i} \left( \frac{K_m \times S}{V_{max}} \right) \]

where \(V_o\) is the initial rate of metabolism, \(K_m\) is the Michaelis-Menten constant, \(V_{max}\) is the maximum rate of metabolism, and \(S\) and \(I\) indicate the concentration of the substrate (simvastatin) and the inhibitor (itraconazole), respectively.

In Vivo Interaction Study. 

\[^{14}\text{C}\]Simvastatin suspended in 0.5% carboxymethylcellulose (CMC) solution was administered orally to fasted male and female rats at a dose of 10 mg/kg immediately after oral administration of itraconazole suspended in 0.5% CMC solution at a dose of 50 mg/kg or 0.5% CMC solution. Blood samples (0.3 ml each) were taken from the jugular vein of each rat using heparinized syringes at various time points after the admin-

\[ [^{14}\text{C}]\text{simvastatin (20 }\mu\text{M)} \text{ was incubated at 37°C for 30 min with human liver microsomes (0.2 mg/ml) or rat liver microsomes (0.2 mg/ml) in the presence of an NADPH-generating system.} \]
by itraconazole in a concentration-dependent manner (Fig. 3a), whereas the formation of M-3 in male rat liver microsomes was scarcely inhibited by itraconazole (Fig. 3b).

The inhibition of simvastatin metabolism by itraconazole was kinetically analyzed with Dixon plots of the data obtained from female rat liver microsomes. As a result, the inhibition of the formation of M-1 (Fig. 4a) and M-2 (Fig. 4b) by itraconazole was demonstrated to be competitive. In addition, the $K_i$ values for the inhibition of the formation of M-1 and M-2 by itraconazole were calculated to be 0.690 and 1.22 $\mu$M, respectively.

Effect of Concomitantly Administered Itraconazole on the Pharmacokinetics of Simvastatin in Male and Female Rats. Figure 6 shows the time courses of the plasma concentration of simvastatin after oral administration of simvastatin (10 mg/kg), with or without concomitant oral administration of itraconazole (50 mg/kg) to male and female rats. The AUC and $C_{\text{max}}$ values and the degree of increase in these parameters produced by itraconazole are summarized in Table 2. The AUC and $C_{\text{max}}$ values of the unchanged drug after oral administration of simvastatin to female rats was increased about 1.6- and 2.0-fold, respectively, by the concomitant administration of itraconazole (Fig. 5a; Table 1). However, the plasma concentration of the unchanged drug after an oral administration of simvastatin to male rats was not affected by the concomitant administration of itraconazole (Fig. 5b, Table 1).

Discussion

Simvastatin is metabolized mainly by CYP3A4 in humans (Vickers et al., 1990; Prueksaritanont et al., 1997). In rats, a sex difference in the pharmacokinetics of simvastatin has been reported (Ohtawa and Uchiyama, 1992); however, the P450 isozyme(s) responsible for simvastatin metabolism have not been identified. In the present study, the main simvastatin metabolites formed by male and female rat liver microsomes were found to be different, and the main metabolites in female rat liver microsomes, M-1 (6\'-hydroxy simvastatin) and M-2 (3\',5\'-dihydrodiol simvastatin), were found to be identical to the main metabolites in human liver microsomes following comparison of their HPLC retention times and TLC $R_f$ (ratio at flow) values (Ishigami et al., 2001) (Fig. 1). In contrast, the main metabolite in male rat liver microsomes, M-3, was shown not to correspond to any of the metabolites
formed in human liver microsomes. The chemical structure of M-3 was proposed to be 3’-hydroxy simvastatin based on the structure of the main metabolite formed in male rat liver microsomes already identified (Ohtawa and Uchiyama, 1992). From the inhibition study with anti-rat P450 antisera, the P450 isozyme responsible for simvastatin metabolism in male rats was demonstrated to be different from that in female rats. In the formation of M-3 by male rat liver microsomes, CYP2C11 was suggested to play the main role, while the CYP3A family was mainly responsible in the formation of M-1 and M-2 in female rat liver microsomes (Fig. 2). Furthermore, itraconazole inhibited the metabolism of simvastatin in female rats but not in male rats (Fig. 4). Considering the previous finding that itraconazole inhibited the metabolism mediated by CYP3A2 in male rats (Yamano et al., 1999), the sex difference in the inhibition by itraconazole was suggested to be attributable to a difference in the ability of itraconazole to inhibit CYP2C11 and CYP3A activity. In addition, the metabolism of simvastatin in human liver microsomes was also inhibited by itraconazole, indicating that female rats rather than male rats reflect the in vitro inhibition in humans. In the investigation of the effect of concomitantly administered itraconazole on the pharmacokinetics of simvastatin in rats, the in vitro metabolism of simvastatin was not inhibited by itraconazole in male rats. In female rats in which inhibition of the in vitro metabolism of simvastatin was observed, the AUC of simvastatin was increased, although the degree of increase was significantly lower than that observed in clinical situations (more than 10-fold) (Neuvonen et al., 1998). As seen in Fig. 1, the amount of M-1 and M-2 formed relative to the acid form

FIG. 4. Dixon plots for the inhibition of the formation of simvastatin metabolites, M-1 (a) and M-2 (b), by itraconazole in female rat liver microsomes. Simvastatin (5–50 μM) was incubated at 37°C for 10 min with pooled female rat liver microsomes (five female rats, 0.2 mg of 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 simvastatin; ●, 10 μM; □, 20 μM; ▲, 50 μM. V̇, M-1 or M-2 formation rate (nmol/min/mg).

FIG. 5. Influence of itraconazole on plasma concentrations of simvastatin in female (a) and male (b) rats. Itraconazole was administrated orally at a dose of 50 mg/kg, and then simvastatin was administrated orally at a dose of 10 mg/kg 1 min later. Each point represents the mean ± S.D. (n = 3). ●, plasma concentration of simvastatin alone; □, plasma concentration of simvastatin in the presence of itraconazole.

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<td>Pharmacokinetic parameters of simvastatin in the absence or presence of itraconazole</td>
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was less in female rat liver microsomes than in human liver microsomes. Because the formation of M-1 and M-2 is more susceptible to inhibition by itraconazole than that of the acid form, the difference in the formation ratio of metabolites in female rats and humans might be one of the causes for the smaller inhibitory effect of the concomitantly administered itraconazole in female rats, compared with that observed in humans.

The drug interaction based on the inhibition of simvastatin metabolism mediated by CYP3A4 in humans could not be reproduced in the study with male rats. On the other hand, in the study with female rats, a drug interaction was observed, although the degree of increase in the AUC of simvastatin was smaller in rats than in humans. These results suggest that female rats are a more appropriate animal model than their male counterparts for the investigation of the drug interaction based on the inhibition of simvastatin metabolism mediated by CYP3A4. Since species and sex differences are observed in P450 isozymes, the establishment of appropriate experimental conditions, taking into account the sex differences are observed in P450 isozymes, the establishment of appropriate experimental conditions, taking into account the sex differences in P450 isozymes responsible for drug metabolism, should be confirmed as far as drug interaction studies using rats as a model animal for humans are concerned.

References


