DRUG INTERACTION BETWEEN SIMVASTATIN AND ITRACONAZOLE IN MALE AND FEMALE RATS

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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 was 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 and Jalava, 1996; Neuvonen et al., 1998). Itraconazole is known as a potent inhibitor of CYP3A4, one of the major cytochrome P450 (P450) isozymes in humans (Olkkola et al., 1994; Varhe et al., 1994).

We have investigated the causes of the increase in the plasma levels of the prodrug-type lactone forms of HMG-CoA reductase inhibitors by concomitantly administered itraconazole. We used a pharmacokinetic model (Ito et al., 1998a,b) involving the pharmacokinetic parameters for itraconazole and the Ki values obtained in in vitro studies using human liver microsomes. The predicted increase in plasma simvastatin levels by the concomitant use of itraconazole agreed reasonably well with those 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 in vitro systems using human 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 (Nelson et al., 1996; Eagling et al., 1998) and sex 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. [14C]-Labeled simvastatin (lactone form), simvastatin (lactone form), and simvastatin acid (Na+ 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 MgCl2) at 37°C for 3 min, an ethanol solution of [14C]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, C18 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) was added to each eluate. The radioactivity was then counted in a liquid scintillation counter (2250 CA, Packard). The TLC system (2.5 mM NADP, 25 mM glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and 10 mM MgCl2) at 37°C for 3 min, an ethanol solution of [14C]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, C18 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) was added to each eluate. The radioactivity was then counted in a liquid scintillation counter (2250 CA, Packard). The TLC analysis of simvastatin and its metabolites was performed under the following TLC conditions: silica-gel plates, 0.25 mm thickness, 60 F254 (Merck KgaA, Darmstadt, Germany); and development solvent system, toluene/acetone/acetic acid (50:50:0.5, v/v/v). The amount of unchanged drug and its metabolites was determined by radioluminography using BAS 2000 equipment (Fuji Photo Film Co., Tokyo, Japan).

To identify the P450 isozymes responsible for the metabolism of simvastatin, an inhibition study using anti-rat P450 antisera was performed. Anti-rat P450 antisera or corresponding control sera (0–0.25 mg of IgG) were added to rat liver microsomes (pooled for five male rats or five female rats, 10 mg of protein/ml) and the resulting mixture was preincubated at room temperature for 30 min. Then, in the same manner as described above, NADPH-generating system was added and preincubated for 3 min at 37°C. Subsequently, an ethanol solution of [14C]simvastatin was added to each microsomal preparation to give a final concentration of 20 μM and then incubated for 5 min at 37°C (0.2 mg of protein/ml, total volume of 0.5 ml). The reaction was stopped by addition of 1 ml of methanol, and the quantity of metabolites in the supernatant was determined by TLC.

Inhibition of Simvastatin Metabolism by Itraconazole. Itraconazole (dimethylacetamide solution; final concentrations: 0–20 μM) and simvastatin (ethanol solution; final concentration: 20 μM) were added to male or female rat liver microsomal preparations (0.2 mg of protein/ml) or rat liver microsomes (0.2 mg/ml) in the presence of an NADPH-generating system. After incubation for 30 s, and a certain volume of scintillation cocktail (Pico-Fluor, Packard) was added to each solubilized sample, and the radioactivity was counted in a liquid scintillation counter (2250 CA, Packard). For quantification of the metabolites, 200 μl of acetonitrile was added to 100 μl of each plasma sample, and after centrifugation of the mixture at 10,000 rpm for 3 min, the supernatant was collected. Furthermore, the metabolites remaining in 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 N2 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'-dihydrodiol 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 antisera, while the formation of these metabolites was scarcely affected by the addition of anti-CYP2C11 antisera (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 antisera, but not by the addition of anti-CYP3A2 antisera (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 by...
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 $R_f$ 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 3'9'-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

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>AUC$_{(+I)}$</th>
<th>AUC$_{(+I)}/$AUC</th>
<th>$C_{\text{max}}$</th>
<th>$C_{\text{max}(+I)}$</th>
<th>$C_{\text{max}(+I)}/C_{\text{max}}$</th>
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<tr>
<td>Female</td>
<td>91.7 ± 8.9</td>
<td>149 ± 8</td>
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<tr>
<td>Male</td>
<td>58.3 ± 8.4</td>
<td>60.1 ± 9.4</td>
<td>1.03</td>
<td>17.0 ± 1.3</td>
<td>16.1 ± 0.7</td>
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**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. $V_0$, 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.
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, is necessary for drug interaction studies using rats as a model animal for humans are concerned.

References