Effect of Chronic Administration of Ritonavir on Function of Cytochrome P450 3A and P-Glycoprotein in Rats

Michiharu Kageyama, Hitomi Namiki, Hiroto Fukushima, Shuichi Terasaka, Tatsuya Togawa, Akina Tanaka, Yukako Ito, Nobuhito Shibata,* and Kanji Takada

Department of Pharmacokinetics, Kyoto Pharmaceutical University; Yamashina-ku, Kyoto 607–8414, Japan.

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Ritonavir (RTV) is well known as an inhibitor of many drugs that are metabolized by cytochrome P450 (CYP) 3A or fluxed via P-glycoprotein (Pgp), although it is also reported that RTV is a potent inducer for them. In this study, to elucidate these contradictory phenomena, functional changes of CYP3A or Pgp during chronic administration of RTV were examined in rats. After pretreatment with RTV for indicated days (day 3–day 14), rats were used in the experiments. The area under the plasma drug concentration vs. time curve (AUC∞) after oral administration of RTV (20 mg/kg) to these rats showed an RTV-treatment period-dependent decrease, and the mean AUC∞ of RTV in Day 14 rats decreased significantly by 57% as compared to the control. The AUC∞ after intravenous (i.v.) administration of RTV to Day 3 and Day 5 rats increased significantly by 28% and 22%, respectively, while there were no significant changes in the AUC∞ in Day 7 and Day 14 rats as compared to the control. As for i.v. administration of erythromycin (EM) or midazolam (MDZ) to RTV-treated rats, the AUC∞ in Day 3 and Day 5 rats increased significantly as compared to the control, while in Day 7 rats and rifampicin-treated rats, the AUC∞ of EM decreased significantly by 82% and 42%, respectively, as compared to the control. For MDZ, there were no significant changes in the AUC∞ in Day 7 or Day 14 rats. After i.v. administration of rhodamine123 (Rho123), the excretion clearances from blood circulation to the intestinal lumen and the biliary excretion clearances in Day 14 rats increased markedly by 2.2-fold and 2.6-fold as compared to the control. It has been confirmed that RTV is not only a potent inhibitor but also a potent inducer of CYP3A, and that RTV is a potent inducer of intestinal Pgp. This property of RTV is responsible for regulating the oral bioavailability of drugs that are mediated by CYP3A and Pgp.

Key words: ritonavir; induction; cytochrome P450 3A; chronic administration; P-glycoprotein; pharmacokinetic

Since the mid-1990s, HIV protease inhibitors (PIs) have been largely responsible for recent successfull results in the treatment of HIV infected patients.1) The combined use of two kinds of reverse transcriptase inhibitors and an HIV PI, namely, highly active anti-retroviral therapy (HAART), has been found to be a better therapy than either drug alone in reducing HIV RNA levels and increasing CD 4 cell counts.2) However, treatment failure has occurred in many cases as a result of poor adherence to therapy, development of viral resistance and pharmacokinetic reasons in HAART.3) Recently, therefore, a combination therapy with two kinds of PIs (dual protease therapy) has been introduced, and has shown clinical effectiveness in preventing the development of tolerance in HIV in clinical practice.4) All PIs are metabolized via cytochrome P450 (CYP) 3A in human or rats, and it has been reported that ritonavir (RTV) inhibits the oxidative metabolism of PIs via CYP3A. RTV, therefore, is used as a booster to increase the bioavailability of another PI in the dual protease therapy.5—8) On the other hand, recent studies demonstrated that all PIs are potent substrates and inhibitors for P-glycoprotein (Pgp). All PIs have higher transport rates from the basolateral to apical direction than the apical to basolateral direction using in vitro models such as Caco-2 or MDR1 transfected LLC-PK1 cells.9—12) Moreover, using Pgp knockout mice, Kim et al. demonstrated that plasma concentrations of indinavir, nelfinavir and saquinavir were elevated 2- to 5-fold as compared to wild type mice after oral administration.9) RTV, therefore, interact with CYP3A and Pgp through the metabolic and efflux processes after oral administration of RTV. Since RTV has a lower metabolic clearance rate among PIs currently used, it has been believed that RTV is a strong inhibitor for other drugs that are metabolized via CYP3A.8) However, in clinical practice, it has been reported that plasma concentrations of RTV or other drugs co-administered with it during repeated chronic administration were reduced as compared to their concentrations in the initial dosing.13,14) Based on these observations, we speculate that RTV has an inducing effect on its disposition of itself and other drugs that are metabolized or fluxed via CYP3A or Pgp. The purpose of this study was to elucidate aspects of inhibitory and inducing effects of RTV during repeated administration to rats in vivo, where we focused on the functions of CYP3A and Pgp in the liver and intestine.

MATERIALS AND METHODS

Materials RTV and rifampicin (RFP) were extracted from capsules commercially available, Norvir® and Rifadin®, respectively. N-methyl-2-pyrroldine (Pharmasolve®) was kindly donated by International Specialty Products (Tokyo, Japan). A surfactant, polyoxyethylene (40) hydrogenated castor oil (HCO-40) was kindly donated by Nikko Chemicals (Tokyo, Japan). Erythromycin (EM) and midazolam (MDZ) were purchased from Nacalai Tesque (Kyoto, Japan). Rhodamine123 (Rho123) was purchased from Sigma Chemical Co. (Steinheim, Germany). All other reagents were used of analytical grade and were used without further purification.

Animals All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Kyoto Pharmaceutical University. Male Wistar rats about 10 weeks old (300±20 g) were obtained from Nippon SLC Co., Ltd. (Hamamatsu, Japan). They were housed individually for

* To whom correspondence should be addressed. e-mail: shibata@mb.kyoto-phu.ac.jp © 2005 Pharmaceutical Society of Japan
at least three days under controlled environmental conditions with free access to general food and water.

Preparation of Solutions Standard stock solutions of RTV, EM, MDZ, Rho123 were prepared by dissolving them in ethanol at various concentrations, and were stored at 4°C in the dark. Samples for the calibration curve for liquid chromatography–mass–mass spectrometry (LC-MS-MS) analysis were prepared by adding a known amount of these standard stock solutions to drug-free plasma in a volume of 1:100. The test solution of RTV for oral administration was prepared by suspending 200 mg of RTV, which was prepared from Norvir® capsules, in 10 ml of 1% (w/v) sodium carboxymethyl cellulose (CMC-Na) suspension. The test solution of RFP for oral administration was prepared by suspending 100 mg of RFP which was prepared from Rifadin® capsules in 10 ml of 1% (w/v) CMC-Na suspension. The test solution of RTV or Rho123 for intravenous administration was dissolved in 50 mg RTV or 2 mg Rho123 with 10 ml of a mixture containing 5% (v/v) ethanol, 5% (v/v) HCO-40, 5% (v/v) Pharmasolve® at a final concentration of 5 mg/kg and 0.2 mg/kg, respectively. EM for intravenous administration was prepared by diluting with saline containing 5% (v/v) dimethylsulfoxide at a final concentration of 3 mg/kg. MDZ for intravenous administration was prepared by dissolving 1 mg MDZ in 10 ml of 0.25% (v/v) ethanol, 0.25% (w/v) HCO-40, 0.25% (v/v) Pharmasolve® at a final concentration of 0.1 mg/kg.

Study Design Rats were divided into five groups for RTV study. All groups were administered orally and study was carried out for a period of 14 d. Group 1 was administered vehicle and this group served as a control. Group 2 (Day 3) received vehicle for the first 11 d followed by RTV from day 12—14; group 3 (Day 5) received vehicle for the first 9 days followed by RTV from day 10—14; group 4 (Day 7) received vehicle for the first 7 d followed by RTV from day 8—14; and group 5 (Day 14) received RTV from day 1 for 14 d. Another group of rats served as a positive control in a concurrent experiment to evaluate Pgp and CYP3A functions. For the positive control study, the rats received vehicle for the first 7 d followed by oral administration of RFP (10 mg/kg/d) for the remaining 7 d (Day 7RFP). All groups of rats were fasted overnight on day 15 and used for either oral, i.v. or excretion studies as mentioned in the subsequent section.

Oral Administration Each group fasted overnight for at least 12 h on day 15, received an oral dose of RTV (20 mg/kg). The drug suspensions in 1% CMC-Na were administered using an oral feeding tube. Then, 0.25 ml aliquots of blood samples were obtained from the external left jugular vein at the designated time, and the plasma was separated by centrifuging at 4°C. All samples were stored at −80°C until analysis.

Intravenous Administration (i.v.) Each group of rats, fasted overnight for at least 12 h on day 15, received an intravenous dose of RTV (5 mg/kg), EM (3 mg/kg) or MDZ (0.1 mg/kg). The drug test solutions were i.v. administered by bolus injection. Then, 0.25 ml aliquots of blood samples were obtained from the external left jugular vein at the designated time, and the plasma was separated by centrifuging at 4°C. All samples were stored at −80°C until analysis.

Method Each group of rats, fasted overnight for at least 12 h on day 15, was anesthetized with an intraperitoneal injection of sodium pentobarbital (32 mg/kg). They were placed in a supine position on a heating pad under a surgical lamp to maintain constant normal body temperature. Cannulation was made with polyethylene tubing, SP-31 into the bile duct to collect bile samples, and then the upper jejunum (15 cm) of intestinal lumen was catheterized with an inlet silicon tube (4.2 mm i.d.). This was perfused with phosphate buffered saline containing 25 mM glucose into the intestinal lumen in a single perfusion manner at a flow rate of 0.5 ml/min. After 30 min of perfusion for stabilization, 0.2 mg/kg of Rho123 was injected via the jugular vein. Simultaneously, intestinal perfusate and bile samples were collected every 15 min over 120 min. Then, 0.25 ml aliquots of blood samples were collected from the jugular vein at designated time, and the plasma was separated by centrifuging at 4°C. All samples were stored at −80°C until analysis.

Assay Methods For extraction procedure of RTV, EM and MDZ, 100 μl of aliquots of plasma, whole blood or bile samples in a 1.5 ml of polyoxyethylene centrifuging tube, 10 μl of indinavir methanol solution (20 μg/ml) was added as an internal standard and vortexed for 15 s. Then, 150 μl of 2% (w/v) ZnSO₄ in 50% (v/v) methanol solution was added to precipitate proteins. The mixture was vortexed for 15 s, and centrifuged at 12000 rpm for 10 min. The clear supernatant was transferred to a new 1.5 ml polyethylene centrifuging tube, 1 ml of diethyl ether was added, and the mixture was vortexed for 30 s. After centrifuging at 12000 rpm for 10 min, the supernatant was decanted to an HPLC sample vial by cooling water layer in an ice-cold bath. After evaporating diethyl ether in a water bath at 40°C, the residue was reconstituted by 100 μl of HPLC mobile phase, and 30 μl was injected into a liquid chromatography-tandem mass spectrometry (LC-MS-MS) system. The LC-MS-MS analysis was carried out using an HPLC system consisting of a LC-10AS quaternary pump (Shimadzu, Kyoto, Japan) equipped with vacuum degasser, and AS-2001 autosampler with a 100-μl loop (Toso, Tokyo, Japan) interfaced with an API 365 SCIEX triple-quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, U.S.A.). RTV, EM, MDZ and the internal standard were separated on an Inertsil ODS-3 (100 mm×2.1 mmφ, GL Science, Tokyo, Japan). The mobile phase which consists of 90% (v/v) acetonitrile containing 10 mM ammonium acetate was degassed before use. The sample was delivered with a flow rate of 0.2 ml/min, and each analysis lasted for 5.0 min. The mass spectrometer was operated in the turbo ion spray mode with positive ion detection. The flow rates of nebulizer gas, curtain gas and collision gas were set at 8, 8 and 21/min, respectively, and the ion spray voltage and temperature were set at 5000 V and 300°C. The declustering potential, the focusing potential, the entrance potential, the collision energy and the collision cell exit potential were set at 20, 20, 10, 30 and 6 V, respectively. Multiple reaction monitoring analysis was performed with the transition m/z 723→296 for RTV, m/z 734→576 for EM, m/z 326→291 for MDZ and m/z 615→422 for indinavir. All raw data were processed with PerkinElmer SCIEX Analyst Software, version 1.0. Taking the peak area ratios of RTV, EM and MDZ against the internal standard (indinavir), the calibration curves for RTV, EM
and MDZ were made. Retention times for RTV, EM, MDZ, and indinavir were 1.64, 2.21, 2.07 and 1.57 min, respectively, and all separations were completed within 5 min. Calibration curves of RTV, EM and MDZ were linear and passed through the origin with correlation coefficients of 0.999 or over. The limits of detection for RTV and EM were 0.005 μg/ml, and for MDZ were 0.0001 μg/ml. The determination of Rho123 concentrations in the samples was performed immediately after the experiment. The perfusate, bile and plasma samples were stored at 4°C after collection. For plasma samples, an equal volume of methanol was added to 100 μl aliquots of plasma to precipitate proteins. The mixture was vortexed for 30 s, and centrifuged at 12000 rpm for 10 min at 4°C. A 100 μl aliquot of the supernatant was then added to the 96 well microplate and Rho123 concentrations were measured plasma concentration, and extrapolated to infinity. The limits of detection for RTV and EM were 0.005 μg/ml, and for MDZ were 0.0001 μg/ml. The determination of Rho123 concentrations in the samples was performed immediately after the experiment. The perfusate, bile and plasma samples were stored at 4°C after collection. For plasma samples, an equal volume of methanol was added to 100 μl aliquots of plasma to precipitate proteins. The mixture was vortexed for 30 s, and centrifuged at 12000 rpm for 10 min at 4°C. A 100 μl aliquot of the supernatant was then added to the 96 well microplate and Rho123 concentrations in the samples were analyzed by a Fluoroskan Acent CF (Dainippon-Pharm. Co., Tokyo, Japan) with fluorimetric detection, an excitation wavelength of 485 nm and an emission wavelength of 527 nm. 100 μl aliquots of both perfusate and bile samples were added directly to the microplate and Rho123 was analyzed as described above.

**Data Analysis** A noncompartmental pharmacokinetic analysis was applied to the plasma concentration vs. time data using the computer program, WinHARMONY.16) The area under the plasma concentration vs. time curve after i.v. or oral administration of RTV, EM, MDZ and Rho123 was calculated using the linear trapezoidal rule up to the last measured plasma concentration, and extrapolated to infinity \((AUC_{0-\infty})\). The terminal elimination half-life, \(t_{1/2}\), was determined by dividing ln 2 by the elimination rate constant at the terminal phase, which was computed by a linear least squared method using at least 3 measured-points. The total body clearance of RTV, EM, MDZ or Rho123 \((CL_{tot})\) and the oral clearance \((CL_{app})\) were calculated by \(D/AUC_{0-\infty}\), where \(D\) represents the i.v. or oral dose of RTV, EM, MDZ or Rho123. The excretion clearance \((CL_{ex})\) of Rho123 from the systemic circulation to the intestinal lumen \((CL_{ex,int})\) and bile duct \((CL_{ex,bile})\) after i.v. administration was calculated by dividing the total amount of Rho123 excreted into the intestinal lumen by \(AUC_{0-\infty}\).

**Statistical Analysis** All values are expressed as the mean±S.E. Statistical differences of the means were assumed to be significant when \(p<0.05\) by one-way ANOVA followed by Turkey’s multiple range test.

**RESULTS**

Plasma trough concentrations of RTV after repeated administration of RTV before the experiments ranged from 0.02 to 0.04 μg/ml throughout the experiments. The time courses of the mean plasma RTV concentration after oral administration of RTV alone to the control, Day 3, Day 5, Day 7 and Day 14 rats are shown in Fig. 1, and the pharmacokinetic parameters of RTV after oral administration alone to these rat groups are listed in Table 1. RTV in plasma gradually increased over 3—6 h, and the time to reach the peak plasma concentration was found to be delayed in accordance with RTV-treatment period. The maximum plasma concentration of RTV, \(C_{max}\), and \(AUC_{0-\infty}\) showed an RTV-treatment period-dependent decrease, and the mean of \(AUC_{0-\infty}\) in Day

![Fig. 1. Effect of Repeated Oral Doses of RTV on Plasma RTV Concentration vs. Time Curves after Oral Administration to RTV-Treated and Control Rats](image-url)

Each rat group was pretreated with oral RTV (20 mg/kg/d) for indicated days. The dose of RTV for oral administration was 20 mg/kg. Each symbol with bar represents the mean±S.E. of 6—10 rats. Key: ○, control; ●, Day 3; ▲, Day 5; ■, Day 7; ●, Day 14.

### Table 1. Pharmacokinetic Parameters of RTV after Oral Administration to RTV-Treated Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(C_{max}) (μg/ml)</th>
<th>(t_{max}) (h)</th>
<th>(AUC_{0-\infty}) (μg·h/ml)</th>
<th>(t_{1/2}) (h)</th>
<th>MRT (h)</th>
<th>(CL_{app}) (l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.88±0.46</td>
<td>3.06±0.57</td>
<td>31.50±2.86</td>
<td>3.99±0.40</td>
<td>5.84±0.36</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>Day 3</td>
<td>4.29±0.37</td>
<td>3.19±0.55</td>
<td>26.90±2.74</td>
<td>3.81±0.63</td>
<td>5.30±0.37</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td>Day 5</td>
<td>4.07±0.43</td>
<td>3.75±0.65</td>
<td>28.61±2.29</td>
<td>3.03±0.12</td>
<td>6.25±0.49</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td>Day 7</td>
<td>2.33±0.30</td>
<td>4.50±1.00</td>
<td>18.90±2.95*</td>
<td>3.84±0.66</td>
<td>7.64±0.51</td>
<td>0.49±0.09*</td>
</tr>
<tr>
<td>Day 14</td>
<td>2.08±0.26*</td>
<td>4.61±0.81</td>
<td>13.89±2.49**</td>
<td>4.30±0.82</td>
<td>7.97±0.53</td>
<td>0.55±0.10*</td>
</tr>
</tbody>
</table>

Dose of RTV for oral administration was 20 mg/kg. Each value represents the mean±S.E. of 6—10 rats. **p<0.01, *p<0.05 compared with appropriate control.
7 and Day 14 rats decreased significantly from 31.50 ± 2.86 (control) to 18.90 ± 0.90 and 13.89 ± 2.49 mg·h/ml, respectively. Moreover, the CL_{app} in Day 7 and Day 14 rats were 0.49 ± 0.09 and 0.55 ± 0.10 l/h, respectively, and increased significantly as compared to the control. Figure 2 shows the time courses of the mean RTV plasma concentration after i.v. administration alone to the control and RTV-treated rats. The pharmacokinetic parameters of RTV after i.v. administration to these rats are listed in Table 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AUC_{0—\infty} (\mu g·h/ml)</th>
<th>t_{1/2} (h)</th>
<th>MRT (h)</th>
<th>Vd_{ss} (l/kg)</th>
<th>CL_{tot} (l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.53 ± 0.89</td>
<td>1.15 ± 0.15</td>
<td>1.27 ± 0.09</td>
<td>1.06 ± 0.03</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>Day 3</td>
<td>8.36 ± 0.60*</td>
<td>1.06 ± 1.03</td>
<td>1.36 ± 0.10</td>
<td>0.83 ± 0.07</td>
<td>0.17 ± 0.01*</td>
</tr>
<tr>
<td>Day 5</td>
<td>7.97 ± 0.40**</td>
<td>1.03 ± 0.06</td>
<td>1.34 ± 0.04</td>
<td>0.86 ± 0.05</td>
<td>0.18 ± 0.01*</td>
</tr>
<tr>
<td>Day 7</td>
<td>6.56 ± 0.44</td>
<td>0.95 ± 0.17</td>
<td>1.15 ± 0.09</td>
<td>0.89 ± 0.08</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>Day 14</td>
<td>6.45 ± 0.72</td>
<td>0.93 ± 0.15</td>
<td>1.20 ± 0.08</td>
<td>0.98 ± 0.08</td>
<td>0.24 ± 0.02</td>
</tr>
</tbody>
</table>

Dose of RTV for intravenous administration was 5 mg/kg. Each value represents the mean ± S.E. of 6—7 rats. *p < 0.05, **p < 0.01 compared with appropriate control.
administration of EM alone to the control and RTV-treated rats. The pharmacokinetic parameters of EM after i.v. administration to these rats are listed in Table 3. The value of $AUC_{0-\infty}$ in Day 3 rats increased significantly as compared to the control from 30.00 to 36.89 ng·h/ml, while that in Day 7 rats decreased significantly from 1.61±0.16 to 0.29±0.03 ng·h/ml. The values of $CL_{\text{tot}}$ and $Vd_{\text{ss}}$ of EM increased markedly. In rats treated with RFP for 7 d, Day 7RFP, the value of $AUC_{0-\infty}$ of EM decreased significantly as compared to the control. Figure 4 shows the time courses of the mean MDZ plasma concentration after i.v. administration of MDZ alone to the control and RTV-treated rats. The pharmacokinetic parameters of MDZ after i.v. administration to these rats are listed in Table 4. The values of $AUC_{0-\infty}$ in Day 3 and Day 5 rats increased significantly as compared to the control from 30.00±1.05 to 36.89±1.69 ng·h/ml and 30.00±1.05 to 52.54±1.40 ng·h/ml, respectively, while there were no significant comparative changes in these values in Day 7 and Day 14 rats. Once the values of $CL_{\text{uni}}$ significantly decreased in Day 3 and Day 5 rats, however, these values in Day 7 and Day 14 rats returned to control levels. Figure 5 shows the intestinal and biliary excretion rates of Rho123 from the blood circulation to the intestinal lumen and bile duct after i.v. administration to the control and RTV-treated rats. The pharmacokinetic parameters of Rho123 are listed in Table 5. There were no notable changes in the plasma Rho123 concentration vs. time courses (Fig. 5a) or its representative pharmacokinetic parameters $AUC_{0-\infty}$ and $CL_{\text{uni}}$. However, total excreted amounts of Rho123 into the intestinal lumen and bile increased significantly in a RTV-treated time dependent manner (Figs. 5b, c). The excretion clearances from systemic circulation to the intestinal lumen ($CL_{\text{ex,int}}$) in Day 14 rats increased markedly by 2.2-fold, and the excretion clearances of Rho123 from the systemic circulation to the bile ($CL_{\text{ex,bile}}$) also increased by 2.6-fold, as compared to the control (Table 5).

### Table 3. Pharmacokinetic Parameters of EM after Intravenous Administration to RTV-Treated Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$AUC_{0-\infty}$ (µg·h/ml)</th>
<th>$t_{1/2}$ (h)</th>
<th>MRT (h)</th>
<th>$Vd_{\text{ss}}$ (l/kg)</th>
<th>$CL_{\text{uni}}$ (l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.61±0.16</td>
<td>1.35±0.23</td>
<td>1.21±0.20</td>
<td>0.65±0.12</td>
<td>0.54±0.04</td>
</tr>
<tr>
<td>Day 3</td>
<td>2.45±0.25*</td>
<td>1.54±0.28</td>
<td>1.59±0.35</td>
<td>0.57±0.09</td>
<td>0.37±0.04*</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.29±0.03***</td>
<td>1.12±0.03</td>
<td>0.93±0.10</td>
<td>2.89±0.62*</td>
<td>3.00±0.37***</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.93±0.17*</td>
<td>1.03±0.11</td>
<td>0.95±0.19</td>
<td>1.00±0.32</td>
<td>0.99±0.16*</td>
</tr>
</tbody>
</table>

Dose of EM for intravenous administration was 3 mg/kg. Each value represents the mean±S.E. of 4—5 rats. **$p<0.001$, $p<0.01$, *$p<0.05$ compared with appropriate control.**

### Table 4. Pharmacokinetic Parameters of MDZ after Intravenous Administration to RTV-Treated Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$AUC_{0-\infty}$ (ng·h/ml)</th>
<th>$t_{1/2}$ (h)</th>
<th>MRT (h)</th>
<th>$Vd_{\text{ss}}$ (l/kg)</th>
<th>$CL_{\text{uni}}$ (l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.00±1.05</td>
<td>0.98±0.05</td>
<td>0.94±0.05</td>
<td>0.93±0.05</td>
<td>0.99±0.03</td>
</tr>
<tr>
<td>Day 3</td>
<td>36.89±1.69**</td>
<td>0.99±0.10</td>
<td>0.94±0.05</td>
<td>0.80±0.07</td>
<td>0.85±0.03*</td>
</tr>
<tr>
<td>Day 5</td>
<td>52.54±1.40***</td>
<td>1.06±0.04</td>
<td>0.93±0.03</td>
<td>0.51±0.02**</td>
<td>0.54±0.02***</td>
</tr>
<tr>
<td>Day 7</td>
<td>33.04±4.00</td>
<td>0.94±0.04</td>
<td>0.87±0.03</td>
<td>0.82±0.09</td>
<td>0.95±0.10</td>
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<tr>
<td>Day 14</td>
<td>29.59±1.35</td>
<td>0.83±0.08</td>
<td>0.86±0.04</td>
<td>0.85±0.06</td>
<td>0.99±0.04</td>
</tr>
</tbody>
</table>

Dose of MDZ for intravenous administration was 0.1 mg/kg. Each value represents the mean±S.E. of 4—5 rats. **$p<0.001$, $p<0.01$, *$p<0.05$ compared with appropriate control.**
Table 5. Pharmacokinetic Parameters of Rho123 after Intravenous Administration to RTV-Treated Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AUC_{0—t last} (ng·h/ml)</th>
<th>t_{1/2} (h)</th>
<th>CL_{tot} (ml/h)</th>
<th>CL_{ex,int} (ml/h)</th>
<th>CL_{ex,bile} (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>87.33±6.54</td>
<td>1.15±0.15</td>
<td>407.6±79.3</td>
<td>5.39±0.91</td>
<td>24.72±1.35</td>
</tr>
<tr>
<td>Day 3</td>
<td>87.35±4.39</td>
<td>1.06±1.03</td>
<td>356.2±29.1</td>
<td>8.77±0.70*</td>
<td>35.87±5.00*</td>
</tr>
<tr>
<td>Day 5</td>
<td>88.25±7.29</td>
<td>1.03±0.06</td>
<td>336.2±31.2</td>
<td>12.04±1.76*</td>
<td>42.36±2.99**</td>
</tr>
<tr>
<td>Day 7</td>
<td>93.43±12.02</td>
<td>0.95±0.17</td>
<td>376.5±74.0</td>
<td>10.02±0.69*</td>
<td>47.10±7.32*</td>
</tr>
<tr>
<td>Day 14</td>
<td>81.44±1.63</td>
<td>0.93±0.15</td>
<td>361.4±26.1</td>
<td>12.02±0.41*</td>
<td>58.92±3.78***</td>
</tr>
</tbody>
</table>

Each rat group was pretreated with oral RTV (20 mg/kg/d) for indicated days. The dosage of Rho123 for intravenous administration was 0.2 mg/kg. Each symbol with bar represent mean±S.E. of 4—5 rats. Key: ( ), control; (■, ○), Day 3; (□, ●), Day 5; (△, ■), Day 7; ( ■, ○), Day 14.

Dose of Rho123 for intravenous administration was 0.2 mg/kg. Each value represents the mean±S.E. of 4—5 rats. ***p<0.001, **p<0.01, *p<0.05 compared with appropriate control.
DISCUSSION

RTV is known to have the most potent inhibitory effect on the metabolism of any PI currently used. Several reports demonstrated that RTV has the lowest metabolic clearance rate among PIs in rat or human liver microsomal fraction, and that inhibition constants of RTV for other PIs, such as nelfinavir, saquinavir, amprenavir or indinavir, show relatively small values. As a result of the strong inhibitory effect of other PIs currently used in clinical practice, RTV is used mainly as a booster drug to increase the bioavailability of other PIs in dual protease therapy. However, results of clinical study showed that a chronic use of RTV decreased the plasma levels of other PIs in dual protease therapy, where RTV is considered to be an inducer for other PIs despite its being known to be a potent inhibitor. In the present study, therefore, we examined the effect of chronic exposure of RTV on its plasma concentration of RTV itself and other drugs that are regulated via CYP 3A and/or Pgp.

As shown in Fig. 1, plasma concentration of RTV after oral administration had no notable change until day 5 after the treatment, whereas in Day 7 and Day 14 rats, the $AUC_{0-\infty}$ values showed significant decreases by 28% and 47%, respectively, as compared to the control, though rats received the same daily dose of RTV throughout the study period (Table 1). On the other hand, the $AUC_{0-\infty}$ values after i.v. administration of RTV in Day 3 and Day 5 rats increased significantly as compared to the control, however, those in Day 7 and Day 14 rats returned to control levels (Fig. 2, Table 2). Since the pharmacokinetic parameters after i.v. administration reflect mainly the contribution of the liver, the metabolism of RTV via CYP3A in the liver in Day 3 or Day 5 rats was inhibited by RTV pretreatment. This phenomenon must be based on the self-inhibition of RTV. In the case of MDZ after i.v. administration, the $AUC_{0-\infty}$ values in Day 3 and Day 5 rats increased by 23% and 75%, respectively, and these observations agreed with the case of RTV (Fig. 4, Table 4). MDZ is a known CYP 3A substrate that is reported as an ideal probe for CYP3A activity not only in vitro but also in vivo, because it is completely excreted through metabolism and is not a substrate of Pgp. Therefore, MDZ is commonly used in vitro to monitor CYP3A activity. Hence, in the early phase of repeated administration of RTV, it inhibits CYP3A-mediated metabolism of drugs and its own originated in mechanism-based inhibition of RTV. The facts that our in vivo data of RTV or MDZ in Day 7 and Day 14 rats after i.v. administration returned to the control levels, on the other hand, suggest that CYP3A induction in the liver would occur on and after Day 5 by repeated administration of RTV. To check the CYP3A mediated-induction in the liver, EM, which is used for the erythromycin breath test as a representative substrate of CYP3A4, was adopted for i.v. study. As shown in Table 3, the $AUC_{0-\infty}$ of EM in Day 3 rats increased by 52% as compared to the control, while, in Day 7 rats, it decreased by 82% as compared to the control. Since EM is a substrate of CYP3A and Pgp, while MDZ is a substrate of CYP3A, a marked decrease in the EM $AUC_{0-\infty}$ in Day 7 rats indicated that an enhanced Pgp function greatly contributed to the elimination of EM. Moreover, in mdr1a knockout mice, the brain/blood concentration ratio of EM showed an 8-fold increase as compared to syngeneic animals, and in human bile is about 10-fold larger than that in plasma. Therefore, it is, therefore, believed that both enhancement of Pgp function and inducing effect of CYP3A by RTV provided a marked reduction in the $AUC_{0-\infty}$ of EM in Day 7 rats. Moreover, in rats treated with a known inducer RFP (Day 7Ref), the $AUC_{0-\infty}$ of EM decreased by 42% as compared to the control. These observations clearly support that RTV has a potent inducing effect on CYP3A in the liver. The in vitro inducing effect of RTV has been confirmed using rat liver microsomes and primary cultured of human hepatocytes. A possible mechanism is that CYP3A protein in the liver is induced due to the activation of a nuclear orphan receptor, namely pregnan X receptor (PXR) by RTV.

Secondly, the effects of repeated administration of RTV on the function of Pgp were examined using a known substrate of Pgp, Rho123. Pgp has been shown to be present and to function as a transporter in the plasma membranes of many normal tissues, and Pgp in the enterocyte brush border membrane limits the bioavailability of many drugs that are metabolized by CYP3A. After i.v. administration of Rho123, the biliary and intestinal excretion clearances increased in a RTV treatment period-dependent manner (Figs. 5b, c, Table 5). To check the remaining effects of RTV during repeated p.o. administration, we measured the trough levels of RTV before experiments. These trough levels in plasma were found to be from 0.02 to 0.05 μM throughout the experiments. Drewes et al. reported that the 50% inhibitory concentration (IC50) of RTV for Pgp in MDR-P388 cells using saquinavir as a Pgp substrate was 0.2 μM. Since the trough levels of RTV were low in our study were far below this IC50, the remaining effect of RTV under repeated administration on the Pgp function was thought to be negligible. Therefore, these observations indicated that the function of Pgp in the gut cells and/or bile duct were enhanced by repeated administration of RTV, or that the amount of Pgp in these tissues was elevated due to the induction of Pgp by RTV.

According to the report of Perloff et al., RTV caused increased expression of CYP3A isofoms and of Pgp with 3 d of its exposure in rats. Our results in Table 5 clearly demonstrated that Pgp had already been induced within this 3 d exposure of RTV, because the $CL_{ex, int}$ and $CL_{bile}$ but not $CL_{int}$ of Rho123 (a substrate of Pgp) increased in Day 3 rats. As shown in Table 4, the $CL_{int}$ of MDZ (a substrate of CYP3A) had already decreased in Day 3 rats. By prediction of our results of i.v. administration, the $AUC_{0-\infty}$ of RTV in Day 3 or Day 5 rats after p.o. administration was not so changed. Based on these observations, RTV had already shown both inhibitory and inducing effects on CYP3A and an inducing effect for Pgp in Day 3 rats, and the inactivation of CYP3A by the mechanism-based inhibition of RTV would mask the induction effect of RTV. Therefore, net balance in the plasma concentrations in Day 3 or Day 5 rats after oral administration seems to even out. In contrast, the inducing effects of RTV for CYP3A or Pgp in the chronic phase of RTV exposure (Day 7 and Day 14) would be superior to the mechanism-based inhibition for CYP3A. In the chronic phase, an induced Pgp function would decrease the absorption of RTV from the intestinal tract, and would enhance the elimination of RTV metabolites that exhibit a mechanism-based inhibition.

It has been reported that coadministration of digoxin (a...
well established Pgp substrate) with RTV after concomitant administration of RTV increased systemic availability of digoxin in healthy volunteers,35,36 where the nonrenal clearance of digoxin was reduced most likely through the inhibitory effect of RTV on the liver CYP3A or effluxed levels of RTV or other drugs that are metabolized by CYP3A. Thereafter, in a chronic administration phase, RTV induces the liver CYP3A increase because of the inhibitory effect of RTV on the liver CYP3A. Moreover, digoxin is primarily excreted via the biliary route, and the inhibitory effect appears in a case in which a drug is administered alone after continuous administration of the inducer, and the inhibitory effect appears in a case in which a next dose of inducer is changed during co-administration.

In summary, we clarified that RTV is not only a potent inducer and a potent inhibitor of CYP3A, and is also a potent inducer of Pgp function, both of which are responsible for regulating oral bioavailability of drugs. In the early phase of repeated administration of RTV, the blood levels of RTV or other drugs that are metabolized via CYP3A increase because of the inhibitory effect of RTV on the liver CYP3A. Thereafter, in a chronic administration phase, RTV induces the liver CYP3A and intestinal Pgp activities, and the blood levels of RTV or other drugs that are metabolized via CYP3A or effluxed via Pgp decrease because of the inducing effect of the liver CYP3A and the intestinal Pgp. In clinical practice, these triple effects of RTV must be considered during chronic oral administration to justify the HAART in HIV-infected patients.

REFERENCES