Mechanisms of Pharmacokinetic Enhancement Between Ritonavir and Saquinavir; Micro/Small Dosing Tests Using Midazolam (CYP3A4), Fexofenadine (p-Glycoprotein), and Pravastatin (OATP1B1) as Probe Drugs

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Abstract

We investigated the mechanisms of ritonavir-mediated enhancement effect on the pharmacokinetics of saquinavir using in vivo probes for CYP3A4 (midazolam), p-glycoprotein (fexofenadine), and OATP1B1 (pravastatin) following oral micro/small dosing. A cocktail of the drugs (2 mg of saquinavir, 100 μg of each probe) was administered to eight healthy volunteers (phase 1), and then coadministered with 20 mg (phase 2) and 100 mg (phase 3) of ritonavir. Plasma concentrations of the drugs were measured by validated LC–MS/MS methods. The mean plasma AUC0-24 (µg·h/mL) of saquinavir at phases 1, 2, and 3 was 101, 2350, and 24,390 (P < 0.01), respectively. The relative area under the plasma concentration-time curve (AUC0-24 ratio) of midazolam and fexofenadine at phases 1, 2, and 3 were 1.09, 1.47 (P < 0.01), and 1.42.2 (P < 0.01–0.05), respectively. In contrast, there was no difference in the pharmacokinetics of pravastatin. Inhibition of intestinal and hepatic CYP3A-mediated metabolism, and intestinal p-glycoprotein-mediated efflux of saquinavir, but not OATP1B1, is involved in the enhancement mechanism. Micro/small dosing is useful for examining the mechanism of drug interactions without safety concern.

Keywords

boosting, fexofenadine, midazolam, ritonavir, saquinavir

Saquinavir and ritonavir are used for the treatment of human immunodeficiency virus infections, and well known inhibitors for cytochrome P450 (CYP) 3A4.1,2 Saquinavir is also a good substrate for CYP3A isozymes.3 Since the bioavailability of saquinavir is extremely low (e.g., 4%), co-administration with ritonavir, so-called ritonavir-mediated pharmacokinetic enhancement (boosting) of saquinavir, has been used to increase the exposure of saquinavir.3,5 Some clinical studies showed that coadministration of ritonavir increased the plasma AUC and Cmax of saquinavir.3,5 In order to evaluate the mechanisms behind this interaction, clinical studies have been conducted.6–8 In healthy volunteers, the i.v. clearance of midazolam, a substrate of CYP3A4, showed a dose-dependent decrease with increasing ritonavir doses.9 Furthermore, oral clearance of midazolam was also decreased (over 13-fold) in HIV-infected patients treated with ritonavir compared with those not treated with ritonavir.10 Thus, a large reduction in the first-pass metabolism and postabsorptive clearance of saquinavir.

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probably through the inhibition of CYP3A4, is thought to be involved in this potential interaction.

Saquinavir and ritonavir are also inhibitors and substrates for p-glycoprotein. Since digoxin and fexofenadine are used as in vivo probes for p-glycoprotein functions without interference with metabolic pathways, the inhibitory potential of multiple administrations of ritonavir and/or saquinavir on the pharmacokinetics of oral digoxin/fexofenadine has been evaluated. Co-administration of ritonavir and saquinavir resulted in a significantly increased \( C_{\text{max}} \) and AUC\(_{0-72} \) without changes in the renal clearance of digoxin. Similarly, single administration of ritonavir increased AUC (infinity) of fexofenadine without changes in the elimination half-life. These results suggest that the mechanism responsible for the interaction between ritonavir and digoxin/ fexofenadine is an inhibition of p-glycoprotein.

Finally, recent in vitro data have suggested that human organic anion transporting polypeptide (OATP) family transporters, which are hepatic uptake transporters, are involved in the pharmacokinetics of some kinds of protease inhibitors. The Xenopus laevis oocyte model showed that saquinavir and lopinavir are substrates for OATP1A2, OATP1B1, and OATP1B3, while darunavir is a substrate for OATP1A2 and OATP1B1. Interestingly, saquinavir and ritonavir strongly inhibited OATP1B1-mediated transport activity with the inhibition constants \( K_i \) of 1.8 \( \mu \)mol/L and 1.4 \( \mu \)mol/L, respectively. Since OATP1B1 is specifically expressed in the basolateral membrane of human hepatocytes, many clinically important drug interactions that can be attributed to OATP1B1 inhibition have been observed. For example, cyclosporine is associated with a remarkable increase in the plasma AUC of some statins through the inhibition of OATP1B1-mediated uptake into the liver.

Taking these observations into consideration, CYP3A4, p-glycoprotein, and OATP1B1, may be involved in the interaction between ritonavir and saquinavir. The aim of the present study was to quantitatively test the inhibitory effect of ritonavir on CYP3A4 and p-glycoprotein in the liver and small intestine, and hepatic OATP1B1 using three substrates as probe, midazolam for CYP3A4, fexofenadine for p-glycoprotein, and pravastatin for OATP1B1. Furthermore, the micro/small dosing approach was incorporated into the study in order to avoid side effects, considering a possibility that administering a cocktail of test drugs at therapeutic dosages may result in high serum concentrations of drugs.

Materials and Methods

Subjects

This study was approved by the Ethics Review Boards of Kyushu University, the Graduate School of Pharmaceuti-
of 50% acetonitrile, and 500 µL of water were added. The samples were loaded onto OASIS HLB 96-Well Plate 10 mg (30 µm; Waters, Milford, MA). The compounds were eluted with 200 µL of methanol and 10 mM ammonium formate/formic acid (1000:1, v/v) solution, and the eluate was injected into the LC–MS/MS system: an LC-20AD system (Shimadzu, Kyoto, Japan) and a QTRAP 5500 mass spectrometer (AB Sciex, Foster City, CA). Quantitation was performed by multiple reaction monitoring (MRM) in the positive ion mode. Chromatographic separation was achieved on a CAPCELL PAK C18 MG column (35 mm x 4.6 mm i.d., particle size 5 µm; Shiseido, Tokyo, Japan). The mobile phase, consisting of 10 mM ammonium formate/formic acid (1000:1, v/v; solution A) and methanol (solution B), was pumped for 6.01 minutes at a flow rate of 0.5 mL/minutes. High performance liquid chromatography (HPLC) separation was done using a linear gradient of 50–90% solution B for 2 minutes, 90% solution B for 2 minutes, 90–50% solution B for 0.01 minutes, and 50% solution B for 2 minutes.

The mass transition was from m/z 671.4 to 570.3 for saquinavir and from m/z 680.4 to 570.3 for the IS. The calibration curve was linear over the standard concentration range of 0.5–100 pg/mL. In this study, plasma samples were diluted within the range by individual blank plasma, when drug concentrations were above the upper limit of the calibration curves. The lower limit of quantification (LLOQ) was defined as the lowest concentration of the calibration curve with acceptable precision (less than 20% for the coefficient of variation) and accuracy (within ±20% for relative error). The LLOQ samples were excluded from the pharmacokinetic analysis.

**Ritonavir.** To each plasma sample (100 µL), IS solution (amprenavir, Toronto Research Chemicals; 2 µmol/L in 50% acetonitrile, 5 µL), 5 µL of 50% acetonitrile, and 500 µL of water were added. The sample was loaded onto OASIS HLB 1 cc (10 mg) columns (Waters). The compound was eluted with 1 mL of acetonitrile. The eluate was evaporated with a CO-105 Centrifuigal Concentrator (TOMY, Tokyo, Japan) for 40 minutes and mixed with 100 µL of 0.1% formic acid. Then, the sample was centrifuged with a 1-13 microcentrifugator (Kubota Corp., Tokyo, Japan) at 16 000 g for 10 minutes at 4°C and 50 µL of supernatant was injected into the LC–MS/MS system: an Alliance 2695 system (Waters) and a Micromass Quattro micro™ API (Waters). Quantitation was performed by MRM in the positive ion mode. Chromatographic separation was achieved on an Atlantis T3 column (50 mm x 2.1 mm i.d., particle size 5 µm; Waters). The mobile phase, consisting of 0.1% formic acid (solution A) and acetonitrile (solution B), was pumped for 3 minutes at a flow rate of 0.4 mL/minutes. HPLC separation was done using a linear gradient of 30–90% solution B for 1 minutes and 90–30% solution B for 2 minutes.

The mass transition was from m/z 721.4 to 197.2 for ritonavir and from m/z 506.1 to 418.2 for the IS. The calibration curve was linear over the standard concentration range of 1.5–4 500 ng/mL.

**Midazolam.** After plasma samples were obtained, 550 µL of 100 mM acetic buffer (pH 5.0) was added immediately to 1.1 mL of each plasma sample. IS solution (rosuvastatin, Toronto Research Chemicals; 20 ng/mL, 10 µL) and 1 mL of 100 mM acetic buffer (pH 5.0) were added to 1.5 mL of the mixture. The samples were loaded onto Inertsil Pharma cartridges (GL Science, Tokyo, Japan). The eluate with methanol was evaporated under nitrogen gas and mixed with acetonitrile containing 1 mM ammonium acetate/water (40:60, v/v) buffered with acetate (pH 5.0). The solution purified by filtration (Centricat W-MO, 0.45 µm; Kurabo Industries, Osaka, Japan) was injected into the LC–MS/MS system: an ACQUITY ultra performance liquid chromatograph (UPLC) system (Waters) and an API 5000 mass spectrometer (AB Sciex). Quantitation was performed by MRM in the positive ion mode. Chromatographic separation was achieved on an ACQUITY UPLC BEH Shield RP18 column (150 mm x 2.1 mm i.d., particle size 1.7 µm; Waters) using acetonitrile/0.1% formic acid (70:30, v/v) as the mobile phase at a flow rate of 0.2 mL/minutes. The mass transition was from m/z 326 to 291 for midazolam and from m/z 482 to 258 for the IS. The calibration curve was linear over the standard concentration range of 5–200 pg/mL.

**Fexofenadine.** To each plasma sample (200 µL), IS solution (terfenadine, Sigma–Aldrich, Tokyo, Japan; 0.5 ng/mL, 20 µL) and 0.4 mL of 10 mM acetic buffer (pH 4.0) were added. The samples were loaded onto OASIS HLB cartridges (Waters). The compounds were eluted with acetonitrile/water (70:30, v/v) and the eluate was injected into the LC–MS/MS system: an ACQUITY UPLC system (Waters) and an API 5000 mass spectrometer (AB Sciex). Quantitation was performed by MRM in the positive ion mode. Chromatographic separation was achieved on an XBridge C18 column (100 mm x 2.1 mm i.d., particle size 3.5 µm; Waters) using acetonitrile/2 mM ammonium acetate (9:1, v/v) as the mobile phase at a flow rate of 0.6 mL/minutes. The mass transition was from m/z 502 to 466 for fexofenadine and from m/z 472 to 436 for the IS. The calibration curve was linear over the standard concentration range of 10–1 000 pg/mL.

**Pravastatin.** To each plasma sample (250 µL), IS solution (R-122798, kindly provided from Daiichi-Sankyo Co. Ltd., Tokyo, Japan; 12.5 ng/mL, 20 µL) and 250 µL of water were added. To each urine sample (500 µL), IS solution (R-122798; 25 ng/mL, 20 µL) and 500 µL of water were added. The samples were loaded onto Oasis MAX cartridges (Waters). The eluate with methanol containing 0.1% acetate was evaporated under
nitrogen gas and mixed with acetonitrile/water (25:75, v/v). The solution purified by filtration (Ultrafree-MC centrifugal filter units, 0.22 μm; Millipore, Billerica, MA) was injected into the LC–MS/MS system: a Prominence UFLC system (Shimadzu, Kyoto, Japan) and an AB Sciex 5500 QTRAP mass spectrometer (AB Sciex). Quantitation was performed by MRM in the negative ion mode. Chromatographic separation was achieved on an Inertsil ODS-3 column (50 mm x 2.1 mm i.d., particle size 2 μm; GL Science). The mobile phase, consisting of acetonitrile (solution A) and acetonitrile/0.001% acetic acid (solution B), was pumped for 11 minutes at a flow rate of 0.2 mL/minute and then for 2 minutes at a flow rate of 0.3 mL/minute. HPLC separation was conducted as follows: 100% solution B for 0.5 minutes, a linear gradient of 100–45% solution B for 4.5 minutes, 45% solution B for 6 minutes, 45–100% solution B for 0.1 minutes, and 100% solution B for 2.9 minutes. The mass transition was from m/z 423 to 321 for pravastatin, m/z 423 to 321 for RMS-416, and m/z 409 to 321 for the IS. The calibration curve was linear over the standard concentration range of 5–10 000 pg/mL for pravastatin and RMS-416 in plasma, and 10–10 000 pg/mL for pravastatin and RMS-416 in urine.

Pharmacokinetic Analysis
The pharmacokinetic analysis was performed using WinNonlin 6.1 (Pharsight, Mountain View, CA). The AUC_{0-24} values of saquinavir, midazolam, fexofenadine, pravastatin, and ritonavir were calculated by the linear trapezoidal rule. C_{max} and T_{max} were obtained directly from the data. The Ke was calculated using least-squares regression from the terminal post-distribution phase of the concentration–time curve. The CL/F and t_{1/2} were calculated as follows: CL/F = dose/AUC_{0-∞} and t_{1/2} = 0.693/Ke.

Statistical Analysis
The pharmacokinetic variables between study phases were compared using the paired t-test. P < .05 was considered statistically significant. With Bonferroni’s correction, the threshold for significance would be P < .0167. The T_{max} data were compared using the Wilcoxon signed-rank test.

Results
No clinically undesirable signs or symptoms possibly attributable to the administration of the drugs were recognized throughout the study. All subjects completed the study successfully according to the protocol.

The mean plasma concentration time profiles of the test drugs at the three phases and corresponding changes in the pharmacokinetic parameters are shown in Figure 1 and Tables 1 and 2, respectively. Ritonavir increased the plasma concentrations of saquinavir significantly for phases 2 and 3, in a dose-dependent manner (Fig. 1). The large increases in AUC_{0-24} and C_{max} values were highly statistically significant (P < .01). The saquinavir AUC_{0-24} ratio in phases 2 and 3 with phase 1 as the reference ranged from 26 to 243, and the corresponding C_{max} ratio ranged from 14 to 82 (Table 1). In contrast, the increase in the t_{1/2} value was small; approximately double of the phase 1 control value.

Co-administration of ritonavir led to an increase in midazolam AUC_{0-24} values by approximately sixfold from 1 420 at baseline to 8 200 pg hour/mL at phase 2, and 15-fold to 19 200 pg hour/mL at phase 3, whereas midazolam C_{max} values increased from 657 pg/mL to 1 350 pg/mL (twofold) and to 2 010 pg/mL (threefold) at phases 2 and 3, respectively (Table 1). Similar to the AUC_{0-24} values, the t_{1/2} of midazolam increased fourfold (from 2.2 hours to 8.5 hours) at phase 2 and 12-fold (to 26.6 hours) at phase 3, demonstrating the impact of ritonavir administration on exposure to midazolam.

Following the co-administration of ritonavir, the plasma concentration of fexofenadine was increased. The mean AUC_{0-24} value increased from 270 at baseline to 4 580 pg hour/mL (1.4-fold) at phase 2, and to 7 120 pg hour/mL (2.2-fold) at phase 3 (Table 1). Similarly, the mean C_{max} value increased from 489 at baseline to 761 pg/mL (1.6-fold) at phase 2, and to 1 250 pg/mL (2.7-fold) at phase 3. While there was clear evidence for an effect of ritonavir on fexofenadine AUC_{0-24} and C_{max} values, this was not the case for the t_{1/2}; the mean t_{1/2} value was comparable among the three study phases.

In contrast to saquinavir, midazolam, and fexofenadine, there was no significant change in any pharmacokinetic parameters (including urinary excretions) of pravastatin and RMS-416 among the three phases (Table 1).

Figure 2 shows individual changes in the AUC_{0-24} of the four test drugs at the three phases. Among three in vivo probe drugs, the AUC_{0-24} values of midazolam and fexofenadine were increased in the most subjects in a dose-dependent manner of ritonavir. Compared with the control values, the change in the AUC_{0-24} was most remarkable for midazolam, followed by fexofenadine at 100 mg of ritonavir.

Table 2 shows pharmacokinetic parameters of ritonavir under two dosage conditions. Increasing the dosage of ritonavir from 20 mg to 100 mg appeared to result in a remarkably non-proportional increase in ritonavir exposure; 235 at 20 mg to 10 737 ng hour/mL at 100 mg for the AUC_{0-24}. A similar trend was observed for the C_{max} value; 44.7 at 20 mg to 1,626 ng/mL at 100 mg.

Discussion
Following simultaneous administration of the three probe drugs, the possible mechanisms of the interaction between
Figure 1. Mean (±SD) plasma concentration–time profiles of the test drugs (saquinavir 2 mg, 100 μg each of midazolam, fexofenadine, and pravastatin) with and without ritonavir (RTV, 20 mg and 100 mg) in eight healthy volunteers.

Table 1. Pharmacokinetic Parameters of Test Drugs at the Three Study Phases

<table>
<thead>
<tr>
<th>Probe Drug</th>
<th>Pharmacokinetic Parameter</th>
<th>Control (A)</th>
<th>RTV + 20 mg (B)</th>
<th>Ratio (B)/A</th>
<th>RTV + 100 mg (C)</th>
<th>Ratio (C)/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saquinavir (2 mg)</td>
<td>AUCO−24 (pg h/mL)</td>
<td>101 ± 30.6</td>
<td>2.540 ± 1.230††</td>
<td>26.0 ± 11.3</td>
<td>23.900 ± 6.400††</td>
<td>243 ± 55.8</td>
</tr>
<tr>
<td></td>
<td>t1/2 (h)</td>
<td>4.60 ± 3.03</td>
<td>7.83 ± 2.22</td>
<td>2.30 ± 1.22</td>
<td>6.34 ± 0.85</td>
<td>1.85 ± 0.94</td>
</tr>
<tr>
<td></td>
<td>Cmax (pg/mL)</td>
<td>69.4 ± 30.2</td>
<td>988 ± 602††</td>
<td>13.8 ± 5.76</td>
<td>5.190 ± 1.760††</td>
<td>81.9 ± 27.5</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>0.5</td>
<td>0.69 ± 0.37</td>
<td>1.38 ± 0.74</td>
<td>1.38 ± 0.52</td>
<td>2.75 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>CL/F (L/h)</td>
<td>19.700 ± 6.960</td>
<td>918 ± 444††</td>
<td>0.05 ± 0.03</td>
<td>86.1 ± 25.8††</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>Midazolam (100 μg)</td>
<td>AUCO−24 (pg h/mL)</td>
<td>1.420 ± 0.496</td>
<td>8.200 ± 3.510††</td>
<td>5.91 ± 1.89</td>
<td>19.200 ± 3.020††</td>
<td>14.7 ± 4.58</td>
</tr>
<tr>
<td></td>
<td>t1/2 (h)</td>
<td>2.23 ± 0.45</td>
<td>8.51 ± 4.04††</td>
<td>3.68 ± 1.30</td>
<td>26.6 ± 9.47††</td>
<td>12.1 ± 3.77</td>
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<tr>
<td></td>
<td>Cmax (pg/mL)</td>
<td>657 ± 170</td>
<td>1.350 ± 449††</td>
<td>2.09 ± 0.60</td>
<td>2.010 ± 4.54††</td>
<td>3.26 ± 1.25</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>0.56 ± 0.18</td>
<td>0.75 ± 0.27</td>
<td>1.38 ± 0.52</td>
<td>1.13 ± 0.44</td>
<td>2.13 ± 0.99</td>
</tr>
<tr>
<td></td>
<td>CL/F (L/h)</td>
<td>75.0 ± 24.5</td>
<td>12.9 ± 7.66††</td>
<td>0.17 ± 0.07</td>
<td>2.71 ± 0.71††</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>Fexofenadine (100 μg)</td>
<td>AUCO−24 (pg h/mL)</td>
<td>3.270 ± 0.656</td>
<td>4.580 ± 1.370†</td>
<td>1.41 ± 0.39</td>
<td>7.120 ± 1.810††</td>
<td>2.19 ± 0.44</td>
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<tr>
<td></td>
<td>t1/2 (h)</td>
<td>7.75 ± 1.59</td>
<td>7.03 ± 1.43</td>
<td>0.94 ± 0.27</td>
<td>7.03 ± 0.88</td>
<td>0.94 ± 0.19</td>
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<tr>
<td></td>
<td>Cmax (pg/mL)</td>
<td>489 ± 183</td>
<td>761 ± 281†</td>
<td>1.61 ± 0.44</td>
<td>1.250 ± 452††</td>
<td>2.69 ± 0.80</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>1.13 ± 0.23</td>
<td>1.13 ± 0.44</td>
<td>1.06 ± 0.50</td>
<td>1.31 ± 0.46</td>
<td>1.21 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>CL/F (L/h)</td>
<td>28.6 ± 4.87</td>
<td>22.0 ± 7.24†</td>
<td>0.76 ± 0.18</td>
<td>13.8 ± 3.35††</td>
<td>0.48 ± 0.10</td>
</tr>
<tr>
<td>Pravastatin (100 μg)</td>
<td>AUCO−24 (pg h/mL)</td>
<td>432 ± 278</td>
<td>515 ± 371</td>
<td>1.18 ± 0.40</td>
<td>567 ± 286</td>
<td>1.42 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>t1/2 (h)</td>
<td>2.70 ± 0.95</td>
<td>2.16 ± 0.69</td>
<td>0.88 ± 0.37</td>
<td>2.10 ± 0.28</td>
<td>0.86 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>Cmax (pg/mL)</td>
<td>142 ± 108</td>
<td>170 ± 118</td>
<td>1.28 ± 0.52</td>
<td>191 ± 73.8</td>
<td>1.67 ± 0.94</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>1.06 ± 0.18</td>
<td>1.06 ± 0.32</td>
<td>1.00 ± 0.27</td>
<td>1.19 ± 0.26</td>
<td>1.15 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>CL/F (L/h)</td>
<td>265 ± 105</td>
<td>245 ± 120</td>
<td>0.92 ± 0.27</td>
<td>201 ± 81.0</td>
<td>0.80 ± 0.20</td>
</tr>
</tbody>
</table>

Parameters are presented as the mean ± SD. RTV, ritonavir.
*P < .05 versus control; †P < .01 versus control; ‡P < .05 versus 20 mg; ‡‡P < .01 versus 20 mg.
saquinavir and ritonavir can be estimated. Ritonavir had a pronounced effect on the pharmacokinetics of midazolam, followed by saquinavir.

Midazolam is known to be significantly metabolized in the intestine\textsuperscript{25}; the CYP3A isoforms (CYP3A4/5) inhibitory effect is pronounced after oral administration of midazolam compared with that after intravenous administration. However, since midazolam clearance was also reported to be decreased significantly following intravenous midazolam plus oral ritonavir administration,\textsuperscript{6} inhibition of hepatic metabolism is also involved in this interaction.\textsuperscript{26} Saquinavir undergoes extensive first-pass metabolism by CYP3A isozymes in the intestine and liver; this is one of the main reasons for its extremely low bioavailability.\textsuperscript{3} So, the most conceivable mechanism behind the potential interaction between ritonavir and saquinavir is inhibition of CYP3A-mediated first-pass metabolism and postabsorptive clearance of saquinavir.

Fexofenadine is often used as an \textit{in vivo} probe to investigate the significance of p-glycoprotein function without interference with metabolic pathways.\textsuperscript{27,28} As shown in Table 1, ritonavir increased fexofenadine \(\text{AUC}_{0-24}\) and \(\text{C}_{\text{max}}\) values, with comparatively minimal effects on the \(t_{1/2}\) value. These results suggest that the most likely mechanism between fexofenadine and ritonavir is the inhibition of intestinal p-glycoprotein function by ritonavir, leading to an enhanced absorption of fexofenadine. In the previous report, the bioavailability of fexofenadine was 0.41 and hepatic clearance was calculated to be 12.0 mL/minutes/kg,\textsuperscript{29} thus the intestinal availability (FaFg) of fexofenadine is estimated as 0.48 when we used 20.7 mL/minutes/kg as hepatic blood flow rate. Therefore, the complete inhibition of p-glycoprotein in the intestine should lead to the AUC increase 2.1-fold at most, which is almost the same as the results of the present clinical study, suggesting that p-glycoprotein function in the intestine is almost fully inhibited by ritonavir. Two weeks of pretreatment with ritonavir and saquinavir resulted in a 1.3-fold and 1.5-fold increase in the \(\text{C}_{\text{max}}\) and \(\text{AUC}_{0-72}\) values of digoxin, another \textit{in vivo} probe for p-glycoprotein.\textsuperscript{8} Other studies also showed that co-administration of ritonavir increased the plasma AUC of digoxin by from 22\% to 86\%.\textsuperscript{6,13} Since ritonavir is a potent inhibitor for p-glycoprotein-mediated drug transport in some tissues, and since p-glycoprotein is responsible for the highly variable oral bioavailability of saquinavir, p-glycoprotein is also involved in the interaction between ritonavir and saquinavir.\textsuperscript{11,12}

Collective evidence from \textit{in vitro} and knockout mouse studies suggests possibilities that other intestinal efflux and uptake transporters might be involved in the interaction between ritonavir and saquinavir\textsuperscript{16-19,30}, including multidrug resistance-associated protein 2 (MRP2) and some OATP members such as OATP1A2, OATP1B1, and OATP1B3. Unfortunately, good \textit{in vivo} probe drugs for these transporters have not yet been identified, except for OATP1B1. So, we added pravastatin, a typical \textit{in vivo} probe substrate for OATP1B1, into the cocktail.\textsuperscript{31,32} As shown in Table 1, there was no change in any pharmacokinetic parameter of pravastatin at any phase, indicating no involvement of OATP1B1 in the interaction with ritonavir. However, a recent interaction study in mice showed that high dose of ritonavir (50 mg/kg) increased pravastatin AUC by sevenfold.\textsuperscript{33} Considering the protein unbound fraction in human plasma (\(f_{\text{up}}\); 0.066)\textsuperscript{34} and predicted blood-to-plasma concentration ratio (0.722)\textsuperscript{35} of ritonavir, the theoretical maximum unbound concentration of ritonavir (100 mg dose) at the inle: to the liver is calculated to be 99.1 nmol/L by using the following equation as previously reported.\textsuperscript{36}

### Table 2. Pharmacokinetic Parameters of Ritonavir (RTV) Under the Two Dosage Conditions

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>RTV (20 mg; A)</th>
<th>RTV (100 mg; B)</th>
<th>Ratio (B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{AUC}_{0-24}) (ng h/mL)</td>
<td>235 ± 218</td>
<td>10 737 ± 4 534</td>
<td>58.0 ± 15.0</td>
</tr>
<tr>
<td>(t_{1/2}) (h)</td>
<td>3.24 ± 1.35</td>
<td>4.48 ± 0.44</td>
<td>1.46 ± 0.81</td>
</tr>
<tr>
<td>(\text{C}_{\text{max}}) (ng/mL)</td>
<td>44.7 ± 22.4</td>
<td>1 626 ± 897</td>
<td>39.9 ± 17.6</td>
</tr>
<tr>
<td>(T_{\text{max}}) (h)</td>
<td>1.06 ± 0.56</td>
<td>2.50 ± 1.28</td>
<td>3.42 ± 3.03</td>
</tr>
<tr>
<td>CL/F (L/h)</td>
<td>105 ± 44.0</td>
<td>10.3 ± 3.60</td>
<td>0.12 ± 0.06</td>
</tr>
</tbody>
</table>

Parameters are presented as the mean ± SD. RTV, ritonavir.
where \( I_{p,max} \), \( K_{a} \), \( F_{r,p} \), \( Q_{h} \) represent the maximum plasma concentration of ritonavir, absorption rate constant (theoretical maximum value = 6 hours\(^{-1}\)), intestinal availability (theoretical maximum value = 1), and hepatic blood flow rate (1.39 L/hour/kg), respectively. The maximum unbound concentration of ritonavir is much less than the reported \( K_{a} \) value of ritonavir for OATP1B1 (1.4 \( \mu \)mol/L).\(^{19}\) Thus, it is reasonable that 100 mg dose of ritonavir should not inhibit OATP1B1-mediated hepatic uptake in humans, though ritonavir surely inhibits OATP-mediated pravastatin transport at high dose of ritonavir in \textit{in vivo} level. Indeed, although not significantly different, the mean AUC\(_{0-24}\) values of pravastatin tended to be larger at interaction phases.

As shown in Table 2, increasing the dosage of ritonavir from 20 mg to 100 mg appeared to result in a non-proportional increase in the plasma AUC of ritonavir. These results were in contrast to those in the previous studies, in which increasing the dose of ritonavir (at higher dose range, 100–600 mg) resulted in an approximately dose-proportional increase in exposure.\(^{4,5}\) In contrast, a 3.6-fold decrease in CL/F was observed across a 10-fold dose range (from 100 mg to 1 000 mg) after single dose condition, and 40% decrease in CL/F was recognized after multiple dosings (from 400 mg to 1 000 mg), suggesting that ritonavir pharmacokinetics were nonlinear.\(^{37,38}\) The 20 mg dose of ritonavir used in the present study was lower than those in the previous studies (1/5–1/50). A possible interpretation is that saturation of the intestinal and/or hepatic metabolism and g-p-glycoprotein-mediated intestinal efflux occurs at the lower therapeutic dosage range of ritonavir.

The micro/small dosing approach was incorporated into the present study. The most relevant in the microdosing experiments is well prediction of pharmacokinetic properties of drugs after therapeutic doses. For this purpose, the linearity in the pharmacokinetic profile between microdosing and therapeutic dose is important. Pharmacokinetic linearity has indeed been shown for the current two test drugs, midazolam and fexofenadine, in microdosing trials.\(^{39,40}\) Thus micro/small dosing of these two drugs reliably described the mechanisms behind interaction between saquinavir and ritonavir.

Ritonavir-mediated enhancement of saquinavir exposure was clearly observed even at an extremely low dosage of saquinavir (2 mg). Saquinavir dose was selected as 2 mg in the present clinical study instead of 100 \( \mu \)g (microdose), because our prediction suggested that 2 mg dose might be necessary to analyze quantitatively the plasma concentration–time profile of saquinavir. Furthermore, changes in the pharmacokinetics of the probe drugs were also detected. A single administration of ritonavir 20 mg and 100 mg resulted in an approximately 26-fold and 243-fold increase in the AUC\(_{0-24}\) of saquinavir. These values are similar to the results obtained with healthy volunteers following therapeutic doses of saquinavir (400 mg and 600 mg) and ritonavir (200–600 mg); the change in the saquinavir AUC ratio ranged from 50 to 132.\(^{4}\) As described previously, microdosing of probe drugs demonstrates possible mechanisms behind extensive drug interactions without safety concerns. These results indicate that the micro/small dosing cocktail test with the use of probe drugs for transporters/metabolic enzymes is useful for evaluating the mechanisms of drug interactions. Obviously such a dosage technique would allow functional characterization of various capabilities (e.g., CYP enzyme activity) individually not only in healthy subjects but also vulnerable populations such as patients with renal or hepatic impairment, or even pediatric patients. However, some basic issues, such as pharmacokinetic linearity and ultrasensitive quantitative methods, should be cleared for its clinical applications and further drug–drug interaction studies.

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Declaration of Conflicting Interests

The authors declare no conflict of interest.

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