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In-vitro and in-vivo pharmacokinetic interactions of amprenavir, an HIV protease inhibitor, with other current HIV protease inhibitors in rats

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Abstract

The drug interactions between a new human immune deficiency virus (HIV) protease inhibitor, amprenavir, and four other protease inhibitors which are presently used have been characterized by in-vitro metabolic studies using rat liver microsomal fractions and in-vivo oral administration studies. The metabolic clearance rates (V_{max}/K_m) of amprenavir, saquinavir, indinavir and nelfinavir in rat liver microsomes were 50.67±3.77, 170.88±15.34, 73.01±2.76 and 126.06±6.23 μL min^{-1} (mg protein)$^{-1}$, respectively, and the degree of metabolic clearance was in the order of saquinavir > nelfinavir > indinavir > amprenavir > ritonavir. The inhibition constants (K_i) of ritonavir for amprenavir, indinavir, nelfinavir and saquinavir were 2.29, 0.95, 1.01 and 1.64 μM, respectively, and that of indinavir for amprenavir was 0.67, indicating that amprenavir metabolism in rat liver microsomes was strongly inhibited by indinavir. The K_i values of amprenavir for indinavir, nelfinavir and saquinavir were 7.41, 2.13 and 16.11 μM, respectively, and those of nelfinavir and saquinavir for amprenavir were 9.15 and 34.57 μM, respectively. The area under the concentration vs time curve (AUC) of amprenavir after oral co-administration with saquinavir, indinavir, nelfinavir or ritonavir (20 mg kg^{-1} for each oral dose in rats) was increased by 1.6-, 2.0-, 1.2- and 9.1-fold, respectively. The AUC values of saquinavir, indinavir and nelfinavir by co-administration with amprenavir showed about 7.3-, 1.3-, and 7.9-fold increase, respectively. These observations suggested that the oral bioavailability of amprenavir was not so affected by co-administration with saquinavir, nelfinavir or indinavir, compared with ritonavir, whereas amprenavir markedly affected the oral bioavailability of saquinavir and nelfinavir. In addition, the in-vivo effects after co-administration of two kinds of HIV protease inhibitors cannot always be predicted from in-vitro data, suggesting the presence of other interaction processes besides metabolism in the liver. However, these results provide useful information for the treatment of AIDS patients when they receive a combination therapy with two kinds of HIV protease inhibitor.

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Introduction

A combination of two kinds of reverse transcriptase inhibitor and an HIV protease inhibitor has been found to be a better therapy than either type of drug alone in reducing HIV RNA levels, increasing CD4 cell counts and preventing the death of AIDS patients (Hoetelmans et al 1998). Thus, the clinical management of patients infected with HIV has been rather affected by the introduction of potent and specific HIV protease inhibitors. Recently, a combination therapy with two kinds

of HIV protease inhibitor has showed a potent clinical effectiveness in preventing development of tolerance by HIV, and this double protease therapy was attempted in clinical practice (Barry et al 1997). It is considered that combination therapy with two kinds of HIV protease inhibitor will be introduced increasingly in the treatment of AIDS patients. However, when selecting a combination of HIV protease inhibitors, preventing drug interactions remains one of the major problems.

To date, five HIV protease inhibitors (amprenavir, ritonavir, indinavir, saquinavir and nelfinavir) have been used in clinical practice for the treatment of AIDS patients. Of them, amprenavir is a newly introduced HIV protease inhibitor (Chiba et al 1997). In our previous studies (Yamaji et al 1999; Shibata et al 2000), we have reported in-vitro and in-vivo interaction between the other four HIV protease inhibitors, except for amprenavir, in rats. The rank order of in-vitro metabolic clearance rate of these drugs was saquinavir > nelfinavir > indinavir > ritonavir, and ritonavir was a stronger inhibitor than the other three protease inhibitors (Shibata et al 2000). Moreover, the degree of in-vivo interaction between any two-drug combinations selected out of the above four was highly variable (Shibata et al 2000). Recent studies have shown that the most influential isozyme involved in the metabolism of these protease inhibitors including amprenavir is CYP3A4 of the cytochrome P-450 system (Williams & Sinko 1999). In this study, we investigated the interactions between amprenavir and other current HIV protease inhibitors, namely ritonavir, indinavir, saquinavir and nelfinavir, in rats both in-vitro and in-vivo.

Materials and Methods

Materials

Amprenavir, indinavir and saquinavir were kindly supplied by Vertex Pharmaceuticals Inc. (Cambridge, MA), Merck Sharp & Dohme Research Laboratories (Rahway, NJ) and Hoffman-LaRoche Inc. (Nutley, NJ), respectively. Ritonavir and nelfinavir were extracted from commercially available capsules or tablets and were purified by a preparative HPLC method. Glucose-6-phosphate (G6P), G6P dehydrogenase (G6PDH) and nicotinamide adenosine dinucleotide phosphate (NADP) were obtained from Sigma Chemicals (St Louis, MO). All other reagents used were of analytical grade and were used without further purification.

Animals and preparation of oral test solutions

Male Wistar rats, about 10 weeks old (300 ± 20 g), were obtained from Nippon SLC Co. Ltd (Hamamatsu, Japan). The rats were housed in pairs for at least seven days under controlled environmental conditions with free access to general food and water before experimentation. Two hundred milligrams of each HIV protease inhibitor was suspended in 10 mL of 2% w/v sodium carmellose (CMC-Na) dispersion. All experiments were performed in accordance with the Guideline for Animal Experimentation in Kyoto Pharmaceutical University.

In-vitro metabolic studies using rat liver microsomal fractions

Under light ether anaesthesia, rat livers (5–10 g) were excised quickly and perfused with ice-cold potassium chloride (KCl) solution (1.15% w/v). Hepatic microsomes were prepared by differential ultracentrifugation (Hayes et al 1995). Briefly, rat liver was homogenized in 5 volumes of 1.15% w/v KCl in a glass–teflon homogenizer kept on ice. The homogenates were centrifuged at 4°C for 15 min at 9000 *g* and the supernatant was centrifuged again at 105 000 *g* for 1 h. Final microsomal pellets were resuspended in 0.1 M phosphate buffer to get a concentration of 8.0 mg protein mL^{-1}. Microsomal protein concentration was determined by the method of Lowry et al (1951) using bovine serum albumin as a standard. The metabolism of HIV protease inhibitor was measured in an NADPH-generating system according to the following method. To a clean 15 mL conical glass tube 100 μL of 5 mM NADPH in 0.1 M phosphate buffer (pH 7.4), 100 μL of 50 mM G6P in 0.1 M phosphate buffer, 4 μL of 500 IU of G6PDH in 0.1 M phosphate buffer, 100 μL of 50 mM $MgCl_2$ in 0.1 M phosphate buffer, 586 μL of 0.1 M phosphate buffer and 10 μL of HIV protease inhibitor solution were added. When the inhibition experiment was performed, 5 μL each of HIV protease inhibitor solution was added. After a 5-min pre-incubation at 37°C in a water bath, the metabolic reaction was initiated by adding 100 μL of rat liver microsomal suspension (final concentration, 0.8 mg protein per mL of microsomal suspension) and incubated for 10 min at 37°C. After a fixed time, the reaction was stopped by the addition of 200 μL of ice-cold 2 M K_3PO_4. The resultant mixture was used for the extraction. In accordance with the universal method, analysis was performed using the mean value of triplicate determinations.

Enzyme kinetic analysis

The initial metabolic reaction rate of HIV protease inhibitor in rat liver microsomes was determined under linear conditions. Judgement of inhibition mode and estimation of kinetic parameters, Michaelis constant (K_m) and maximum reaction rate (V_{max}), were performed by Lineweaver–Burk plot analysis (Fitzsimmons & Collins 1997). The reciprocal of reaction rate for a substrate (V) in the absence or presence of an inhibitor was plotted against the reciprocal of substrate concentrations, [S], and then regression lines were fitted to each data set using the Excel program. The values of the inhibition constant (K_i) were determined by Dixon plot (Nebbia et al 1999). The reciprocal of the V was plotted against the concentrations of an inhibitor, [I], and similarly each data set was fitted to a regression line. After detecting intersection points between every two regression lines, the K_i value was given by calculating the mean of x-coordinates of those intersection points.

In-vivo pharmacokinetic studies using rats

Under light ether anaesthesia, three or four rats, weighing 300 ± 20 g, fasted overnight with free access to water for at least 12 h, received an oral dose of HIV protease inhibitors. Thirty minutes before the administration of drug, blank blood samples (0.25 mL) were withdrawn from the external left jugular vein. The oral dose of each HIV protease inhibitor was 20 mg kg^{-1}, and drug suspensions (20 mg mL^{-1}) in 2% CMC-Na were administered by using an oral feeding tube. Then, 0.25-mL blood samples were collected into heparinized centrifuging tubes at 0.5, 1, 1.5, 2, 3, 4, and 6 h after administration. The plasma samples were obtained by centrifuging the blood samples at 9000 g for 10 min and were immediately frozen in a deep freezer at -80°C until analysis.

Extraction procedure and analytical conditions for HIV protease inhibitors

The extraction procedure and HPLC technique used for plasma samples (100 μL) were described previously in our reports (Yamaji et al 1999; Shibata et al 2000). For the microsomal samples, to the resultant aqueous layer after adding 200 μL of ice-cold 2 M K_3PO_4 in the rat microsomal experiment, 4 mL of diethyl ether was added to extract HIV protease inhibitors. The mixture was shaken vigorously for 10 min and centrifuged at 14000 g for 10 min. The aqueous phase was frozen in a cold bath at -10°C and the ether extract was transferred to a clean test tube. The organic phase was evaporated to dryness at 50°C under a stream of nitrogen gas. The determination of HIV protease inhibitors in the microsomal samples was performed by a liquid chromatography–mass spectrometry (LC–MS). The LC–MS consists of a SIL-10A system controller (Shimadzu, Kyoto Japan), a LC-10ADvp pump (Shimadzu, Kyoto Japan), a SPD-10A UV detector (Shimadzu, Kyoto Japan), a SIL-10ADvp automatic injector (Shimadzu, Kyoto Japan), a CTO-10A column oven (Shimadzu, Kyoto Japan), and LCMS-QP8000α mass spectrometer (Shimadzu, Kyoto Japan). The analytical column for the separation of saquinavir, nelfinavir, indinavir and amprenavir was a Cadenza CD-C18 (2.0 mm i.d. $\times$ 100 mm). The column was maintained at 60°C for all separations. Elution was carried out isocratically at a flow-rate of 0.2 mL min^{-1} with 50% acetonitrile containing 1% acetic acid. The mobile phase was degassed before use. Mass spectrometry was performed utilizing atmospheric pressure chemical ionization (APCI) at negative mode. The voltages of APCI prove and CDL were set at -5 kV and 0 V, respectively, and the flow rate of nebulizing gas (N_2) flow rate was set at 2.5 L min^{-1}. The temperatures of APCI prove and CDL were set at 400°C and 230°C, respectively. All elutions of four HIV protease inhibitors were finished within 5 min, where the voltages of deflectors were set at -60 V from the beginning of injection to 2.25 min and thereafter at -50 V. The final extraction residues were reconstituted with 50 μL of the mobile phase and 20 μL of sample was injected to the LC–APCI–MS system. Mass chromatograms of indinavir, saquinavir, amprenavir and nelfinavir were detected at 614, 669, 564 and 626 m/z, respectively.

Pharmacokinetic analysis

A non-compartmental pharmacokinetic analysis was applied to the plasma concentration–time data using a computer program, WinHARMONY (Yoshikawa et al 1998). The terminal elimination rate constant, λ_z, was determined by a linear regression of at least three data points from the terminal portion of the plasma concentration–time plots. The area under the plasma concentration–time curve (AUC) after oral administration was calculated using the linear trapezoidal rule up to the last measured plasma concentration, $C_{p(last)}$, and extrapolated to infinity using a correction term, namely $C_{p(last)}\lambda_z^{-1}$. The area under the first-moment curve to the last measured plasma concentration (AUMC) was also calculated using the linear trapezoidal rule and the addition of the correction term after the last measured point ($t_{(last)}$) to infinity, namely,

$t_{(last)}C_{p(last)}\lambda_z^{-1}+C_{p(last)}\lambda_z^{-2}$. The terminal elimination half-life, $t_{\frac{1}{2}}$, was determined by dividing ln2 by λ_z. The mean residence time, MRT, was calculated by dividing AUMC by AUC. The apparent clearance, CL_{app}, was calculated by $D_{oral}AUC^{-1}$, where D_{oral} represents the oral dose.

Statistics

Values from in-vivo and in-vitro studies are expressed as mean$\pm$s.d. Statistical comparisons of in-vivo pharmacokinetic parameters were determined using Wilcoxon's signed-rank test with a significance level of 0.05.

Results

In the in-vitro metabolism of indinavir in the presence of amprenavir, indinavir concentrations employed as a substrate, [S], were 1.0–8.0 μM, and amprenavir concentrations as an inhibitor, [I], were 2.0–20.0 μM. The regression lines of Lineweaver–Burk plot for in-vitro metabolism of indinavir in the presence of amprenavir (0, 2.0, 10.0 and 20.0 μM) were $1/V = 13.6/[S]+3.1$, $1/V = 26.2/[S]+3.6$, $1/V = 38.8/[S]+3.4$ and $1/V = 47.0/[S]+3.1$, respectively. The kinetic parameter values (i.e., Michaelis constant, K_m and maximum velocity, V_{max}) of indinavir were found to be 4.52 ± 0.11 μM and 0.33 ± 0.04 nmol min^{-1} (mg protein)$^{-1}$, respectively, and the metabolic clearance rate (V_{max}/K_m) of indinavir was 73.01 ± 2.76 μL min^{-1} (mg protein)$^{-1}$. The regression lines of the Dixon plot for indinavir (1.0, 2.0, 4.0 and 8.0 μM) in the presence of amprenavir were $1/V = 1.7[I]+16.2$, $1/V = 0.86[I]+9.7$, $1/V = 0.40[I]+6.6$ and $1/V = 0.19[I]+5.3$, respectively, and the K_i value of amprenavir for indinavir was found to be 7.41 μM.

Conversely, for amprenavir metabolism in the presence of indinavir, employed concentrations of amprenavir as a substrate and indinavir as an inhibitor were 0.4–4.0 μM and 1.0–4.0 μM, respectively. The regression lines of Lineweaver–Burk plot for amprenavir in the presence of indinavir (0, 1.0, 2.5 and 4.0 μM) were $1/V = 13.1/[S]+16.7$, $1/V = 27.5/[S]+16.2$, $1/V = 46.3/[S]+15.8$ and $1/V = 78.3/[S]+18.7$, respectively. The K_m, V_{max} and V_{max}/K_m values of amprenavir were found to be 2.84 ± 0.37 μM, 0.14 ± 0.03 nmol min^{-1} (mg protein)$^{-1}$ and 50.67 ± 3.77 μL min^{-1} (mg protein)$^{-1}$, respectively. The regression lines of the Dixon plot for amprenavir (0.4, 1.0, 2.0 and 4.0 μM) in the presence of indinavir were $1/V = 40.0[I]+44.6$, $1/V = 17.3[I]+27.1$, $1/V = 8.7[I]+21.9$ and $1/V = 3.6[I]+18.9$, respectively, and the K_i value of indinavir for amprenavir was found to be 0.67 μM.

For amprenavir metabolism in the presence of ritonavir, concentrations of amprenavir as a substrate were 0.4–4.0 μM, and the concentrations of ritonavir as the inhibitor were 2.5–10.0 μM. The concentration range of ritonavir employed here was relatively large in comparison with those used in the combination of ritonavir/nelfinavir or ritonavir/saquinavir in our previous reports (Yamaji et al 1999; Shibata et al 2000). The regression lines of Lineweaver–Burk plot for amprenavir in the presence of ritonavir (0, 2.5, 5.0 and 10.0 μM) were $1/V = 15.6/[S]+11.1$, $1/V = 46.0/[S]+9.2$, $1/V = 71.3/[S]+5.1$ and $1/V = 108.3/[S]+4.8$, respectively. The regression lines of the Dixon plot for amprenavir (0.4, 1.0, 2.0 and 4.0 μM) in the presence of ritonavir were $1/V = 22.1[I]+61.2$, $1/V = 8.8[I]+30.9$, $1/V = 3.6[I]+20.2$ and $1/V = 1.7[I]+15.7$, respectively, and the K_i value of ritonavir for amprenavir was found to be 2.29 μM.

For other combinations of HIV protease inhibitors, amprenavir/saquinavir and amprenavir/nelfinavir, the in-vitro kinetic parameters were determined in the same way as described above. The K_m, V_{max} and V_{max}/K_m values of saquinavir were 8.31 ± 0.25 μM, 1.42 ± 0.10 nmol min^{-1} (mg protein)$^{-1}$, and 170.88 ± 15.34 μL min^{-1} (mg protein)$^{-1}$, respectively. The K_i values of amprenavir and saquinavir against each other were 16.11 and 34.57 μM, respectively. The K_m, V_{max} and V_{max}/K_m values of nelfinavir were 5.87 ± 0.72 μM, 0.74 ± 0.07 nmol min^{-1} (mg protein)$^{-1}$, and 126.06 ± 6.23 μL min^{-1} (mg protein)$^{-1}$, respectively. The K_i values of amprenavir and nelfinavir against each other were 2.13 and 9.15 μM, respectively.

Table 1 summarizes mutual K_i values between five HIV protease inhibitors, where the K_i values obtained in our previous studies (Yamaji et al 1999; Shibata et al 2000) and in this study were combined. Ritonavir exhibited small values of K_i for indinavir (0.95 μM), saquinavir (1.64 μM) and nelfinavir (1.01 μM), while the K_i value of ritonavir for amprenavir was 2.29 μM. The K_i values of amprenavir for indinavir, nelfinavir and saquinavir were 7.41, 2.13, and 16.11 μM, respectively. Ritonavir showed strong inhibitory effects on indinavir, nelfinavir and saquinavir with relatively small K_i values below 2.0 μM. However, it was considered that the inhibitory effect of ritonavir for amprenavir was relatively weaker than that for indinavir, nelfinavir or saquinavir. On the other hand, the K_i value of indinavir for amprenavir was 0.67 μM, which was the smallest value.

Figure 1 shows the effects of saquinavir, indinavir, nelfinavir and ritonavir on the plasma concentration versus time profile of amprenavir after oral admin-

Table 1 Summary of the inhibition constants (μM) of five HIV protease inhibitors in rat liver microsomes.

Substrate	Inhibitor				
	Amprenavir	Indinavir	Nelfinavir	Saquinavir	Ritonavir
Amprenavir	–	0.67	9.15	34.57	2.29
Indinavir	7.41	–	2.14	9.05	0.95
Nelfinavir	2.13	2.76	–	5.22	1.01
Saquinavir	16.11	3.55	2.35	–	1.64
Ritonavir	–	–	–	–	–

Values represent the inhibition constants, K_is. The values of K_is, except for the mutual values between amprenavir and the other four drugs, were quoted from our previous reports (Yamaji et al 1999; Shibata et al 2000).

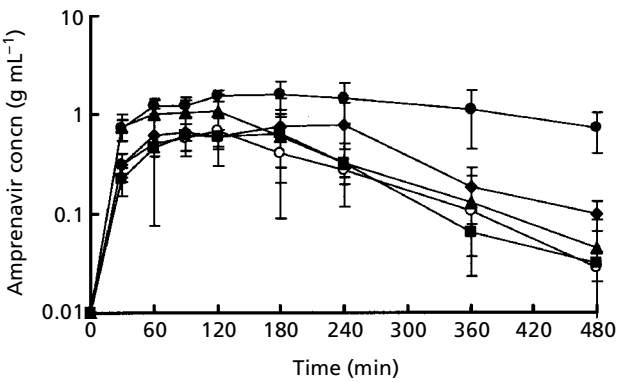

Figure 1 Effect of HIV protease inhibitors (saquinavir, indinavir, nelfinavir, ritonavir) on the plasma concentration–time profiles of amprenavir after co-administration to rats. ○, amprenavir alone; ◆, with saquinavir; ▲, with indinavir; ■, with nelfinavir; ●, with ritonavir. The oral dose of each HIV protease inhibitor was 20 mg kg^{-1}. Each symbol with bar represents mean±s.d. of six experiments.

istration. The pharmacokinetic parameters based on the non-compartmental pharmacokinetic analysis are summarized in Table 2. When amprenavir was administered alone to rats, the maximum plasma drug concentration, C_{max}, was 0.71±0.24 μg mL^{-1}, the AUC was 2.23 ±0.84 μg h mL^{-1}, the $t_{\frac{1}{2}}$ was 1.02±0.20 h, the mean residence time (MRT) was 2.52±0.40 h, the CL_{app} was 3.76±1.61 L h^{-1}. As a result of co-administration of amprenavir with saquinavir and nelfinavir, there were no changes in the C_{max} and AUC of amprenavir. However, the $t_{\frac{1}{2}}$ and MRT with saquinavir increased significantly ($P < 0.05$) from 1.02±0.20 to 2.37±0.44 h and from 2.52±0.40 to 3.44±0.87 h, respectively. With indinavir, the C_{max} and AUC values increased significantly ($P < 0.05$) by about 2.0-fold from 0.71±0.24 to 1.40±0.26 μg mL^{-1} and from 2.23±0.84 to 4.32±0.98 μg h mL^{-1}, respectively, and the CL_{app} decreased significantly from 3.76±1.61 to 2.39±0.73 L h^{-1} ($P < 0.05$). It was noted that ritonavir showed a marked increase ($P < 0.05$) in the C_{max}, AUC, $t_{\frac{1}{2}}$ and MRT of amprenavir, and a marked decrease in the CL_{app} of amprenavir ($P < 0.05$).

Figure 2 shows the effects of amprenavir or ritonavir on the plasma concentration vs time profiles of saquinavir (Figure 2A), indinavir (Figure 2B) and nelfinavir (Figure 2C) after oral administration. The pharmaco-

Table 2 Pharmacokinetic parameters of amprenavir in combination with other HIV protease inhibitors administered orally to rats.

	C_{max} (μg mL^{-1})	AUC (μg h mL^{-1})	$t_{\frac{1}{2}}$ (h)	MRT (h)	CL_{app} (L h^{-1})
Amprenavir alone	0.71±0.24	2.23±0.84	1.02±0.20	2.52±0.40	3.76±1.61
+Saquinavir	1.08±0.44	3.61±0.99	2.37±0.44*	3.44±0.87*	3.15±1.12
+Indinavir	1.40±0.26*	4.32±0.98*	1.32±0.37*	2.71±0.43	2.39±0.73*
+Nelfinavir	0.78±0.24	2.77±0.88	1.26±0.18	3.03±0.41	4.17±1.34
+Ritonavir	1.88±0.48*	20.22±1.18*	7.47±2.33*	12.07±3.31*	0.53±0.07*

Values were tested by Wilcoxon's signed-rank test. Each value represents the mean±s.d. of six experiments. *$P < 0.05$ compared with values when drug administered alone.

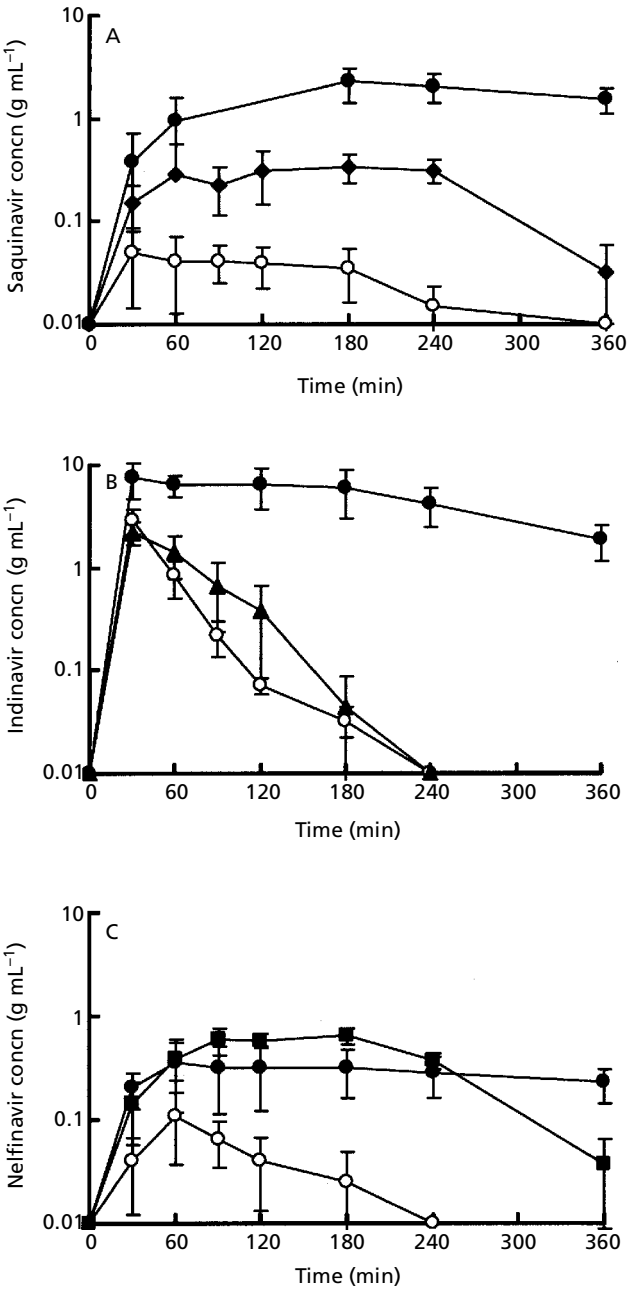

Figure 2 Effect of amprenavir or ritonavir on the plasma concentration–time profiles of other HIV protease inhibitors (saquinavir, indinavir, nelfinavir) after co-administration to rats. A. ○, saquinavir alone; ◆, with amprenavir; ●, with ritonavir. B. ○, indinavir alone; ▲, with amprenavir; ●, with ritonavir. C. ○, nelfinavir alone; ■, with amprenavir; ●, with ritonavir. The oral dose of each HIV protease inhibitor was 20 mg kg^{-1}. Each symbol with bar represents mean±s.d. of six experiments.

kinetic parameters based on the non-compartmental pharmacokinetic analysis are summarized in Table 3. When co-administered with amprenavir, the C_{max} values of saquinavir and nelfinavir increased significantly ($P < 0.05$) from 0.06±0.03 to 0.44±0.14 μg mL^{-1} and from 0.13±0.06 to 0.73±0.11 μg mL^{-1}, respectively. The AUC values of saquinavir and nelfinavir with amprenavir also increased significantly ($P < 0.05$) from 0.21±0.06 to 1.43±0.24 μg h mL^{-1} and from 0.29±0.11 to 2.29±0.39 μg h mL^{-1}, respectively. The values of CL_{app} for saquinavir and nelfinavir with amprenavir decreased significantly ($P < 0.05$) from 37.97±13.34 to 4.13±0.56 L h^{-1} and from 25.97±7.03 to 2.01±0.35 L h^{-1}, respectively. On the other hand, when co-administered with ritonavir, the values of C_{max}, AUC, $t_{\frac{1}{2}}$, MRT and CL_{app} for saquinavir, indinavir and nelfinavir fluctuated more markedly as compared with those arising from co-administration with amprenavir.

Discussion

Increase in plasma concentrations of saquinavir, nelfinavir and indinavir because of interaction with ritonavir is well established (Kumar et al 1999). This positive interaction is believed to be due to the inhibition of CYP3A4-mediated metabolism by ritonavir. The values of metabolic clearance rate (V_{max}/K_m) and CL_{app} after mono-treatment with ritonavir were reported to be 21.7 μL min^{-1} (mg protein)$^{-1}$ (Molla et al 1996) and 0.351 L h^{-1} (Kempf et al 1997), respectively. Combining these observations with our results, the rank order of the V_{max}/K_m and CL_{app} after mono-treatment with five HIV protease inhibitors was saquinavir > nelfinavir > indinavir > amprenavir > ritonavir, and the AUCs of saquinavir, nelfinavir, indinavir and amprenavir were increased markedly by co-administration with ritonavir. Several investigators have also studied the inhibition of saquinavir, indinavir and nelfinavir metabolism by ritonavir, but none of these HIV protease inhibitors altered the metabolism of ritonavir in-vitro or in-vivo (Koudrikova et al 1998). In addition, our previous studies concluded that amprenavir did not inhibit the metabolism of ritonavir in rat liver microsomes (Yamaji et al 1999; Shibata et al 2000). Therefore, ritonavir is the strongest inhibitor among those five drugs tested, and co-administration with ritonavir decreased the CL_{app} of saquinavir, nelfinavir, indinavir and amprenavir markedly. As a consequence of this, the C_{max}, AUC and $t_{\frac{1}{2}}$ of these drugs increased.

Based on the results of the in-vitro rat liver microsome studies, the rank order for intensity of metabolic in-

Table 3 Pharmacokinetic parameters of saquinavir, indinavir and nelfinavir administered orally to rats in combination with amprenavir or ritonavir.

	C_{max} (μg mL^{-1})	AUC (μg h mL^{-1})	$t_{\frac{1}{2}}$ (h)	MRT (h)	CL_{app} (L h^{-1})
Saquinavir alone	0.06±0.03	0.21±0.06	1.80±0.41	3.20±0.65	37.97±13.34
+Amprenavir	0.44±0.14*	1.43±0.24*	1.33±0.52	3.20±0.67	4.13±0.56*
+Ritonavir	3.23±1.50*	17.63±4.07*	2.42±0.46	5.48±1.02*	0.29±0.10*
Indinavir alone	2.75±0.81	1.99±0.57	0.65±0.08	0.77±0.03	4.45±1.22
+Amprenavir	2.41±0.48	2.51±0.54	0.36±0.03*	0.94±0.21	2.93±0.81
+Ritonavir	7.95±2.56*	38.46±10.97*	2.75±1.26*	3.71±2.10*	0.20±0.06*
Nelfinavir alone	0.13±0.06	0.29±0.11	0.69±0.08	1.78±0.41	25.97±7.03
+Amprenavir	0.73±0.11*	2.29±0.39*	1.04±0.28	2.88±0.21*	2.01±0.35*
+Ritonavir	0.31±0.11*	3.25±1.33*	5.94±1.49*	9.60±2.26*	2.14±0.73*

Values were tested by Wilcoxon's signed-rank test. Each value represents the mean±s.d. of six experiments. *$P< 0.05$ compared with values when drug administered alone.

hibition was ritonavir > nelfinavir > amprenavir > saquinavir for indinavir as substrate, ritonavir > amprenavir > indinavir > saquinavir for nelfinavir as substrate, and ritonavir > nelfinavir > indinavir > amprenavir for saquinavir as substrate. Thus, the in-vitro inhibitory intensity of amprenavir on saquinavir, nelfinavir and indinavir was below the middle position. However, the degree of inhibitory intensities of the other four drugs on amprenavir were in the rank order of indinavir > ritonavir > nelfinavir > saquinavir, with indinavir showing the strongest inhibition for amprenavir in the in-vitro metabolism. On the other hand, based on the in-vivo pharmacokinetic parameters, the increasing effects on the C_{max} and AUC of amprenavir after co-administration were in the rank order of ritonavir > indinavir > saquinavir > nelfinavir, with ritonavir showing the strongest effects on amprenavir pharmacokinetics, and indinavir being second strongest. The C_{max} and AUC of saquinavir and nelfinavir after co-administration with amprenavir increased significantly, while their CL_{app} decreased significantly despite amprenavir's relatively weak inhibitory intensity of effect on saquinavir and nelfinavir metabolism in-vitro. Accordingly, for the combination of amprenavir with saquinavir, nelfinavir or indinavir, the in-vitro results do not always reflect the in-vivo results, suggesting the involvement of other interaction processes besides metabolism in the liver. The liver and the intestine are possible sites for induction of a drug–drug interaction via CYP3A4. We therefore speculated that saquinavir–amprenavir or nelfinavir–amprenavir interactions might be mainly due to intestinal CYP3A4 metabolism, because the half-lives of saquinavir and nelfinavir did not change when amprenavir was co-administered with them.

Recently, von Moltke et al (1998) reported that amprenavir and ritonavir showed potent mechanism-based inhibition of CYP3A in human liver microsomes. The biotransformation of triazolam, a substrate for CYP3A to its α-hydroxy and 4-hydroxy metabolites by human liver microsomes in-vitro was strongly inhibited by amprenavir and ritonavir with an IC50 (50% inhibitory concentration) value of 2.5–2.9 and 0.14 μM, respectively, and pre-incubation of microsomes with amprenavir or ritonavir, alone or together, increased their inhibitory potency (i.e., their IC50 values were reduced to 1.4 and 0.07 μM, respectively). Hence, it can be said that ritonavir has a stronger potency with mechanism-based inhibition than amprenavir. With co-administration of the two kinds of drug in-vivo, ritonavir not only increased the values of C_{max} and AUC of saquinavir, indinavir, nelfinavir and amprenavir but also increased the values of $t_{\frac{1}{2}}$ and MRT. Although amprenavir increased the values of C_{max} and AUC of saquinavir and nelfinavir, significant increases were not observed in the $t_{\frac{1}{2}}$ values. Taking these observations into consideration, the marked increase and prolongation in the pharmacokinetic parameters of amprenavir after co-administration with ritonavir was due to a strong effect of the mechanism-based inhibition of CYP3A by ritonavir, and the in-vivo potency of drug interaction based on the mechanism-based inhibition of CYP3A by amprenavir seemed to be weaker than that seen with ritonavir. The clinical importance of mechanism-based CYP3A inhibition is not clearly established, although this inhibitory mechanism indicates the likelihood of

greater complexity in extrapolating in-vitro results to the in-vivo condition.

The oral bioavailability of HIV protease inhibitors is relatively low and is variable within, and between, patients (Noble et al 1996; Palkama et al 1999). The decrease in absorption by an active efflux pump in the intestine such as P-glycoprotein (P-gp) is one of the reasons for this low oral bioavailability. P-gp is a 170-kDa transmembrane protein that is a member of the ATP-binding cassette transporter family, and is localized at the apical surface of secretion in the intestine and other tissues (Pajeva et al 1996). It appears to act as a general detoxification system protecting tissues from endogenous or exogenous lipophilic compounds (Ecker & Chiba 1997). Alsenz et al (1998) confirmed that saquinavir and ritonavir were both substrates for an efflux mechanism in Caco-2 cells, most likely P-gp, which acts as a counter-transporter for both drugs. Profit et al (1999) reported that saquinavir is a substrate for P-gp and that ritonavir, nelfinavir and indinavir modulate P-gp function in both human lymphocytes and Caco-2 cells. In addition, Choo et al (2000) reported that changes in brain-to-plasma distribution ratio were noted after intravenous administration of saquinavir, indinavir and amprenavir following pretreatment with LY-335979, a P-gp inhibitor. These observations indicate that ritonavir, saquinavir, nelfinavir, indinavir and amprenavir are all potent modulators of P-gp. However, the degree of intensity of these drugs as P-gp modulators that is localized at the apical surface of the small intestine is still unclear. In this regard, to explain contradictions observed between in-vivo and in-vitro results of the interactions of amprenavir with the other four HIV protease inhibitors, it is also necessary to conduct further detailed studies on the absorption process via P-gp of those HIV protease inhibitors.

From the in-vitro and in-vivo results in a series of our previous studies (Yamaji et al 1999; Shibata et al 2000), we proposed that the best combination of two kinds of protease inhibitors for the treatment of AIDS patients is firstly nelfinavir–saquinavir, and secondly nelfinavir–indinavir, because of the relatively large metabolic clearance of nelfinavir and there being no marked change in their pharmacokinetic parameters when co-administered. In the case of co-administration with amprenavir, however, it should be noted that amprenavir has the potential to induce drug interactions with other HIV protease inhibitors or other drugs that are metabolized via CYP3A4, such as benzodiazepines, calcium-channel blockers or HMG-CoA reductase inhibitors, because amprenavir has a relatively small metabolic clearance in rat liver microsomes.

In summary, in-vitro findings from this study suggest that amprenavir, next to ritonavir, has a relatively strong inhibitory potency for nelfinavir, and indinavir has the strongest inhibitory potency on amprenavir metabolism in rat liver microsomes. The in-vivo findings demonstrated that amprenavir increases the bioavailability of saquinavir and nelfinavir but not indinavir. Conversely, the bioavailability of amprenavir was not so affected by co-administration of nelfinavir, saquinavir or indinavir, compared with ritonavir. In the combinations of amprenavir–saquinavir, amprenavir–indinavir, amprenavir–nelfinavir and amprenavir–ritonavir, dosage adjustments should be required to optimize therapeutic efficacy for patients with HIV infections. This study provides useful information for the treatment of AIDS patients when they receive a combination therapy of two kinds of HIV protease inhibitor. Nevertheless, further detailed studies are required to ascertain sites of interactions between HIV protease inhibitors.

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