J. Pharm. Pharmacol. 2000, 52: 1239–1246
Received April 3, 2000
Accepted April 4, 2000

Pharmacokinetic Interactions between HIV-1 Protease Inhibitors in Rats: Study on Combinations of Two Kinds of HIV-1 Protease Inhibitors

NOBUHITO SHIBATA, YASUHIRO MATSUMURA, HIROYUKI OKAMOTO, YUKO KAWAGUCHI, AKIKO OHTANI, YUKAKO YOSHIKAWA AND KANJI TAKADA

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

Abstract

The drug interactions between four human immune deficiency virus (HIV-1) protease inhibitors have been characterized by in-vitro metabolic studies using rat liver microsomal fractions and in-vivo oral administration. In this study, a new HPLC analytical method developed by us was used for the simultaneous determination of saquinavir and nelfinavir in rat plasma and microsomes.

The metabolic clearance rates (V_{max}/K_m) of saquinavir, nelfinavir, and indinavir were $170{\cdot}9 \pm 10{\cdot}9$, $126{\cdot}1 \pm 4{\cdot}4$, and $73{\cdot}0 \pm 2{\cdot}0$ μL min^{-1} (mg protein)$^{-1}$, respectively. Ritonavir was the strongest inhibitor with inhibition constants (K_i) of 1·64 μM for saquinavir, 0·95 μM for indinavir, and 1·01 μM for nelfinavir. Nelfinavir was the second strongest inhibitor with K_i's of 2·35 μM for saquinavir and 2·14 μM for indinavir. Indinavir was the third strongest inhibitor with K_i's of 2·76 μM for nelfinavir and 3·55 μM for saquinavir. Saquinavir was the weakest inhibitor for the other three HIV-1 protease inhibitors. After oral co-administration in combination with another HIV-1 protease inhibitor, the AUCs of saquinavir, indinavir, and nelfinavir were significantly increased compared with mono-treatment. The AUCs of saquinavir were increased about 10·1-, 3·1- and 45·9-fold in the presence of indinavir, nelfinavir and ritonavir, respectively. The AUCs of indinavir were increased about 6·8-, 5·9- and 9·4-fold in the presence of nelfinavir, saquinavir and ritonavir, respectively. The AUCs of nelfinavir were increased about 2·2-, 6·6- and 8·5-fold in the presence of indinavir, saquinavir and ritonavir, respectively.

The in-vivo effects observed after co-administration of two kinds of HIV-1 protease inhibitor were not always expected from in-vitro data, suggesting the presence of other interaction processes besides metabolism in the liver. These results provide useful information for the treatment of AIDS patients receiving combination therapy with two HIV-1 protease inhibitors.

The recent discovery of HIV-1 protease inhibitors has introduced a new class of first-line drug therapies for mid-stage and advanced-stage acquired immunodeficiency syndrome (AIDS) patients (Chiba et al 1997). In particular, combination therapy with two kinds of reverse transcriptase inhibitor and an HIV-1 protease inhibitor has been found to be more effective than either drug alone in reducing levels of HIV-1 RNA, increasing CD4 cell counts and preventing the death of AIDS patients (Hoetelmans et al 1998). Currently, four HIV-1 protease inhibitors, indinavir, saquinavir, ritonavir and nelfinavir, are used in clinical practice (Williams & Sinko 1999). Recent studies on the metabolic fate of the HIV-1 protease inhibitors showed that the most influential isozyme involved in the metabolism of the protease inhibitors is CYP3A4, with the isomers of CYP2C9 and CYP2D6 also contributing (von Molttke et al 1998). For instance, ritonavir is the strongest inhibitor in-vitro, with an estimated K_i from 0·95 to 1·64 μM to the other three HIV-1 protease inhibitors in experiments with rat liver microsomes (Yamaji

Correspondence: N. Shibata, Department of Pharmacokinetics, Kyoto Pharmaceutical University, 5 Nakauchi-cho Misasagi Yamashina-ku, Kyoto 607-8414, Japan.

et al 1999). Ritonavir also potently inhibited the CYP-mediated metabolites of indinavir, saquinavir and nelfinavir in human liver microsomes (Koudriakova at al 1998). Combination therapy with two kinds of HIV-1 protease inhibitor has showed a potent clinical effectiveness in preventing tolerance by HIV-1 (Barry et al 1997). Therefore, it is considered that combination therapy with two HIV-1 protease inhibitors will be introduced increasingly for the treatment of AIDS patients in clinical practice. To establish the optimum combination, it is necessary to examine the effects of drug interactions on the metabolism of each combination of two HIV-1 protease inhibitors.

In this report, using our newly developed assay method for saquinavir and nelfinavir by HPLC, we investigated the aspects of drug interactions between combinations of two kinds of HIV-1 protease inhibitor in rat both in-vitro and in-vivo. We will refer to an optimal combination of two HIV-1 protease inhibitors.

Materials and Methods

Materials

Glucose-6-phosphate (G6P), G6P dehydrogenase (G6PDH) and nicotinamide adenosine dinucleotide phosphate (NADP) were obtained from Sigma Chemicals (St Louis, MO). Saquinavir was kindly supplied from Hoffman-LaRoche Laboratory, and nelfinavir was extracted from capsules and was purified by preparative HPLC. Acetonitrile (HPLC grade) and diethyl ether were supplied by Kanto Chemical Co. Inc. (Tokyo, Japan). Bovine serum albumin and sodium carmellose (CMC-Na) were obtained from Nacalai Tesque Co. (Kyoto, Japan). All other reagents used were of analytical grade and were used without further purification. The standard stock solutions of protease inhibitors were prepared by dissolving in methanol at a concentration of 250–1000 $\mu g\,mL^{-1}$. These solutions were used to prepare standards for the calibration curves of HPLC analysis. The calibration curve samples were prepared by adding known amounts of protease inhibitors to appropriate materials.

Animals and preparation of oral test solutions

Male Wistar rats, about 10 weeks old, were obtained from Nippon SLC Co. Ltd. (Hamamatsu, Japan). The rats were housed in pairs at least seven days under controlled environmental conditions; general food and water were freely available. Each HIV-1 protease inhibitor (5 g) was suspended with 10 mL of 2% (w/v) CMC-Na solution.

In-vitro metabolic studies using rat liver microsomal fractions

After the dislocation of neck vertebrae, rat liver (5–10 g) was 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 at 9000 *g* for 15 min and the supernatant were centrifuged again at 105 000 *g* for 1 h. Final microsomal pellets were resuspended in 0·1 M phosphate buffer to a concentration of 8·0 mg protein mL^{-1}. Microsomal protein concentrations were determined by method of Lowry et al (1951) using bovine serum albumin as a standard. The metabolism of HIV-1 protease inhibitor was measured in an NADPH-generating system according to the method specified below. 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 units 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-1 protease inhibitor solution were added. When the inhibition experiment was performed, each 5 μL of HIV-1 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 mL^{-1} of microsomal suspension). After incubation for 10 min, the reaction was stopped by the addition of 100 μL of ice-cold 2 M K_3PO_4. The mixture was vortexed vigorously and centrifuged at 14 000 *g* for 10 min to precipitate protein. The resultant supernatant was used for the HPLC analysis of saquinavir and nelfinavir.

Enzyme kinetic analysis

The initial reaction rate of HIV-1 protease inhibitor in rat liver microsomes was determined under linear conditions. Kinetic parameters, Michaelis constant (K_m) and maximum reaction rate (V_{max}), were determined by Lineweaver-Burk plot. The estimates of the inhibition constant (K_i) were determined by plotting the slopes of the Lineweaver-Burk plot versus inhibitor concentrations (Fitzsimmons & Collins 1997).

In-vivo pharmacokinetic studies using rats

Three or four rats, 350 ± 10 g, fasted overnight with free access to water for at least 12 h, received an

oral dose of HIV-1 protease inhibitors. Rats were anaesthetized with light ether anaesthesia. At 30 min before the administration of drug, 0·2 mL of blank blood samples were withdrawn from the external left jugular vein by direct puncture using a syringe equipped with a 26-gauge needle after incision. The oral dose of each HIV-1 protease inhibitor was 20 mg kg^{-1}, and drug suspensions (0·5 mg mL^{-1}) with 2% CMC-Na were orally administered by a feeding tube within a consecutive 3-min interval. Then, 0·2-mL samples of blood were collected into heparinized centrifuging tubes from the external left jugular vein at 0·5, 1, 1·5, 2, 3, 4, and 6 h after the injection. Plasma samples were obtained by centrifuging blood samples at 9000 *g* for 30 min, and were immediately frozen in a deep freezer at −80°C until analysis.

Extraction procedure and analytical conditions for HIV-1 protease inhibitors

The resultant aqueous layer (380 μL) from the rat microsomal experiment and plasma (100 μL) were diluted with phosphate buffer to make 1 mL of sample solution. These specimens were extracted with 3 mL of diethyl ether. The mixture was shaken for 10 min at 9000 *g*. After the aqueous phase had been frozen in a cold bath at −10°C, the ether extract was transferred to a clean test tube. The organic liquid was evaporated to dryness at 50°C under a stream of nitrogen gas. The determinations of saquinavir and nelfinavir in sample specimens were performed by an HPLC method. The liquid chromatograph (Shimadzu LC-10AS, Kyoto, Japan) was equipped with an automatic injector (Toso Model 8020), a UV detector (Shimadzu SPD-10A) and a chromatographic terminal (Chromatec, Kyoto, Japan). The analytical column for saquinavir and nelfinavir was a Chemcosorb 5-ODS-UH (4·6 mm i.d. × 250 mm). The column was maintained at 65°C for all separations. Elution was carried out isocratically at a flow-rate of 1·1 mL min^{-1} with a mixture of acetonitrile and water (32·5 : 67·5, v/v). The mobile phase was degassed before use. The detection of saquinavir and nelfinavir was performed at a UV wavelength of 210 nm and 245 nm, respectively, and the retention times of these drugs were 15 min and 12·5 min, respectively. The final extraction residues were reconstituted with 200 μL of the mobile phase and 100 μL of sample was injected to the HPLC system. For the quantitative determination of drugs, the absolute calibration method was used. The correlation coefficients for the calibration curves were 0·999 or better, and the detection limits for each drugs was 0·1 μg mL^{-1}. The extraction and the detection of indinavir were performed using our method reported previously.

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 after oral administration, AUC, 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 concentration 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, $t_{1/2}$, was determined by dividing ln2 by λ_z. The mean residence time, MRT, was calculated as AUMC/AUC. The apparent clearance, CL_{app}, was calculated by D_{oral} AUC^{-1}, where D_{oral} represents the oral dose.

Statistics

All values are expressed as means ± s.e.m. unless otherwise noted. Statistical differences of the means were assumed to be significant when $P < 0{\cdot}05$ (two-sided *t*-test).

Results

Figure 1 shows Lineweaver-Burk and Dixon plots for the inhibition of saquinavir metabolism in rat liver microsomes by nelfinavir. The saquinavir concentrations employed as the substrate were 0·75–6·0 μM, and the nelfinavir concentrations as the inhibitor were 7·5–30·0 μM. The kinetic parameter values (i.e., Michaelis constant, K_m and maximum velocity, V_{max}) of saquinavir were estimated by a linear regression analysis and were 8·31 ± 0·18 μM and 1·42 ± 0·07 nmol min^{-1} (mg protein)$^{-1}$, respectively. The metabolic clearance rate (V_{max}/K_m) of saquinavir was 170·9 ± 10·9 μL min^{-1} (mg protein)$^{-1}$ (Figure 1A). With the addition of nelfinavir, the metabolism of saquinavir was inhibited, and the inhibition

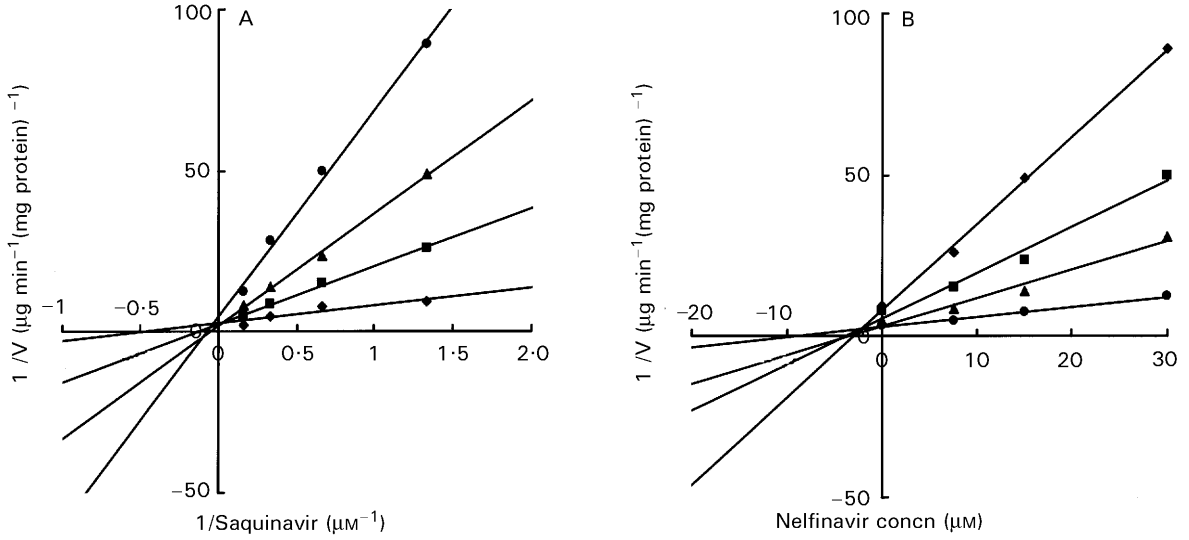

Figure 1. Lineweaver-Burk (A) and Dixon (B) plots for inhibition of saquinavir metabolism by nelfinavir in rat liver microsomes. Lineweaver-Burk plot revealed a competitive interaction between saquinavir and nelfinavir. The K_i value of nelfinavir for saquinavir is 2·35 μM. A. ◆, saquinavir alone; ■, with 7·5 μM nelfinavir; ▲, with 15 μM nelfinavir; ●, with 30 μM nelfinavir. B. ◆, 0·75 μM saquinavir; ■, 1·5 μM saquinavir; ▲, 3·0 μM saquinavir; ●, 6·0 μM saquinavir.

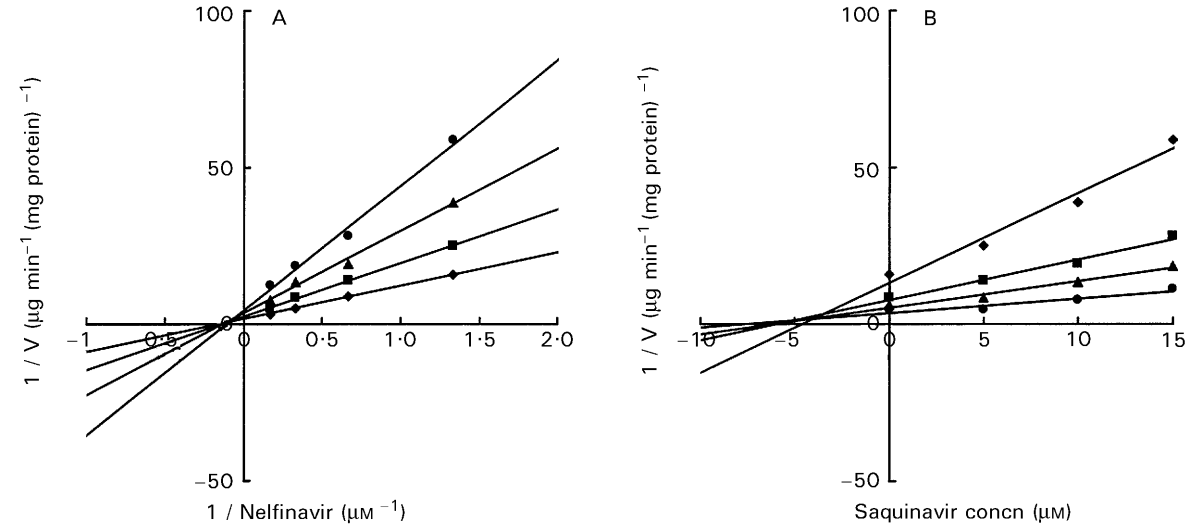

Figure 2. Lineweaver-Burk (A) and Dixon (B) plots for inhibition of nelfinavir metabolism by saquinavir in rat liver microsomes. Lineweaver-Burk plot revealed a competitive interaction between nelfinavir and saquinavir. The K_i value of saquinavir for nelfinavir is 5·22 μM. A. ◆, saquinavir alone; ■, with 7·5 μM nelfinavir; ▲, with 15 μM nelfinavir; ●, with 30 μM nelfinavir. B. ◆, 0·75 μM saquinavir; ■, 1·5 μM saquinavir; ▲, 3·0 μM saquinavir; ●, 6·0 μM saquinavir.

constant, K_i of nelfinavir was estimated to be 2·35 μM (Figure 1B). Figure 2 shows Lineweaver-Burk and Dixon plots for the inhibition of nelfinavir metabolism in rat liver microsomes by saquinavir. The nelfinavir concentrations employed as the substrate were 0·75–6·0 μM, and the saquinavir concentrations as the inhibitor were 5·0–15·0 μM. The estimated values of K_m and V_{max} of nelfinavir were $5{\cdot}87 \pm 0{\cdot}51$ μM and $0{\cdot}74 \pm 0{\cdot}05$ nmol min^{-1} (mg protein)$^{-1}$, respectively. The value of V_{max}/K_m for nelfinavir was $126{\cdot}1 \pm 4{\cdot}4$ μL min^{-1} (mg protein)$^{-1}$ (Figure 2A). With the addition of saquinavir, the metabolism of nelfinavir was also inhibited, and the value of K_i was estimated to be 5·22 μM for nelfinavir (Figure 2B). As previously reported (Yamaji et al 1999), the values of K_m and V_{max} for indinavir were $4{\cdot}52 \pm 0{\cdot}08$ μM and $0{\cdot}33 \pm 0{\cdot}03$ nmol min^{-1} (mg protein)$^{-1}$, respectively, and the value of V_{max}/K_m was $73{\cdot}0 \pm 2{\cdot}0$ μL min^{-1} (mg protein)$^{-1}$. From these parameters, the metabolic clearance rates of saquinavir, nelfinavir and indinavir were deter-

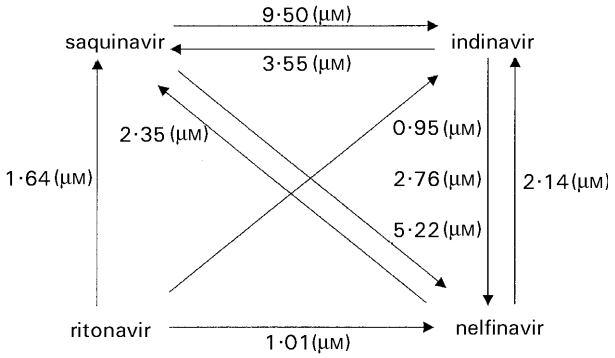

Figure 3. Summary of the inhibition constants, K_i's, of four HIV-1 protease inhibitors in the rat liver microsomes. The values of K_i, except for the mutual values between saquinavir and nelfinavir, were quoted from our previous report (Yamaji et al 1999).

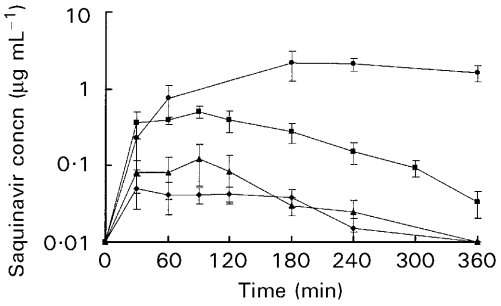

Figure 4. Effect of HIV-1 protease inhibitors (indinavir, nelfinavir, ritonavir) on the plasma concentration-time profiles of saquinavir after co-administration to rats. ◆, saquinavir alone; ■, with indinavir; ▲, with nelfinavir; ●, with ritonavir. The oral doses of saquinavir, indinavir, nelfinavir and ritonavir were 20 mg kg^{-1}, respectively. Each symbol with bar represents the mean ± s.e. of three or four experiments.

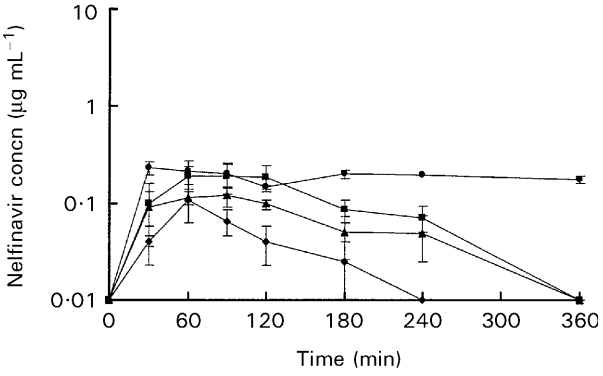

Figure 5. Effect of HIV-1 protease inhibitors (saquinavir, indinavir, ritonavir) on the plasma concentration-time profiles of nelfinavir after co-administration to rats. ◆, saquinavir alone; ■, with indinavir; ▲, with nelfinavir; ●, with ritonavir. The oral doses of saquinavir, indinavir, nelfinavir and ritonavir were 20 mg kg^{-1}, respectively. Each symbol with bar represents the mean ± s.e.m. of three or four experiments.

mined and the rank order of those values was saquinavir > nelfinavir > indinavir.

Figure 3 summarizes all K_i values between the four HIV-1 protease inhibitors. In this figure, we combined the K_i values obtained in both our previous study (Yamaji et al 1999) and in this study. Ritonavir is a potent metabolic inhibitor of CYP3A4, and several investigators have studied the metabolic inhibition of ritonavir on saquinavir, indinavir and nelfinavir; however, none of these HIV-1 protease inhibitors altered the metabolism of ritonavir in-vitro or in-vivo (Koudriakova et al 1998). Our results (Figure 3) were consistent with those in the literature, that is, ritonavir exhibited smallest values of K_i: 1·64 μM for saquinavir, 1·01 μM for nelfinavir and 0·95 μM for indinavir. The K_i values of nelfinavir were in a middle position of the four HIV-1 protease inhibitors: 2·35 μM for saquinavir and 2·14 μM for indinavir. The K_i values of indinavir for saquinavir and nelfinavir were 3·55 μM and 2·76 μM, respectively, and these values were relatively large.

On the basis of in-vitro metabolic studies, we examined the in-vivo pharmacokinetic interactions between various combinations of two HIV-1 protease inhibitors. Figure 4 shows the effects of indinavir, nelfinavir and ritonavir on the pharmacokinetics of saquinavir after oral administration and Figure 5 shows the effects of indinavir, saquinavir and ritonavir on the pharmacokinetics of nelfinavir after oral administration to rats. The pharmacokinetic parameters based on the non-compartmental pharmacokinetic analysis are summarized in Table 1, where the data of pharmacokinetic parameters for indinavir after co-administration with nelfinavir, saquinavir or ritonavir in our previous report (Yamaji et al 1999) were included. When saquinavir, indinavir or nelfinavir were administered alone to rats: the maximum plasma drug concentration (C_{max}) values, were 0·05 ± 0·02, 0·80 ± 0·29 and 0·09 ± 0·01 μg mL^{-1}; the $t_{1/2}$ values were 2·14 ± 0·28, 0·58 ± 0·06 and 1·36 ± 0·11 h; the AUCs were 0·15 ± 0·03, 1·07 ± 0·31 and 0·17 ± 0·02 μg h mL^{-1}; and the CL_{app} values were 34·9 ± 5·2, 7·8 ± 0·1 and 32·1 ± 2·5 L h^{-1}, respectively. As a result of co-administration of ritonavir, the AUCs of saquinavir, indinavir and nelfinavir were significantly increased from 0·15 ± 0·03 to 6·88 ± 1·87 ($P < 0·01$), from 1·07 ± 0·31 to 10·01 ± 2·88 ($P < 0·05$) and from 0·17 ± 0·02 to 1·44 ± 0·41 μg h mL^{-1} ($P < 0·05$), respectively, while the CL_{app} was significantly decreased ($P < 0·01$) from 34·9 ± 5·2 to 0·9 ± 0·3, from 7·8 ± 0·1 to 0·5 ± 0·1 and from 32·1 ± 2·5 to 1·3 ± 0·6 L h^{-1}, respectively. When co-adminis-

Table 1. Pharmacokinetic parameter values of saquinavir, indinavir and nelfinavir in combination.

	Pharmacokinetic parameter				
	C_{max} ($\mu g\,mL^{-1}$)	AUC ($\mu g\,h\,mL^{-1}$)	$t_{\frac{1}{2}}$ (h)	MRT (h)	CL_{app} ($L\,h^{-1}$)
Saquinavir alone	0·05 ± 0·02	0·15 ± 0·03	2·14 ± 0·28	3·90 ± 0·65	34·9 ± 5·2
+ Indinavir	0·61 ± 0·02**	1·52 ± 0·25**	1·26 ± 0·09	2·39 ± 0·27	4·2 ± 0·7**
+ Nelfinavir	0·10 ± 0·02	0·47 ± 0·07	1·96 ± 0·54	3·17 ± 0·80	27·1 ± 5·5**
+ Ritonavir	2·62 ± 0·79*	6·88 ± 1·87**	2·85 ± 1·21	5·04 ± 1·63*	0·9 ± 0·3**
Indinavir alone	0·80 ± 0·29	1·07 ± 0·31	0·58 ± 0·06	1·62 ± 0·17	7·8 ± 0·1
+ Nelfinavir	7·16 ± 1·19**	7·27 ± 1·92*	0·51 ± 0·02**	0·91 ± 0·06*	0·9 ± 0·2*
+ Saquinavir	6·76 ± 1·08**	6·27 ± 1·27**	0·53 ± 0·02**	0·81 ± 0·04*	1·0 ± 0·2*
+ Ritonavir	4·22 ± 2·33*	10·01 ± 2·88*	2·94 ± 0·72*	4·85 ± 1·25*	0·5 ± 0·1**
Nelfinavir alone	0·09 ± 0·01	0·17 ± 0·02	1·36 ± 0·11	2·95 ± 0·26	32·1 ± 2·5
+ Indinavir	0·15 ± 0·03	0·37 ± 0·06	1·20 ± 0·09	2·42 ± 0·25	16·6 ± 2·2*
+ Saquinavir	0·39 ± 0·05	1·12 ± 0·18	1·10 ± 0·07	2·26 ± 0·06	5·4 ± 0·7**
+ Ritonavir	0·33 ± 0·10**	1·44 ± 0·41*	8·67 ± 1·96**	13·18 ± 3·17**	1·3 ± 0·6**

The data for indinavir after co-administration with nelfinavir, saquinavir or ritonavir in our previous report (Yamaji et al 1999) were included. Each value represents the mean ± s.e.m. of three or four experiments. *$P < 0·05$, **$P < 0·01$, compared with appropriate control.

tered with indinavir, the C_{max} of saquinavir was significantly increased ($P < 0·01$) from 0·05 ± 0·02 to 0·61 ± 0·02 $\mu g\,mL^{-1}$ and the AUC also increased from 0·15 ± 0·03 to 1·52 ± 0·25 $\mu g\,h\,mL^{-1}$, while the CL_{app} was markedly decreased ($P < 0·01$) from 34·9 ± 5·2 to 4·2 ± 0·7 $L\,h^{-1}$. However, the effect of nelfinavir on the plasma concentration–time profile of saquinavir was less than that of indinavir, namely, the C_{max} and AUC of saquinavir were increased about 2- and 3·1-fold with co-administration of nelfinavir, although there were no differences in values of CL_{app}. With co-administration of saquinavir, the C_{max} of nelfinavir was increased from 0·09 ± 0·01 to 0·39 ± 0·05 $\mu g\,mL^{-1}$ and the AUC also increased from 0·17 ± 0·02 to 1·12 ± 0·18 $\mu g\,h\,mL^{-1}$, while the CL_{app} significantly ($P < 0·01$) decreased from 32·1 ± 2·5 to 5·4 ± 0·7 $L\,h^{-1}$. However, the effect of indinavir on the plasma concentration-time profile of nelfinavir was less than that of saquinavir. The effect of nelfinavir on the pharmacokinetic profiles of indinavir were of a comparable degree to that of saquinavir. The time to reach peak concentrations of saquinavir and nelfinavir tended to increase as their AUCs were increased in the presence of other HIV-1 protease inhibitors.

Discussion

In our previous report on drug interactions of HIV-1 protease inhibitors (Yamaji et al 1999), we assayed the HIV-1 protease inhibitors in rat plasma and liver microsomes by HPLC methods using a reversed-phase column with a 20% carbon content and silica gel with a pore size of 100 Å (Chemcosorb 5-ODS-H). Using this column, however, the peaks of saquinavir and nelfinavir could not be separated on the chromatograms. In this study, good simultaneous separation of saquinavir and nelfinavir on a chromatogram was attained by use of another kind of reversed-phase column, namely, Chemcosorb 5-ODS-UH with a 30% carbon content and silica gel of 60 Å pore size. Therefore, we could describe the intensity of interaction between combinations of two kinds of HIV-1 protease inhibitor: ritonavir/saquinavir, ritonavir/nelfinavir, ritonavir/indinavir, saquinavir/nelfinavir, saquinavir/indinavir and nelfinavir/indinavir.

The Eadie-Hofstee plots analysis for the metabolism of saquinavir, indinavir and nelfinavir showed that only one enzyme took part in the metabolism of these HIV-1 protease inhibitors by rat liver microsomes in the concentration range employed in this study. Therefore, the metabolic competitive inhibition between these four HIV-1 protease inhibitors could be elucidated by their metabolic clearance rates. Increases in plasma concentrations of HIV-1 protease inhibitors as a result of drug interaction between ritonavir and the other three HIV-1 protease inhibitors is well established and is believed to be due to inhibition of CYP3A4-mediated metabolism by ritonavir. Saquinavir, nelfinavir and indinavir also have inhibitory effects on CYP3A4-mediated metabolism (Kempf et al 1997). The values of metabolic clearance (V_{max}/K_m) for ritonavir were reported to be 21·7 $\mu L\,min^{-1}\,mg^{-1}$ (Molla et al 1996). The rank order of the V_{max}/K_m for the four HIV-1 protease inhibitors are saquinavir > nelfinavir > indinavir > ritonavir. Therefore, co-administration of ritonavir would decrease the metabolic clearance of saquinavir, nelfinavir and indinavir. In addition, the value of CL_{app} reported for ritonavir is

0·351 L h^{-1} (Kempf et al 1997). The rank order of CL_{app} was saquinavir > nelfinavir > indinavir > ritonavir, and this order is the same as that of metabolic clearance in-vitro. Therefore, the co-administration of ritonavir decreased the CL_{app} values of saquinavir, nelfinavir and indinavir, and consequently increased their C_{max} and $t_{1/2}$. Moreover, von Molttke et al (1998) reported that the rank order of the inhibitory effects of these HIV-1 protease inhibitors on the 4-hydroxylation of triazolam, which is mainly metabolized by CYP3A4 in human hepatic microsomes, was ritonavir > indinavir > nelfinavir > saquinavir. Hence, it was assumed that the rank order of inhibitory effects of these drugs gained from our in-vitro data in rat liver microsomes essentially agreed with the human in-vitro data.

Using an HPLC method we newly developed, we could make the drug interactions between the combinations of two kinds of HIV-1 protease inhibitor clear. Judging by the degree of inhibitory intensity in the in-vitro rat liver microsomes, the inhibitory effect for the metabolism of saquinavir as a substrate was in the rank order of ritonavir > nelfinavir > indinavir, while that for indinavir as a substrate was in the rank order of ritonavir > nelfinavir > saquinavir. Judging by the pharmacokinetic parameters, the increasing effect for the bioavailability of saquinavir after co-administration in-vivo was in the rank order of ritonavir $\gg$ nelfinavir > indinavir, while that of indinavir was in the rank order of ritonavir > nelfinavir > saquinavir. Accordingly, for combinations of saquinavir with the other three HIV-1 protease inhibitors (ritonavir, nelfinavir, indinavir) or indinavir with the other three HIV-1 protease inhibitors (ritonavir, nelfinavir, saquinavir), the in-vitro results reflect the in-vivo results. For nelfinavir, however, the inhibitory effect on the metabolism of nelfinavir as a substrate in-vitro was in the rank order of ritonavir > indinavir > saquinavir. However, the enhancement of nelfinavir bioavailability after co-administration in-vivo was in the rank order of ritonavir > saquinavir > indinavir. Clearly, in the case of nelfinavir with the other three HIV-1 protease inhibitors (ritonavir, saquinavir, indinavir), the in-vitro results did not reflect the in-vivo results, suggesting the participation of other interactions besides the metabolic process in the liver.

The oral bioavailability of saquinavir is variable within and between patients (Noble & Faulds 1996). As one reason for this low oral bioavailability, the absorption is decreased by an active efflux pump in the intestine such as P-glycoprotein. P-glycoprotein 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 various 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-glycoprotein, which acts as a counter-transporter for both drugs. In addition, Profit et al (1999) reported that saquinavir is a substrate for P-glycoprotein and that ritonavir, nelfinavir and indinavir modulate P-glycoprotein function in both human lymphocytes and Caco-2 cells. These observations indicate that ritonavir, saquinavir, nelfinavir and indinavir are all potent modulators for P-glycoprotein. However, the degree of intensities of these drugs as modulators of P-glycoprotein that is an localized at the apical surface of the small intestine are unclear. It is evidenced that the co-function of P-glycoprotein and CYP3A4 in small intestine is an important factor to oral drug delivery. P-glycoprotein increases the exposure to drug-metabolizing enzymes and enhances intestinal metabolism of drugs by prolonging the intercellular residence time through the repetitive processes of excretion and reabsorption (Benet et al 1996). The possible contributions of P-glycoprotein in the intestinal metabolism was further supported by the observations that the time to reach peak concentrations of saquinavir and nelfinavir were increased as their AUCs were increased in the presence of other HIV-1 protease inhibitors. Consequently, to explain the contradictions that exist between in-vivo and in-vitro results of the interactions of nelfinavir with the other three HIV-1 protease inhibitors, it is necessary to perform further detailed experiments on the absorption process of these HIV-1 protease inhibitors via P-glycoprotein traversing the plasma membrane and CYP3A4 existing in the inside of enterocytes.

With the clinical use of HIV-1 protease inhibitors, the chemotherapy of AIDS patients has progressed day by day. Indeed, combination therapy with two kinds of reverse transcriptase inhibitors and an HIV-1 protease inhibitor, namely, hyperactive anti-retroviral therapy, has markedly improved the survival of AIDS patients (Finzi et al 1999). Combination therapy using HIV-1 protease inhibitors provides greater viral suppression, and several regimens with co-administration of two kinds of protease HIV-1 inhibitors, including quadruple therapy, are now undergoing clinical trial (Barry et al 1997). Ritonavir, saquinavir and indinavir are peptide-mimetic compounds, and their metabolic clearances are very extensive in the

body. Therefore, chronic use of these compounds will provide undesirable side-effects on the liver. Nelfinavir, however, is not a peptide-mimetic compound, and is thought to exhibit less side-effects on the liver. From this point of view, we recommend nelfinavir as a drug of first choice among these four HIV-1 protease inhibitors. Moreover, taking both the in-vitro and in-vivo results into consideration, the best combination of two protease inhibitors for the therapy of AIDS patients is firstly nelfinavir/saquinavir, and secondly nelfinavir/indinavir. This study provides useful information for the treatment of AIDS patients when they receive a combination therapy of two kinds of HIV-1 protease inhibitors.

References

Alsenz, J., Steffen, H., Alex, R. (1998) Active apical secretory efflux of the HIV protease inhibitors saquinavir and ritonavir in Caco-2 cell monolayers. Pharm. Res. 15: 423–428

Barry, M., Gibbons, S., Back, D., Mulcahy, F. (1997) Protease inhibitors in patients with HIV disease. Clin. Pharmacokinet. 32:194–209

Benet, L. Z., Wu, C. Y., Hebert, M. F., Wacher, V. J. (1996) Intestinal drug metabolism and antitransport process: a potential shift in oral drug delivery. J. Control. Release 39: 139–143

Chiba, M., Hensleighh, M., Lin, J. H. (1997) Hepatic and intestinal metabolism of indinavir, an HIV protease inhibitor, in rat and human microsomes. Biochem. Pharm. 53: 1187–1194

Ecker, G., Chiba, P. (1997) Structure–activity-relationship studies on modulators of the multidrug transporter P-glycoprotein—an overview. Wien. Klin. Wochenschr. 107/22: 681–686

Finzi, D., Blankson, J., Siliciano, J. D. (1999) Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat. Med. 5: 512–519

Fitzsimmons, M. E., Collins, J. M. (1997) Selective biotransformation of the human immunodeficiency virus protease inhibitor saquinavir by human small-intestinal cytochrome P4503A4. Drug Metab. Dispos. 25: 256–666

Hoetelmans, R. M. W., Reijers, M. H. E., Weverling, G. J., Kate, R. W., Wit, F. W. N. M., Mulder, J. W., Weigel, H. M., Frissen, P. H. J., Lange, J. M. A. (1998) The effect of plasma drug concentrations on HIV-1 clearance rate during quadruple drug therapy. AIDS 12: F111–F115

Kempf, D. J., Marsh, K. C., Kumar, G., Rodrigues, A. D., Denissen, J. F., McDonald, E., Kukulka, M. J., Hsu, A., Granneman, G. R., Baroldi, P. A., Sun, E., Pizzuti, D., Plattner, J. J., Norbeck, D. W., Leonard, J. M. (1997) Pharmacoknetic enhancement of inhibitors of the human immunodeficiency virus protease by coadministration with ritonavironavir. Antimicrob. Agents Chemother. 41: 654–660

Koudriakova, T., Iatsimirskaia, E., Utkin, I., Gangl, E., Vouros, P., Storozhuk, E., Orza, D., Marinina, J., Gerber, N. (1998) Metabolism of the human immunodeficiency virus protease inhibitors indinavir and ritonavir by human intestinal microsomes and expressed cytochrome P45503A4/3A5: mechanism-based inactivation of cytochrome P4503A by ritonavir. Drug Metab. Dispos. 26: 552–561

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the folin phenol agent. J. Biol. Chem. 193: 265–275

Molla, A., Korneyeva, M., Gao, Q., Vasavanonda, S., Schipper, P. J., Mo, H. M., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N., Hsu, A., Grannerman, G. R., Ho, D. D., Boucher, C. A. B., Leonard, J. M., Norbeck, D. W., Kempf, D. J. (1996) Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. Nat. Med. 2: 760–766

Noble, S., Faulds, D. (1996) Saquinavir, a review of its pharmacology and clinical potential in the management of HIV infection. Drugs 52: 93–112

Pajeva, I. K., Wiese, M., Cordes, H. P., Seydel, J. K. (1996) Membrane interactions of some catamphiphilic drugs and relation to their multidrug-resistence-reversing ability. J. Cancer Res. Clin. Oncol. 122: 27–40

Profit, L., Eagling, V. A., Back, D. J. (1999) Modulation of P-glycoprotein function in human lymphocytes and Caco-2 cell monolayers by HIV-1 protease inhibitors. AIDS 13: 1623–1627

Yamaji, H., Matsumura, Y., Yoshikawa, Y., Takada, K. (1999) Pharmacokinetic interactions between HIV-protease inhibitors in rats. Biopharm. Drug Dispos. 20: 241–247

Yoshikawa, Y., Kato, K., Sone, H., Takada, K. (1998) Development and evaluation of noncompartmental pharmacokinetic analysis program 'WinHARMONY' using Visual BASIC language having a function of automatic recognition v time profile. Jpn. J. Clin. Pharmacol. Ther. 29: 475–487

von Molttke, L. L., Greenblatt, D. J., Grassi, J. M., Granda, B. W., Duan, S. X., Fogelmmann, S. M., Daily, J. P., Harmatz, J. S., Shader, R. I. (1998) Protease inhibitors as inhibitors of human cytochromes P450: high risk aassociated with ritonavir. J. Clin. Pharmacol. 38: 106–111

Williams, G. C., Sinko, P. J. (1999) Oral absorption of the HIV protease inhibitors: a current update. Adv. Drug Del. Rev. 39: 211–238