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Docosahexaenoic acid (DHA) inhibits saquinavir metabolism in-vitro and enhances its bioavailability in rats

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Abstract

This study investigated the effect of docosahexaenoic acid (DHA) on the metabolism of saquinavir by cytochrome P450 3A (CYP3A) in-vitro using rat liver microsomes and in-vivo using rats. DHA showed a concentration-dependent inhibition of in-vitro saquinavir metabolism with K_m, V_{max} and K_i values of 2.21 μM, 0.054 μmol h^{-1} (mg protein)$^{-1}$ and 149.6 μM, respectively. After oral co-administration with 250 μg kg^{-1} DHA, the bioavailability of saquinavir significantly increased approximately 4 fold ($P < 0.01$) without affecting the elimination half-life, as compared with the control. In contrast, oral administration of DHA did not affect the kinetic parameters of saquinavir administered intravenously. These results suggest that the inhibitory effect of DHA on saquinavir metabolism predominantly takes place in the gut and imply that DHA impairs the function of enteric, but not of hepatic, CYP3A. The pharmacokinetic interaction occurred only when DHA was taken simultaneously with oral administration of saquinavir. These results considered together with the lack of time-dependent saquinavir metabolism inactivation effects in-vitro, imply that the inhibitory effect of DHA is primarily reversible. It is concluded that DHA inhibited saquinavir metabolism in-vitro and enhanced the oral bioavailability of saquinavir in rats.

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Introduction

Saquinavir is a potent human immunodeficiency virus (HIV)-protease inhibitor, and its major elimination pathway is reported to be hydroxylation by cytochrome P450 3A (CYP3A). It has also been shown that saquinavir is extensively metabolized by microsomes in the small intestine (Fitzsimmons & Collins 1997). Since its bioavailability is approximately 4% in the fed and 1% in the fasted state (Noble & Faulds 1996), it has been used clinically in combination with other antiretroviral drugs such as ritonavir, an antiretroviral drug that has an inhibitory effect on both CYP3A and P-glycoprotein (P-gp) (Eagling et al 1997). Because of the high cost of anti-HIV drugs, a method to increase their low bioavailability is highly desirable.

A major barrier for oral drug absorption is the first-pass metabolism by cytochrome P450, mostly CYP3A in the small intestine. In addition, P-glycoprotein (P-gp), an active efflux transporter classified as one of the ATP-binding cassette (ABC) transporters, has been reported to act synergistically with CYP3A on pre-systemic gut metabolism of drugs given orally (Benet et al 1999). Therefore, bioavailability of substrates of both CYP3A and P-gp, such as midazolam, felodipine, ciclosporin and saquinavir, may be limited. It has been suggested that co-administration with inhibitors of CYP3A or P-gp may effectively improve bioavailability of such drugs (Benet et al 2004).

Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), an essential polyunsaturated fatty acid (PUFA) present in fish oil, has been commercially available as a supplementary food ingredient since its health benefits were reported (Horrocks & Yeo 1999). We recently reported that DHA competitively inhibited the CYP3A-mediated 6β-hydroxylation of testosterone in rat liver microsomes and enhanced the oral bioavailability of ciclosporin in rats (Hirunpanich et al 2006). However, the possibility of using DHA as a bioavailability enhancer has been shown only with ciclosporin in our previous

paper, and further evidence was considered to be necessary using other CYP3A substrates. Therefore, the current study was conducted to examine the effect of DHA on CYP3A-mediated metabolism of saquinavir in-vitro, and to investigate the effect of DHA on the bioavailability of saquinavir in rats.

Materials and Methods

Materials

Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) was kindly supplied by FANCL (Yokohama, Japan). Saquinavir was kindly provided by Chugai Pharmaceutical Co. Ltd (Tokyo, Japan). β-Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G6P) disodium salt and glucose-6-phosphate dehydrogenase (G6PDH) from yeast were supplied by Oriental Yeast Co. Ltd (Tokyo, Japan). Magnesium chloride hexahydrate, dibasic potassium phosphate, monobasic potassium phosphate, potassium chloride (KCl), potassium phosphate tribasic, ethyl ether, carboxymethyl cellulose sodium (CMC-Na), sodium hydroxide, sodium carbonate, potassium tartrate, methanol, methylene chloride (dichloromethane), acetonitrile, polyoxyethylene (10) hydrogenated castor oil and 1-methyl-2-pyrrolidone were purchased from Wako Pure Chemical Industry Ltd (Osaka, Japan). Bovine serum albumin, 8-methoxypsoralen (8-MOP) and Folin-Ciocalteu's phenol were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other reagents used were of analytical grade and were available commercially.

Preparation of solutions

DHA stock solution was freshly prepared by dissolving in methanol to produce a concentration of 0.1 M and further dilution to obtain final concentrations of 10, 50, 100, 200 and 500 μM for the in-vitro experiments. The oral solution of saquinavir was prepared by suspending 200 mg saquinavir in 10 mL of 2% CMC-Na solution. The intravenous solution of saquinavir was prepared by dissolving 50 mg saquinavir in 10 mL of a mixture containing 5% ethanol, 5% polyoxyethylene (10) hydrogenated castor oil and 5% 1-methyl-2-pyrrolidone. The standard stock solution of saquinavir and 8-methoxypsoralen (8-MOP) was prepared by dissolving in methanol to produce a concentration of 1 mg mL^{-1}, and stored at 4°C. Samples for producing the calibration curve for HPLC analysis were prepared by adding known amounts of standard stock solution to drug-free plasma in the volume ratio of 1:10.

Liver microsomes preparation

Liver microsomes were prepared by a differential ultracentrifugation method (Hayes et al 1995) using the livers of male Wistar rats (Nihon Ikagaku Doubutsu, Saitama, Japan), 250–300 g. The protein content of the microsomal preparation was measured by the method of Lowry et al (1951) with bovine serum albumin as the standard.

Effect of DHA on metabolism of saquinavir in-vitro

The metabolism of saquinavir was measured in a system consisting of an NADPH-generating system and microsomes according to the method of Yamaji et al (1999) with some modifications. Incubation mixtures containing 5 mM $NADP^+$, 50 mM G6P, 500 U G6PDH, saquinavir solution (1.95, 6.3 and 13 μM) and various concentrations of DHA (0, 10, 50, 100, 200 and 500 μM) or 1 μM ketoconazole (a potent CYP3A inhibitor ($K_i = 0.1\,\mu$M) used as a positive control), were added into glass tubes and pre-incubated for 5 min at 37°C. The metabolic reaction was initiated by the addition of rat liver microsomal fractions yielding a final protein concentration of 0.4 mg mL^{-1}. Incubation time and temperature were controlled at 10 min and 37°C, respectively. Two-hundred microlitres of the resultant mixture was transferred into a glass tube containing 1500 μL of methylene chloride and 1 μM internal standard 8-MOP, at 0 and 10 min, respectively, followed by vigorous mixing for 20 s. The analysis was performed and the mean values of triplicate determinations was used. The percentage of metabolized saquinavir was determined from the difference between saquinavir concentrations before (0 min) and after (10 min) the reaction with the microsomes, according to the following equation:

$$M = [(SQV_{0min} - SQV_{10min})/SQV_{0min}] \times 100 \quad (1)$$

where M and SQV represent metabolized saquinavir (%) and saquinavir concentration (μM), respectively.

Time-dependent inactivation of saquinavir metabolism by DHA

Pre-incubation of DHA (0 (control), 75 μM) and 40 μM erythromycin, an irreversible CYP3A inhibitor (Zhou et al 2005) used as a positive control, with mixtures containing 5 mM $NADP^+$, 50 mM G6P, 500 U G6PDH and rat liver microsomal fractions (final protein concentration of 0.4 mg mL^{-1}) were conducted at 37°C for 0, 5, 10 and 20 min. After the incubation periods, 1.95 μM saquinavir was added and further incubated for 10 min. The reaction was terminated by the addition of 1500 μL methylene chloride containing 1 μg mL^{-1} 8-MOP, followed by vigorous mixing for 20 s. The concentration of saquinavir metabolism was measured and the mean value of triplicate determinations was used. The residual saquinavir concentration at each pre-incubation time was determined from the difference between saquinavir concentrations in the metabolic reaction at 0 and 10 min. The inhibitory effect of pre-incubation with DHA or erythromycin on saquinavir metabolism was expressed as the percentage of residual saquinavir. To determine the extent of saquinavir metabolism inactivation, residual saquinavir concentration at pre-incubation 0 min was arbitrarily set as 100%. The percentage of residual saquinavir at each pre-incubation time was determined according to the following equation:

$$R = (R_{SQVtmin}/R_{SQV0min}) \times 100 \quad (2)$$

where R, $R_{SQV0min}$ and $R_{SQVtmin}$ represent residual saquinavir (%) and residual saquinavir concentrations at the pre-incubation time 0 min (μM) and at each pre-incubation time (μM), respectively.

The semilogarithmic plot of the percentage of residual saquinavir was plotted against the pre-incubation time. The rate of saquinavir degradation was determined from the slope of the lines.

Extraction procedure for saquinavir from microsomes

The resultant mixture with added methylene chloride and 8-MOP was shaken on a high-speed shaker for 20 min and centrifuged at 2500 rev min^{-1} for 10 min. The lower organic layer was removed and evaporated to dryness at 50°C. The residue was reconstituted with 200 μL of the mobile phase (35% acetonitrile and 50 mM phosphate buffer, pH 5.0) and used for the determination of saquinavir concentration by HPLC analysis.

Enzyme-kinetic parameters

The velocity of saquinavir metabolic reaction (v) was estimated from the loss of saquinavir in the reaction mixture of rat liver microsomes, according to the following equation:

$$v = [(SQV_{0min} - SQV_{10min})/TPt \quad (3)$$

where T and Pt represent the time of reaction (h) and the protein concentration of microsomes ((mg protein) L^{-1}), respectively.

The Michaelis–Menten constant (K_m), inhibition constant (K_i) and maximum reaction rate (V_{max}) were calculated by non-linear least-squares regression analysis using the computer program, WinNonlin Professional v.4.01 (Mountain View, CA), according to the following equation:

$$v = V_{max}SQV/\{K_m[1 + DHA/K_i)] + SQV\} \quad (4)$$

where DHA represents the DHA concentration (μM).

Effect of DHA on saquinavir metabolism in-vivo

Male Wistar rats (Nihon Ikagaku Doubutsu Saitama, Japan), 220–250 g, were used throughout the in-vivo pharmacokinetic studies. The rats had free access to general food and water and were maintained in a temperature-controlled facility with a 12-h light–dark cycle for at least one week. Before starting the experiment, the rats were fasted but allowed free access to water for at least 12 h. The experiment was performed in accordance with the guidelines for Animal Experimentation in the Faculty of Pharmaceutical Sciences, Showa University.

Under light ether anaesthesia, the rats were placed in a supine position on a surgery pad. The left femoral artery of fasted-rat was cannulated with a polyethylene tube (SP-31; Natsume Seisakusho, Tokyo, Japan) to facilitate blood sampling. The cannulated rat was kept in a Bolman cage after the operation and studied after recovery from anaesthesia under a heating lamp to maintain normal body temperature. The rats received an oral dose of saquinavir, 50 mg kg^{-1}. Since the concentration of oral saquinavir solution was 20 mg mL^{-1}, the volume for administration to rats per body weight was 2.5 mL kg^{-1}. To examine the effect of DHA, 125 or 250 μg $kg^{-1}$of DHA, which corresponded to final concentrations 5 and 10% v/w of DHA, respectively, was added to 2.5 mL kg^{-1} of oral saquinavir solution. An oral dose of saquinavir (50 mg kg^{-1}) in combination with 125 or 250 μg kg^{-1} of DHA or 2% CMC-Na solution (DHA-treated or control rats, respectively) was administered by using an oral plastic feeding catheter.

To examine the reversibility of DHA's effect on saquinavir metabolism, 2% CMC-Na with or without 250 μg kg^{-1} DHA was administered intragastrically (DHA-treated and control rats, respectively) at 2 h before the oral administration of 50 mg kg^{-1} saquinavir. Two hours was selected as the time in which DHA should have a marked inhibitory effect on the pharmacokinetic profile of saquinavir. Blood samples (300 μL) were collected from the femoral artery into heparinized centrifuge tubes at 1, 2, 4, 6, 8 and 10 h after administration.

Fasted-rats were cannulated with polyethylene tube in the right femoral vein and left femoral artery for intravenous administration and collecting blood samples, respectively. The intravenous dose of saquinavir in rats was 10 mg kg^{-1}. Since the concentration of intravenous saquinavir solution was 5 mg mL^{-1}, the volume for administration to rats per body weight was 2 mL kg^{-1}. To determine the effect of DHA, 200 μg kg^{-1} DHA, equivalent to a final concentration of 10% v/w, was orally administered. After recovery from anaesthesia, 2% CMC-Na solution alone or containing 200 μg kg^{-1} DHA was orally administered (nominated as control or DHA-treated rats) for 2 h before 10 mg kg^{-1} saquinavir was administered intravenously through the femoral vein. Then, blood samples (300 μL) were collected from the femoral artery into heparinized centrifuge tubes at 0.167, 0.5, 1, 2, 4 and 6 h.

Plasma samples were obtained by centrifuging the blood samples at 3000 rev min^{-1} for 10 min and immediately frozen at −80°C until analysis. The concentration of saquinavir in plasma samples was analysed by HPLC. The plasma concentration–time profiles of saquinavir after oral and intravenous administration in each experimental group were determined.

Extraction of saquinavir from plasma

Extraction of saquinavir from plasma samples was performed according to the method of Walson et al (2003). The frozen plasma samples were thawed and vortexed. A volume of 150 μL plasma was transferred to a glass tube containing 1 mL of methylene chloride and 1 μM 8-MOP, as an internal standard. The tubes were shaken at high speed on a shaker for 20 min and centrifuged for 5 min at 2500 rev min^{-1} and 750 μL of the lower organic layer was transferred to a glass tube and evaporated to dryness at 50°C. The residue was reconstituted with 200 μL of the mobile phase (35% acetonitrile and 50 mM phosphate buffer, pH 5.0) and used for HPLC injection.

High-performance liquid chromatography (HPLC) analysis

Saquinavir concentration was determined by an HPLC system which consisted of a dual pump system (DP-8020; Tosoh, Tokyo, Japan), a UV detector (UV-8020; Tosoh), a column oven (CO-8000; Tosoh), a degasser (SD 8022; Tosoh) and a digital recorder (Chromatocorder 21; Tosoh). The HPLC column used was Nova-Pak C18 (4.6×150 mm, Waters, MA, USA). The two mobile phase components (A and B) were as follows: A, 750 mL of acetonitrile and 250 mL of 50 mM phosphate buffer (pH 5.0); B, 1000 mL of 50 mM phosphate buffer (pH 5.0). These mobile phases were degassed before use.

Elution of saquinavir with a linear gradient was performed with 62–45% mobile phase B (0–16 min) for separation, 45–0% (16–18 min) then 0% (18–22 min) for column wash and 0–62% (22–22.5 min) then 62% (22.5–25 min) for column re-equilibration. The analysis was maintained at 30°C, with a flow rate of 1 mL min^{-1} over a 25-min run time. The absorbance wavelength was 210 nm. The volume of the reconstituted saquinavir samples applied onto the HPLC system was 70 μL.

Pharmacokinetic analysis

Pharmacokinetic parameters of saquinavir (total clearance (Cl_{tot} for intravenous dose and Cl_{tot}/F for oral dose), half-life (t½), volume of distribution (V_{dss} for intravenous dose and V_{dss}/F for oral dose), mean residence time (MRT) and the areas under the whole blood concentration–time curve from zero to infinity (AUC_∞)) were estimated by a non-compartmental analysis using a computer program, WinNonlin (version 4.0.1; Pharsight Corporation, NC, USA).

Statistical analysis

Data are expressed as the mean ± standard error of mean (s.e.m.). Levene's test was used to test for variance homogeneity. Treatment groups were compared with the control group by using a standard statistical procedure – Student's *t*-test when two groups were compared and by analysis of variance followed by Dunnett's post-hoc test for multiple comparison among the groups. However, in cases where only 3 replicates were employed, non-parametric statistical methods (Kruskal–Wallis test and Mann–Whitney *U*-test) were used. Differences were considered to be statistically significant if $P < 0.05$.

Results

Effect of DHA on metabolism of saquinavir in-vitro

Table 1 shows the effect of DHA on the metabolism of saquinavir in rat liver microsomes. Saquinavir was extensively metabolized in rat liver microsomes and the rate of metabolism was decreased when the saquinavir concentration increased, indicating the saturation of the enzymatic reaction. Ketoconazole (1 μM), as a positive control, consistently inhibited the amount of metabolized saquinavir by about 50% in all experimental groups as compared with the control. After incubation with various concentrations of DHA, the amount of metabolized saquinavir was decreased in a dose-dependent manner. In addition, our results demonstrate that the inhibitory effect of 500 μM DHA was equivalent to that of 1 μM of ketoconazole in all experimental groups. Lower concentrations of DHA (10 and 50 μM) did not show a significant inhibitory effect as compared with the control. The in-vitro kinetic parameters, K_m, V_{max} and K_i, as estimated by a linear regression analysis, were 2.21 ± 0.04 μM, 0.054 ± 0.030 μmol h^{-1} (mg protein)$^{-1}$ and 149.6 ± 4.2 μM, respectively.

To investigate the reversibility of the effect of DHA on CYP 3A activity in-vitro, the time-dependent inhibition of saquinavir metabolism was examined. Erythromycin, a potent irreversible CYP3A inhibitor, strongly inhibited the saquinavir metabolism in a time-dependent manner with a rate of saquinavir degradation of 0.134 min^{-1} (Figure 1). The inhibitory effect of DHA on saquinavir metabolism was not significantly increased after elongation of pre-incubation times, with the rate of saquinavir degradation being similar to that of a control group (0.048 vs 0.040 min^{-1}, respectively).

Table 1 Inhibitory effect of DHA and ketoconazole on metabolism of saquinavir in rat liver microsomes

Initial saquinavir concn (μM)	Metabolized saquinavir (%)						
	Control	10 μM DHA	50 μM DHA	100 μM DHA	200 μM DHA	500 μM DHA	1 μM Ketoconazole
1.95	75.6 ± 0.6	74.5 ± 0.6	67.8 ± 4.7	58.5 ± 3.8*	53.0 ± 3.8*	42.0 ± 4.4**	38.3 ± 6.0**
6.3	68.8 ± 3.2	63.6 ± 4.7	59.4 ± 0.5	48.8 ± 3.3*	48.8 ± 3.3*	31.8 ± 3.1**	31.3 ± 13.0**
13	46.9 ± 2.2	37.9 ± 5.0	32.9 ± 5.4	32.6 ± 2.4	32.6 ± 2.4*	23.5 ± 4.6**	18.0 ± 7.7**

DHA (0, 10, 50, 100, 200 and 500 μM) or 1 μM ketoconazole were pre-incubated with saquinavir in the NADPH-generating system for 5 min at 37°C. The metabolic reaction was initiated by the addition of rat liver microsomal fractions and controlled at 37°C for 10 min. The inhibitory effect was determined from metabolized saquinavir (%) (M), which was calculated from equation 1. Values are mean ± s.e.m. of three independent assays. *$P < 0.05$, **$P < 0.01$, compared with control.

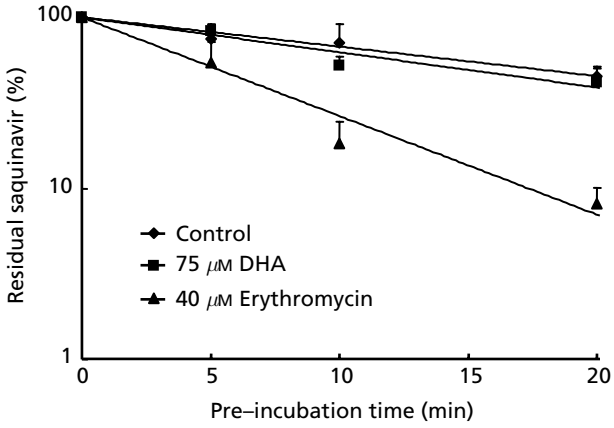

Figure 1 Time-dependent inactivation of saquinavir metabolism by DHA and 40 μM erythromycin in rat liver microsomes. Pre-incubation of microsomal fractions in NADPH-generating system with 0 μM and 75 μM DHA solution or 40 μM erythromycin was conducted at 37°C for 0, 5, 10 and 20 min, followed by adding saquinavir and incubating for 10 min. The residual saquinavir concentration was determined from the difference between saquinavir concentrations in the metabolic reaction at 0 and 10 min. To determine the extent of inactivation, residual saquinavir concentration at pre-incubation (0 min) was arbitrarily set as 100%. Values are mean ± s.e.m. of three independent assays.

Effect of DHA on bioavailability of saquinavir

Based on the in-vitro metabolism study, we examined the in-vivo pharmacokinetic effect of DHA in rats. The effect of oral administration of DHA on the pharmacokinetic parameters after oral and intravenous saquinavir administration in rats are shown in Table 2.

The plasma concentration–time profiles of saquinavir after oral administration of 50 mg kg^{-1} saquinavir in combination with 125 or 250 μg kg^{-1} DHA are shown in Figure 2A. When saquinavir was orally co-administered with DHA, saquinavir plasma concentrations were markedly increased in a dose-dependent manner. After oral co-administration of saquinavir and 250 μg kg^{-1} DHA, the values of AUC and C_{max} were significantly increased by about 3.3 and 2.9 fold, respectively, as compared with the control group (3.09 vs 0.72 μg h mL^{-1} and 0.70 vs 0.18 μg mL^{-1}, $P < 0.01$, respectively). The value of Cl/F and V_{dss}/F were significantly decreased from 122.6 to 24.6 L h^{-1} kg^{-1} and 271.5 to 55.4 mL kg^{-1}, ($P < 0.05$), respectively. The bioavailability (F) values of saquinavir, calculated as (AUC(oral)/Dose(oral)) · (Dose(i.v.)/AUC(i.v.)), were 6.5% and 26.4% in the control and 250 μg kg^{-1} DHA-treated groups, respectively. When corrected by these F values, the CL and V_{dss} after oral administration were found to be similar in both the control and DHA groups (7.70 vs 6.49 L h^{-1} kg^{-1} and 17.65 vs 14.62 mL kg^{-1}, respectively). In contrast, co-administration of saquinavir with 125 μg kg^{-1} DHA gave pharmacokinetic parameters (AUC, C_{max}, Cl/F and V_{dss}/F), which were not significantly different when compared with the control (1.16 μg h mL^{-1}, 0.22 μg mL^{-1}, 82.4 L h^{-1} kg^{-1} and 280.9 mL kg^{-1}, respectively). Moreover, the values of t½, T_{max} and MRT were not significantly different between each group.

The effect of orally administered DHA on the plasma concentration of saquinavir after intravenous administration with 10 mg kg^{-1} saquinavir is shown in Figure 2B. There were no significant differences in the saquinavir concentrations or in the pharmacokinetic parameters between the control and DHA-treated groups.

To determine the reversibility effect of DHA on saquinavir metabolism in-vivo, DHA was pre-administered before saquinavir was given. The effect of pre-treatment with DHA on the plasma saquinavir concentration in rats is shown in Figure 3. Pre-treatment with 250 μg kg^{-1} DHA for 2 h, before oral administration of 50 mg kg^{-1} saquinavir, did not affect the plasma saquinavir concentrations, and there were no statistical differences in

Table 2 Effect of DHA co-administration on the pharmacokinetic parameters of saquinavir after oral (50 mg kg^{-1}) and intravenous (10 mg kg^{-1}) administrations of saquinavir to rats

Parameters	Oral administration			Intravenous administration	
	Control (without DHA) (n = 7)	Co-administered with 125 μg kg^{-1} DHA (n = 4)	Co-administered with 250 μg kg^{-1} DHA (n = 9)	Control (without DHA) (n = 5)	Co-administered with 200 μg kg^{-1} DHA (n = 5)
AUC_∞ (μg h mL^{-1})	0.72 ± 0.27	1.16 ± 0.36	3.09 ± 0.44**	2.20 ± 0.47	2.34 ± 0.42
t½ (h)	1.50 ± 0.19	1.96 ± 0.47	1.96 ± 0.39	1.50 ± 0.18	2.63 ± 1.25
Cl_{tol}/F, Cl (L h^{-1} kg^{-1})	122.6 ± 32.9	82.4 ± 46.7	24.6 ± 9.2*	5.31 ± 0.97	5.15 ± 1.31
V_{dss}/F, V_{dss} (mL kg^{-1})	271.5 ± 79.8	280.9 ± 19.4	55.4 ± 13.2*	12.4 ± 3.2	15.60 ± 4.72
T_{max} (h)	3.86 ± 0.83	4.50 ± 0.50	4.00 ± 0.47	—	—
C_{max} (μg mL^{-1})	0.18 ± 0.07	0.22 ± 0.07	0.70 ± 0.14**	—	—
MRT (h)	5.11 ± 0.51	5.74 ± 0.79	5.33 ± 0.69	1.40 ± 0.13	2.59 ± 1.21

AUC_∞, area under the whole blood concentration–time curve from zero to infinity; t½, half life; CL_{tot}, total clearance; F, bioavailability; Cl, clearance; V_{dss}, volume of distribution; T_{max}, time for maximum concentration; C_{max}, maximum drug concentration; MRT, the mean residence time. Values are mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, compared with control.

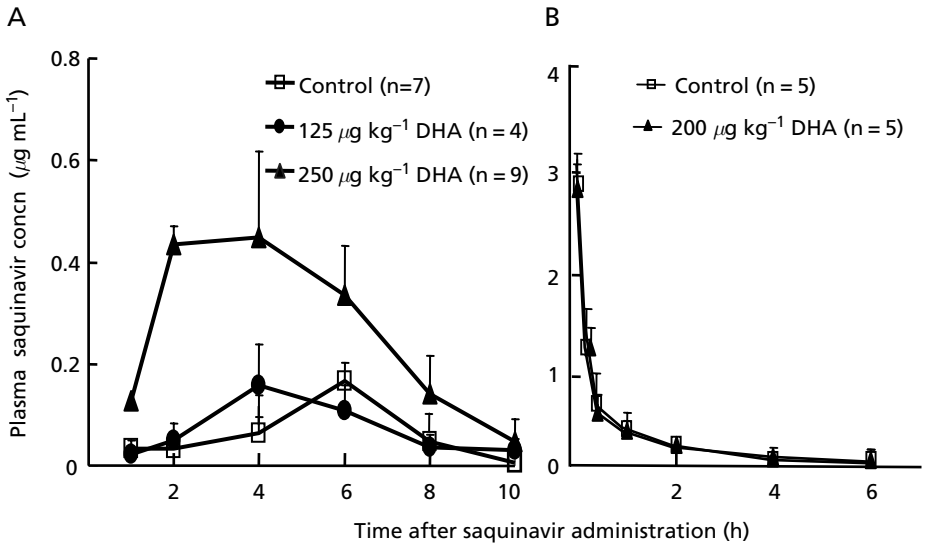

Figure 2 Effect of orally administered DHA on the saquinavir plasma concentration after oral (A) and intravenous (B) administration of saquinavir to rats. Rats were orally administered 50 mg kg^{-1} saquinavir with 0, 125 and 250 μg kg^{-1} DHA (A) and intravenously administered 10 mg kg^{-1} saquinavir after oral administration with 2% CMC-Na (control) or 200 μg kg^{-1} DHA for 2 h (B), respectively. Values are mean ± s.e.m. for 4–9 rats.

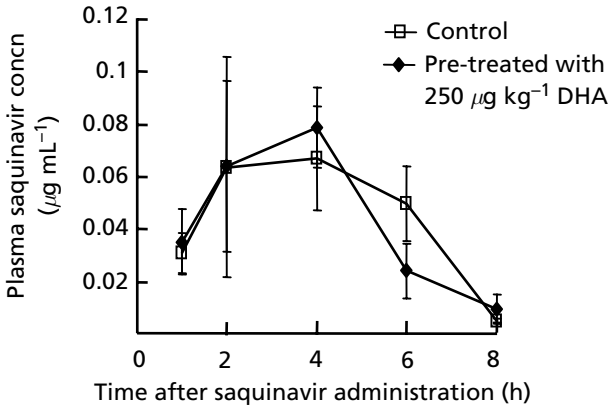

Figure 3 Effect of orally pre-administered DHA on the saquinavir plasma concentration after oral administration of saquinavir in rats. Rats were orally administered without (control) or with 250 μg kg^{-1} DHA for 2 h before orally administering 50 mg kg^{-1} saquinavir. Values are mean ± s.e.m. for 3 rats.

AUC_∞, C_{max} and T_{max} between DHA-treated and control rats (0.36 vs 0.58 μg h mL^{-1}, 0.080 vs 0.103 L h^{-1} kg^{-1} and 2.8 vs 3.3 h, respectively).

Discussion

This study was performed to determine the effect of DHA on saquinavir metabolism in-vitro and in-vivo. It has been reported that saquinavir is metabolized by cytochrome P450, and that more than 90% of its metabolism is mediated by CYP3A (Noble & Faulds 1996). Fitzsimmons & Collins (1997) reported that saquinavir is extensively metabolized by gut in its pre-systemic first-pass elimination and the metabolism of saquinavir is qualitatively the same in both hepatic and small intestinal microsomes. We therefore employed liver microsomes as a source of cytochrome P450 instead of using intestinal microsomes. The K_m and V_{max} of saquinavir in rat liver microsomes obtained in this study (2.21 μM and 0.054 μmol h^{-1} (mg protein)$^{-1}$, respectively) were comparable with those reported in a previous study (Yamaji et al 1999).

Our in-vitro studies showed that DHA inhibited saquinavir metabolism in a dose-dependent manner. These results are consistent with our previous study, which demonstrated that DHA inhibited the oxidation of testosterone to 6β-hydroxytestosterone, a well-known probe of CYP3A metabolism, in rat liver microsomes with a K_i of 5.52 ± 0.83 μM (Hirunpanich et al 2006). In our study, the K_i value of DHA for saquinavir metabolism was 149.6 μM, which is higher than that for testosterone metabolism. The fact that saquinavir was oxidized in the small intestine, or by hepatic microsomes, to about 7 metabolites (Fitzsimmons & Collins 1997) may indicate that this apparently higher K_i value is representative of the overall metabolites of saquinavir but not for any specific metabolite.

Mechanism-based inhibition (MBI) of CYP3A is characterized by nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-, time- and concentration-dependent enzyme inactivation. The possibility of MBI could be excluded by at least one piece of negative evidence of the above characteristics of MBI (i.e., the lack of time-dependency). In this study, the inhibitory effect of DHA did not change after elongation of the pre-incubation times of DHA with the microsomal fractions and NADPH-generating system before the addition of saquinavir, with the

rate of saquinavir degradation being similar to that of the control group but lower than that of erythromycin, a potent mechanism-based inhibitor of CYP3A enzyme. These lines of evidence suggest that the inhibitory effect of DHA on saquinavir metabolism is different from that of erythromycin and imply that the inhibitory effect of DHA on saquinavir metabolism is primarily reversible and not a mechanism-based inhibition. However, further studies should be undertaken on this issue.

In the in-vivo studies in rats, oral co-administration with 250 μg kg^{-1} DHA significantly increased the AUC and C_{max} by about three fold without affecting the elimination half-life and volume of distribution, as compared with the control. In contrast, DHA did not change the kinetic parameters of saquinavir after intravenous administration. In addition, Fitzsimmons & Collins (1997) reported that saquinavir is extensively metabolized by human small-intestinal microsomes and that CYP3A is the principal enzyme involved in the intestinal biotransformation of saquinavir. This suggests that oral administration of DHA affects saquinavir metabolism in the intestine but not liver. When combined with our in-vitro results, it is, therefore, conceivable that CYP3A enzyme in the gut is prone to inactivation and contributed to the increase in oral bioavailability of saquinavir in the rat. Moreover, pre-treatment with DHA did not affect the oral kinetic parameters of saquinavir, indicating that their interaction occurs only when DHA and saquinavir are administered concomitantly. Taken together with the lack of time-dependent saquinavir metabolism inactivation effect in-vitro, these data suggest that DHA has a reversible inhibitory effect on CYP3A activity. However, the type of reversibility inhibition of DHA on CYP3A enzyme should be evaluated. These observations suggest that DHA acts as a modulator of the absorption process of saquinavir by inhibiting the cytochrome P450 system, mainly CYP3A, in the small intestine.

It has been reported that saquinavir bioavailability is influenced not only by intestinal cytochrome metabolism but also by P-gp, which is co-localized at the apical membrane in which cytochrome P450 is expressed (Wacher et al 1998; Benet et al 1999). Liu & Tan (2000) showed that DHA did not change P-gp expression in the P-388 mouse leukaemia cell line. Our previous study (Hirunpanich et al 2006) reported that DHA did not affect the transportation of ciclosporin across Caco-2 cells. However, Pereira de Oliveira et al (2005) examined the oral bioavailability of Ind(8)-Val, a valine derivative of indinavir with a P-gp inhibitory effect, in a Caco-2 model and found that it was not improved as compared with indinavir after administration in rats, suggesting an inconsistency between data obtained from the Caco-2 cell model and in-vivo tests. The effect of DHA on P-gp was thought to be negligible in this study, although it should be clarified further both in-vitro and in in-vivo models.

Many studies have investigated methods to increase saquinavir bioavailability and thus to overcome the large intra-individual variability in its pharmacokinetics. Cremophor EL has been examined to determine its ability to increase the AUC and C_{max} of saquinavir in a randomized, placebo-controlled study, but the safety of cremophor EL for oral use remains to be elucidated (Martin-Facklam et al 2002). Kupferschmidt et al (1998) determined that co-administration of grapefruit juice and saquinavir produced a 2-fold increase in the AUC in healthy subjects. This was consistent with evidence that grapefruit components inhibited CYP3A4-mediated saquinavir metabolism in human liver microsomes and P-gp-mediated saquinavir transport in Caco-2 cell monolayers (Eagling et al 1999). However, because of the irreversible inhibition and down-regulation of CYP3A4 protein by grapefruit juice (Lown et al 1997), as well as the large variation in its effects (Kupferschmidt et al 1998), the clinical use of grapefruit juice to improve the bioavailability of saquinavir is questionable.

DHA is a long-chain n-3 polyunsaturated fatty acid found in fish oil, which is popular as a supplementary health diet. Busnach et al (1998) reported that DHA improved ciclosporin absorption by increasing AUC but the mechanism of action has not been elucidated. Recently, DHA has been shown to enhance oral absorption of ciclosporin in rats by inhibiting CYP3A, and without affecting P-gp in gut (Hirunpanich et al 2006). Moreover, Sethabouppha et al (unpublished data) indicated that several polyunsaturated fatty acids (e.g., DHA, γ-linolenic acid, arachidonic acid and ecosapentaenoic acid (EPA)), inhibit CYP3A in rat liver microsomes. From our in-vitro and in-vivo observations, DHA showed a reversible inhibition of CYP3A metabolism that resulted in a 3-fold increase in oral bioavailability of saquinavir without affecting its elimination half-life or volume of distribution. DHA exerts an inhibitory effect on saquinavir metabolism when co-administered with the efficacious dose, 250 μM kg^{-1}, a very low dose but one that can effectively inhibit CYP3A activity in gut. Since DHA has no noticeable toxicity and has already been used as a supplementary food ingredient, it might be concluded that the inhibitory effect of DHA on CYP3A provides an attractive method to enhance the bioavailability of saquinavir.

Conclusions

This study is the first to show that DHA inhibits saquinavir metabolism in-vitro and in-vivo. In addition, these observations strongly support the findings of our previous study (Hirunpanich et al 2006), which suggested that DHA can be employed as a bioavailability enhancer for drugs subject to extensive pre-systemic gut elimination by CYP3A.

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