

## DEMONSTRATION OF DOCOSAHEXAENOIC ACID AS A BIOAVAILABILITY ENHANCER FOR CYP3A SUBSTRATES: IN VITRO AND IN VIVO EVIDENCE USING CYCLOSPORIN IN RATS

Vilasinee Hirunpanich, Jun Katagi, Benjaporn Sethabouppha, and Hitoshi Sato

Department of Clinical and Molecular Pharmacokinetics/Pharmacodynamics, Faculty of Pharmaceutical Science, Showa University, Tokyo, Japan (V.H., J.K., B.S., H.S.); Faculty of Pharmacy, Mahasarakham University, Mahasarakham, Thailand (V.H.); Pharmacokinetic Research Section, Ono Pharmaceutical Co., Ltd., Osaka, Japan (J.K.); and Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand (B.S.)

Received August 28, 2005; accepted November 10, 2005

### ABSTRACT:

To investigate the pharmacokinetic interaction between cyclosporin A (CsA) and docosahexaenoic acid (DHA) in vivo, 5 mg/kg CsA was orally or intravenously coadministered with DHA (50–200  $\mu$ g/kg) to rats. The effect of DHA on CYP3A activity was determined using rat liver microsomes in vitro. Moreover, the effect of DHA on P-glycoprotein (P-gp) function was examined using cultured Caco-2 cells in vitro. After oral coadministration of CsA with 100  $\mu$ g/kg and 200  $\mu$ g/kg DHA, bioavailability (BA) was significantly increased, compared with control rats. In contrast, no pharmacokinetic interaction was observed when CsA was intravenously administered in rats dosed orally with DHA, suggesting that DHA did

not affect hepatic metabolism. The formation of 6 $\beta$ -hydroxytestosterone from testosterone in rat liver microsomes was competitively inhibited by DHA. The  $K_m$ ,  $V_{max}$ , and  $K_i$  values were 25.5  $\mu$ M, 2.45 nmol/min/mg protein, and 5.52  $\mu$ M, respectively. Moreover, basal-to-apical transport of [ $^3$ H]CsA in the Caco-2 cell monolayer was not affected by DHA but was decreased by valspodar (PSC 833), a P-gp inhibitor. Our finding is the first to indicate that DHA inhibits intestinal CYP3A both in vitro and in vivo, but not P-gp. It was thus demonstrated that DHA could be used as a BA enhancer for the drugs that are extensively metabolized by CYP3A in the gut.

*cis*-4,7,10,13,16,19-Docosahexaenoic acid (DHA) is one of the polyunsaturated fatty acids (PUFAs) classified as an omega-3 fatty acid. DHA is present in fatty fish (mackerel, salmon, tuna) and mother's milk. In addition, flaxseed oil, canola oil, walnuts, and phytoplankton are good sources of DHA (Horrocks and Yeo, 1999). DHA plays a vital role in brain development in infants and maintenance of normal brain function in adults, skin integrity, eicosanoid signaling, and vision function (Williard et al., 2001). Epidemiological evidence showed beneficial effects of DHA on plasma lipids (Connor et al., 1993; Tsai and Lu, 1997; Calabresi et al., 2004), hypertension (Engler et al., 1999; Das, 2004), depression (Naliwaiko et al., 2004), rheumatoid arthritis (Covington, 2004), stroke (Iso et al., 2002), and cardiovascular diseases (Harper and Jacobson, 2003). DHA is commercially available as a dietary supplement in the form of a mixture with eicosapentaenoic acid (EPA, C20:5n-3) and/or a crude extract from fish oil (Covington, 2004). However, no interactions of DHA with drugs have been reported so far.

Cyclosporin A (CsA) is a lipophilic compound and is frequently

used after solid organ transplant surgery as an immunosuppressive agent. It has low oral absorption and its bioavailability is increased with a high-fat meal (Mueller et al., 1994). Several studies have indicated that the use of fish oil, PUFAs containing DHA and EPA, as the vehicle for CsA can limit the toxicity and decrease the therapeutic dose of CsA without decreasing its immunosuppressive effect (Thakkar et al., 2000). Furthermore, a prospective, randomized placebo-controlled, double-blind study determined that a mixture of *n*-3 PUFAs (85% EPA and DHA) and vitamin E increased bioavailability of CsA in kidney graft recipients (Busnach et al., 1998), and suggested that fish oil or the substances contained in fish oil might play a major role in increasing the bioavailability of CsA. However, the mechanism of the increased bioavailability remains to be clarified.

CsA is a cytochrome isoenzyme 3A (CYP3A) and P-glycoprotein (P-gp) substrate. Previous studies have demonstrated that the bioavailability of CsA can be increased by concomitant administration of ketoconazole, a potent CYP3A inhibitor and a moderate inhibitor of P-gp. CsA bioavailability decreased when coadministered with rifampin, a potent inducer of CYP3A and P-gp (Benet et al., 1999). There has been a strategic notion that CYP3A and/or P-gp inhibitor may be utilized clinically as a drug absorption enhancer due to the increase in the drug's bioavailability. Therefore, the present study was designed to investigate the effects of DHA on CYP3A metabolism

This work was supported in part by a research grant from the Nagai Foundation Tokyo.

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.105.007088.

**ABBREVIATIONS:** DHA, *cis*-4,7,10,13,16,19-docosahexaenoic acid; PUFA, polyunsaturated fatty acid; AUC<sub>∞</sub>, area under the whole blood concentration-time curve from zero to infinity; EPA, eicosapentaenoic acid; CsA, cyclosporin A; P-gp, P-glycoprotein; PSC 833, valspodar; HPLC, high-performance liquid chromatography; HBSS, Hanks' balanced buffered saline;  $t_{1/2}$ , half-life;  $V_{dss}$ , volume of distribution; CL<sub>tot</sub>, total clearance;  $F$ , bioavailability;  $K_m$ , Michaelis-Menten constant,  $K_i$ , inhibition constant;  $V_{max}$ , maximum metabolic rate;  $S$ , substrate concentration;  $I$ , inhibition concentration;  $v$ , metabolic reaction; TEER, transepithelial electrical resistance; B to A, basolateral to apical.

and P-gp function in vitro and in vivo to provide an insight into the possibility of DHA as a bioavailability enhancer.

### Materials and Methods

**Chemicals.** DHA was a gift from FANCL Corp. (Yokohama, Japan). Cyclosporin A and PSC 833 were kindly supplied by Novartis (Basel, Switzerland). A cyclosporine measurement kit (TDxFLx) was purchased from Abbott Japan Co. Ltd. (Tokyo, Japan). Corn oil, polyethylene glycol 200, and testosterone were obtained from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin, 6 $\beta$ -hydroxytestosterone, and 17 $\alpha$ -methyltestosterone were purchased from Sigma-Aldrich (St. Louis, MO). Enflurane was purchased from Dainabot Co. Ltd. (Tokyo, Japan). All other reagents used in this experiment were of analytical grade.

**Liver Microsome Preparation.** Liver microsomes were prepared by using the livers of male Wistar rats (Nihon Ikagaku Doubutsu, Saitama, Japan) weighing 220 to 250 g, according to the method of Sanwald et al. (1995). The protein content of the microsomal preparation was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard.

**Effect of DHA on CYP3A Activity in Vitro.** Testosterone, 6 $\beta$ -hydroxytestosterone, and 17 $\alpha$ -methyltestosterone were dissolved in methanol to a final concentration of 1 mM, 10 mM, and 20 mM, respectively, and kept as the stock solution at 4°C. NADPH was dissolved in distilled water to a final concentration of 10 mM and stored at -20°C. The inhibitory effect of DHA on CYP3A was evaluated by using the method of Ikeda et al. (2001). The oxidation of testosterone to 6 $\beta$ -hydroxytestosterone was used to assay CYP3A activity. Testosterone (15, 30, 100, and 300  $\mu$ M) was preincubated for 5 min in an incubation mixture consisting of 0.25 M potassium phosphate buffer containing 0.25 mM EDTA (pH 7.4), distilled water, rat liver microsomes (19.2 mg protein/ml), and DHA (5, 10, 20, 50, and 100  $\mu$ M). The reaction was initiated by the addition of 50  $\mu$ l of an NADPH-generating system (final concentrations: 3.3 mM glucose 6-phosphate, 0.4 unit/ml glucose-6-phosphate dehydrogenase, 1.3 mM NADP<sup>+</sup>, and 3.3 mM MgCl<sub>2</sub>) and terminated by the addition of 2 ml of ethyl acetate containing a 10 nM concentration of the internal standard, 17 $\alpha$ -methyltestosterone. Incubation time and temperature were controlled at 10 min and 37°C, respectively. The samples were centrifuged at 2500 rpm for 10 min, followed by storing at -80°C for 10 min, and 1 ml of the upper organic layer was transferred and evaporated at 40°C under a stream of nitrogen. The residue was reconstituted with 150  $\mu$ l of methanol and distilled water, and 100  $\mu$ l of the sample was used for injection in the high-performance liquid chromatography (HPLC) system.

**HPLC Analysis.** The samples obtained from microsome activity assays were analyzed for 6 $\beta$ -hydroxytestosterone concentrations by HPLC. The HPLC system was composed 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 column used was a TSG Gel SuperODS 2  $\mu$ m (100 mm  $\times$  4.6 mm). The two mobile phase components were as follows: mobile phase A, 580 ml of distilled water mixed with 2 ml of acetonitrile and 400 ml of methanol; and mobile phase B, 280 ml of distilled water mixed with 320 ml of acetonitrile and 400 ml of methanol. These mobile phases were degassed before used.

A linear gradient was programmed. The elution mode was 0 to 27% mobile phase B (0–10 min) for separation, 27–100% (10–20 min), then 100% (20–25 min) for column wash and 100–0% (25–30 min) for column reequilibration. The analysis was performed at 40°C, with a gradient mobile phase flow rate of 1 ml/min over the 30-min run time. The absorbance wavelength was set at 245 nm.

The extraction efficiency (recovery) of the sample preparation procedure was tested by comparing the peak-area ratio obtained from chromatography of aqueous standards with those of extracted incubation mixtures. The standard, 6 $\beta$ -hydroxytestosterone, concentrations, ranging from 10 to 1000  $\mu$ M, were used for the calibration curve.

**Enzyme-Kinetic Parameters.** The Michaelis-Menten constant ( $K_m$ ), inhibition constant ( $K_i$ ), and maximum metabolic rate ( $V_{max}$ ) were calculated by nonlinear least-squares regression analysis using the computer program, WinNonlin Professional v.4.01 (Pharsight, Mountain View, CA), according to the following equation, which represents the initial velocity of the metabolic reaction ( $v$ ):

$$v = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]}$$

where  $S$  and  $I$  represent the substrate and inhibition concentrations, respectively.

**Effect of DHA on Bioavailability of CsA in Vivo.** Male Wistar rats (Nihon Ikagaku Doubutsu) weighing 220 to 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 1 week. Before starting the experiment, the animals were fasted but allowed free access to water for 24 h. Under enflurane anesthesia, the femoral artery of each rat was cannulated with a polyethylene tube (SP-31; Natsume Seisakusho, Tokyo, Japan) to facilitate blood sampling. Some rats were cannulated in the femoral vein for i.v. administration. The cannulated rat was kept in a Bolman cage after the operation and studied after recovery from anesthesia.

Fifty milligrams of CsA was dissolved in 10 ml of corn oil, and the rats received an oral dose of CsA at 5 mg/kg body weight, i.e., 1 ml of the CsA-containing corn oil per kg. To examine the effect of DHA, 50, 100, or 200  $\mu$ g/kg DHA was added to a 1 ml/kg dose of the CsA-containing corn oil. An oral dose of CsA (5 mg/kg), alone or in combination with various concentrations of DHA, was administered by using an oral feeding tube. Then, blood samples (120  $\mu$ l) were collected from the femoral artery at 1, 2, 3, 4, 6, 9, 12, and 24 h.

Moreover, to examine the effect of a lag time between the DHA and CsA doses, corn oil with or without 200  $\mu$ g/kg DHA were intragastrically administered to rats nominated as control and DHA-treated rats, respectively, and after 3 h, 5 mg/kg CsA was given orally. Blood samples (120  $\mu$ l) were collected 1, 2, 3, 4, 6, 9, 12, and 24 h after the CsA administration.

For i.v. administration, 50 mg of CsA was dissolved in the mixture of polyethylene glycol 200 and 10% ethanol. After the rats recovered from anesthesia, corn oil alone or containing 100  $\mu$ g/kg DHA was orally administered (nominated as control or DHA-treated rats), 3 h before 5 mg/kg CsA was intravenously administered through the femoral vein. Then, blood samples (120  $\mu$ l) were collected from the femoral artery at 0.167, 0.5, 1, 2, 4, 8, 12, and 24 h.

All blood samples were collected in heparinized anticoagulant tubes, thoroughly mixed, and stored at 4°C until analysis.

**Determination of CsA Concentration in Blood.** CsA concentrations in whole blood were assayed by a commercially available fluorescence polarization immunoassay kit (TDxFLx; Abbott Japan Co. Ltd.) using the monoclonal antibody against CsA. Calibration curves (10–10,000 ng/ml) were obtained at each time for a set of samples, and the method was evaluated by analyzing the quality control samples provided by the manufacturer.

**Pharmacokinetic Analysis.** Pharmacokinetic parameters of CsA, total body clearance ( $CL_{tot}$  for i.v. dose and  $CL_{tot}/F$  for oral dose), half-life ( $t_{1/2}$ ), volume of distribution ( $V_{dss}$  for i.v. dose and  $V_{dss}/F$  for oral dose), and the areas under the whole blood concentration-time curve from zero to infinity ( $AUC_{\infty}$ ) were estimated by a noncompartmental analysis using the computer program, WinNonlin Professional (v.4.01; Pharsight).

**Caco-2 Cell Culture.** The Caco-2 cells were obtained from The American Type Culture Collection (Manassas, VA). Cells were grown in the culture medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air, with medium change on alternate days. For transport studies, the Caco-2 cells were seeded in a Transwell with 1-cm<sup>2</sup> permeable polycarbonate inserts (3- $\mu$ m pore size; Corning Life Sciences, Acton, MA) in 12-well tissue culture plates for 22 to 24 days before the transport study.

The integrity of the cell monolayer was measured by the transepithelial electrical resistance (TEER) measurements. After 3 weeks in cell culture, the monolayers that developed a TEER of approximately 500  $\Omega$  cm<sup>2</sup> were used for the transport studies.

**Effect of DHA on P-gp-Mediated Transport of [<sup>3</sup>H]CsA across Caco-2 Monolayers.** Filter-grown Caco-2 cells, after removal of the culture medium, were washed twice with Hanks' balanced buffered saline (HBSS; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). After each wash, the plate was

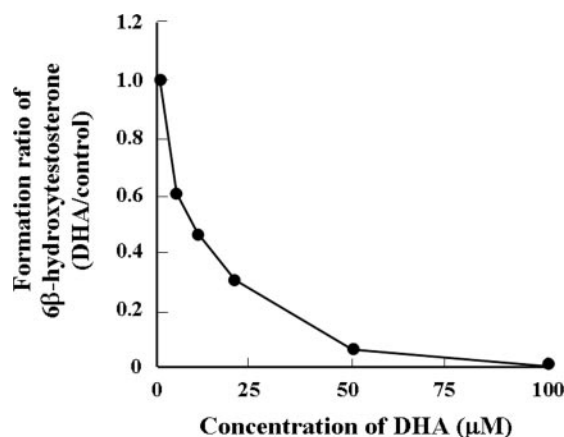


FIG. 1. Effect of DHA on the formation of 6 $\beta$ -hydroxytestosterone compared with control. Microsomal fractions were incubated with testosterone with or without DHA (5, 10, 20, 50, and 100  $\mu$ M). Each point represents the mean of duplicate determinations.

incubated for 30 min. TEER was measured to ensure the integrity of monolayers after the last wash. Then, 2 ml of HBSS was pipetted into the basolateral side (well) and another 1 ml of HBSS into the apical side of the cell layer (insert) (Augustijns et al., 1993).

CsA transport was initiated by adding 50 nM [ $^3$ H]CsA (3Ci/mmol) in HBSS buffer to either the apical or the basolateral side of the monolayers in the absence or presence of 100  $\mu$ M DHA or PSC 833. The basolateral to apical (B to A) transport of CsA was measured by aliquots of 50  $\mu$ l of incubation mixture from the receiver chambers, and an equal volume of buffer was replenished at 30, 60, 90, and 120 min. The radioactivity in these samples was determined by liquid scintillation counting.

**Statistical Analysis.** Data are expressed as the mean  $\pm$  S.D. Comparisons between two groups and among more than three groups were performed using Student's *t* test and one-way analysis of variance for multiple comparisons among the groups, respectively. The difference was considered to be statistically significant if the probability value was less than 0.05 ( $p < 0.05$ ).

## Results

**Effect of DHA on CYP3A Activity in Vitro.** Figure 1 shows the effect of DHA on the biotransformation of testosterone to 6 $\beta$ -hydroxytestosterone in rat liver microsomes. The ratios of 6 $\beta$ -hydroxytestosterone concentrations in DHA-containing tubes/control were extensively decreased with increasing DHA concentrations; in particular, 100  $\mu$ M DHA completely inhibited the formation of 6 $\beta$ -hydroxytestosterone.

The enzyme-kinetic parameters ( $K_m$  and  $V_{max}$ ) were  $25.47 \pm 3.60$   $\mu$ M and  $2.45 \pm 0.14$  nmol/min/mg protein, respectively. The inhibitory effect for DHA ( $K_i$ ) was calculated to be  $5.52 \pm 0.83$   $\mu$ M. As shown in the Lineweaver-Burk plots (Fig. 2), the slope of the line ( $K_m/V_{max}$ ) increased with the increase in the inhibitor (DHA) concentration, whereas the y-intercept ( $1/V_{max}$ ) did not change.

**Effect of DHA on Bioavailability CsA in Vivo.** Pharmacokinetic interaction between DHA and CsA in vivo was studied in rats. The mean blood concentration-time profiles of CsA after oral administration of 5 mg/kg CsA in combination with DHA (50, 100, and 200  $\mu$ g/ml) are shown in Fig. 3. The pharmacokinetic parameters obtained by a noncompartmental analysis are summarized in Table 1. When CsA was coadministered with DHA, CsA concentrations in whole blood were markedly increased in a dose-dependent manner with DHA. In groups of rats dosed with 100 and 200  $\mu$ g/ml DHA, the values of  $AUC_{\infty}$  significantly increased by 2.3- and 2.4-fold, respectively, when compared with the control group. In addition, the  $CL_{tot}/F$  and  $V_{dss}/F$  values were significantly decreased about 2.7-fold, whereas  $t_{1/2}$  values did not show any notable changes.

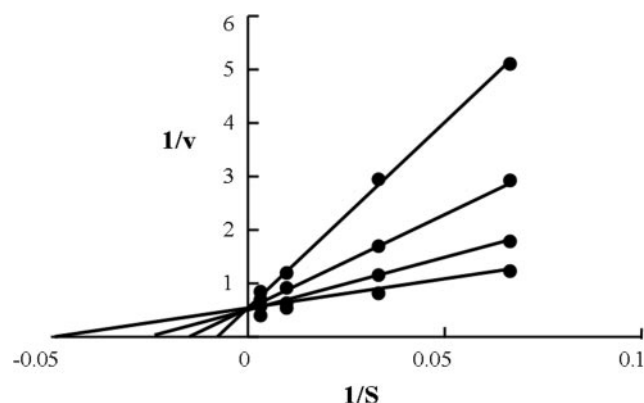


FIG. 2. Lineweaver-Burk plot for inhibition of testosterone oxidation by DHA in rat liver microsomes. Rat liver microsomes were incubated with various concentrations (15, 30, 100, and 300  $\mu$ M) of testosterone with 0, 10, 20, or 30  $\mu$ M DHA in the presence of an NADPH-generating system at 37°C for 10 min. [S] and [V] represent testosterone concentration and rate of oxidation, respectively. Each point represents the mean of duplicate determinations.

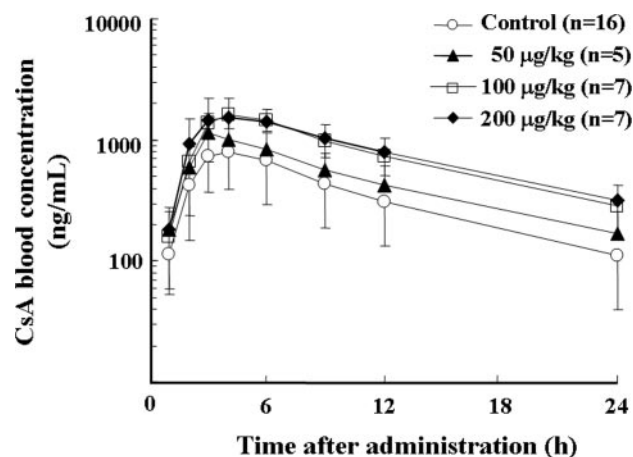


FIG. 3. Effect of orally administered DHA on blood CsA concentrations after oral administration of CsA to rats. Rats were orally administered 5 mg/kg CsA with 0, 50, 100, or 200  $\mu$ g/kg DHA. Each symbol with bar represents the mean  $\pm$  S.D. ( $n = 5-16$ ).

In contrast, in the case of i.v. administration, there were no differences in the blood CsA concentrations,  $AUC_{\infty}$ ,  $CL_{tot}$ ,  $V_{dss}$ , or  $t_{1/2}$  between control and DHA-treated rats (Fig. 4; Table 2).

Since CsA is eliminated predominantly by hepatic metabolism after i.v. administration (Parekh et al., 2004), the  $CL_{tot}$  of CsA (approximately 0.1 l/h/kg) can be attributed to hepatic clearance, which is far smaller than the hepatic blood flow rate in rats and thus limited by its intrinsic clearance. The bioavailability (*F*) values of CsA, as calculated as  $AUC(oral)/AUC(i.v.)$  at the same dosage, were 20.3%, 27.9%, 46.5%, and 48.6% at the DHA doses of 0 (control), 50, 100, and 200  $\mu$ g/ml, respectively. When corrected by these *F* values, the  $CL_{tot}$  (i.e., hepatic clearance) and  $V_{dss}$  values of CsA after oral administration turned out to be similar (range 0.11–0.15 l/h/kg and 1.5 to 1.8 l/kg, respectively) among DHA doses used in the combination with CsA.

Figure 5 shows the time courses of the mean blood CsA concentrations after pretreatment with 200  $\mu$ g/kg DHA 3 h before oral administration of 5 mg/kg CsA. There was no difference in blood CsA concentrations between control and DHA-treated groups.

**Effect of DHA on P-gp-Mediated Transport of [ $^3$ H]CsA across Caco-2 Monolayers.** [ $^3$ H]CsA was loaded on basolateral side of Caco-2 cell monolayers. The transport of [ $^3$ H]CsA in the basolateral



TABLE 1

Effect of DHA coadministration on the pharmacokinetic parameters of CsA after oral administration of CsA (5 mg/kg) to rats

Data are expressed as mean  $\pm$  S.D.

Parameters	Control (without DHA) (n = 16)	Oral Dose of DHA		
		50 $\mu$ g/kg (n = 6)	100 $\mu$ g/kg (n = 7)	200 $\mu$ g/kg (n = 7)
AUC <sub>∞</sub> (mg/l · h)	9.78 $\pm$ 5.32	13.4 $\pm$ 5.78	22.41 $\pm$ 7.40**	23.41 $\pm$ 6.55**
t <sub>1/2</sub> (h)	7.68 $\pm$ 1.04	8.00 $\pm$ 0.96	8.30 $\pm$ 1.65	8.52 $\pm$ 0.68
CL <sub>tot</sub> /F (l/h/kg)	0.748 $\pm$ 0.598	0.456 $\pm$ 0.271	0.248 $\pm$ 0.093*	0.230 $\pm$ 0.070*
V <sub>dss</sub> /F (l/kg)	8.61 $\pm$ 6.34	5.57 $\pm$ 2.86	3.20 $\pm$ 0.85*	3.14 $\pm$ 0.86*

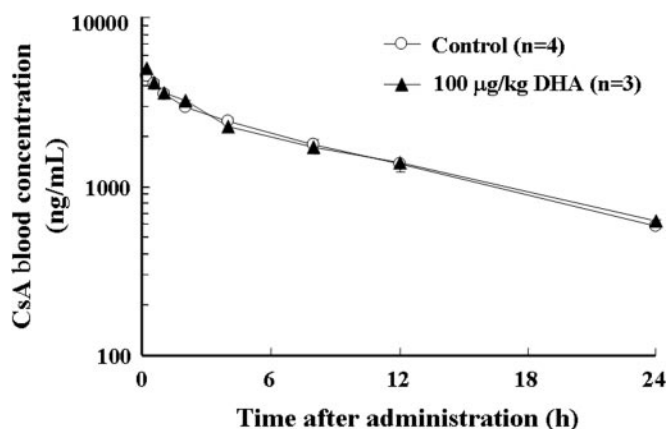
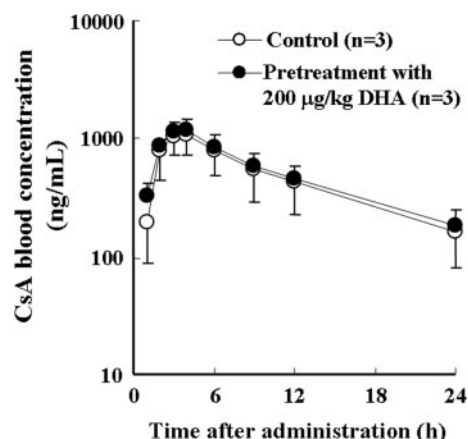
\* Statistically different from the corresponding value in the control group ( $p < 0.05$ ).\*\* Statistically different from the corresponding value in the control group ( $p < 0.01$ ).FIG. 4. Effect of orally administered DHA on blood CsA concentrations after intravenous administration of CsA to rats. Rats were either orally administered 100  $\mu$ g/kg DHA or not given DHA before intravenous administration of 5 mg/kg CsA. Each symbol with bar represents the mean  $\pm$  S.D. (n = 3–4).FIG. 5. Effect of pretreatment with 200  $\mu$ g/kg DHA on blood CsA concentrations 3 h before oral administration of CsA to rats. Rats were orally administered corn oil with or without 200  $\mu$ g/kg DHA before oral administration of 5 mg/kg CsA. Each symbol with bar represents the mean  $\pm$  S.D. (n = 3).

TABLE 2

Effect of DHA coadministration on the pharmacokinetic parameters of CsA after i.v. administration of CsA (5 mg/kg) to rats

Data are expressed as mean  $\pm$  S.D.

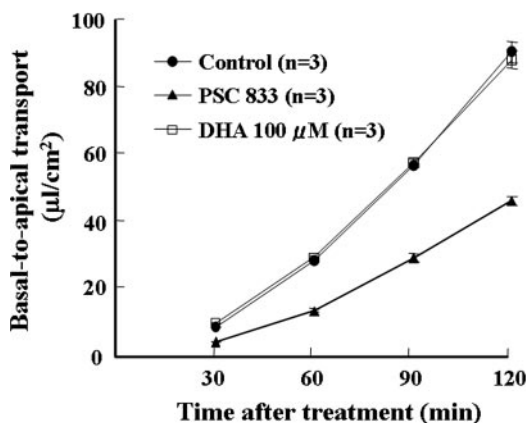
Parameters	Control (without DHA) (n = 4)	Coadministered with DHA 100 $\mu$ g/kg (n = 3)
AUC <sub>∞</sub> (mg/l · h)	48.2 $\pm$ 3.0	50.7 $\pm$ 4.7
t <sub>1/2</sub> (h)	9.62 $\pm$ 0.81	11.0 $\pm$ 0.4
CL <sub>tot</sub> (l/h/kg)	0.104 $\pm$ 0.006	0.0991 $\pm$ 0.0088
V <sub>dss</sub> (l/kg)	1.36 $\pm$ 0.10	1.43 $\pm$ 0.09

(B) to apical (A) direction across Caco-2 cell monolayers is shown in Fig. 6. A strong P-gp inhibitor, PSC 833, decreased the transportation of CsA from the B to A direction, but was not affected by 100  $\mu$ M DHA. These results suggest that DHA does not inhibit the P-gp-mediated transport of [<sup>3</sup>H]CsA.

### Discussion

Intestinal phase I metabolism and active extrusion of absorbed drug have recently been recognized as major determinants of oral drug bioavailability. CYP3A, the major phase I drug-metabolizing enzyme in humans, and the multidrug efflux pump, P-gp, are present at high concentration in the villus tip of enterocytes, the primary site of absorption for orally administered drugs. The importance of CYP3A and P-gp in limiting oral drug delivery was suggested by Benet et al. (1999). Therefore, this study was undertaken to explore the possibility that DHA, a PUFA in fish oil, may enhance the absorption of substrates of CYP3A and/or P-gp.

In the in vitro study using rat liver microsomes, DHA decreased the ratio of the formation of testosterone metabolite in a dose-dependent

FIG. 6. Effect of DHA and PSC 833 on [<sup>3</sup>H]CsA transport across Caco-2 monolayers in the basolateral to apical direction. Data represent the mean  $\pm$  S.D. (n = 3).

manner in DHA concentrations up to 100  $\mu$ M, suggesting that there was a decreased conversion of testosterone to its metabolite, 6 $\beta$ -hydroxytestosterone. The Lineweaver-Burk plots clearly indicated that DHA competitively inhibited CYP3A in vitro.

CsA is also the substrate of P-gp that acts as an absorption barrier by transporting drug from intestinal cells into the lumen, and is known to be coexpressed with the CYP3A system in the intestine (Lin, 2003). The Caco-2 cell line, a model for human intestine drug absorption, which expresses the P-gp efflux transport, was used to examine the additional effect of DHA on the bioavailability of CsA. Our results showed that DHA did not affect the [<sup>3</sup>H]CsA B to A transport across Caco-2 cell monolayers. Moreover, in a previous study reported by

Liu and Tan (2000), DHA did not affect P-gp expression in P388 or P388/DOX cell lines. These lines of evidence suggest that DHA inhibits the activity of CYP3A but not P-gp function.

In addition, oral coadministration of DHA and CsA in rat increased bioavailability of CsA by about 130%, whereas the hepatic clearance and volume of distribution did not change. In contrast, pretreatment with DHA did not alter the pharmacokinetics of CsA after i.v. administration. After oral administration of CsA alone, CsA was only partially absorbed in the small intestine with a bioavailability of approximately 20% in rats, which is close to that in human (approximately 30%) (Parekh et al., 2004). In other words, DHA affected the pharmacokinetics of orally administered CsA, but not intravenously administered CsA. From these lines of evidence in vivo and in vitro, inhibition of intestinal but not hepatic CYP3A enzymes is suggested to be the major mechanism for the enhancement of CsA bioavailability by DHA.

CsA absorption is variable and incomplete when administered orally (Wu et al., 1995). A clinical study in kidney transplant patients indicated that variability of intestinal expression of P-gp in humans may be a more important determinant of CsA bioavailability than the intestinal CYP3A metabolism (Lown et al., 1997). However, the presence of CYP3A is believed to be responsible for the decrease of CsA bioavailability in many studies. Evidence has been provided in many in vitro and in vivo studies that the low oral bioavailability of CsA is due to extensive cytochrome-mediated metabolism in the gastrointestinal tract rather than liver (Kolars et al., 1991; Wu et al., 1995).

Because of the poor oral absorption of CsA, an oral microemulsion has been developed which offers greatly improved and more predictable CsA bioavailability (Trull et al., 1995). However, a recent study suggested that CsA is quite well absorbed from the conventional formulation and that the low bioavailability is due to extensive cytochrome metabolism in gut wall. If this were the case, the improved bioavailability of CsA seen with the microemulsion formulation would presumably be due to protection from metabolism rather than to improved intestinal absorption.

In an attempt to improve the oral bioavailability, the cytochrome inhibitor and/or P-gp inhibitor has been coadministered with low-bioavailability drugs. Woo et al. (2003) determined that oral bioavailability of paclitaxel, a CYP3A and P-gp substrate, was enhanced by coadministration with the P-gp inhibitor, KR-30031, a verapamil analog with fewer cardiovascular effects, although the reversibility of its effects has not yet been determined.

Several studies have demonstrated that the bioavailability of CsA can be increased by concomitant administration of ketoconazole, a CYP3A and P-gp inhibitor (Gomez et al., 1995). However, the usefulness of this drug as an oral drug absorption enhancer is limited because many side effects such as transient anorexia, vomiting, lethargy, increased shedding of hair, and gingival hyperplasia have been reported (Mouatt, 2002).

Grapefruit juice is well known to increase the bioavailability of many drugs, including CsA. Experimental studies have demonstrated that grapefruit juice not only inhibits drug metabolism via CYP3A in the intestine but also enhances drug absorption via P-gp in Caco-2 cells (Spahn-Langguth and Langguth, 2001). Several studies revealed that the inhibitory effect of grapefruit juice on CYP3A in gut wall resulted in markedly increased bioavailability of oral, but not intravenous, CsA (Dahan and Altman, 2004). Grapefruit juice produces irreversible CYP3A inhibition; therefore, it does not need to be taken simultaneously with medication to produce the interaction (Yee et al., 1995). The irreversible inhibition of CYP3A by grapefruit juice is consistent with the observation that drug interactions occur in vivo

well after grapefruit juice consumption (Dahan and Altman, 2004). In contrast, the interaction between DHA and CsA is likely to require the presence of both substrate and DHA in the intestine, suggesting that the inhibitory activity of DHA on CYP3A is reversible.

Furthermore, our in vivo result, which demonstrated the increase in oral bioavailability of CsA when coadministered with DHA, is consistent with the data of Busnach et al. (1998), who reported that the pharmacokinetic profile of CsA was improved in kidney graft recipients supplemented with *n*-3 polyunsaturated fatty acid containing EPA and DHA. The present study should be the first to provide more mechanistic and comprehensive insight into the effect of a PUFA on CsA bioavailability. The concentrations of CsA were not significantly different from the 100 and 200  $\mu\text{g/ml}$  DHA doses in vivo. This can occur, presumably, because DHA showed a saturable effect on its inhibition of CYP3A in the rat intestine, and the dose range of 100 to 200  $\mu\text{g/kg}$  DHA might result in its concentration in the gut being above its  $K_i$  value (5.52  $\mu\text{M}$  or 1.8  $\mu\text{g/ml}$ ) determined in our experiment.

No serious toxic effects of DHA have been reported (Horrocks and Yeo, 1999). Instead, many health benefits of DHA have been observed, and it is now available in some foods, especially infant formula. Clinical studies have reported that dietary supplementation with fish oil containing DHA can potentiate the efficacy and limit the toxicity of CsA. It has been shown that the use of a fish oil vehicle for CsA can decrease CsA-induced hypertension, decrease CsA nephrotoxicity, and enhance the immunosuppressive effect of CsA. The protective mechanism of action of DHA has been determined in several studies. For example, Thakkar et al. (2000) explained that DHA decreases the toxicity of CsA by reversing the membrane-perturbing effect of CsA and increasing the endothelial macromolecular permeability. It is considered from the above-mentioned properties of DHA that DHA may be used as a bioavailability enhancer in the clinical setting, with the added assurance of the improvement of both the safety and efficacy of CsA.

In summary, we have demonstrated that DHA has a reversible inhibitory activity on CYP3A in vitro. Moreover, DHA potently inhibited intestinal CYP3A metabolism but did not interfere with P-gp function and hepatic metabolism. To our knowledge, there have been no reports describing the mechanism of drug bioavailability enhancement by PUFAs (e.g., DHA), from the viewpoint of their effect on presystemic drug metabolism, which may significantly affect the pharmacokinetic profiles of CYP3A substrates. A comprehensive study of various PUFA-inhibitory effects on CYP3A is now undertaken in our laboratory. This study, therefore, is the first to clearly demonstrate that DHA can inhibit CYP3A metabolism both in vitro and in vivo. Selective and reversible inhibition of CYP3A by DHA in the intestinal lumen aiming to increase drug bioavailability should be an attractive method, because DHA may be clinically utilized as an effective bioavailability enhancer without notable toxicities, when combined with CYP3A substrates.

**Acknowledgments.** We appreciate Pharsight Corporation for providing us a license for WinNonlin software as part of the Pharsight Academic License (PAL) program. We sincerely thank FANCL Corporation (Yokohama, Japan) for their generous gift of pure DHA.

## References

- Augustijns PF, Bradshaw TP, Gan LS, Hendren RW, and Thakker DR (1993) Evidence for a polarized efflux system in Caco-2 cells capable of modulating cyclosporin A transport. *Biochem Biophys Res Commun* 197:360–365.
- Benet LZ, Izumi T, Zhang Y, Silverman JA, and Wachter VJ (1999) Intestinal MDR transport proteins and P-450 enzymes as barriers to oral drug delivery. *J Controlled Release* 62:25–31.
- Busnach G, Stragliotto E, Minetti E, Perego A, Brando B, Broggi ML, Nefrologia GC, Renale DTT, and Granda ONC (1998) Effect of *n*-3 polyunsaturated fatty acids on cyclosporine

- pharmacokinetics in kidney graft recipients: a randomized placebo-controlled study. *J Nephrol* **11**:87–93.
- Calabresi L, Villa B, Canavesi M, Sirtori CR, James RW, Bernini F, and Franceschini G (2004) An omega-3 polyunsaturated fatty acid concentrate increases plasma high-density lipoprotein cholesterol and paraoxonase levels in patients with familial combined hyperlipidemia. *Metabolism* **53**:153–158.
- Connor WE, DeFrancesco CA, and Connor SL (1993) N-3 fatty acids from fish oil. Effects on plasma lipoproteins and hypertriglyceridemic patients. *Ann NY Acad Sci* **683**:16–34.
- Covington MB (2004) Omega-3 fatty acids. *Am Fam Physician* **70**:133–140.
- Dahan A and Altman H (2004) Food-drug interaction: grapefruit juice augments drug bioavailability—mechanism, extent and relevance. *Eur J Clin Nutr* **58**:1–9.
- Das UN (2004) Long chain polyunsaturated fatty acids interact with nitric oxide, superoxide anion and transforming growth factor-beta to prevent human essential hypertension. *Eur J Clin Nutr* **58**:195–203.
- Engler MM, Engler MB, Goodfriend TL, Ball DL, Yu Z, Su P, and Kroetz DL (1999) Docosahexaenoic acid is an antihypertensive nutrient that affects aldosterone production in SHR. *Proc Soc Exp Biol Med* **221**:32–38.
- Gomez DY, Wachter VJ, Tomlanovich SJ, Hebert MF, and Benet LZ (1995) The effects of ketoconazole on the intestinal metabolism and bioavailability of cyclosporine. *Clin Pharmacol Ther* **58**:15–19.
- Harper CR and Jacobson TA (2003) Beyond the Mediterranean diet: the role of omega-3 fatty acid in the prevention of coronary heart disease. *Prev Cardiol* **6**:136–146.
- Horrocks LA and Yeo YK (1999) Health benefits of docosahexaenoic acid (DHA). *Pharmacol Res* **40**:211–225.
- Ikeda T, Nishimura K, Taniguchi T, Yoshimura T, Hata T, Kashiya E, Kudo S, Miyamoto G, Kobayashi H, Kobayashi S, et al. (2001) HAB protocol: in vitro evaluation of drug interaction caused by enzyme inhibition. *Xenobio Metab Dispos* **16**:115–126.
- Iso H, Sato S, Umemura U, Kudo M, Koike K, Kitamura A, Imano H, Okamura T, Naito Y, and Shimamoto T (2002) Linoleic acid, other fatty acids and the risk of stroke. *Stroke* **33**:2086–2093.
- Kolars JC, Awni WM, Merion RM, and Watkins PB (1991) First-pass metabolism of cyclosporin by the gut. *Lancet* **338**:1488–1490.
- Lin JH (2003) Drug-drug interaction mediated by inhibition and induction of P-glycoprotein. *Adv Drug Deliv Rev* **55**:53–81.
- Liu QY and Tan BK (2000) Effects of cis-unsaturated fatty acids on doxorubicin sensitivity in P388/DOX resistant and P388 parental cell lines. *Life Sci* **67**:1207–1218.
- Lown KS, Mayo RR, Leichtman AB, Hsiao HL, Turgeon DK, Schmiedlin-Ren P, Brown MB, Guo W, Rossi SJ, Benet LZ, et al. (1997) Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. *Clin Pharmacol Ther* **62**:248–260.
- Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275.
- Mouatt JG (2002) Cyclosporin and ketoconazole interaction for treatment of perianal fistulas in the dog. *Aust Vet J* **80**:207–211.
- Mueller EA, Kovarik JM, VanBree JB, Grevel J, Luckner PW, and Kutz K (1994) Influence of a fat-rich meal on the pharmacokinetics of a new oral formulation of cyclosporine in a crossover comparison with the market formulation. *Pharm Res (NY)* **11**:151–155.
- Naliwaiko K, Araujo RL, Da Fonseca RV, Castilho JC, Andreatini R, Bellissimo MI, Oliveira BH, Martins EF, Curi R, Fernandes LC, et al. (2004) Effects of fish oil on the central nervous system: a new potential antidepressant? *Nutr Neurosci* **7**:91–99.
- Parekh K, Trulock E, and Patterson GA (2004) Use of cyclosporine in lung transplantation. *Transplant Proc* **36**:318S–322S.
- Sanwald P, Blankson EA, Dulery BD, Schoun J, Huebert ND, and Dow J (1995) Isocratic high-performance liquid chromatographic method for the separation of testosterone metabolites. *J Chromatogr B Biomed Appl* **672**:207–215.
- Spahn-Langguth H and Langguth P (2001) Grapefruit juice enhances intestinal absorption of the P-glycoprotein substrate talinolol. *Eur J Pharm Sci* **12**:361–367.
- Thakkar RR, Wang OL, Zerouga M, Stillwell W, Haq A, Kissling R, Pierce WM, Smith NB, Miller FN, and Ehringer WD (2000) Docosahexaenoic acid reverses cyclosporin A-induced changes in membrane structure and function. *Biochim Biophys Acta* **1474**:183–195.
- Trull AK, Tan KK, Tan L, Alexander GJ, and Jamieson NV (1995) Absorption of cyclosporin from conventional and new microemulsion oral formulations in liver transplant recipients with external biliary diversion. *Br J Clin Pharmacol* **39**:627–631.
- Tsai PJ and Lu SC (1997) Fish oil lowers plasma lipid concentrations and increases the susceptibility of low density lipoprotein to oxidative modification in healthy men. *J Formos Med Assoc* **96**:718–726.

---

**Address correspondence to:** Dr. Hitoshi Sato, Department of Clinical and Molecular Pharmacokinetics/Pharmacodynamics, Faculty of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo, 142-8555, Japan. E-mail: sato-tky@umin.ac.jp

---