DEMONSTRATION OF DOCOSAHEXAENOIC ACID (DHA) AS A

BIOAVAILABILITY ENHANCER FOR CYP3A SUBSTRATES: *IN VITRO* AND

IN VIVO EVIDENCE USING CYCLOPORIN IN RATS

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Abbreviations used are:

DHA, cis-4,7,10,13,16,19-docosahexaenoic acid; CYP 3A, cytochrome isoenzyme 3A;

PUFAs, polyunsaturated fatty acids; AUC_∞, areas under the whole blood concentration-time

curve from zero to infinity; $T_{1/2}$, half life; V_{dss}, volume of distribution; CL_{tot}, total clearance; F,

bioavailability; K_m, Michaelis-Menten constant, K_i, inhibition constant and V_{max}, maximum

metabolic rate; S, substrate concentrations; I, inhibition concentrations; v, metabolic reaction;

TEER, transepithelial electrical resistant; B to A, basolateral to apical

ABSTRACT:

In order to investigate pharmacokinetic interaction between cyclosporin A (CsA) and docosahexaenoic acid (DHA) *in vivo*, 5 mg/kg CsA was orally or intravenously co-administered with DHA (50-200 µg/kg) into rats. 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 co-administration of CsA with 100 µg/kg and 200 µg/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 6β-hydroxytestosterone from testosterone in rat liver microsomes was competitively inhibited by DHA. The K_m, V_{max} and K_i values were 25.5 µM, 2.45 nmol/min/mg-protein and 5.52 µM, 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 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 which are extensively metabolized by CYP 3A 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 also 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 adult, skin integrity, eicosanoid signaling and vision function (Williard et al., 2001). Epidemiological evidence showed beneficial effects of DHA on plasma lipids (Tsai and Lu, 1997; Connor et al., 1993; 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 (Haper 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 frequently used after solid organ transplant surgery as an immunosuppressive agent. It has low oral absorption and its bioavailability is increased with 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 co-administration 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 drug's bioavailability. Therefore, the present study was designed to investigate

the effects of DHA on CYP3A metabolism 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. Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) was a gift from FANCL Corp. (Yokohama, Japan). Cyclosporin A and PSC 833 were kindly supplied by Novartis Pharmaceutical company (Tokyo, Japan). Cyclosporin measurement kit (TDxFLx^{TM}) 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, 6β-hydroxytestosterone, and 17α-methyltestosterone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 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 220-250 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, 6β-hydroxytestosterone, and 17α-methyltestosterone were dissolved in methanol to a final concentration 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 6β-hydroxytestosterone was used to assay CYP3A activity. Testosterone (15,

30, 100, and 300 μM) was pre-incubated 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 μM). Reaction was

initiated by the addition of 50 μl NADPH-generating system (final concentrations: 3.3 mM

glucose 6-phosphate, 0.4 units/ml of glucose 6-phosphate dehydrogenase, 1.3 mM $NADP^+$

and 3.3 mM $MgCl_2$) and terminated by the addition of 2 ml of ethyl acetate containing 10 nM

of the internal standard, 17α-methyltestosterone. Incubation time and temperature were

controlled at 10 min and 37°C, respectively. The samples were centrifuged at 2,500 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 μl methanol and distilled water and 100 μl of the sample was used for injection in the

HPLC system.

High-Performance Liquid Chromatography (HPLC) Analysis. The samples obtained

from microsome activity assays were analyzed for 6β-hydroxytestosterone concentrations by

HPLC. The HPCL system 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

Super-ODS 2 μm (100 mm x 4.6 mm). The two mobile phase components were as follows:

mobile phase (A): 580 ml of distilled water was mixed with 2 ml of acetonitrile and 400 ml of

methanol. *Mobile phase (B):* 280 ml of distilled water was mixed with 320 ml of acetonitrile

and 400 ml of methanol. These mobile phases were degassed before used.

A linear gradient was programmed. The elusion mode was 0-27% 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 re-equilibration. The analysis was performed at 40°C, with a

gradient mobile phase flow rate 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, 6β-hydroxytestosterone, concentrations

ranging from 10 to 1,000 μM, were used for calibration curve.

Enzyme-Kinetic Parameters. Michaelis-Menten constant (K_m), inhibition constant (K_i), and maximum metabolic rate (V_{max}) were calculated by non-linear least-squares regression analysis using the computer program, WinNonlin Professional v.4.01 (Mountain View, CA, USA), 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 CsA *In Vivo*. Male Wistar rats (Nihon Ikagaku Doubutsu Saitama, Japan) weighing 220-250g were used throughout *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 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 polyethylene tube (SP-31, Natsume Seisakusho, Tokyo, Japan) to facilitate blood sampling. Some rats were cannulated in the femoral vein for intravenous (*i.v.*) administration. The cannulated rat was

kept in a Bolman cage after the operation and studied after recovery from anesthesia.

Fifty mg of CsA was dissolved in 10 ml corn oil, and the rats received an oral dose of

CsA at 5 mg per kg body weight (5 mg/kg), i.e., 1 ml of the CsA-containing corn oil per kg. To

examine the effect of DHA, 50, 100, or 200 µg/kg of DHA was added to 1 ml/kg 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 µl) were collected from the femoral artery at 1, 2, 3, 4, 6, 9, 12, and 24 h.

Moreover, in order to examine the effect of a lag-time between the DHA and CsA doses,

corn oil with or without 200 µg/kg DHA were intragastrically administered into rats

nominated as control and DHA-treated rats, respectively, and after 3 h, 5 mg/kg CsA was

orally given. Blood samples (120 µl) were collected 1, 2, 3, 4, 6, 9, 12, and 24 h after the CsA

administration.

For *i.v.* administration, 50 mg 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 µg/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 µl) 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[TM], Abott Laboratories, IL, USA) using the monoclonal antibody against CsA. Calibration curves (10-10,000 ng/ml) were obtained each time of 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_∞) were estimated by a non-compartmental analysis using a computer program, WinNonlin[TM] (version 4.0.1, Pharsight Corporation, North Carolina, USA).

Caco-2 Cell Culture. The Caco-2 cells were obtained from American type culture collection (Manassas, USA). Cells were grown in the culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine, 100 U/ml penicillin and 100 µg/ml

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streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2

and 95% air, with medium change on alternate days. For transport studies, the Caco-2 cells

were seeded in transwell with 1 cm^2 permeable polycarbonate inserts (3 µm pore size; Coring

Costar Corp., Cambridge, MA) in 12-well tissue culture plates for 22-24 days prior to the

transport study.

The integrity of the cell monolayer was measured by the transepithelial electrical

resistant (TEER) measurements. After 3 weeks in cell culture, the monolayers developed a

TEER approximately 500 Ωcm^2 were used for the transport studies.

Effect of DHA on P-gp-mediated Transport of [³H]-CsA across Caco-2 Monolayers.

Filter-grown Caco-2 cells, after removal of the culture medium, were washed twice with

Hank's balanced buffered saline (HBSS, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan). After

each wash, the plate was 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 [³H]-CsA (3Ci/mmol) in HBSS buffer to

either the apical or basolateral side of the monolayers in absence or presence 100 µM DHA or

PSC833. The basolateral to apical (B to A) transport of CsA were measured by aliquots of 50

μl 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±standard deviation (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 (ANOVA) for multiple comparisons among

the groups, respectively. 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 6β-hydroxytestosterone in rat liver microsomes. The ratios of 6β-hydroxytestosterone concentrations in DHA-containing tubes/control were extensively decreased with increasing DHA concentrations; especially, 100 μM DHA completely inhibited the formation of 6β-hydroxytestosterone.

The enzyme-kinetic parameters (K_m and V_{max}) were 25.47 ± 3.60 μM and 2.45 ± 0.14 nmol/min/mg-protein, respectively. The inhibitory effect for DHA (K_i) was calculated to be 5.52 ± 0.83 μM. 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 while 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 μg/ml) are shown in Fig. 3. The pharmacokinetic parameters obtained by a non-compartmental analysis are summarized in Table 1. When CsA was co-administered 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 µg/ml DHA, the values of AUC_∞ significantly

increased by 2.3- and 2.4-fold, respectively, when compared with control group. In addition,

the CL_{tol}/F and V_{dss}/F were significantly decreased about 2.7-fold, while $T_{1/2}$ values showed

any notable changes.

On the other hand, in the case of *i.v.* administration, there were no differences in the

blood CsA concentrations, AUC_∞, 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 µg/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 (ranged 0.11 to 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 µg/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 [³H]-CsA across Caco-2 Monolayers.

[³H]-CsA was loaded on basolateral side of Caco-2 cell monolayers. The transport of

[³H]-CsA in the basolateral (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 B to A

direction, but was not affected by 100 µM DHA. These results suggest that DHA does not

inhibit the P-gp mediated transport of [³H]-CsA.

Discussion

Intestinal phase I metabolism and active extrusion of absorbed drug have recently been recognised as major determinants of oral drug bioavailability. CYP3A, the major phase I drug metabolising 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 PUFAs 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 dose-dependently in DHA concentrations up to 100 μM, suggesting that there was a decreased conversion of testosterone to its metabolite, 6β-hydroxytestosterone. The Lineweaver-Burk plots clearly indicated that DHA competitively inhibited CYP3A *in vitro*.

CsA is also the substrate of P-gp which acts as an absorption barrier by transporting drug from intestinal cells into the lumen, and is known to be co-expressed 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 employed to examine the additional effect of

DHA on the bioavailability of CsA. Our results showed that DHA did not affect the [^{3}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 co-administration of DHA and CsA in rat increased bioavailability of

CsA by about 130%, while the hepatic clearance and volume of distribution did not change. In

contrast, pretreatment with DHA did not alter the pharmacokinetic 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 human may be 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 (Wu et al., 1995, Kolars et al., 1991).

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 is presumably due to protection from metabolism rather than to improve 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 P-gp inhibitor, KR-30031, 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 inhibit drug metabolism via CYP3A in the intestine but also enhance 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 in order 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, suggested that the inhibitory activity of DHA on CYP3A is reversible.

Furthermore, our *in vivo* result demonstrated the increase in oral bioavailability of CsA when co-administered with DHA, in 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 between 100 and 200 µg/ml DHA doses *in vivo*. It can be presumably because DHA

showed a saturable effect on its inhibition of CYP3A in the rat intestine and the range of

100-200 µg/kg DHA dose might result in its concentration in the gut above its *Ki* value (5.52

µM or 1.8 µg/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 food,

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 an bioavailability enhancer in clinical setting, with additive assurance of the improvement of both 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 pre-systemic 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.

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Footnotes

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Legends for figures

Fig. 1   Effect of DHA on the formation of 6β-hydroxytestosterone compared with control.

Microsomal fractions were incubated with testosterone with or without DHA (5, 10, 20, 50

and 10 μM). Each point represents the mean of duplicate determinations.

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 μM) testosterone with 0, 10, 20, or 30 μM DHA in the presence of a

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 μg/kg DHA. Each symbol with bar represents the mean ± SD (n=5-16).

Fig. 4   Effect of orally administered DHA on blood CsA concentrations after intravenous

administration of CsA to rats. Rats were orally administered with or without 100 μg/kg DHA

before intravenous administration of 5 mg/kg CsA. Each symbol with bar represents the mean

± SD (n=3-4).

Fig. 5 Effect of pre-treatment with 200 µg/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

µg/kg DHA before oral administration of 5 mg/kg CsA. Each symbol with bar represents the

mean ± SD (n=3).

Fig. 6 Effect of DHA and PSC 833 on [^{3}H]-CsA transport across Caco-2 monolayers in the

basolateral to apical direction. Data represents the mean ± SD (n=3).

Table 1 Effect of DHA co-administration on the pharmacokinetic parameters of CsA after oral administration of CsA (5mg/kg) to rats

Parameters	Control (without DHA) (n=16)	Oral dose of DHA		
		50 µg/kg (n=6)	100 µg/kg (n=7)	200 µg/kg (n=7)
AUC_∞ (mg/L•h)	9.78 ± 5.32	13.4 ± 5.78	22.41 ± 7.40[**]	23.41 ± 6.55[**]
$T_{1/2}$ (h)	7.68 ± 1.04	8.00 ± 0.96	8.30 ± 1.65	8.52 ± 0.68
CL_{tol}/F (L/h/kg)	0.748 ± 0.598	0.456 ± 0.271	0.248 ± 0.093[*]	0.230 ± 0.070[*]
V_{dss}/F (L/kg)	8.61 ± 6.34	5.57 ± 2.86	3.20 ± 0.85[*]	3.14 ± 0.86[*]

Data are expressed as mean ± SD.

[*] Statistically different from the corresponding value in the control group ($p < 0.05$).

[**] Statistically different from the corresponding value in the control group ($p < 0.01$).

Table 2 Effect of DHA co-administration on the pharmacokinetic parameters of CsA after *i.v.* administration of CsA (5mg/kg) to rats

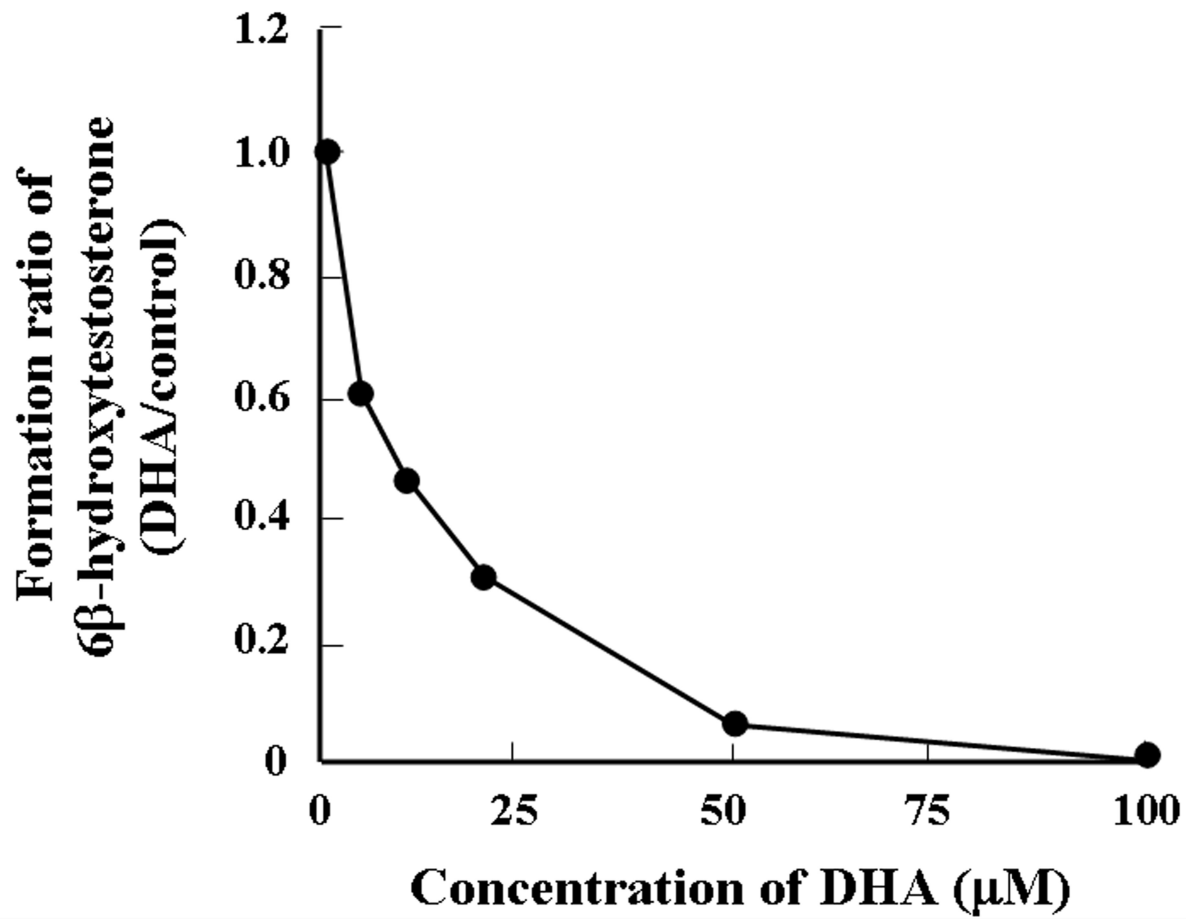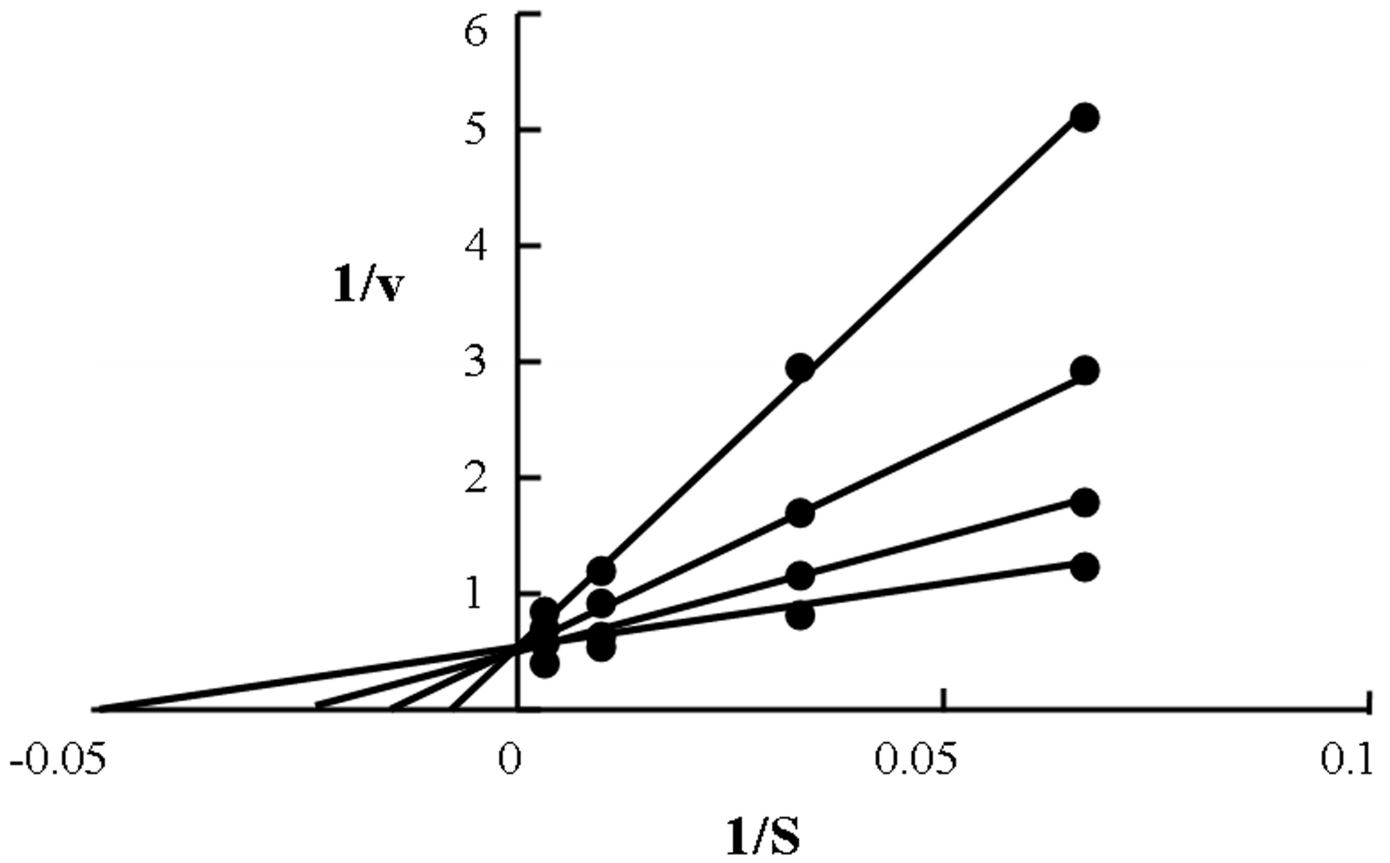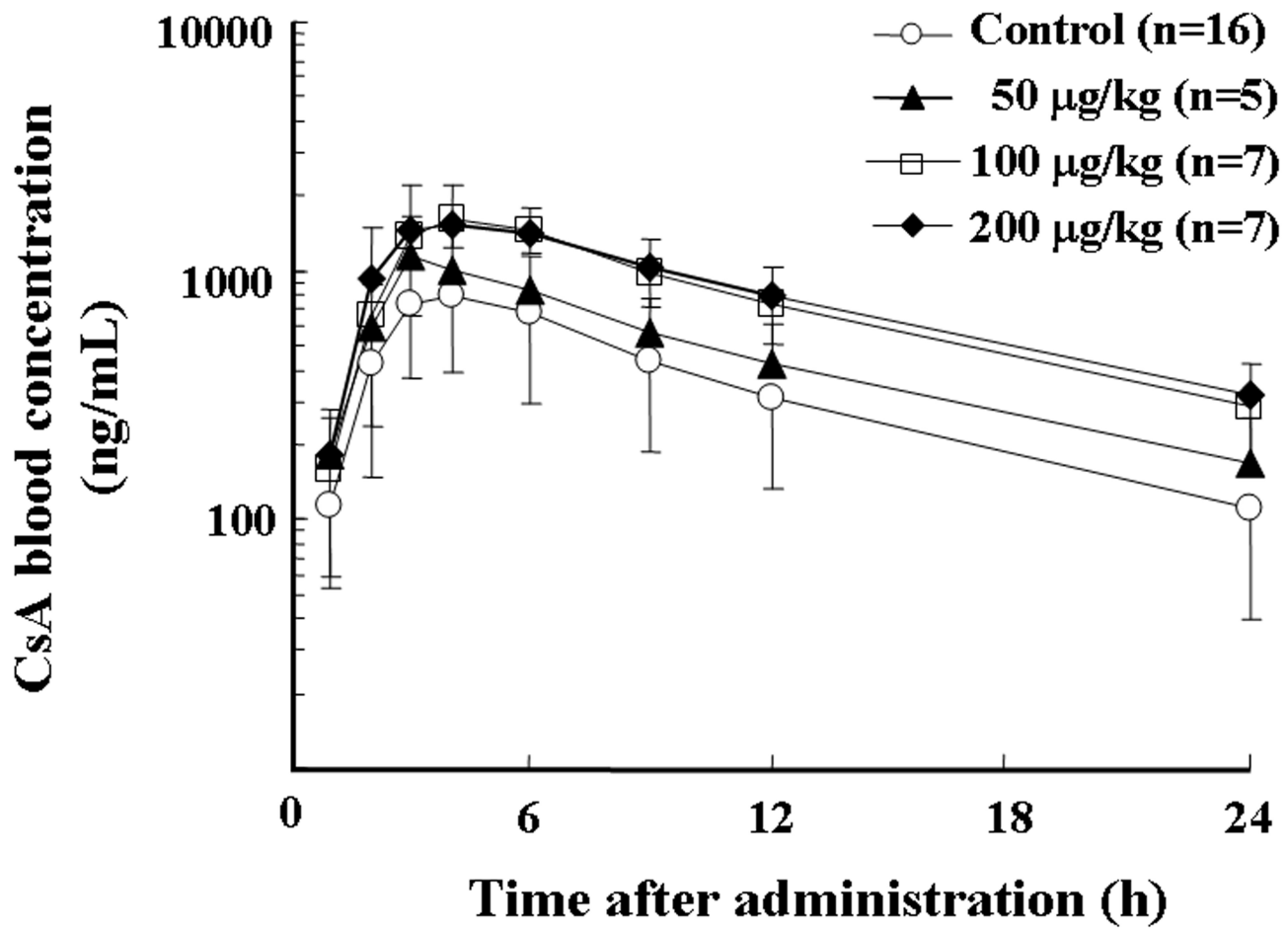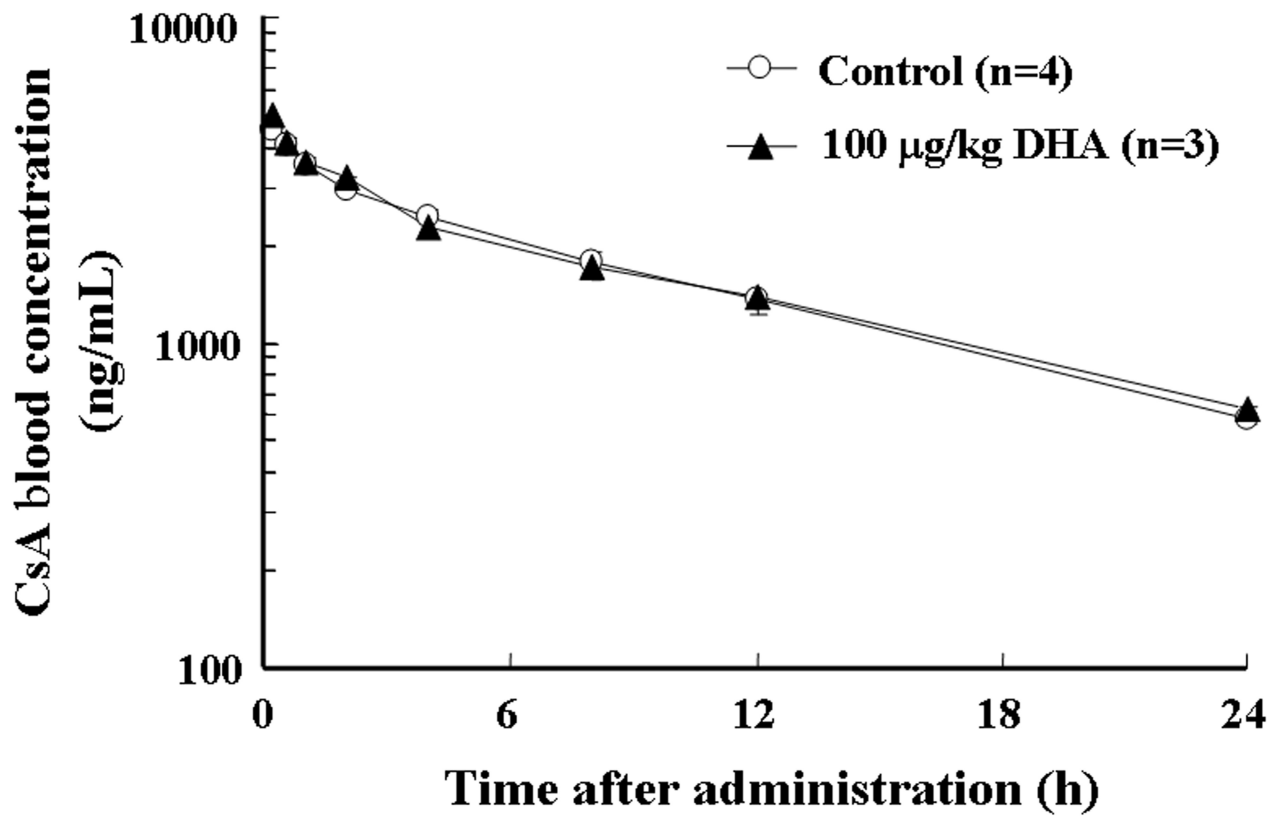| Parameters	Control (without DHA) (n=4)	Co-administered with DHA 100 μg/kg (n=3)
AUC_∞ (mg/L•h)	48.2 ± 3.0	50.7 ± 4.7
$T_{1/2}$ (h)	9.62 ± 0.81	11.0 ± 0.4
CL_{tol} (L/h/kg)	0.104 ± 0.006	0.0991 ± 0.0088
V_{dss} (L/kg)	1.36 ± 0.10	1.43 ± 0.09

Data are expressed as mean ± SD.

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6