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Inhibitory effect of docosahexaenoic acid (DHA) on the intestinal metabolism of midazolam: *In vitro* and *in vivo* studies in rats

V. Hirunpanich[b], K. Murakoso[a], H. Sato[a,*]

[a] *Department of Clinical and Molecular Pharmacokinetics/Pharmacodynamics, Faculty of Pharmaceutical Science, Showa University, Shinagawa-ku, Tokyo 142-8555, Japan*
[b] *Faculty of Pharmacy, Mahasarakham University, Kantharavichai, Mahasarakham 44150, Thailand*

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Abstract

The aim of this study was to evaluate the effects of docosahexaenoic acid (DHA) on the intestinal cytochrome P450 isoenzyme (CYP3A) and P-glycoprotein (P-gp) functions using midazolam and rhodamine-123 as specific substrates of CYP3A and P-gp, respectively. Perfused everted intestinal segments from rats were employed to determine the effects of DHA on midazolam metabolism and rhodamine-123 transport. In addition, the effects of DHA on *in vitro* midazolam metabolism in rat intestinal microsomes and on midazolam bioavailability in rats were examined. The intestinal extraction ratio (ER_G) of midazolam was determined to be 0.43 and decreased significantly to 0.12, 0.07, and 0.06 in the presence of 50, 100, and 200 μM DHA, respectively, in a concentration-dependent manner. The results from an *in vitro* study using rat intestinal microsomes demonstrated that DHA competitively inhibited the intestinal CYP3A activity with K_i of 15.7 and 27.1 μM for the formations of 1′-OH midazolam and 4-OH midazolam, respectively. Moreover, the oral administration of DHA (100 mg/kg) increased the AUC_∞, C_{max}, and oral bioavailability (F) of midazolam by about 50% in rats, without affecting the $T_{1/2}$, V_{dss}/F, or CL_{tot}/F. In contrast, DHA did not change the serosal-to-mucosal transport of rhodamine-123 in the perfused everted intestine and oral administration of DHA (100 mg/kg) had no influence on the pharmacokinetics of intravenously administered midazolam in rats, thus suggesting that DHA has little effect on the intestinal P-gp activity and hepatic clearance of midazolam. This study provided the first direct evidence to show that DHA has an inhibitory effect on the intestinal pre-systemic metabolism of a CYP3A substrate and that DHA has little, if any, effect on the P-gp activity in the gut.
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Keywords: Docosahexeanoic acid (DHA); Midazolam; Rhodamine-123; CYP3A; P-glycoprotein (P-gp); Perfused everted intestinal segment

1. Introduction

Both intestinal cytochrome P450 isoenzyme (CYP3A) and the multidrug efflux pump, MDR or P-glycoprotein (P-gp), are present at high levels in the enterocytes at the villus tip of the small intestine. They act as a concerted barrier to the absorption of many drugs (Benet et al., 1999). Therefore, a possible strategy to increase the bioavailability of a drug includes the use of inhibitors for CYP3A and/or P-gp.

Docosahexaenoic acid (DHA) is an essential polyunsaturated, 22-carbon fatty acid, which is taken from food such as fish and marine animal oils. DHA is absorbed orally and taken up into serum phospholipids. DHA may act as an inhibitor in the gut tissue, but not in the liver tissue, because DHA is incorporated into a triglycerides fraction in all fat types and free form of DHA is almost undetectable in the circulation (FDA, 2004), which suggests that DHA has little inhibitory effect on the CYP3A activity in the liver. After oral administration, DHA appears in each organ (plasma, erythrocyte and the fat body of the orbit, liver, intestine, skin, brain, heart and muscle) within 0.5 h and was observed until 24 h with maximum plasma concentrations (T_{max}) 2–3 h (Tago and Teshima, 2002).

Abbreviations: DHA, docosahexaenoic acid; CYP3A, cytochrome P450 isoenzyme; P-gp, P-glycoprotein; ER_G, extraction ratio; F, oral bioavailability; AUC_∞, areas under the whole blood concentration–time curve from zero to infinity; $T_{1/2}$, half-life; T_{max}, time to maximum plasma concentration; V_{dss}, volume of distribution; CL_{tot}, total clearance; K_m, Michaelis–Menten constant; K_i, inhibition constant; V_{max}, maximum reaction velocity; S, substrate concentration; I, inhibitor concentration; v, reaction velocity; *p.o.*, per oral; *i.v.*, intravenous.

* Corresponding author. Tel.: +81 3 3784 8611; fax: +81 3 5498 1148.
E-mail address: sato-tky@umin.net (H. Sato).

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Several studies (Hirunpanich et al., 2005; Hirunpanich and Sato, 2006; Yao et al., 2006) have reported that DHA inhibits CYP3A activity in rat "liver" microsomes *in vitro*.

A recent study (Hirunpanich et al., 2005; Hirunpanich and Sato, 2006) reported that DHA increased the oral bioavailabilities of cyclosporin (CsA) and saquinavir 2.4- and 3.3-fold, respectively, in rats, without affecting their hepatic clearances, thus suggesting these effects to occur *via* the inhibition of the pre-systemic metabolism of the drugs. However, there is no direct evidence to show a DHA's effect on "gut" metabolism of a CYP3A substrate.

Previously, to determine the possible effect of DHA on P-gp activity, [^{3}H]-CsA transport across the cultured Caco-2 cells was examined. These cells provide a model for human intestinal drug absorption in the presence of P-gp efflux transport activity. The results showed that 100 μM DHA displayed no inhibitory effect on P-gp (Hirunpanich et al., 2005). However, cultured Caco-2 cells may not necessarily represent the intestinal handling of drugs in rats. Furthermore, Barthe et al. (1999) reported that there are some disadvantages in using the Caco-2 system because it is a static system with small closed receptor compartments, which has a very low transport rate, and exaggerates the paracellular absorption compared with the small intestine. In addition, Thummel et al. (1996) suggested that hepatic and intestinal CYP3A4 are not coordinately regulated, since there is no significant correlation between hepatic and intestinal CYP3A activity. Therefore, the lack of direct evidence of the effects of DHA on CYP3A and P-gp activities in the intestine indicates that is necessary to quantitatively evaluate the effects of DHA on CYP3A-mediated midazolam metabolism and P-gp-mediated efflux of rhodamine-123 in the small intestine. A reported by Gandia et al. (2004) reported mucosal-to-serosal transport and extraction of a drug can be determined in a time-dependent manner using an everted segment where the serosal side is constantly perfused and mucosal-to-serosal transport and extraction of a drug can be determined at the designated time points. In the present study this model was used to determine the effects of different DHA concentrations on CYP3A and P-gp activities located in the intact intestinal tissue.

Midazolam, a preoperative anesthetic agent, is a selective CYP3A4 substrate and has been shown to undergo extensive gut metabolism, while the drug does not show bidirectional transported by P-gp in the gut (Kim et al., 1999; Polli et al., 2001). Midazolam has often been used as an *in vivo* CYP3A probe to investigate the intestinal CYP3A-mediated metabolism of drugs (Benet et al., 2004; Paine et al., 1996). Although midazolam is not commercially available as an oral drug in Japan, it has been used as an oral drug for anesthetic pre-medication and anxiety, in both adult and pediatric patients in many countries including USA and Thailand. A number of studies have reported interactions between midazolam and CYP3A-inhibitors such as clarithromycin (Quinney et al., 2007), grapefruit juice (Goho, 2001; Kim et al., 2006). Therefore, the effects of DHA on the *in vitro* midazolam metabolism in rat intestinal microsomes and on midazolam bioavailability in rats were also examined in this study.

2. Materials and methods

2.1. Materials

cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA) was purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA). A potent P-gp inhibitor, PSC-833, was generously supplied by Novartis Pharmaceutical Company (Tokyo, Japan). A glucose measurement kit, midazolam, 1′-OH midazolam, phenacetin, diazepam, rhodamine-123, and ketoconazole were obtained from Wako Pure Chemical Industries (Osaka, Japan). Dulbecco's modified Eagle's medium supplement with nutrient mixture F-12 (DMEM/F12) culture medium was purchased from Gibco Trade Mark (Grand Island, NY, USA). All other reagents used were of analytical grade.

2.2. Perfused everted intestinal segment study

Male Wistar rats (Nihon Ikagaku Doubutsu, Saitama, Japan) weighing 250–300 g were fasted overnight with free access to water prior to conducting the experiments. Experiments with animals were performed in accordance with the guidelines for Animal Experimentation in the Faculty of Pharmaceutical Sciences, Showa University. The perfusion of the everted intestinal segments was performed as previously reported (Gandia et al., 2004) with some modifications. Briefly, under light ether anesthesia, the rats were placed in a supine position on a surgery pad. The rats were exsanguinated and the entire small intestine was quickly excised and flushed three times with pre-warmed saline. An approximately 10 cm section, of the proximal and distal end of the whole small intestine was removed. CYP3A proteins are predominantly localized in duodenal or jejunal mucosa of small intestine and that the expression P-gp mRNA levels increases longitudinally along the intestine with the highest level in the mucosa of ileum and colon (Benet et al., 1999; Iida et al., 2005; Kivisto et al., 2004). Therefore, in order to determine the CYP3A activity, two 12-cm segments were removed from the duodenal end and designated sites 1 and 2, respectively. Another two 6-cm intestinal segments were removed above the distal end of the ileum and designated as sites 3 and 4, respectively. Silicone tubes (outer diameter; 0.8 mm, inner diameter; 0.3 mm) were inserted into the both ends of an everted intestinal segment and secured with surgical silk. These silicone tubes were then used to connect each everted intestinal segment to a peristaltic pump (Masterflex®, Illinois, USA). DMEM/F-12 culture medium was used as a perfusion solution and medium in both mucosal and serosal sides. Next, 15 ml of medium, pre-oxygenated and pre-incubated at 37 °C, was added into the chamber of mucosal side where the everted intestinal segment was immersed. The serosal side was perfused with medium at a flow rate of 2.0 ml/min for 10 min for stabilization, and the in- and out-flows of the medium perfusing through the serosal side were mixed in another chamber filled with 7.0 ml of the medium. Throughout the experiment, the chambers of mucosal and serosal sides were maintained at 37 °C, and the chamber of mucosal side was continuously bubbled with a mixture of 5% CO_2 and 95%. A simplified diagram of the everted intestinal perfusion study is illustrated in Fig. 1.

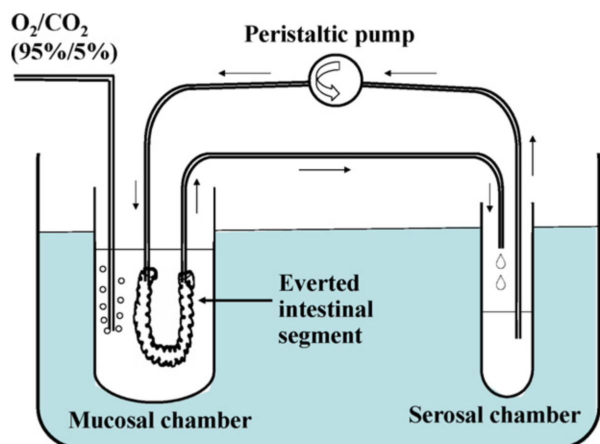

Fig. 1. A simplified diagram of the perfused everted intestinal segment study. An everted segment of rat intestine is immersed in the mucosal chamber containing the perfusion medium in which the testing compounds (*i.e.* DHA, midazolam, rhodamine-123) are dissolved. The medium is perfused from the serosal chamber through the everted intestinal segment in the mucosal chamber using a peristaltic pump with a flow rate of 2 ml/min.

2.2.1. Effect of DHA on CYP3A in the perfused everted intestinal segment

After 10 min of perfusion of the everted intestinal segments 1 or 2, midazolam (30 μM as final concentration) was added into the mucosal medium with or without DHA (10, 50, 100 and 200 μM) or 10 μM ketoconazole, a potent CYP3A inhibitor. An aliquot (200 μl) of the serosal medium was taken at 30, 60, 90, and 120 min and replaced with the same volume of the blank medium. After centrifugation at 10,000 rpm for 5 min, 70 μl of each sample was injected into a high performance chromatographic (HPLC) system as described later to determine the concentrations of midazolam and its metabolites. In addition, after concluding the perfusion, the intestinal segments were washed three times with ice-cold 0.9% saline solution, blotted dry, weight, and kept at −80 °C for extraction.

The extraction of midazolam, 1′-OH midazolam and 4-OH midazolam from the intestinal tissue, was done using a modification of the method described by Sawada et al. (2002) with some modification. The intestinal segments were cut in to the small pieces and homogenized using a glass homogenizer, then transferred to glass tubes containing 1000 μl 1N NaOH and incubated for 30 min. After vortexing for 5 min, 3 ml ethyl acetate, and 10 μl internal standard (10 μg/ml diazepam in methanol) were added. The tubes were shaken vigorously on a shaker for 20 min and centrifuged at 2500 rpm for 5 min and 2000 μl of the upper 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, and 70 μl was used for HPLC analysis.

2.2.2. Effect of DHA on P-gp activity in the perfused everted intestinal segment

To determine the effect of DHA on P-gp activity in the gut, rhodamine-123 and PSC-833, a well-known P-gp substrate and P-gp inhibitor (Achira et al., 1999), respectively, were used in the everted intestinal segment perfusion where the segment sites 3 and 4 were employed. After the stabilization period, rhodamine-123 (5 μM) was added in the absence or presence of DHA (10, 50, 200, and 1000 μM) or PSC-833 (10 μM) into the mucosal side chamber. In order to determine the transport of rhodamine-123 across the everted intestinal segment, an aliquot (200 μl) of the medium were taken from the serosal side at 0, 10, 30, 45, and 60 min and replaced with the same volume of the blank medium. After centrifugation for 5 min at 10,000 rpm, 10 μl of each sample was injected into the HPLC system for analysis as described later. The time course of the cumulative amounts of rhodamine-123 transported from the mucosal to serosal sides across the everted intestinal segments were examined.

DMSO was used as solvent for midazolam, ketoconazole, DHA, and PSC-833 and the final concentrations of DMSO after its addition to the mucosal medium were 0.6%, 0.05%, 0.5% and 0.1%, respectively.

2.3. Preparation of rat intestinal microsomes

Rat intestinal microsomes were prepared using a modification of the techniques described by Cotreau et al. (2001). An intestinal segment (40 cm from the pyloric region) was excised quickly under light anesthesia with ether, and flushed twice with 25 ml solution A (1.5 mM KCl, 96 mM NaCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, and 27 mM sodium citrate) on an ice-cooled petri dish. The proximal end of the intestinal segment was then clamped and filled with 10 ml ice-cold solution B (phosphate buffer saline (pH 7.4), 5 mM Na_2EDTA, and 0.5 mM dithiothreitol), clamped at the distal end, shaken gently, and incubated on ice for 10 min in 10 ml of the same solution. The intestine was incised longitudinally from the distal end. The intestine and solution B were mixed 1 min by hand. The cloudy solution was collected into a clean centrifuge tube and the remaining intestine was combined with 10 ml solution B and mixed by hand. This process was repeated three times. The collected solutions were centrifuged (1500 rpm, for 5 min at 4 °C) and the supernatant was removed and the pellet was washed twice with 15 ml solution C (5 mM histidine, 0.25 M sucrose, and 0.5 mM Na_2EDTA). The intestinal cells were homogenized in 5 ml solution C in a glass–telflon homogenizer and kept on ice. The homogenates were centrifuged at 20,000 × *g* for 10 min at 4 °C and supernatant was transferred to a clean centrifuge tube. The pellets were centrifuged again at 20,000 × *g* for 10 min at 4 °C. The collected supernatant was combined with 52 mM $CaCl_2$ (0.19 ml of supernatant), then mixed gently for 5 s, kept on ice for 15 min, and centrifuged at 2500 × *g* for 10 min at 4 °C. Finally, microsome pellets were suspended in 0.1 ml of solution D (100 mM KH_2PO_4, 10 mM Na_2EDTA, and 20% glycerol). Microsomal protein concentration was quantified using the Bradford assay (1976) with bovine serum albumin as a standard.

2.4. Effect of DHA on midazolam metabolism in rat intestinal microsomes

The oxidation of midazolam to yield 1′-OH midazolam and 4-OH midazolam was used to evaluate the CYP3A activity in the intestinal microsomes. Midazolam (0, 5, 10, 50, 100, 150, and 200 μM) was pre-incubated for 5 min in an incubation mixture

consisting of 50 mM KH_2PO_4 (pH 7.6), 5 mM $MgCl_2$, intestinal microsomes (final concentration 0.5 mg protein/ml), with or without DHA (5, 10, 20, 50 and 100 μM) dissolved in DMSO (final concentration 0.2%). The metabolic reaction was initiated by the addition of 4.4 mM NADPH (total volume 1 ml) and incubating the mixture at 37 °C. After 10 min the reaction was terminated by the addition of 100 μl 1N NaOH. Next, 20 μl of 50 nM phenacetin (in methanol), as an internal standard, and 3 ml ethyl acetate were added to the resultant mixture. After the tubes were mixed for 20 min and centrifuged at 3000 rpm for 10 min, 2 ml of the upper organic layer was transferred to a glass tube and evaporated to dryness at 40 °C for 1 h. The residue was reconstituted with 200 μl of mobile phase, and 70 μl of each sample was injected into HPLC system for analysis.

2.5. *In vivo study using rats*

2.5.1. *Animals and surgery*

Male Wistar rats (Nihon Ikagaku Doubutsu, Saitama, Japan) weighing 240–250 g were acclimatized to environmentally controlled (a temperature-controlled facility with a 12-h light/12-h dark cycle) for at least 1 week. The rats were fasted for approximately 12 h with water given *ad libitum*. The femoral artery was cannulated with polyethylene tubing (SP-31, Natsume Seisakusho, Tokyo, Japan) to facilitate blood sampling under light ether anesthesia. Some rats were cannulated at the femoral vein for intravenous (*i.v.*) administration. After concluding the operation, each cannulated rat was kept in a Bolman cage and the experiment was started following recovery from anesthesia.

2.5.2. *Effect of DHA on the pharmacokinetics of midazolam in rats*

The effect of DHA on the pharmacokinetics of midazolam was evaluated after midazolam was orally or intravenously administered in rats. For oral administration, midazolam was dissolved in physiologic saline at a concentration of 10 mg/ml, and 0.1 M hydrochloric acid was used to obtain a pH of 3.3. DHA (100 mg/kg) or olive oil (as a control) was orally pre-administered 15 min before the midazolam (10 mg/kg) was orally administered into the rat. Blood samples (about 250 μl) were collected from the cannulated femoral artery at 5, 10, 20, 30, 45, 60, 90, 120, and 150 min. An equivalent volume of saline was then injected through the femoral artery.

For *i.v.* administration, midazolam was dissolved in a mixture of 25% ethanol, 25% polyethylene glycol 400, and 50% water at a concentration 5 mg/ml. Olive oil with or without DHA (100 mg/kg) was administered orally 2 h before the midazolam (5 mg/kg) was introduced intravenously through a femoral vein *via* the cannula. Blood samples were taken from the femoral artery at 5, 10, 30, 60, 90, and 120 min.

All blood samples were collected in heparinized tubes, and the plasma was obtained by centrifuging the blood samples at 8000 rpm for 8 min at 4 °C. The extraction of midazolam, 1′-OH midazolam, and 4-OH midazolam in plasma samples were performed according to a modification of the method described by Carrillo et al. (1998). An aliquot (100 μl) plasma samples was combined with 10 μl of 10 μg/ml diazepam (in methanol), 1 ml glycerine buffer (0.75 M, pH 9), and 4 ml ethyl acetate. The mixture was shaken vigorously for 30 min, centrifuged at 3000 rpm for 10 min, and the upper organic layer was transferred to a glass tube containing 1 ml acetate buffer (0.1 M, pH 4.7). The tubes were mixed for 20 min and centrifuged at 3000 rpm for 10 min. After the second extraction, 3 ml of the organic layer was transferred to a conical glass tube and evaporated to dryness at 40 °C for 1 h. The residue was reconstituted with 180 μl of mobile phase, and 70 μl of each sample was injected into the HPLC system for analysis.

2.6. *HPLC analysis*

2.6.1. *HPLC analysis of midazolam, 1′-OH midazolam, and 4-OH midazolam*

Each sample (70 μl) was injected into the HPLC system composed of a dual pump (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 reversed-phase column used was a Cadenza CD-C18, 4.6 mm × 100 mm, 3 μm (Imtakt Co., Kyoto, Japan) equipped with a Nova-Pak® C18 guard column (Waters, Massachusetts, USA). The mobile phase consisted of a mixture of acetonitrile, methanol, and 0.1 M sodium acetate buffer (pH 4.7) (32:5:63, v/v, for mobile phase A for *in vitro* and 40:5:55, v/v, for mobile phase B for *in vivo* experiments) and was pumped by an isocratic program at a flow rate 1.0 min/ml. The absorbance wavelength was set at 245 nm. The retention times of the reagents used were as follows: phenacetin (4.2 min); 4-OH midazolam (7.4 min); 1′-OH midazolam (9.6 min); and midazolam (14.2 min, Fig. 2A) for the mobile phase A and midazolam (7.3 min) and diazepam (10 min) for the mobile phase B

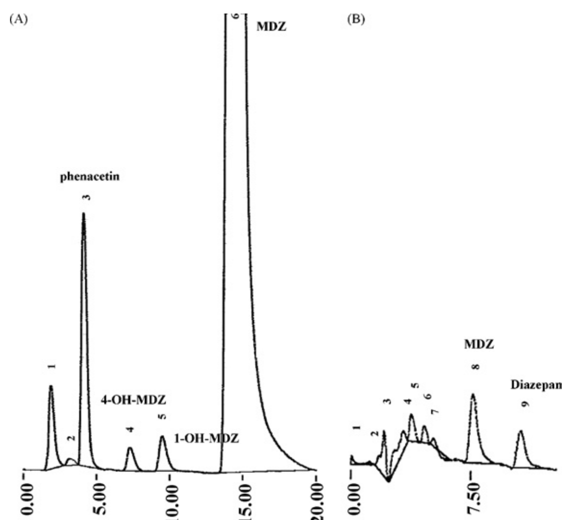

Fig. 2. A representative HPLC chromatogram of midazolam, 1′-OH midazolam, 4-OH midazolam, and phenacetin (internal standard) from the rat intestinal microsomes model (A); and a representative HPLC chromatogram of midazolam, and diazepam (internal standard) from the plasma samples in the *in vivo* study in rats model (B). Using the HPLC analytical methods described in the text, calibration curves were plotted as the peak area *vs*. drug concentration and obtain r^2 were over 0.99 ($n = 6$). MDZ represents midazolam.

(Fig. 2B). Ketoconazole (used as an inhibitory positive control) was not observed to interfere with the assay.

2.6.2. HPLC analysis of rhodamine-123

The concentration of rhodamine-123 in the transport medium was determined using the HPLC system with a fluorescence detector (FS-8020, Tosoh) as reported previously (Yumoto et al., 1999) with minor modifications. Briefly, the reversed-phase column used was a Nova-Pak® C18, 4.6 mm × 150 mm (Waters, Massachusetts, USA). The mobile phase was a mixture of acetonitrile, 20 mM sodium acetate (pH 4.0) and water with 1.5 mM triethylamide (50:20:30, v/v) and pumped at a flow rate of 1 ml/min. Detection was set at wavelength of 485 nm for excitation and 546 nm for emission. PSC-833 (used as an inhibitory positive control) was not observed to interfere with the assay.

The calibration curves were prepared each time in a concentration range from 0.1 to 2 μg/ml for midazolam, 0.05–1 μg/ml for 1′-OH midazolam and 4-OH midazolam and 0.25–2.5 ng/ml for rhodamine-123, and within- and between-run precision was below 5% and 7%, respectively, for each compound. The accuracy of the procedure was in the range 0.4–6%. The correlation coefficients (linearity) were always more than 0.99 and the detection limits defined as S/N = 3 were 0.05 μg/ml for midazolam, 0.01 μg/ml for 1′-OH midazolam, 0.01 μg/ml for 4-OH midazolam, and 0.1 ng/ml for rhodamine-123.

2.7. Data analysis in the perfused everted intestinal segment study

2.7.1. Extraction ratio of midazolam in the perfused everted intestinal segment study

Midazolam, 1′-OH midazolam and 4-OH midazolam were determined from both the mucosal and serosal chambers at each time point. In addition, intracellular 1′-OH midazolam and 4-OH midazolam were also determined at the final time point to assess the accurate total amount of metabolites accumulated in the intestine tissue. Extraction ratio in the intestine (ER_G), a marker referring to the extent of metabolism relative to the amount of drugs which interacted with the enzyme, was calculated by the following equation (Eq. (1)) (Benet et al., 2004):

$$ER_G = \frac{\sum \text{Metabolite}_{(\text{mucosal, serosal, intracellular})}}{\sum \text{Parent}_{(\text{serosal, intracellular})} + \sum \text{Metabolite}_{(\text{mucosal, serosal, intracellular})}} \quad (1)$$

where "Metabolite (mucosal, serosal, intracellular)" refers to the amount of 1′-OH midazolam and 4-OH midazolam in the 120 min samples from mucosal side, serosal side and intracellular cells, respectively, and "Parent (serosal, intracellular cells)" represents the amount of midazolam amount in the 120 min samples in the serosal side and intracellular cells, respectively.

2.7.2. Transport of rhodamine-123 in the perfused everted intestinal segment study

The transport rate of rhodamine-123 from the mucosal to the serosal surfaces across the everted intestinal segment was calculated from the slope of the cumulative amount of rhodamine-123 transport with time and expressed as a unit of concentration/time/length of intestinal segment (pmol/min/6 cm-intestinal segment).

2.8. Data analysis in the rat intestinal microsomes study

The Michaelis–Menten constant (K_m), maximum reaction velocity (V_{max}) and inhibition constant (K_i) were calculated using a non-linear least-squares regression analysis using the computer program, WinNonlin® Professional v.4.01 (version 4.0.1, Pharsight Corporation, North Carolina, USA), according to the following equation (Eq. (2)):

$$v = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \quad (2)$$

where v represents the reaction velocity (pmol/min/mg protein), and S and I represent the substrate and inhibitor concentrations (μM), respectively.

2.9. Data analysis in the in vivo study in rats

The pharmacokinetic parameters of midazolam in rat, *i.e.* total clearance (CL_{tot} for *i.v.* dose and CL_{tot}/F for oral dose), elimination 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® (version 4.0.1, Pharsight Corporation, North Carolina, USA). Oral bioavailability (F) was determined using the following equation (Eq. (3)):

$$F\,(\%) = \frac{AUC_{oral}}{AUC_{i.v.}} \times \frac{Dose_{i.v.}}{Dose_{oral}} \times 100 \quad (3)$$

where AUC_{oral} and $AUC_{i.v.}$ are the AUC after oral and *i.v.* administrations, respectively.

2.10. Statistical analysis

The data are expressed as the mean ± standard error of the mean (S.E.M.). Comparisons between two groups and among more than three groups were performed using Student's *t*-test and one-way analysis of variance (ANOVA) followed by Dennett's post hoc test, respectively. A difference was considered to be statistically significant if the probability value was less than 0.05 ($p < 0.05$).

3. Results

3.1. Perfused everted intestinal segment studies

3.1.1. Effect of DHA on CYP3A activity in the perfused everted intestinal segment

The intestinal extraction ratio (ER_G) was taken as a surrogate marker for the functional activity of CYP3A which indicated the fraction of parent drug that is metabolized as it crosses the

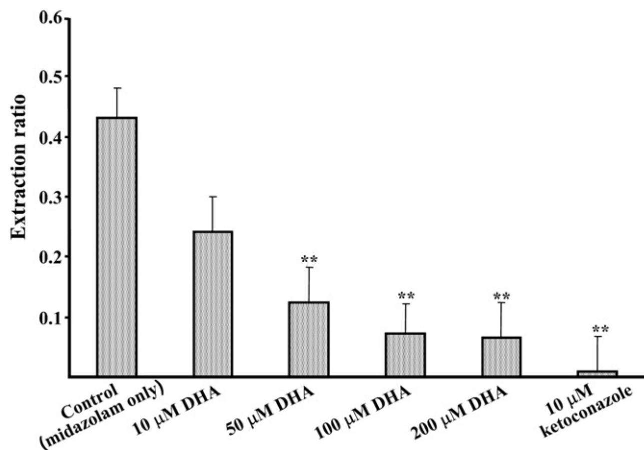

Fig. 3. The extraction ratio of midazolam in the absence or presence of DHA or ketoconazole after incubated for 120 min at 37 °C in the perfused everted intestinal segment study. Midazolam (30 μM) with or without DHA (10, 50, 100 and 200 μM) or ketoconazole (10 μM) were applied into the medium in the mucosal chamber and samples were taken from the serosal medium at the indicated time points. Midazolam, 1′-OH midazolam, and 4-OH midazolam concentrations in the intestinal tissue were determined at the last time point of the incubation. The extraction ratio was calculated according to Eq. (1). One-way ANOVA with post hoc test was conducted and statistically significant differences ($p < 0.01$) from control group were shown by the asterisks (**). Each column represents the average of four determinations, and the vertical bar represents the standard error of the mean (S.E.M.).

intestinal epithelial cells (Benet et al., 2004). Fig. 3 shows the ER_G of midazolam after incubation for 120 min at 37 °C in the perfused everted intestinal segment. The ER_G of midazolam was 0.43 ± 0.05 in the control group, but it was extensively decreased by approximately 40-fold to a value of 0.008 ± 0.057 when midazolam was co-incubated with 10 μM ketoconazole. The ER_G of midazolam also decreased significantly in the presence of 50, 100, and 200 μM DHA to 0.12 ± 0.06, 0.07 ± 0.05, and 0.06 ± 0.06, respectively ($p < 0.01$).

3.1.2. Effect of DHA on P-gp activities in the perfused everted intestinal segment

Fig. 4 shows the time courses of the cumulative rhodamine-123 transport from the mucosal to the serosal sides across the everted intestinal segment in the absence or presence of DHA (10, 50, 200, and 1000 μM) or PSC-833 (10 μM). The mucosal-to-serosal transport of rhodamine-123 increased significantly with PSC-833, but was not affected by DHA. The transport rate of rhodamine-123 was calculated from the slope of the cumulative amount of rhodamine-123 transport *vs.* time curves (Fig. 5). PSC-833 significantly enhanced the intestinal transport rate of rhodamine-123 compared to the control (74.83 ± 21.7 *vs.* 4.34 ± 0.69 pmol/min/6 cm-intestinal segment, respectively, $p < 0.05$). However, no differences were observed between the transport rates of rhodamine-123 the DHA-treated and control groups as well, as regardless of the concentrations of DHA.

3.2. Effect of DHA on midazolam metabolism in rat intestinal microsomes

The ratios of the rate of 1′-OH midazolam and 4-OH midazolam formation in DHA-containing tubes/control (without DHA) decreased significantly with increasing DHA concentrations, thus indicating the existence of a concentration-dependent inhibition of midazolam metabolism by DHA. As clearly shown in Fig. 6, the formation rates of both 1′-OH midazolam and 4-OH midazolam were completely inhibited by 100 μM DHA.

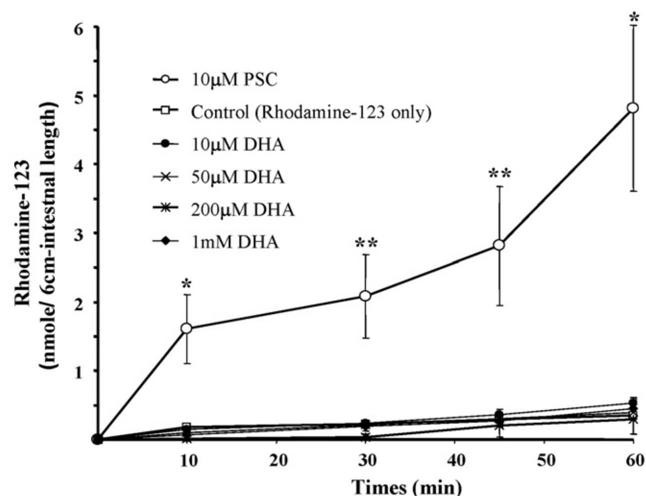

Fig. 4. The time course of rhodamine-123 transport from the mucosal to serosal sides across the everted intestinal segment in the absence or presence of DHA or PSC-833. Rhodamine-123 (5 μM) with or without DHA (10, 50, 200 and 1000 μM) or PSC-833 (10 μM) were applied into the medium in the mucosal chamber and samples were taken from the serosal medium at the designated time points. One-way ANOVA with post hoc test was performed and statistically significant differences $p < 0.05$ and $p < 0.01$ from control group were shown by the asterisks (*) and (**), respectively. Each data point is the average of four determinations, and the vertical bar represents the standard error of the mean (S.E.M.).

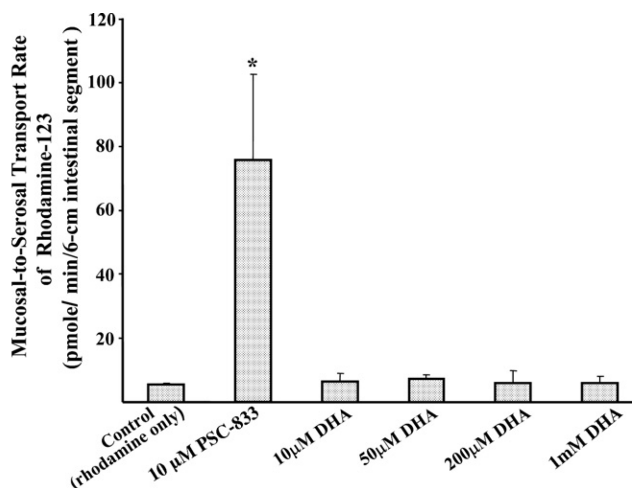

Fig. 5. Transport rate of rhodamine-123 from the mucosal to serosal sides across the everted intestinal segment in the absence or presence of DHA (10, 50, 200 and 1000 μM) or PSC-833 (10 μM). Transport rates were calculated from the slope of the cumulative amount of rhodamine-123 transport *vs.* time curves (shown in Fig. 4). The asterisks (*) indicates a statistically significant difference ($p < 0.05$) when compared to the control group according to a one-way ANOVA with post hoc test. Each column represents the average of four determinations, and the vertical bar represents the standard error of the mean (S.E.M.).

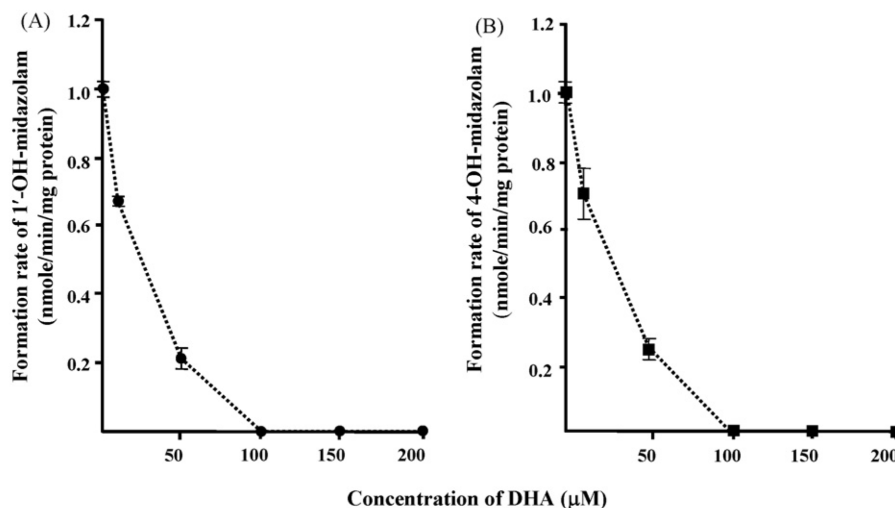

Fig. 6. Ratios of the rate of formation of 1′-OH midazolam (A) and 4-OH midazolam (B) in DHA-containing tubes/control (without DHA). Rat intestinal microsomes were incubated with midazolam (50 μM) in the absence or presence DHA (10, 50, 100, 150, and 200 μM). The activities of 1′-OH midazolam and 4-OH midazolam in absence of DHA were 0.064 ± 0.00138 and 0.090 ± 0.00158 nmol/min/mg protein, respectively. Each data point is the average of three determinations, and the vertical bar represents the standard error of the mean (S.E.M.).

Fig. 7 shows the Lineweaver–Burk plots for the inhibition of 1′-OH midazolam and 4-OH midazolam synthesis, respectively, by DHA in rat intestinal microsomes. They demonstrate that DHA caused a competitive inhibition of the CYP3A activity in the intestine, since increasing the DHA concentration did not change the intercept of the plot lines on the *y*-axis ($1/V_{max}$) but changed the slope of the lines (apparent K_m). The enzyme-kinetic parameters (V_{max}, K_m, and K_i) of 1′-OH midazolam and 4-OH midazolam in rat intestinal microsomes, as calculated by a non-linear least squares regression, are summarized in Table 1.

Table 1
Kinetics parameters of midazolam metabolites formation in rat intestinal microsomes

Parameters	1′-OH midazolam	4-OH midazolam
V_{max} (nmol/min/mg protein)	0.122 ± 0.0057	0.256 ± 0.034
K_m (μM)	62.3 ± 8.21	148.05 ± 37.62
DHA K_i (μM)	15.7	27.1

Midazolam (50, 100, and 150 μM) was incubated at 37 °C in an incubation mixture consisting of 50 mM KH_2PO_4 (pH 7.6), 5 mM $MgCl_2$, intestinal microsomes (0.5 mg protein/ml), 4.4 mM NADPH, and DHA (0, 10, 25, 50, 100, and 200 μM) for 10 min. The formations of 1′-OH midazolam and 4-OH midazolam were determined and the enzyme-kinetic parameters of 1′-OH midazolam and 4-OH midazolam were calculated according to Eq. (2). Data are expressed as mean ± S.E.M. ($n = 3$).

3.3. *Effect of DHA on pharmacokinetics of midazolam in rats*

Fig. 8A shows the plasma concentrations of midazolam orally administered in rats with or without oral pre-treatment of DHA (100 mg/kg). DHA significantly increased the AUC of mida-

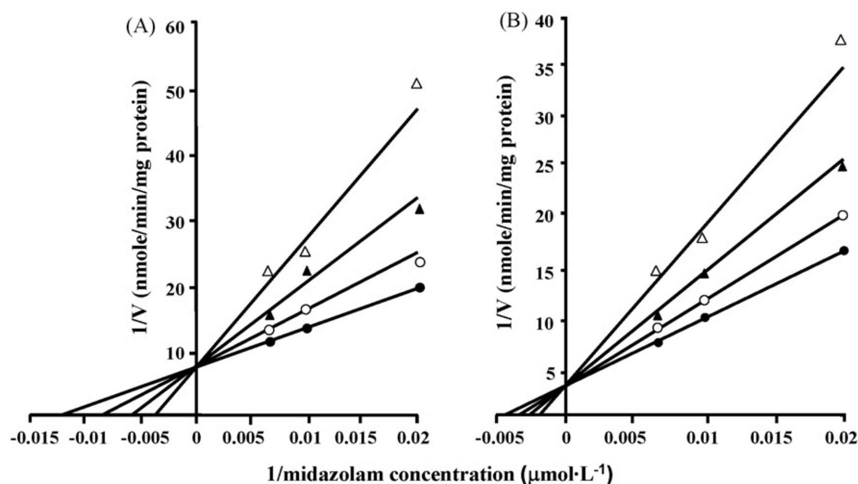

Fig. 7. The Lineweaver–Burk plots for the inhibition of 1′-OH midazolam (A) and 4-OH midazolam (B) by DHA in rat intestinal microsomes. Rat intestinal microsomes were incubated with several concentrations of midazolam (50, 100, and 150 μM) and DHA (0 (●), 10 (○), 25 (▲), and 50 (△) μM) in buffer consisting of 50 mM KH_2PO_4 (pH 7.6), 5 mM $MgCl_2$, distilled water, and 0.44 mM NADPH at 37 °C for 10 min. Each point represents the mean of duplicate determinations.

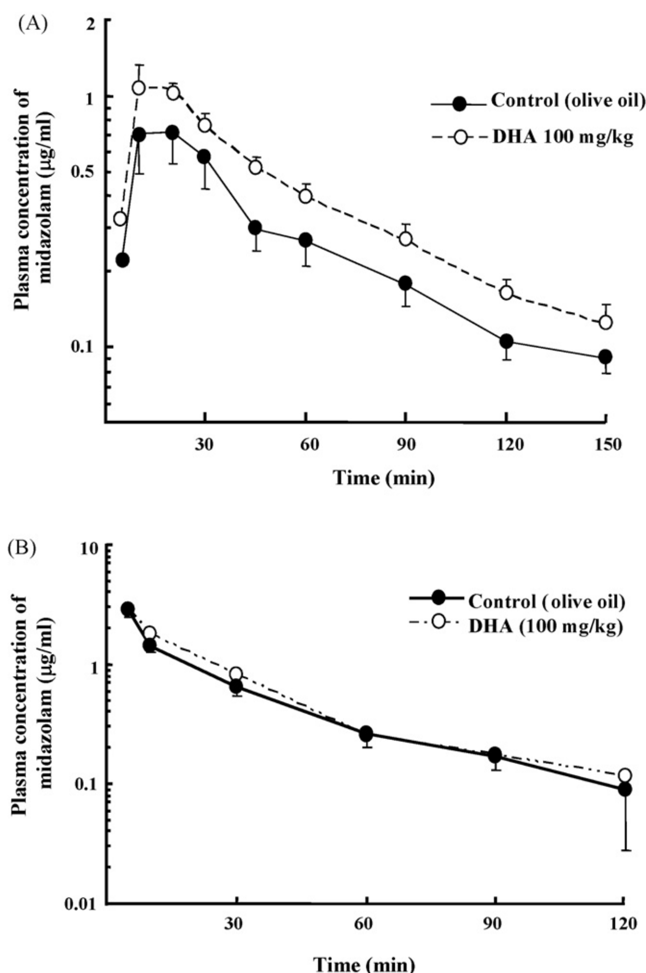

Fig. 8. Plasma midazolam concentrations *vs.* time course after oral (A) and intravenous (B) administrations in rats with or without pre-dosage of DHA. The doses of midazolam for oral and *i.v.* administrations were 10 and 5 mg/kg, respectively. Pretreatment of rats with DHA was conducted with a dose of 100 mg/kg for 15 and 120 min before the oral (10 mg/kg) or intravenous (5 mg/kg) administrations of midazolam to rats; control rats received an equal volume of vehicle (olive oil). Each data point is the average of four to seven determinations, and the vertical bar represents the standard error of the mean (S.E.M.). In some cases, the S.E.M. falls within the circle.

zolam by about 50%, above that of the control group. The maximum concentration (C_{max}) was enhanced by 43% (Fig. 8A), but the differences were not in a significant level. However, DHA caused no significant change in the midazolam elimination half-life ($T_{1/2}$), V_{dss}/F, T_{max}, or CL_{tol}/F (Table 2). The oral bioavailability of midazolam in the DHA-treated group was about 53% higher than that in the control group, indicating that DHA enhanced the bioavailability of midazolam.

The oral administration with DHA (100 mg/kg) prior to an *i.v.* dosage of midazolam had no detectable influence on the pharmacokinetic parameters of midazolam, *i.e.* the AUC_∞, $T_{1/2}$, V_{dss}, and CL (Table 2) and the plasma midazolam concentrations were almost identical in the control and DHA-treated groups (Fig. 8B).

4. Discussion

A recent study reported that oral administration of DHA significantly increased the oral bioavailabilities of cyclosporine A and saquinavir in rats, without altering their elimination half-lives (Hirunpanich et al., 2005; Hirunpanich and Sato, 2006). This suggested that DHA could be used to enhance the bioavailability of drugs with limited availability due to CYP3A-mediated, intestinal first-pass metabolism. In order to examine this hypothesis, a perfused everted intestinal segment model (Gandia et al., 2004) was employed. This model has been shown to have several advantages over the everted intestinal sac model because the serosal compartment has a large volume and constantly perfused as to allow serial sampling of the medium. Segments of the everted rat intestine were incubated at 37 °C in a pre-oxygenated culture containing both glutamine and glucose as substrates to produce ATP for the intestinal mucosa. The segment continued to show peristaltic contraction for at least 2 h after excision, which is consistent with the observations of Gandia et al. (2004). In addition, the glucose consumption by the intestinal segment increased over the course of experiment (data not shown), suggesting that tissue viability was maintained.

Both intestinal CYP3A and P-gp have been reported to reduce the intracellular concentrations of their substrates, by metabolism and by transmembrane efflux, respectively (Benet et al., 1999). Various reports indicate that regional differences

Table 2
Pharmacokinetic parameters of midazolam in rats

Parameters	Oral administration (10 mg/kg)		IV administration (5 mg/kg)	
	Control (without DHA) ($n=7$)	Pre-administered with DHA (100 mg/kg) ($n=7$)	Control (without DHA) ($n=4$)	Pre-administered with DHA (100 mg/kg) ($n=3$)
AUC_∞ (μg/ml × min)	48.8 ± 7.55	71.4 ± 5.91*	83.5 ± 4.9	81.5 ± 4.81
$T_{1/2}$ (h)	0.820 ± 0.049	0.748 ± 0.139	0.417 ± 0.064	0.374 ± 0.046
CL_{tot}/F, CL_{tot} (ml/min/kg)	244.7 ± 44.8	145.3 ± 12.1	60.6 ± 4.0	61.9 ± 3.64
V_{dss}/F, V_{dss} (l/kg)	15.87 ± 5.83	9.17 ± 1.74	2.15 ± 0.28	1.99 ± 0.22
T_{max} (h)	0.309 ± 0.057	0.262 ± 0.034	ND	ND
C_{max} (mg/ml)	0.913 ± 0.220	1.378 ± 0.170	ND	ND
F (%)	27.9	43	ND	ND

DHA (100 mg/kg) was pre-administered orally 15 min or 120 min before the oral (10 mg/kg) or intravenous (5 mg/kg) administration of midazolam to rats. Pharmacokinetic parameters of midazolam were calculated as described in Section 2. Each value is expressed as mean ± S.E.M. The asterisks (*) indicates a statistically significant difference at $p < 0.05$ when compared to the control group according to a Student t-test. ND: not determined.

exist in the expression of CYP3A and P-gp along the length of the small intestine (Benet et al., 1999; Carrillo et al., 1998). Iida et al. (2005) reported that the expression MDR1 mRNA in the rat intestine is concentrated in the ileum and that the localization of the CYP3A enzyme is mainly observed in the duodenum and the distal jejunum. This is consistent with kinetic studies reporting that the rate of rhodamine-123 transport mediated by P-gp in the ileum is higher than that in the jejunum (Yumoto et al., 1999), and that the portions of jejunum and ileum of small intestine display selective expression of CYP3A and P-gp, respectively (Berggren et al., 2003; Johnson et al., 2002). In this study, therefore, the isolated jejunum and ileum of rat's small intestine we employed as a source of CYP3A and P-gp, respectively.

To determine the effect of DHA on the bioavailability of drugs, midazolam was chosen as the CYP3A substrate because it is extensively metabolized by intestinal CYP3A to form 1′-OH midazolam and 4-OH midazolam. There is conflicting evidence concerning the possibility that midazolam is a substrate of P-gp (Benet et al., 2004; Tolle-Sander et al., 2003). Since midazolam is a highly permeable drug, the contribution of P-gp efflux may not be observed in intact cells or tissues, although its ability to activate P-gp ATPase was demonstrated in an *in vitro* ATPase assay system (Tolle-Sander et al., 2003). In the present study, the concentrations of 1′-OH midazolam and 4-OH midazolam in both the mucosal and serosal environments continued to increase over the course of the experiment, indicating that midazolam metabolism was taking place in this experimental system with the structure of the intestine. Midazolam, 1′-OH midazolam, and 4-OH midazolam concentrations in the intestinal segment were measured at the last time point to determine the total amount of metabolites while measuring the extraction ratio of midazolam through the intestinal tissue (ER_G).

The ER_G of midazolam was determined to be 0.43 ± 0.05, and this similar to the results reported in human by Thummel et al. (1996), Paine et al. (1996) and Lee et al. (1994) who determined the ER_G values of midazolam to be in the range of 0.4–0.5 after oral administration. The present result is also comparable to the ER_G value of 0.25 ± 0.07 determined in rats (Higashikawa et al., 1999) using a dose of 0.05 μmol midazolam (which corresponds to 50 μM midazolam injected into the jejunum loop). DHA (10–200 μM) exhibited a concentration-dependent inhibition of the ER_G of midazolam (ranging from 32% to 73%). The maximum inhibitory effect evoked by DHA was comparable to the inhibitory effect of 10 μM ketoconazole, a potent inhibitor of CYP3A.

Previous studies described that high concentrations of midazolam lead to a saturation of the hydroxylation reaction and, therefore, a reduction in the pre-systemic first-pass through the intestine and liver (Thummel et al., 1996; Higashikawa et al., 1999; Fisher et al., 1999). Saturation in the formation of midazolam into 1′-OH midazolam and 4-OH midazolam in a modified Caco-2 monolayer model occur when the midazolam concentration in the apical layer exceeded 25 and 80 μM, respectively (Fisher et al., 1999). In this study 30 μM of midazolam was added into the mucosal (receiver) side which is higher than its K_m value (6 μM) *in vitro* (Kotegawa et al., 2002). However, the intracellular concentration of midazolam was found to be only about 0.5 μM which is much lower than K_m. The lower concentration of midazolam in the intestinal tissue compared to the extracellular medium concentration may be the result of intracellular metabolism of the drug by CYP3A, high permeability of the drug across the tissue, and flow of medium perfusing the mucosal side of an everted intestinal segment (*i.e*. sink-effect).

The inhibitory effect of DHA on midazolam metabolism was also demonstrated in the *in vitro* study using rat "intestinal" microsomes in contrast to the previous studies (Hirunpanich et al., 2005; Hirunpanich and Sato, 2006) that employed rat "liver" microsomes. DHA decreased the ratio of the formation of both 1′-OH midazolam and 4-OH midazolam in a dose-dependent manner. The calculated V_{max} values for 1′-OH and 4-OH midazolam formations in the intestinal microsomes were similar to the previously reported values (0.11 and 0.21 nmol/min/mg protein, respectively) but the K_m values for 1′-OH and 4-OH midazolam were higher (6 and 55 μM, respectively) (Kotegawa et al., 2002). The latter difference may be due to differences in the preparation of the intestinal microsome, which could alter the nonspecific binding of the drug. This would cause differences in the unbound fractions of the drug in the incubation mixture, and K_m is reported to be influenced by microsomal protein binding (Obach et al., 2006). The Lineweaver–Burk plots clearly indicated that DHA competitively inhibited the formation of 1′-OH midazolam and 4-OH midazolam with a K_i of 15.7 and 27.1 μM, respectively. These results are consistent with the observation in the perfused everted intestinal segments that DHA decreased the ER_G of midazolam. Recently, Yao et al. (2006) reported that DHA has an inhibitory effect not only CYP3A4 with a value K_i of 34 μM but also CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 in human liver microsomes with K_i values of 41, 2.9, 6.7, 122 and 65 μM, respectively. However, CYP3A is the most prevalent (greater than 70%) enzyme involved in oxidative drug metabolism present in the small intestine, and therefore, the principle factor in the first-pass metabolism of substrates for the P450 system (Benet et al., 1999). In addition, Zhang et al. (1999) found that CYP3A4 is the major form of CYP expressed in human small intestine enterocytes with minor amounts of CYP2C and CYP1A1. Therefore, the strategy to use DHA as to enhance the bioavailability of CYP3A substrates may be promising, its application for non-3A CYP isoforms may not be feasible.

The pharmacokinetic parameters for midazolam obtained in this study without DHA are comparable with a previous study (Kotegawa et al., 2002). A pre-dosing of DHA (100 mg/kg) markedly increased the F, C_{max}, and AUC of midazolam by about 50%, without affecting the elimination half-life ($T_{1/2}$). Moreover, DHA did not show any effect on the pharmacokinetics of intravenously injected midazolam, thus indicating a lack of effect on the hepatic clearance of midazolam. Therefore, the concentration of DHA in the portal vein of the liver may not have reached a sufficient level to inhibit the midazolam metabolism in the liver, after absorption from the intestine.

Oral bioavailability and intestinal drug absorption can be significantly limited by CYP3A and P-gp in the intestine, especially for dual substrates of CYP3A and P-gp such as sirolimus, cyclosporin A and saquinavir (Benet et al., 1999, 2004). P-gp is expressed at high levels in the villus tip enterocytes of the

small intestine and may work in conjunction with CYP3A to reduce oral bioavailability by actively pumping substrate from an intracellular location back across the apical plasma membrane (Benet et al., 1999; Thummel et al., 1996). Therefore, the effect of DHA on the intestinal absorption of a specific P-gp substrate, rhodamine-123 was examined. The ileum portion of the intestine was used to negate the regional differences of P-gp activity, considering the highest expression of P-gp mRNA in the lower part of intestine, especially at ileum (Iida et al., 2005; Yumoto et al., 1999). Since no trace of rhodamine metabolites were found in the HPLC analysis, the transport of rhodamine was evaluated without consideration of its metabolism. The transport of rhodamine-123 from the mucosal side to the serosal side across the everted intestinal segment increased with time and the transport rate was enhanced by approximately 18-fold when PSC-833 was added, indicating that P-gp was functioning properly in the intestinal segment used, consistent with previous reports (Achira et al., 1999; Cho et al., 2000). The present study demonstrated that DHA has little, if any, effect on the intestinal P-gp activity, because there was no significant change in the transport rate of rhodamine-123 even at a high concentrations of DHA (1000 μM). These results collectively suggest that P-gp is not involved in the increased bioavailability of midazolam observed with the pre-dosing of DHA.

F is the product of the fraction of the administered drug that is absorbed intact from gastrointestinal lumen (F_a), the fraction of the absorbed drug that is not metabolized during passage through the gastrointestinal epithelium (F_g), and the fraction of the absorbed dose delivered to the liver through the portal vein that is not subsequently lost during first-pass hepatic metabolism (F_h) (Benet et al., 1999), as given in Eq. (4):

$$F = F_a \cdot F_g \cdot F_h \tag{4}$$

Our results demonstrated that DHA did not influence P-gp activity or hepatic metabolism of midazolam; hence, only F_g is the dominant factor to affect the oral bioavailability of midazolam. The F_g is defined as one minus the extraction ratio (ER_G), as shown in Eq. (5):

$$F_g = 1 - ER_G. \tag{5}$$

Substituting the data in Fig. 3 into Eq. (5) yielded values of F_g of midazolam alone and in the presence of DHA (10, 50, 100, and 200 μM) *in vitro* of 0.57, 0.76, 0.88, 0.93, and 0.94, respectively, thus indicating that DHA increases the F_g of midazolam by about 33–65%. These results are comparable with the bioavailability-enhancing effect of DHA for midazolam observed *in vivo* in rats. Taken together, the present results of both the *in vitro* and *in vivo* experiments demonstrated that DHA enhanced the midazolam bioavailability by decreasing the ER_G of the drug. In this context, this study therefore provides direct evidence that demonstrate DHA's inhibitory effect on the intestinal CYP3A enzyme and pre-systemic intestinal first-pass of a CYP3A substrate, while previous studies (Hirunpanich et al., 2005; Hirunpanich and Sato, 2006) have only suggested it.

The effects of dietary components on drug bioavailability are an on-going concern in clinical drug therapy. Much interest has been focused on grapefruit juice and related flavonoids, since they increase bioavailabilities of a large number of CYP3A substrates by inactivation of CYP3A, a so-called mechanism-based inhibition, as well as by inhibiting P-gp activity in the intestine (Hare and Elliott, 2003). These effects may not be restored until CYP3A proteins are newly synthesized. The hepatic clearance of these substrates remains unchanged. Therefore, their *in vivo* effects may be variable.

Although DHA toxicity was not directly determined in this study, no toxic effects and symptoms have not been observed. Arterburn et al. (2000) reported that docosahexaenoic acid single cell oil (DHASCO oil) which contains a high concentration of DHA (about 52%) was safely administered in pregnant rats at concentrations up to 1250 mg/kg body weight/day, which represents about 650 mg/kg body weight/day of DHA. The efficacious DHA doses (50–100 mg/kg) used in the present experiment are much less than that reported toxic dose, suggesting the safety of DHA. Our dosage of DHA in rats (100 mg/kg), corresponding to 6 g/60 kg human subject, is somewhat higher than a typical therapeutic dose of DHA for human, *e.g.* 1.5 g/day in the case of Omacor® (Bays, 2006) which is a concentrated formulation of omega-3 PUFAs including DHA and EPA. Therefore, a human clinical study should be further conducted in order to confirm the possible effects of DHA for CYP3A substrates in clinical situations.

In conclusion, this study provided "first direct" evidence to show that DHA inhibited intestinal drug metabolism by reversibly interfering with intestinal CYP3A enzymes but not with the efflux transporter, P-gp, thus providing a basis for future clinical research to access the potential of DHA as an active ingredient to enhance the oral bioavailability of drugs which have a low bioavailability because of the CYP3A-mediated first-pass intestinal metabolism.

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