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Intestinal Absorption Mechanism of Tebipenem Pivoxil, a Novel Oral Carbapenem: Involvement of Human OATP Family in Apical Membrane Transport

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Abstract: Tebipenem pivoxil (TBPM-PI) is an oral carbapenem antibiotic for treating otolaryngologic and respiratory infections in pediatric patients. This agent is a prodrug to improve intestinal absorption of TBPM, an active form, and an absorption rate of TBPM-PI is higher than those of other prodrug-type β-lactam antibiotics. In the present study, we hypothesized that a certain mechanism other than simple diffusion is involved in the process of improved intestinal absorption of TBPM-PI and examined the mechanism. TBPM-PI uptake by Caco-2 cells was decreased by ATP-depletion and lowering the temperature to 4 °C, suggesting the contribution of carrier-mediated transport mechanisms. This uptake was partially decreased by ACE inhibitors, and the reduction of the absorption by captopril was observed by *in vivo* study and *in situ* single-pass intestinal perfusion study in rat, supporting the contribution of influx transporters. Since some ACE inhibitors and β-lactam antibiotics are reported to be substrates of PEPT and OATP families, we measured transporting activity of TBPM-PI by intestinally expressed transporters, PEPT1, OATP1A2, and OATP2B1. As a result, significant transport activities were observed by both OATP1A2 and OATP2B1 but not by PEPT1. Interestingly, pH dependence of TBPM-PI transports was different between OATP1A2 and OATP2B1, showing highest activity by OATP1A2 at pH 6.5, while OATP2B1-mediated uptake was higher at neutral and weak alkaline pH. OATP1A2 exhibited higher affinity for TBPM-PI ($K_m = 41.1$ μM) than OATP2B1 ($K_m > 1$ mM) for this agent. These results suggested that TBPM-PI has high intestinal apical membrane permeability due to plural intestinal transport routes, including the uptake transporters such as OATP1A2 and OATP2B1 as well as simple diffusion.

Keywords: Tebipenem pivoxil; oral carbapenem; intestinal absorption; OATP1A2 and OATP2B1

Introduction

Tebipenem pivoxil (TBPM-PI, Figure 1) is an oral carbapenem antibiotic for treating otolaryngologic and respiratory infections such as persistent otitis media, upper respiratory infection and bacterial pneumonia in pediatric patients.[1−3] TBPM, an active form, is esterified to TBPM-PI as a prodrug with a pivaloyloxymethyl group to carboxylic acid at the C-2 position to improve its oral absorption. When TBPM-PI is administered orally, this agent is absorbed from the gastrointestinal tract and converted to TBPM by carbox-

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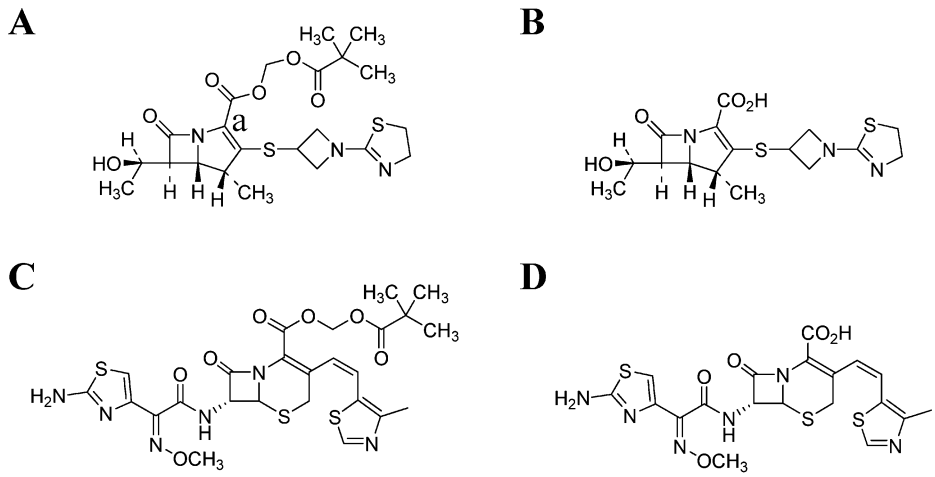

Figure 1. Chemical structures of test compounds: (A) tebipenem pivoxil (TBPM-PI); (B) tebipenem (TBPM); (C) cefditoren pivoxil (CDTR-PI); (D) Cefditoren (CDTR). "a" shows [^{14}C]-labeled position.

yesterase localized at the intestinal epithelial cells and then TBPM is transferred into blood.

Most β-lactam antibiotics have high hydrophilicity, and they usually do not show good oral absorption due to poor membrane permeability. Prodrug which has been modified through chemical introduction of a lipophilic group is one of the methods to improve bioavailability of β-lactam antibiotics. Bioavailability of prodrugs is, however, limited to 30−50% in most cases, showing unsatisfactory oral absorption (Table 1).[4−10] When TBPM-PI is administered orally, the cumulative amount of urinary excreted TBPM is 54−73% of a dose in human, and the amount including metabolites is about 80% of a dose.[10] From this clinical observation, TBPM-PI is considered to exhibit higher intestinal absorption than other similar prodrugs. In the process of intestinal absorption of TBPM-PI, simple diffusion is accelerated by application of prodrug strategy, which is one of the factors to improve oral absorption. Moreover, we thought that active transport might be involved, because the absorption of TBPM-PI is remarkably high compared to other prodrugs.

Table 1. Human Oral Absorption of β-Lactam Antibiotics (Prodrug Type)

drug	human F (%)	ref
cefditoren pivoxil	20	4
cefcapene pivoxil	33−41[a]	5
cefetamet pivoxil	41−51	6
cefuroxime axetil	32−45	7
cefpodoxime proxetil	36−45[a]	8
cefteram pivoxil	27	9
tebipenem pivoxil	54−73[a]	10

[a] Values represent cumulative urinary excretion of active form.

Recent advance in drug transporter research demonstrated that drug transporters play important roles in pharmacokinetics of various drugs. These previous studies, however, mainly targeted drug transports in the brain, kidney, and liver. The knowledge about transporters involved in the intestinal absorption of drugs other than MDR1 (P-glycoprotein) and peptide transporter 1 (PEPT1) is very limited.

Previously, it was reported that PEPT1 and organic anion transporting polypeptides (OATP) contributed to influx transport in the intestinal absorption process of several drugs. Some β-lactam antibiotics (cephalexin, cefadroxil, cefixime, etc.) are transported by PEPT1 which localized at the apical

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membrane of intestinal epithelial cells, and these agents exhibited fairly good absorption.[11−15] Moreover, PEPT1 has been used as a target to improve oral absorption of, for example, valacyclovir, a valine ester of acyclovir with poor oral absorption.[16] Other chemical derivations and improved formulation have been used to maximize the PEPT1-mediated intestinal absorption of some drugs.[17−19] Fexofenadine, pravastatin, and talinolol are transported by OATP1A2 and OATP2B1 in humans and Oatp1a5 in rats, and these transporters contribute to the intestinal absorption of those drugs at least partially.[20−24]

Based on these backgrounds, we examined the intestinal absorption mechanism of TBPM-PI, which exhibits high oral absorption, focusing on influx transporters, PEPT1 and OATPs. As a result, we found that TBPM-PI is transported by OATP1A2 and OATP2B1 but not by PEPT1. This is the first report showing the contribution of the OATP family to intestinal absorption of a β-lactam antibiotic, especially of its ester prodrug, and this knowledge is thought to be useful for novel drug research.

Materials and Methods

Chemicals. Cefditoren pivoxil (CDTR-PI), CDTR (an active form of CDTR-PI), TBPM-PI and TBPM were synthesized by Meiji Seika Kaisha, Ltd. [^{14}C]TBPM-PI (2.19 MBq/mg) was customized by Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). [^{3}H]Estrone-3-sulfate (2120 GBq/mmol) and [^{3}H]glycyl-sarcosine (1480 GBq/mmol) were purchased from PerkinElmer (Boston, MA) and American Radiolabeled Chemical, Inc. (St. Louis, MO), respectively. 2,4-Dinitrophenol was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were commercial products of reagent grade.

Animals. Male Sprague−Dawley (SD) rats were supplied by Nippon Charles River (Yokohama, Japan). Animals were housed and handled according to the "Principles of Laboratory Animal Care" by National Institutes of Health and the "Guidance for the Care and Use of Laboratory Animals" (Pharmaceutical Research Center, Meiji Seika. Kaisha Ltd.).

Uptake Study of TBPM-PI by Caco-2 Cells. Caco-2 cells (human colonic carcinoma cell line) were obtained from American Type Culture Collection (Manassas, VA) and cultured in a 75 cm^2 flask with DMEM containing 50 μg/mL streptomycin, 50 units/mL penicillin G and 10% fetal bovine serum at 37 °C in a 5% CO_2 humidified atmosphere. For uptake study, cells were seeded to a 24-well multiplate (1×10^5 cells/well) and cultured for 18−24 h. Cells were washed with phosphate buffered saline (PBS) and combined with 100 μM TBPM-PI or [^{14}C]TBPM-PI (0.2 μCi/mL) in uptake buffer (Hanks balanced salt solution supplemented with 110 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.2)). In the case of adding transporter substrates or inhibitors, these were added with TBPM-PI simultaneously. Dimethyl sulfoxide (DMSO) used to dissolve TBPM-PI and transporter substrates or inhibitors did not exceed 2.0% at a final concentration. ATP-depletion of Caco-2 cells was carried out by adding 1 mM 2,4-dinitrophenol at 15 min before the initiation of uptake measurement and preincubation of the cells. When CDTR-PI was used,

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the same method was applied as for TBPM-PI. Uptake was performed at 37 °C for 15 min. Then the cells were washed with ice-cold PBS three times and added with 250 μL of lysis buffer containing 100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 6.0)−acetonitrile−methanol in the volume ratio 50:45:5. Cell lysate was centrifuged (8000*g*, 10 min, 4 °C) and the supernatant was recovered and filtrated. One hundred microliters of distilled water and 200 ng/mL cefotaxime in 100 mM MOPS buffer (pH 6.0) as internal standard were added to 100 μL of the supernatant or standard curve solution in lysis buffer. An aliquot of this solution was loaded to LC/MS (MS-2010A, Shimadzu corporation (Kyoto, Japan)) to quantify TBPM-PI and TBPM (converted from TBPM-PI in the cells). The conditions for LC/MS analysis were as follows: column, SanFire C_{18} 5 μm (2.1 × 150 mm; Waters (Milford, MA)); mobile phase, A, 0.1% formic acid, and B, acetonitrile; gradient condition, 100% A from 0 to 1 min, 100 to 20% A from 1 to 5 min, 20% A from 5 to 6 min, 20 to 100% A from 6 to 6.01 min, and 100% A from 6.01 to 12 min; column temperature, 40 °C; flow rate, 0.3 mL/min; ionization mode, ESI (positive); monitor ions, TBPM-PI (m/z = 498), TBPM (m/z = 384), CDTR-PI (m/z = 311), CDTR (m/z = 507) and cefotaxime (m/z = 456). When [^{14}C]TBPM-PI was used, AQUASOL 2 (PerkinElmer) was added to the cell lysate and radioactivity was measured by a liquid scintillation counter (LS-5000TD, Beckman Coulter, K. K. (Tokyo, Japan)).

Rat *in Vivo* Study. SD male rats (8 to 9 weeks old) fasted more than 15 h were cannulated in the femoral artery with polyethylene tubing (PE-50) under anesthesia with diethyl ether, and then rats were fixed in a Borrmann's cage. TBPM-PI dosing solution was prepared according to the method described below. At first, TBPM-PI was dissolved in 1 M hydrochloric acid and neutralized with sodium hydroxide to pH 6.5. Then, 5 w/v % PEG-60 hydrogenated castor oil (HCO-60, Nikko Chemicals Co., Ltd., Tokyo, Japan) was added to bring the TBPM-PI concentration to 2 mg/mL (solution A). On the other hand, captopril was dissolved in 1 M sodium hydroxide, and the pH of this solution was adjusted with sodium hydroxide to pH 6.5. 5 w/v % HCO-60 solution was added to this solution to bring the captopril concentration to 2 mg/mL (solution B). The dosing solution was prepared by mixing equal volume of solution A and solution B. For control group, 5 w/v % HCO-60 solution (pH 6.5) was used instead of solution B. In the case of CDTR-PI, dosing solution was prepared by the same method of TBPM-PI. Dosing solution was administered to rats orally with or without captopril (300 mg/kg) at a dose of 5 mg/kg TBPM-PI or CDTR-PI. Blood sampling was performed from the femoral artery at 5, 15, 30, 45, 60, 60, 90, and 120 min after administration, respectively. Plasma was obtained by centrifugation from the blood sample, and TBPM concentration in the samples was measured by the same method of uptake study by Caco-2 cells.

Rat *in Situ* Intestinal Single-Pass Perfusion Study. This experiment was performed according to the method of Okudaira et al.[25] with minor modifications. In brief, SD male rats (8 to 9 weeks old) fasted more than 15 h were anesthetized with nembutal (Abbott, Chicago, IL). The femoral vein was cannulated with polyethylene tubing (PE-50) to transfuse fresh blood collected previously from another rat. The abdominal cavity was opened, and a 5 cm intestinal segment was prepared at the upper part of the ileum. The intestinal contents were flushed out with 5 mL of ice-cold saline followed by 1 mL of air. The segment was cannulated with silicon tubing connected to a perfusion pump. The outflow from the segment was led to polyethylene tubing (PE-240). The segment was perfused with DPBS (pH 6.5) containing 100 μM TBPM-PI with or without 10 mM captopril in 100 mM HEPES/DPBS (pH 6.5). DMSO used to dissolve TBPM-PI and captopril did not exceed 2.0% at a final concentration. The perfusion flow rate was 0.2 mL/min. After starting the perfusion, venous blood from the mesenteric vein was collected by inserting an indwelling needle. Blood sampling was performed at 4 min intervals for 28 min. Plasma was obtained by the centrifugation of blood, and TBPM concentration in plasma was measured by the same method of uptake study by Caco-2 cells. The absorption rate of TBPM in the venous outflow (V) was calculated according to the following equation:

$$V = Q \times C_{\text{last}}$$

where Q and C_{last} are blood flow rate draining the segment and the concentration at final point (28 min) of TBPM in the venous blood, respectively.

Uptake Study of TBPM-PI by *Xenopus* Oocyte Expression Systems. OATP1A2 and OATP2B1-expressing oocytes were prepared by microinjecting complementary RNA (cRNA) of each transporter (cDNA sequences: Genbank No. 6579 and 11309, respectively) and cultivating for three days as described previously.[14] Human PEPT1-expressing oocytes were purchased from BD Gentest (Woburn, MA). PEPT1 uptake study was performed according to the manufacturer's instructions. In the uptake study, cRNA of OATP1A2 or OATP2B1 was microinjected, which was followed by three days cultivation in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$ and 10 mM HEPES, pH 7.4). After washing with modified Barth's solution (pH 5.0 to 8.0), the oocytes were incubated in modified Barth's solution containing TBPM-PI at room temperature. Being washed three times with ice-cold modified Barth's solution, each oocyte was added to 200 μL of lysis buffer (used in Caco-2 uptake study). Then oocytes were disrupted with sonication and the lysate was centrifuged (8000*g*, 10 min, 4 °C). The supernatant was recovered and filtrated. This supernatant or standard curve solution was diluted with the lysis buffer or

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control supernatant by 2-fold, respectively. One hundred microliters of distilled water and internal standard solution (used in Caco-2 uptake study) were added to 100 μL of the diluted supernatant or standard curve solution in lysis buffer, respectively. LC/MS analysis to measure the uptake amount of TBPM-PI (containing the TBPM) was performed by the same method in Caco-2 uptake study. Expression level of each transporter was confirmed by uptake of known substrates, estrone-3-sulfate for OATP1A2 and OATP2B1, and glycylsarcosine (Gly-Sar) for PEPT1.

Analytical Methods. The results are expressed as means (± standard deviations). Statistical analyses were performed by Student's *t* test in the uptake study by *Xenopus* oocyte expression systems and analysis of variance (ANOVA) in rat *in vivo* and *in situ* experiments using Microsoft Excel and SAS system ver. 8.02 (SAS institute Inc., Cary, NC), respectively. The criterion of significance was $p < 0.05$. In rat *in vivo* and *in situ* experiments, the plasma concentration−time profile in each rat was analyzed based on a noncompartment model using WinNonlin Professional ver. 4.1 (Pharsight Corporation, Mountain View, CA) to obtain pharmacokinetic parameters. Elimination half-life ($t_{1/2}$), the area under the plasma concentration−time curve (AUC) from zero to the last observable concentration (AUC_{0-obs}), and the AUC from zero to infinity (AUC_{0-inf}) were calculated in the rat *in vivo* study. AUC_{0-obs} was calculated in the rat *in situ* study. To calculate the kinetic parameters on TBPM-PI transport, the uptake rate and concentration of substrate were fitted to the Michaelis−Menten equation using SYSTAT ver.5.04 (SYSTAT Software Inc. (Chicago, IL)).

Results

Uptake of TBPM-PI by Caco-2 Cells. To investigate the involvement of a certain mechanism rather than passive diffusion in intestinal membrane permeability of TBPM-PI, uptakes of this agent were evaluated using Caco-2 cells. TBPM-PI uptakes by Caco-2 cells were decreased at 4 °C and by ATP depletion to 73% and 53% of the control, respectively (Figure 2A). These results suggested the involvement of carrier-mediated transport as well as simple diffusion in the intestinal absorption process of TBPM-PI.

Next, the effect of various transporter substrates and inhibitors was investigated in TBPM-PI uptakes by Caco-2 cells. Captopril and ascorbic acid had potent inhibitory effects in a dose-dependent manner (Figure 2B). A weak inhibitory effect was observed by adding cholic acid and valproic acid, however this effect was dose independent (Figure 2B). Moreover, cationic compounds (tetraethylammonium and L-carnitine) had no effect (Figure 2C). From these results, we additionally investigated the inhibitory effects on ACE inhibitors and ascorbic acid. Enalapril had potent inhibitory effect as well as captopril, and lisinopril had a weak effect (Figure 2C). In contrast, no effect was observed by cephalexin and sulpiride, substrates of the PEPT family by which

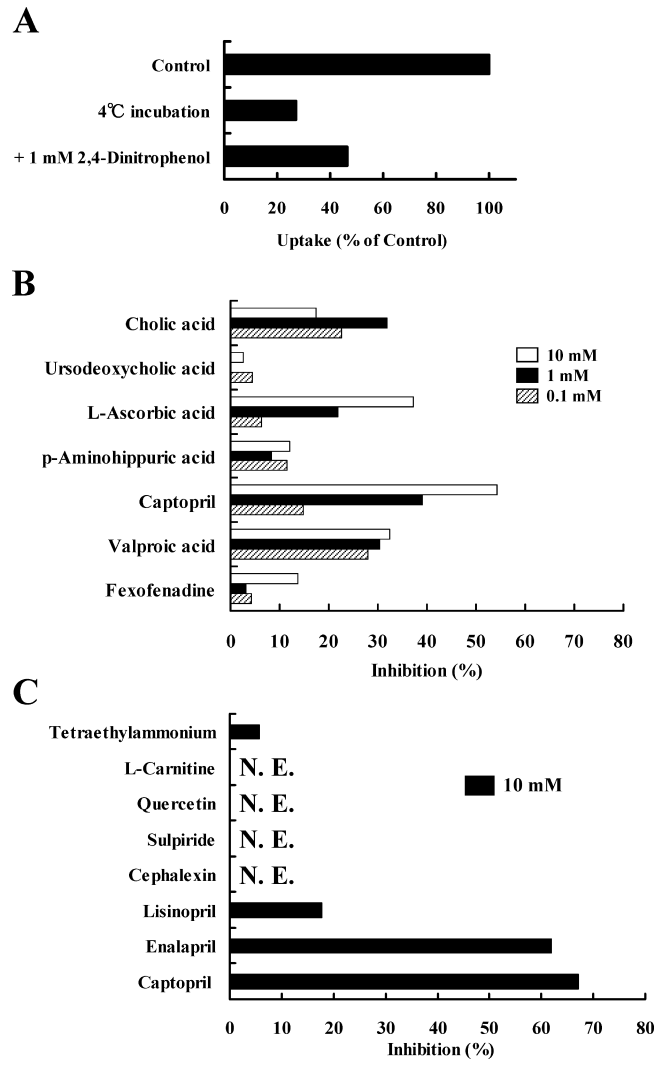

Figure 2. Energy dependence (A) and effects of various transporter substrates and inhibitors (B and C) on TBPM-PI uptake by Caco-2 cells. The concentration of TBPM-PI was 100 μM. Transporter substrates and inhibitors were combined with TBPM-PI simultaneously. All uptakes were performed for 15 min. Each value represents the mean ($n = 2$). Incubation time of 1 mM 2,4-dinitrophenol pretreatment was 15 min. The concentration of quercetin was 100 μM. "N. E." means not effective in panel C.

transport ACE inhibitors and quercetin, an inhibitor of the sodium-dependent vitamin C transporter (SVCT) family (Figure 2C).

Then, we investigated the specificity of the inhibitory effect of captopril and ascorbic acid in TBPM-PI uptake by Caco-2 cells. Inhibitory effects of these compound were reproduced in TBPM-PI uptakes (Figure 3). However, no inhibitory effect was observed in the case of CDTR-PI, which is one of the prodrugs of β-lactam antibiotics (Figure 3).

Inhibitory Effect by Captopril in Rat Intestinal Absorption of TBPM-PI. We investigated the inhibitory effect on rat intestinal absorption by captopril which had the most potent effect in Caco-2 study. When coadministered orally with captopril, plasma concentration of TBPM was appar-

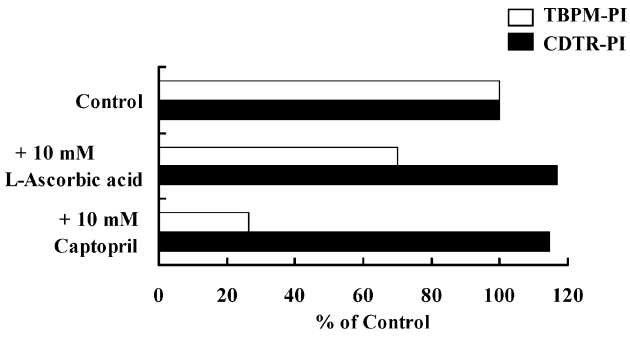

Figure 3. Effects of captopril and L-ascorbic acid on TBPM-PI and CDTR-PI uptake into Caco-2 cells. The concentration of TBPM-PI or CDTR-PI was 100 μM. Captopril or L-ascorbic acid was added with the test compound simultaneously. All uptakes were performed for 15 min. Each value represents the mean ($n = 2$).

ently reduced (Figure 4A). C_{max} and AUC_{inf} were significantly reduced to about one-third of the control group (Table 2A). On the other hand, captopril coadministration was not effective in the case of CDTR-PI (Figure 4B). To elucidate this inhibitory effect by captopril in TBPM-PI, rat *in situ* intestinal single-pass perfusion study was performed. Distinct difference of TBPM concentration in plasma from mesenteric vein was detected between the absence and presence of captopril (Figure 5). AUC_{0-obs} and absorption rate were significantly reduced to about half of the control group (Table 3). These data supported that the inhibitory effect of captopril is expressed on the process of TBPM-PI intestinal absorption.

Uptake of TBPM-PI by Influx Transporters. Some ACE inhibitors and β-lactam antibiotics have been reported to be substrates of influx transporters such as PEPT family and OATP family.[11,14,26,27] Therefore TBPM-PI is considered to be a possible substrate of these transporters. We evaluated the transport activity of TBPM-PI mediated by PEPT1, OATP1A2, and OATP2B1 which are expressed in the small intestine by using *Xenopus* oocyte gene-expression systems.[11,20,22] The uptake of TBPM-PI in *Xenopus* oocytes expressing PEPT1 was comparable to that in water-injected oocytes (Figure 6A), whereas the uptake of Gly-Sar, a typical PEPT1 substrate, was significantly higher in *Xenopus* oocytes expressing PEPT1 than that in water-injected oocytes (Figure 6B). Accordingly, TBPM-PI is not considered to be a substrate of PEPT1.

On the other hand, the uptakes of TBPM-PI by *Xenopus* oocytes expressing OATP1A2 and OATP2B1 were both significantly increased compared with those by water-injected oocytes (Figure 7). Moreover, uptakes of estrone-3-sulfate via OATP1A2 and OATP2B1 were inhibited by TBPM-PI in a dose-dependent manner. When the concentration of estrone-3-sulfate was 10 nM, IC_{50} values at pH 6.0 were 8.0 μM and 410 μM in OATP1A2 and OATP2B1, respectively. These results suggested that TBPM-PI is a substrate of both OATP1A2 and OATP2B1. On the other hand, no uptakes of TBPM, an active form of TBPM-PI, were observed by OATP1A2 and OATP2B1 (data not shown). These results suggested that esterification of carboxylic acids at the C-2 position is important for recognition of substrates of OATP1A2 and OATP2B1.

Effect of pH on TBPM-PI Transport by OATP1A2 and OATP2B1. To consider the impact of OATP1A2 and OATP2B1 on the intestinal absorption process of TBPM-PI, the uptakes of TBPM-PI in *Xenopus* oocytes expressing these transporters were measured at various pH conditions (pH 5.0−8.0) (Figure 8). OATP1A2-mediated uptakes of TBPM-PI showed a bell shape, and maximal activity was observed at a weak acidic condition (pH 6.5, Figure 8A). In contrast, the uptake of TBPM-PI by OATP2B1 was increased with increasing pH (Figure 8B). Thus, the characteristics of pH dependence in the TBPM-PI transport by these transporters were different, even though these transporters are classified as the same family. It is speculated that TBPM-PI is absorbed from the upper part of the intestine because this agent is absorbed quickly showing the time to peak concentration (T_{max}) around 30 min in human.[10] In the present study, significant transport activities mediated by both OATP1A2 and OATP2B1 were observed under the condition from weak acidic to neutral pH, which is thought to be physiological pH in the small intestine.

Kinetic Analysis of TBPM-PI Transport by OATP1A2 and OATP2B1. Kinetic analysis of TBPM-PI transports mediated by OATP1A2 and OATP2B1 was performed at pH 6.0, which is close to the pH in the small intestine. As shown in Figure 9A, the uptake of TBPM-PI by OATP1A2 was saturable with a K_m value of 41.1 μM. On the other hand, the uptake of TBPM-PI by OATP2B1 was not saturated up to 1 mM. Considering this result, the K_m value in OATP2B1 is estimated over 1 mM (Figure 9B). Due to the solubility problem of TBPM-PI, an uptake study could not be performed at a concentration over 1 mM. Our findings suggested that the TBPM-PI transport is saturated by OATP1A2 but hardly saturated by OATP2B1 in the small intestine even when the concentration of TBPM-PI is high enough.

Discussion

When TBPM-PI was administered orally, high blood concentration of TBPM, an active form, is rapidly acquired, and its absorption reaches about 80% of dose in human. It is speculated that TBPM-PI is mainly absorbed from the upper part of the small intestine, because its absorption is rapid (T_{max} is around 30 min), when TBPM-PI is administered orally to human.[10] The membrane permeation by simple diffusion alone may not be enough to explain such good absorption, because TBPM-PI is a cationic compound having

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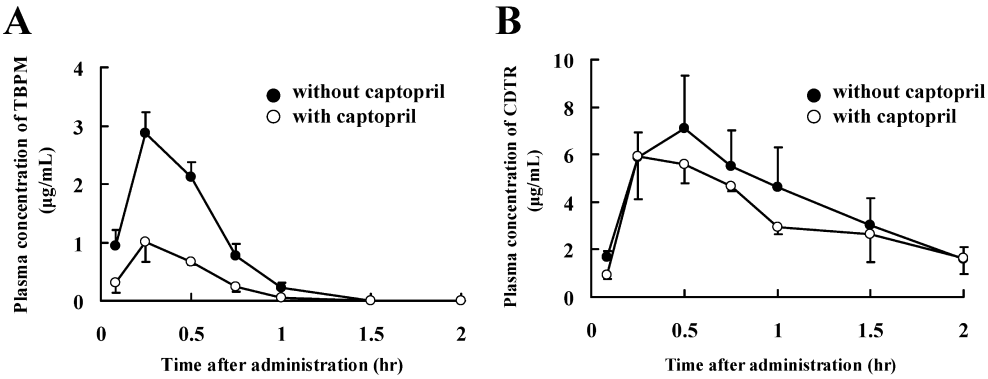

Figure 4. Effect of captopril on plasma TBPM or CDTR concentrations after oral administration of TBPM-PI (A) or CDTR-PI (B) in rat. TBPM-PI or CDTR-PI was administered to fasted male rats orally at a dose of 5 mg/kg TBPM-PI or CDTR-PI with or without captopril (300 mg/kg). Each value represents the mean ± SD ($n = 3$).

Table 2. Pharmacokinetic Parameters of TBPM or CDTR after Oral Administration of TBPM-PI or CDTR-PI at a Dose of 5 mg/kg in Rat[a]

group	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (μg/mL)	AUC_{0-inf} (μg·h/mL)
TBPM-PI alone	0.15 ± 0.02	0.25 ± 0.00	2.88 ± 0.35	1.52 ± 0.12
TBPM-PI + captopril	0.13 ± 0.06	0.25 ± 0.00	1.01 ± 0.34[b]	0.50 ± 0.07[b]
CDTR-PI alone	0.68 ± 0.11	0.42 ± 0.14	7.12 ± 2.18	9.78 ± 2.75
CDTR-PI + captopril	1.18 ± 0.61	0.42 ± 0.14	6.46 ± 1.53	9.88 ± 3.06

[a] TBPM-PI or CDTR-PI was administered to fasted male rats orally with or without captopril (300 mg/kg). Each value represents the mean ± SD ($n = 3$). [b] Significant difference between both groups ($P < 0.05$).

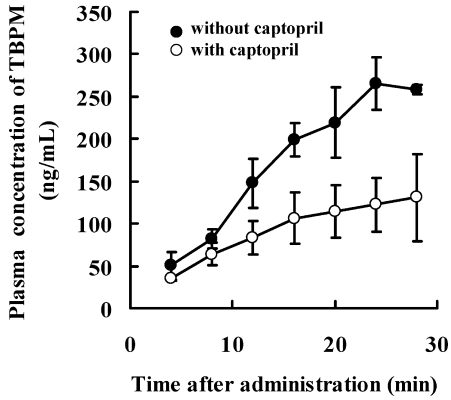

Figure 5. Effect of captopril on TBPM-PI intestinal absorption in rat *in situ* intestinal single-pass perfusion study. A 5 cm intestinal segment at the upper part of the ileum was perfused with 100 μM TBPM-PI with or without 10 mM captopril in 100 mM HEPES/DPBS (pH 6.5). The perfusion flow rate was 0.2 mL/min. The concentration of TBPM in venous blood from mesenteric vein was measured at 4 min intervals for 28 min. Each value represents the mean ± SD ($n = 3$).

Table 3. A Pharmacokinetic Parameter and Absoption Rate of TBPM in Rat *in Situ* Intestinal Single-Pass Perfusion Study[a]

group	AUC_{0-obs} (ng·min/mL)	absorption rate (V) (nmol/min)
TBPM-PI alone	4372 ± 333	0.10 ± 0.00
TBPM-PI + captopril	2363 ± 606[b]	0.05 ± 0.02[b]

[a] Each parameter was calculated based on the concentrations of TBPM in venous blood from mesenteric vein. Each value represents the mean ± SD ($n = 3$). [b] Significant difference between both groups ($P < 0.05$).

a thiazolinylazetidine group at the C-3 position (apparent $pK_a = 7.72$) and exists mainly in a cationic form under weak acidic conditions in the small intestine. We therefore investigated the involvement of influx transporters in addition to simple diffusion as a factor for the high absorption of TBPM-PI.

First, energy dependency was investigated by an uptake study using Caco-2 cells. TBPM-PI uptake was reduced by ATP-depletion and the incubation at 4 °C (Figure 2), suggesting the contribution of influx transporters in intestinal absorption. However simple diffusion was thought to be contributing to TBPM-PI uptake to some degree because a significant uptake was still observed under ATP-depleted or low-temperature conditions. Next, the effect of various transporter substrates and inhibitors was investigated in TBPM-PI uptakes by Caco-2 cells. Captopril and L-ascorbic acid had inhibitory effects with a dose-dependent manner (Figures 2B and 3). However, the inhibitory effect of L-ascorbic acid was weaker than that of captopril and the uptake was not decreased by quercetin, an inhibitor of SVCT family which localized in intestine.[28,29] On the other hand, ACE inhibitors except for captopril also had the inhibitory

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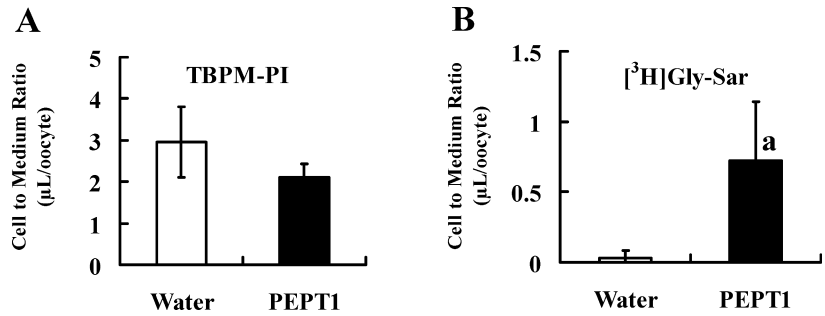

Figure 6. Uptake of TBPM-PI (A) and [^{3}H]Gly-Sar (B) by *Xenopus* oocytes expressing PEPT1. Uptake of TBPM-PI (100 μM) or [^{3}H]Gly-Sar (20 μM) was measured at room temperature and pH 6.5 for 60 min. Open and closed bars represent the results obtained with *Xenopus* oocyte injected with water and PEPT1 cRNA, respectively. Each value represents the mean $\pm$ SD ($n = 7-8$). "a" indicates a significant difference of the uptake by PEPT1-expressing oocytes from that by water-injected oocytes ($P < 0.05$).

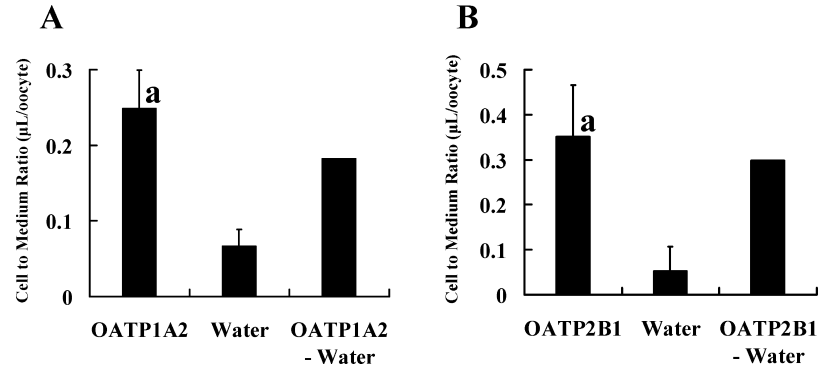

Figure 7. Uptake of TBPM-PI by *Xenopus* oocytes expressing OATP1A2 (A) and OATP2B1 (B). Uptake of TBPM-PI (10 μM) was measured at room temperature and pH 6.0 for 60 min. Each value represents the mean $\pm$ SD ($n = 10-12$). "a" indicates a significant difference of the uptake by OATP1A2 or OATP2B1-expressing oocytes from that by water-injected oocytes ($P < 0.05$).

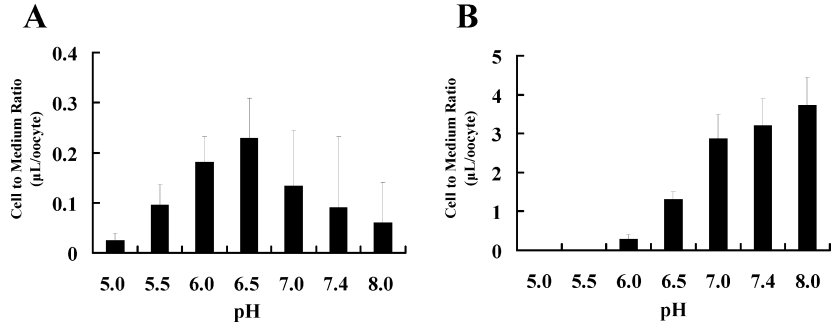

Figure 8. The effect of pH on TEPM-PI uptake by *Xenopus* oocyte expressing OATP1A2 (A) and OATP2B1 (B). Uptake of TBPM-PI (10 μM) was measured at room temperature for 60 min. The pH ranged from 5.0 to 8.0. Closed bars represent the transport activity by OATP1A2 and OATP2B1. Each value represents the mean $\pm$ SD ($n = 8-13$).

effects (Figure 2C). From these results, we focused on the influx transporters which may be involved in the effect of ACE inhibitors.

We selected the candidate influx transporters that are responsible for TBPM-PI intestinal absorption of TBPM-PI based on the results of Caco-2 uptake experiments and the chemical structure of TBPM-PI. Captopril and enalapril are reported to be substrates of PEPT1 and OATP families, respectively.[11,26] Moreover, some cephalosporin antibiotics (cephalexin, cefadroxil, etc.) exhibit high oral absorption due to the contribution of an influx transporter PEPT1.[11−15] Benzylpenicillin is reported to be a substrate of OATP family as well as PEPT1.[27] OATP1A2 and OATP2B1 are influx transporters which localize at the brush-border membrane of intestinal epithelial cells as well as PEPT1.[11,20,22,30] We therefore focused on these three transporters and investigated the transport of TBPM-PI by them.

No significant transport of TBPM-PI was observed by PEPT1 (Figure 6A). Although TBPM transport by PEPT1 was not measured directly, TBPM is not considered to be a substrate of PEPT1 since Gly-Sar uptake by PEPT1 was not affected by TBPM (data not shown). Moreover, substrates of PEPT family (cephalexin[31] and sulpiride[32]) had no inhibitory effect on TBPM-PI uptake by Caco-2 cells

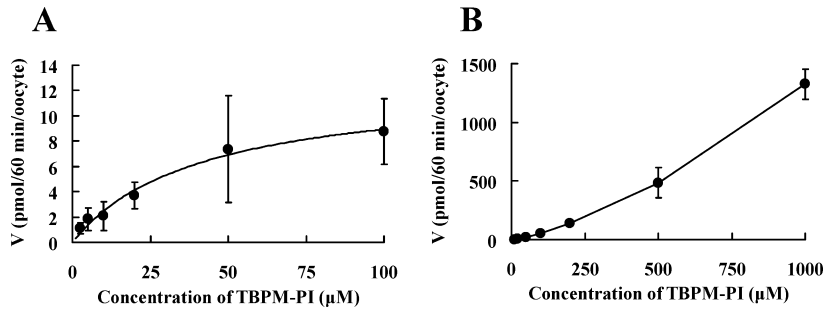

Figure 9. Concentration−rate curve of TBPM-PI uptake by OATP1A2 (A) and OATP2B1 (B) expressed in *Xenopus* oocyte. Uptake of TBPM-PI was measured at room temperature and pH 6.0 for 60 min. The concentrations of TBPM-PI ranged from 2.5 to 100 μM for OATP1A2 and from 10 to 1000 μM for OATP2B1, respectively. Each value represents the mean ± SD ($n = 7-12$).

(Figures 2B and 2C). Accordingly, a contribution of PEPT1 to the absorption of TBPM-PI is considered to be negligible. In contrast, a significant transport activity of TBPM-PI was observed by OATP1A2 and OATP2B1 (Figure 7) and no significant transport activity of TBPM by these transporter was detected. So, this kind of transport activity was selective for prodrug TBPM-PI but not for active form TBPM.

Interestingly, both OATP1A2 and OATP2B1 exhibited pH dependence in TBPM-PI transport, but the optimal pH differed between them. While the highest transport activity was observed at pH 6.5 for OATP1A2, OATP2B1 exhibited higher activity at neutral and weak alkaline pH showing significant activity at weakly acidic pH (Figure 8). As already mentioned, TBPM-PI was estimated to be absorbed from the upper part of the intestine, where the luminal pH is acidic. Accordingly, although the optimal pH for the transport of TBPM-PI is different between OATP1A2 and OATP2B1, both of them should contribute to the intestinal absorption of TBPM-PI. OATP1A2 had relatively high affinity for TBPM-PI ($K_m = 41.1$ μM), while OATP2B1 was not saturated up to 1 mM. Maximum TBPM-PI concentration in the gastrointestinal tract estimated from a clinical dose of this agent (4 or 6 mg/kg, b.i.d.) is very high (probably mM order). Therefore, OATP1A2 might be saturated at a clinical dose, while OATP2B1 might not be saturated even at such a high concentration. These results suggested that OATP2B1 is more likely to contribute to the influx transport of TBPM-PI *in vivo*. At present, it is difficult to estimate the *in vivo* contribution of OATP1A2 and OATP2B1 to TBPM-PI absorption, since there is insufficient information on the expression level and the activity of these transporters in the small intestine. Although these results suggested the carrier-mediated permeation of TBPM-PI in the small intestine, an *in vivo* verification of the contribution of transporters to oral absorption of TBPM-PI has not been carried out. We have preliminarily observed that TBPM-PI is transported by rat Oatp1a5, a transporter localized at the apical membrane of small intestinal epithelial cells in rats (data not shown). Accordingly, further studies in animals should clarify the *in vivo* contribution of these transporters to TBPM-PI absorption.

The OATP family in general is involved in the transport of various endogenous compounds such as bile acids, thyroid hormones, prostanoids, and conjugated steroids, and many drugs.[33] Currently a structure−activity relationship (SAR) study on OATP family and their substrates is under development. Yarim et al. have constructed a 3D-QSAR model by comparative molecular field analysis (CoMFA) of Oatp1a5.[34] Yarim et al. reported that the important pharmacophores in the substrate structure are (1) a negatively charged group, (2) a relatively extended hydrophobic region, and (3) a hydrogen bond donor. Fexofenadine and pravastatin, which are transported by OATP1A2 and OATP2B1,[20,22,23] have the structures that fit to the pharmacophore of Oatp1a5. However, there is a lack of consistency between this pharmacophore and TBPM-PI, of which we found the transport activity in the present study. The new knowledge obtained from the present study should be useful for future studies on the substrate recognition of OATP family.

Generally, patients are at risk of drug−drug interaction by taking the two or more medicines which are transported by the common transporter.[35] There are some reports on the drug−drug interaction on PEPT1. When cefadroxil was administered (at a dose of 5 mg/man) with cephalexin (45

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mg/man), the maximum concentration (C_{max}) and the area under the plasma concentration−time curve (AUC) of cefadroxil were significantly decreased.[36] In contrast, in the combination of valacyclovir and cephalexin of 500 mg each/man, plasma concentration profiles of both medicines were not changed.[37] The food−drug interaction has been also reported in intestinal absorption of OATP substrates. Oral absorption of the drugs which are transported by OTP1A2 and OATP2B1 is reduced when the drugs are administered with grapefruit juice or orange juice.[20,38,39] However, the risk of such interaction with TBPM-PI may be lower than that with drugs which are mainly absorbed by an influx transporter, since in TBPM-PI there are plural routes, including at least two OATPs and simple diffusion. More data of clinical use of TBPM-PI should be accumulated to verify the possible drug interactions in its intestinal absorption. The antibiotics with low oral absorption have a higher risk of not acquiring sufficient pharmacological effects in clinical use and expanding the antibiotics-resistant microorganism. Moreover, these agents are thought to cause a high frequency of intestinal side effects such as diarrhea and microbial substitution. From these considerations, the oral antibiotics with high absorption such as TBPM-PI have clinical benefits.

In conclusion, we found that OATP1A2 and OATP2B1 are involved in the intestinal absorption of TBPM-PI with high oral absorption. This is the first report showing the contribution of OATP family to the intestinal absorption of β-lactam antibiotics. It is also interesting that the ester prodrug but not the active form, TBPM, was a substrate of the influx transporters, since usually this kind of ester prodrug is expected to have higher membrane permeability due to its increased simple diffusion. The knowledge obtained from this study is useful for creating new drugs with better oral absorption and improving the absorption rate of drugs by the prodrug strategy.

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