Oseltamivir (Tamiflu) Is a Substrate of Peptide Transporter 1

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ABSTRACT:
Oseltamivir, an ester-type prodrug of the neuraminidase inhibitor [3R,4R,5S]-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate (Ro 64-0802), has been developed for the treatment of A and B strains of the influenza virus but has neuro-psychiatric and other side effects. In this study, we characterized the transport across intestinal epithelial cells and the absorption of oseltamivir in rats. Uptake by Caco-2 cells (human carcinoma cell line) and HeLa cells transfected with peptide transporter 1 (HeLa/PEPT1) was time- and temperature-dependent and was inhibited by typical PEPT1 inhibitors such as glycyl-sarcosine (Gly-Sar). The uptake by Caco-2 cells and HeLa/PEPT1 was saturable, with similar \( K_{m} \) values. Oseltamivir absorption in adult rats was greatly reduced by simultaneous administration of milk, casein, or Gly-Sar. Furthermore, the plasma and brain concentrations of oseltamivir were higher in fasting than in nonfasting rats after oral administration. These results suggest that oseltamivir is a substrate of PEPT1 and that PEPT1 is involved in its intestinal absorption.

Various transporters are expressed on apical and basolateral membranes of intestinal epithelial cells, serving to take up nutrients and to excrete xenobiotics into the lumen. Influx transporters are able to accept nutrients and also various drugs as substrates. In particular, peptide transporter 1 (PEPT1, SLC15A1), localized at brush-border membranes of human small intestine (Saito et al., 1995), plays important roles in the absorption of not only di-/tripeptides (Tamai et al., 1994) but also peptide-mimetic compounds, such as orally administered \( \beta \)-lactam antibiotics (Ganapathy et al., 1995; Sai et al., 1996) and the anticancer agent bestatin (Tomita et al., 1990; Inui et al., 1992). Several researchers recently found that intestinal PEPT1 can transport \( t \)-valine ester prodrugs, such as valacyclovir and valganciclovir (Bailmame et al., 1998; Han et al., 1998; Sugawara et al., 2000). Therefore, such structural modification of drugs may result in increased intestinal absorption, mediated by PEPT1.

Oseltamivir phosphate (oseltamivir), manufactured under the trade name Tamiflu as an ester-type prodrug of the neuraminidase inhibitor Ro 64-0802, has been developed for the treatment of A and B strains of the influenza virus. This drug has been reported to be associated with neuropsychiatric side effects (http://www.fda.gov/cder/drug/infopage/tamiflu/QA20051117.htm and http://www.mhlw.go.jp/english/index.html), which are likely to be caused by distribution of oseltamivir and/or its metabolite(s) to the central nervous system. We recently examined the possible role of P-glycoprotein (P-gp) as the determinant of brain distribution of oseltamivir and Ro 64-0802 both in vitro using LLC-GA5-COL150 cells, which overexpress human multidrug resistance (or resistant) 1 (MDR1) P-gp on the apical membrane, and in vivo using mdr1a/1b knockout mice (Morimoto et al., 2008). The permeability of oseltamivir in the basolateral-to-apical direction was significantly greater than that in the opposite direction. The brain distribution of oseltamivir was increased in mdr1a/1b knockout mice compared with wild-type mice. In contrast, negligible transport of Ro 64-0802 by P-gp was observed in both in vitro and in vivo studies. These results showed that oseltamivir, but not Ro 64-0802, was a substrate of P-gp. Accordingly, low levels of P-gp activity or drug-drug interactions at P-gp may lead to enhanced brain accumulation of oseltamivir, and this in turn may account for the central nervous system effects of oseltamivir observed in some patients (Morimoto et al., 2008). During that research, we noticed that increased plasma concentration and toxicity were observed in fasted infant rats compared with nonfasted ones. Therefore, we speculated that transporters involved in the uptake of food components also take part in the absorption of oseltamivir.

The purpose of the present study was to characterize the transport of oseltamivir across intestinal epithelial cells. We first examined whether oseltamivir is a substrate of PEPT1 by using a human carcinoma cell line, Caco-2 cells, and HeLa cells stably expressing human PEPT1 (HeLa/PEPT1). A rat in vivo study was then conducted to confirm involvement of PEPT1 in oseltamivir absorption in the small intestine. Our findings indicate that a drug-food interaction with...
potential clinical significance is likely to occur between oseltamivir and milk.

Materials and Methods

Chemicals and Animals. Oseltamivir phosphate was purchased from Sequoia Research Products (Pangbourne, UK). Ro 64-0802 was biologically synthesized from oseltamivir using porcine liver esterase (Sigma-Aldrich, St. Louis, MO) as described previously (Morimoto et al., 2008). The human colon adenocarcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD). HeLa/PEPT1 and HeLa transfected with vector alone (mock) were established as described previously (Nakanishi et al., 2000). Dulbecco’s modified Eagle’s medium (DMEM), nonessential amino acids, penicillin, streptomycin, and Hanks’ balanced salt solution (HBSS) were all from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Nitta Gelatin (Osaka, Japan). Glycyl-sarcosine (Gly-Sar), bis(4-nitrophenyl) phosphate (BNPP), and bovine serum albumin were from Sigma-Aldrich. NaN3 was from Nitta Gelatin (Osaka, Japan). Glycyl-sarcosine (Gly-Sar), bis(4-nitrophenyl) phosphate (BNPP), and bovine serum albumin were from Sigma-Aldrich. The protein assay kit was purchased from Bio-Rad (Hercules, CA). All the other chemicals and solvents were commercial products of analytical, high-performance liquid chromatography, or liquid chromatography/mass spectrometry grade as appropriate.

The animal study was performed according to the Guidelines for the Care and Use of Laboratory Animals at the Takasaki University of Health and Welfare and approved by the Committee of Ethics of Animal Experimentation of the university. One- and 8-week-old Wistar rats were purchased from SLC Japan (Hamamatsu, Japan).

Cell Culture and Cellular Quality Assessment. Caco-2 cells were cultured in DMEM containing 10% FBS, 1% nonessential amino acids, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 1 mg/ml genetin (G418) as described previously (Nakanishi et al., 2000).

Uptake study with HeLa cells was also performed at 37°C in HBSS adjusted to pH 6.0. Cultured cells were washed and preincubated in the buffer without oseltamivir or Ro 64-0802 for 10 min at 37°C. The uptake was initiated by adding HBSS (300 μl) containing oseltamivir or Ro 64-0802. After incubation for designated times at 37°C, the experiment was terminated by removing the medium, followed by washing five times with 2 ml of ice-cold HBSS. For quantitation of the drug taken up by the cells, the cells were suspended in acetonitrile/methanol (25:75; v/v), collected with a cell scraper (Asahi Techno Glass Corporation Co. Ltd., Chiba, Japan), and then sonicated for 30 min, followed by centrifugation at 12,000 rpm and 4°C for 10 min. The supernatant was evaporated under centrifugal evaporator at 30°C. The residue was dissolved in 0.1 ml of 10 mM ammonium acetate buffer, pH 7.0, and filtered by passing through a 0.45-μm pore size membrane filter (Millipore Corporation, Billerica, MA). The supernatants were then subjected to liquid chromatography/mass spectrometry analysis. Cellular protein was determined using a protein assay kit with bovine serum albumin as a standard.

In Vitro Data Analysis. The permeability (microliter per milligram of protein) was expressed as the apical-to-basolateral side obtained by dividing...
the transported amount (micromole per milligram of protein) at the basolateral side by the initial concentration (micromolar) in the apical side. The permeability coefficient \( P_{\text{app}} \) (centimeter per second) was calculated from the linear portion of an uptake versus time plot using the following equation: \( P_{\text{app}} = \frac{dQ}{dt}/A/C_0 \), where \( dQ/dt \) is the initial permeation rate across the Caco-2 cell monolayer (micromole per second), \( A \) is the surface area of the filter (square centimeter), and \( C_0 \) is the initial concentration of the solution in the apical side (micromolar).

Cell/medium ratio was obtained by dividing the cellular uptake amount by the concentration of test compound in the medium. Kinetic parameters for transport activity were estimated by nonlinear least-squares fitting of the data to the following equation using the MULTI program: \( V = V_{\text{max}} \cdot S/(K_m + S) + K_p + S \), where \( V \), \( S \), \( K_m \), \( V_{\text{max}} \), and \( K_p \) represent the initial uptake rate, substrate concentration, Michaelis constant, maximum uptake rate, and first-order rate constant, respectively.

**Pharmacokinetic Study.** The 8-week-old male rats (weight, 185–206 g) were deprived of food for 12 h before experiments. Oseltamivir was dissolved in distilled water, milk (commercially available cows’ milk), Gly-Sar solution (20 or 125 mM), or casein solution (300 mg/10 ml) and was orally administered to rats at a single dose of 30 mg/kg (dosing volume, 10 ml/kg). For the intravenous injection study, 8-week-old male rats (weight, 177–186 g) were administered 30 mg/kg oseltamivir via the jugular vein (the dosing volume, 1 ml/kg). Blood samples were withdrawn from the jugular vein of rats with a heparinized syringe at designated times under anesthesia induced with diethyl ether. In the case of infant rats, 1-week-old infant rats (weight, 16.5–20.6 g) from the same parental female rat were used. Three of them (two male, one female) were separated from the parental female rat and starved overnight, and the others (three male, two female) remained with the parental rat and were fed milk. Oseltamivir dissolved in distilled water was orally administered to infant rats at a single dose of 30 mg/kg (the dosing volume, 10 ml/kg). Blood samples were collected from the jugular vein at 30 min after the start of administration under anesthesia induced with diethyl ether, and then the infant rats were decapitated. The brain was quickly excised, rinsed with ice-cold saline, blotted dry, and weighed. Samples were stored at -30°C until analysis. Blood samples were centrifuged (1700g) for 15 min at 4°C to obtain plasma. As it has been reported that rat intestinal PEPT1 expression shows a diurnal rhythm, all the in vivo studies were conducted at the same time of the day (Pan et al., 2004). Quantification of oseltamivir and Ro 64-0802 in plasma and brain tissues was performed using reported methods (Wiltshire et al., 2000) with some modifications. In brief, aliquots of brain tissues (100 mg) were homogenized with 1 ml of 5 mM ammonium acetate buffer, followed by centrifugation at 1700g, and 0.9 ml of the supernatant was subjected to solid-phase extraction (Empore Mixed Phase Cation, 7 mm/3 ml; 3M Bioanalytical Technologies, St. Paul, MN). The methods used for the extraction of plasma and brain homogenate were the same. The maximum plasma concentration \( C_{\text{max}} \) and time to \( C_{\text{max}} \) \( T_{\text{max}} \) were determined directly from the observed data. The area under the plasma concentration-time curve from time 0 to 6 h (AUC from 0 to 6 h) was estimated by the linear trapezoidal method.

**Analytical Methods.** Aliquots (5 µl) of samples containing oseltamivir and Ro 64-0802 were injected into a high-performance liquid chromatography system (LC-20A system; Shimadzu, Kyoto, Japan) equipped with a Capcellpak OD column (150 × 2.0 mm i.d., Shiseido, Tokyo, Japan) using isocratic elution at 0.1 ml/min with 0.05% formic acid. Analytes were detected using a quadrupole mass spectrometer (LCMS-2010EV; Shimadzu) fitted with an electrospray ionization source. Analytes were detected in the positive mode, and protonated molecular ions at \( m/z = 313 \) for oseltamivir and \( m/z = 285 \) for Ro 64-0802 were monitored. Each value presented is the mean ± S.E.M. of three samples. Statistical analysis was performed by means of Student’s t test.
A difference between means was considered to be significant when the p value was less than 0.05.

**Results**

**Characterization of Osel tamivir Transport across Caco-2 Cell Monolayers.** We have shown that oseltamivir is a substrate of P-gp (Morimoto et al., 2008), and it was speculated that it might be a substrate of human carboxylesterase 1 (hCE-1, CES1A1, HU1) and/or carboxylesterase 2 (hCE-2, hiCE, HU3), which are present in several organs, including small intestine (Imai, 2006). Therefore, we first examined the effects of carboxylesterase and P-gp on the permeability of oseltamivir across Caco-2 cell monolayers. Figure 1A shows the permeability coefficient of oseltamivir without BNPP pretreatment and absence of verapamil (○) was 0.45 ± 0.02 × 10⁻⁶ cm/s (control value). The permeability coefficient of oseltamivir after pretreatment BNPP (▲) or in the presence of verapamil (□) was not significantly changed (0.61 ± 0.07 and 0.60 ± 0.06 × 10⁻⁶ cm/s, respectively). The permeability of oseltamivir after pretreatment with BNPP and in the presence of verapamil (●, 1.07 ± 0.10 × 10⁻⁶ cm/s) was significantly higher than the control value (p < 0.05). Consequently, we decided that subsequent influx studies using Caco-2 cells should be done after preincubation with BNPP and after adding verapamil to the apical chamber. Figure 1B shows the inhibitory effects of dipeptide and temperature on oseltamivir permeability across Caco-2 cell monolayers. Gly-Sar and Trp-Gly, which are substrates of PEPT1, significantly decreased the permeability coefficient of oseltamivir to 0.39 ± 0.07 and 0.49 ± 0.04 × 10⁻⁶ cm/s, respectively, from 0.61 ± 0.04 × 10⁻⁶ cm/s (Fig. 1B). The permeability coefficient was greatly decreased to 0.12 ± 0.01 × 10⁻⁶ cm/s at 4°C. Although we examined the concentration dependence of oseltamivir transport across Caco-2 cell monolayers, kinetic parameters could not be determined (data not shown).

**Characterization of Osel tamivir Uptake by Caco-2 Cells.** The time course, concentration dependence, effect of PEPT1 substrates, and temperature dependence of oseltamivir uptake by Caco-2 cells were studied, and the results are shown in Figs. 2 and 3. The uptake of oseltamivir increased linearly up to 1 min (Fig. 2A). Thus, the initial uptake rate was obtained as the slope of the uptake over 1 min, and the incubation time of 1 min was used in subsequent studies. The concentration dependence of the initial uptake of oseltamivir was studied over the range from 30 μM to 10 mM (Fig. 2B). The uptake was saturable, and the Kₘ, Vₘₐₓ, and Kₐ values were 6.54 ± 2.03 mM, 45.6 ± 12.0 nmol/min/mg protein, and 0.470 ± 0.517 μmol/min/mg protein, respectively. The uptake was greatly reduced at 4°C. Both Gly-Sar and Trp-Gly significantly and concentration-dependently inhibited the uptake of oseltamivir (Fig. 3).

**Characterization of Osel tamivir Uptake in HeLa/PEPT1.** The uptake of oseltamivir by HeLa/PEPT1 increased in a time-dependent manner and was higher than that by HeLa/mock (Fig. 4A). Kinetic
parameters of oseltamivir transport via PEPT1 were evaluated. The uptake of oseltamivir by PEPT1 was estimated after subtracting the uptake by HeLa/mock from those by HeLa/PEPT1. The uptake was saturable, and the \( K_m \) and the \( V_{\text{max}} \) values were estimated to be 8.59 ± 1.98 mM and 11.4 ± 1.68 nmol/10 min/mg protein, respectively (Fig. 4B). The uptake was markedly reduced when the temperature was lowered to 4°C (Fig. 5). In addition, the uptake of oseltamivir was decreased in the presence of PEPT1 substrates, 20 mM Gly-Sar or Trp-Gly (Fig. 5). On the other hand, the cell/medium ratio of Ro 64-0802, an active metabolite of oseltamivir, was 0.034 ± 0.02 %/10 min/mg protein, which was not significantly different from that of mock cells. This result suggested that the active metabolite is not a substrate of PEPT1.

Plasma Concentration of Oseltamivir in Rats. Fig. 6 shows the plasma concentration of unchanged drug after a single oral administration of oseltamivir (30 mg/kg) in rats (8-week-old). The pharmacokinetic parameters of oseltamivir are summarized in Table 1. Co-administration of 20 mM Gly-Sar did not affect oseltamivir pharmacokinetics. However, when 125 mM Gly-Sar was administered concurrently, the plasma concentration of oseltamivir was dramatically reduced (Fig. 6), and the bioavailability (BA) was decreased from 31.5% to 5.5%. When oseltamivir dissolved in milk was orally administered to rats, the plasma concentration of oseltamivir was dramatically reduced (Fig. 6), and the bioavailability (BA) was decreased to 8.5%. Concurrent administration of casein (300 mg/kg) also significantly decreased the plasma concentration of oseltamivir, and the BA was decreased to 5.5% (Table 1).

Plasma and Brain Concentrations of Oseltamivir in Pups. Oseltamivir was administered to fasting (non–breast-fed) rats, which were separated from the parental female rats overnight, and to non-fasting (breast-fed) rats born from the same mother. The plasma and brain concentrations of oseltamivir in fasting rats (13.4–15.3 µg/ml and 830–1344 ng/g brain, respectively) were higher than those in nonfasting ones (0.34–0.88 µg/ml and 48.3–172 ng/g brain, respectively) after oral administration of the drug (Fig. 7). The plasma concentration was well correlated with the brain concentration.

Discussion

We have already shown that oseltamivir is transported by P-gp and that the brain distribution is significantly affected by P-gp (Morimoto et al., 2008). During our study we noticed that the plasma concentration of oseltamivir, as well as its toxicity, was enhanced in fasted baby rats compared with nonfasted ones. Because baby rats are always fed milk, their gastrointestinal tract contains large amounts of di- and tripeptides, and we speculated that a peptide transporter might take part in the absorption of oseltamivir. Accordingly, we examined the intestinal transport of oseltamivir using Caco-2 cells and PEPT1-expressing HeLa cells. Initial uptake of oseltamivir by Caco-2 cells was saturable and temperature-dependent and was inhibited by typical PEPT1 substrates (Figs. 2 and 3). The transport properties of oseltamivir across a Caco-2 cell monolayer (Fig. 1) were consistent with the results of the uptake studies. These findings strongly suggested that oseltamivir is a substrate of PEPT1 and that its absorption is mediated, at least in part, by PEPT1. We directly confirmed that PEPT1 transports oseltamivir using PEPT1-expressing HeLa cell (Figs. 4 and 5). The \( K_m \) value obtained in HeLa/PEPT1 cells was similar to that obtained in Caco-2 cells (Fig. 4). All these results suggested that the transport of oseltamivir across a Caco-2 cell monolayer was mediated by PEPT1. It is interesting to note that Ro 64-0802, which is an active metabolite of oseltamivir, was not a substrate of PEPT1, although the metabolite with a carboxylic acid moiety superficially seems to be more similar than oseltamivir to a dipeptide.

To estimate the effects of milk, as well as protein and peptides derived from milk, on absorption of oseltamivir, we conducted an in vivo study using 8-week-old and infant rats. Casein is a major protein in milk, and 300 mg/kg casein is equivalent of 10 ml/kg milk as protein content. Moreover, 1.25 mmol/kg (125 mM) dipeptide is equivalent to 10 ml/kg milk, if milk protein is completely digested to di- and tripeptides. Therefore, we concurrently administered oseltamivir with 10 ml/kg milk, 300 mg/kg casein, or 125 mM Gly-Sar to 8-week-old infant rats. Casein is a major protein in milk, and 300 mg/kg casein is equivalent to 10 ml/kg milk, if milk protein is completely digested to dipeptides. Therefore, we concurrently administered oseltamivir with 10 ml/kg milk, 300 mg/kg casein, or 125 mM Gly-Sar to 8-week-old rats. Oseltamivir absorption was greatly reduced by these treatments (Fig. 6), suggesting that PEPT1 is involved in gastric absorption of
oseltamivir in rats. Such a peptide-drug interaction can directly affect the therapeutic efficacy and safety of substrate drugs, especially in infants, which are routinely fed milk. We then examined PEPT1-mediated peptide-drug interaction in 1-week-old infant rats. The plasma and brain concentrations of oseltamivir were both higher in fasting rats than in nonfasting rats after oral administration of the drug (Fig. 7). These results suggest that milk peptides interacted with oseltamivir on PEPT1 and thereby inhibited absorption of oseltamivir in infant rats.

In this study, we showed that oseltamivir is a substrate of PEPT1 and that this drug was absorbed at least in part via PEPT1 in small intestine. This result has two important implications. In general, it is thought that intestinal absorption of ester-type prodrugs of carboxylic acids occurs via simple diffusion, and hence can be improved by increasing the lipophilicity. However, it now appears that PEPT1 may also play a role. This is consistent with previous reports on PEPT1-mediated transport of the L-valine ester prodrug valacyclovir (Balmiane et al., 1998; Han et al., 1998; Sugawara et al., 2000). Absorption of various ester-type prodrugs might also be mediated by the influx transporter PEPT1. Second, the absorption of PEPT1 substrates might be influenced by eating, although so far there are few examples concerning the influence of food components on the absorption of medicines that are substrates of PEPT1. If other such peptide-drug interactions occur, they could directly affect the therapeutic efficacy and safety of substrate drugs, especially in infants. Further studies, including clinical studies, are needed.

References

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