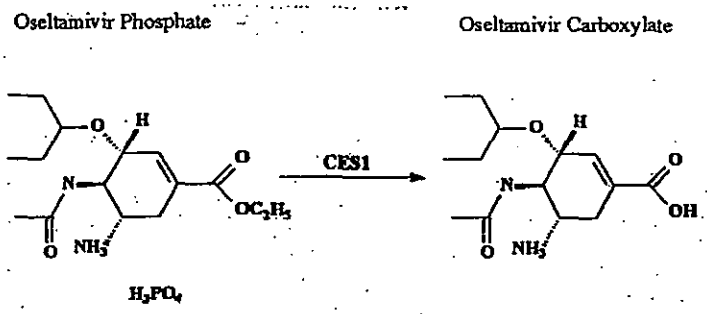

FIG. 1. CES1-mediated activation of oseltamivir phosphate.

substrate of CES1 (Shi et al., 2006), making it an excellent candidate compound to assess the effect(s) of the identified *CES1* mutations on prodrug activation. In addition, accumulating evidence has indicated that the biotransformation of oseltamivir phosphate to the primary active form oseltamivir carboxylate is not only related to its antiviral efficacy but also associated with potential toxicity (http://www.fda.gov/cder/foi/nda/2000/21-246_Tamiflu_Pharmr.pdf).

In the present study, we investigated the influence of the two newly identified CES1 variants on the metabolism (i.e., activation) of the prodrug oseltamivir phosphate. The results suggested that genetic variants of *CES1* that result in dysfunctional enzyme activity could likewise play an important role in both therapeutic efficacy as well as tolerability or toxicity during oseltamivir therapy.

Materials and Methods

Materials. Oseltamivir phosphate and its active metabolite oseltamivir carboxylate were obtained from Toronto Research Chemicals Inc. (North York, ON. Canada). *p*-nitrophenyl acetate (PNPA) and *p*-nitrophenol (PNP) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of the highest analytical grade and were commercially available.

Enzymatic Study. The establishment of Flp-In-293 cells (Invitrogen, Carlsbad, CA) stably expressing WT and p.Gly143Glu and p.Asp260fs CES1 has been described previously (Zhu et al., 2008). The transfected cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 μg/ml hygromycin B. After reaching approximately 95% confluence, cells were then washed and harvested in reaction buffer (phosphate-buffered saline containing 10 mM HEPES, pH 7.4). Afterward, cells were sonicated and then centrifuged at 9000*g* for 30 min at 4°C. The supernatant (S9 fraction) was collected and stored at −70°C until use. The liver tissues were obtained from a healthy liver donor and determined to express neither p.Gly143Glu nor the p.Asp260fs mutation and served as a native CES1 control. The liver samples (~300 mg) were homogenized, and the S9 fraction was obtained after centrifugation at 9000*g* for 30 min at 4°C. The protein concentrations were determined using a Pierce BCA assay kit (Pierce, Rockford, IL).

The oseltamivir hydrolysis study was carried out in 1.5-ml tubes at a total volume of 100 μl. Before incubations, oseltamivir phosphate solutions were freshly prepared in 50 μl of reaction buffer. The reaction was initiated by mixing oseltamivir phosphate with 50 μl of S9 fractions. The final oseltamivir phosphate concentrations ranged from 10 to 5000 μM. Our preliminary study indicated that the formation of oseltamivir carboxylate was linear with a series of S9 protein concentrations (0.05–0.5 mg/ml) and incubation times (5–15 min) that we tested. In the present study, the enzymatic reactions were performed with the final S9 protein concentration standardized at 0.1 mg/ml and an incubation period of 10 min at 37°C. After incubation, the reaction was terminated by adding 500 μl of methanol containing 40 μM ritalinic acid as the internal standard. The mixture was centrifuged at 16,000*g* for 5 min to precipitate protein, and the supernatants were then analyzed using an established high-performance liquid chromatography (HPLC) assay. Enzyme kinetic data of oseltamivir hydrolysis were fit to the Michaelis-Menten equation, and kinetic parameters K_m and V_{max} were calculated using nonlinear regression analysis with GraphPad Prism software (GraphPad Software Inc., San Diego, CA). In addition, PNPA, a widely used esterase substrate (including CES1), was included in the study as a positive control using a method described previously (Zhu et al., 2008).

HPLC Analysis. An HPLC method was used to measure oseltamivir carboxylate formation as a consequence of oseltamivir phosphate hydrolysis. The HPLC system consisted of an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a diode-array detector with the wavelength set at 220 nm. The mobile phase was a mixture of methanol and 20 mM KH_2PO_4 (pH2.5). A gradient elution was applied for the separation with the time program set as follows: from 0 to 4 min, methanol was 44%, and increased to 50% from 4 to 14 min, then maintained at 50% until 16 min, where methanol was returned to the initial condition (44%). Ritalinic acid, oseltamivir carboxylate, and oseltamivir were eluted at 5.1, 6.0, and 15.7 min, respectively, with the flow rate set at 1 ml/min. In Fig. 2, a typical chromatogram is represented of 100 μM of oseltamivir hydrolyzed by WT *CES1* S9 fractions after incubation. The intraday and interday relative standard deviations were determined to be less than 10%. The lower limit of quantification of oseltamivir carboxylate was 0.25 μM.

Results

PNPA is a sensitive and established model substrate of CES1 as well as other human esterases. The PNPA hydrolysis assay demonstrated that WT CES1 prepared from the cells transfected with WT *CES1* gene rapidly hydrolyzed PNPA to PNP with a catalytic efficiency comparable with that of normal human liver tissues (Fig. 3). Consistent with our previous observations, the enzymatic activity of both p.Gly143Glu and p.Asp260fs toward PNPA was dramatically reduced relative to WT enzyme (Zhu et al., 2008).

The oseltamivir phosphate incubation study demonstrated that the S9 preparations of both WT CES1-transfected cells and human liver tissues efficiently convert oseltamivir to its active antiviral component, oseltamivir carboxylate, suggesting that oseltamivir serves as an excellent substrate of CES1 (Fig. 3). The V_{max} and K_m values were determined to be 145 ± 5 nmol/min/mg protein and 1.38 ± 0.13 mM, respectively, under our experimental conditions (Fig. 4). The CES1 variants p.Gly143Glu and p.Asp260fs displayed poor catalytic activity toward oseltamivir hydrolysis (Figs. 3 and 4). The V_{max} value of p.Gly143Glu was found to be 37 ± 1 nmol/min/mg protein, which is approximately 25% of that of WT CES1. The K_m value of p.Gly143Glu was estimated to be 2.15 ± 0.18 mM. In addition, p.Asp260fs failed to produce any detectable hydrolysis of oseltamivir as measured by the formation of oseltamivir carboxylate (Fig. 3). The human liver S9 fractions prepared from a healthy donor specimen produced similar catalytic activity toward both PNPA and oseltamivir phosphate, which was in excellent agreement with that of our W *CES1*-transfected cells (Fig. 3).

Discussion

CES1 is the predominant hydrolase in the liver and plays an important role in the biotransformation of drugs and prodrugs that contains ester bonds. CES1 genetic variants and their potential for having therapeutic implications have been increasingly reported recently. Our previous study identified two nonsynonymous coding region variants, p.Gly143Glu and p.Asp260fs. In vitro functional studies have shown that the catalytic function mediating the typically efficient and rapid hydrolysis of methylphenidate was clearly disrupted in both the p.Gly143Glu variant and the p.Asp260fs mutation. The potential for clinically significant outcomes in the presence of these two mutations was investigated in the original subject found to carry both CES1 variants. That subject displayed an extremely abnormal pharmacokinetic profile after the administration of methylphenidate, displaying vastly higher overall blood concentrations of methylphenidate and an unprecedented distortion in the disposition of the respective isomers of the drug (Patrick et al., 2007; Zhu et al., 2008).

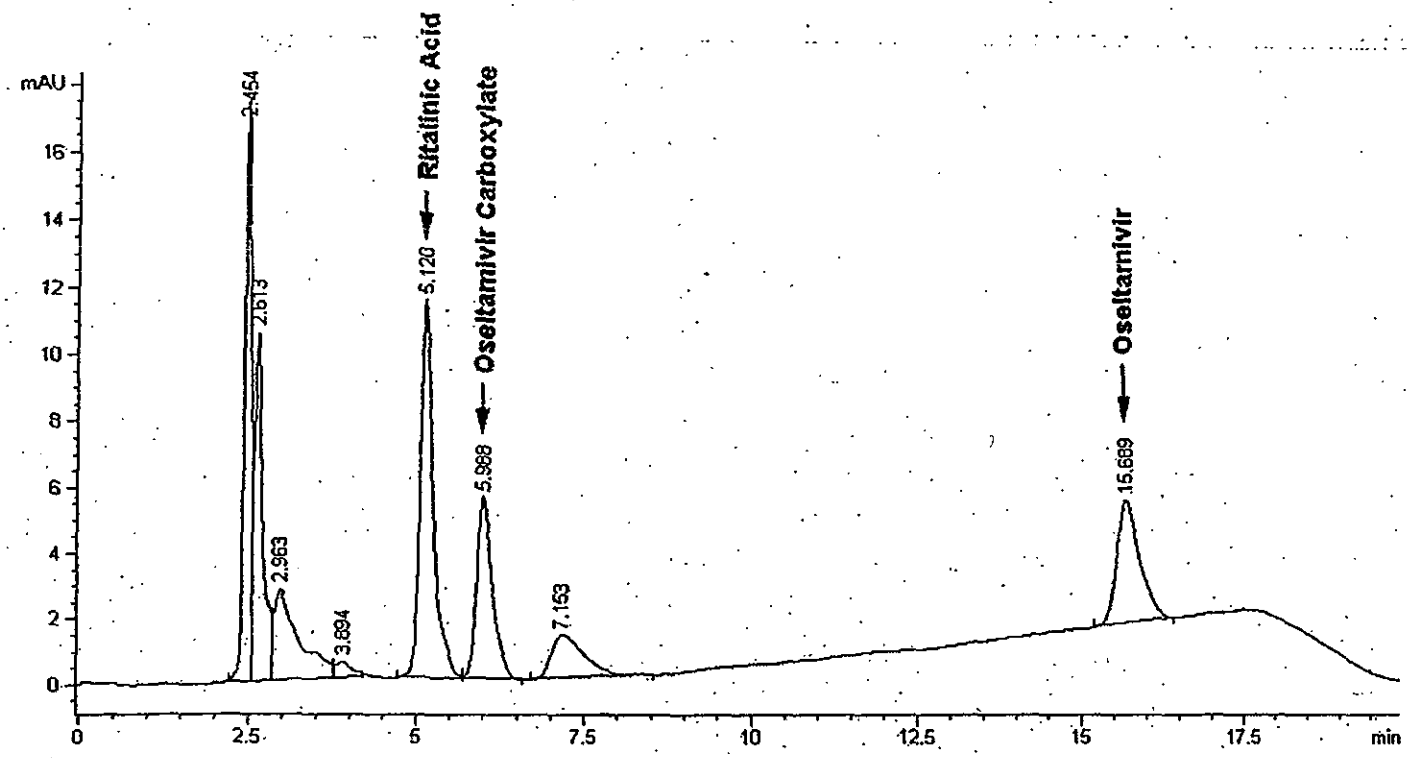

FIG. 2. Representative chromatograph of oseltamivir carboxylate. Oseltamivir carboxylate was analyzed by the HPLC assay after incubation of oseltamivir phosphate (100 μM) and WT CES1 S9 fractions at 37°C for 10 min.

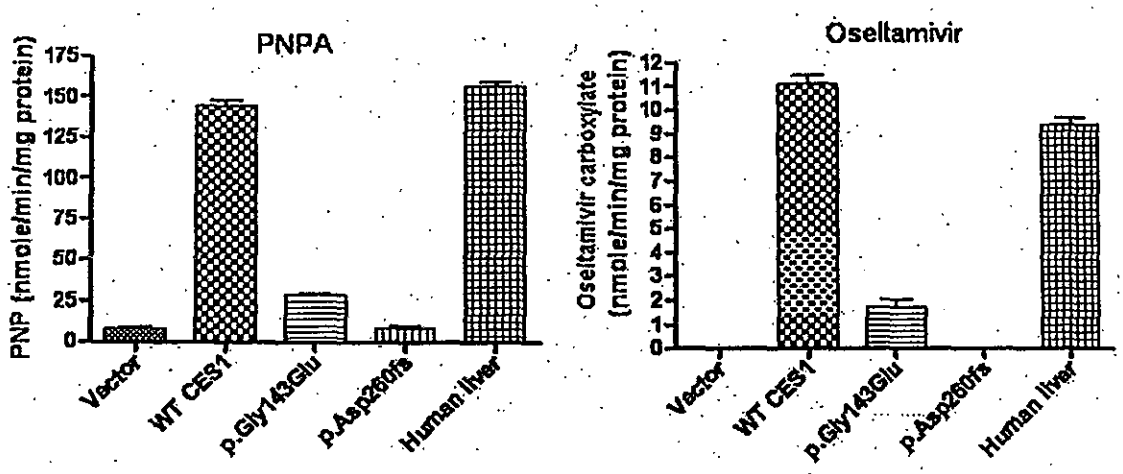

FIG. 3. Hydrolysis of PNPA and oseltamivir by human liver microsomal, WT CES1, and its mutants p.Gly143Glu and p.Asp260fs. The hydrolytic products of PNPA and oseltamivir were determined after incubating the substrates with the enzymes at 37°C for 10 min. Data were expressed as the mean ± S.D. (n = 4).

In addition, the subject experienced significantly higher cardiovascular vital signs relative to other 19 study subjects serving as a pharmacodynamic correlate to the pharmacokinetic observations (Zhu et al., 2008).

Because the activation of many ester prodrugs depends to a great degree upon functional CES1 enzyme to produce the therapeutic moiety, dysfunctional CES1 variants could hinder prodrug activation and lead to the alteration of therapeutic effects and accumulation of the parent prodrug with continued dosing. Such an outcome could lead to therapeutic failure and, depending on the compound administered, unanticipated adverse effects or toxicities. As a prodrug, oseltamivir does not exhibit activity toward the influenza virus unless it is converted to its active metabolite oseltamivir carboxylate by CES1 (Fig. 1). In the present study, the catalytic activity of p.Gly143Glu and p.Asp260fs toward oseltamivir hydrolysis (i.e., activation) was investigated using transfected cell lines stably expressing WT and individual mutant CES1 enzyme. The data indicated that the enzymatic activity of p.Gly143Glu is substantially decreased with a V_{max} value approximately one fourth that of WT CES1, whereas p.Asp260fs failed to show any measurable hydrolytic activity toward oseltamivir. Acknowledging the limitations of in vitro methodologies, this fundamental alteration in the catalytic activity of CES1 strongly suggests that the activation of oseltamivir would be compromised in patients who express such *CES1* variants. In addition to these two mutations, several other natural nonconservative *CES1* variants were recently determined to also have functional significance (Shi et al., 2006; Tang et al., 2006). Furthermore, beyond the coding area mutations, a number of functional variants have been reported in the transcriptional regulation region of *CES1* gene (Geshi et al., 2005; Hosokawa et al., 2008; Yoshimura et al., 2008). Among those, a single nucleotide

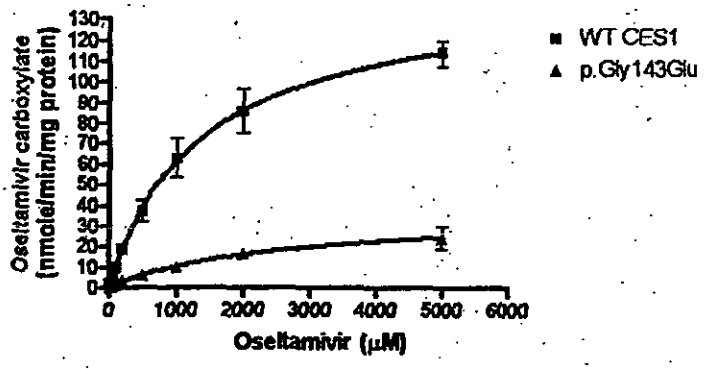

FIG. 4. Enzymatic kinetics study of oseltamivir hydrolysis catalyzed by WT CES1 and its variant p.Gly143Glu. The hydrolysis of oseltamivir (10–5000 μM) was determined after incubation with the cell S9 fractions at 37°C for 10 min. The V_{max} and K_m values were calculated using nonlinear regression analysis with GraphPad Prism software. Data present means ± S.D. for four independent experiments.

polymorphism, −816A/C of the *CES1A2* gene was found to be associated with an improved therapeutic response to an angiotensin-converting enzyme inhibitor imidapril, which is a prodrug and selectively activated by CES1 (Geshi et al., 2005).

It was noted that the observed V_{max} value of WT CES1 is consistent with that reported by Shi et al. (2006), whereas the K_m value is seven times higher. We suspect this difference is more likely than not the result of different experimental conditions used in these two independent studies. For example, the reaction buffer used in the present study was phosphate-buffered saline containing 10 mM HEPES (pH 7.4), whereas a Tris buffer was used in the study by Shi et al. (2006). Indeed, a recent in vitro study addressing this very issue indicates that different enzymatic activity of CES could be observed when different assay buffers were used (Williams et al., 2008). Finally, the S9 fractions used in the present study were prepared from a stable CES1 cell line rather than a transient expression assay.

Functional CES1 is not only critical for the conversion of oseltamivir to its active metabolite to achieve a favorable therapeutic response, but it is also related to the toxicity during oseltamivir therapy. Converging evidence suggests that CES1 function in juvenile animals remain at a significantly lower level than that of adult animals (Kadner et al., 1992; Morgan et al., 1994; Moser et al., 1998; Karanth and Pope, 2000; Padilla et al., 2004; Anand et al., 2006). Animal studies demonstrated that juvenile rats did not hydrolyze oseltamivir efficiently, and they are more susceptible than adults to oseltamivir toxicity (http://www.fda.gov/cder/foi/nda/2000/21-246_Tamiflu_Pharmr.pdf). The present study suggests that, in addition to age, genetic variation is potentially an important factor influencing the enzymatic function of CES1 and could play a role in the therapeutic outcome and toxicity of pharmacotherapy with oseltamivir as well as other known CES1 substrates. Our previously published data with the psychostimulant methylphenidate indicate that the effects of *CES1* variants on drug disposition are already advanced beyond the realm of speculation.

In summary, two newly identified *CES1* mutations p.Gly143Glu and p.Asp260fs were determined to be dysfunctional enzymes with respect to the activation of the prodrug oseltamivir. Impaired enzymatic function could have significant implications with regard to both the therapeutic efficacy and tolerability of oseltamivir. It should be noted that the extremely low prevalence of p.Asp260fs mutation relegates its clinical significance to being very minor even though it results in a nonfunctional enzyme. However, p.Gly143Glu is a common variant in all populations assessed thus far, with the exception of Asians. A clinical study, particularly one assessing patients who have been genotyped and found to be heterozygously expressing p.Gly143Glu, is warranted to elucidate the influence of the p.Gly143Glu mutation on the pharmacological disposition and potential toxicities of oseltamivir.

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Limited Brain Distribution of [3*R*,4*R*,5*S*]-4-Acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate Phosphate (Ro 64-0802), a Pharmacologically Active Form of Oseltamivir, by Active Efflux across the Blood-Brain Barrier Mediated by Organic Anion Transporter 3 (Oat3/*Slc22a8*) and Multidrug Resistance-Associated Protein 4 (Mrp4/*Abcc4*)

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ABSTRACT:

[3*R*,4*R*,5*S*]-4-Acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate (Ro 64-0802) is a pharmacologically active form of the anti-influenza virus drug oseltamivir. Abnormal behavior is a suspected adverse effect of oseltamivir on the central nervous system. This study focused on the transport mechanisms of Ro 64-0802 across the blood-brain barrier (BBB). Ro 64-0802 was found to be a substrate of organic anion transporter 3 (OAT3/*SLC22A8*) and multidrug resistance-associated protein 4 (MRP4/*ABCC4*). Human embryonic kidney 293 cells expressing OAT3 exhibited a greater intracellular accumulation of Ro 64-0802 than mock-transfected cells (15 versus 1.2 μl/mg protein/10 min, respectively). The efflux of Ro 64-0802 was 3-fold greater when MRP4 was expressed in MDCKII cells and was significantly inhibited by indomethacin. After its microinjection into the cerebrum, the amount of Ro 64-0802 in brain was significantly greater in both *Oat3*$^{-/-}$ mice and *Mrp4*$^{-/-}$ mice compared with the corresponding wild-type mice (0.36 versus 0.080 and 0.32 versus 0.060 nmol at 120 min after injection, respectively). The brain/plasma concentration ratio ($K_{p,brain}$) of Ro 64-0802, determined in wild-type mice after subcutaneous continuous infusion for 24 h, was close to the capillary volume (approximately 10 μl/g brain). Although the $K_{p,brain}$ of Ro 64-0802 was unchanged in *Oat3*$^{-/-}$ mice, it was significantly greater in *Mrp4*$^{-/-}$ mice (41 μl/g of brain). These results suggest that Ro 64-0802 can cross the BBB from the blood, but its brain distribution is limited by its active efflux by Mrp4 and Oat3 across the BBB. The transporter responsible for the brain uptake of Ro 64-0802 remains unknown, but Oat3 is a candidate transporter.

Oseltamivir is an ester-type prodrug of Ro 64-0802, a potent and selective inhibitor of viral neuraminidase, a key enzyme involved in the release of influenza virus from host cells. Oseltamivir is used for the treatment and prophylaxis of infectious diseases caused by both Influenzavirus A and Influenzavirus B (Bardsley-Elliot and Noble, 1999). In recent studies, abnormal behavior, such as jumping and falling from balconies, has been reported in teenagers or younger people who are taking oseltamivir (http://www.fda.gov/cder/drug/infopage/tamiflu/QA20051117.htm; Fuyuno, 2007). In response to these reports, the Ministry of Health, Labor and Welfare has issued a warning regarding the use of oseltamivir as a medication for teenagers or younger people and has prohibited the prescribing of oseltamivir for them in Japan.

The pharmacological actions of oseltamivir on the central nervous system have been reported in several animal studies (Izumi et al., 2007; Satoh et al., 2007; Usami et al., 2008; Yoshino et al., 2008), although the association between such pharmacological actions and abnormal behavior remains an open question. The systemic administration of oseltamivir increases dopamine levels in the rat medial prefrontal cortex (Yoshino et al., 2008), and oseltamivir and Ro

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ABBREVIATIONS: Ro 64-0802, [3*R*,4*R*,5*S*]-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate; BBB, blood-brain barrier; CES1A1, carboxylesterase 1A1; P-gp, P-glycoprotein; Oat/OAT, organic anion transporter; Mrp/MRP, multidrug resistance-associated protein; HEK, human embryonic kidney; MDCK, Madin-Darby canine kidney; LC, liquid chromatography; MS, mass spectrometry; GFP, green fluorescent protein; ANOVA, analysis of variance; PCR, polymerase chain reaction; Mdr, multidrug resistance; Bcrp, breast cancer resistance protein; Oatp, organic anion transporter peptide.

64-0802 enhance spike synchronization between hippocampal CA3 pyramidal cells and evoked synchronized population bursts, which recruit virtually all of the neurons in the network (Usami et al., 2008). It has also been demonstrated that oseltamivir and Ro 64-0802 affect neuronal excitability in rat hippocampal slices and that Ro 64-0802 is 30 times more potent than oseltamivir (Izumi et al., 2007).

Whether oseltamivir and Ro 64-0802 cross the blood-brain barrier (BBB) is an important issue, considering their pharmacological actions on the central nervous system. In clinical studies, both oseltamivir and Ro 64-0802 were detected in the plasma after oral administration of oseltamivir. Oseltamivir is converted to Ro 64-0802 by carboxylesterase 1A1 (CES1A1) in the liver (Shi et al., 2006). Most of the administered dose is recovered in the urine as Ro 64-0802 by glomerular filtration and tubular secretion by organic anion transporters in the kidney (He et al., 1999). The penetration of drugs to the brain from the circulating blood is limited by the BBB, which is formed by endothelial cells connected tightly to adjacent cells. It has been shown that oseltamivir can cross the BBB, but P-glycoprotein (P-gp) limits its brain penetration at the BBB (Morimoto et al., 2008; Ose et al., 2008). In contrast, Ro 64-0802 exhibits only a limited distribution in the brain because it is close to the brain capillary volume. Therefore, the permeability of Ro 64-0802 across the BBB has been considered to be quite low because of its hydrophilic nature and anionic charge at neutral pH.

In this study, we hypothesized that the low distribution of Ro 64-0802 in the brain is attributable to active efflux at the BBB. We focused on two organic anion transporters—organic anion transporter 3 (OAT3/*SLC22A8*) and multidrug resistance-associated protein 4 (MRP4/*ABCC4*)—as the candidate transporters involved. Oat3 is expressed on the abluminal membrane of the brain capillary endothelial cells in rodents (Kikuchi et al., 2003; Mori et al., 2003; Roberts et al., 2008). Cumulative in vivo studies suggest that Oat3 plays a significant role in the uptake of hydrophilic organic anions into the endothelial cells, the first step in its overall elimination from the brain to the blood (Ohtsuki et al., 2002; Kikuchi et al., 2003, 2004; Mori et al., 2003, 2004). Ro 64-0802 has been identified as a substrate of OAT1/*SLC22A6* (Hill et al., 2002). Considering the overlapping substrate specificities of OAT1 and OAT3, it is possible that OAT3 accepts Ro 64-0802 as substrate. MRP4 is an ATP-binding cassette transporter localized in the luminal membrane of the brain capillary endothelial cells (Leggas et al., 2004). MRP4 accepts anionic drugs as substrates (Ci et al., 2007; Hasegawa et al., 2007; Imaoka et al., 2007) and mediates their unidirectional efflux into the circulating blood (Leggas et al., 2004). It has been demonstrated that the elimination of topotecan from the brain was delayed and that the concentration of topotecan in the cerebrospinal fluid was greatly enhanced in *Mrp4*$^{-/-}$ mice compared with the corresponding wild-type mice (Leggas et al., 2004). It has also been demonstrated that the brain/plasma concentration ratio of 9′-(2′-phosphonylmethoxyethyl)-adenine 3 h after intravenous administration was greater in *Mrp4*$^{-/-}$ mice than in wild-type mice (Belinsky et al., 2007).

In this study, in vivo experiments were undertaken using wild-type, *Oat3*$^{-/-}$, and *Mrp4*$^{-/-}$ mice to examine the involvement of Oat3 and Mrp4 in the uptake and efflux of Ro 64-0802 across the BBB.

Materials and Methods

Reagents. Oseltamivir phosphate and its active metabolite, Ro 64-0802 (purity >95%), were synthesized according to a previous report (Yamatsugu et al., 2007). All other chemicals used in the experiments were of analytical grade.

Animals. *Mrp4*$^{-/-}$ mice had been established previously (Leggas et al., 2004). *Oat3*$^{-/-}$ mice were obtained from Deltagen, Inc. (San Carlos, CA). Male C57BL/6J, *Mrp4*$^{-/-}$, and *Oat3*$^{-/-}$ mice were maintained by CLEA Japan, Inc. (Tokyo, Japan). All mice (10–18 weeks old) were maintained under standard conditions with a reverse dark-light cycle. Food and water were available ad libitum. All experiments using animals in this study were performed according to the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, University of Tokyo).

Uptake of Ro 64-0802 by Human OAT3-Expressing HEK293 Cells. An in vitro transport experiment was performed as described previously (Deguchi et al., 2004). After the cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min, drug uptake was initiated by the addition of Krebs-Henseleit buffer containing Ro 64-0802 (10 μM). The Krebs-Henseleit buffer consisted of 118 mM NaCl, 23.8 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM $CaCl_2$, adjusted to pH 7.4. Uptake was terminated at the designated times by the addition of ice-cold Krebs-Henseleit buffer after the removal of the incubation buffer. The cells were then washed twice with 1 ml of ice-cold Krebs-Henseleit buffer, solubilized in 500 μl of 1 mM Tris-HCl buffer (pH 7.4), and stored overnight at 4°C. After sonication, aliquots (250 μl) were subjected to liquid chromatography (LC)-mass spectrometry (MS) analysis. The remaining 20 μl of cell lysate was used to determine the protein concentration by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Construction of Human MRP4/CES1A1-Expressing MDCKII Cells. CES1A1 cDNA was subcloned into the pTARGET vector (Promega, Madison, WI) (Mori et al., 1999) and transfected into MDCKII cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The transfectants were selected by culturing them in the presence of neomycin (1600 μg/ml) (Invitrogen) and were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic (Invitrogen), and neomycin (400 μg/ml) at 37°C with 5% CO_2 and 95% humidity. MDCKII cells with sufficient CES1A1 activity (CES1A1-MDCKII) were cloned and used as the hosts for infection with recombinant adenovirus carrying the human *MRP4* gene, which had been established previously (Ci et al., 2007; Hasegawa et al., 2007; Imaoka et al., 2007). CES1A1-MDCKII cells were infected with recombinant adenovirus containing human MRP4 transporter cDNA at a multiplicity of infection of 10 for 48 h to overexpress human MRP4 (MRP4/CES1A1-MDCKII). Green fluorescent protein (GFP) was used as the negative control (GFP/CES1A1-MDCKII).

The expression of MRP4 protein was confirmed by Western blotting. The cell lysates were loaded onto a SDS-polyacrylamide gel (7.5%) with a 3.75% stacking gel. *N*-Linked carbohydrate groups were cleaved from the MRP4 protein in the cell lysates with *N*-glycosidase F (PNGase F; New England Biolabs, Ipswich, MA). Digestion was performed according to the manufacturer's instructions, except that the samples were incubated for 30 min at 37°C in denaturing buffer rather than for the recommended 10 min at 100°C. To minimize protein degradation, protease inhibitors were included in all the steps. After incubation at 37°C for 30 min, the samples were separated by SDS-polyacrylamide gel electrophoresis (7.5%). The proteins were electroblotted on to a polyvinylidene difluoride membrane (Pall Corporation, East Hills, NY). The membrane was blocked with blocking buffer [Tris-buffered saline containing 0.05% Tween 20 (TTBS) and 3% skimmed milk] for 1 h at room temperature. After it had been washed with TTBS, the membrane was incubated overnight at 4°C with monoclonal anti-MRP4 M4I-10 antibody (1:1000 in blocking buffer; Abcam Inc., Cambridge, MA). The protein was detected by binding horseradish peroxidase-labeled anti-rat IgG antibody (1:5000 in blocking buffer; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Immunoreactivity was detected with an ECL Plus Western Blotting Detection Kit (GE Healthcare).

***Efflux of Ro 64-0802 Formed Intracellularly from Oseltamivir in* MRP4/CES1A1-MDCKII and GFP/CES1A1-MDCKII Cells.** After the cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min, oseltamivir (10 μM) was added to the incubation buffer in the presence or absence of indomethacin (50 μM). At the designated times, the incubation buffer was collected. Ice-cold Krebs-Henseleit buffer was then added, and the cells were washed four times. After the cells had been collected, they were frozen in liquid nitrogen and stored at −80°C until use. The efflux

clearance of Ro 64-0802 from the transfectants was determined using the integration plot method. The amount of Ro 64-0802 effluxed to the buffer at time t [X_{buffer} (t), nanomoles per milligram of protein] can be described by the following equation:

$$dX_{buffer}(t)/dt = CL_{efflux} \times C_{cell}(t)$$

where CL_{efflux} (microliters per minute per milligram of protein) represents the efflux clearance of Ro 64-0802 from the cells, and C_{cell} (t) (micromolar concentration) is the cellular concentration of Ro 64-0802. Cellular volume was assumed to be 4 μl/mg protein. The integration of this equation from time 0 to time t yields the following equation:

$$X_{buffer}(t) = CL_{efflux} \times AUC_{cell}(0-t)$$

where AUC_{cell} (0–t) (micromolar concentration × min) represents the area under the cellular concentration-time curve for Ro 64-0802 from time 0 to time t. Because the amount of Ro 64-0802 in buffer [A_{buffer} (t), nanomoles per milligram of protein] is given by the sum of X_{buffer} (t) and the amount of Ro 64-0802 existing in the buffer at time 0 (X_0), A_{buffer} (t) is described by the following equation:

$$A_{buffer}(t) = CL_{efflux} \times AUC_{cell}(0-t) + X_0$$

Thus, the CL_{efflux} value can be obtained by fitting A_{buffer} (t) versus AUC_{cell} (0–t) using a least-squares regression program (MULTI) (Yamaoka et al., 1981).

Efflux of Ro 64-0802 from the Cerebral Cortex of Wild-Type, *Oat3*$^{-/-}$, and *Mrp4*$^{-/-}$ Mice after Microinjection. The efflux of the test compounds from the brain after their microinjection into the cerebral cortex was investigated using the brain efflux index method, as described previously (Kakee et al., 1996). Ro 64-0802 (1 mM) in 0.5 μl of ECF buffer (122 mM NaCl, 25 mM $NaHCO_3$, 10 mM *d*-glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, and 10 mM HEPES, pH 7.4) was injected into the cerebral cortex (4.5 mm lateral to the bregma and 2.5 mm in depth). After the intracerebral microinjection, the mice were decapitated, and the amount of Ro 64-0802 that remained in the ipsilateral cerebrum was determined with LC-MS analysis.

Brain/Plasma Concentration Ratio of Ro 64-0802 after Subcutaneous Infusion of Oseltamivir or Ro 64-0802 in Wild-Type, *Oat3*$^{-/-}$, and *Mrp4*$^{-/-}$ Mice. Male C57BL/6J, *Mrp4*$^{-/-}$, and *Oat3*$^{-/-}$ mice (10–18 weeks old), weighing approximately 25 to 30 g, were used for these experiments. An osmotic pump (8 μl/h; Alzet, Cupertino, CA) was implanted under the skin in the backs of the mice under pentobarbital anesthesia (30 mg/kg). The mice received a continuous subcutaneous infusion of oseltamivir or Ro 64-0802 at doses of 400 or 80 nmol/h/mouse, respectively. Blood samples were collected from the postcaval vein at 24 h after treatment under pentobarbital anesthesia, and the brain was excised immediately. Plasma was obtained by centrifugation of the blood samples (10,000g). The esterase inhibitor, dichlorvos (200 μg/ml), was used to prevent ex vivo hydrolysis of the oseltamivir to Ro 64-0802 in the blood and plasma (Wiltshire et al., 2000; Lindegardh et al., 2006). The plasma and brain concentrations of Ro 64-0802 were determined with LC-MS analysis.

Quantification of Ro 64-0802 in Plasma and Brain Specimens. The brain was homogenized with a 4-fold volume of phosphate-buffered saline to obtain a 20% brain homogenate. Plasma specimens (10 μl) were mixed with 40 μl of ethanol, and the brain homogenates (100 μl) were mixed with 400 μl of ethanol. All of these mixed solutions were centrifuged at 15,000g for 10 min. The supernatants of the plasma specimens were mixed with an equal volume of water and subjected to LC-MS analysis. The supernatants of the brain specimens (350 μl) were evaporated, and the pellets were reconstituted with 50 μl of 20% ethanol. The reconstituted specimens were centrifuged at 15,000g for 10 min, and an aliquot of the supernatant was subjected to LC-MS analysis.

An LCMS2010EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used for the analysis. Samples were separated on a CAPCELL PAK C18 MGII column (3 μm, 2 mm × 50 mm; Shiseido, Tokyo, Japan) in binary gradient mode at a flow rate of 1 ml/min. Formic acid (0.05%) and acetonitrile were used for the mobile phase. The acetonitrile concentration was initially 10% and then increased linearly to 40% over 2 min. Finally, the column was reequilibrated at an acetonitrile concentration of 10% for 3 min. The total run time was 5 min. Ro 64-0802 was eluted at 2.5 min. In the mass analysis, Ro 64-0802 was detected at a mass-to-charge ratio of 285.15 under positive electron spray ionization conditions. The interface voltage was −3.5 kV, and the nebulizer gas (N_2) flow was 1.5 l/min. The heat block and curved desolvation line temperatures were 200 and 150°C, respectively.

Statistical Analysis. Data are presented as means ± S.E. of three to six animals, unless otherwise specified. Student's two-tailed unpaired t test and one-way ANOVA followed by Tukey's multiple comparison test were used to identify significant differences between groups when appropriate. Statistical significance was set at $P < 0.05$.

Results

Uptake of Ro 64-0802 into Human OAT3-Expressing HEK293 Cells. To show that Ro 64-0802 is an OAT3 substrate, an in vitro transport experiment was performed. The intracellular accumulation of Ro 64-0802 was significantly greater in HEK293 cells expressing human OAT3 than in mock-transfected cells (1.2 ± 0.2 and 15.1 ± 0.2 μl/10 min/mg protein for vector-transfected and OAT3-expressing HEK293 cells, respectively).

Construction of Human MRP4/CES1A1-Expressing MDCKII Cells. A clone of the MDCKII cells exogenously expressing CES1A1 was selected by measuring the hydrolytic activity against *p*-nitrophenyl acetate (data not shown). The subsequent study was performed using this clone as the host. After infection with the recombinant adenovirus, the protein expression of MRP4 in the MRP4/CES1A1-expressing MDCKII cells was confirmed by Western blot analysis (Fig. 1A). An anti-MRP4 monoclonal antibody recognized a 175-kDa protein, which is larger than the molecular mass for MRP4 (149 kDa) predicted from the sequence in the Swiss-Prot database. Deglycosylation of the cell lysates with *N*-glycosidase F resulted in a reduction in the molecular mass, suggesting that the molecular mass of MRP4 in the MRP4/CES1A1-expressing MDCKII cells was increased by glycosylation (Fig. 1B).

Efflux of Ro 64-0802 Formed Intracellularly from Oseltamivir in Human GFP/CES1A1 and MRP4/CES1A1-Expressing MDCKII Cells. An efflux transport experiment was conducted using MRP4/CES1A1- and GFP/CES1A1-MDCKII cells. When MRP4/CES1A1- and GFP/CES1A1-MDCKII cells were incubated with oseltamivir, Ro 64-0802 was detected in both the buffer and the cells in a time-dependent manner (Fig. 1, C and D). The concentration of Ro 64-0802 in the buffer was higher for the MRP4/CES1A1-MDCKII cells than for the GFP/CES1A1-MDCKII cells, and cellular Ro 64-0802 was lower in the MRP4/CES1-MDCKII cells (Fig. 1, C and D). Indomethacin, an inhibitor of MRP4 (Reid et al., 2003; Nozaki et al., 2007), reversed the effects of exogenous MRP4 expression. Integration plots of the efflux transport of Ro 64-0802 are shown in Fig. 2A. The efflux clearance of Ro 64-0802 from MRP4/CES1A1-MDCKII cells was 3.1-fold greater than that from GFP/CES1A1-MDCKII cells and was significantly inhibited by indomethacin (Fig. 2B).

Efflux of Ro 64-0802 from the Cerebral Cortex of Wild-Type and *Oat3*$^{-/-}$ Mice after Microinjection. Real-time PCR was performed to check the adaptive regulation of efflux transporters at the BBB of *Oat3*$^{-/-}$ mice. There were no significant differences in the mRNA expression of Mdr1a, Bcrp, Mrp4, organic anion transporter peptide 1a4 (Oatp1a4), or Oatp1c1 in the cerebral cortex, quantified by real-time PCR, between wild-type and *Oat3*$^{-/-}$ mice (data not shown).

To examine the involvement of Oat3 in the efflux transport of Ro 64-0802 across the BBB, Ro 64-0802 was directly injected into the mouse cerebral cortex, and the amount of Ro 64-0802 remaining in the brain was determined at 60 and 120 min after injection. The amount of Ro 64-0802 remaining in the brain was compared between wild-type and *Oat3*$^{-/-}$ mice (Fig. 3). As shown in Fig. 3, *Oat3*$^{-/-}$