capsule was suspended in saline. Five hundreds milligrams of oseltamivir phosphate are dissolved in 1 ml of water,[3] and the maximal concentration of OP used for oral administration (1000 mg/kg) was 100 mg/ml. Thus, insoluble substances were considered to be additives, and not OP, and the suspension was injected after shaking. A Relenza blister (5 mg) contains zanamivir hydrate and lactose, and these were completely dissolved in saline. A Plavix tablet, which contains 75 mg of clopidogrel and additives, was made into an emulsion by grinding in a mortar and pestle containing saline. Diclofenac sodium was dissolved in saline. Doses of drugs were expressed as a free base and administered intraperitoneally (i.p.), subcutaneously (s.c.) or orally (*p.o.*) at 0.1 ml volume/10 g body weight.

Statistical Analysis Mean core body temperature before drug administration was 38.2±0.03 °C (S.E.M., n=146), the range was 37—39.3 °C, and drug effects were expressed as the decrease in body temperature (Δ°C). All data were expressed as mean±S.E.M. (n=6 or 8). Multiple t-test with Bonferroni correction following ANOVA was used for multiple comparison between control and treated groups.[13] Student's t-test or Welch's procedure was also applied to the same group because multiple comparison can overslip side effects (adverse reaction) (known casual relationship) of drugs.[14] Differences at $p<0.05$ (two-tailed) were considered to be significant.

RESULTS

Effects of OP on Core Body Temperature OP (30, 100, 300 mg/kg, i.p.) dose-dependently lowered the body temperature (Fig. 1). The peak effects were observed 10, 20 and 30—40 min after administration of 30, 100 and 300 mg/kg of OP, respectively. Variations in the effects of intraperitoneal OP were smaller than those of oral OP (Fig. 2), and there were many significant time points between the saline and OP groups (multiple t-test with Bonferroni correction) (Fig. 1). $AUCs_{0-60\,min}$ of hypothermia (Δ°C×min) values were: −3.2±3.6 (n=6) (saline), −20.7±6.4 (30 mg/kg), −79.3±5.7 (100 mg/kg) and −164.1±15.9 (300 mg/kg). Significant differences in effects were observed between saline and the 100 mg ($p<0.05$) and 300 mg/kg ($p<0.01$) groups (multiple t-test with Bonferroni correction).

Oral administration of OP (100, 300, 1000 mg/kg) also lowered the core body temperature in a dose-dependent manner (Fig. 2). Saline lowered the body temperature, but significant hypothermia was observed at doses of 300 and 1000 mg/kg, *p.o.* (Fig. 2, multiple t-test with Bonferroni correction). When non-corrected Student's t-test was employed, the effect of 100 mg/kg OP was statistically significant at some time points (Fig. 2). The peak effects were observed at 30—60 min after administration, and recovery was not evident at 2 h after administration. $AUCs_{0-120\,min}$ of hypothermia (Δ°C×min) values were: −93.9±10.9 (n=8) (saline), −149.5±27.9 (100 mg/kg), −219.2±51.3 (300 mg/kg) and −300.6±39.0 (1000 mg/kg). Significant effects were observed between saline and the 300 ($p<0.05$) and 1000 mg/kg ($p<0.01$) groups (multiple t-test with Bonferroni correction). Non-corrected Student's t-test showed the same degrees of significance as those from the multiple t-test. When compared by peak effects, approximately triple doses of oral oseltamivir were needed to produce the same peak effects as intraperitoneal oseltamivir.

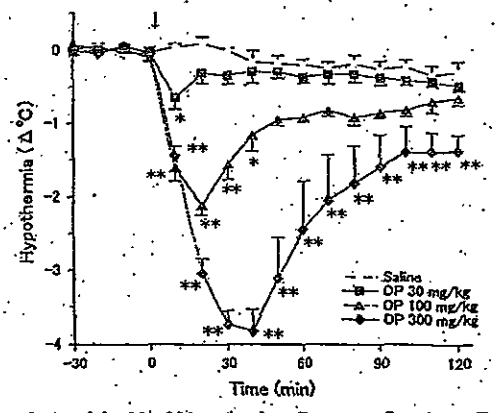

Fig. 1. Oseltamivir (30—300 mg/kg, i.p.) Decreases Core Body Temperature in a Dose-Dependent Manner in Mice

Each point represents the mean±S.E.M. of 6 mice. Ordinate: decrease in body temperature from the baseline (mean of −30—0 min). Abscissa: time in minutes after administration of the drug. Significance of differences between control and test values was determined by the two-tailed multiple t-test with Bonferroni correction following one-way analysis of variance (3 comparisons in 4 groups). $*p<0.05$ and $**p<0.01$. OP, oseltamivir.

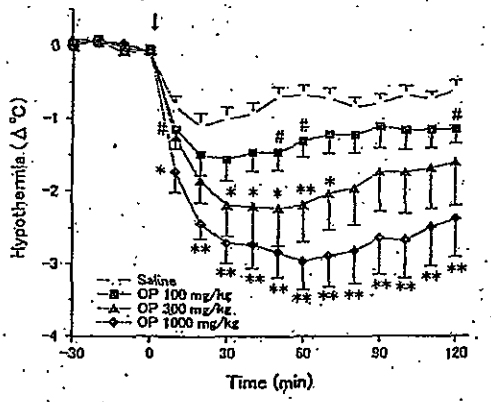

Fig. 2. Oseltamivir (100—1000 mg/kg, *p.o.*) Decreases Core Body Temperature in a Dose-Dependent Manner in Mice

Each point represents the mean±S.E.M. of 8 mice. Ordinate: decrease in body temperature from the baseline (mean of −30—0 min). Abscissa: time in minutes after administration of the drug. $*p<0.05$ and $**p<0.01$ (multiple t-test between control and test values). #$p<0.05$ (non-corrected Student's t-test was applied to those groups (see Materials and Methods). OP, oseltamivir.

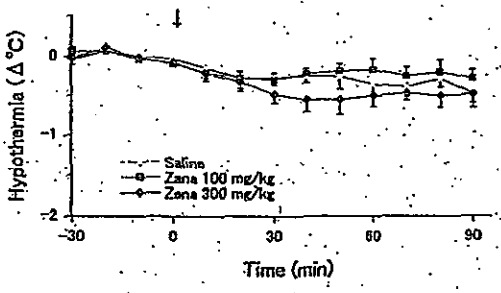

Fig. 3. Zanamivir (100, 300 mg/kg, i.p.) Does Not Alter Core Body Temperature in Mice

Each point represents the mean±S.E.M. of 6 mice. Ordinate: decrease in body temperature from the baseline (mean of −30—0 min). Abscissa: time in minutes after administration of the drug. No significant differences were seen by multiple t-test or Student's t-test. Zana, zanamivir.

Effects of Zanamivir on Core Body Temperature Zanamivir (100, 300 mg/kg, i.p.) slightly lowered the core body temperature (Fig. 3). No statistical significance was observed (multiple *t*-test with Bonferroni correction) and Student's *t*-test).

Effects of Clopidogrel on Hypothermic Effects of OP Since the hydrolysis of OP is strongly inhibited by clopidogrel,[9] clopidogrel was used to inhibit the hydrolysis of OP. Clopidogrel (300 mg/kg, s.c.), which alone slightly lowered body temperature, only tended to reduce the hypothermic effects of OP (Fig. 4).

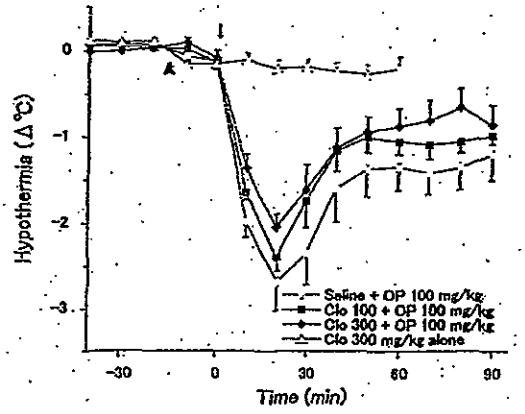

Fig. 4. Effects of Clopidogrel (100, 300 mg/kg, s.c.) on Hypothermia Induced by Oseltamivir (100 mg/kg, i.p.)

Each point represents the mean±S.E.M. of 6 mice. Ordinate: decrease in body temperature from the baseline (mean of −40–0 min). Abscissa: time in minutes after administration of the oseltamivir. Clopidogrel was administered at the point shown by the upward arrow (−15 min). No significant differences were seen by multiple *t*-test or Student's *t*-test. OP, oseltamivir, Clo, clopidogrel.

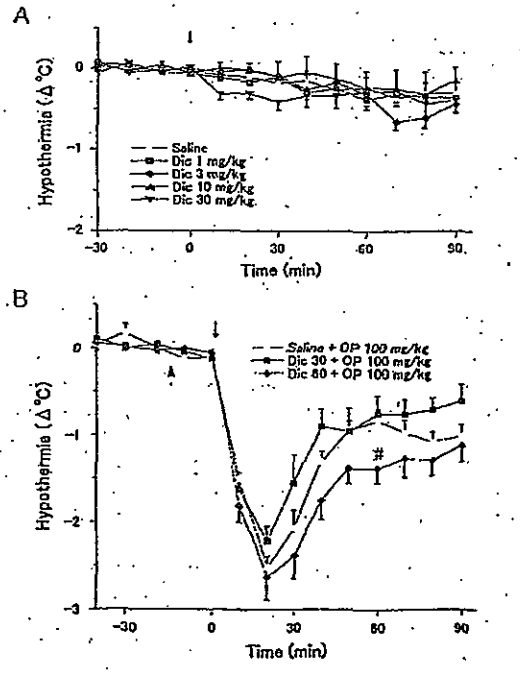

Fig. 5. Effects of Diclofenac on Core Body Temperature and the Interaction with Oseltamivir

Diclofenac (1—30 mg/kg, i.p.) does not alter core body temperature in mice (A). Diclofenac (30, 60 mg/kg, s.c.) does not affect the hypothermic effects of oseltamivir (100 mg/kg, i.p.) (B). Each point represents the mean±S.E.M. of 6 mice. Ordinates: decrease in body temperature from the baseline (mean of −30—0 min (A) and −40–0 min (B)). Abscissae: time in minutes after the administration of oseltamivir. Diclofenac was administered at the point shown by the upward arrow (−15 min). *$p<0.05$ (non-corrected Student's *t*-test). Dic, diclofenac.

Effects of Diclofenac on Core Body Temperature and Its Interaction with OP Diclofenac failed to produce hypothermia at doses of 1—30 mg/kg, i.p. (Fig. 5A). Since non-steroidal anti-inflammatory drugs (NSAIDs) are sometimes prescribed with OP for influenza infection in Japan, the drug interaction between OP and the strong NSAID diclofenac was studied. Diclofenac (30, 60 mg/kg, s.c.) administered 15 min before OP did not affect the latter's hypothermic effects (100 mg/kg, i.p.) (Fig. 5B).

DISCUSSION

Tamiflu interview form and New Drug Application (NDA) data summary describe that OP at 7.6, 76.1 and 761 mg/kg, *p.o.* does not affect body temperature in adult rats,[3,15] whereas at 533 mg/kg, *p.o.* it lowered the body temperature of rats aged 7 or 14 d.[16] A recent study by Izumi *et al.*[17] has provided supplementary data indicating that OP at 50 mg/kg, i.p. significantly augmented the hypothermic effects of ethanol in 30-d-old rats. Here, we have demonstrated that OP alone generates potent hypothermic effects in mice, consistent with our preliminary study obtained using adult rats (data not shown).

Brain/plasma C_{max} ratios for OP and OC in mice administered OP at 10 mg/kg, *p.o.* were 0.42 and 0.22, respectively.[18] Thus, it is considered that both OP and OC penetrated the blood–brain barrier in the present study using a high dose of OP. Body temperature is usually regulated by opposing controls of heat production and heat loss. The preoptic anterior hypothalamus (POAH) is a thermoregulation center.[19] The organum vasculosum laminae terminalis (OVLT), part of the circumventricular organs (CVO), is located near the POAH and is the target site of endogenous pyrogens.[20] In addition, the CVO lacks the blood–brain barrier[21,22] These circumstances suggest that OP or OC can affect body temperature regardless of whether or not the target site of either drug is located within the blood–brain barrier.

OP (30—300 mg/kg, i.p. and 100—1000 mg/kg, *p.o.*) dose-dependently lowered the normal core body temperature in mice (Figs. 1, 2). The Tamiflu NDA data summary describes very rapid hydrolysis of OP in mice,[23] and the present study indicated that the hypothermic effects of OP were relatively sustained (Figs. 1, 2). Therefore, it is suggested that the active compound that lowered body temperature was metabolized OC, and not the parent compound OP. However, a further study using OC powder will be required before a final conclusion can be reached. Tamiflu capsule contains insoluble additives. In our preliminary study, a filtrate of Tamiflu suspension lowered the body temperature (data not shown). As the soluble additive povidone is a very high-polymer compound (molecular weight several tens of thousands) and its molar dose included in a Tamiflu capsule is very low, it seems unlikely that additives other than OP or OC lowered the body temperature. Oral saline (10 ml/kg) administration unexpectedly produced hypothermia (Fig. 2). In the preliminary study (n=6 or 7), oral water administration to fasted

mice and oral saline administration to nonfasted mice lowered the body temperature by about 1 °C. In addition, insertion alone of an oral probe to the stomach *via* esophagus produced mild hypothermia (0.5 °C). Although pyrogenic messages *via* peripheral (largely vagal) afferent nerves activated by the cytokines induces hyperthermic response by direct afferent transmission to the POAH,[24] evidences of reflex hypothermia by mechanical vagal afferent stimuli could not be found in literatures. Nevertheless, these findings suggest that mechanical messages from esophagus and stomach *via* vagal afferents reflexly lower body temperature.

Zanamivir as well as OC inhibit the influenza virus-specific neuraminidase and also the release of virus from host cells.[25] As the hydrolyzed compound zanamivir cannot be absorbed by the gastrointestinal tract, a fine powder of zanamivir is inhaled (10 mg, twice a day) when used for treatment of humans. After inhalation of zanamivir powder, distribution of the drug is restricted to an upper respiratory tract, a target site of influenza virus. In order to elevate its plasma concentration, the same doses of zanamivir (100, 300 mg/kg) as OP were intraperitoneally administered. Zanamivir (100, 300 mg/kg, i.p.) did not produce hypothermia (Fig. 3). From these results, it is considered that hypothermia after OP administration is not due to neuraminidase inhibition at a thermoregulation center or a peripheral organ that is involved in thermoregulation.

CESs are classified into 5 subfamilies (CES1—CES5), and CES1 is subclassified into CES1A—CES1H.[26] The CESs that hydrolyze OP are suggested to be the CES1A and CES1B isozymes in human liver and mouse plasma, respectively.[4,26,27] Clopidogrel, a substrate of CES1, inhibits the hydrolysis of OP to OC *in vitro*: hydrolysis of OP (50 μM) is inhibited by 5 and 50 μM clopidogrel to 50% and 10%, respectively, in human CES1-expressing cells.[9] *In vivo*, a large proportion of clopidogrel is rapidly metabolized by CES to the non-active metabolite SR26334 in humans,[28,29] and T_{max} of the hydrolyzed metabolite SR26334 after oral administration of clopidogrel (75 mg) is 1.9 h in humans.[29,30] T_{max} of OP administered orally at a dose of 75 mg is 1 h, and T_{max} of its metabolite OC is 4 h,[3,31] suggesting that clopidogrel competitively inhibits the hydrolysis of OP to OC in humans. Clopidogrel 300 mg/kg, s.c., which alone slightly lowered body temperature and did not affect behavior of mice, tended to inhibit the hypothermic effects of OP, although not to a significant degree (Fig. 4). Conversion of OP to OC is very rapid in mouse plasma; the concentration of OC after oral OP administration in mice attains a near C_{max} value within 15 min after administration[25] and high amounts of CES are present in mouse plasma.[27] These findings support the negative non-significant interaction of clopidogrel with OP in the present *in vivo* mouse study. The tendency for clopidogrel to exert inhibitory effects on OP-induced hypothermia (Fig. 4) and the prolonged effect of OP on temperature (Figs. 1, 2) suggest that hypothermia is induced by OC, but hypothermic effects induced by OP also cannot be ruled out. In fact, it has been demonstrated that both OP and OC facilitate neuronal firing in hippocampal slices, OC being 30 times more potent than OP in this respect.[17]

Diclofenac, a strong NSAID, is used for treating high fever. Although diclofenac does not lower the normal body temperature in animals, it effectively reduces fever due to pyrogens; the ED_{50} is 0.13 mg/kg, *p.o.* in rats.[32] Voltaren package insert and interview form give a warning of severe hypothermia if used in children and the elderly with high fever.[10,11] In this study, very high doses of diclofenac (1—30 mg/kg, i.p.) did not decrease the body temperature (Fig. 5A) and at 30 and 60 mg/kg, s.c. it did not interact with the hypothermic effects of OP (100 mg/kg, i.p.) (Fig. 5B). Thus, no drug interaction between diclofenac and OP was evident, at least in terms of normal body temperature. However, as diclofenac can be used in patients with high fever, further studies using pyrexia mice are needed to investigate drug interaction between diclofenac and OP.

In the present study, intraperitoneal and oral administration of OP induced dose-dependent hypothermic effects in normal mice. However, since recent clinical studies have shown that the antipyretic effect of OP on type A influenza is stronger than that on type B influenza,[33,34] the antipyretic effect of OP is considered to be due to not only direct pharmacological effects on thermoregulation, but also anti-influenza virus activity.

Severe hypothermia as an adverse event has been reported to the MHLW from manufacturer of OP and also from medical institutions.[7] The proportion of hypothermia cases in Japanese patients below 10 years old relative to all reported cases is 40.1% (18/44 cases), and this ratio is higher than those for other adverse reactions (*i.e.* 16.7% for anaphylaxis (6/36 cases)), based on initial data made available to the public by the MHLW.[7] Since the body weight of children below 10 years old is low, it is considered that more severe hypothermia may occur in comparison with that in the elderly when heat production decreases or heat loss increases after OP ingestion. Thus, it is possible that the hypothermic effects observed in mice are related to the severe hypothermia in humans after OP ingestion. Further studies are needed to elucidate the mechanisms of hypothermia in mice and their relationship to the adverse events reported in humans.

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Short Communication

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Oseltamivir (Tamiflu) Efflux Transport at the Blood-Brain Barrier via P-Glycoprotein

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ABSTRACT:

Oseltamivir (Tamiflu, Roche, Nutley, NJ), an ester-type prodrug of the anti-influenza drug Ro 64–0802 (oseltamivir carboxylate), has been reported to be associated with neuropsychiatric side effects, which are likely to be caused by distribution of oseltamivir and/or its metabolite into the central nervous system. Enhanced toxicity and brain distribution of oseltamivir in unweaned rats led us to hypothesize that the low level of distribution of oseltamivir and/or Ro 64–0802 in adult brain was caused by the presence of a specific efflux transporter at the blood-brain barrier. We 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 protein 1 P-gp on the apical membrane, and in vivo using mdr1a/1b knockout mice. The permeability of oseltamivir in the basal-to-apical direction was significantly greater than that in the opposite direction. The directional transport disappeared on addition of cyclosporin A, a P-gp inhibitor. 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 show that oseltamivir, but not Ro 64–0802, is 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 may in turn account for the central nervous system effects of oseltamivir observed in some patients.

Oseltamivir phosphate (oseltamivir) (Fig. 1), manufactured under the trade name Tamiflu (Roche, Nutley, NJ) as an ester-type prodrug of the neuraminidase inhibitor Ro 64–0802 (oseltamivir carboxylate) (Fig. 1), has been developed for the treatment of A and B strains of the influenza virus, whereas the typical anti-influenza drug amantadine is used only for the A strain. However, the drug exhibits several adverse effects, not only in the digestive system (abdominalgia, diarrhea, and nausea) but also in the central nervous system (CNS); the latter include headache, vertigo, somnolence, insomnia, numbness, and behavioral excitement (basic product information of Tamiflu from Roche). Recently, there has been concern that the drug may be associated with suicidal or abnormal behavior especially in younger patients (http://www.fda.gov/cder/drug/infopage/tamiflu/QA20051117.htm and http://www.mhlw.go.jp/english/index.html). At present, the U.S. label of the drug specifies that the drug is not to be administrated to patients less than 1 year of age, whereas the label in Japan only mentions that the safety in the patients is not confirmed and includes the caution that administration to patients older than 10 years of age is possibly at risk to develop neurological side effect.

In general, CNS effects are caused by distribution of a drug and/or its metabolite(s) into the CNS through the blood-brain barrier (BBB). When oseltamivir was administered to rats at the high dose of 1000 mg/kg in safety examinations, the brain concentrations of the unchanged drug in 7-, 14-, and 24-day-old rats were 1540, 650, and 2 times greater than that in 48-day-old ones, whereas the brain concentration of the active metabolite Ro 64–0802 was lower than the plasma concentration in all the groups. In addition, brain unchanged drug concentration-dependent toxicity was observed (basic product information of Tamiflu from Roche). Those reports suggested that oseltamivir causes CNS side effects in younger animals in which the BBB is immature (Johanson, 1980), although it is not clear which compound is responsible to cause the CNS side effect. Drug concentration in the brain may be determined not only by passive diffusion but also by active transport and/or specific accumulation. Accordingly, BBB function is partially maintained by efflux transporters such as P-glycoprotein (P-gp), which is expressed at the luminal membrane in brain capillaries. Several P-gp substrates are known to exhibit low apparent permeability from the blood to the brain. Permeation of these P-gp substrates into the brain is increased in animals in which P-gp activity is reduced or abrogated, e.g., as a result of drug-drug interaction, mdr1a/1b deficiency (mdr1a/1b$^{-/-}$ mice), or immature BBB function (Tsuji, 1998; Demeule et al., 2002; Ebinger and Uhr, 2006). In the present study, we examined whether oseltamivir and its active metabolite Ro 64–0802 are substrates of P-gp, using P-gp-overexpressing cells and mdr1a/1b knockout mice, to clarify the possible involvement of P-gp in controlling their brain distribution.

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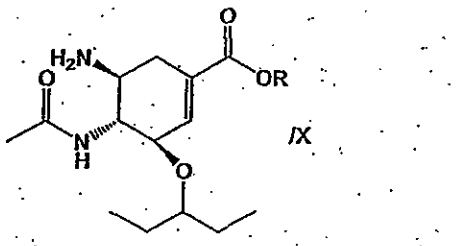
Materials and Methods

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, St. Louis, MO). All the other chemicals and solvents were commercial products of analytical, high-performance liquid chromatography (HPLC), or liquid chromatography/mass spectrometry grade. The LLC-PK1 (wild-type) and P-gp-overexpressing LLC-GA5-COL150 cells were obtained from Japan Health

ABBREVIATIONS: CNS, central nervous system; BBB, blood-brain barrier; P-gp, P-glycoprotein; HPLC, high-performance liquid chromatography.



	R	X
Oseltamivir phosphate	C_2H_5	H_3PO_4
Ro 64-0802	H	—

FIG. 1. Chemical structures of oseltamivir phosphate and its active metabolite Ro 64–0802.

Science Research Resources Bank (Osaka, Japan) and Riken Gene Bank (Tsukuba, Japan), respectively (Tanigawara et al., 1992; Ueda et al., 1992). 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. Male FVB wild-type mice and mdr1a/1b knockout mice were purchased from Taconic Farms (Germantown, NY) and used at 8 to 9 weeks of age.

Cell Culture and Transport Experiments. LLC-PK1 and LLC-GA5-COL150 cells were cultured, passaged, and grown as described previously (Ishiguro et al., 2004). Cells were cultured at 37°C in a 5% CO_2 atmosphere. For transport studies, cells were seeded onto Transwell filter membrane inserts (Costar, Bedford, MA) at a density of 2.5×10^5 cells/cm^2. Medium 199 supplemented with 10% fetal bovine serum, 14.3 mM $NaHCO_3$, and 2 mM L-glutamine (additionally, 150 ng/ml colchicine for LLC-GA5-COL150 cells) was used as culture medium. The culture medium was replaced with fresh medium after 2 days, and cell monolayers cultured for 5 days were used for transport studies. The cell monolayers were preincubated in transport medium (Hanks' balanced salt solution; 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM NaCl, 0.385 mM Na_2HPO_4, 25 mM D-glucose, and 10 mM HEPES, pH 7.4) for 10 min at 37°C. After preincubation, transport was initiated by adding the test drug to the donor side and transport medium to the receiver side. Drug transport was observed in two directions [apical (A) to basal (B) and B to A] over 150 min at 37°C. The permeability (P_{app}, cm/s) of the compounds across cell monolayers was evaluated by dividing the slope of the time course of the transport from A to B or from B to A by concentration at the donor side as $P_{app(AB)}$ or $P_{app(BA)}$, respectively. The permeability ratio was obtained by dividing $P_{app(BA)}$ by $P_{app(AB)}$. Kinetic parameters, V_{max}, and apparent K_m for P-gp-mediated drug transport were calculated by nonlinear least-squares analysis (MULTI program) using the following equation, assuming that A to B flux (V_{AB}) can be expressed as the difference between passive (V_{PD}) and P-gp-mediated flux ($V_{P\text{-}gp}$) (Yamaoka et al., 1981; Shirasaka et al., 2007).

$$V_{AB} = V_{PD} - V_{P\text{-}gp} = P_{app,PD} \cdot S \cdot C_u - \frac{V_{max} \cdot C_u}{K_m + C_u} \quad (1)$$

where $P_{app,PD}$ is the membrane permeability by passive diffusion [$P_{app,PD}$ of the test compounds in monolayers can be evaluated by using potent P-gp inhibitor, cyclosporin A (10 μM)], and C_u is the drug concentration in the apical solution. K_m value represents the apical concentration of the drug at which the decreased permeability by P-gp-mediated efflux became half of its maximal value.

Brain Distribution of Oseltamivir. Oseltamivir was dissolved in water and administered to FVB mice and mdr1a/1b knockout mice at single oral doses of 30, 100, and 300 mg/10 ml/kg (each $n = 3$). At 1 h after dosing (corresponding to T_{max}) (Li et al., 1998), blood was withdrawn from the heart with heparinized syringes. Subsequently, residual systemic blood was washed out by 3 ml of saline that was injected from the heart and discharged by cutting abdominal vein, and then the brain was removed. Blood was centrifuged (1700g) for 15 min at 4°C to obtain plasma. Quantitation of oseltamivir and Ro 64–0802 in plasma and brain tissues was performed using reported methods (Wiltshire et al., 2000) with some modifications. Briefly, 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 identical.

Analytical Methods. Aliquots (20 μl) of oseltamivir and Ro 64–0802 samples were injected into an HPLC system (LC-20A system, Shimadzu, Kyoto, Japan) equipped with Inertsil CN-3 column (4.6 × 100 mm, 5 mm, GL Sciences Inc., Tokyo, Japan) using isocratic elution at 0.5 ml/min with 80 mM 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 monitored were m/z = 313 for oseltamivir and m/z = 285 for Ro 64–0802. Some oseltamivir samples were analyzed with an HPLC system (Alliance System, Waters, Milford, MA) consisting of the 2690 separation module with an analytical column, 250 × 4.6-mm i.d. Mightysil RP-18 Aqua column (Kanto Chemical, Tokyo, Japan), and mobile phases consisting of a mixture of 10 mM phosphate buffer, pH 6.0, and methanol in ratios of 40 and 60%, at a flow rate of 1 ml/min and at 40°C. Detection was done at the wavelength of 230 nm with a 2487 dual-wavelength absorption detector (Waters). Samples for calibration were prepared in a similar manner to that described above for the preparation of analytical samples. Statistical analysis of kinetic parameters 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 and Discussion

In the present study, we examined the transport of oseltamivir and its active metabolite Ro 64–0802 via P-gp using P-gp-overexpressing cells and mdr1a/1b gene-knockout mice to evaluate the factors that affect the BBB distribution of these compounds. The permeability of oseltamivir in the basal-to-apical direction in LLC-GA5-COL150 cells was significantly higher than that in the opposite direction, whereas the permeability in wild-type cells was comparable in the two directions, with the permeability ratios of approximately 7.8 and 1.2 in LLC-GA5-COL150 and wild-type cells, respectively (Table 1). In the presence of cyclosporin A, the permeability ratio in LLC-GA5-COL150 cells became approximately unity. These in vitro results indicate that oseltamivir is a substrate of P-gp, and its overall permeability is significantly affected by the efflux transporter. When oseltamivir was administered at various doses to mdr1a/1b knockout mice, an increase in the accumulation of oseltamivir in the brain was observed compared with that in wild-type mice (Table 2). Therefore, it is likely that brain distribution of oseltamivir is controlled by P-gp. Basal-to-apical transport of oseltamivir in P-gp-overexpressing cells was saturable with the K_m and V_{max} values of 1.3 mM and 0.203 nmol/min/cm^2, respectively. Because the K_m value is much higher than the free plasma concentration of oseltamivir in the clinical situation (about 50 nM; basic product information of Tamiflu from Roche), it is reasonable to consider that P-gp is not saturated by usual clinical doses of oseltamivir, and the brain accumulation of oseltamivir should be affected by P-gp in humans. These results suggest that variation in P-gp activity in the brain resulting from genetic differences or coadministered drugs may affect the brain distribution of oseltamivir, leading to CNS side effects.

As shown in Table 2, a dose-dependent increase in the $K_{p,app}$ value of oseltamivir was observed in both mdr1a/1b knockout and wild-type mice, in the range from 30 mg/kg to the highest dose of 300 mg/kg. The $K_{p,app}$ ratio ($K_{p,app}$ value in knockout mice/$K_{p,app}$ value in wild-type mice) of oseltamivir was also increased in a dose-dependent manner. The dose-dependent increase of $K_{p,app}$ value of oseltamivir in wild-type mice may be explained by the saturation of P-gp. However,

TABLE 1

Apparent permeability and permeability ratio of oseltamivir and Ro 64–0802 in LLCPK1-GA5-COL150 and LLC-PK1 cells (wild-type)

Condition	Cell Line	P_{app} cm/s ($\times 10^{-6}$)		Permeability Ratio
		Apical to Basal	Basal to Apical	
		mean ± S.E.	*mean ± S.E.*	
Oseltamivir	LLCPK1-GA5-COL150	2.47 ± 2.47	19.2 ± 1.40	7.77
	Wild-type	6.10 ± 0.18	7.24 ± 0.40	1.19
With 10 μM CysA	LLCPK1-GA5-COL150	6.06 ± 0.10	7.06 ± 0.60	1.17
Ro 64–0802	LLCPK1-GA5-COL150	1.55 ± 0.14	1.72 ± 0.14	1.11
With 10 μM CysA	LLCPK1-GA5-COL150	1.73 ± 0.06	1.49 ± 0.07	0.86

Permeability ratio = (P_{app} B to A)/(P_{app} A to B); CysA, cyclosporin A.
The initial concentrations of test compounds were *100 μM*.
Data are expressed as mean or mean ± S.E. of three experiments.

TABLE 2

$K_{p,app}$ (plasma-brain concentration ratio) of oseltamivir and Ro 64–0802 between mdr1a/1b knockout and wild-type mice

Dose	N	Oseltamivir			Ro 64–0802		
		Mdr1a/1b KO	Wild-type	Ratio	Mdr1a/1b KO	Wild-type	Ratio
mg/kg		*mean ± S.E.*	*mean ± S.E.*	*KO/wild*	*mean ± S.E.*	*mean ± S.E.*	*KO/wild*
30	3	0.647 ± 0.059**	0.137 ± 0.016	4.7	0.003 ± 0.005	0.005 ± 0.005	0.5
100	3	0.847 ± 0.176*	0.171 ± 0.056	4.9	0.007 ± 0.001	0.016 ± 0.013	0.4
300	3	6.505 ± 2.843	0.689 ± 0.250	9.6	0.017 ± 0.002	0.022 ± 0.003	0.5

KO, knockout.
$K_{p,app,brain}$ were measured at 1 h (T_{max}) after p.o. administration to mdr1a/1b KO and wild-type mice.
Each value represents the mean ± S.E. of three animals.
* $P < 0.05$; ** $P < 0.01$, *by Student's t test.*

$K_{p,app}$ value of oseltamivir in mdr1a/1b knockout mice also increased dose-dependently. Because this observation cannot be explained by P-gp, other transporters may be involved in the transport of oseltamivir across the BBB (Hill et al., 2002). Another possibility is that free fraction of oseltamivir, which would affect the brain penetration, was increased because of the saturation of its plasma protein binding. However, because plasma protein binding of oseltamivir administered orally at 30 and 300 mg/kg (corresponding to plasma concentration of 0.5 and 5 mg/ml, respectively) was found to be 36.3 and 32.0%, respectively (data not shown), it is reasonable to consider that plasma protein binding of oseltamivir is not saturated in this dosing range. On the other hand, plasma concentrations of oseltamivir were comparable in mdr1a/1b knockout mice and wild-type mice after oral administration of oseltamivir; those at a dose of 30, 100, and 300 mg/kg were 1.05 ± 0.21, 3.80 ± 1.89, and 2.82 ± 0.67 μg/ml for wild-type mice and 0.95 ± 0.09, 4.02 ± 0.47, and 2.27 ± 0.61 μg/ml for mdr1a/1b knockout mice, respectively. This may be because the elimination of oseltamivir occurs mainly via hydrolysis by esterase in blood and liver, and intestinal P-gp may have relatively little effect than BBB (Li et al., 1998; Ogihara et al., 2006).

In the case of the active metabolite Ro 64–0802, the permeability in LLC-GA5-COL150 cells was comparable in both directions. The permeability ratio of Ro 64–0802 was approximately unity in both the presence and absence of cyclosporin A, suggesting that the active metabolite is not a substrate of P-gp (Table 1). These results are consistent with the finding that brain accumulation of Ro 64–0802 in mdr1a/1b knockout mice was very limited and comparable with that in wild-type mice. These findings suggested that brain distribution of this active metabolite is unlikely to be affected by P-gp in humans.

In conclusion, our in vivo and in vitro results indicate that oseltamivir, but not its active metabolite Ro 64–0802, is a substrate of P-gp and that the brain distribution of oseltamivir is significantly affected by P-gp. Accordingly, interindividual variation of P-gp activity may be an important factor determining susceptibility to the CNS side effects of this drug, in addition to the genetic polymorphisms of carboxylesterase 1 (Shi et al., 2006) and sialidase (Li et al., 2007). Various factors, such as genetic polymorphisms of P-gp, drug-drug interactions at P-gp, and altered expression by inflammatory cytokines, could influence apparent P-gp activity and therefore might play a role in increasing the accumulation of oseltamivir in the brain, thereby contributing to the occurrence of CNS side effects of oseltamivir in humans.

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