

Oseltamivir (Tamiflu™) efflux transport at the blood-brain barrier via P-glycoprotein

Kaori Morimoto, Masanori Nakakariya, Yoshiyuki Shirasaka, Chihaya Kakinuma, Takuya Fujita, Ikumi

Tamai, Takuo Ogihara

Faculty of Pharmacy, Takasaki University of Health and Welfare, Takasaki, Gunma 370-0033, Japan

(K.M., C.K., T.O.), Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda, Chiba

278-8510, Japan (M.N., Y.S., I.T., T.O.) and College of Information Science and Engineering,

Ritsumeikan University, Kusatsu, Shiga 525-8577, Japan (T.F.)

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Corresponding author:

Takuo Ogihara, Ph.D., Prof.

Laboratory of Biopharmaceutics, Department of Pharmacology, Faculty of Pharmacy, Takasaki University of

Health and Welfare, Takasaki, Gunma 370-0033, Japan,

Phone; +81-27-352-1180, Fax: ;+81-27-352-1118; E-mail: togihara@takasaki-u.ac.jp

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BBB, blood-brain barrier; P-gp, P-glycoprotein; CNS, central nervous system; FDA, Food and Drug

Administration; T_{max}, time of the maximum concentration

ABSTRACT

Oseltamivir (Tamiflu™), an ester-type pro-drug of the anti-influenza drug Ro 64-0802, 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 due to 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 over-express human MDR1 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 upon 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 demonstrate 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.

Introduction

Oseltamivir phosphate (oseltamivir, Fig.1), manufactured under the trade name Tamiflu™ as an ester-type pro-drug of the neuraminidase inhibitor Ro 64-0802 (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 US label of the drug specifies that the drug is not to be administered 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 year 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

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 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., due to 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 or not oseltamivir and its active metabolite Ro 64-0802 are substrates of P-gp, using P-gp over-expressing cells and *mdr1a/1b*-knockout mice, in order to clarify the possible involvement of P-gp in controlling their brain distribution.

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 other chemicals and solvents were commercial products of analytical, HPLC or LC/MS grade. The LLC-PK1 (wild type) and P-gp-overexpressing

LLC-GA5-COL150 cells were obtained from Japan Health 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% CO₂ atmosphere. For transport studies, cells were seeded onto Transwell filter membrane inserts (Costar, Bedford, MA) at a density of 2.5×10^5 cells/cm². Medium 199 supplemented with 10% fetal bovine serum, 14.3 mM NaHCO₃, 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 pre-incubated in transport medium (TM, Hanks' Balanced Salt Solution; 0.952 mM CaCl₂, 5.36 mM KCl, 0.441 mM KH₂PO₄, 0.812 mM MgSO₄, 136.7 mM NaCl, 0.385 mM Na₂HPO₄, 25 mM D-glucose and 10 mM HEPES, pH 7.4) for 10 min at 37°C. After pre-incubation, transport was initiated by adding the test drug to the donor side and TM 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 parameter, V_{max} and apparent K_m , for P-gp-mediated drug transport was 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-gp}) (Yamaoka K et al., 1981, Shirasaka Y et al., 2007).

$$\begin{aligned} V_{AB} &= V_{PD} - V_{P-gp} \\ &= P_{app,PD} \cdot S \cdot C_a - \frac{V_{max} \cdot C_a}{K_m + C_a} \end{aligned} \quad (1)$$

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, cyclosporine A (10 μ M)), and C_a is the drug concentration in the apical solution. K_m value represents the AP 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 hr after dosing (corresponding to T_{max}) (Li W 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 (1700 x g) 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 1,700 $\times g$, and 0.9 mL of the supernatant was subjected to solid phase extraction (Empore Mixed Phase Cation-MPC, 7 mm/3 mL). 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 x 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, Kyoto, Japan) 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 x 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, Milford, MA). 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 over-expressing 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, while 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 over-expressing cells was saturable with the K_m and V_{max} values of 1.3 mM and 0.203 nmol/min/cm², 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 TamifluTM 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 due to genetic differences or co-administered 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, $K_{p,app}$ value of oseltamivir in *mdr1a/1b*-knockout mice also increased dose-dependently. Since this observation cannot be explained by P-gp, other transporters might 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 due to the saturation of its plasma protein-binding. However, because plasma protein-binding of oseltamivir administrated orally at 30 and 300 mg/kg (corresponding to plasma concentration of 0.5 mg/mL and 5 mg/mL, respectively) 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 these 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 $\mu\text{g/mL}$ for wild-type mice and 0.95 ± 0.09 , 4.02 ± 0.47 and 2.27 ± 0.61 $\mu\text{g/mL}$ 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, inter-individual variation of P-gp activity might be an important factor determining susceptibility to the CNS side effects of this drug, in addition to the genetic polymorphisms of carboxylesterase1 (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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Figure Legends and Tables

Figure 1. Chemical structures of oseltamivir phosphate and its active metabolite Ro 64-0802

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	\pm S.E.	mean	\pm S.E.	
Oseltamivir	LLCPK1-GA5-COL150	2.47	\pm 2.47	19.2	\pm 1.40	7.77
	Wild-type	6.10	\pm 0.18	7.24	\pm 0.40	1.19
with 10 μ M CysA	LLCPK1-GA5-COL150	6.06	\pm 0.10	7.06	\pm 0.60	1.17
Ro 64-0802	LLCPK1-GA5-COL150	1.55	\pm 0.14	1.72	\pm 0.14	1.11
with 10 μ M CysA	LLCPK1-GA5-COL150	1.73	\pm 0.06	1.49	\pm 0.07	0.86

P_{app} , Apparent permeability; (Permeability ratio) = (P_{app} , B to A) / (P_{app} , A to B); CysA, cyclosporin A

The initial concentrations of test compounds were 100 μ M.

Data are expressed as mean or mean \pm 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

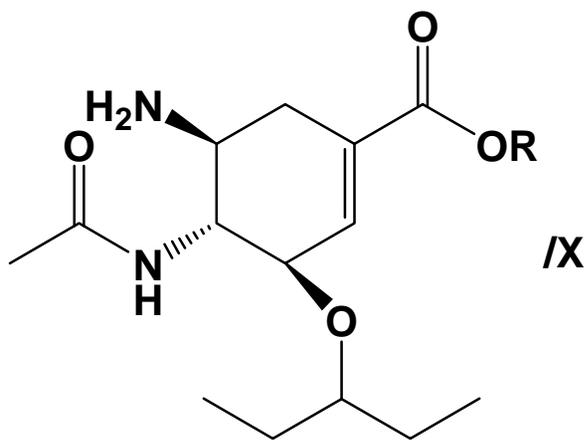
Dose mg/kg	N	Oseltamivir			Ro 64-0802						
		Mdr1a/1b KO		Wild-type	Ratio	Mdr1a/1b KO		Wild-type	Ratio		
		mean	\pm S.E.	mean	\pm S.E.	KO/wild	mean	\pm S.E.	mean	\pm S.E.	KO/wild
30	3	0.647	\pm 0.059	^b 0.137	\pm 0.016	4.7	0.003	\pm 0.005	0.005	\pm 0.005	0.5
100	3	0.847	\pm 0.176	^a 0.171	\pm 0.056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

KO, knockout.

$K_{p,app,brain}$ were measured at 1 h (T_{max}) after oral administration to mdr1a/1b knockout and wild-type mice.

Each value represents the mean \pm S.E. of three animals.

a; $p < 0.05$, b; $p < 0.01$, by Student's t-test.



	R	X
Oseltamivir phosphate	C₂H₅	H₃PO₄
Ro 64-0802	H	—

Figure 1