Original Article

Developmental changes of brain distribution and localization of oseltamivir and its active metabolite Ro 64-0802 in rats

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ABSTRACT — Oseltamivir, a prodrug of the neuraminidase inhibitor [3R, 4R, 5S]-4-Acetamide-5-amino-3-(1-ethylpropyl)-1-cyclohexene-1-carboxylate phosphate (Ro 64-0802), is widely used for treatment of influenza infections in Japan, but may be associated with mental instability and suicidal tendencies as a rare side effect, especially in infants and young patients. We examined developmental changes in the brain distribution of oseltamivir and Ro 64-0802, and in the expression of P-glycoprotein (P-gp) at the blood-brain barrier (BBB) in rats by 8 weeks. Brain concentration and $K_{p,app,brain}$ (brain-to-plasma concentration ratio) of oseltamivir were highest in 2-week-old rats (1.45 $\mu\text{g/g}$ brain and 0.14, respectively), and were negatively correlated with both age and P-gp expression at the BBB. In contrast, brain concentration and $K_{p,app,brain}$ of Ro 64-0802 after oral gavage of oseltamivir were lowest in 2-week-old rats (0.02 $\mu\text{g/g}$ brain and 0.02), and increased with age. Mass imaging analysis revealed that both compounds were distributed homogenously in brain cross-sections, including the hippocampus. From these results, it was estimated that oseltamivir concentration throughout the brain cross-sections was 70-fold and 0.9-fold higher than that of Ro 64-0802 in 2-week-old and 8-week-old rats, respectively. Such developmental changes of prodrug/drug concentration ratio, if they also occur in humans, may provide a rational basis for the putative central nervous system (CNS) side effects in young patients.

Key words: Brain, Distribution, Age, Oseltamivir, Rat

INTRODUCTION

Oseltamivir is an ester-type prodrug of the neuraminidase inhibitor [3R,4R,5S]-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid (Ro 64-0802), which targets the A and B strains of influenza virus. This drug has been widely used in Japan and is stored on a large scale for use if an influenza pandemic occurs. However, there have been reports, though cases are rare, that infants and young patients taking oseltamivir have developed neuropsychiatric side effects, including mental instability and suicidal tendencies. Accordingly, The Ministry of Health, Labor and Welfare in Japan has restricted the use of this drug in teenagers and younger patients. The mechanisms of this putative side effect are not presently clear, though several studies have demonstrated that oseltamivir and Ro 64-0802 have an excitatory effect on hippocampal neurons (Izumi *et al.*, 2007; Usami *et al.*, 2008), a dopamine-releasing effect in the

prefrontal cortex (Yoshino *et al.*, 2008), and a hypothermia-promoting effect (Ono *et al.*, 2008) in experimental animals.

We have been studying the brain distribution of oseltamivir and Ro 64-0802 in animals (Morimoto *et al.*, 2008). The distribution volume of Ro 64-0802 in the brain was equivalent to the vascular space. This suggested that the low lipophilicity of Ro 64-0802 (calculated clogP value of -0.97) restricts its penetration through the BBB by passive diffusion (Morimoto *et al.*, 2008). On the other hand, the ester-type prodrug oseltamivir showed a more than ten-fold higher brain distribution compared with Ro 64-0802, reflecting its higher clogP value of 1.29. We have also demonstrated that the brain distribution of oseltamivir is limited by P-glycoprotein (P-gp)-mediated efflux transport at the blood-brain barrier: the brain concentration of oseltamivir in *mdr1a/1b* knockout mice was five-fold higher than that of wild-type mice (Morimoto *et al.*, 2008). These results indicate that low levels of

P-gp activity or drug-drug interaction at P-gp may lead to enhanced brain accumulation of oseltamivir, and consequently oseltamivir rather than Ro 64-0802 may cause the putative neuropsychiatric effects. However, because Ro 64-0802 has a ten-fold stronger *in vitro* neuroexcitatory potential than oseltamivir (Usami *et al.*, 2008), it is important to investigate the localization of oseltamivir and Ro 64-0802 in the brain, which contains many different functional regions. In addition, because young patients appear to be more at risk than adults for neuropsychiatric symptoms, and the expression of P-gp at the BBB is proportional to age, it is also possible that the brain distribution of oseltamivir is affected by age.

Therefore, the purpose of this study was to elucidate the age-dependent changes in the brain distribution of oseltamivir and Ro64-0802 and their relation to P-gp expression at the BBB in rats. We also examined the brain localization of oseltamivir and Ro 64-0802 using mass imaging analysis in rats.

MATERIALS AND METHODS

Animals and materials

Male Wistar rats (Slc:Wistar, Japan SLC, Inc., Shizuoka, Japan) were used. The animals were acclimated to and maintained at $23 \pm 2^\circ\text{C}$ on a twelve-hour/twelve-hour light/dark cycle. All animals were housed in standard laboratory cages and had free access to food and water throughout the study period. 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.

Mouse anti-Pgp antibody (clone C219, Signet Laboratories, Dedham, MA, USA), goat anti-mouse IgG peroxidase conjugate (Sigma, Saint Louis, MO, USA) and an enhanced chemiluminescence detection (ECL) kit (Amersham, Buckinghamshire, UK) were used. Oseltamivir was purchased from Sequoia Research Products (Pangbourne, UK). Ro 64-0802 was synthesized from oseltamivir using porcine liver esterase (Sigma). All other chemicals and solvents were commercial products of analytical, high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS) grade.

Western blotting

Brain membrane fractions from 2-, 3-, 4- and 8-week-old rats were prepared according to the reported procedure (Mateny *et al.*, 2004). Briefly, rats were anesthetized under ether inhalation, and the brain was excised. The

brain tissues were homogenized on ice in 15 volumes of Tris-HCl buffer (0.01 mol/l, pH 7.4) containing protease inhibitors leupeptin (10 $\mu\text{g/ml}$), pepstatin A (1 $\mu\text{g/ml}$), and phenylmethylsulfonyl fluoride (50 $\mu\text{g/ml}$) using a glass-Teflon homogenizer (30,000 rpm, 45 sec). The homogenates were centrifuged at 4,000 $\times g$ for 10 min at 4°C and the pellets were discarded. The supernatants were centrifuged at 100,000 $\times g$ for 30 min at 4°C . The resulting pellets were suspended in 7.5 volumes of Tris-HCl buffer with protease inhibitors by brief homogenization (30,000 rpm, 20 sec) and stored at -20°C until analysis. Protein concentrations were determined by duplicate in the Lowry method with bovine serum albumin as the standard.

Samples were normalized to equal protein concentrations, loaded at 30 $\mu\text{g/lane}$ onto 3.5% polyacrylamide gel, electrophoresed at 40 mA for 1 hr and transferred onto polyvinylidene difluoride membranes at 120 mA for 12 hr at 4°C . Blots were blocked for nonspecific binding by overnight incubation at 4°C in Tris-buffered saline containing 0.1% Tween 20 and 5% dry milk. P-gp was detected with the anti-P-gp antibody C219 followed by anti-mouse IgG using the ECL system (GE Healthcare, Buckinghamshire, UK).

Developmental changes of brain distribution of oseltamivir and Ro 64-0802

Oseltamivir was dissolved in water and administered to fasted 2-, 3-, 4- and 8-week-old rats at a dose of 300 mg/10 ml/kg. One hour after dosing (this time corresponds to T_{max} ; Li *et al.*, 1998), blood was withdrawn from the heart with a heparinized syringe. Residual blood was washed out of the brain, i.e., the appropriate volume of saline (5% of body weight) was injected from the heart and discharged by cutting abdominal vessels, and then the brain was excised. Plasma samples were obtained by immediate centrifugation of blood. Samples were stored at -20°C until assay.

Analytical method

The concentrations of oseltamivir and Ro 64-0802 in plasma and brain were measured using a high-performance liquid chromatography-tandem mass spectrometry system as previously described (Morimoto *et al.*, 2011). Briefly, an API3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) with electrospray ionization in the positive mode was used. The multiple reaction monitor was set at 313-225 m/z for oseltamivir and 285-197 m/z for Ro64-0802.

The samples and standards for oseltamivir and Ro 64-0802 were prepared by solid-phase extraction. Plasma

(10 μ l) and 10% brain homogenate (100 μ l) were diluted 100- and 10-fold with 5 mM ammonium acetate buffer (pH 3.5), respectively, and 1 ml aliquots were subjected to solid-phase extraction using InertSepTM MPC (Mixed-phase cation, GL Science Inc., Tokyo, Japan). After application of a sample, the column was rinsed with 4 ml of methanol, followed with 4 ml of 90% methanol. Then, the analytes were eluted with 4 ml of methanol containing 10 vol% of 50 mM ammonium acetate pH 9.0. The eluate was evaporated to dryness at below 50°C with nitrogen gas-blowing. The residue was dissolved in 100 μ l of 20% acetonitrile containing 0.05% formic acid for analysis. Aliquots (10 μ l) of samples were injected into a high-performance liquid chromatography system (HP1100 system, Hewlett Packard, Waldbronn, Germany) equipped with a Capcell pak C18 MGII column (50 mm x 2.0 mm i.d., 3 μ m, Shiseido Co., Tokyo, Japan) and a guard column (C18 MGII S-3, Shiseido) using isocratic elution at 0.2 ml/min with 20% acetonitrile containing 0.05% formic acid. The concentration ranges for quantitative measurement of oseltamivir and Ro 64-0802 were from 0.01 to 5 μ g/ml or g sample.

MALDI-TOF/MS imaging

Serial 10 μ m coronal sections including the hippocampus were cut with a cryomicrotome and thaw-mounted on indium tin oxide-coated glass slides (Sigma) at -20°C. Subsequent operations were entrusted to Shimadzu Corporation (Kyoto, Japan). Homogeneous cocrystallization of the analytes with the matrix is crucial for high sensitivity of MS analysis. Prior to matrix deposition, tissue slices were kept for 20 min with desiccant to equilibrate at room temperature and then for another 20 min in a vacuum chamber. Glass slides were mounted on a mass target plate (Shimadzu) with conductive adhesive double-sided tape (3M, St Paul, MN). Freshly prepared 2,5-dihydroxybenzoic acid (25 mg/ml solution in 50% acetonitrile containing 0.1% trifluoroacetic acid) was used as the matrix solution and an aliquot of 300 μ l was deposited per spot at a pitch of 0.25 mm on the specimens by using an automatic chemical inkjet printer (CHIP-1000, Shimadzu). This process was repeated 30 times. After matrix deposition, the slides were kept in a vacuum chamber for 20 min to dry the matrix. Images were typically collected from 2,501 spots separated by 0.25 mm pitch for an image size of 10.6 mm x 7.0 mm. Mass imaging experiments were performed in the positive ionization mode using a MALDI mass spectrometer (AXIMA Performance, Shimadzu). Laser power was adjusted as required. MALDI mass spectra were acquired using an AXIMA Resonance (Shimadzu) equipped with a

337 nm N₂ laser in the reflectron positive mode. Mass spectra were obtained under the following conditions: more than 200 laser shots per spectrum; scanning mass range of 1-1,000 Da.

Analysis

The pharmacokinetic parameters of the two treatments were compared using a paired t-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Developmental changes of P-gp level in rat brain

We previously reported that brain penetration of oseltamivir was restricted by the efflux transporter P-gp at the BBB in mice. Here, to study the developmental changes of P-gp expression at the BBB in rats, we employed semi-quantitative western blotting of membrane fraction of rat forebrain. As shown in Fig. 1, P-gp protein was detected at two weeks after birth and subsequently increased in proportion to development. The expression level at 2 neonatal weeks was only 40% of that at 8 neonatal weeks.

Fig. 1. Developmental changes of P-gp expression in rat brain. (A) Immunoblotting using mouse P-gp monoclonal antibody (C219) against crude membrane fractions of forebrain prepared from 2-, 3-, 4- and 8-week-old rats. (B) The protein levels are expressed as percentages of those in membrane fraction of adult rat (eight weeks old).

Developmental changes in brain penetration of oseltamivir and Ro 64-0802

Plasma and brain concentrations of oseltamivir and Ro 64-0802 were measured one hour after oral administration of oseltamivir at a dose of 300 mg/kg. The brain concentration and $K_{p,app,brain}$ value of oseltamivir in 2-week old rats took the highest values, i.e., 1.45 ± 0.22 $\mu\text{g/g}$ brain and 0.14 ± 0.02 , and then gradually decreased with age (Fig. 2). In contrast, the brain concentration and $K_{p,app,brain}$ value of Ro 64-0802 in 2-week old rats were the lowest, i.e., 0.02 ± 0.01 $\mu\text{g/g}$ brain and not detectable, and then gradually increased with age (Fig. 2).

Brain localization of oseltamivir and Ro 64-0802

To understand the cause of the putative neuropsychiatric effect, it is important to characterize the localization of oseltamivir and Ro 64-0802 in the brain. To obtain good contrast between the background and compound-related signals, we analyzed coronal sections prepared from rat brain after oral administration of the vehicle, single oral

administration of oseltamivir and repeated oral administration of oseltamivir. Imaging mass revealed that oseltamivir and Ro 64-0802 were homogeneously distributed in the coronal sections, including the hippocampus (Fig. 3). Signal intensities of oseltamivir and Ro 64-0802 increased in proportion to the brain concentrations. To identify the ion peaks at m/z 313 and 285, we conducted tandem mass spectrometry (MS/MS), comparing fragments acquired from the tissue and from standard compounds. The fragments at m/z 313 and 285 were assigned to oseltamivir and Ro 64-0802, respectively (Fig. 4).

DISCUSSION

We and other groups have demonstrated that brain distribution of oseltamivir is limited by P-gp-mediated efflux transport at the BBB in animals (Morimoto *et al.*, 2008; Ose *et al.*, 2008). To investigate why young patients and infants might be particularly susceptible to side effects, we examined developmental changes of P-gp expres-

Fig. 2. Developmental changes of brain concentrations of oseltamivir and its metabolite Ro 64-0802 in rats. Plasma and brain concentrations were measured at one hour after oral dosing of oseltamivir (300 mg/kg). $K_{p,app,brain}$ is the brain-to-plasma concentration ratio. Data are expressed as the mean $\pm$ S.E. of three animals. Asterisks indicate significant difference versus 8-week-old rats; * $p < 0.05$; ** $p < 0.01$.

Brain distribution of oseltamivir in rats

Fig. 3. Homogeneous distribution of oseltamivir and Ro 64-0802 in rat brain. Mass imaging of oseltamivir and Ro 64-0802 in brain slices of 2-week-old rats obtained one hour after single or repeated (4 times at 1.5 hr intervals) oral administration of oseltamivir at a dose of 300 mg/kg ($n = 1$). Brain concentrations and K_p values are expressed as the means of three animals.

Fig. 4. Comparisons of tissue MS/MS spectra of ion peaks at m/z 313 and 284 with those of authentic oseltamivir (A) and Ro 64-0802 (B); a, authentic; b, repeated administration; c, single administration; d, control. Comparison of the three spectra allowed assignment of m/z 313 and 284 to oseltamivir and Ro 64-0802, respectively. Chemical structures assigned to the fragments are shown.

sion at the BBB and the brain distribution of oseltamivir and Ro 64-0802 after oral administration of oseltamivir. In agreement with previous reports (Ose *et al.*, 2008, 2009; Matsuoka *et al.*, 1998), the protein level of P-gp in crude membrane fraction of rat brain increased with age; in 2-week-old rats, the level was only 40% of that in 8-week-old rats. In addition, the brain concentration and $K_{p,app,brain}$ value of oseltamivir in 2-week-old rats after oral administration of oseltamivir took the highest values, and subsequently decreased with age. This negative correlation between brain distribution of oseltamivir and age-dependent P-gp expression suggests that P-gp level at the BBB is one of the determinants of brain distribution of oseltamivir. Similar results have been found in primates: P-gp level at human BBB increased with age (Daood *et al.*, 2008) and brain distribution of ^{14}C -oseltamivir in infant rhesus monkeys was higher than that in adults (Takashima *et al.*, 2011). Brain exposure to oseltamivir might be higher in human infants than in adults.

On the other hand, the $K_{p,app,brain}$ of Ro 64-0802 was comparable to the brain vascular volume. This indicates that Ro 64-0802 hardly penetrates the BBB. However, $K_{p,app,brain}$ of Ro 64-0802 increased with age. Since we flushed out the capillary space of rat brain before tissue measurement, this result indicates that a small amount of Ro 64-0802 does penetrate the BBB. Indeed, Ose *et al.* (2009) showed that transporters such as Mrp4 and Oat3 contribute to the active efflux of Ro 64-0802 at the BBB, and speculated that Oat3 is also involved in the brain uptake of Ro 64-0802, at least in mice. In addition, it has been demonstrated that mouse brain homogenate hydrolyzes ^{14}C -oseltamivir into Ro 64-0802 (Hatori *et al.*, 2009). Although the *in vitro* enzymatic activity in mouse brain was 250-fold lower than that in plasma, it is possible that Ro64-0802 is formed and accumulated in brain, because its low lipophilicity would prevent diffusion across lipid bilayers. The cause of the age-dependent increase of brain distribution of Ro 64-0802 is presently unknown, but Mrp4 and Oat3 may play a role.

For the first time, we separately visualized the rat brain distributions of oseltamivir and Ro 64-0802 by means of mass imaging analysis. The agreement of tissue MS/MS spectra of ion peaks at m/z 313 and 284 with those of authentic oseltamivir and Ro 64-0802 proves the validity of mass imaging analysis of the compounds. Both compounds were homogeneously distributed in brain cross-sections, including the hippocampus. Therefore, it was estimated that the concentration of oseltamivir throughout the brain cross-sections was 70-fold and 0.9-fold higher than that of Ro 64-0802 in 2-week-old and 8-week-old rats, respectively. Recently, it was reported that Ro

64-0802 inhibits not only influenza viral neuraminidase but also one of four human sialidases, Neu2. The latter enzyme plays a role in the regulation of the length of sialic acid chains, which are abundant in the hippocampus. Dysfunction of this enzyme is known to be related to epileptic conditions. Usami *et al.* (2008) reported that oseltamivir and Ro 64-0802 enhance spike synchronization between hippocampal CA3 neurons, producing a 'population burst phenomenon', for which the ED50 values were 10.2 μM and 0.7 μM , respectively. In addition, Ro 64-0802 exhibits stronger cytotoxicity than oseltamivir (Shi *et al.*, 2006). Although the relationship between the population burst phenomenon, cytotoxicity, and neuropsychiatric effects is unknown, our findings raise the possibility that the putative central nervous system (CNS) effects might be due to different compounds, depending upon age; i.e., oseltamivir at 2 weeks of age and Ro 64-0802 at 8 weeks of age, at least in rats. However, the implications of these findings for the putative neuropsychiatric effects in infants and young humans remain unclear, because there is no comparative information on BBB development in rats and humans, despite the similarities of tight junction formation (Johanson, 1980) and P-gp expression changes (Daood *et al.*, 2008) during the course of development. Nevertheless, if such developmental changes of prodrug/drug concentration ratio also occur in humans, they may provide a rational basis for the putative CNS side effects in young patients.

During our study, a report on rat brain autoradiography after [^{14}C]oseltamivir injection appeared (Hatori *et al.*, 2011). In that report, the highest radioactivity was found in the choroid plexus, the pineal body, and the median eminence, where the tight junctions of the BBB or the blood-cerebrospinal fluid barrier are incomplete. Our brain sections did not include these areas, except for the choroid plexus. However, both their results and ours suggest that brain concentrations of oseltamivir and Ro 64-0802 are highly restricted by the BBB and therefore small functional changes of transporters at the BBB may greatly affect the concentrations of oseltamivir and Ro 64-0802 in brain parenchyma.

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