Pharmacokinetic mechanism involved in the prolonged high retention of laninamivir in mouse respiratory tissues after intranasal administration of its prodrug laninamivir octanoate

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Running title: Retention mechanism of laninamivir in the lungs

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Number of text pages: 35
Number of tables: 2
Number of figures: 7
Number of references: 34
Number of words in abstract: 246 words
Number of words in introduction: 611 words
Number of words in discussion: 1427 words

Abbreviations: LO, laninamivir octanoate; ELF, epithelial lining fluid; Radio-HPLC, high performance liquid chromatography with radioactive flow detection; LC-MS, liquid chromatography-mass spectrometry; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; BAL, bronchoalveolar lavage; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; HBSS, Hank’s balanced salt solution; FBS, fetal bovine serum
Abstract

Laninamivir octanoate (LO) is an ester prodrug of the neuraminidase inhibitor laninamivir. We previously reported that a prolonged high retention of laninamivir in mouse respiratory tissues was achieved by intranasal administration of LO. In this study, we evaluated intrapulmonary pharmacokinetics both in vivo and in vitro in order to investigate the potential mechanism involved in such a preferable retention. After intranasal administration of LO to mice (0.5 μmol/kg), the drug was distributed from the airway space into the lungs, and laninamivir remained in the lung at 24 h post-dose (2680 pmol/g), with a higher concentration than that in the epithelial lining fluid. The laninamivir was localized mainly on the epithelial cells of airway tracts, determined by microautoradiography using 14C-labeled LO. In mouse airway epithelial cells, the cellular uptake and hydrolysis of LO were observed over incubation time without any apparent saturation at the highest concentration tested (1000 μM). Furthermore, after additional incubation in drug-free medium, the intracellular laninamivir was released very slowly into the medium with an estimate rate constant of 0.0707 h⁻¹, which was regarded as a rate-limiting step in the cellular retention. These results demonstrated that the prolonged high retention of laninamivir in the respiratory tissues was attributed to a consecutive series of three steps: uptake of LO into the airway epithelial cells, hydrolysis of LO into laninamivir by intracellular esterase(s), and limited efflux of the generated laninamivir due to its poor membrane permeability. This prodrug approach could be useful for lung-targeting drug delivery.
Introduction

Laninamivir octanoate (LO, Fig. 1), a long-acting neuraminidase inhibitor, has been licensed in Japan for the treatment of influenza virus infection in both adult and pediatric patients. In a double-blind, randomized controlled clinical trial in adult patients with infection of seasonal influenza viruses including oseltamivir-resistant viruses, a single inhaled 40 mg dose of LO had a non-inferior efficacy compared with oseltamivir (75 mg) which was administered orally twice daily over 5 days (Watanabe et al. 2010). In addition, a single dose of LO markedly reduced the time to alleviation of influenza illness in comparison with oseltamivir, in pediatric patients infected with oseltamivir-resistant influenza A (H1N1) virus (Sugaya et al. 2010).

LO is an octanoyl ester prodrug of the active form laninamivir (Fig. 1), which shows potent neuraminidase inhibitory activities in vitro against various influenza A and B viruses, including oseltamivir-resistant viruses (Yamashita et al. 2009), 2009 pandemic H1N1 viruses (Itoh et al. 2009), and highly pathogenic H5N1 influenza viruses (Kiso et al. 2010). In a mouse influenza virus A/Puerto Rico/8/34 infection model, the intranasal dose of laninamivir demonstrated a prolonged survival effect, and this effect was drastically improved by derivatization into the octanoyl ester prodrug, LO (Yamashita et al. 2009, Honda et al. 2009). In the same model, a single intranasal dose of LO showed an antiviral effect which is similar to that after the repeated intranasal dose of zanamivir and superior to that after the repeated oral dose of oseltamivir (Kubo et al. 2010).

These long-acting characteristics of LO can be partly explained by the following favorable pharmacokinetic property. After a single intranasal/intratracheal administration
of LO to mice and rats, the LO was efficiently converted/hydrolyzed to its active form laninamivir, and thereafter the generated laninamivir was retained over long periods in the respiratory tissues (Koyama et al. 2009, Koyama et al. 2010). Moreover, it was confirmed in mice that this retention level was much higher than those after intranasal administration of laninamivir itself and zanamivir (Koyama et al. 2009). Such preferable pharmacokinetic characteristics were observed in humans as well. After a single inhaled administration of LO in healthy male volunteers, plasma laninamivir reached a C_max at 4 h and then slowly decreased with an elimination half-life (t_1/2) of approximately 3 days (Ishizuka et al. 2009, Yoshiba et al. 2011). Furthermore, highly retained laninamivir was confirmed in the pulmonary epithelial lining fluid (ELF) with a longer t_1/2 than that observed in the plasma (Ishizuka et al. 2012).

The ELF is a thin layer that covers the airway surface and is often considered to be the extracellular site of pulmonary infections (Baldwin et al. 1992). Bronchoalveolar lavage (BAL) has become a widely used technique for collecting the ELF in order to determine sufficient drug concentrations achieved at the infected site. This technique has been commonly applied to antibacterial agents (Rodvold et al. 2011a) and is also recently applicable to other anti-infective agents such as antifungal, antitubercular, antiparasitic, and antiviral agents (Rodvold et al. 2011b). In the case of LO as well, it is likely that the ELF is a relevant site to determine drug concentrations from the standpoint of PK/PD relationship, considering viral surface location of the neuraminidase enzyme.

In the present study, we investigated intrapulmonary pharmacokinetics and disposition after a single intranasal administration of LO in mice utilizing the BAL technique and microautoradiography, in comparison with that after a single intranasal
administration of the active form, laninamivir. Furthermore, we examined the LO transport and subsequent conversion/hydrolysis into laninamivir in primary cultured mouse lung epithelial cells, with a corresponding compartmental kinetic analysis in order to investigate the prolonged high retention mechanism of laninamivir in the respiratory tissues after intranasal administration of LO.
Materials and methods

Materials. Both LO and its active form laninamivir were synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Their internal standards (IS; [3H3]LO and [3H3]laninamivir) were also synthesized at the same company. [14C]-labeled LO ([14C]LO, 40.4 µCi/mg) was synthesized at GE Healthcare UK Ltd. (Little Chalfont, UK) and its radiochemical purity was guaranteed to be more than 97% by Radio-HPLC. The mouse epithelial cell enrichment kit was obtained from Stem Cell Technologies Inc. (Vancouver, Canada). Dispase was purchased from Invitrogen Corp. (Carlsbad, CA) and collagenase A was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The Urea Nitrogen B test kit and Ham’s F-12K medium were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents and solvents used were commercially available and were of extra pure, guaranteed or LC-MS grade.

Animals. Female BALB/cAnNCrlCrlj mice at 6 weeks of age, purchased from Charles River Japan, Inc. (Kanagawa, Japan), were used after 1 week of acclimatization in a controlled animal area with a temperature of 23±2°C and a relative humidity of 55±10% under a 12-h cycle of light/dark artificial lighting. A laboratory diet and drinking water were given ad libitum throughout the experiments. All animal experimental procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

Pharmacokinetic study. Under anesthesia with pentobarbital (50 mg/kg i.p.), each test
compound (LO or laninamivir) dissolved in physiological saline at 0.2 μmol/ml was administered intranasally to the mice at a volume of 2.5 ml/kg, corresponding to 0.5 μmol/kg (N=3–4/time point). At designated time points up to 24 h post-dose, blood was collected by cardiac puncture under diethyl ether anesthesia. Subsequently, the trachea was surgically exposed and cannulated with a polyethylene tube attached to a guide needle on a 1-ml syringe. Three 1-ml aliquots of phosphate buffered saline (pH 7.4) were slowly instilled through the trachea into the airway space, and bronchoalveolar lavage (BAL) fluid was collected by gentle aspiration. After that, whole lung was isolated from each carcass. To minimize further hydrolysis of LO after the sample collection, the protease inhibitor AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) was added to each of the blood and BAL fluids at the final concentration of 5 mM. The blood was then centrifuged at 18800g for 3 min at 4°C to obtain plasma, and the BAL fluid was centrifuged at 650g for 10 min at 4°C to remove cells and debris. The lungs that were individually weighed were added to 9-fold volume of 50% acetonitrile and homogenized using a Mixer Mill MM300 (Retsch GmbH, Haan, Germany) to prepare a 10% lung homogenate. All of the samples were stored frozen at −20°C until analysis.

**Determination of LO and laninamivir.** All of the biological samples (BAL fluid, lung homogenate and plasma) were analyzed by LC-MS/MS as follows. The sample (100 μL), acetonitrile (100 μL) and internal standards (10 μL of 20 ng/ml [2H3]LO and [2H3]laninamivir) were mixed and centrifuged at 18800g for 3 min at 4°C. An 8-μL aliquot of the supernatant was injected into the LC-MS/MS system consisting of API 4000 (Applied Biosystems, Foster City, CA) coupled to a Shimadzu 20A (Shimadzu Corp.,
Kyoto, Japan). The analytes were separated on an analytical column, a PC HILIC Silica (5 μm, 2.0 × 150 mm; Shiseido Co., Ltd., Tokyo, Japan), at a column oven temperature of 40°C. The flow rate was 0.6 ml/min with a gradient of 10 mM ammonium acetate and acetonitrile. Ionization was conducted in the positive-ion mode at a source temperature of 650°C, using nitrogen nebulizing and heating gas. LO and its IS ([2H3]LO) were analyzed in the multiple reaction monitoring (MRM) mode using the mass transitions of m/z 473→60 and 476→60, respectively. Laninamivir and its IS ([2H3]laninamivir) were analyzed in MRM mode using the mass transitions of m/z 347→60 and 350→60, respectively. The calibration curves were generated using the analyte to IS peak area ratios by weighted (1/x) least-squares linear regression over the concentration ranges of 1–100 ng/ml (2.12–212 pmol/ml) and 0.5–100 ng/ml (1.44–288 pmol/ml) for LO and laninamivir, respectively. Intra-assay precision and accuracy were less than 15% for the quality control samples prepared at low, medium and high concentrations of each analyte. Some of the samples were diluted with blank matrices to fall within the calibration curve range described above.

**Urea assay.** In order to correct the dilution of ELF by BAL fluid, both BAL fluid and plasma were tested for urea concentration using a commercially available urea assay kit (Urea Nitrogen B test kit, Wako Pure Chemical Industries, Ltd.). The BAL fluid was analyzed undiluted, whereas the plasma was diluted 1:5 with distilled water to fall within the calibration curve range (0.5–10 mg/dL). Based on the urea concentrations determined, drug concentrations in ELF (C_{ELF}) were calculated from the following formula:

\[ C_{ELF} = C_{BAL} \times \left( \frac{\text{Urea}_{\text{plasma}}}{\text{Urea}_{\text{BAL}}} \right) \]
where $C_{\text{BAL}}$ is the measured drug concentration in BAL fluid, $\text{Urea}_{\text{plasma}}$ is the urea concentration in plasma, and $\text{Urea}_{\text{BAL}}$ is the urea concentration in BAL fluid. This calculation is based on the assumption that urea concentrations in the vascular and ELF compartments are equivalent because of the freely diffusible nature of urea (Rennard SI et al. 1992).

**Pharmacokinetic analysis.** The pharmacokinetic parameters of LO and laninamivir were calculated from the ELF, lung and plasma concentrations using Phoenix WinNonlin (Version 6.1; Pharsight Corp., Mountain View, CA) based on a non-compartmental method. The maximum concentration ($C_{\text{max}}$) and the time to $C_{\text{max}}$ ($T_{\text{max}}$) were obtained by observation. The elimination half-life ($t_{1/2}$) was calculated by the regression analysis of 3 or more log-transformed data points in the terminal phase. The area under the concentration versus time curve up to the last quantifiable time ($\text{AUC}_{\text{last}}$) was calculated by the trapezoidal method.

**Microautoradiography.** $[^{14}\text{C}]\text{LO}$ was administered intranasally to mice, in a similar manner as described above. At 0.25, 1, 6, and 24 h (N=1/time point), the mice were sacrificed by exsanguination under anesthesia with diethyl ether. The bronchi and lungs were excised as representatives of the respiratory tissues, and then fixed in 10% neutral buffered formalin. The formalin-fixed tissues were dehydrated in ethanol, transferred to xylene, and embedded in paraffin. The paraffin sections were cut at 4-μm thickness on a microtome (Yamato Kohki Industrial Co., Ltd., Saitama, Japan) and the slices were mounted on MAS-coated slides (Matsunami Glass Ind., Ltd., Osaka, Japan). After
deparaffinization, the slides were dipped in NTB-3 autoradiography emulsion (Carestream Health, Inc., Rochester, NY) and stored for 4 weeks at 4°C in the dark. The film was then developed, and the sections were stained with hematoxylin-eosin. The sections were evaluated qualitatively for the distribution of silver grains, appearing as black dots after magnification by microscopy.

**Isolation and primary culture of mouse airway epithelial cells.** Airway epithelial cells were isolated from BALB/c mice, according to the procedure reported previously (Mandavi et al. 2011), with slight modifications. Briefly, lungs were perfused with physiological saline from pulmonary vasculature to clear residual blood. The lungs were then excised and cut into ca. 1-mm³ pieces. Epithelial cells were dissociated by incubation of the tissue pieces with dispase (2 mg/ml) for 30 min at room temperature, followed by a solution of collagenase A (2 mg/ml) in Ham’s F-12K medium supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) for 2 h at 37°C. After centrifugation at 350g for 5 min, the cells were treated with Ham’s F-12K medium containing DNase I (ca. 100 U/ml, Invitrogen Corp.) for 5 min at room temperature, and filtered successively through 70-μm and 40-μm cell strainers. After centrifugation at 350g for 5 min, the cells were resuspended in HBSS with 10 mM HEPES and 2% (v/v) FBS. The epithelial cells were isolated by using a mouse epithelial enrichment kit containing immunomagnetic beads and antibodies against CD45, TER119, CD31 and BP-1 to remove hematopoietic, endothelial, and fibroblast cells by negative selection, in accordance with the instruction manual. Then, the isolated cells were seeded at 3 × 10⁵ cells/well on a 12-well collagen I-coated plate (Becton Dickinson Biosciences, Franklin Lakes, NJ) and cultured in Ham’s
F-12K medium supplemented with 15% (v/v) FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a 5% CO₂ incubator until reaching confluence (5-7 days). The culture medium was changed every 2–3 days. The epithelial lineage was confirmed by immunohistochemical staining with anti-mouse pan-cytokeratin HRP (Santa Cruz Biotechnology, Inc., CA), using its isotype antibody as a negative control.

**Drug uptake assay.** The cells grown on the 12-well plate were washed twice with Ham’s F12-K medium (without serum and additives), and pre-incubated in the medium (1 ml/well) for 30 min at 37°C. For the time course of LO uptake into the cells, 50 μM LO dissolved in the medium (1 ml/well) was added to each well and incubated up to 120 min at 37°C in a 5% CO₂ incubator. For concentration dependency of the LO uptake, 10–1000 μM LO (1 ml/well) was added and incubated in a similar manner. After the incubation, the cells were washed three times with 3 ml of ice-cold phosphate buffered saline (pH 7.4) and then solubilized with 0.5% (v/v) Triton X-100 (0.2 ml/well). All of the solubilized samples were subjected to the determination of intracellular LO and laninamivir by LC-MS/MS as described above. Protein concentrations in the samples were determined by the Lowry method using bovine serum albumin as a standard. All of the assays were conducted in triplicate.

**Drug release and disposition assay.** After cells were incubated with 50 μM LO (1 ml/well) for 60 min, the cells were washed three times with 3 ml of pre-warmed medium and subsequently further incubated with drug-free medium at 37°C in the 5% CO₂ incubator. At designated time points, all of the medium was collected from each well and
pre-warmed drug-free medium was newly added for further incubation up to 24 h. To terminate the incubation, the cells were washed three times with 3 ml of ice-cold phosphate buffered saline and solubilized as described above (each time point in triplicate). All of the medium and solubilized cell samples were subjected to the LC-MS/MS analysis. Protein concentrations in the cell samples were also determined by the Lowry method.

**Compartmental kinetic modeling.** Based on the drug release assay data in the primary cultured mouse airway epithelial cells, compartmental kinetic modeling was performed using nonlinear ordinary least-squares regression. The regression and parameter estimation were achieved using Phoenix WinNonlin, and the model was simultaneously fitted to data of the drug release experiment. Model scheme is shown in Fig. 6, which was developed to estimate rate constants describing the following events: the release of intracellular LO into the extracellular compartment ($k_{LO}$), hydrolysis of LO to laninamivir within the intracellular compartment ($k_{Hydrolysis}$), and release of intracellular laninamivir into the extracellular compartment ($k_{Lani}$). Equations used to fit the model to the data set are shown below.

\[
\frac{dLO_M}{dt} = k_{LO} \cdot LO_C
\]

\[
\frac{dLO_C}{dt} = -k_{LO} \cdot LO_C - k_{Hydrolysis} \cdot LO_C
\]

\[
\frac{dLani_C}{dt} = k_{Hydrolysis} \cdot LO_C - k_{Lani} \cdot Lani_C
\]

\[
\frac{dLani_M}{dt} = k_{Lani} \cdot Lani_C
\]

where LO$_M$ and LO$_C$ are the concentration of LO in the medium and cells, respectively, and Lani$_M$ and Lani$_C$ are the concentration of laninamivir in the medium and cells, respectively. The uptake transport rates of LO and laninamivir were considered as zero in
this model, since the drug-free medium was newly exchanged at each time point, therefore drug concentration in the medium was quite low compared to that in the cells. All of the rate constants in this model were assumed to be first order.
Results

Pharmacokinetics in ELF, lungs and plasma after intranasal administration of LO and laninamivir. Drug concentration-time profiles in ELF, lungs, and plasma after intranasal administration of LO (0.5 μmol/kg) are shown in Fig. 2, and those after intranasal administration of laninamivir (0.5 μmol/kg) are shown in Fig. 3. Pharmacokinetic parameters of LO and/or laninamivir for each administration are listed in Table 1. After LO administration, ELF concentration of LO rapidly decreased with a t1/2 of 1.1 h, whereas that of laninamivir reached a Cmax (1520 pmol/ml) at 1 h post-dose and then slowly declined with a t1/2 of 25.1 h. In a similar fashion, lung concentration of LO rapidly declined, while that of laninamivir was observed soon after administration with Cmax (4350 ng/g) being reached at 6 h post-dose, followed by being gradually eliminated with a t1/2 of 30.9 h. In plasma, LO appeared rapidly with a tmax of 0.5 h and decreased with a t1/2 of 0.6 h. Plasma laninamivir reached a Cmax at 1 h post-dose and then eliminated in a bi-exponential manner with a terminal t1/2 of 13.1 h. Among the biological fluids and tissues tested, the lungs exhibited the highest retention level of laninamivir, being 2680 pmol/g at the final time point (24 h).

On the other hand, after laninamivir administration, laninamivir in ELF decreased with more rapid elimination rate than LO administration and fell down to a concentration of 51.3 pmol/ml at 4 h post-dose, which was approximately 7- and 22-fold lower than LO and laninamivir concentrations (348 and 1150 pmol/ml) at the same time point after LO administration, respectively. Subsequently, ELF concentration of laninamivir gradually
decreased with a t1/2 of 25.4 h. Lung concentration of laninamivir showed a Cmax (1020 ng/g) at 0.25 h, and then gradually decreased with a t1/2 of 25.0 h. In plasma, laninamivir concentration reached a Cmax at 0.5 h post-dose and then declined over time, not being detected after 8 h post-dose. When comparing the lung retention levels at 24 h between the two test compounds administered, laninamivir concentration was 9.5-fold higher in LO administration than laninamivir administration. Similarly, laninamivir concentrations in both ELF and plasma were quite higher in LO administration, compared with laninamivir administration.

**Microautoradiographic localization in the respiratory tissues after intranasal administration of [14C]LO.** After a single intranasal administration of [14C]LO to mice, the localization of radioactivity in the respiratory tissues was investigated at a cellular level by microautoradiography, using both bronchi and lungs as representatives. At 0.25 h post-dose, the radioactivity detected as black-silver grains was confirmed to be widely distributed in the airway space and epithelial layer of both tissues (data not shown). Thereafter, the [14C]LO-derived radioactivity was clearly present for a long period, as shown representatively in the microautoradiograms at 24 h post-dose (Fig. 4). In the bronchi (Fig. 4A), the radioactivity was mainly located on the epithelial cells (ciliated columnar epithelial cells and goblet cells), and also observed on the bronchial gland to some extent. In the lungs (Fig. 4B), a distinct radioactivity was seen over the epithelial cells (cuboidal epithelial cells) of bronchioli, terminal bronchiole, and/or alveolar bronchiole. A slight level of radioactivity was detected on the pulmonary alveoli and pulmonary arterial blood as well.
Uptake of LO in primary cultured mouse airway epithelial cells. Time course of LO uptake in primary cultured mouse airway epithelial cells are shown in Fig. 5A. When the cells were incubated with 50 μM LO, the cellular uptake of LO increased with incubation time for up to 90 min and thereafter nearly reached a steady state. The active form laninamivir was produced with time almost linearly for up to 90 min. In a concentration dependence study, where the incubation was terminated at 60 min, the cellular uptake of LO increased with the concentration added (10–1000 μM) without any apparent saturation even at the highest concentration (Fig. 5B). Furthermore, no saturation was observed in the cellular hydrolysis over the LO concentration range tested.

Drug release and disposition in primary cultured mouse airway epithelial cells pre-exposed with LO. Fig. 7 shows the experimental result, in which the cells were pre-exposed with LO (50 μM) for 60 min, followed by being further incubated in drug-free medium up to 24 h. At an early period of the incubation with drug-free medium, intracellular LO was mainly released to the medium. In addition, a portion of the intracellular LO was hydrolyzed within the cells, which resulted in the increase of intracellular concentration of laninamivir over approximately 1 h. The laninamivir generated was released very slowly to the medium, still being observed in the cells at 24 h.

Compartmental kinetic modeling of drug release and disposition in primary cultured mouse airway epithelial cells. The parameter estimates generated by compartmental modeling (Fig. 6) are listed in Table 2. The dotted lines shown in Fig. 7 represent the fit of
model to all data. The $k_{LO}$ and $k_{Lani}$ were 0.950 and 0.0707 h$^{-1}$, respectively. The $k_{Hydrolysis}$ was 0.561 h$^{-1}$, which was slightly smaller than $k_{LO}$ and considerably greater than $k_{Lani}$. Sampling distributions of these rate constant estimates had the coefficients of variation (CV) less than 30%.
Discussion

These experiments represent that a prolonged high retention of laninamivir in the respiratory tissues could be achieved by utilizing a prodrug approach and that the airway epithelial cells were considered to be an important component involved in this retention. Furthermore, a potential mechanism of such a high retention is proposed based on in vivo and in vitro studies.

After a single intranasal administration of LO (0.5 μmol/kg), the active metabolite laninamivir was measureable in ELF, lungs and plasma shortly after dosing (2 min), and the elimination of laninamivir was considerably slow over the t_{1/2} range of 13.1 to 30.9 h (Fig. 2 and Table 1). Among the biological fluids and tissues analyzed, the lungs showed the highest retention of laninamivir, being 2680 pmol/g at 24 h post-dose. This retention level was 9.5-fold higher than that after a single administration of laninamivir itself (282 pmol/g, Fig. 3). Furthermore, at 24 h after a single intranasal administration of [^{14}C]LO, a majority of the radioactivity was localized in the epithelial cells of airway tracts, as shown in Fig. 4. We previously demonstrated that laninamivir was determined as a predominant metabolite in mouse respiratory tissues (trachea and lungs) at 24 h after intranasal administration of [^{14}C]LO (Koyama et al. 2009), therefore all of the radioactivity seen in both sections in Fig. 4 was considered to be present as the active form, laninamivir. These results demonstrated that the airway epithelial cells played a significant role in the prolonged high retention of laninamivir after LO administration.

Pharmacokinetic mechanism involved in such a preferable retention can be explained by a consecutive series of the following three steps: 1) uptake of LO from the airway
space into airway epithelial cells, 2) hydrolysis of LO into laninamivir by esterase(s) localized in the cells, 3) trapping of the generated laninamivir within the cells due to its poor membrane permeability. First of all, the cellular uptake of LO is considered to be achieved by its lipophilic property, which is accomplished by introducing octanoyl moiety to the active form laninamivir. According to previous reports, inhaled compounds are generally absorbed by passive diffusion from the airway space into the circulating blood, and the absorption rate is increased with an increase in lipophilicity for compounds (Brown et al. 1983, Tronde et al. 2003). Furthermore, the absorption of lipophilic compounds is considered to be mediated primarily by membrane diffusion (Effros et al. 1983), whereas hydrophilic compounds appear to be absorbed by passive diffusion through the intercellular junction pores (Schneeberger, 1991). In fact, the log D<sub>7.4</sub> of LO and laninamivir were −0.01 and < −2.18, respectively (unpublished data), therefore LO is likely to diffuse more easily into the airway epithelial cells, compared to laninamivir. On the other hand, the involvement of transporters in the pulmonary cellular uptake of LO has not been clarified yet; however, it is unlikely that any transporters contribute to the LO uptake, considering the result that no apparent saturation was observed in the LO uptake into primary cultured mouse airway epithelial cells even at the highest concentration tested (1000 μM, Fig. 5B), which is close to the aqueous solubility of LO (approximately 0.6 mg/ml, corresponding to 1270 μM).

The drug concentration level in the airway space might also affect the drug amount diffused into the airway epithelial cells. As shown in Figs. 2 and 3, when focusing on the ELF concentrations over early time periods, LO administration exhibited slower drug elimination than laninamivir administration, resulting in the higher drug concentration
observed in ELF. Actually, at 4 h after LO administration, the ELF concentration of LO was 348 pmol/ml, which was approximately 7-fold higher than that of laninamivir at the same time point after laninamivir administration (51.3 pmol/ml). This slow elimination would maintain high drug concentration exposing to the airway epithelial cells, leading to the increase of intracellular drug amounts. A possible factor for generating the different drug elimination rate in ELF between LO and laninamivir is considered to be paracellular transport. In the case of hydrophilic compounds, pulmonary absorption is thought to be accomplished by passive diffusion through tight junctions (Schneeberger, 1991), as described above. Moreover, most exogenous macromolecules with a molecular weight less than 40 kDa are also thought to be absorbed from the airway space through tight junctions (Matsukawa et al. 1997, Patton 1996), and the absorption rate is inversely related to the molecular weight (Hastings et al. 1992, Matsukawa et al. 1997, Schanker et al. 1983). From these reports, it is considered that the paracellular pathway has a significant role on air-to-blood transport, especially for hydrophilic compounds. As shown in Fig. 3, soon after laninamivir administration, the plasma concentration of laninamivir increased in response to the rapid elimination in ELF, whereas the lung concentration of laninamivir was almost constant, not showing any considerable increase. These results support the finding that the laninamivir administered would pass through the circulating blood via paracellular pathway.

Furthermore, the intracellular hydrolysis plays an important role on the onset and duration of the pharmacological effect after LO administration. In addition to metabolic enzymes such as CYP enzymes and conjugation enzymes, esterase is currently known to be expressed in the respiratory tissues (Zhang et al. 2006, Somers et al. 2007) and engage
in the hydrolysis/activation of several inhaled prodrugs such as beclomethason dipropionate (Wurthwein et al. 1990) and ciclesonide (Nave et al. 2010). In the case of LO as well, hydrolysis in the respiratory tissues was observed both in vivo (Fig. 2) and in vitro (Fig. 5), and a part of the LO diffused into the airway epithelial cells was continuously hydrolyzed to laninamivir during additional incubation in drug-free medium (Fig. 7). In addition, the generated laninamivir was still present within the cells over 24 h (Fig. 7A), possibly due to its poor membrane permeability. On compartmental kinetic analysis, \( k_{\text{Lani}} \) (0.0707 h\(^{-1}\)) was considerably lower than \( k_{\text{Hydrolysis}} \) (0.561 h\(^{-1}\), Table 2), indicating that a rate-limiting step for prolonged high retention of the generated laninamivir would be the membrane permeability, not the LO hydrolysis. This phenomenon was consistent with in vivo observation, in which laninamivir was generated rapidly after LO administration and then gradually eliminated with a \( t_{1/2} \) of 30.9 h (\( k_{\text{el}} = 0.0224 \) h\(^{-1}\)). The LO hydrolysis was non-saturable with high catalytic capacity of the esterase(s), since the generation of intracellular laninamivir increased with the LO concentration added (10–1000 \( \mu \)M, Fig. 5B).

With regard to the relationship with the pharmacological effect, the airway epithelial cells might serve as a source of laninamivir for supplying sufficient laninamivir concentration to the ELF, a possible site of action of neuraminidase inhibitors. In mice infected with influenza virus A/Puerto Rico/8/34, a single intranasal administration of LO (0.5 \( \mu \)mol/kg) demonstrated a significant life-prolonging effect (Yamashita et al. 2009). At the same dose of LO, ELF concentration of laninamivir was highly maintained over time (842 pmol/mL at 24 h post-dose, Fig. 2A), exceeding more than 100-folds of 50% inhibitory concentrations for the same virus type (5.97 nM, Yamashita et al. 2009). These
suggest the possibility that the slow efflux of laninamivir from the airway epithelial cells into the extracellular fluids would be helpful for exhibiting long-acting neuraminidase inhibition.

A similar prodrug approach has been advocated by Bodor et al., for drug-targeting of blood-brain barrier (BBB). They showed that several hydrophilic compounds including opioid peptides were successfully delivered to the central nervous system by using specific prodrug approaches (Prokai et al. 1999, Bodor et al. 2002, Tapfer et al. 2004), one of which was to incorporate a lipophilic ester into the active form for the increase of permeability through BBB and the retention of the generated active form within the target site. Similarly, Pang and co-workers have suggested that there is a diffusional barrier that prevents enalaprilat from permeating across the hepatic cell membrane, whereas its ester prodrug enalapril easily crosses the cell membrane and then the generated active metabolite enalaprilat accumulate inside the cells (Pang et al. 1984, de Lannoy et al. 1986). It is also demonstrated from our studies that this prodrug approach can be used for pulmonary acting drugs as well, especially in local administration via intranasal and/or inhaled route.

In summary, the prodrug LO administered intranasally to mice was transported into the airway epithelial cells by passive diffusion and efficiently converted/hydrolyzed to its active metabolite laninamivir. Subsequently, the generated laninamivir was retained within the cells over the long period by less penetration into the airway and/or systemic circulation due to its hydrophilic property. This could be one of the rational prodrug approaches for lung-targeting drug delivery and might be applicable for other pulmonary acting drugs.
Acknowledgements

We gratefully acknowledge Mr. Shin-ichi Inaba (Daiichi Sankyo) for useful discussion regarding the compartmental kinetic model.
Authorship Contributions

Participated in research design: Koyama and D. Nakai

Conducted experiments: Koyama and N. Nakai

Contributed new reagents or analytical tools: Koyama and Takahashi

Performed data analysis: Koyama

Wrote or contributed to the writing of manuscript: Koyama, D. Nakai, Kobayashi, Imai, and Izumi
References


long-acting prodrug of the novel neuraminidase inhibitor laninamivir in rats. 

*Xenobiotica* **40**: 207–216.


Figure legends

Figure 1. Chemical structures of laninamivir octanoate (LO) and its active metabolite laninamivir.

An asterisk indicates the $^{14}$C-labeled position. When dissolved in water, LO is equilibrated at 9:1 (3-acyl form:2-acyl form). Hence, LO is defined as a mixture of the 3-acyl form (major) and the 2-acyl form (minor).

Figure 2. Drug concentration-time profiles in ELF (A), lungs (B), and plasma (C) after a single intranasal administration of LO at a dose of 0.5 μmol/kg to mice.

Concentrations of LO (closed circle) and laninamivir (open circle) in ELF, lungs and plasma are expressed as the mean ± S.D. of three or four animals at each time point.

Figure 3. Drug concentration-time profiles in ELF (A), lungs (B), and plasma (C) after a single intranasal administration of laninamivir at a dose of 0.5 μmol/kg to mice.

Concentrations of laninamivir (open circle) in ELF, lungs and plasma are expressed as the mean ± S.D. of three or four animals at each time point.

Figure 4. Microautoradiograms of respiratory tissues at 24 h after a single intranasal administration of [14C]LO to mice.

Bronchi (A) and lungs (B) are shown as representatives for the respiratory tissues. The distribution of radioactivity is observed on the tissue sections as black dots after magnification (200×) by microscopy.
BG, bronchial gland; BL, bronchial lumen; TB, terminal bronchiole; AV, alveoli; PB, pulmonary arterial blood.

**Figure 5.** Time course (A) and concentration dependency (B) of LO uptake into primary cultured mouse airway epithelial cells.

For the time course of LO uptake (A), cells were incubated with 50 μM LO for up to 120 min at 37°C and then intracellular concentrations of LO (closed bar) and laninamivir (open bar) were determined. For concentration dependency of the LO uptake (B), cells were incubated with 10–1000 μM LO for 60 min at 37°C and then intracellular drug concentrations were also determined. Data represent the mean±S.E. in triplicate at each point.

**Figure 6.** Model scheme depicting drug release and disposition in primary cultured mouse airway epithelial cells pre-exposed with LO.

$k_{LO}$, rate constant of the release of LO into extracellular compartment; $k_{Hydrolysis}$, rate constant of the hydrolysis of LO to laninamivir within intracellular compartment; $k_{Lan}$, rate constant of the release of laninamivir into extracellular compartment.

**Figure 7.** Drug release and disposition in primary cultured mouse airway epithelial cells pre-exposed with LO.

Cells were incubated in drug-free medium for up to 24 h after being pre-exposed with 50 μM LO for 60 min. Concentrations of LO (closed circle) and laninamivir (open circle) in the cells (A) and medium (B) were determined at each time point. Data represent the mean.
± S.E. in triplicate. Dashed lines represent the computer-generated best fit of the equations describing the compartmental model (Fig. 6) to the data.
<table>
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<th>Matrix</th>
<th>Analyte</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;</th>
<th>T&lt;sub&gt;max&lt;/sub&gt;</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt;</th>
<th>AUC&lt;sub&gt;last&lt;/sub&gt;</th>
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<td>ELF</td>
<td>LO</td>
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<td>0.033*</td>
<td>1.1</td>
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<td></td>
<td></td>
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<tr>
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<td>0.6</td>
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<tr>
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<tr>
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<tr>
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<td>Laninamivir</td>
<td>478</td>
<td>0.5</td>
<td>NC</td>
<td>782</td>
<td></td>
</tr>
</tbody>
</table>

Each value was calculated based on the mean concentration derived from three or four animals at each time point.
NC, not calculated.
* initial sampling point, ** drug concentration at the initial sampling point.
Table 2. Kinetic parameters associated with drug release and disposition in primary cultured mouse airway epithelial cells.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>S.E.</th>
<th>CV</th>
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<tr>
<td>$k_{LO}$</td>
<td>0.950</td>
<td>0.148</td>
<td>15.5</td>
</tr>
<tr>
<td>$k_{Hydrolysis}$</td>
<td>0.561</td>
<td>0.107</td>
<td>19.0</td>
</tr>
<tr>
<td>$k_{Lani}$</td>
<td>0.0707</td>
<td>0.0177</td>
<td>25.1</td>
</tr>
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</table>

Primary cultured mouse airway epithelial cells were incubated with LO (50 μM) for 60 min, followed by drug-free medium for up to 24 h as described in Materials and Methods. Parameter estimates were generated by nonlinear regression of the data based on the equations described in Materials and Methods according to the model scheme shown in Fig. 6.
Laninamivir octanoate (LO)

3-acyl form (major)

and

2-acyl form (minor)

Laninamivir
Fig. 3

A) ELF

B) Lungs

C) Plasma

Drug concentration (pmol/mL)

Time (h)
Fig. 5

A

B

Cellular uptake (pmol/mg) vs. Time (min)

Cellular uptake (pmol/mg) vs. LO concentration (μM)

Inset: Convex plots showing the effect of LO concentration on cellular uptake.
Fig. 6

\[ \begin{align*}
L_{OM} & \xrightarrow{k_{LO}} L_{OC} \\
L_{OM} & \xleftarrow{k_{Lani}} L_{OM}
\end{align*} \]

\[ L_{OC} \xrightarrow{k_{Hydrolysis}} L_{OC} \]

\[ L_{OM} \xleftarrow{k_{Lani}} L_{OM} \]

\[ L_{OC} \xrightarrow{k_{Hydrolysis}} L_{OC} \]