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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ABSTRACT

Laninamivir octanoate (LO) (Inavir; Daiichi Sankyo, Japan) 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 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 hours postdose (2860 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 (Tamiflu; F. Hoffmann-La Roche Ltd., Basel, Switzerland)-resistant viruses, a single inhaled 40-mg dose of LO had a noninferior 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 and Ohashi, 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 that 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 partly be 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, 2010). Moreover, it was confirmed in mice that this retention level was much higher than those after intranasal administration of laninamivir itself and zanamivir (Relenza; GlaxoSmithKline plc, London, UK) (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 Cmax at 4 hours and then slowly decreased with an elimination half-life (t1/2) of approximately 3 days (Ishiizuka et al., 2009; Yoshihara et al., 2011). Furthermore, highly retained laninamivir was confirmed in the pulmonary epithelial lining fluid (ELF) with a longer t1/2 than that observed in the plasma (Ishiizuka 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

ABBREVIATIONS: BAL, bronchoalveolar lavage; ELF, epithelial lining fluid; FBS, fetal bovine serum; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LO, laninamivir octanoate.
Retention Mechanism of Laninamivir in the Lungs

Laninamivir octanoate (LO) and Laninamivir

![Chemical structures of 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).](Image)

Laninamivir octanoate (LO) and Laninamivir

Materials and Methods

Materials. Both LO and its active form laninamivir were synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Their internal standards (IS; $[2H_3]$LO and $[2H_3]$laninamivir) were also synthesized at the same company. $^{14}$C-labeled LO ($^{14}$C-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 high-performance liquid chromatography with radioactive flow detection. The mouse epithelial cell enrichment kit was obtained from Stem Cell Technologies Inc. (Vancouver, BC, Canada). Dispase was purchased from Invitrogen (Carlsbad, CA), and collagenase A was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The Urea Nitrogen B test kit (Urea Nitrogen B test kit, Wako Pure Chemical Industries, Ltd.) and its radiochemical purity were guaranteed to be more than 97% by high-performance liquid chromatography with radioactive flow detection. The mouse epithelial cell enrichment kit was obtained from Stem Cell Technologies Inc. (Vancouver, BC, Canada). Dispase was purchased from Invitrogen (Carlsbad, CA), and collagenase A was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The Urea Nitrogen B test kit (Urea Nitrogen B test kit, Wako Pure Chemical Industries, Ltd.) and its radiochemical purity were guaranteed to be more than 97% by high-performance liquid chromatography with radioactive flow detection. The mouse epithelial cell enrichment kit was obtained from Stem Cell Technologies Inc. (Vancouver, BC, Canada). Dispase was purchased from Invitrogen (Carlsbad, CA), and collagenase A was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The Urea Nitrogen B test kit (Urea Nitrogen B test kit, Wako Pure Chemical Industries, Ltd.) and its radiochemical purity were guaranteed to be more than 97% by high-performance liquid chromatography with radioactive flow detection.

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-hour 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. With mice under anesthesia with pentobarbital (50 mg/kg i.p.), each test compound (LO or laninamivir) dissolved in physiologic 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 hours postdose, 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 4-2-aminobenzene sulfonyl fluoride hydrochloride was added to each of the blood and BAL fluids at the final concentration of 5 nM. The blood was then centrifuged at 18,800g for 3 minutes at 4°C to obtain plasma, and the BAL fluid was centrifuged at 650g for 10 minutes at 4°C to remove cells and debris. The lungs that were individually weighed were added to ninefold 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 biologic 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 $[^3H]_{LO}$ and $[^3H]_{laninamivir}$) were mixed and centrifuged at 18,800 × g for 3 minutes 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 HILC 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 65°C using nitrogen nebulizing and heating gas. LO and its IS ($[^3H]_{LO}$) were analyzed in the multiple reaction monitoring mode using the mass transitions of $m/z$ 473→60 and 350→60, respectively. Laninamivir and its IS ($[^3H]_{laninamivir}$) were analyzed in multiple reaction monitoring mode using the mass transitions of $m/z$ 476→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. 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). On
the basis of the urea concentrations determined, drug concentrations in ELF (C_{ELF}) were calculated from the following formula:

\[
C_{ELF} = C_{BAL} \times \frac{(Urea_{BAL}/Urea_{ELF})}{C_{2}}
\]

where C_{BAL} is the measured drug concentration in BAL fluid, Urea_{ELF} is the urea concentration in plasma, and Urea_{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 et al., 1986).

**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 noncompartmental method. The maximum concentration (C_{max}) and the time to C_{max} (T_{max}) were obtained by observation. The elimination half-life (t_{1/2}) was calculated by the regression analysis of three or more log-transformed data points in the terminal phase. The area under the concentration versus time curve up to the last quantifiable time (AUC_{0-\infty}) was calculated by the trapezoidal method.

**Microautoradiography.** [^{14}C]LO was administered intranasally to mice in a similar manner as described above. At 0.25, 1, 6, and 24 hours (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 slides 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 (Kumari and Saxena, 2011) with slight modifications. Briefly, lungs were perfused with physiologic saline from pulmonary vasculature to clear residual blood. The lungs were then excised and cut into approximately 1-mm^2 pieces. Epithelial cells were dissociated by incubation of the tissue pieces with dispase (2 mg/ml) for 30 minutes at room temperature, followed by a solution of collagenase A (2 mg/ml) in Hams’ F-12K medium supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) for 2 hours at 37°C. After centrifugation at 350g for 5 minutes, the cells were treated with Hams’ F-12K medium containing DNase I (approximately 100 U/ml; Invitrogen) for 5 minutes at room temperature and filtered successively through 70-μm and 40-μm cell strainers. After centrifugation at 350g for 5 minutes, the cells were resuspended in Hanks’ balanced salt solution with 10 nM 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 x 10^5 cells/well on a 12-well collagen I-coated plate (BD Biosciences, Franklin Lakes, NJ) and cultured in Hams’ 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., Santa Cruz, CA) using its isotype antibody as a negative control.

**Drug Uptake Assay.** The cells grown on the 12-well plate were washed twice with Hams’ F12-K medium (without serum and additives) and preincubated in the medium (1 ml/well) for 30 minutes 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 minutes 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 minutes, the cells were washed three times with 3 ml of prewarmed 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 prewarmed drug-free medium was newly added for further incubation up to 24 hours. 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.** On the basis of 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. 7, 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}). Equation used to fit the model to the data set is shown below.

\[
\frac{dC_{LO}}{dt} = -k_{LO} \cdot C_{LO} - k_{Hydrolysis} \cdot C_{LO} + k_{LANI} \cdot C_{LANI}
\]

where \(C_{LO}\) and \(C_{LANI}\) are the concentration of LO and laninamivir in the medium and cells, respectively, and \(k_{LO}\) and \(k_{LANI}\) are the concentration of laninamivir in the intracellular and extracellular compartments, respectively. The uptake transport rates of LO and laninamivir were calculated using the method using bovine serum albumin as a standard. All of the assays were conducted in triplicate.

**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 \(t_{1/2}\) of 1.1 hours, whereas that of laninamivir reached a \(C_{max}\) (1520 pmol/ml) at 1 hour postdose and then slowly declined with a \(t_{1/2}\) of 25.1 hours. In a similar fashion, lung concentration of LO rapidly declined, whereas that of laninamivir was observed soon after administration with \(C_{max}\) (4350 ng/g), being reached at 6 hours postdose followed by being gradually eliminated with a \(t_{1/2}\) of 30.9 hours. In plasma, LO appeared rapidly with a \(t_{max}\) of 0.5 hours and decreased with a \(t_{1/2}\) of 6.0 hours. Plasma laninamivir reached a \(C_{max}\) at 1 hour postdose and then was eliminated in a biexponential manner with a terminal \(t_{1/2}\) of 13.1 hours. Among the biologic fluids and tissues tested, the lungs exhibited the highest retention level of laninamivir, being 2680 pmol/g at the final time point (24 hours).
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 hours postdose, 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 \( t_{1/2} \) of 25.4 hours. Lung concentration of laninamivir showed a \( C_{\text{max}} \) (1020 ng/g) at 0.25 hours, and then gradually decreased with a \( t_{1/2} \) of 25.0 hours. In plasma, laninamivir concentration reached a \( C_{\text{max}} \) at 0.5 hours postdose and then declined over time, not being detected after 8 hours postdose. When comparing the lung retention levels at 24 hours 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 \( [\text{14C}] \text{LO} \). After a single intranasal administration of \( [\text{14C}] \text{LO} \) 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 hours postdose, 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 \( [\text{14C}] \) LO-derived radioactivity was clearly present for a long period, as shown representatively in the microautoradiograms at 24 hours postdose (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 is 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 minutes and thereafter nearly reached a steady state. The active form laninamivir was produced with time almost linearly for up to 90 minutes. In a concentration dependence study, where the incubation was terminated at 60 minutes, 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. Figure 6 shows the experimental result, in which the cells were pre-exposed with LO (50 μM) for 60 minutes, followed by being further incubated in drug-free medium up to 24 hours. 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 hour. The laninamivir generated was released very slowly to the medium, still being observed in the cells at 24 hours.

Compartmental Kinetic Modeling of Drug Release and Disposition in Primary Cultured Mouse Airway Epithelial Cells. The parameter estimates generated by compartmental modeling (Fig. 7) are listed in Table 2. The dotted lines shown in Fig. 6 represent the fit of model to all data. The \( k_{\text{LO}} \) and \( k_{\text{Lani}} \) was 0.950 and 0.0707 h\(^{-1}\), respectively. The \( k_{\text{Hydrol}} \) was 0.561 h\(^{-1}\), which was slightly smaller than \( k_{\text{Lani}} \) and considerably greater than \( k_{\text{LO}} \). 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 measurable in ELF, lungs, and plasma shortly after dosing (2 minutes), and the elimination of laninamivir was considerably slow over the \( t_{1/2} \) range of 13.1 to 30.9 hours (Fig. 2; Table 1). Among the biologic fluids and tissues analyzed, the lungs showed the highest retention of laninamivir, being 2680 pmol/g at 24 hours postdose. 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 hours after a single intranasal administration of \([^{14}\text{C}]\text{LO}\), 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 hours after intranasal administration of \([^{14}\text{C}]\text{LO}\) (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:

### TABLE 1
Pharmacokinetic parameters of LO and/or laninamivir in ELF, lungs, and plasma after a single intranasal administration of LO and laninamivir to mice.

<table>
<thead>
<tr>
<th>Test Compound</th>
<th>Matrix</th>
<th>Analyte</th>
<th>( C_{\text{max}} ) pmol/ml or g</th>
<th>( T_{\text{max}} ) h</th>
<th>( t_{1/2} ) h</th>
<th>AU/Cmax pmol·h/ml or g</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO</td>
<td>ELF</td>
<td>LO</td>
<td>20,400**</td>
<td>0.033*</td>
<td>1.1</td>
<td>23,600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laninamivir</td>
<td>1520</td>
<td>1</td>
<td>25.1</td>
<td>26,500</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>LO</td>
<td>1760</td>
<td>0.5</td>
<td>1.3</td>
<td>4480</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Laninamivir</td>
<td>4350</td>
<td>6</td>
<td>30.9</td>
<td>75,800</td>
</tr>
<tr>
<td>Laninamivir</td>
<td>ELF</td>
<td>Laninamivir</td>
<td>19,900**</td>
<td>0.033*</td>
<td>25.4</td>
<td>19,600</td>
</tr>
<tr>
<td></td>
<td>Lungs</td>
<td>Laninamivir</td>
<td>1020</td>
<td>0.25</td>
<td>25.0</td>
<td>9890</td>
</tr>
<tr>
<td></td>
<td>Plasma</td>
<td>Laninamivir</td>
<td>478</td>
<td>0.5</td>
<td>NC</td>
<td>782</td>
</tr>
</tbody>
</table>

NC, not calculated.
* Initial sampling point.
** Drug concentration at the initial sampling point.

**Fig. 4.** Microautoradiograms of respiratory tissues at 24 hours after a single intranasal administration of \([^{14}\text{C}]\text{LO}\) 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.
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 and Schanker, 1983; Tronde et al., 2003). Furthermore, the absorption of lipophilic compounds is considered to be mediated primarily by membrane diffusion (Effros and Mason, 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 with 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

![Graph A](image1)

**Fig. 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 minutes 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 minutes at 37°C, and then intracellular drug concentrations were also determined. Data represent the mean ± S.E. in triplicate at each point.

![Graph B](image2)

![Graph C](image3)

**Fig. 6.** 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 hours after being pre-exposed with 50 µM LO for 60 minutes. 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. 7) to the data.
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>
</tr>
</thead>
<tbody>
<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>23.1</td>
</tr>
</tbody>
</table>

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 hours after LO administration, the ELF concentration of LO was 348 pmol/ml, which was approximately sevenfold 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 exposed 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 and Hemberger, 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 P450 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 beclomethasone dipropionate (Becotide; GlaxoSmithKline plc) (Wörthwein and Rohdewald, 1990) and ciclesonide (Alvesco; Nycomed International Management GmbH, Zurich, Switzerland) (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. 6). In addition, the generated laninamivir was still present within the cells over 24 hours (Fig. 6A), possibly due to its poor membrane permeability. Compartmental kinetic analysis, $k_{Lani}$ (0.0707 h$^{-1}$) was considerably lower than $k_{Hydrolysis}$ (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 hours ($k_{d1} = 0.0224$ h$^{-1}$). The LO hydrolysis was nonsaturable with high catalytic capacity of the esterase(s), because the generation of intracellular laninamivir increased with the LO concentration added (10–1000 µM; 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 µmol/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 hours postdose; Fig. 2A), exceeding more than 100-fold 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. (2002), for drug-targeting of blood-brain barrier. 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 blood-brain barrier and the retention of the generated active form within the target site. Similarly, Pang and coworkers (Pang et al., 1984; de Lamoy and Pang, 1986) suggested that there is a diffusion barrier that prevents enalaprilat from permeating across the hepatic cell membrane, whereas its ester prodrug enalapril (Vasotec; Merck & Co., NJ) easily crosses the cell membrane and then the generates active metabolite enalaprilat accumulate inside the cells. 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.

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References


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