CS-8958, a Prodrug of the Novel Neuraminidase Inhibitor R-125489, Demonstrates a Favorable Long-Retention Profile in the Mouse Respiratory Tract

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CS-8958 is a prodrug of the pharmacologically active form R-125489, a selective neuraminidase inhibitor, and has long-acting anti-influenza virus activity in vivo. In this study, the tissue distribution profiles after a single intranasal administration of CS-8958 (0.5 μmol/kg of body weight) to mice were investigated, focusing especially on the retention of CS-8958 in the respiratory tract by comparing it with R-125489 and a marketed drug, zanamivir. After administration of [14C]CS-8958, radioactivity was retained in the respiratory tract over long periods. At 24 h postdose, the radioactivity concentrations after administration of [14C]CS-8958 were approximately 10-fold higher in both the trachea and the lung than those of [14C]R-125489 and [14C]zanamivir. The [14C]CS-8958-derived radioactivity present in these two tissues consisted both of unchanged CS-8958 and of R-125489 at 1 h postdose, while only R-125489, and no other metabolites, was detected at 24 h postdose. After administration of unlabeled CS-8958, CS-8958 was rapidly eliminated from the lungs, whereas the lung R-125489 concentration reached a maximum at 3 h postdose and gradually declined, with an elimination half-life of 41.4 h. The conversion of CS-8958 to R-125489 was observed in mouse trachea and lung S9 fractions and was inhibited by esterase inhibitors, such as diisopropylfluorophosphate and bis-p-nitrophenylphosphate. These results demonstrated that CS-8958 administered intranasally to mice was efficiently converted to R-125489 by a hydrolase(s) such as carboxylesterase, and then R-125489 was slowly eliminated from the respiratory tract. These data support the finding that CS-8958 has potential as a long-acting neuraminidase inhibitor, leading to significant efficacy as an anti-influenza drug by a single treatment.

Influenza is a contagious illness caused by influenza viruses that infect the respiratory tract. The illness can be debilitating and at times can lead to hospitalization and death. Some people, such as the elderly, young children, and individuals with other health problems, are at greater risk of developing more-severe illnesses or of suffering from the complications of influenza, including pneumonia.

Two classes of drugs are available for the treatment of influenza: M2 ion channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir). The therapeutic use of the M2 ion channel inhibitors is limited by their side effects (more common with amantadine), the emergence of antiviral resistance, and the lack of activity against new swine-origin H1N1 strains, such as A/California/04/09 infection model (17, 18). In contrast, after intranasal administration of CS-8958, the prolonged survival effect was drastically improved in the same model relative to those of R-125489 and zanamivir (17, 18). Furthermore, it was reported recently that CS-8958 was also sensitive against new swine-origin H1N1 strains, such as A/California/04/09 (10).

In the present study, in order to examine whether or not the pharmacokinetic profile of CS-8958 contributes to the increased pharmacological effect, we investigated tissue distribution after a single intranasal administration of CS-8958 to mice, focusing especially on retention in the respiratory tract (the primary site of viral infection and replication), in comparison with the distribution of R-125489 and zanamivir. This study includes not only the in vivo metabolism of CS-8958 in...
the mouse respiratory tract but also the in vitro metabolism (hydrolysis) of CS-9858 by using S9 fractions prepared from mouse trachea and lung. These data provide fundamental information indicating that the long retention of R-125489 in the respiratory tract after administration of the prodrug CS-9858 probably contributes to the increased pharmacological effect.

MATERIALS AND METHODS

Chemicals and reagents. [14C]CS-8958 (20.8 mCi/mmol) and [14C]R-125489 (14.9 mCi/mmol) were synthesized at GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, United Kingdom). The radiochemical purities of these compounds were guaranteed to be more than 97% by high-performance liquid chromatography with radioactive flow detection. [14C]Zanamivir (19 mCi/mmol; 99.8% radiochemical purity) and unlabeled zanamivir were also synthesized. Unlabeled CS-8958 and R-125489 were synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan) according to published procedures (8, 9). [3H]CS-8958 and [3H]R-125489 were also synthesized at the same company. Diisopropylphosphoro- phosphate (DFP), bis-p-nitrophenylphosphate (BNPP), and p-chloromercuribenzoate (PCMBS) were purchased from EMD Chemicals Inc. (Darmstadt, Germany), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and Funakoshi Corp. (Tokyo, Japan), respectively. Eserine, 5,5'-dithiobis-2-nitrobenzonic acid (DTNB), and EDTA were from Sigma-Aldrich Co. All other reagents and solvents used were commercially available and were of extra-pure, guaranteed, or liquid chromatography–mass spectrometry (LC-MS) grade.

Animals. Female BALB/cAnNcrCrlj mice (6 weeks old; Charles River Japan, Inc., Yokohama, Japan) were used after 1 week of acclimatization. Under anesthesia with a 1:1 mixture of diethyl ether and chloroform, each test compound was administered intranasally to the mice at a volume of 2.5 ml/kg of body weight, corresponding to 0.5 μmol/kg (3 or 4 mice/time point), the whole lung was isolated as described above. The lung was individually weighed, added to a ninefold volume of CH3CN–H2O (1/1, vol/vol) and homogenized using a Polytron homogenizer (PT-MR300S, Kinematica AG) on ice to prepare a 10% lung homogenate. After centrifugation (18,800 × g, 4°C, 3 min), the supernatant was subjected to solid-phase extraction. For the extraction of CS-8958, 0.1 ml CH3OH was applied to an Oasis HLB 96-well plate (30 mg/well, 30 μl) and subjected to radioactivity measurement using a model 2300TR liquid scintillation counter (Packard Instrument Company, Meriden, CT). The radioactivity concentration was expressed as an equivalent (eq) value of 14C-labeled compound per gram.

Metabolite identification in the trachea and lung. At 1 and 24 h after a single intranasal administration of [14C]CS-8958 at a dose of 3 μmol/kg (2 mice/time point), the trachea and whole lung were each isolated as described above, pooled, and weighed. Subsequently, a threefold volume of ethanol was added to each of the pooled trachea and lung samples, and they were homogenized using a Polytron homogenizer (PT10/35, Kinematica AG, Littau, Switzerland) on ice. The extracted fractions were analyzed using a high-performance liquid chromatography system (an Alliance 2695 separation module coupled with a 2996 photodiode array detector; Waters Corp., Milford, MA) equipped with a radio- active detector (Radiomatic 500TR, Perkin-Elmer, Inc.) and a mass spectro- metry detector (Ultima; Waters Corp.). The analytical conditions were as follows: analytical column, Hydrosphere C18 (6.0 mm by 150 mm, 5 μm; YMC Co., Ltd., Kyoto, Japan); column oven temperature, 30°C; mobile phase A, 0.1% (vol/vol) CH3OH–H2O; mobile phase B, 0.1% (vol/vol) CH3OH in CH3CN; flow rate, 1 ml/min; gradient of mobile phase B, 0% from 0 to 5 min (constant), 0% to 50% from 5 to 25 min (linear), 50% to 80% from 25 to 26 min (linear), and 80% from 26 to 30 min (constant); injection volume, 10 or 40 μl; capillary voltage, 3 kV; collision energy, 50 eV for LC–MS analysis and 25 eV for LC–tandem MS (LC–MS-MS) analysis.

Lung concentration-time profile. At 0.25, 1, 3, 6, 24, 48, 72, and 120 h after a single intranasal administration of unlabeled CS-8958 at a dose of 0.5 μmol/kg (3 or 4 mice/time point), the whole lung was isolated as described above. The lung was individually weighed, added to a ninefold volume of CH3CN–5% CH3COOH (1/1, vol/vol), and homogenized using a Polytron homogenizer (PT- MR300S, Kinematica AG) on ice to prepare a 10% lung homogenate. After centrifugation (18,800 × g, 4°C, 3 min), the supernatant was subjected to solid-phase extraction. For the extraction of CS-8958, 0.1 ml supernatant mixed with 0.65 ml H2O was applied to an Oasis HLB 96-well plate (30 mg/well, 30 μl) and subjected to radioactivity measurement using a model 2300TR liquid scintillation counter (Packard Instrument Company, Meriden, CT). The radioactivity concentration was expressed as an equivalent (eq) value of 14C-labeled compound per gram.

FIG. 1. Chemical structures of CS-8958 and R-125489. The asterisk indicates the 14C-labeled position. When dissolved in water, CS-8958 is equilibrated at 1:1 (3-acetyl form–2-acetyl form). Hence, CS-9858 is defined as a mixture of the 3-acetyl form (major) and the 2-acetyl form (minor).
parameters were used for R-125489 and the IS: declustering potential, 60 V each; collision energy, +40 V each; collision cell exit potential, +22 V each. The optimal source parameters were as follows: curtain gas, 20 lb/in²; collision gas, 6 arbitrary units; ion spray voltage, +4,500 V; ion source temperature, 700°C; ion source gas 1, 50 lb/in²; and ion source gas 2, 60 lb/in². The calibration curves were generated using the analyte-to-IS peak area ratios by weighted (1/x²) least-squares linear regression over the concentration range of 10 (lower limit of quantification) to 5,000 ng/g for both CS-8958 and R-125489. The assay was well validated, and the accuracies of the quality control samples prepared at low, medium, and high concentrations of each compound were within 85 to 115% at every measurement.

Pharmacokinetic analysis. Pharmacokinetic parameters were calculated from the mean lung CS-8958 and R-125489 concentrations, using a noncompartment model, by WinNonlin Professional computer software (version 4.0.1; Pharsight Corp., Mountain View, CA). The elimination half-life (t1/2) was calculated for both CS-8958 and R-125489 and was expressed as the apparent half-life calculated by least-squares regression using the values observed in a designated period. The maximum concentration in the lung (Cmax) and the time to reach Cmax (tmax) were calculated only for R-125489. The Cmax was obtained as the highest concentration among the observed values, and the tmax was obtained as the time showing Cmax.

Preparation of trachea and lung S9 fractions. After 50 mice were euthanized by exsanguination under diethyl ether anesthesia, the tracheae and whole lungs were removed and each pooled. Each sample was weighed, homogenized in a threefold volume of 50 mM phosphate buffer (pH 7.4) containing 0.15 M KCl using a Polytron homogenizer (PT-MR3000; Kinematica AG), and centrifuged at 9,000 rpm for 15 min at 4°C to prepare S9 fractions. The protein concentrations were determined by the Lowry method using bovine serum albumin as a standard. The samples were flash-frozen in liquid nitrogen and maintained at −80°C until use.

Effects of esterase inhibitors on hydrolysis of CS-8958 in trachea and lung S9 fractions. An aliquot of each trachea and lung S9 fraction (2 mg/ml diluted with 50 mM phosphate buffer [pH 7.4]) was preincubated with various esterase inhibitors (final concentration, 1 mM) for 10 min at 37°C. Then CS-8958 (final concentration, 10 μM) was added to initiate the reaction. After incubation for the designated time at 37°C, the reaction was terminated with an equal volume of CH3CN. After centrifugation at 18,800 × g for 3 min at 4°C, 5 μl of each supernatant was subjected to R-125489 determination by LC–MS-MS analysis using [³H]R-125489 as the IS. The analytical conditions for R-125489 were the same as those described above. All of the experiments were performed in the presence of 1% dimethyl sulfoxide. The inhibitors used in the experiment were as follows: DFP (serine hydrolase inhibitor), eserine (cholinesterase inhibitor), BNPP (carboxylesterase inhibitor), EDTA (metal-chelating agent), and DTNB and PCMB (arylesterase inhibitors). The results were expressed as percentages of the control activity in the absence of the inhibitors.

RESULTS

Whole-body autoradiography. After a single intranasal administration of [¹⁴C]CS-8958, [¹⁴C]R-125489, or [¹⁴C]zanamivir at a dose of 0.5 μmol/kg, a certain level of radioactivity reached the respiratory tract (nasal cavity, trachea, and lung), while the remaining radioactivity was swallowed and entered the gastrointestinal tract through the esophagus. Radioactivity was observed in the blood, kidney, and urine in the bladder. In the case of [¹⁴C]CS-8958, radioactivity was also distributed in the liver.

In the respiratory tract, the primary site of influenza virus infection and replication, [¹⁴C]CS-8958 showed considerably higher radioactivity than [¹⁴C]R-125489 and [¹⁴C]zanamivir following 1 h postdose, as shown representatively in the autoradiograms at 6 h postdose (Fig. 2). Moreover, the [¹⁴C]CS-
8958-derived radioactivity was still clearly observed in the lung at 72 h postdose (data not shown).

Radioactivity concentrations in trachea and lung. The concentrations of radioactivity in the trachea and lung after a single intranasal administration of \[^{14}C\]CS-8958, \[^{14}C\]R-125489, or \[^{14}C\]zanamivir at a dose of 0.5 \(\mu\)mol/kg are shown in Fig. 3. At 0.25 h postdose, the radioactivity concentrations were almost the same for the three compounds in either tissue: approximately 4 and 30 nmol eq/g in the trachea and lung, respectively. Subsequently, the concentrations in both tissues slowly declined after administration of \[^{14}C\]CS-8958, while rapidly decreasing after administration of \[^{14}C\]R-125489 or \[^{14}C\]zanamivir. The mean radioactivity concentrations at 24 h after the administration of \[^{14}C\]CS-8958 were 0.990 and 5.57

FIG. 4. Radiochromatograms of trachea and lung extracts after a single intranasal administration of \[^{14}C\]CS-8958 to mice at a dose of 3 \(\mu\)mol/kg.
nmol eq/g in the trachea and lung, respectively, concentrations that were 11.6- and 9.5-fold higher than that of $[^{14}C]R$-125489 and 11.1- and 13.4-fold higher than that of $[^{14}C]$zanamivir. The amount of $[^{14}C]$CS-8958-derived radioactivity remaining in the lung at 24 h postdose was 0.702 nmol eq, which corresponded to 8.10% of the dose.

**Metabolite identification in the trachea and lung.** Radiochromatograms of the trachea and lung extracts after a single intranasal administration of $[^{14}C]$CS-8958 at a dose of 3 μmol/kg are shown in Fig. 4. At 1 h postdose, two and three distinct radioactive peaks were observed in the trachea and lung, respectively. By comparing their chromatographic retention times and LC–MS-MS spectra to those of the authentic standards, the 3-acyl form of the unchanged form CS-8958 and R-125489 were identified in the trachea, whereas the 2-acyl form of CS-8958, the 3-acyl form of CS-8958, and R-125489 were identified in the lung. The 2-acyl form of CS-8958 was not apparently detected in the trachea, probably due to the limitation of the radioactivity. On the other hand, at 24 h postdose, only R-125489 was detected in both the trachea and the lung, and no other metabolites were detected.

**Pharmacokinetics in the lung.** The lung concentration-time profiles of CS-8958 and R-125489 after a single intranasal administration of CS-8958 at a dose of 3 μmol/kg are shown in Fig. 5, and the pharmacokinetic parameters calculated from these profiles are presented in Table 1. After administration, the lung CS-8958 concentrations declined with $t_{1/2}$ of 0.833 h. At 12 h postdose or later, the CS-8958 concentrations were below the lower limit of quantification (10 ng/g). On the other hand, the lung R-125489 concentrations increased soon after administration and reached the $C_{\text{max}}$ of 6.41 nmol/g at 3 h postdose. Subsequently, R-125489 was slowly eliminated from the lung, with a $t_{1/2}$ of 41.4 h. Even at 120 h postdose, R-125489 remained in the lung at a concentration of 0.915 nmol/g, which is considerably higher than the $C_{\text{max}}$ of 6.41 nmol/g. The control values for the hydrolyase activities were 4.51 and 1.01 pmol/min/mg in lung and trachea S9 fractions, respectively.

**Effects of esterase inhibitors on hydrolysis of CS-8958 in trachea and lung S9 fractions.** The effects of various esterase inhibitors on the hydrolysis of CS-8958 were examined in mouse trachea and lung S9 fractions. In Table 2, the effects of various esterase inhibitors on the conversion of CS-8958 to R-125489 in mouse trachea and lung S9 fractions are shown. In general, R-125489 hydrolyase activity was strongly inhibited by DFP and BNPP, whereas EDTA and PCMB showed no inhibitory effects. The inhibitory effects of DFP and BNPP could not be conducted using other esterase inhibitors due to the limitation of the material. The hydrolyase activity was preliminarily confirmed to increase linearly with CS-8958 concentrations ranging from 2 to 1,000 μM (data not shown).

**DISCUSSION**

Whole-body autoradiography and quantitative determination of concentrations in the trachea and lung showed that radioactivity was retained in the trachea and lung after a single intranasal administration of $[^{14}C]$CS-8958 to mice, with a level reasonably higher than those of $[^{14}C]$R-125489 and $[^{14}C]$zanamivir (Fig. 2 and 3). Furthermore, from the results of the metabolite identification, it was demonstrated that CS-8958 administered to mice was metabolized/hydrolyzed to R-125489 and then stayed in the respiratory tract for a long time as R-125489 (Fig. 4). Actually, when unlabeled CS-8958 was intranasally administered to mice, R-125489 was slowly elimi-

![FIG. 5. Concentration-time profiles of CS-8958 and R-125489 in the lung after a single intranasal administration of CS-8958 to mice at a dose of 0.5 μmol/kg (3 or 4 mice/time point; data are means ± standard deviations). The filled circles and open circles represent CS-8958 and R-125489, respectively.](http://aac.asm.org/)

**TABLE 1. Pharmacokinetic parameters of CS-8958 and R-125489 in the lung**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$C_{\text{max}}$ (nmol/g)</th>
<th>$t_{\text{max}}$ (h)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-8958</td>
<td>NC</td>
<td>NC</td>
<td>0.833</td>
</tr>
<tr>
<td>R-125489</td>
<td>6.41</td>
<td>3.0</td>
<td>41.4</td>
</tr>
</tbody>
</table>

$^a$ After a single intranasal administration of CS-8958 to mice at a dose of 0.5 μmol/kg.

$^b$ NC, not calculated.

**TABLE 2. Effects of various esterase inhibitors on the conversion of CS-8958 to R-125489 in mouse trachea and lung S9 fractions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>CS-8958 hydrolyase activity (% of control) in S9 fractions $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trachea</td>
</tr>
<tr>
<td>Control</td>
<td>100.0</td>
</tr>
<tr>
<td>DFP</td>
<td>44.1</td>
</tr>
<tr>
<td>Eserine</td>
<td>—</td>
</tr>
<tr>
<td>BNPP</td>
<td>—</td>
</tr>
<tr>
<td>DTNB</td>
<td>—</td>
</tr>
<tr>
<td>EDTA</td>
<td>—</td>
</tr>
<tr>
<td>PCMB</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$ Various esterase inhibitors (final concentration, 1 mM) were each added to the trachea and lung S9 fractions 10 min before the addition of $[^{14}C]$CS-8958 (final concentration, 10 μM) in order to examine their effects on its hydrolysis at 37°C. The experiment was performed in the presence of 1% dimethyl sulfoxide.
nated from the lung, with a t$_{1/2}$ of 41.4 h after reaching the C$_{\text{max}}$ at 3 h postdose (Fig. 5 and Table 1). In addition to the target sites, [14C]CS-8958 was distributed in the liver as well, in which the level of the radioactivity was lower than that in the lung (Fig. 2). This suggested that CS-8958 would be deposited not only in the respiratory tract but also in the liver and then would be hydrolyzed there to R-125489. A similar tissue distribution profile was observed in rats; however, there were no notable abnormalities in a 2-week repeated-dose inhalation toxicity study with rats, even at the highest dose technically achievable, 173 μmol/kg (unpublished data).

It has been reported that zanamivir shows a short serum t$_{1/2}$ after a single intranasal administration to mice (15). Similar findings have been obtained for humans, with a median serum t$_{1/2}$ ranging between 2.5 and 5.05 h after intranasal or inhaled administration to healthy volunteers (3). In parallel with these findings, rapid elimination from the respiratory tract has been reported for humans, with t$_{1/2}$ values of 2.8 h (13) and approximately 1.5 h (2), which are calculated based on the drug concentrations determined in sputum and nasal washings after inhaled administration of zanamivir and positron emission tomography imaging in the respiratory tract after intranasal administration of [11C]zanamivir, respectively. In accordance with these previous reports (2, 3, 13, 15), [14C]zanamivir administered intranasally to mice was rapidly cleared from the respiratory tract (Fig. 2 and 3). Additionally, similarly rapid elimination was observed after administration of [14C]R-125489 (Fig. 2 and 3), which is structurally related to zanamivir. In contrast to these two compounds, CS-8958 exhibited a long retention in the respiratory tract as the active form R-125489, and this phenomenon is likely to contribute to a long-acting efficacy in vivo against influenza viruses.

In fact, in a model of influenza virus A/Puerto Rico/8/34 infection in mice, R-125489 and zanamivir were reported to exhibit similar survival effects on the mice, whereas CS-8958 improved the life-prolonging effect significantly over those of R-125489 and zanamivir. Furthermore, the life-prolonging effect of CS-8958 was still observed when it was administered intranasally to mice at a single dose of 0.5 μmol/kg even 7 days before the virus infection, while the same effect was not observed with zanamivir (18). Based on the lung concentration-time profiles after a single intranasal administration of CS-8958 at the same dose (Fig. 5), the R-125489 concentration at 7 days postdose was extrapolated to be 0.51 nmol/g, which corresponds to 610 nM based on the assumption that 1 g of lung is equal to 0.83 ml (4). This value is approximately 100-fold higher than the 50% inhibitory concentration (5.97 nM) of R-125489 in the same virus type. From these results, there was a good relationship between the lung pharmacokinetic profile and the antiviral effect in mice, and the lung retention of R-125489 by the administration of the prodrug CS-8958 was considered to contribute efficiently to its improved survival effect.

The detailed retention mechanism of CS-8958 has not been clarified yet. However, one of the key factors is considered to be the increase in lipophilicity due to acylation of the active form, based on results where retention was observed for the prodrug CS-8958 and not for the active form, R-125489 (Fig. 2 and 3). Generally, prodrugs have become established tools for improving the physicochemical, biopharmaceutical, or pharmacokinetic properties of pharmacologically active agents, and about 5 to 7% of drugs approved worldwide are reported to be classified as prodrugs (14). From this point of view, the lipophilic moiety (octanoyl form) of CS-8958 might lead to an increased ability to permeate the epithelial cells located in the respiratory tract. In addition, the hydrolysis in the respiratory tract is considered to be another important factor. In the process of transport from the respiratory tract to the circulating blood, CS-8958 is considered to be hydrolyzed to R-125489, which has difficulty penetrating into the circulating blood due to its high hydrophilicity. In fact, the hydrolysis of CS-8958 was observed in mouse trachea and lung S9 fractions (Table 2). The hydrolysis in mouse lung S9 fractions was strongly inhibited by DFP and BNPP, indicating the contribution of carboxylesterase. Cholinesterase might also contribute to this hydrolysis, since eserine showed inhibitory effects on it. On the other hand, CS-8958 hydrolysis activity was affected by DFP, but not by EDTA and PCMB, suggesting that arylesterase contributed less, or not at all. Further investigations regarding the retention mechanism of CS-8958 in the respiratory tract are being conducted in our laboratories.

In summary, CS-8958 was efficiently converted to R-125489 by a hydrolase(s) such as carboxylesterase after intranasal administration to mice, and then R-125489 was slowly eliminated from the respiratory tract. These data support the finding that CS-8958 has potential as a long-acting neuraminidase inhibitor, which probably leads to its significant efficacy as an anti-influenza drug by a single treatment. It is expected that a single inhalation of CS-8958 might be sufficient to treat influenza, in contrast to the dosage regimens of currently available drugs.

REFERENCES


