

Penetration of GS4071, a Novel Influenza Neuraminidase Inhibitor, into Rat Bronchoalveolar Lining Fluid following Oral Administration of the Prodrug GS4104

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GS4071 is a novel potent inhibitor of influenza neuraminidase ($K_i < 1$ nM) with low (<5%) oral bioavailability in animals. An ethyl ester prodrug of GS4071, GS4104, has exhibited good oral bioavailability in rat, mouse, and dog models and is currently being developed for the treatment of influenza A and B virus infections. Since influenza virus replicates primarily in the surface epithelial cells of the respiratory tract, the ability of the prodrug to deliver GS4071 to the bronchoalveolar lining fluid (BALF) following an oral dose of GS4104 should be an important indicator of its potential efficacy. In the present study, we determined the concentration-time profiles of GS4071 in the BALF and plasma of rats following oral administration of GS4104. The BALF was sampled by bronchoalveolar lavage with endogenous urea as a dilution marker. The concentration of GS4071 in BALF reached a peak at 2 h (1 h after the plasma peak) and declined at a slower rate than plasma levels, suggesting slow clearance of drug from the lung acini. The ratios of the area-under-the-curve (AUC) values of GS4071 in BALF to those in plasma were 1.05 for AUC from 0 to 6 h (AUC_{0-6}) and 1.51 for $AUC_{0-\infty}$, indicating significant penetration of the parent drug into the lower respiratory tracts of rats following oral administration of the prodrug. No unchanged GS4104 was detected in BALF.

Influenza viruses are responsible for respiratory illness with significant morbidity and mortality. Currently, influenza is managed mainly by preventive immunization or by symptomatic treatment. The success of immunization is limited by continuous mutations of the influenza virus. The therapeutic use of existing anti-influenza drugs, such as amantadine and ribavirin, is limited by their significant adverse side effects, emergence of resistant viral strains, and lack of activity against influenza B.

Viral neuraminidase (sialidase) is an enzyme responsible for the release of new virus particles from infected cells. Several inhibitors of viral neuraminidase have been reported to be powerful anti-influenza agents (6, 8, 9). A direct (intranasal) application of the neuraminidase inhibitor GG167 (5-acetyl-amino-2,6-anhydro-4-guanidino-3,4,5-trideoxy-D-glycerol-D-galactonon-2-enoic acid) has been reported to produce suppression of the virus in vivo. However, the compound was not effective when given orally or intraperitoneally (6).

GS4071 [(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid] is a novel potent and selective inhibitor of influenza viral neuraminidase ($K_i < 1$ nM) (5). GS4071 was highly effective in animal (mouse) models of infection. GS4104 [ethyl(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate] is an orally bioavailable ethyl ester prodrug of GS4071. Following absorption from the gastrointestinal tract, GS4104 undergoes rapid enzymatic conversion to the active form, GS4071 (4).

Influenza virus replicates primarily in the surface epithelial cells of the respiratory tract (6). Therefore, the presence of active drug in the lung interstitium may be necessary to produce the desired antiviral response. The objective of the present study was to determine the extent of penetration of the neuraminidase inhibitor GS4071 into the lower respiratory

tracts of rats following oral administration of the prodrug. To measure the penetration, the concentration-time profile of GS4071 in the bronchoalveolar lining fluid (BALF) was determined by bronchoalveolar lavage (BAL) and then compared to the plasma concentration-time profile following oral administration of GS4104. Because of the high cellular content of BALF, which includes alveolar macrophages and many types of epithelial cells (1), the measured GS4071 concentration presumably represents both intracellular and extracellular drug. The BAL technique with urea as a dilution marker was validated by measuring BALF and plasma concentrations of the aminoglycoside antibiotic tobramycin after intravenous administration and comparing the results to published data.

MATERIALS AND METHODS

Reagents. GS4071 (RO 64-0802/000), GS4104 (RO 64-0796/001), and GS4057 [(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-cyclopentanoxy)-1-cyclohexene-1-carboxylic acid] were synthesized by Gilead Sciences, Inc. Naphthalenedialdehyde (NDA) and potassium cyanide were purchased from Fluka (Ronkonkoma, N.Y.). All other chemicals and solvents (analytical grade) were purchased from Baxter (Muskegon, Mich.). Water was purified with a Milli-Q UF purification system (Millipore Corp., Bedford, Mass.).

Study design. The in-life phase of the study was conducted in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 86-23) and was approved by an Institutional Animal Care and Use Committee. A group of rats received an intravenous bolus injection of GS4071 (30 mg/kg of body weight) (group 1), and another group of rats received an oral dose of GS4104 (30 mg eq/kg) (group 2). Blood samples were obtained at intervals over 6 h postdosing from each rat in group 1. At specified time points postdosing, the animals in group 2 were anesthetized with an intramuscular injection of ketamine-xylazine. Samples of lavage fluid (containing BALF) and blood were collected from group 2 over the 6 h postdosing (two rats per time point). Blood samples (~0.5 ml) were collected via the orbital plexus, placed in heparinized tubes, and immediately processed for plasma. Plasma samples were frozen at $\leq 20^\circ\text{C}$ until analyzed. The BALF samples were obtained by instillation of a known volume of saline into the lung followed by aspiration of the fluid as described below. The lavage samples were frozen at $\leq 20^\circ\text{C}$ until analyzed.

BALF sample collection. The anesthetized animal was placed dorsally on a dissection board and secured in position by taping its limbs to the board. The trachea was exposed in the midcervical region, and a small incision was made in the trachea (tracheostomy), ensuring that the region was free of blood. A 5-ml

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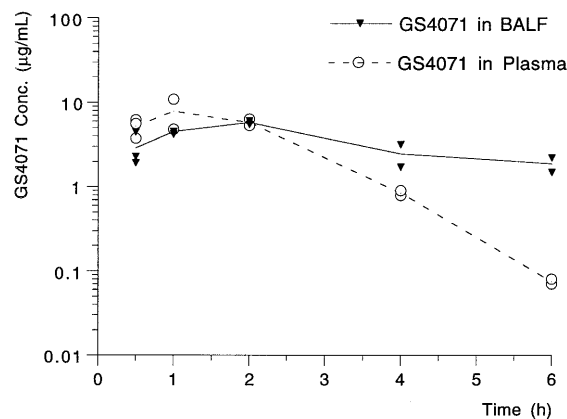


FIG. 1. Concentrations of GS4071 in BALF and plasma following oral administration of 30 mg eq of GS4104/kg (two rats per time point).

Luer-Lok syringe was filled with 4 ml of phosphate-buffered saline (PBS) and fitted with an 18-gauge stainless-steel gavage cannula. The cannula was introduced into the trachea as far as the branching of the bronchioles and temporarily secured in place with a ligature in order to prevent any back flushing of the lavage fluid or possible tissue damage caused by movement of the cannula. The dissection board was placed at a 45° angle, and BAL was performed by slowly instilling the PBS into the lungs via the tracheal cannula, followed by gentle aspiration. The recovered lavage fluid was thus a mixture of BALF and PBS. The dwell time of the instilled fluid was 30 s (from start of instillation to start of aspiration). The exact position of the cannula was verified perfunctorily after instilling a small volume of an aqueous solution of Coomassie brilliant blue G dye (0.5%, wt/vol) followed by the gross examination of the stained lung tissue postmortem. To ensure that BALF was not contaminated with blood from the tracheostomy or damaged lung tissue, concentrations of hemoglobin in the recovered lavage fluid samples were measured by a commercially available colorimetric assay (catalog no. 525-A; Sigma, St. Louis, Mo.).

Determination of GS4071 and GS4104 levels in lavage fluid and plasma.

Plasma samples were diluted 1:10 with PBS, and lavage fluid samples were analyzed undiluted. The samples were analyzed by a fluorescence derivatization high-pressure liquid chromatography (HPLC) assay. An analog of GS4071, GS4057, was used as the internal standard. Samples (80 µl) were placed in 1.5-ml-diameter polypropylene centrifuge tubes, and 300 µl of internal standard (0.5 µg of GS4057/ml in 0.1% trifluoroacetic acid-acetonitrile) was added. The resulting mixture was briefly vortex mixed and then centrifuged for 5 min (Biofuge; 15,000 × g). The supernatants were transferred to screw-cap polypropylene centrifuge tubes and evaporated to dryness under reduced pressure at room temperature (SpeedVac sample concentrator; Savant, Farmingdale, N.Y.). Fifty microliters of a 2-mmol/liter potassium cyanide solution in a 0.25-mol/liter borate-phosphate buffer, pH 6.5, was added, followed by 20 µl of a 10-mmol/liter NDA solution in acetonitrile. The tubes were sealed and incubated at 40°C for 45 min. After incubation, the samples were briefly vortex mixed and then centrifuged for 5 min, and the supernatants were transferred into autosampler vials for HPLC analysis. Separations were performed by a gradient reversed-phase HPLC method. The column was an Inertsil octyldecyl silane-3 column (100 by 4.6 mm; particle size, 5 µm). The column temperature was set at 40°C, the fluorescence detector was set at a 420-nm excitation wavelength and a 472-nm emission wavelength, and the injection volume was 40 µl. Mobile phase A consisted of 50 mM sodium acetate in acetonitrile-water (27:73, vol/vol), and mobile phase B consisted of 50 mM sodium acetate in acetonitrile-water (60:40, vol/vol). The gradient was 100% A for 8 min followed by 100% B for 8 min. The flow rate was maintained at 3 ml/min, and the total run time was 20 min. The retention times were 4.8 min for GS4057, 5.6 min for GS4071, and 14.1 min for GS4104. The method was linear for both GS4071 and GS4104 over the range 0.1 to 5.12 µg/ml ($r^2 > 0.999$), the limit of quantitation was 0.1 µg/ml, and the limit of detection was 0.05 µg/ml for either GS4104 or GS4071. The intrarun precision and accuracy were <10.3 and <12.1%, respectively.

Determination of urea in BALF and plasma. Plasma samples were diluted 1:10 with PBS, and BALF samples were analyzed undiluted. Concentrations of urea in plasma and BALF samples were determined by an enzymatic UV assay with a commercial diagnostic kit (catalog no. 66-UV; Sigma). The ratio of sample volume to reagent volume was 1:50. The method was linear over the range 8 to 256 µg/ml ($r^2 > 0.999$), the limit of quantitation was 8 µg/ml, and the limit of detection was 2 µg/ml. The intrarun precision and accuracy were <5.0 and <7.4%, respectively.

Determination of dilution factor. Following introduction of saline into the lung, the applied volume is subject to change due to absorption of water or dilution with endogenous BALF. In order to assess the true volume of BALF

TABLE 1. Pharmacokinetic parameters for GS4071 in BALF and plasma following oral administration of GS4104 or intravenous administration of GS4071 to rats^a

Agent and body fluid	C_{max} (µg/ml)	T_{max} (h)	$t_{1/2}$ (h) ^b	AUC ₀₋₆ (µg · h/ml)	AUC _{0-∞} (µg · h/ml)	Bioavailability (%)
GS4104						
BALF	5.75	2.00	3.18	19.9	28.7	
Plasma	7.81	1.00	0.73	18.9	19.0	36
GS4071, plasma			1.32	51.8	52.6	100

^a Doses were 30 mg eq of GS4104 per kg and 30 mg of GS4071 per kg. Two rats were tested for GS4071, and two rats per time point were tested for GS4104.

^b Apparent elimination half-lives ($t_{1/2}$) were estimated from 2 to 6 h following oral administration and from 1 to 6 h following intravenous administration.

recovered in the aspirated fluid sample, an endogenous dilution marker can be employed. Urea is distributed evenly throughout the body water, and its local concentrations in BALF and plasma should be identical. Dilution of the BALF with saline was therefore corrected by using endogenous urea as the dilution marker. The recovered lavage fluid, which comprises the BALF diluted with saline, was analyzed for GS4071 and GS4104 by a suitable analytical method. Then the lavage fluid and plasma were analyzed for urea. The concentration of a drug in BALF was calculated by using simple dilution principles as $C_L \times U_{PL}/U_L$, where C_L and U_L are the concentrations of the drug and urea, respectively, in lavage fluid, and U_{PL} is the concentration of urea in plasma. The technique has been used previously to determine the penetration of aminoglycoside antibiotics (tobramycin and gentamicin) into rat BALF (5).

Pharmacokinetic calculations. The pharmacokinetic parameters for GS4071 in BALF and plasma were assessed by using the concentration data determined by the fluorescence derivatization HPLC assay and corrected for dilution by using endogenous urea as a dilution marker. The apparent half-lives of the elimination phase and values for the area under the plasma or BALF concentration-versus-time curve from 0 to 6 h and from 0 h to infinity (AUC₀₋₆ and AUC_{0-∞}, respectively) were calculated by noncompartmental methods.

RESULTS

No unchanged GS4104 was detected in either lavage fluid or plasma following oral administration of GS4104. The concentration-time profiles for GS4071 in the BALF and plasma following a 30-mg eq/kg oral dose of GS4104 are shown in Fig. 1. Figure 2 shows the plasma concentration-time profile following an intravenous dose of 30 mg of GS4071/kg. The pharmacokinetic data for GS4071 in BALF and plasma following oral GS4104 administration are summarized in Table 1 and compared to data for intravenous GS4071.

GS4071 penetrated into BALF following oral administration of GS4104 to rats. From the gross postmortem examination of

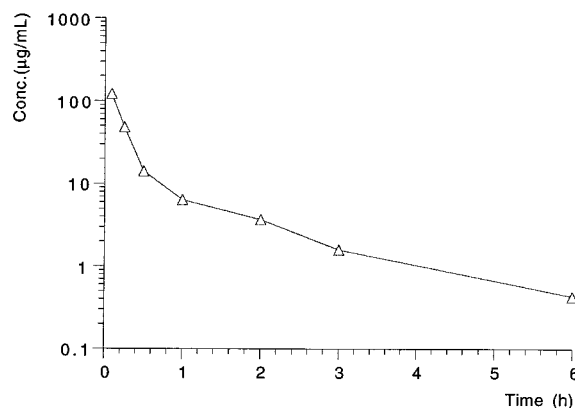


FIG. 2. Mean concentrations of GS4071 in plasma following an intravenous dose of 30 mg of GS4071/kg to rats ($n = 2$).

the stained lung following administration of Coomassie brilliant blue G dye, we found that when the cannula was inserted into the retracted trachea as described in Materials and Methods, it was located in the area of the lower primary bronchus before its branching to the secondary bronchi. A volume of 4 ml of saline instilled during lavage distends the lung, filling the available space. As a result, the technique allows one to sample BALF over nearly the entire area of the lower respiratory tract. Contamination of BALF by blood was shown to be negligible (<0.15%, vol/vol) based on measurement of hemoglobin concentrations. The maximum mean concentration of GS4071 in BALF following oral administration of GS4104 was 5.75 µg/ml (the maximum concentration in BALF was reached 2 h postdosing compared with 1 h in plasma) compared to 7.81 µg/ml in plasma. At 6 h postdosing, concentrations of GS4071 in BALF were approximately 20-fold higher than those in plasma. The penetration ratio, calculated as the ratio of the AUC₀₋₆ value for GS4071 concentration in BALF to that for GS4071 concentration in plasma following oral administration of GS4104, was 1.05. Based on AUC_{0-∞} values, the ratio was 1.51. The apparent terminal half-life for GS4071 in BALF was more than fourfold longer than that in plasma. However, the data were limited to 6 h postdosing and probably underestimate the true elimination half-lives. The plasma clearance of GS4071 following an intravenous dose of GS4071 was 570 ml/h/kg, and the apparent elimination half-life was 1.32 h. The estimated oral bioavailability of GS4071 following administration of a 30-mg eq/kg dose of GS4104 was approximately 36%. This value is consistent with the previously reported value of 35.4 ± 10.6% in rats for an oral dose of 10 mg eq of GS4104/kg.

DISCUSSION

BAL has been used to study both the volume and the cellular and chemical composition of the BALF in animals and humans (5, 14). A number of studies have used BAL for measuring the concentrations of drugs such as antimicrobial agents in the alveolar lining fluid (3, 8, 11, 12). Although BAL is a very useful technique for measuring the concentration of chemicals in the BALF, two major drawbacks have prevented wider use of the technique. First, the instilled saline causes unpredictable dilution of BALF. To compensate for this dilution, a dilution marker has to be used. In the present study, endogenous urea was used as a marker of dilution. Urea has been shown to be a reliable marker for the determination of the BALF volume and allows one to calculate the concentration of drugs by using simple dilutional principles (5). Second, concentrations of the investigated compound in the lavage fluid, which is typically diluted at least 20- to 40-fold, are very low, requiring highly sensitive detection techniques. In the present study, we used a sensitive fluorescence derivatization HPLC assay for determination of GS4071 and GS4104. The assay provided sufficient sensitivity for detection of the drugs in either plasma or diluted BALF.

From our study, the penetration of GS4071 into the BALF of rats, expressed as the ratio of the AUC₀₋₆ in BALF to the AUC₀₋₆ in plasma, was 1.05. The shape of the concentration-time profile in BALF was different from that in plasma, suggesting that the pharmacokinetics of GS4071 in the two fluids may be different. Apparently, the elimination of GS4071 from plasma occurs at a faster rate than that from the lung interstitium. The observed levels of GS4071 in BALF suggest that the antiviral effect may be more sustained than would be anticipated from monitoring plasma levels.

There could be many reasons for the different elimination rates of GS4071 from lung and plasma. It has been reported,

for instance, that a large number of basic, lipophilic drugs are efficiently removed from the circulation and accumulate in the lung (11). The uptake of these compounds has been reported to take place through passive, concentration-dependent diffusion from blood to the BALF, across the alveolar capillary membrane. It is followed by intracellular binding within the lung (7). These compounds may be eliminated from the lower respiratory tract by lymphatic drainage, the mucociliary transport mechanism, or reabsorption and back diffusion into the blood (2), as well as through enzymatic degradation. It has also been proposed that the accumulation of basic amines in the lung may involve distribution into lysosomes, where the lower pH causes their ionization and thus prevents further diffusion (9).

Viral infection of the lung is usually accompanied by inflammation that is characterized by various changes in pulmonary tissue. Some of these changes may also enhance the transport of drugs across the inflamed barriers (7). For instance, increased blood flow, vascular dilation, and resulting increased vascular permeability may increase passive, gradient-driven diffusion of a drug into the lung epithelium. A clear relationship between the degree of inflammation and the degree of drug concentration in the bronchial secretions was shown for erythromycin, clindamycin, oleandomycin, gentamicin, and tobramycin. Based on these studies, it can be expected that the concentration of GS4071 in BALF may be even higher in subjects with infected lung tissues.

In conclusion, the present study has demonstrated that oral administration of the prodrug GS4104 leads to significant penetration of the active influenza neuraminidase inhibitor GS4071 into the fluid lining the lungs of rats. The pharmacokinetic behavior of GS4071 in BALF differs from that in plasma, with a slower elimination from alveolar acini. Since influenza virus replicates primarily in the surface epithelial cells of the respiratory tract, the presence of GS4071 in BALF may be an important indicator of its potential antiviral efficacy. These data suggest that oral GS4104 will achieve a sustained antiviral concentration of GS4071 in the fluid lining the lungs. The data also suggest that monitoring of GS4071 plasma levels may underestimate the duration of its activity in lungs.

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