Identification of GS 4104 as an Orally Bioavailable Prodrug of the Influenza Virus Neuraminidase Inhibitor GS 4071

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GS 4071 is a potent carbocyclic transition-state analog inhibitor of influenza virus neuraminidase with activity against both influenza A and B viruses in vitro. GS 4116, the guanidino analog of GS 4071, is a 10-fold more potent inhibitor of influenza virus replication in tissue culture than GS 4071. In this study we determined the oral bioavailabilities of GS 4071, GS 4116, and their respective ethyl ester prodrugs in rats. Both parent compounds and the prodrug of the guanidino analog exhibited poor oral bioavailability (2 to 4%) and low peak concentrations in plasma (Cmax; Cmax < 0.06 μg/ml). In contrast, GS 4014, the ethyl ester prodrug of GS 4071, exhibited good oral bioavailability (35%) as GS 4071 and high Cmax's of GS 4071 (Cmax = 0.47 μg/ml) which are 150 times the concentration necessary to inhibit influenza virus neuraminidase activity by 90%. The bioavailability of GS 4014 as GS 4071 was also determined in mice (30%), ferrets (11%), and dogs (73%). The plasma of all four species exhibited high, sustained concentrations of GS 4071 such that at 12 h postdosing the concentrations of GS 4071 in plasma exceeded those necessary to inhibit influenza virus neuraminidase activity by 90%. These results demonstrate that GS 4014 is an orally bioavailable prodrug of GS 4071 in animals and that it has the potential to be an oral agent for the prevention and treatment of influenza A and B virus infections in humans.

Influenza virus infections, which have plagued humans throughout history (14), continue to be a serious health concern in terms of both morbidity and mortality (1). Current options for the control of influenza virus infections have limitations. Vaccines provide only partial protection due to their underutilization and the variability in the antigenic determinants of the surface glycoproteins (5, 29). Amantadine and rimantadine, the only antiviral agents approved for use for the treatment of influenza A virus infections, are not active against influenza B viruses, and virulent strains resistant to these agents develop quickly in the clinical setting (2, 7). Thus, there remains a need to identify new antiviral agents that can be used to prevent and treat influenza virus infections.

Recently, there has been a great deal of interest in the influenza virus neuraminidase (sialidase) as a potential antiviral target. This enzyme, which is expressed on the surfaces of influenza A and B viruses, hydrolyzes terminal sialic acid residues from glycoproteins, glycolipids, and oligosaccharides. The influenza virus neuraminidase is thought to be required for the elution of newly synthesized virions from infected cells and thus is essential for virus replication (17, 21, 22). In addition, the neuraminidase may facilitate movement of the virus through the mucus of the respiratory tract (4, 13).

Several sialic acid-based neuraminidase inhibitors have been shown to inhibit influenza A and B virus replication in vitro (22, 32, 35). Zanamivir (GG167) (Fig. 1), the most potent of these sialic acid-based inhibitors, has demonstrated efficacy in animal models of influenza virus infection (26, 27, 32, 35) and in studies with humans (9, 10), and it is under clinical development for the treatment of influenza A and B virus infections. However, due to its poor oral bioavailability, zanamivir is applied topically to the respiratory tract as an intranasal spray or inhalant (9, 10, 26, 27).

In an attempt to identify potentially orally bioavailable influenza virus neuraminidase inhibitors, we have designed and synthesized a series of carbocyclic transition-state analog inhibitors of the influenza virus neuraminidases in which lipophilic side chains replace the polar glycerol moiety of the sialic acid-based inhibitors (12). GS 4071 (Fig. 1), the lead candidate from this series, is comparable to zanamivir in terms of its ability to inhibit influenza virus neuraminidase activity (Ki, ~1 nM) and virus replication when tested in vitro (12, 20). However, because GS 4071 lacks the polar guanidino and glycerol groups present in zanamivir, we postulated that GS 4071 or a prodrug of GS 4071 might be orally bioavailable.

In this study we have investigated the oral bioavailability of GS 4071 and, for comparison, its more potent guanidino analog, GS 4116 (12, 19) (Fig. 1). On the basis of the observation that esterification of carboxylic groups has increased the oral bioavailabilities of many compounds (30), we have also determined the bioavailabilities of GS 4071 and GS 4116 following oral administration of their ethyl ester prodrugs.

MATERIALS AND METHODS

Compounds and reagents. The bioavailabilities of the following five compounds (Fig. 1) were determined in this study: GS 4071; GS 4104, an ethyl ester prodrug of GS 4071; GS 4116, the guanidino analog of GS 4071; GS 4109, an ethyl ester prodrug of GS 4116; and zanamivir (GG167). These compounds were synthesized at Gilead Sciences by previously published procedures (12, 33). Rat plasma and pooled human plasma were purchased from Pel-Freez Biologicals (Rogers, Ark.) and George King Bio-medicals (Overland Park, Kans.), respectively. Influenza A/PR/8/34 (H1N1) virus was from the American Type Culture Collection (Rockville, Md.). The fluorescent neuraminidase substrate 2′-[(methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUN) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless indicated.

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Quantitative neuraminidase assay to determine inhibitor concentration. Neuraminidase activity was determined in an enzymatic assay with purified influenza A/PR/8/34 (H1N1) virus as the source of viral neuraminidase. The virus was grown in embryonated hen eggs and was purified from allantoic fluid with a sucrose gradient (15). A purified virus was harvested, resuspended in 10 mM Tris (pH 7.5) containing 0.1 M NaCl and 60% (vol/vol) glycerol, and stored at −70°C until use.

The fluorescent substrate MUN was used to measure the enzymatic activity of the viral neuraminidase (25, 23). Briefly, 10 μl of virus, diluted in 2× assay buffer (66 mM MES [2-[N-(morpholino)ethanesulfonic acid; pH 6.5] containing 8 mM CaCl2), was mixed with an equal volume of water or animal plasma containing inhibitor, and the mixture was preincubated at room temperature for 30 min. The enzymatic reaction was initiated with the addition of 80 μl of assay buffer containing 12.5 μM MUN. After 8-sec incubation at 37°C, the reaction was terminated with the addition of 150 μl of 0.014 N NaOH in 83% ethanol. The stopped reaction mixtures were immediately transferred to a 96-well, white, flat-bottom plate, and the relative amount of the fluorescent product 4-methylumbelliferone produced in each sample was determined with a LS 50B luminescence spectrometer (Perkin-Elmer Limited, Beaconsfield, United Kingdom) with an excitation wavelength of 360 nm and an emission wavelength of 448 nm.

The enzymatic assay is most sensitive at about the IC50 of each inhibitor, the samples were diluted until the neuraminidase activity fell between 30 and 70% of that of the uninhibited activity, i.e., near the IC50 of the parent compound. A standard curve for the parent compound was constructed each time that the plasma samples were assayed. Duplicate aliquots of a limited number of samples were assayed for GS 4071 and GS 4104 to their respective parent compounds. Since the quantitative enzymatic assay detects parent compound, this conclusion is also supported by the fact that we have not identified metabolites of GS 4104, and zanamivir (GG167). Ac, acetyl.

The plasma samples from animals receiving oral prodrug (GS 4104 or GS 4109) were assayed in two ways to determine the concentration of parent compound and total compound. One aliquot was diluted and assayed in buffer to detect parent compound. A second aliquot was diluted in rat plasma and was incubated at 37°C for 30 min to hydrolyze any remaining prodrug and allow the detection parent compound. A second aliquot was diluted in rat plasma and was incubated at 37°C for 30 min to hydrolyze any remaining prodrug and allow the detection of parent compound. A second aliquot was diluted in rat plasma and was incubated at 37°C for 30 min to hydrolyze any remaining prodrug and allow the detection of parent compound.

assay, with an IC50 of approximately 100 nM (Fig. 2). GS 4109 ethyl ester prodrug of GS 4071, exhibited poor activity in this vitro neuraminidase assay (12, 20). In contrast, GS 4104, the prodrug of GS 4116, the guanidino derivative from animals given drug orally (or values obtained at 4 and 12 h for orally administered zanamivir). AUCi.v. is the AUC of GS 4071 after i.v. administration, and dose i.v. is the i.v. was calculated as CL

\[ \text{F} = \frac{\left[ \text{AUC}_{\text{p.o.}} \right]}{\left[ \text{dose}_{\text{p.o.}} \right]} \times \frac{\left[ \text{AUC}_{\text{i.v.}} \right]}{\left[ \text{dose}_{\text{i.v.}} \right]} \]

where \( \text{AUC}_{\text{i.v.}} \) is the dose of GS 4104 in milligram equivalents of GS 4071 per kilogram, \( \text{AUC}_{\text{p.o.}} \) is the AUC of GS 4071 after oral (p.o.) administration of GS 4104, \( \text{dose}_{\text{p.o.}} \) is the i.v. dose of GS 4071. Total clearance (CL) from plasma was calculated as dose/ \( \text{AUC}_{\text{i.v.}} \). The volume of distribution at steady-state (\( V_{\text{ss}} \)) of drug administered i.v. was calculated as CL \times MRT.

### RESULTS

Neuraminidase inhibitor prodrugs are readily hydrolyzed to parent compound in rat plasma. As shown in Fig. 2, GS 4071 is a potent inhibitor of influenza virus neuraminidase activity, with an IC50 and an IC90 of 2 and 10 nM, respectively, in an in vitro neuraminidase assay (12, 20). In contrast, GS 4104, the ethyl ester prodrug of GS 4071, exhibited poor activity in this assay, with an IC50 of approximately 100 nM (Fig. 2). GS 4109 is an ethyl ester prodrug of GS 4116, the guanidino derivative of GS 4071, and also was a poor inhibitor of influenza virus neuraminidase activity in the in vitro enzymatic assay, with no inhibitory activity detected at a concentration of 100 nM; for comparison, the IC50 and the IC90 of GS 4116 in this assay were 0.9 and 5 nM, respectively. The parent compounds and the prodrugs were stable under the assay conditions, with no change in potency observed for either of the compounds after 24 h at room temperature (data not shown).

Ethyl ester prodrugs were chosen because, once absorbed, they are readily converted to the parent compound by esterase activity in plasma and tissues (30). To test whether plasma esterase activity could convert the neuraminidase inhibitor prodrugs to the parent compounds, the prodrugs were incubated with rat plasma at 37°C for 30 min prior to assaying their inhibitory activities in the enzymatic assay. As indicated in Fig. 2, the inhibitory activity of GS 4104 was indistinguishable from that of the parent compound following incubation with rat plasma, indicating that the esterase activity in rat plasma can efficiently convert the prodrug to GS 4071. GS 4104 is rapidly hydrolyzed to the parent compound, as indicated by the observation that full conversion occurred in less than 5 min at 37°C. Similar results were obtained for the prodrug of GS 4116 (data not shown). The presence of rat plasma at a final concentration of 10% of the total reaction volume did not affect the inhibitory activity of GS 4071, GS 4116, or zanamivir as indicated by the fact that the IC50 of these compounds were identical when rat plasma was present or absent from the reaction mixture (data not shown).

In contrast to the rapid and complete conversion of the prodrugs to their respective parent compounds in rat plasma, the prodrugs were relatively stable in human, ferret, and dog plasma. In human and ferret plasma, 15 and 10% of the prodrug was converted to the parent compound, respectively, during a 30-min incubation at 37°C. Essentially none of the prodrug was converted to the parent compound during a 30-min incubation in dog plasma. None of the animal plasmas had an effect on the activity of either parent compound or zanamivir in the enzymatic assay when the plasma was present at a level of 10% of the final reaction volume.

GS 4104 is orally bioavailable in rats. To determine the oral bioavailabilities of GS 4071, GS 4116, and their respective ethyl ester prodrugs, GS 4104 and GS 4109, the four compounds were administered to rats by the i.v. and oral routes. The oral bioavailability of zanamivir was also determined for comparison. A summary of the values of the pharmacokinetic

### TABLE 1. Values of pharmacokinetic parameters for neuraminidase inhibitors administered as 10 mg-eq/kg doses to rats

<table>
<thead>
<tr>
<th>Compound</th>
<th>Route</th>
<th>( \text{AUC}_{0-24} ) (mg·h/liter)</th>
<th>( \text{C}_{\text{max}} ) (µg/ml)</th>
<th>( t_{\text{max}} ) (h)</th>
<th>CL (liters/h/kg)</th>
<th>( t_{1/2} ) (h)</th>
<th>( V_{\text{ss}} ) (liters/kg)</th>
<th>( F ) as parent compound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS 4071</td>
<td>i.v.</td>
<td>8.4 ± 1.4</td>
<td>0.3 ± 0.1</td>
<td>4.0 ± 1.6</td>
<td>1.5 ± 0.3</td>
<td>1.6 ± 0.4</td>
<td>1.3 ± 0.6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>0.3 ± 0.1</td>
<td>0.03 ± 0.00</td>
<td>4.0 ± 1.6</td>
<td>1.6 ± 0.4</td>
<td>1.0 ± 0.5</td>
<td>1.3 ± 0.6</td>
<td>4.3 ± 1.6</td>
</tr>
<tr>
<td>GS 4104</td>
<td>i.v.</td>
<td>6.6 ± 1.2</td>
<td>3.0 ± 0.9</td>
<td>1.6 ± 1.6</td>
<td>1.8 ± 0.3</td>
<td>6.2 ± 2.3</td>
<td>3.1 ± 0.9</td>
<td>79 ± 14</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>3.0 ± 0.9</td>
<td>0.47 ± 0.13</td>
<td>1.6 ± 1.6</td>
<td>6.2 ± 2.3</td>
<td>7.0 ± 0.6</td>
<td>3.1 ± 0.9</td>
<td>35 ± 11</td>
</tr>
<tr>
<td>GS 4116</td>
<td>i.v.</td>
<td>9.0 ± 1.7</td>
<td>0.4 ± 0.1</td>
<td>1.9 ± 1.5</td>
<td>1.3 ± 0.2</td>
<td>5.7 ± 0.8</td>
<td>1.1 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>0.4 ± 0.1</td>
<td>0.06 ± 0.01</td>
<td>1.9 ± 1.5</td>
<td>20.1 ± 7.0</td>
<td>21.0 ± 7.0</td>
<td>4.0 ± 1.0</td>
<td>100</td>
</tr>
<tr>
<td>GS 4109</td>
<td>i.v.</td>
<td>9.2 ± 0.9</td>
<td>0.2 ± 0.1</td>
<td>0.8 ± 0.8</td>
<td>1.2 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>1.6 ± 0.3</td>
<td>102 ± 10</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>0.2 ± 0.1</td>
<td>0.03 ± 0.00</td>
<td>0.8 ± 0.8</td>
<td>6.0 ± 0.1</td>
<td>18.0 ± 5.3</td>
<td>21.0 ± 1.1</td>
<td>102 ± 10</td>
</tr>
<tr>
<td>Zanamivir (GG167)</td>
<td>i.v.</td>
<td>5.5 ± 1.5</td>
<td>0.2 ± 0.2</td>
<td>1.2 ± 0.7</td>
<td>1.9 ± 0.6</td>
<td>1.1 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>0.2 ± 0.2</td>
<td>0.06 ± 0.01</td>
<td>1.2 ± 0.7</td>
<td>1.8 ± 0.6</td>
<td>1.8 ± 0.6</td>
<td>3.7 ± 2.3</td>
<td>100</td>
</tr>
</tbody>
</table>

*Values are means ± standard deviations for four animals on the basis of the levels of parent compound in plasma.

The terminal \( t_{\text{1/2}} \) was estimated on the basis of the values at 6 and 12 h for the samples from animals given drug i.v. and the values at 12 and 24 h for the samples from animals given drug orally (or values obtained at 4 and 12 h for orally administered zanamivir).
administration of a single 10-mg/kg dose (datum points represent the means ± standard deviations for four animals). (B) Concentration of GS 4116 following i.v. (○) or oral (□) administration of a 10-mg/kg dose of GS 4116 or oral administration of a 10-mg-eq/kg dose of the prodrug GS 4104 (□) (datum points represent the means ± standard deviations for four animals). (C) Concentration of zanamivir following i.v. (○) or oral (□) administration of a single 10-mg/kg dose (datum points represent the means ± standard deviations for three animals). A horizontal line indicating a concentration in plasma of 10 nM, the IC₉₀ of GS 4071 in the influenza virus neuraminidase enzymatic assay (Fig. 2), is shown for reference.

The plasma concentration-versus-time profile of the parent compound following the i.v. administration of GS 4071 to rats, the concentrations of GS 4071 in plasma declined in a multieponential manner (Fig. 3A), with an apparent elimination t₁/₂ of 1.6 h. The CL of GS 4071 was 1.47 liters/kg and the V₅ₐₓ was 1.26 liters/kg. Following oral administration of a 10-mg/kg dose of GS 4071 to rats, the Cₘₕₗₓ of GS 4071 was 0.03 µg/ml (105 nM) at 4.0 h postdosing (Fig. 3A). The F of GS 4071, based on a comparison of the AUC of GS 4071 for the oral and i.v. doses, was 4.3%.

The plasma concentration-versus-time profile of the parent compound following the i.v. administration of GS 4104 was similar to that following the administration of the parent compound (data not shown), as were most of the pharmacokinetic parameters. However, V₅ₐₓ was much greater for GS 4104 (3.05 liters/kg) than for the parent compound (1.26 liters/kg). The larger V₅ₐₓ for GS 4104 may indicate that it is distributed outside the vascular space more efficiently than the parent compound. Following oral administration of GS 4104, the Cₘₕₗₓ (0.52 µg/ml; 1,830 nM) and F (35%) of the parent compound were approximately 10-fold higher than those observed following the oral administration of GS 4071 itself. The concentration of parent compound in plasma 12 h after the oral administration of GS 4104 was 0.009 µg/ml (32 nM), which is three times the concentration required to inhibit 90% of the enzymatic activity in the in vitro assay (see above). No prodrug was detected in any of the samples.

Figure 3B indicates the plasma concentration-versus-time profile for GS 4116, the guanidino analog of GS 4071, following the i.v. or oral administration of GS 4116 or the oral administration of the prodrug GS 4109. The results obtained for the parent compound are similar to those obtained for GS 4071, with GS 4116 having an F of 4.0%. However, in contrast to what was observed for GS 4104, the bioavailability of GS 4116 from its oral prodrug was poor (2.1%). Again, no prodrug was detected in any of the samples.

The plasma concentration-versus-time profiles for zanamivir administered by the i.v. and oral routes are presented in Fig. 3C and are consistent with the results obtained following the i.v. or oral administration of zanamivir to mice (26). The F of zanamivir (3.7%) was comparable to those of GS 4071 and GS 4116 (Table 1).

GS 4104 is detected in the plasma of dogs and ferrets. To ensure that the bioavailability of the parent compound from orally administered GS 4104 was not specific to rats, similar experiments were performed with mice, dogs, and ferrets. Mice and ferrets were included because they are used in animal models of influenza virus infection, and dogs were chosen because they are often used as a nonrodent species in preclinical studies. A comparison of the values of the pharmacokinetic parameters determined for the four species are summarized in Table 2.

Compared to the rat, the plasma concentration-versus-time profile for the parent compound in mice administered an oral dose of GS 4104 shows a comparable Tₘₕₗₓ and a comparable rate of decline from the Cₘₕₗₓ (data not shown). Mice were also similar to rats in that GS 4104 was not detected in any of the samples. The bioavailability of GS 4071 from orally administered GS 4104 in mice was 30%.

The plasma concentration-versus-time profile for the parent compound in dogs orally given the prodrug was different from those observed in mice and rats (Fig. 4) in that the concentrations of the parent compound in plasma were sustained for a longer period in dogs than in rats or mice. Dogs also differed from rats and mice in that substantial amounts of GS 4104 were detected in the plasma samples; this was especially true during the first 4 h postdosing, during which time GS 4104 was the predominant species. The F of the parent compound from GS 4104 in dogs was 73%.

Among the four animal species tested, ferrets had the lowest bioavailability of parent compound (11%) from orally administered GS 4104. In ferrets the rate of elimination of GS 4071 derived from oral prodrug was similar to that seen in dogs (Fig. 4). Ferrets were also similar to dogs in that intact GS 4104 was present in their plasma samples. However, whereas the peak concentrations of GS 4071 and prodrug in plasma were not dramatically different in dogs, the concentration of prodrug in the ferret plasma samples obtained during the first 2 to 3 h postdosing was substantially greater than that of GS 4071.
DISCUSSION

The influenza viruses are well understood but poorly controlled human pathogens which are responsible for approximately 30,000 deaths each year in the United States alone (18). The demonstration that zanamivir, a potent and selective inhibitor of the influenza virus neuraminidases in vitro, is effective both in animal models of influenza infection and in challenge studies with humans (10, 26, 27, 32, 35) indicates that the influenza virus neuraminidase is a valid antiviral target. However, due to its poor oral bioavailability and rapid elimination, zanamivir is administered topically to the respiratory tract as a nasal spray or inhalant. We have identified GS 4071, a member of a series of novel carbocyclic transition-state analog inhibitors of the influenza virus neuraminidases, as a lead compound with the potential to maintain potency while being orally bioavailable (12).

Although GS 4071 is more lipophilic than zanamivir, with an amino group replacing the guanidino group of zanamivir and a lipophilic 3-pentyloxy group replacing the polar glycerol group, GS 4071 itself exhibited poor oral bioavailability in rats. However, the parent compound was bioavailable (F = 11 to 73%) in rats, mice, dogs, and ferrets following the oral administration of GS 4104, the ethyl ester prodrug of GS 4071. Ester prodrugs have been used to increase the bioavailability of carboxylic acid-containing compounds (30). Once absorbed, these prodrugs are readily hydrolyzed by a variety of esterases present in the blood and tissues of many species and the parent compound is released (16, 24). Notably, esterification of the carboxylic group of GS 4116, the more polar guanidino analog of GS 4071, did not increase its bioavailability.

The results presented in this report indicate that oral administration of GS 4104 led to higher and more sustained concentrations of the active form of the drug in plasma than did oral administration of GS 4071 itself. This was particularly evident in dogs and ferrets, the species with detectable levels of intact prodrug in their plasma. The observation that the apparent terminal t1/2 of GS 4071 following oral administration to rats (10.6 h) was significantly longer than that following intravenous dosing (1.6 h) indicates that the elimination of GS 4071 is rate limited by slow oral absorption of the polar parent mole-

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound</th>
<th>Dose (mg-eq/kg), route of administration</th>
<th>AUC0–24 (mg • h/liter)</th>
<th>Cmax (µg/ml)</th>
<th>Tmax (h)</th>
<th>CL (liters/h/kg)</th>
<th>t1/2 (h)</th>
<th>VSS (liters/kg)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse (n = 3)</td>
<td>GS 4071 10, i.v.</td>
<td>13</td>
<td>0.77</td>
<td>NDa</td>
<td>3.9</td>
<td>ND</td>
<td>3.9</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GS 4104 10, oral</td>
<td>3.9</td>
<td>1.2</td>
<td>1.0</td>
<td>0.77</td>
<td>ND</td>
<td>3.9</td>
<td>30</td>
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<tr>
<td>Rat (n = 4)</td>
<td>GS 4071 10, i.v.</td>
<td>8.4</td>
<td>4.7</td>
<td>1.6</td>
<td>1.5</td>
<td>1.6</td>
<td>1.3</td>
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<td>GS 4104 10, oral</td>
<td>3.0</td>
<td>0.47</td>
<td>2.0</td>
<td>0.33</td>
<td>0.6</td>
<td>0.28</td>
<td>11</td>
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<td>Ferret (n = 3)</td>
<td>GS 4071 1, i.v.</td>
<td>2.2</td>
<td>2.2</td>
<td>1.6</td>
<td>0.32</td>
<td>1.8</td>
<td>0.56</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GS 4104 5, oral</td>
<td>1.2</td>
<td>0.20</td>
<td>2.0</td>
<td>7.0</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Dog (n = 5)</td>
<td>GS 4071 5, i.v.</td>
<td>18</td>
<td>1.2</td>
<td>3.8</td>
<td>11</td>
<td>3.7</td>
<td>3.7</td>
<td>73</td>
<td></td>
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<tr>
<td></td>
<td>GS 4104 5, oral</td>
<td>11</td>
<td>1.2</td>
<td>3.8</td>
<td>11</td>
<td>3.7</td>
<td>3.7</td>
<td>73</td>
<td></td>
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</table>

a Values are means and are based on the levels of GS 4071 in plasma.
b ND, not determined.

FIG. 4. Concentration-time profiles of GS 4071 and the prodrug GS 4104 in dog and ferret plasma. (A) Concentration of GS 4071 (●) in dog plasma following administration of an i.v. dose (5 mg/kg) of GS 4071 or the concentration of GS 4071 (○) and GS 4104 (▲) in dog plasma following administration of an oral dose (5 mg-eq/kg) of the prodrug GS 4104 (datum points represent the means ± standard deviations for five animals). (B) Concentration of GS 4071 (●) in ferret plasma following administration of an i.v. dose (1 mg/kg) of GS 4071 or the concentration of GS 4071 (○) and GS 4104 (▲) in ferret plasma following administration of an oral dose (5 mg-eq/kg) of the prodrug GS 4104 (datum points represent the means ± standard deviations for three animals). A horizontal line indicating a concentration in plasma of 10 nM, the IC50 of GS 4071 in the influenza virus neuraminidase enzymatic assay (Fig. 2), is shown for reference.
cule (flip-flop kinetics). In the case of the prodrug GS 4104, i.v. and oral administration to rats gave similar apparent terminal \( t_{1/2} \) for the parent (6.2 and 7.0 h, respectively). These data suggest that elimination of the parent compound after administration of prodrug is rate limited by conversion to GS 4071 rather than by absorption. While the stability of the prodrug in plasma would be expected to affect its rate of conversion to GS 4071, the ability of prodrug to aid delivery of the compound to extravascular tissues may also play a role in maintaining the concentrations of GS 4071 in plasma. For example, it has recently been demonstrated that GS 4071 binds selectively to the lung tissue of rats (6), and slow release of prodrug from this or other tissue compartments may also contribute to the longer \( t_{1/2} \) of the parent compound observed after the administration of prodrug.

The ability of GS 4104, like several other basic lipophilic compounds containing a primary amino group (3, 25, 31), to accumulate in lung tissue may also affect its antiviral activity at the primary site of influenza virus infection and replication. Consistent with this hypothesis, Eisenberg et al. (6) have demonstrated that GS 4071 is present in bronchoalveolar lavage fluid following oral administration of the prodrug to rats. Of particular interest, the peak concentration of GS 4071 in the bronchoalveolar lavage fluid was similar to that in the plasma, but it declined more slowly compared to the rate of decline in plasma, indicating that the antiviral activity in lung tissue may be more persistent than is suggested by the plasma concentration-versus-time profile (6). Because these experiments were carried out with rats, which do not have detectable levels of circulating prodrug in plasma, we would anticipate that the accumulation of prodrug in lung tissue might be even more pronounced in animal species such as dogs or ferrets which have greater amounts of circulating prodrug. In this respect, it is noteworthy that prodrug is detected in human plasma samples following the oral administration of GS 4104 (34).

In summary, we have demonstrated that GS 4104 is an orally bioavailable prodrug of the potent influenza virus neuraminidase inhibitor GS 4071 in each of the four animal species tested. We have also demonstrated that in each of the four animal species, including ferrets, in which the lowest bioavailabil-

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