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Antiviral Research 37 (1998) 107–120

Antiviral Research

Inhibition of influenza virus infections in mice by GS4104, an orally effective influenza virus neuraminidase inhibitor

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Received 26 September 1997; accepted 14 November 1997

Abstract

The carbocyclic transition state sialic acid analog GS4071 ([3*R*,4*R*,5*S*]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexane-1-carboxylic acid), a potent influenza virus neuraminidase inhibitor, was highly inhibitory to influenza A/NWS/33 (H1N1), A/Victoria/3/75 (H3N2), A/Shangdong/09/93 (H3N2) and B/Hong Kong/5/72 viruses in Madin Darby canine kidney (MDCK) cells. The 50% effective concentrations in these experiments ranged from 1.8 to 59.5 μM, with no cytotoxicity evident at 1000 μM, using inhibition of viral cytopathic effect determined visually and by neutral red dye uptake. The ethyl ester prodrug of GS4071, GS4104, administered by oral gavage (p.o.), had significant inhibitory effects on infections in mice induced by these viruses. Antiviral effects were seen as prevention of death, increase in mean day to death, inhibition of decline of arterial oxygen saturation, lessened lung consolidation and inhibition of infectious virus recovered from the lungs. No toxicity was seen in dosages up to 100 mg/kg/day (highest evaluated). Comparison experiments run versus the influenza A (H1N1) virus-induced infection using GS4104, GS4071 and the neuraminidase inhibitor zanamivir (GG167, 4-guanidino-Neu5Ac2en), all administered p.o., indicated a 10-fold or greater potency for inhibiting the infection by GS4104. The minimum effective dosage for GS4104 was 0.1 mg/kg/day, with the compound administered twice daily for 5 days beginning 4 h pre-virus exposure. Oral therapy with GS4104 could be delayed from 48 to at least 60 h after exposure of mice to influenza A (H1N1) virus and still render a significant antiviral effect, the time of delay being dependent on the viral challenge dose. Intranasal instillation of GS4071 and GG167 to mice infected with influenza virus was highly inhibitory to the infection, the minimum effective dosages to significantly prevent death being 0.01 mg/kg/day for GS4071 and 0.1 mg/kg/day for GG167. Caging of infected mice treated with 10 mg/kg/day of GS4104 with infected saline-treated animals did not transfer any influenza-inhibitory effect to the latter animals. These data provide strong evidence of the potential of orally administered GS4104 for treatment of influenza A and B virus infections in humans.

Keywords: Influenza virus; GS4104; GS4071; Neuraminidase inhibitor; Zanamivir; GG167; Antiviral

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0166-3542/98/$19.00
PII S0166-3542(97)00065-X

1. Introduction

Control of human influenza virus infections continues to be a major public health goal. The only antiviral drugs now available for prophylaxis and treatment of influenza are amantadine and rimantadine and these are ineffective against influenza B virus infections (Douglas, 1990). In addition, their clinical use is further limited by the rapid emergence of resistant virus mutants in the clinic (Hayden and Couch, 1992). Due to the rapid changes which occur in the antigenic determinants of the influenza virus, vaccine efficacy is also curtailed (Couch et al., 1996).

Recent studies have indicated that the potent inhibitor of influenza A and B viral neuraminidases, 4-guanidino-Neu5Ac2en (GG167, zanamivir, Fig. 1), has strong antiviral efficacy against both influenza A and B viruses in vitro and inhibits influenza virus infections in animal models (Von Itzstein et al., 1993; Woods et al., 1993; Hayden et al., 1994; Thomas et al., 1994; Ryan et al., 1994, 1995). Clinical influenza challenge studies using zanamivir have also demonstrated significant disease-inhibitory efficacy (Hayden et al., 1996). Due to poor oral bioavailability and rapid renal excretion, however, the clinical use of this material has been limited to topical application to the respiratory tract (Ryan et al., 1994, 1995; Hayden et al., 1996).

A series of carbocyclic transition-state sialic acid analogs have been synthesized in order to develop a potentially orally bioavailable influenza virus neuraminidase inhibitor. Replacement of the polar glycerol moiety of zanamivir with lipophilic side chains has resulted in the analog [3*R*,4*R*,5*S*]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexane-1-carboxylic acid, (GS4071, Fig. 1) which is as potent an inhibitor of influenza virus neuraminidase activity and in vitro influenza virus replication as zanamivir (Hayden and Rollins, 1997; Kim et al., 1997; Mendel et al., 1997 and Mendel et al., manuscript submitted). The ethyl ester derivative of GS4071, GS4104 (Fig. 1) has been shown to be well absorbed orally, with high and persisting serum levels of the parent compound, GS4071 occurring (Li et al., 1997 and Li et al., manuscript submitted). This report will focus primarily on the effect of both GS4071 and GS4104 on infections induced in mice by influenza A and B viruses.

2. Materials and methods

2.1. Compounds

GS4071, GS4104 and GG167 were synthesized by Gilead Sciences (Foster City, CA) using previously published procedures (Von Itzstein et al., 1994; Kim et al., 1997). Ribavirin was provided by ICN Pharmaceuticals (Costa Mesa, CA). Each was prepared in tissue culture medium for in vitro studies and in sterile physiological saline solution (PSS) for animal experiments.

2.2. Cells, culture media

Madin Darby canine kidney (MDCK) cells were from the American Type Culture Collection

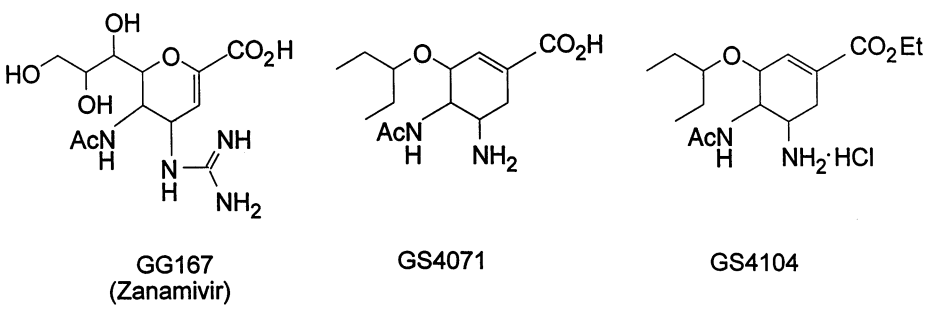

Fig. 1. Structures of GG167 (Zanamivir, M.W. 446), GS4071 (M.W. 284) and GS4104 (M.W. 348).

(ATCC, Rockville, MD). They were grown in Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum (FBS; HyClone, Logan, UT). When used with influenza viruses, the MEM was supplemented with 10 units/ml of trypsin (Sigma), 1 μg/ml of EDTA, 0.18% $NaHCO_3$ and 50 μg/ml of gentamycin. For in vitro antiviral experiments and for virus titrations, 96-well flat-bottomed microplates (Corning, NY) were used.

2.3. Viruses

Influenza A/NWS/33 (H1N1) virus was provided by K.W. Cochran (University of Michigan, Ann Arbor, MI). Influenza A/Victoria/3/75 (H3N2) and B/Hong Kong/5/72 viruses were obtained from the ATCC. The recent clinical isolate, influenza A/Shangdong/09/93 (H3N2), was provided by Helen Regnery of the Centers for Disease Control and Prevention (Atlanta, GA). A pool of the latter virus was initially prepared in the allantoic cavity of fertilized pathogen-free hen eggs, then passaged through 8–10 g BALB/c mice seven times to increase its murine virulence. A final pool was prepared as a mouse lung homogenate. The other viruses were propagated in MDCK cells for use in the present experiments. All were frozen in cryovials at -80°C. Each was titrated in MDCK cells and in mice prior to use in antiviral studies.

2.4. Mice

Female specific pathogen-free BALB/c mice weighing 8–10, 13–15 or 18–21 g, depending on the virus used, were obtained from Simonsen Laboratories (Gilroy, CA). Housing and care of the animals were as previously reported (Sidwell et al., 1986, 1992).

2.5. In vitro antiviral assays

Activity of each test compound was evaluated by inhibition of visually discerned viral cytopathic effect (CPE) as has been previously described (Huffman et al., 1997). Various one-half $\log_{10}$ concentrations of GS4071 were added to an 18 h monolayer of cells ≈ 5 min prior to virus exposure and remained on the cells until the test was read (72–96 h). A 50% virus-inhibitory (effective) concentration (EC50) was determined. Cytotoxicity, determined by visual examination and neutral red dye uptake in both resting and rapidly dividing cells, was expressed as the 50% cell-inhibitory concentration (CC50). Selectivity indexes (SI) were determined as CC50 (resting cells) $\div$ EC50.

2.6. Determination of arterial oxygen saturation (SaO_2)

An Ohmeda Biox 3740 pulse oximeter (Ohmeda, Louisville, OH) was used to determine daily SaO_2 values in mice as has been described previously (Sidwell et al., 1992).

2.7. Determination of lung virus titers

Each mouse lung was homogenized and varying dilutions assayed in triplicate for infectious virus in MDCK cells as described previously (Sidwell et al., 1986).

2.8. General procedure for in vivo antiviral experiments

Mice were anesthetized by intraperitoneal injection of phenobarbital and exposed to 90 μl of virus by intranasal instillation (i.n.). Treatments with test compounds varied according to the experiment, with doses selected to range from what was anticipated to produce a strong antiviral effect to doses that were marginal or inactive. This dose selection was also dependent upon the quantity of the compound available at the time. Twice daily treatment schedules were generally selected based on pharmacokinetic data (Li et al., 1997). Other treatment schedules are currently under study. In each infected, drug-treated group, 8–10 mice were used per drug dosage, with 18–20 infected animals utilized as PSS-treated controls. Three to five mice were used in toxicity control groups and as normal controls. Parameters for evaluation of antiviral activity included prevention of death and/or increase in mean day to death (MDD) determined through 21 days and

SaO_2 determined on days 3–10 of the infection. In certain experiments, additional infected mice were used at each dosage which were killed at various days after initiation of infection and their lungs removed, weighed, assigned a consolidation score ranging from 0 (normal) to 4 (maximal consolidation), depending on the percentage of the lung exhibiting typical plum coloration and each lung was assayed for infectious virus titer.

Toxicity control mice were weighed prior to initiation of treatment and again 18 h after termination of therapy. They were observed daily for occurrence of death and for obvious signs of toxicity for 21 days. Normal control mice were similarly weighed and observed. In addition, SaO_2 values were determined in these animals in parallel with the infected animals. When mice were killed for examination of lung parameters, additional normal control mice were included in the experiment and these animals were also sacrificed and their lungs handled in the same manner as those from infected animals.

2.8.1. Experiment design to compare the in vivo anti-influenza efficacy of orally administered GS4071, GS4104 and GG167

Mice weighing 18–21 g, infected with the NWS influenza virus in a 50% cell culture infectious dose (CCID50) of $10^{3.6}$, were divided into groups and treated by oral gavage (p.o.) in parallel with 10, 1 and 0.1 mg/kg/day of each compound dissolved in PSS or with PSS only. Treatment was twice daily (08:00, 18:00) for 5 days, beginning 4 h pre-virus exposure. Deaths were recorded daily and SaO_2 values determined. Toxicity controls were not included due to shortage of available compound.

2.8.2. Experiment design to compare the effects of p.o.-administered GS4104 and GG167 on lung infection parameters

Mice infected with the NWS virus as above were treated p.o. with 100, 32 or 10 mg/kg/day of GS4104, GG167 or PSS using the same treatment schedule described earlier. Five animals were sacrificed on days 1, 2, 4 and 6 post-virus exposure. Their lungs were removed on each day and assayed for consolidation and virus titer. Toxicity controls were included with this experiment.

2.8.3. Experiment design to determine the effect of p.o.-administered GS4104 on influenza A (H3N2) and B virus infections

In this series of experiments, 13–15 g mice were infected with the A/Victoria or B/Hong Kong viruses and 8–10 g animals were infected with the A/Shangdong virus. Treatment was p.o. as above with three dosages of GS4104, the range of dosages varying with the virus used. Deaths, SaO_2, lung consolidation and virus titers were determined in each experiment as above. Toxicity controls were also run with each experiment.

2.8.4. Experiment design to determine the influence of delay of p.o. GS4104 treatment on inhibition of influenza virus infection

Three experiments were run to determine how long twice daily for 5 days' p.o. treatment with GS4104 could be delayed and still provide significant antiviral efficacy in A/NWS influenza virus-infected mice.

In the first experiment treatment did not begin until 24, 36, 48 or 60 h after virus exposure, with occurrence of deaths and SaO_2 values determined. The viral dose utilized was an approximate LD85, which was equivalent to $10^{3.6}$ CCID50/ml. In the second and third experiments, the virus dose was increased to $10^{5.6}$ CCID50/ml, which was highly lethal to the animals. In these latter experiments, ribavirin was evaluated in parallel with GS4104. In the second experiment, treatment with GS4104 began 48, 60 or 72 h post-virus exposure, whereas p.o. ribavirin treatment began 24, 36 or 48 h after viral challenge. In the third experiment, GS4104 treatment was started 24 or 36 h post- and ribavirin therapy 4 h pre- or 4 h post-virus exposure. The same disease parameters as used in the first experiment were assayed in these latter studies. Due to an inadequate supply of GG167, this compound could not be run in parallel in this delayed treatment experiment.

2.8.5. Experiment design to compare efficacies of GS4079, GG167 and ribavirin administered i.n.

Dosages of 1, 0.1, 0.01, 0.001 and 0.0001 mg/kg/day of GS4071 and GG167 and 75 mg/kg/day of ribavirin, all in PSS vehicle, were administered to mice i.n. in a volume of 60 μl twice daily for 5

Table 1
In vitro effect of GS4071 and GG167 on influenza viruses used for animal experiments

Virus	GS4071			GG167		
	EC50[a,b] (μM)	CC50[c] (μM)	SI[d]	EC50 (μM)	CC50 (μM)	SI
A/NWS/33 (H1N1)	26.0	>1000	>39	60.0	>1000	>16
A/Victoria/3/75 (H3N2)	0.2	>1000	>5000	0.4	>1000	>2500
A/Shangdong/09/93 (H3N2)	1.8	>1000	>555	10.0	>1000	>100
B/HongKong/5/72	2.3	>1000	>435	1.2	>1000	>833

[a] Mean of CPE inhibition and NR uptake data.
[b] EC50: 50% virus-inhibitory (effective) concentration.
[c] CC50: 50% cell-inhibitory concentration.
[d] SI: Selectivity index (CC50 ÷ EC50).

days beginning 4 h pre-virus exposure. Each i.n. treatment was achieved by first anesthetizing the mice by i.p. injection of sodium pentobarbital. The animals were infected with the A/NWS virus which had been pre-titrated in animals treated i.n. as above with PSS since such i.n. saline treatment has been shown to enhance the infection (Judd et al., 1997). A viral challenge dose of $10^{2.4}$ CCID50/ml was used, which resulted in 95% death rate in the i.n. PSS-treated mice. Parameters studied were occurrence of death and SaO_2 decline. Toxicity controls were included with this i.n. therapy experiment.

2.8.6. Experiment design to investigate the potential for production of a viral infection-inhibiting aerosol during p.o. GS4104 treatment.

An experiment was run to determine if either the act of multiple p.o. treatments with GS4104 to influenza A/NWS-infected mice, or urinary and fecal excretions from such treated animals, would produce an aerosol capable of protecting the animals from the infection. The infected mice were treated p.o. twice daily for 5 days beginning 4 h pre-virus exposure with either GS4104 used at a dose of 10 mg/kg/day, or with PSS. Ten of the PSS-treated infected mice were marked and caged with the GS4104-treated animals. An additional 18 PSS-treated infected mice were also caged separately, with the cages located at least 20 ft apart. Occurrence of death and SaO_2 values were determined.

2.9. Statistical analysis

Increases in survivor numbers were evaluated using χ^2 analysis with Yates' correction. Lung score reductions were analyzed using Wilcoxon ranked sum analysis. Differences in MDD, mean lung weights, lung virus titers and SaO_2 were compared with control values using the *t*-test.

3. Results

3.1. In vitro anti-influenza virus efficacy of GS4071

An experiment was run to determine the sensitivity of the viruses used in the mouse experiments to GS4071. The results, summarized in Table 1, indicated that while all appeared to be inhibited by this compound, the sensitivity of each varied appreciably, with the A/NWS/33 virus being least inhibited. The in vitro experiments were all repeated at least once with similar findings each time. No cytotoxicity was observed in either resting or rapidly dividing cells at dosages as high as 1000 μM. In general, the EC50 values using neutral red were 2–5 times higher than using visually discerned CPE. No viral inhibition or cytotoxicity was seen at this dose level.

Table 2
Comparison of the effect of orally administered[a] GS4071, GS4104 and GG167 on survival and arterial oxygen saturation in influenza A (H1N1)[b] virus infected mice

Compound	Dosage (mg/kg/day)	Survival/total	Mean day to death[c] ± S.D.	Mean day 10 SaO_2 [d] (% ± S.D.)
GS4071	10	6/8**	13.5 ± 0.9**	85.6 ± 2.5**
	1	3/8	11.8 ± 1.7**	84.0 ± 5.3*
	0.1	0/8	10.0 ± 0.7	78.5 ± 4.2
GS4104	10	8/8**	>21.0 ± 0.0**	86.4 ± 2.1**
	1	7/8**	14.0 ± 0.0**	86.3 ± 2.1**
	0.1	2/8	11.1 ± 2.4**	83.0 ± 4.9*
GG167	10	5/8*	12.3 ± 2.0**	86.2 ± 2.1**
	1	2/8	11.7 ± 1.2**	84.0 ± 4.9*
	0.1	0/8	9.8 ± 1.1	76.2 ± 3.5
PSS	0	2/16	9.6 ± 1.2	77.8 ± 3.7

[a] Bid × 5 beginning 4 h pre-virus exposure.
[b] A/NWS/33.
[c] Mice dying prior to day 21.
[d] Normal control SaO_2: 87.7 ± 1.6.
*$P<0.05$; **$P<0.01$ compared to PSS-treated control.

3.2. Comparison of efficacy of p.o.-administered GS4071, GS4104 and GG167 on an influenza A (H1N1) virus infection in mice

As seen in Table 2, each compound exerted significant antiviral effect as shown by significantly increased numbers of survivors, increased MDD and less SaO_2 decline on day 10, the last day this value was determined. The daily SaO_2 values using the 1 mg/kg/day dose of each compound are seen in Fig. 2. GS4104 exerted the greatest protective effect, with significant prevention of death occurring at both the 10 and 1 mg/kg/day dose. A significant increase in MDD and reduced decrease in SaO_2 were seen also at the 0.1 mg/kg/day dose. Significant efficacy with GS4071 and GG167 was seen down to 1 mg/kg/day only, with prevention of death occurring only at 10 mg/kg/day.

3.3. Comparison of effects of p.o.-administered GS4104 and GG167 on lung infection parameters

When p.o.-administered GS4104 and GG167 were compared for their ability to inhibit lung consolidation and virus titers, the superiority of GS4104 was readily evident. Effects on development of lung score and on increasing lung weight on day 6 are shown in Table 3, with virus titer inhibition data shown graphically in Fig. 3. At the earlier times in which lungs were examined for consolidation, scores were quite low and weights

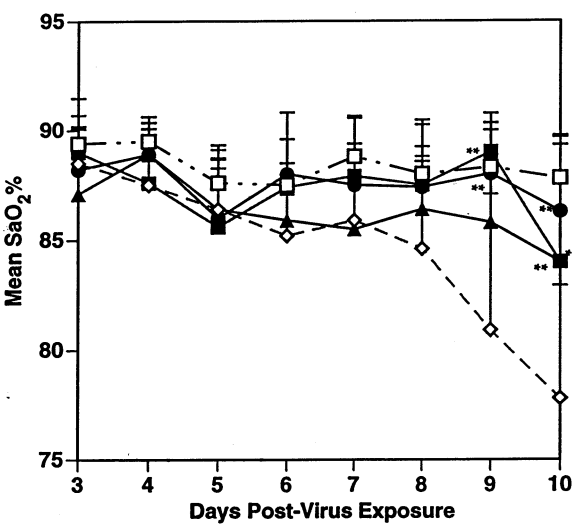

Fig. 2. Comparison of the effect of oral treatment (bid × 5 beginning 4 h pre-virus exposure) with 1 mg/kg/day of GS4071, GS4104 and GG167 on inhibition of arterial oxygen saturation decline in influenza A/NWS/33 (H1N1) virus-infected mice. ■ GS4071; ● GS4104; ▲ GG167; ◇ PSS; □ Normal controls. *$P<0.05$, **$P<0.01$ compared to PSS-treated controls.

Table 3
Effects of orally administered[a] GS4104 and GG167 on lung consolidation in influenza A (H1N1)[b] virus-infected mice

Compound	Dose (mg/kg/day)	Toxicity controls		Infected, treated mean day 6 lung data	
		Survival/total	Mean host wt. change[c] (g)	Score ± S.D.	Weight (mg ± S.D.)
GS4104	100	3/3	0.4	0.0 ± 0.0**	115 ± 11**
	32	3/3	1.3	0.0 ± 0.0**	115 ± 14**
	10	3/3	1.2	0.0 ± 0.0**	105 ± 8**
GG167	100	3/3	−0.2	0.2 ± 0.3**	142 ± 21**
	32	3/3	−0.2	0.4 ± 0.4**	137 ± 21**
	10	3/3	0.7	1.2 ± 0.5*	167 ± 10**
PSS	0	—	—	2.6 ± 0.5	239 ± 36
Normal controls	—	3/3	1.0	—[d]	—[d]

[a] Bid × 5 beginning 4 h pre-virus exposure.
[b] A/NWS/33.
[c] Difference between initial weight and weight 18 h after final treatment.
[d] Normal control mean lung score: 0.0; mean lung weight: 110 ± 10 mg.
*$P < 0.05$; **$P < 0.01$

had increased to a much lesser extent than the day 6 data shown in the table. No evidence of influenza virus-induced lung consolidation was seen in any infected mice treated with any dose of GS4104, with all lungs having scores of '0' and no increase in lung weight. The lung weight inhibition at the 100 and 10 mg/kg/day doses was statistically better ($P < 0.05$, $P < 0.01$, respectively) in the GS4104-treated animals than in those receiving GG167. Animals treated with GG167 significantly inhibited both development of lung scores and increase in lung weight, but scores as high as 3.0 were seen in some lungs and the mean lung weights increased up to 50% above normal values (data not shown).

GS4104 appeared well tolerated by the toxicity control mice, with all surviving and gaining weight. GG167-treated toxicity control animals all survived, but those receiving the 100 and 32 mg/kg/day doses lost weight during treatment.

3.4. Effect of p.o.-administered GS4104 on influenza a (H3N2) and B virus infections

The results of this experiment, using two strains of influenza A (H3N2) virus and one strain of influenza B virus, are shown in Table 4. To conserve space, only the day 6 lung data are shown. Effects on lung score and increased lung weight at other times were quite similar to those seen using the H1N1 influenza A virus described above (data not shown), but the A/Victoria and A/Shangdong lung viruses were inhibited to the greatest degree (0.5–1.0 $\log_{10}$, $P < 0.05$) on day 2 rather than on day 6. Both the A/Shangdong and the B/Hong Kong virus infections appeared more sensitive to treatment with GS4104 than the A/Victoria virus, with significant inhibitory effects against these infections seen at lower doses than was observed using the A/Victoria virus. Since only 69% of the PSS-treated infected mice died of the A/Victoria virus challenge, while 89–100% of the PSS-treated infected mice died when exposed to the B and A/Shangdong viruses, this lesser antiviral effect was considered not to be due to virus challenge dose used. In no experiment were lung virus titers inhibited more than ≈ 1 $\log_{10}$.

3.5. Influence of delay of p.o. treatment on inhibition of influenza virus infection

These data are summarized in Table 5. In the first experiment, using delayed initiation of p.o. therapy with GS4104 against a moderate virus

Table 4
Effect of orally administered[a] GS4104 on influenza A (H3N2) and B virus infections in mice

Virus	Treatment	Dose (mg/kg/day)	Survival/total	Mean day to death ± S.D.	Mean day 10 SaO_2 (% ± S.D.)	Mean day 6 lung data		
						Score ± S.D.	Weight (mg ± S.D.)	Virus titer (log_{10}/g ± S.D.)
A/Victoria/3/79	GS4104	10	8/8**	>21.0 ± 0.0**	86.4 ± 2.8**	1.8 ± 1.1	206 ± 13	5.7 ± 0.4
		1	5/8	10.0 ± 1.9	83.3 ± 4.6	2.3 ± 0.4	184 ± 30	6.3 ± 0.4
		0.1	3/8	8.0 ± 1.7	78.6 ± 4.6	2.0 ± 1.7	198 ± 47	5.3 ± 1.7
	PSS	0	5/16	9.7 ± 3.8	80.8 ± 4.0	2.5 ± 0.8	228 ± 26	6.0 ± 0.3
A/Shangdong/09/93	GS4104	100	10/10**	>21.0 ± 0.0**	86.5 ± 1.6**	1.3 ± 1.3*	180 ± 26*	5.0 ± 0.3*
		10	9/10**	10.0 ± 0.0	85.5 ± 3.8**	1.4 ± 1.3*	216 ± 39	5.8 ± 0.4
		1	3/10*	12.2 ± 3.3*	84.7 ± 5.5**	2.6 ± 0.4	214 ± 47	5.3 ± 0.5
	PSS	0	0/20	10.4 ± 2.4	77.7 ± 4.7	3.0 ± 0.4	236 ± 34	6.0 ± 0.5
B/HongKong/5/72	GS4104	10	8/9**	11.0 ± 0.0	86.9 ± 2.3**	1.0 ± 0.4**	182 ± 11**	3.5 ± 1.3**
		3.2	9/10**	10.0 ± 0.0	85.3 ± 4.3**	nd[b]	nd	nd
		1	1/10	11.1 ± 3.3	80.9 ± 6.8	nd	nd	nd
	PSS	0	2/18	10.5 ± 3.8	78.4 ± 4.5	3.0 ± 0.4	224 ± 20	4.8 ± 0.3

[a] Bid × 5 beginning 4 h pre-virus exposure.
[b] Not done.
*$P < 0.05$; **$P < 0.01$, compared to PSS-treated controls run with the same virus.

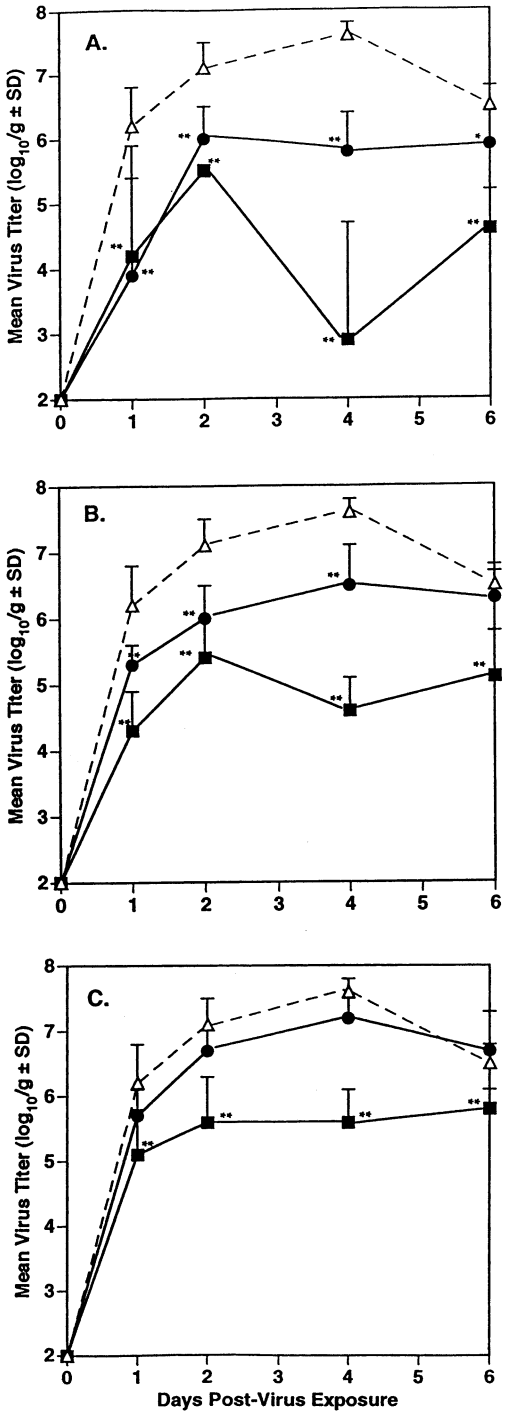

Fig. 3. Effect of oral treatment (bid × 5 beginning 4 h pre-virus exposure) with GS4104 and GG167 on lung virus titers in influenza A/NWS/33 (H1N1) virus-infected mice. A: 100 mg/kg/day; B: 32 mg/kg/day; C: 10 mg/kg/day. ■ GS4104; ● GG167; Δ PSS. $^{**}P < 0.01$ compared to PSS-treated controls.

challenge which killed 85% of the PSS-treated control mice, beginning of therapy could be delayed to at least 60 h after virus exposure (last time assayed), with all treated mice surviving the infection and literally no decline in SaO_2 seen. In the second and third experiments, a 100-fold higher viral challenge was used and this markedly affected the efficacy of delayed GS4104 therapy, with only a moderate disease-inhibitory effect seen when therapy began 48 h after viral exposure as seen by a 20% survival rate, significantly increased MDD and less decline in SaO_2. Ribavirin was not effective in this second experiment when treatment began as early as 24 h after virus inoculation. When time of therapy initiation was decreased for each compound in the third experiment, significant inhibition of the disease was seen with each.

3.6. Comparison of the efficacies of GS4071, GG167 and ribavirin administered intranasally

Using the parent compound, GS4071, and GG167 in this experiment wherein the compounds were applied directly to the pulmonary tissues via i.n. treatment, remarkably lower dosages of both GS4071 and GG167 were found significantly inhibitory to this virus infection (Table 6). GS4071 was considered highly effective down to a dose of 0.01 mg/kg/day; GG167 was only weakly inhibitory to the infection at this dose. Ribavirin, used at a single dose of 75 mg/kg/day, was also highly effective. Two of the three toxicity control animals receiving ribavirin died during therapy and in view of this apparent toxicity, it was surprising that all infected mice treated with this compound survived.

3.7. Investigation of the potential for production of a viral infection-inhibiting aerosol during p.o. GS4104 therapy

The potent activity of the i.n.-instilled neuraminidase inhibitors suggested that possibly p.o. therapy was producing an aerosol of the compound of sufficient magnitude to exert an influenza disease-protective effect in the mice. When infected animals treated p.o. with GS4104 were caged with infected, PSS-treated mice, however,

Table 5
Effect of delayed oral treatments[a] with GS4104 or ribavirin on influenza A (H1N1)[b] virus infections in mice

Compound	Expt. no.	Beginning of therapy (h)	Surv/total	Mean day to death ± S.D.	Mean SaO_2 (% ± S.D.)
Moderate virus challenge ($10^{3.6}$ CCID50/ml)					Day 10
GS4104[c]	1	24	10/10**	>21.0**	86.2 ± 1.6**
	1	36	10/10**	>21.0**	86.3 ± 1.6**
	1	48	10/10**	>21.0**	86.8 ± 1.3**
	1	60	10/10**	>21.0**	86.9 ± 2.9**
PSS	1	24	4/26	11.3 ± 3.3	79.8 ± 3.8
High virus challenge ($10^{5.6}$ CCID50/ml)					Day 7
GS4104[c]	3	24	10/10**	>21.0 ± 0.0**	85.9 ± 3.5**
	3	36	5/10**	7.2 ± 3.2	82.0 ± 6.2**
	2	48	2/10	8.5 ± 1.8**	82.0 ± 5.4**
	2	60	0/10	7.6 ± 1.6	78.6 ± 4.7
	2	72	0/10	7.5 ± 2.3	77.2 ± 3.3
Ribavirin[d]	3	−4	10/10**	>21.0 ± 0.0**	86.4 ± 2.2**
	3	4	10/10**	>21.0 ± 0.0**	87.7 ± 2.4**
	2	24	0/10	7.0 ± 1.0	79.8 ± 5.6
	2	36	0/10	6.1 ± 1.3	76.5 ± 4.7
	2	48	0/10	6.9 ± 0.7	77.6 ± 5.6
PSS	3	24	0/20	5.2 ± 1.1	75.6 ± 2.7
	2	48	0/20	7.0 ± 1.1	78.3 ± 5.5

[a] Bid × 5 beginning at times indicated.
[b] A/NWS/33.
[c] 10 mg/kg/day.
[d] 75 mg/kg/day.
**$P < 0.01$ compared to PSS controls run in the same experiment.

no evidence of disease inhibition was seen (Table 7), since the placebo-treated mice caged with the GS4104-treated animals died at approximately the same rate as the control animals caged separately.

4. Discussion

These data indicate that orally administered GS4104, the ethyl ester prodrug derivative of GS4071, is highly effective against infections induced in mice by a spectrum of influenza viruses. The antiviral efficacy of GS4104 administered p.o. significantly exceeded that of GS4071 and of GG167; these oral efficacy data correlate well with the oral bioavailability of each of these compounds. GS4071 and GG167, when administered orally to rats in a single dose of 10 mg/kg, induces plasma levels of between 0.03 and 0.07 μg/ml; GS4104 treatment results in a maximum plasma level of GS4071 approximately 10 times higher. The levels of GG167 drop to undetectable limits within 6–8 h, whereas GS4071 levels drop slower, with detectable compound persisting for 24 h whether administered as the original material or in the GS4104 prodrug form (Li et al., 1997 and Li et al., manuscript submitted). These pharmacokinetic properties of GS4071 and GG167 may account for the moderate in vivo activity they exhibited when administered p.o.

The SaO_2 measurements provide interesting insights into the effects of therapy on pulmonary function in influenza virus-infected mice. As illustrated in Fig. 2, the PSS-treated animals infected with an approximate LD90 of virus began to display discernible reduction in SaO_2 values by day 8. Treatment with all compounds inhibited this decline, indicating less inhibition in lung function was occurring in the treated infected animals. Mice treated with GG167 had lower SaO_2 values than those treated with the other compounds, however, indicating a lesser antiviral effect proba-

Table 6
Effect of intranasal treatment[a] with GS4071, GG167 and ribavirin on influenza A (H1N1)[b] virus infections in mice

Compound	Dosage (mg/kg/day)	Toxicity controls		Infected, treated mice		
		Surv/total	Mean wt change[c] (g)	Surv/total	Mean day to death ± S.D.	Mean day 10 SaO_2 (% ± S.D.)
GS4071	1	3/3	0.3	10/10**	>21.0 ± 0.0**	86.8 ± 2.3**
	0.1	3/3	0.2	10/10**	>21.0 ± 0.0**	84.8 ± 2.6**
	0.01	3/3	0.9	8/10**	8.0 ± 0.0	83.3 ± 4.9**
	0.001	3/3	0.4	3/10	7.6 ± 4.1	79.0 ± 3.9
	0.0001	3/3	1.1	0/10	8.2 ± 2.3	76.4 ± 3.6
GG167	1	3/3	−0.2	9/9**	>21.0 ± 0.0**	86.1 ± 3.0**
	0.1	3/3	−0.6	9/10**	9.0 ± 0.0	85.4 ± 4.6**
	0.01	3/3	0.2	3/10	9.7 ± 1.9**	81.2 ± 5.2**
	0.001	3/3	−0.7	1/10	7.3 ± 2.7	77.2 ± 4.2
	0.0001	3/3	−0.5	1/10	7.1 ± 2.0	75.7 ± 2.3
Ribavirin	75	1/3	0.7	10/10**	>21.0 ± 0.0**	86.5 ± 3.6**
PSS	0	—	—	1/20	6.9 ± 1.6	75.5 ± 2.7
Normal Controls	—	3/3	0.6	—	—	—[d]

[a] Bid × 5 beginning 4 h pre-virus exposure.
[b] A/NWS/33.
[c] Difference between initial weight and weight 18 h after final treatment.
[d] Normal controls mean day 10 SaO_2: 87.2 ± 2.4%.
**$P < 0.01$.

bly due to the rapid clearance of the compound from the blood. Those animals receiving GS4071 exhibited a rapid drop in SaO_2 on day 10. As cited above, GS4071, while not achieving high blood levels in orally treated animals, did persist in the blood longer than GG167. The rapid SaO_2 decline on day 10 seen in mice treated with this compound may be a manifestation of clearance of the compound from the blood. We have shown only the mean day 10 SaO_2 data in the tables, since at this point the differences in levels of this parameter were always greatest.

The effects of orally administered GS4104 on inhibition of lung virus titers did not always correlate with the survival data. In experiments with all influenza viruses, infectious virus titers in the lungs increased quickly, often with titers exceeding 10^6/g seen by day 2. It is possible that evaluation of the effects of treatment on day 1 may have shown more inhibition in titer. In our experience, reduction of challenge virus by 0.5 $\log_{10}$ can significantly lessen the number of mice succumbing to influenza virus infection, so it is possible that this compound, as well as GG167, may exert their protective effects by reducing the viral challenge by only this amount. This explanation of antiviral effect may also apply to the delayed therapy studies, to be considered later.

GS4104 appeared well tolerated by the mice in these studies at dosages up to 100 mg/kg/day, which was the maximum dose used. Other toxicity experiments run in rats using GS4104 administered p.o. have indicated the compound to cause no deaths, clinical signs, changes in organ appearance, or histopathological alterations in dosages up to 800 mg/kg/day for 14 days (personal communication, Choung Kim, Gilead Sciences). Using equivalent surface area conversion (Freirich et al., 1966), 800 mg/kg/day in the rat equates to a dose of 1600 mg/kg/day in the mouse. Thus the in vivo margin of safety of GS4104 against influenza virus infections in mice appears to be high.

It was interesting that although all the influenza viruses used in animal studies were also sensitive

Table 7
Failure of oral treatment[a] with GS4104 to produce an influenza A (H1N1)[b] virus infection-inhibiting aerosol

Treatment	Dose (mg/kg/day)	Surv/total	Mean day to death ± S.D.	Mean day 7 SaO_2 (% ± S.D.)
GS4104	10	9/10**	8.0 ± 0.0	85.6 ± 2.0**
PSS (mice caged with GS4104-treated animals)	0	0/10	6.0 ± 1.3	76.3 ± 4.2
PSS (mice caged separately from GS4104-treated animals	0	0/18	6.6 ± 0.8	75.1 ± 0.8

[a] Bid × 5 beginning 4 h pre-virus exposure.
[b] A/NWS/33.
** $P < 0.01$.

to GS4071 in vitro, this sensitivity was quite virus-dependent, with the A/NWS/33 virus being approximately 30-fold less sensitive to the compound than the A/Victoria/3/75 virus. Since the multiplicity of infection (0.0001) in the in vitro experiments was approximately the same for all viruses used, this effect could not be attributed to differences in viral challenge. Mendel et al. (1997), and manuscript submitted) have shown the A/Victoria virus to be over 250-fold more sensitive to GS4071 than the B/Hong Kong virus using plaque reduction assay, whereas the A/Victoria and B/Hong Kong viruses were approximately equal in sensitivity in the present experiment. The viruses used by Mendel et al. (1997), while obtained from the same source as those used by us, have had different passage histories in the respective laboratories. In addition, the in vitro assay procedures were different in the two laboratories. Neuraminidase inhibition data, included in the Mendel et al. (1997) report, also show the enzymes from the influenza NWS and B viruses to be less sensitive than that from the Victoria virus, but the IC50 differences to be only 2- to 3-fold. It is acknowledged that since no cytotoxicity was seen in the in vitro experiments, the SI determinations shown were only approximate, although do indicate the large in vitro margin of safety for these compounds. Despite the variations in sensitivity to this compound in vitro, murine infections induced by all of these viruses appeared to be sensitive to treatment with GS4104, with the infection induced by the A/Victoria virus appearing to be the least inhibited by this compound in mice.

It was important to demonstrate that GS4104 was highly effective when treatment began late in the infection. Such data indicate the potential therapeutic, in contrast to prophylactic, use of this compound for treatment of influenza virus infections. The decreased time allowed to begin therapy and still achieve an antiviral effect was obviously dependent upon the dose of the viral challenge. It can be seen in Table 5 that the MDD of the saline-treated mice receiving the high virus challenge was 7.0 days in experiment 2 and 5.2 days in experiment 3. In similarly treated mice exposed to the moderate virus dose, however, the MDD was 11.3 days. Thus in the experiments with the high virus challenge, the infection was progressing much faster and would require earlier introduction of the antiviral drug to significantly influence the course of the disease. If consideration is given to how the milder influenza infection usually occurring in humans would respond to therapy with GS4104 compared to the lethal murine infections used in the present experiments, the prediction may be made that late start of therapy in the human disease would probably still be highly inhibitory to the progress of the infection.

Since the neuraminidase (sialidase) of influenza virus plays a key role in the release of progeny virus from infected cells (Palese and Compans, 1976), which is a relatively late step in the viral replicative process, it is not surprising that this strong and selective inhibitor of the enzyme would have efficacy when administered late in the infection. Ribavirin, which was also evaluated for its effect when therapy was delayed, is thought to be

primarily inhibitory to influenza virus through its 5′-triphosphate, which selectively inhibits influenza virus RNA polymerase (Eriksson et al., 1997) and RNA transcriptase, the latter leading to prevention of the synthesis of structural and non-structural viral polypeptides (Oxford, 1976). Such a mechanism indicates a need to begin therapy earlier in the infection process with ribavirin, which was illustrated in the present experiments.

The parent compound, GS4071, exhibited an approximate 1000-fold more potency for inhibiting the virus infection when the compound was administered i.n. This compound was used rather than the prodrug, GS4104, since the drug application was directly onto the infected tissues, not requiring blood levels to be achieved. This highly magnified antiviral potential suggested that if even a small percentage of the parent drug became aerosolized, the antiviral effects shown may be due to this aerosol treatment rather than an actual parenteral therapy. The design of the experiment summarized in Table 7 would appear to provide the answer to this concern, since PSS-treated infected mice caged with the GS4104-treated infected animals did not display any indication of protection.

These experiments demonstrate that the carbocyclic, transition-state sialic acid analog inhibitor of influenza virus neuraminidase, GS4071, is a potent inhibitor of influenza A (H1N1 and H3N2) and influenza B virus replication in vitro. Oral administration of the ethyl ester prodrug of GS4071, GS4104, will strikingly inhibit infections in mice induced by these same viruses while being well tolerated by the animals. This oral treatment can be started relatively late in the infection, the actual time to begin being dependent of the viral challenge dose. This ability to inhibit in vivo infections induced by representatives of all viruses currently involved in influenza epidemics, using oral administration that may begin after disease signs have been established, provide strong evidence for the potential for GS4104 to be used in the treatment of human influenza A and B virus infections.

Acknowledgements

Supported by contracts N01-AI-35178, N01-AI-15097 and N01-AI-65291 from the Virology Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health and by a grant from Gilead Sciences, Foster City, CA.

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