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The neuraminidase inhibitor GS4104 (oseltamivir phosphate) is efficacious against A/Hong Kong/156/97 (H5N1) and A/Hong Kong/1074/99 (H9N2) influenza viruses

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Abstract

In 1997, an H5N1 avian influenza A/Hong Kong/156/97 virus transmitted directly to humans and killed six of the 18 people infected. In 1999, another avian A/Hong/1074/99 (H9N2) virus caused influenza in two children. In such cases in which vaccines are unavailable, antiviral drugs are crucial for prophylaxis and therapy. Here we demonstrate the efficacy of the neuraminidase inhibitor GS4104 (oseltamivir phosphate) against these H5N1 and H9N2 viruses. GS4071 (the active metabolite of oseltamivir) inhibited viral replication in MDCK cells (EC_{50} values, 7.5–12 μM) and neuraminidase activity (IC_{50} values, 7.0–15 nM). When orally administered at doses of 1 and 10 mg/kg per day, GS4104 prevented death of mice infected with A/Hong Kong/156/97 (H5N1), mouse-adapted A/Quail/Hong Kong/G1/97 (H9N2), or human A/Hong Kong/1074/99 (H9N2) viruses and reduced virus titers in the lungs and prevented the spread of virus to the brain of mice infected with A/Hong Kong/156/97 (H5N1) and mouse-adapted A/Quail/Hong Kong/G1/97 (H9N2) viruses. When therapy was delayed until 36 h after exposure to the H5N1 virus, GS4104 was still effective and significantly increased the number of survivors as compared with control. Oral administration of GS4104 (0.1 mg/kg per day) in combination with rimantadine (1 mg/kg per day) reduced the number of deaths of mice infected with 100 MLD_{50} of H9N2 virus and prevented the deaths of mice infected with 5 MLD_{50} of virus. Thus, GS4104 is efficacious in treating infections caused by H5N1 and H9N2 influenza viruses in mice.

Keywords: Human influenza A viruses; Avian influenza viruses; Antiviral agents

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1. Introduction

Influenza virus infection is a serious uncontrolled disease that causes death in elderly people and young children. The morbidity and mortality associated with influenza infection result in economical losses (Kaiser et al., 1998). Vaccines and antiviral agents are the most promising for controlling a potential influenza epidemic; however, preparation of vaccine against new strains of influenza virus is a time-consuming process, and some persons are not adequately protected by vaccination (Ruben, 1990; Powers and Belshe, 1993; Ohmit et al., 1999). In such situations, antiviral agents would have potential for preventing death due to infection.

Amantadine (1-aminoadamantane hydrochloride) and its derivative rimantadine (α-methyl-1-adamantane methylamine hydrochloride) have been shown to be therapeutically and prophylactically effective against human influenza A viruses (Zlydnikov et al., 1981; Douglas, 1990). However, the drugs have limited utility because of their side effects and the rapid emergence of resistant viruses during treatment. In addition, amantadine and rimantadine are ineffective against influenza B viruses (Hayden, 1996).

To find better therapies for influenza virus infection, investigators have targeted neuraminidase (NA) in the design of new antiviral drugs (von Itzstein et al., 1993; Bamford, 1996; Kim et al., 1997; Li et al., 1997; Gubareva and Webster, 1999). As a result of their studies, two NA inhibitors were developed for prophylaxis and treatment of influenza A and B infections. These inhibitors are zanamivir, which must be inhaled, and oseltamivir (GS4104), which is taken orally, and is the ethyl ester prodrug of GS4071 (Kim et al., 1997; Li et al., 1997; Mendel et al., 1997). Zanamivir has demonstrated a significant antiviral effect in cell culture, in animal models, and in human studies (Woods et al., 1993; Gubareva et al., 1995; Hayden et al., 1996). When administered orally, GS4104 protects mice and ferrets against influenza infection (Sidwell et al., 1998; Mendel et al., 1998) and is efficacious against currently circulating human strains of influenza A and B viruses in clinical trials (Gubareva et al., 2000).

Since 1997, two avian influenza viruses (H5N1 and H9N2) have been transmitted directly from birds to humans. A highly virulent strain of avian influenza virus (H5N1) entered the human population in May of 1997 and caused an outbreak of influenza in Hong Kong (De Jong et al., 1997; Subbarao et al., 1998; Claas et al., 1998). This strain was the first reported avian influenza virus to cause clinical respiratory illness in humans. One year later, an avian H9N2 virus was isolated from humans (Peiris et al., 1999). Both of these incidents suggest that a human pandemic caused by an avian influenza virus or by an influenza virus containing avian influenza genes remains a possibility, and an appropriate strategy must be developed for dealing with this possible event. The aims of our study were to evaluate the efficacy of GS4071 against H5N1 and H9N2 influenza viruses in vitro and to evaluate the efficacy of the prodrug derivative oseltamivir (GS4104) against H5N1 and H9N2 influenza virus infections in mice.

2. Materials and methods

2.1. Compounds

GS4104 and GS4071 were provided by Gilead Sciences, Inc. (Foster City, CA) and rimantadine was purchased from DuPont, Delaware. The antiviral drugs were dissolved in Eagle's minimal essential medium (MEM) for in vitro studies and in distilled water for in vivo studies.

2.2. Cells and viruses

Madin–Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were grown in MEM containing 10% fetal bovine serum (FBS; Summit Biotech, Ft. Collins, CO) and antibiotics. The influenza viruses used in this study were H5N1 virus A/Hong Kong/156/97 (A/HK/156/97) and H9N2 viruses A/Hong Kong/1074/99 (A/HK/1074/99) and A/Quail/Hong Kong/G1/97) (A/Qa/HK/G1/97). We used A/HK/156/97 (H5N1) and A/HK/1074/99 (human)

(H9N2) viruses that were initially isolated from a patient in MDCK cells. Then viruses were propagated in embryonated chicken eggs to make a stock of virus. Virus stocks were aliquoted and stored at −70°C until use. Influenza A/Qa/HK/G1/97 (H9N2) virus was isolated from quail in Hong Kong (Guan et al., 1999), cloned two times and propagated in embryonated chicken eggs. Influenza A/Qa/HK/G1/97 (H9N2) virus was serially passed three times in mouse lungs, propagated in embroynated chicken eggs, and used in in vivo experiments in mice. This virus was designated as A/Qa/HK/G1/97 (P_3). Each virus was titrated in embryonated chicken eggs prior to use and virus titers were ranged from 6.0 to 8.0 $\log_{10}$ egg infectious dose (EID_{50}/0.1 ml). All the experiments were conducted at St. Jude Children's Research Hospital in the biosafety level 3 containment facility approved for use in the study of these viruses.

2.3. Antiviral assay

A modified enzyme-linked immunoassay (ELISA) (Belshe et al., 1988; Hayden et al., 1990) was used to determine the antiviral effects of the anti-NA compounds. This assay detected expression of viral nucleoprotein (NP) in infected cells. Briefly, MDCK cells were seeded in 96-well culture plates at a density of 3000 cells per well in MEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μg/ml kanamycin sulfate. Cells were incubated at 37° with 5% CO_2 until 90% confluency was achieved. Cells were then washed twice with serum-free MEM, and residual medium was removed. Each microtiter plate included uninfected control cultures, virus-infected control wells and virus-infected cultures to which antiviral compounds were added. The cultures were overlaid with MEM (100 μl) containing 2.5 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma Chemical Co., St. Louis, MO) and twice the concentration of antiviral drug being studied. After incubation for 30 min at 37°C 100 μl of virus containing allantoic fluid (approximately 0.1 pfu per cell) was added to all wells except the wells with the cell control. After incubation for 18 h at 37°C in a humidified atmosphere of 5% CO_2 cells were fixed by adding 100 μl of cold acetone–PBS mixture (80:20). Expression of NP protein was measured by ELISA as described previously (Belshe et al., 1988). The percent inhibition of virus replication by the antiviral compound was calculated after correction for the background (cell control) values as follows: Percent inhibition = $100 \times [1 - (OD_{450})$ treated sample/(OD_{450}) virus control sample]. The EC_{50} value (i.e. the concentration of compound required to inhibit virus replication by 50%) was determined by plotting the percent inhibition of virus replication as a function of compound concentration.

To study sensitivity to GS4104 of the virus isolated from mouse lungs at the end of treatment the plaque assay in MDCK cells was utilized. MDCK monolayers were infected with viruses from lungs of mice treated and untreated with GS4104 ($\sim$50 pfu per well for 6-well plates) and were overlaid with agar containing the antiviral compound GS4071 at concentrations ranging from 0.03 to 10 μM. After 4 days of incubation, the agar was removed, and the plaques were visualized by crystal violet staining and were counted.

2.4. NA inhibition assay

NA activity was measured by the colorimetric assay (Aymard-Henry et al., 1973) with fetuin (Sigma Chemical Co., St. Louis, MO) as a substrate. The inhibitory effect of GS4071 on NA activity was determined by assaying for enzyme activity in the presence of range of concentrations of the compound. Viruses used in NA inhibition tests were diluted in phosphate-buffered saline to standardize enzyme activity (0.5 OD units at $\lambda = 540$ nM). Ten-fold dilutions ranging from 0.0001 to 100 nM of the antiviral compound were incubated with viruses for 30 min at room temperature and were then incubated with fetuin overnight. The inhibition assays were performed in triplicate. The IC_{50} values were determined for each virus.

2.5. Infection and drug administration in mice

Female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized by inhalation of metofane and were inoculated intranasally with 100 μl of diluted virus for the appropriate virus challenge. GS4104 was administered by oral gavage in volume of 100 μl. Treatment with compounds varied according to the experiments. In most cases, five to ten mice were given the drugs twice daily for 5 days; the first dose was administered 4 h before the mice were infected with virus (Mendel et al., 1998). The weight of each mouse and the number of deaths were recorded. The mean survival day (MSD) was calculated by the following formula: $MSD = [f(d-1)]/n$, were f is the number of survivors on day d (survivors on day 16 were included in f for that day) and n is the number of mice in the group (Grunert et al., 1965).

2.6. Virus titration in mice

Three mice from each group were killed at various days after infection; under sterile conditions, the brains were removed first, and then the lungs. The organs were washed three times in phosphate buffered saline (PBS), ground and suspended in a total volume of 1 ml of PBS. After centrifugation at 2000 rpm for 10 min the supernatants were diluted in 10-fold steps, and the virus titers were assayed in embryonated chicken eggs. Titers of infectious virus were presented as $\log_{10}$ (EID_{50}/0.1 ml). The level of detection was 0.1 $\log_{10}$ EID_{50}/0.1 ml.

2.7. Statistical analysis

Effect of the different doses of drug in mice infected with different viruses on survival of animals was estimated by the Kaplan–Meier method. Survival was measured from the day of the virus infection until the death of the animal. Any deaths occurring after day 16 were not observed, and those were censored at day 16. Comparisons between groups were made using the log-rank test. The Fisher's, exact test was applied to compare mouse survival rate between the groups. Logistic regression was used to explore the relationship between the survival rate and dosage of the particular drug as well as the virus type. Differences in lung and brain virus titers between groups were compared using multiple comparison method (Dunnett's two-tailed t-test) available with analysis of variance procedure. P-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Pathogenicity of A/HK/156/97 (H5N1) and H9N2 viruses in mice

All the genes segments of the A/HK/1074/99 virus isolated from human are genetically highly related to those of A/Qa/HK/G1/97. In addition, A/Qa/HK/G1/97 (H9N2) virus has genes encoding internal proteins (PB2, PB1, PA, NP, M, NS) that are genetically closely related to A/HK/156/97 (H5N1) virus (Guan et al., 1999). It was previously shown that an H5N1 avian influenza virus was highly pathogenic in mice without adaptation (Gubareva et al., 1998; Gao et al., 1999; Lu et al., 1999; Dybing et al., 2000). The virus replicated in mouse lungs and brains and caused highly lethal systemic disease (Gubareva et al., 1998; Lu et al., 1999). There have been no reports about the pathogenicity of H9N2 influenza viruses in mice. For this reason, we first studied the pathogenicity of H9N2 viruses in mice, in comparison with that of A/HK/156/97 (H5N1) virus. Our experiments confirmed that the A/HK/156/97 (H5N1) virus is highly pathogenic in mice. All the mice infected with 10-fold dilutions of A/HK/156/97 virus starting with 10 EID_{50} and higher lost up to 10% of their body weight by day 4 and 15% by day 7 after infection (Table 1). All the mice infected with 1 mouse lethal dose (MLD_{50}) and higher died by day 8 after infection. An MLD_{50} for A/HK/156/97 virus was estimated at 9 EID_{50}. Undiluted A/HK/1074/99 virus that contained 10^5 EID_{50} killed only two of the five infected mice. The remaining mice showed signs of disease: they had lost 11% of their weight by day 4 and 23% by day 7 after infection. Eight days after infection,

Table 1
Pathogenicity of influenza A/HK/156/97 (H5N1) and H9N2 viruses in mice

Virus/virus dose (EID_{50})	Dead/total	Loss or gain of mean weight on postinfection day (%)				MLD_{50}/0.1 ml (EID_{50})[a]	Mean virus titer (log_{10} EID_{50}/0.1 ml) ± S.D.[b]	
		4	7	11	16		Lung	Brain
A/HK/156/97 (H5N1)						~9	7.5 ± 0.5	1.0 ± 0.5
10	5/5	−10.2 ± 0.5	+15.0 ± 0.5	NA[c]	NA			
1	3/5	−9.1 ± 0.5	−12.0 ± 0.5	−18.7 ± 1.3	0 ± 0.5			
10^{-1}	0/5	−8.5 ± 1.0	−11.3 ± 1.2	−16 ± 2.0	+1.1 ± 0.5			
A/Qa/HK/G1/97 (P_0) (H9N2)						$10^{4.9}$	4.5 ± 0.7	<0.1
10^5	3/5	−10.2 ± 0.9	−15.4 ± 1.2	−2.7 ± 1.8	+1.2 ± 0.7			
10^4	0/5	−8.2 ± 1.1	−10.1 ± 1.2	−1.0 ± 0.2	+1.4 ± 0.9			
10^3	0/5	−5.3 ± 0.6	−5.8 ± 0.8	+1.6 ± 0.7	+2.7 ± 0.6			
A/Qa/HK/G1/97 (P_3) (H9N2)						$10^{2.9}$	6.0 ± 1.0	3.0 ± 1.0
10^4	5/5	−21.3 ± 1.0	−32.4 ± 2.9	NA	NA			
10^3	3/5	−22.3 ± 2.1	−30.0 ± 1.6	−20.5 ± 1.7	0 ± 0.4			
10^2	0/5	−15.1 ± 1.8	−28.2 ± 3.2	−20.1 ± 1.7	+1 ± 0.9			
A/HK/1074/99 (H9N2)						$10^{4.7}$	4.0 ± 1.0	<0.1
10^5	3/5	−11.1 ± 1.4	−23.4 ± 1.8	−21.1 ± 0.9	+0.5 ± 0.1			
10^4	1/5	−5.3 ± 0.6	−10.0 ± 1.2	−7.0 ± 1.0	+0.8 ± 0.3			
10^3	0/5	−1.0 ± 1.9	−1.2 ± 0.2	−0.3 ± 0.5	+1.1 ± 0.9			

[a] Expressed as the EID_{50}/0. 1ml required to give 1 MLD50/0.1 ml.

[b] Mice were infected with 1 MLD_{50}/0.1 ml of A/HK/156/97 and A/Qa/HK/G1/97 (P3) viruses or with 100 μl of allantoic fluid of A/Qa/HK/G1/97 (P_0) and A/HK/1074/99 viruses. Mean virus titers were determined on day 4 after infection and represent means ± S.D. of three mice per group.

[c] NA, not applicable (all mice in group were dead, so weight could not be measured).

the surviving mice started gaining weight, and the other signs of disease disappeared. An MLD_{50} for A/HK/1074/99 virus was estimated at $10^{4.7}$ EID_{50}. Undiluted A/Qa/HK/G1/97 that contained 10^5 EID_{50} did not kill all the mice in the group. However, after three passages in mouse lungs, A/Qa/HK/G1/97 (P_3) had greater pathogenicity in mice. A representative dose-response curve showed that 10^4 EID_{50} of A/Qa/HK/G1/97 (P_3) killed all mice. These mice had lost between 20 and 30% of their body weight by day 4 and 7 after infection, respectively. An MLD_{50} for A/Qa/HK/G1/97 (P_3) was estimated at $10^{2.9}$ EID_{50} compared to $10^{4.9}$ for A/Qa/HK/G1/97 virus.

To further examine the pathogenicity of H9N2 viruses in mice we examined the degree of viral replication in mouse lungs and brains (Table 1). A/HK/156/97 (H5N1) virus replicated in high titer in mouse lungs (7.5 $\log_{10}$ EID_{50}/0.1 ml) and was found in mouse brain in lower titer (1.0 $\log_{10}$ EID_{50}/0.1 ml). These results confirmed data of Gubareva et al. (1998) Lu et al. (1999) about systematic spread of A/HK/156/97 virus in mice. Titers of A/HK/1074/99 and A/Qa/HK/G1/97 viruses in the lungs were lower than those of A/HK/156/97 virus and they were not detected in the mouse brains. After three passages in mouse lungs A/Qa/HK/G1/97 (P_3) virus replicated in mouse lungs to high titers as compared with unadapted virus and was detected in mouse brain at a titer 3.0 $\log_{10}$ EID_{50}/0.1 ml. It was noteworthy, that A/Qa/HK/G1/97 (P_3) was recovered from mouse brain at higher titers than A/HK/156/97 (H5N1), whereas an opposite situation was observed in the lungs.

3.2. In vitro activity of GS4071 against H5N1 and H9N2 influenza viruses

In vitro experiments were done to determine the sensitivity of H5N1 and H9N2 viruses to the anti-NA inhibitor GS4071. Using the cell-ELISA technique, we studied the effect of GS4071 on viral NP expression in MDCK cells. The EC_{50} values of GS4071 against the H5N1 and H9N2 viruses were similar and ranged from 7.5 to 12 μM (Table 2). In cell–ELISA, the human and quail isolates of H9N2 and a human isolate of H5N1 were approximately 2-fold less sensitive to GS4071 than the human A/Singapore/1/57 (H2N2) influenza virus.

We evaluated the efficacy of GS4071 against the H5N1 virus A/HK/156/97 and the H9N2 influenza viruses in NA inhibition assays. GS4071 efficiently inhibited the enzyme activity of the H5N1 and H9N2 viruses (Table 2). The sensitivities to the NA inhibitor did not differ among the viruses and the IC_{50} values (i.e. concentration of drug that is required to inhibit the enzyme activity of NA by 50%) were 15 nM against A/HK/1074/99 virus, 10 nM against A/Qa/HK/G1/97 viruses, and 7 nM against A/HK/156/97 virus.

3.3. Efficacy of GS4104 against H5N1 and H9N2 influenza virus infection in mice

To determine the efficacy of orally administered GS4104, we measured the amount of weight loss and determined the mean survival day and the number of mice which died after infection with H5N1 and H9N2 influenza viruses. GS4104 exerted a significant dose-dependent antiviral effect

Table 2
In vitro effect of GS4071 on influenza H5N1 and H9N2 viruses

Virus	In vitro inhibition	
	Mean $EC_{50} \pm$ S.D. (μM)[a]	Mean $IC_{50} \pm$ S.D. (nM)[b]
A/HK/156/97 (H5N1)	7.5 ± 2.5	7.0 ± 0.9
A/Qa/HK/G1/97 (P_0) (H9N2)	10.0 ± 1.0	10.0 ± 0.4
A/Qa/HK/G1/97 (P_3) (H9N2)	10.0 ± 3.0	10.0 ± 2.0
A/HK/1074/99 human (H9N2)	12.0 ± 2.0	15.0 ± 0.7
A/Singapore/1/57 (H2N2)	5.0 ± 0.6	9.0 ± 1.0

[a] Determined by ELISA and represents the concentration of the compound required to inhibit virus replication in MDCK cells by 50% compared with that of controls without drug.

[b] Determined in NA inhibition assay and represents the drug concentration required to reduce the NA activity to 50% compared with that of controls without drug.

in mice infected with A/HK/156/97 (H5N1) virus (Table 3). All mice given the drug, lost less weight than mice in the control group. Mice given doses of 1 and 10 mg/kg per day did not die; mice given doses of 0.1 mg/kg per day survived longer and the number of survivors was also increased.

We next studied the effect of orally administered GS4104 on the human influenza A/HK/1074/99 (H9N2) infection in mice. No deaths occurred among mice infected with undiluted virus and treated with doses of 1 and 10 mg/kg each day. The number of survivors and the survival were greater among infected mice treated with 0.1 mg/kg per day than among the infected and untreated mice. However, the differences neither in survival rate, not in survival were not statistically significant (Table 3). Since the A/HK/1074/99 virus isolated from human did not kill all of the mice in the control group, we studied the effect of GS4104 on A/Qa/HK/G1/97 (P_3) virus, which is genetically closely related to the A/HK/1074/99 virus and causes death in 100% of the infected mice. Mice infected with 10 MLD_{50} of A/Qa/HK/G1/97 (P_3) virus and treated with orally administered GS4104 were fully protected from death at doses of 1, 10 and 100 mg/kg per day. Mice given these doses lost less weight than mice in the control group. Comparison of the results from the control group and the group treated with a dose of 0.1 mg/kg per day GS4104 showed that the number of survivors and the MSD were greater in the treated group.

3.4. Effect of orally administered GS4104 on the replication of H5N1 and H9N1 influenza viruses in the lungs and brains of mice

Since both the H5N1 and mouse adapted H9N2 influenza viruses used in this study replicate to high titers in the lungs and to lower titers in the brains of mice, we wished to determine the extent of reduction in virus levels that could be achieved with GS4104. After infection with 5 MLD_{50} of A/HK/156/97 (H5N1) or A/Qa/HK/G1/97 (P_3) viruses, groups of mice were treated with different doses of GS4104. Three mice from each group were humanely killed on each day after infection, and the virus from their lungs and brains was titrated. The titers of both viruses in the lungs of mice treated with GS4104 decreased in a dose-dependent fashion with the lowest effective dose being 0.1 mg/kg per day in both cases (Fig. 1 A, B). In mice infected with A/HK/156/97 (H5N1), GS4104 significantly decreased the titer of virus in the lungs, but at doses of 1 and 10 mg/kg per day, the reduction in virus titer was approximately the same as that of mice treated with 100 mg/kg per day; residual virus at titers of 3–4 $\log_{10}$ EID_{50} was detected in the lungs of these animals (Fig. 1A). In mice infected with A/Qa/HK/G1/97 (P_3), doses of 1 and 10 mg/kg per day reduced the virus titer in lungs significantly and a dose of 100 mg/kg per day completely inhibited replication (Fig. 1B).

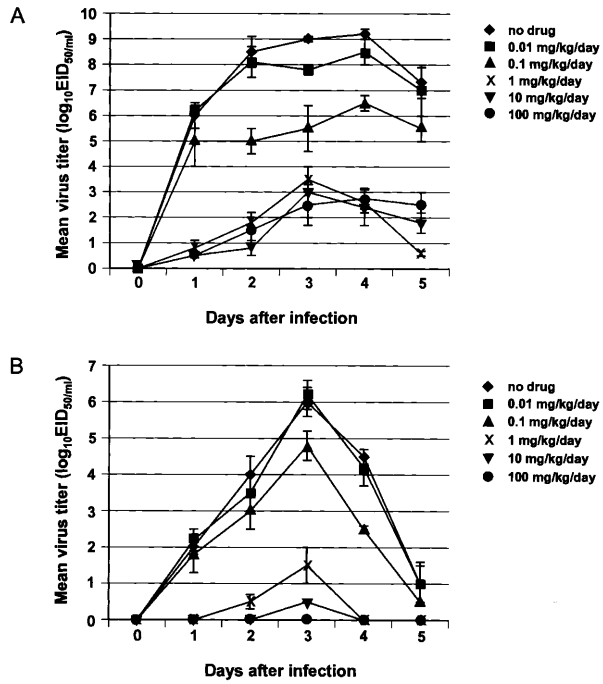

Fig. 1. Effect of different doses of GS4104 on titers of A/HK/156/97 (H5N1) (A) and A/Qa/HK/G1/97 (P_3) (H9N2) (B) viruses from the lungs of infected mice. BALB/c mice were infected intranasally with 5 MLD_{50} of A/HK/156/97 or A/Qa/HK/G1/97 (P_3) viruses and treated with different doses of GS4104 or no drug. Three mice from each group were sacrificed on each day after infection for virus titration in embryonated chicken eggs (EID_{50}/ml). $P < 0.01$ for groups of mice treated with 0.1, 1, 10 and 100 mg/kg per day of GS4104 compared with the untreated mice.

Table 3
The effect of orally administered GS4104 on influenza H5N1 and H9N2 virus infection in mice[a]

Virus	Virus dose (MLD_{50})	Treatment[b]	Dose (mg/kg per day)	Survival/total[c]	Loss or gain of mean weight on postinfection day (%)				Mean survival day ± S.D.[d]
					3	7	11	15	
A/HK/156/97 (H5N1)	5	GS4104	0.1	4/5*	−1.2 ± 0.9	−4.4 ± 0.7	+1.1 ± 0.5	+9.9 ± 0.3	14.0 ± 0**
			1	5/5**	−2.7 ± 0.6	−7.3 ± 0.5	+7.8 ± 0.2	+17.0 ± 1.3	>16**
			10	5/5**	−0.5 ± 0.8	−6.4 ± 0.7	+4.4 ± 0.3	+13.9 ± 1.2	>16**
Control		PBS		0/4	−7.6 ± 0.2	−11.0 ± 1.5	NA[e]	NA	7.5 ± 0.5
A/Qa/HKG1/97 (P_3) (H9N2)	10	GS4104	0.1	5/10*	−15.5 ± 1.2	−25.6 ± 1.9	−10.0 ± 0.9	+1.0 ± 0.5	9.8 ± 0.8**
			1	9/9**	−10.8 ± 1.8	−15.8 ± 1.4	−10.1 ± 0.5	+1.9 ± 0.2	>16**
			10	10/10**	−7.7 ± 1.1	−10.0 ± 0.8	−10.0 ± 0.3	+2.2 ± 0.6	>16**
			100	10/10**	−12.2 ± 0.9	−5.0 ± 0.2	−3.1 ± 0.5	+2.5 ± 0.6	>16**
Control		PBS		0/10	−18.4 ± 1.2	−26.2 ± 1.9	NA	NA	4.0 ± 1.0
A/HK/1074/99 human(H9N2)	<1	GS4104	0.1	3/5	−8.4 ± 0.3	−19.1 ± 1.5	−15.4 ± 0.9	+2.0 ± 0	11.2 ± 0.3
			1	5/5	+0.4 ± 0.1	−2.3 ± 0.4	+1.4 ± 0.1	+2.2 ± 0.1	>16
			10	5/5	+0.5 ± 0.1	−1.3 ± 0	+2.2 ± 0.2	+2.2 ± 0	>16
Control		PBS		2/5	−11.3 ± 0.3	−23.2 ± 1.3	−21.1 ± 2.0	+2.2 ± 0.1	8.8 ± 0.7

[a] *$P<0.05$, **$P<0.01$ compared with PBS controls run in the same experiments.
[b] Bid × 5 beginning 4 h pre-virus exposure.
[c] The Fisher's Exact test of survival rate between the control and treatment groups.
[d] The log-rank test for differences in survival curves between the control and treatment groups.
[e] NA, not applicable (all mice in the group were dead, so weight could not be measured).

The H5N1 and H9N2 viruses were undetectable in the brain of mice after treatment with doses of GS4104 as low as 0.1 mg/kg per day (Table 4). When the mice were treated with 0.01 mg/kg per day, the level of virus on days 3 and 4 were lower than those of the control animals.

3.5. Sensitivity of virus from murine lungs to GS 4071

The presence of virus in the lungs of mice infected with A/HK/156/97 (H5N1) and treated with high doses of GS4104 suggested that drug-resistant viruses might have been emerging. To determine whether the virus from lungs of mice treated with 100 mg/kg per day GS4104 and killed on 5 day after infection was resistant to GS4071, we studied the virus's sensitivity to GS4071 in plaque assay and cell-ELISA. In plaque assay original and isolated from mouse lungs A/HK/156/97 viruses did not differ in their sensitivity to GS4071 and the EC_{50} value for plaque size (i.e. concentration required to reduce the number of plaques by 50%) was approximately 3 μM for both the viruses. At concentrations of 10 μM, GS4071 completely inhibited the formation of plaques of both viruses even pinpoint plaques were not observed (data not shown). In cell-ELISA, GS4071 inhibited the replication of A/HK/156/97 (H5N1), in a dose-dependent fashion that was indistinguishable from that associated with the original virus (Fig. 2). Therefore, GS4104-resistant viruses did not appear to emerge in these studies.

3.6. Influence of delay of treatment with GS4104 on virus infection in mice

Since the NA of influenza virus plays a key role in the release of progeny virus from infected cells, (Air and Laver, 1989; Palese and Compans, 1976) we hypothesized that GS4104 would be efficacious when administered late in the infection. We, therefore, determined how long the treatment with GS4104 could be delayed and still provide an antiviral effect. In our experiments, twice-daily treatment of mice infected with 10 MLD_{50} of A/HK/156/97 (H5N1) virus began at 24, 36, 48, 60, and 72 h after infection. GS4104 was efficacious in mice when therapy was initiated 24 or 36 h after infection (Fig. 3). When treatment was delayed until 48 h after virus exposure all of the mice died, but the mean length of survival was extended to 9.8 days as compared with 8 days for the control group. GS4104 was not effective in preventing death and extending the length of sur-

Table 4
Efficacy of treatment with GS4104 on the spread of influenza A/HK/156/97 (H5N1) and mouse adapted A/Qa/HK/G1/97 (P_3) (H9N2) viruses to the brain of mice[a]

Day after infection	Virus infectivity (log_{10} EID_{50}/0.1ml ± S.D.)[b] in the brain of mice infected with:					
	A/HK/156/97 (H5N1)[c]			A/Qa/HK/G1/97 (P_3) (H9N2)[c]		
	No drug	Treated with GS4104 (mg/kg per day)		No drug	Treated with GS4104 (mg/kg per day)	
		0.01	0.1		0.01	0.1
1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
3	1.0 ± 0	0.6 ± 0.1*	<0.1	3.0 ± 0.5	1.5 ± 0.5*	<0.1
4	1.5 ± 0.5	1.1 ± 0.4*	<0.1	3.3 ± 0.3	1.5 ± 0*	<0.1
6	1.0 ± 0.4	0.2 ± 0*	<0.1	0.2 ± 0	0.2 ± 0.2	<0.1

[a] *$P < 0.05$ compared with control mice untreated with drug.
[b] Samples from groups of three mice treated with different doses of GS4104 or not treated were investigated.
[c] Bid × 5 beginning 4 h pre-virus exposure.

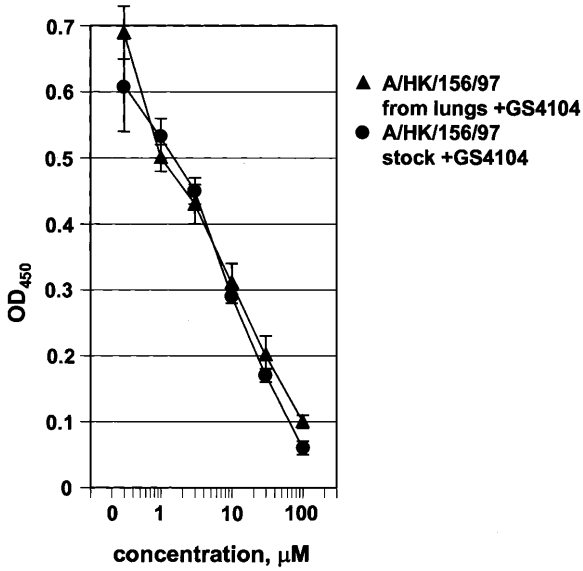

Fig. 2. Effect of GS4071 on replication in MDCK cells of influenza A/HK/156/97 (H5N1) virus recovered from mouse lungs. The A/HK/156/97 (H5N1) virus was isolated from lungs of mice that were untreated or treated with 100 mg/kg per day of GS4104 on day 5 after infection. The samples were assayed in cell-ELISA. Each point represents the mean of NP expression (OD at $\lambda = 450$ nm) in four replicate wells in one experiment.

vival when treatment began 60 h or more after virus inoculation.

3.7. Efficacy of orally administered GS4104 and rimantadine in mice infected with H9N2 virus

Since rimantadine and GS4104 target different proteins in the process of influenza virus replication, namely the M2 ion channel (rimantadine) and the NA (GS4104), we hypothesized that a combination of these drugs would be more efficacious than drugs administered singly. To test this hypothesis, we used high (i.e. 100 MLD_{50}) and low (i.e. 5 MLD_{50}) doses of H9N2 virus in the mouse model.

When administered singly, GS4104 (0.1 and 1 mg/kg per day) and rimantadine (10 mg/kg per day) were not effective in preventing the death of mice infected with high doses of virus (Table 5). The only beneficial effect of these drugs when given individually at these doses was an increase in the length of mean survival day as compared with that of the control mice. The number of survivors and the length of mean survival day were greater among mice infected with high doses of virus and treated with 10 mg/kg per day GS4104 than among control mice. The combination of GS4104 and rimantadine at all doses studied significantly increased the number of survivors and the survival as compared with those of controls and with those of mice treated with the same doses of drugs administered singly. The group of mice that received 10 mg/kg per day of GS4104 and 10 mg/kg per day of rimantadine demonstrated the best survival rate and the highest length of mean survival day. However, when the high dose of virus (100 MLD_{50}) was used, the combination of drugs did not completely protect mice from weight loss or death.

When administered individually, GS4104 (0.01 mg/kg per day) and rimantadine (1 mg/kg per day) did not prevent weight loss and death of mice infected with low dose of H9N2 virus (Table 5). However, combinations of the two agents (0.01 mg/kg per day GS4104 and 1 mg/kg per day rimantadine; 0.1 mg/kg per day GS4104 and 10 mg/kg per day rimantadine) resulted in an increase in the survival. Combinations of the inhibitors at other doses fully protected the mice from death. The mice that received the combination of 0.1 mg/kg per day GS4104 and of 1 mg/kg per day rimantadine had the best survival rate when compared with those mice that received only one of the drugs. Thus, these two inhibitors are more effective when given in combination than when given singly.

4. Discussion

In the present study we established that GS4071 inhibits the replication of the influenza viruses A/HK/156/97 (H5N1) and A/HK/1074/99 (H9N2) and the avian influenza virus A/Qa/HK/G1/97 (H9N2) in MDCK cells. In addition, GS4071 inhibits the NA activity of these viruses. The mean EC_{50} measured by ELISA and the mean IC_{50} measured by NA inhibition tests were similar against A/HK/156/97 (H5N1), A/HK/

1074/99 (H9N2), and A/Qa/HK/G1/97 (H9N2) influenza viruses, and the viruses did not differ in their sensitivities to the drug in vitro. Our in vitro results concur with those of previous studies in which GS4107 inhibited viral replication of different strains of human influenza A and B viruses in cell culture (Hayden and Rollins, 1997; Sidwell et al., 1998). Finding from studies of zanamivir, another neuraminidase inhibitor against avian influenza viruses in vitro (Gubareva et al., 1995; Thomas et al., 1994), showed that zanamivir and GS4071 had similar IC_{50} values against the neuraminidase of A/HK/156/97; for zanamivir the IC_{50} value was 1 nM (Gubareva et al., 1998); for GS4107, 7 nM. Although studies of the efficacy of zanamivir against H9N2 viruses have not been done, zanamivir has been shown to effectively inhibit the NA activity of an avian A/Ty/Min/833/80 (H4N2) virus (Gubareva et al., 1996). The IC_{50} value was 11 nM. In the present study, we found that the IC_{50} value of GS4071 against H9N2 viruses ranged from 10 to 15 nM.

The results of our in vivo experiments indicate that orally administered GS4104 is highly effective in treating H5N1 and H9N2 infections in mice. In earlier studies of the efficacy of GS4104 in mice infected with human strains of influenza viruses, Sidwell et al. (1998) showed that oral administration of GS4104 at a dosage of 1 mg/kg per day resulted in an increase in the number of survivors and in the mean survival time, but did not fully protect the mice from death. In our experiments, the same dose of GS4104 fully protected mice from death. The differences in results probably reflect the different properties of the human and avian viruses.

Zanamivir administered intranasally is effective in treating mice infected with A/HK/156/97 (H5N1) influenza virus (Gubareva et al., 1998). Although the sensitivities of A/HK/156/97 to zanamivir and GS4107 do not differ in vitro, the dose of zanamivir required to prevent the death of mice infected with this virus is 25-fold greater than that of GS4104. Since the type of virus and method and quantity of virus challenge were the same in the previous and current studies, this difference is thought to be due to differences in the drug's pharmacokinetic properties in the mouse model. Zanamivir acts at local sites of administration to the respiratory tract, whereas

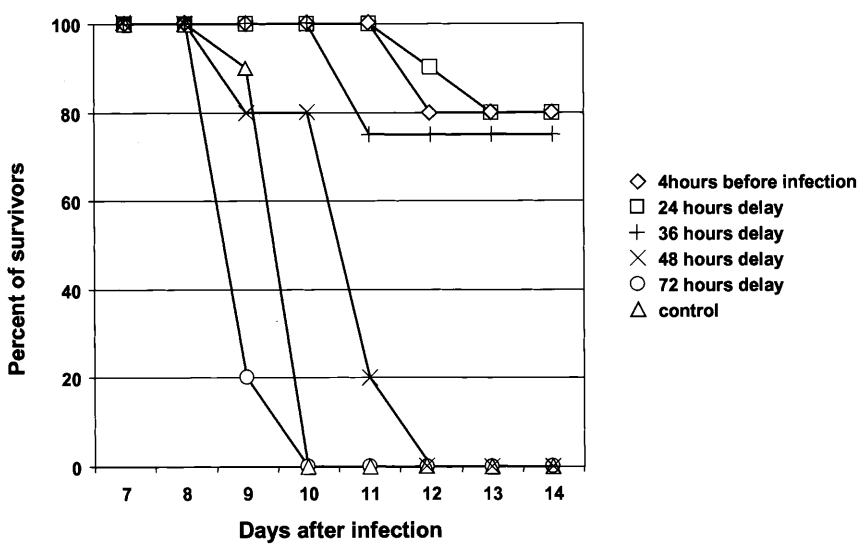

Fig. 3. Effect of delayed treatment with GS4104 on A/HK/156/97 (H5N1) virus infection in mice. Groups of mice were infected with 10 MLD_{50} of A/HK/156/97 (H5N1) virus, and twice-daily treatment with GS4104 (1 mg/kg per day) was begun at intervals 24, 36, 48, 60 and 72 h after infection and continued for 5 days. Each group contained ten mice except one group which contained eight mice. In this group treatment was started 36 h after infection.

Table 5
Efficacy of combination of GS4104 and rimantadine in mice infected with high and low doses of influenza A/Qa/HK/G1/97 (P_3) (H9N2) virus[a]

Virus dose/drugs (mg/kg per day)		Survivors/total[b]	Loss or gain of mean weight on postinfection day (%)			Mean survival day ± S.D.[c]
			3	5	11	
High virus dose	(100 MLD_{50})					
GS4104[d]	Rimantadine[e]					
0.1	–	0/10	−18.3 ± 1.7	−26.2 ± 2.2	NA[f]	4.9 ± 0.4**
1	–	0/10	−15.7 ± 1.3	−22.4 ± 2.3	NA	5.1 ± 0.7*
10	–	5/10*	−15.6 ± 1.7	−19.0 ± 1.7	+1.1 ± 0.5	10.5 ± 0.5***
–	10	0/10	−18.9 ± 1.1	−21.7 ± 2.0	NA	4.5 ± 0.7
0.1	10	7/10**	−15.8 ± 1.9	−20.2 ± 2.2	+1.3 ± 0.2	12.0 ± 1.0***
1	10	7/10**	−14.5 ± 0.5	−20.8 ± 0.8	+1.4 ± 0.4	12.1 ± 1.4***
10	10	9/10***	−13.3 ± 0.9	−20.1 ± 0.5	+1.3 ± 0.1	14.1 ± 0***
Control		0/10	−18.0 ± 0.3	−28.0 ± 2.9	NA	4.2 ± 0.5
Low virus dose *(5 MLD_{50})*						
0.01	–	0/10	−18.4 ± 3.9	−23.0 ± 3.6	NA	9.0 ± 0
0.1	–	4/10	−16.2 ± 1.6	−28.4 ± 2.5	+1.2 ± 0.2	12.0 ± 0***
–	1	0/10	−17.0 ± 0.7	−28.4 ± 4.8	NA	8.0 ± 0.6
–	10	8/10***	−11.6 ± 2.1	−19.5 ± 2.6	+1.1 ± 0.1	14.0 ± 0***
0.01	1	2/10	−11.2 ± 2.1	−19.1 ± 0.3	+1.7 ± 0.9	11.2 ± 3.7***
0.01	10	10/10***	+11.6 ± 2.2	−19.4 ± 5.1	+1.9 ± 0.2	>16***
0.1	1	10/10***	−12.8 ± 1.9	−19.8 ± 2.9	+2.0 ± 0.8	>16***
0.1	10	10/10***	−11.5 ± 1.4	−18.5 ± 1.6	+1.9 ± 0.1	>16***
Control		0/10	−19.6 ± 2.7	−35.0 ± 1.9	NA	9.0 ± 0

[a] *$P<0.05$ **$P<0.01$, ***$P<0.001$ compared to control mice untreated with drugs.
[b] The Fisher's Exact test of survival rate between the control and treatment groups.
[c] The log-rank test for differences in survival curves between the control and treatment groups.
[d] Bid × 5 beginning 4 h pre-virus exposure.
[e] Bid × 5 beginning 24 h pre-virus exposure.
[f] NA, not applicable (all mice in group were dead, so weight could not be measured.

GS4104 administered orally crosses the cells lining the intestine and migrates into the blood stream (Li et al., 1997). Thus, GS4104 can reach cells outside the respiratory tract and may be more effective than zanamivir against highly pathogenic systemic infection caused by A/HK/156/97 virus.

Oral administration of GS4104 at doses 0.1 mg/kg per day and higher reduced the titers of the A/HK/156/97 (H5N1) and A/Qa/HK/G1/97 (P_3) (H9N2) viruses to undetectable levels in the brains of infected mice. In contrast, both the H5N1 and H9N2 viruses were found in the brains of untreated mice. It has been postulated that in previous pandemics during 1889, 1918 and 1957 (Potter, 1998) and has been demonstrated in recent H3N2 epidemics in Japan (Togashi et al., 1997) that encephalitis is sometimes associated with influenza infection of humans. The ability of GS4104 to limit or prevent the spread of virus to the central nervous system (CNS) may be useful in treating patients in an emerging pandemic situation and in treating the sporadic cases of encephalitis associated with influenza A and B viruses.

Treatment with GS4104 greatly reduced the titers of A/Qa/HK/G1/97 (P_3) (H9N2) virus in a dose-dependent fashion in the lungs of mice. However, in the case of mice infected with A/HK/156/97 (H5N1), GS4104 reduced the titer in the lungs significantly, and in a dose-dependent fashion up to 1 mg/kg per day but further dose increases failed to produce an additional reduction in viral titer. The virus isolated from the mice in the end of treatment with 100 mg/kg per day of

GS4104 was not resistant to GS4071 in MDCK cells. These results are similar to the findings of studies in which A/HK/156/97 virus was isolated in the lungs of infected mice after treatment with zanamivir (Gubareva et al., 1998).

The passage of influenza viruses in cell culture with zanamivir and GS4071 eventually leads to the emergence of resistant mutants (Gubareva et al., 1996; Tai et al., 1998), but this emergence requires many more cycles of replication than those achieved in the mouse experiments described above. Unlike the selection of amantadine- and rimantadine-resistant mutants, the selection of mutants resistant to NA inhibitors is difficult, and the NA of these viruses is often unstable (McKimm-Breschkin et al., 1996; Gubareva et al., 1996). The finding that GS4104, when given 36 h after H9N2 infection, was still effective and increased the number of survivors as compared with controls indicates that it is possible to markedly influence the outcome of infection in time to prevent systemic spread. Studies with human influenza viruses (Sidwell et al., 1998) indicate that the efficacy of GS4104 following delayed therapy depended on dose of virus that was used to infect mice. When Sidwell and colleagues (1998) used $10^{3.6}$ $CCID_{50}$ for infection in mice treatment could be delayed for up to 60 h after infection. When virus dose increased to $10^{5.5}$ $CCID_{50}$ which corresponds to approximately 10 MLD_{50} the time in which treatment could be delayed decreased to 36 h, that concurs with our results.

These limited studies of the use of GS4104 and rimantadine to treat mice infected with A/Qa/HK/G1/97 (P_3) (H9N2) established the proof of principal that these inhibitors are more effective when used in combination than when used individually. It remains to be established whether rimantadine-resistant variants appeared in the mice. On the basis of rimantadine's mode of action on the M2 ion channel (Hay, 1996) and the appearance of resistant mutants during treatment of influenza A (Zlydnikov et al., 1981; Hayden and Cough, 1992; Hayden, 1996), it is probable that such rimantadine-resistant mutants would occur. However, the reduction in the virus load and the difference in two drug's sites of action could potentially reduce the emergence of rimantadine-resistant mutants, and, in more practical terms, the amount of drug needed in a pandemic situation when supplies of any influenza drug are likely to be critically short.

In conclusion, the anti-NA compound GS4071 inhibited in MDCK cells the replication of H5N1 and H9N2 viruses, that are transmitted directly from birds to humans. Oral administration of the prodrug of GS4071, GS4104, is highly efficacious against infection in mice induced by these viruses. Our experiments showed that GS4104 given orally at least 36 h after virus infection still render a significant antiviral effect and that those viruses that grow in the presence of the drug are not resistant to inhibitor in MDCK cells. In addition, the combination of GS4104 and rimantadine decreased the dose of rimantdine required and may, therefore, reduce the side effects caused by rimantadine. Taken together, the data provide strong evidence for the efficacy of GS4104 against systemic avian influenza viruses in mammals.

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