

Virulence May Determine the Necessary Duration and Dosage of Oseltamivir Treatment for Highly Pathogenic A/Vietnam/1203/04 Influenza Virus in Mice

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Background. Control of highly pathogenic avian H5N1 influenza viruses is a major public-health concern. Antiviral drugs could be the only option early in the pandemic.

Methods. BALB/c mice were given oseltamivir (0.1, 1, or 10 mg/kg/day) twice daily by oral gavage; the first dose was given 4 h before inoculation with H5N1 A/Vietnam/1203/04 (VN1203/04) virus. Five- and 8-day regimens were evaluated.

Results. Oseltamivir produced a dose-dependent antiviral effect against VN1203/04 in vivo ($P < .01$). The 5-day regimen at 10 mg/kg/day protected 50% of mice; deaths in this treatment group were delayed and indicated the replication of residual virus after the completion of treatment. Eight-day regimens improved oseltamivir efficacy, and dosages of 1 and 10 mg/kg/day significantly reduced virus titers in organs and provided 60% and 80% survival rates, respectively ($P < .05$). Overall, the efficacy of the 5- and 8-day regimens differed significantly (death hazard ratio, 2.658; $P < .01$). The new H5N1 antigenic variant VN1203/04 was more pathogenic in mice than was A/HK/156/97 virus, and a prolonged and higher-dose oseltamivir regimen may be required for the most beneficial antiviral effect.

Conclusions. Oseltamivir prophylaxis is efficacious against lethal challenge with VN1203/04 virus in mice. Viral virulence may affect the antiviral treatment schedule.

Since 1997, highly pathogenic avian H5N1 influenza viruses have caused concern about their human pandemic potential [1]. Although the mass slaughter of poultry in Hong Kong stopped the H5N1 outbreak in 1997, the precursors of the virus continue to circulate in southern China [2, 3]. Reassortment of these precursor viruses with other avian influenza viruses has generated multiple genotypes of H5N1 viruses in recent years [4]. The continued cocirculation of these viruses in wild aquatic birds

and poultry in China has created the potential for avian-to-human and human-to-human transmission [4, 5]. Although only 2 cases of human H5N1 infection were reported in early 2003 [6], a new H5N1 antigenic variant has spread widely across Asia since December 2003, causing deaths in ~50% of confirmed cases, including probable human-to-human transmission in a family cluster of the disease in Thailand [7].

Vaccination and antiviral treatment are the 2 options for the control of influenza. Although vaccine is the preferred method of prophylaxis, at least 6 months are required to produce vaccine against currently circulating influenza viruses, including new H5N1 antigenic variants [8]. The application of antiviral drugs during the early stages of a pandemic should help to control it [9]. Two classes of drugs are currently available for prophylaxis and treatment of influenza virus infection: M2 ion channel blockers (amantadine and rimantadine) and neuraminidase inhibitors (NAIs; oseltamivir and zanamivir). Long-term amantadine or rimantadine prophylaxis was effective against pandemic influenza in

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1968 and 1977 [10, 11]. However, H5N1/04 influenza viruses that have been isolated in Thailand and Vietnam have asparagine at position 31 of the M2 protein that invariably confers resistance to amantadine and rimantadine [6, 12]. Therefore, NAIs may be the only effective antiviral option for the control of H5N1/04 variants in Asia. As part of pandemic preparedness, the efficacy of NAIs against highly pathogenic H5N1 viruses should be investigated.

Prophylactic oseltamivir was used in 2003 during an H7N7 avian influenza virus outbreak in the Netherlands; however, its efficacy is unclear because the initiation of treatment was delayed. Lack of data about widespread, prolonged prophylactic use of oseltamivir was mentioned as an obstacle faced by health planners during the H7N7 outbreak [13]. Although several studies have provided information on the effective dosages of oseltamivir and the appropriate length of treatment during winter epidemic influenza seasons [14–16], only a few strains of avian influenza viruses have been tested with NAIs. A/HK/156/97 (HK156/97) is the only H5N1 strain that has been tested in a mouse model [17–19]. Different influenza virus strains reportedly have different *in vivo* sensitivities to NAIs [20]. Recently isolated H5N1 variants have differed from earlier H5N1 isolates in their pathogenicity in ducks and ferrets [21, 22]. Therefore, it is imperative to determine the sensitivity of the H5N1/04 isolates to NAIs, and we report studies that evaluated the efficacy in mice of oseltamivir against the new antigenic H5N1 variant A/Vietnam/1203/04 (VN1203/04) virus. This virus was isolated from the throat swab of a fatally infected human, and it is highly pathogenic to mice without adaptation.

MATERIALS AND METHODS

NA inhibitors. Oseltamivir carboxylate [(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid] and the prodrug oseltamivir phosphate (oseltamivir)[ethyl(3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate] were provided by Hoffmann–La Roche. Zanamivir (4-guanidino-Neu5Ac2en) was provided by the R. W. Johnson Pharmaceutical Research Institute.

Viruses and cells. The H5N1 influenza viruses VN1203/04 and HK156/97 were obtained through the World Health Organization network. VN1203/04 virus was passaged 3 times in embryonated chicken eggs. HK156/97 virus was passaged twice in mouse lungs and once in embryonated chicken eggs in our laboratory [18]. The sensitivity to NAIs of the same stock of HK156/97 virus has been previously evaluated *in vitro* and *in vivo* [17–19]. Experiments with VN1203/04 and HK156/97 viruses were conducted in a biosafety level (BSL) 3+ containment facility. To determine the sensitivity of the highly pathogenic viruses to NAIs *in vitro* in BSL2 laboratories, the reverse-genetics VN1203 × PR8 and HK156 × PR8 viruses, each of

which contain the NA from the VN1203/04 virus or the NA from the HK156/97 virus in the background of A/Puerto Rico/8/34 (H1N1) virus, were rescued as described elsewhere [23]. MDCK and 293T human embryonic kidney cells were obtained from the American Type Culture Collection. MDCK cells transfected with cDNA encoding human 2,6-sialyltransferase (MDCK-SIAT1 cells) were provided by Dr. M. Matrosovich (Philipps University, Marburg, Germany) and were maintained as described elsewhere [24].

NA enzymatic activity and NA-inhibition assay. A modified fluorometric assay was used to determine the NA activity of the virus with the fluorogenic substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (Sigma) [25, 26]. The fluorescence of the released 4-methylumbelliferone was quantified in a Fluoroskan II spectrophotometer (Labsystems) at excitation and emission wavelengths of 355 and 460 nm, respectively. NA inhibition was assayed with viruses standardized to equivalent NA activity and incubated with NAIs at concentrations of 0.00005–10 μ mol/L [25]. The IC₅₀ of NA enzymatic activity was determined by plotting the percentage of inhibition of NA activity as a function of the compound concentration, calculated from the dose-response curve.

Cell-based virus reduction assay. Confluent MDCK-SIAT1 cells were preincubated for 1 h with serial 10-fold dilutions of the NAIs (0.0001–500 μ mol/L). The cells were infected with 0.001 pfu/cell of virus for 2 h at 37°C and were then overlaid with infection medium that contained NAIs (0.0001–500 μ mol/L). Virus replication was determined by measurement of hemagglutinin (HA) activity after 72 h of incubation at 37°C. The 50% effective concentration (EC₅₀) of the compound was determined by plotting the percentage of inhibition of virus replication as a function of the compound concentration, calculated from the dose-response curve.

Drug efficacy *in vivo*. Female 6-week-old BALB/c mice (Jackson Laboratories) were anesthetized with isoflurane and intranasally inoculated with 50 μ L of 10-fold serial dilutions of VN1203/04 virus in PBS. The mouse lethal dose (MLD₅₀) was calculated after a 16-day observation period. Oseltamivir was administered by oral gavage twice daily for 5 or 8 days to groups of 10 mice at dosages of 0.1, 1, and 10 mg/kg/day. Control (infected but untreated) mice received sterile PBS (placebo) on the same schedule. Four hours after the first dose of oseltamivir, the mice were inoculated intranasally with 5 MLD₅₀ of VN1203/04 virus in 50 μ L of PBS. Survival and weight change were observed for 24 days. Virus titers in the mouse organs were determined on days 3, 6, and 9 after inoculation. Three mice from each experimental and placebo group were killed, and the lungs and brains were removed. The organs were homogenized and suspended in 1 mL of PBS. The cellular debris was cleared by centrifugation at 2000 g for 5 min. The limit of virus detection was 0.75 log₁₀ EID₅₀. For calculation of the

mean, samples with a virus titer $<0.75 \log_{10} \text{EID}_{50}/\text{mL}$ were assigned a value of 0. Virus titers in each organ were calculated by use of the method of Reed and Muench [27] and are expressed as mean $\log_{10} \text{EID}_{50}/\text{mL} \pm \text{SE}$. All studies were conducted under applicable laws and guidelines of and after approval from the St. Jude Children's Research Hospital Animal Care and Use Committee.

Replication efficiency of the VN1203/04 and HK156/97 influenza viruses in mice. To compare the pathogenicity of the VN1203/04 and the HK156/97 viruses, we inoculated mice with $\sim 3 \text{EID}_{50}$ and $\sim 3 \text{MLD}_{50}$ of each virus. The stock HK156/97 virus used in the study has been characterized elsewhere [17, 18]. Mouse organs (brain and lungs) and blood were collected on days 3, 6, and 9 after inoculation, and virus was titered (in $\log_{10} \text{EID}_{50}/\text{mL}$). Serum was collected on day 21 after inoculation and tested by HA-inhibition assay to quantify anti-HA antibodies.

Sequence analysis. The HA and NA sequences of the viruses isolated from the lungs of mice on days 6 and 9 after inoculation were amplified by reverse-transcription polymerase chain reaction, as described elsewhere [28]. The sequences were determined by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital, as described elsewhere [29].

Statistical analysis. Virus titers in brain and lungs on days 3, 6, and 9 after inoculation were compared by analysis of variance. The Kaplan-Meier method was used to estimate the probability of survival. The log-rank test was used to compare outcomes of the placebo and the 3 treatment groups. The proportional hazards model [30] was used to estimate the death hazard ratio between each treatment and placebo group.

RESULTS

Sensitivity of VN1203/04 virus to NAIs in vitro. NA-inhibition and virus-reduction assays were used to evaluate the sensitivity of the NA of VN1203/04 NA virus to zanamivir and

oseltamivir carboxylate in vitro (table 1). Both assays used the reverse genetics-derived VN1203 \times PR8 virus that contained the NA of the VN1203/04 wild-type virus in a background of PR8 virus. To compare the sensitivity of VN1203/04 virus to a previously characterized H5N1 virus and to a common H1N1 virus, the reverse genetics-derived HK156 \times PR8 and PR8 viruses were also included. Comparable concentrations of zanamivir were required to inhibit the NA enzymatic activity of all 3 viruses, whereas the VN1203 \times PR8 virus was more sensitive than the HK156 \times PR8 and PR8 viruses to oseltamivir carboxylate (mean IC_{50} , 0.4, 4.1, and 4.5 nmol/L, respectively) (table 1). In virus-reduction assays in MDCK-SIAT1 cells, which allow a more sensitive evaluation of influenza virus sensitivity to NAIs than do MDCK cells [24], the mean EC_{50} values of zanamivir were comparable for all 3 viruses tested, but VN1203/04 virus was the most sensitive to oseltamivir carboxylate (table 1).

Efficacy of oseltamivir against VN1203/04 virus in vivo. To evaluate the pathogenicity of VN1203/04 virus, we inoculated mice with 10-fold dilutions of the virus and observed clinical symptoms and survival. The virus was highly pathogenic in mice without prior adaptation; a virus dose as low as $10^{1.2} \text{EID}_{50}$ caused the deaths of all 5 infected mice. The symptoms preceding death were more pronounced at higher virus doses (virus inocula $10^{2.2-3.2} \text{EID}_{50}$): by day 7 after inoculation, the mice had lost $>25\%$ of their body weight, were inactive, and had developed hind-limb paralysis. The $\text{MLD}_{50}/\text{mL}$ of the stock virus was $10^{9.3}$.

We evaluated the prophylactic efficacy of 5 and 8 days of oseltamivir treatment of mice against challenge with 5 MLD_{50} of VN1203/04 virus. Oseltamivir treatment for 5 days had previously been shown to be efficacious against A/NWS/33 (H1N1) influenza virus infection in mice [31]. Treatment of mice with NAIs (oseltamivir or peramivir) at dosage of 1–10 mg/kg/day for 5 days was effective against lethal challenge with HK156/97 virus [17].

Table 1. Sensitivity of reverse genetics-derived influenza viruses to neuraminidase (NA) inhibitors (NAIs) in NA-inhibition and virus-reduction assays.

Reverse-genetics virus	IC_{50} , nmol/L ^a		EC_{50} , $\mu\text{mol/L}$ ^b	
	Zanamivir	Oseltamivir carboxylate	Zanamivir	Oseltamivir carboxylate
VN1203 \times PR8 (H1N1)	0.8 \pm 0.1	0.4 \pm 0.1	0.9 \pm 0.1	0.1 \pm 0.1
HK156 \times PR8 (H1N1)	0.7 \pm 0.1	4.1 \pm 0.2	0.5 \pm 0.1	1.0 \pm 0.1
PR/8/34 (H1N1)	0.7 \pm 0.1	4.5 \pm 0.2	1.1 \pm 0.1	4.6 \pm 1.2

NOTE. Data are presented as mean \pm SD.

^a NA inhibition was assayed with viruses standardized to equivalent NA activity and incubated with NAIs at concentrations of 0.00005–10 $\mu\text{mol/L}$ with 2-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid as substrate [25]. The IC_{50} was determined by plotting the percentage of inhibition of NA activity as a function of the compound concentration, calculated from the dose-response curve. Values are from 3 independent determinations.

^b The 50% effective concentration of the compound in MDCK-SIAT1 cells, based on the measurement of hemagglutination activity 72 h after infection with 0.001 pfu/cell of virus.

The administration of 1 and 10 mg/kg/day of oseltamivir for 5 days had significantly inhibited virus replication in the lungs at days 3 and 6 after inoculation ($P < .05$) but not at day 9 after inoculation (figure 1A). All mice, except those that received 10 mg/kg/day, had detectable brain virus titers at days 3, 6, and 9 after inoculation (figure 1C). The 5-day regimen at a dosage of 0.1 mg/kg/day did not show any significant antiviral effect, compared with that shown with the placebo. Only mice that received the 5-day regimen at 10 mg/kg/day survived challenge (50% survival rate) (figure 2A), whereas mice that received other 5-day regimens died during the observation period. It was noted that most mice developed severe neurological symptoms before death. The increase in lung virus titers at day 9 after inoculation in the mice that received 1 and 10 mg/kg/day (figure 1A) indicated that there was replication of the residual virus after the 5-day treatment regimen ended. It was also noted that death occurred at later days after inoculation for the mice that received 1 and 10 mg/kg/day of oseltamivir, compared with the groups that received placebo and 0.1 mg/kg/day of oseltamivir (figure 2A).

The administration of oseltamivir for 8 days improved survival rates in all treatment groups (figure 2B). A dose-dependent survival outcome was observed: the highest survival rate was achieved at 10 mg/kg/day (80%), followed by 1 mg/kg/day (60%) and 0.1 mg/kg/day (10%) (table 2). Treatment with 1 and 10 mg/kg/day of oseltamivir for 8 days had significantly inhibited virus replication in the lungs by day 9 after inoculation ($P < .05$) (figure 1B), which indicates a greater inhibition of residual virus replication than that with the 5-day regimens.

Our results also suggested that weight change is prognostic of mouse survival. In the 8-day regimens, mice that received 1 or 10 mg/kg/day of oseltamivir had higher survival rates and less weight loss (<6%) than those that received 0.1 mg/kg/day or placebo (>15%). Furthermore, treatment with 10 mg/kg/day of oseltamivir for 8 days, rather than 5 days, resulted in decreased weight loss, from 13.5% to only 5%, and an increased survival rate, from 50% to 80%.

Extending the duration of oseltamivir treatment from 5 to 8 days significantly enhanced survival. In the proportional hazards model, mice that received the 5-day regimens had a

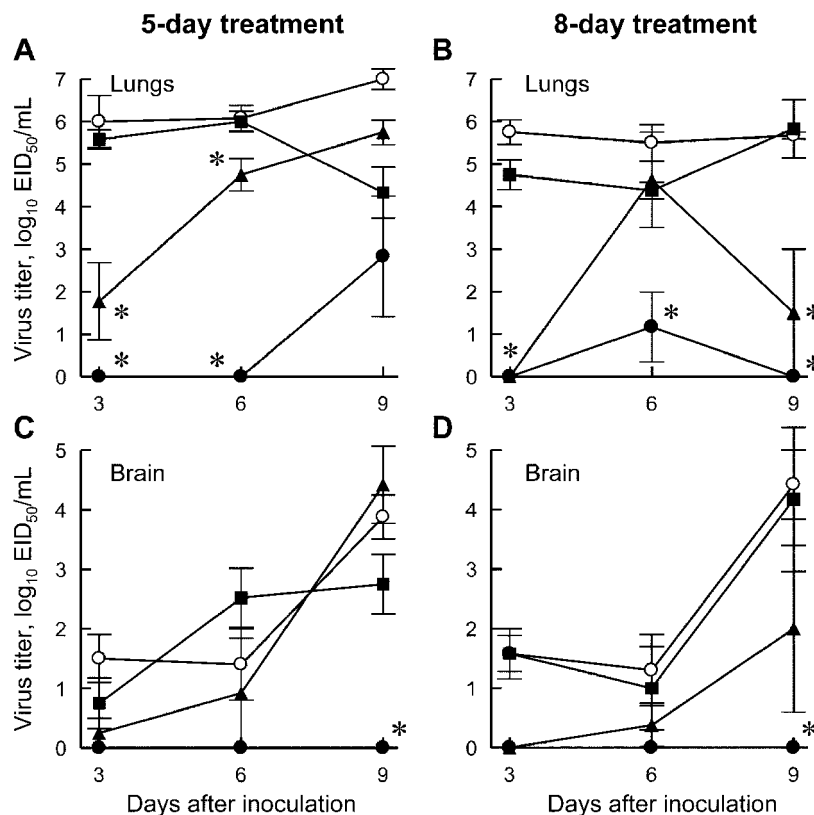


Figure 1. Effect of 5- and 8-day oseltamivir regimens on virus titers in the lungs and brains of mice inoculated with 5 mouse 50% lethal doses of VN1203/04 (H5N1) influenza virus. BALB/c mice were treated with 0.1 (black squares), 1 (black triangles), or 10 (black circles) mg/kg/day of oseltamivir for 5 (A and C) or 8 (B and D) days, beginning 4 h before virus exposure. Control mice received PBS on the same schedule (placebo; white circles). Each data point represents the mean virus titer \pm SE (\log_{10} EID₅₀/mL) obtained from the lungs (A and B) or brains (C and D) of 3 mice. The virus titers of the 4 dosage groups were compared by analysis of variance. Asterisks indicate $P < .05$.

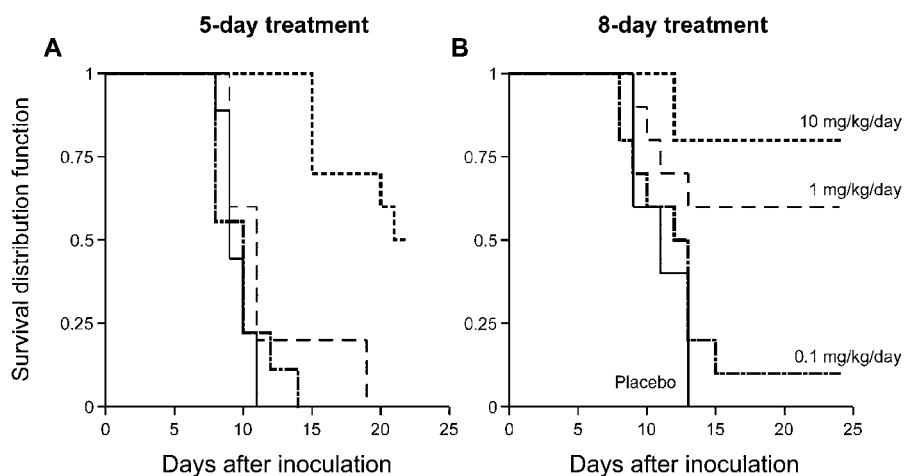


Figure 2. Effect of 5- and 8-day oseltamivir regimens on survival of mice inoculated with 5 mouse 50% lethal doses of VN1203/04 (H5N1) influenza virus. BALB/c mice were treated with 0.1, 1, or 10 mg/kg/day of oseltamivir for 5 (A) or 8 (B) days, beginning 4 h before virus exposure. The Kaplan-Meier method was used to estimate the probability of survival.

2.7-fold greater risk of death than did mice that received the 8-day regimens ($P < .01$). However, the 8-day oseltamivir treatment regimen did not completely protect mice challenged with the VN1203/04 virus.

Infectivity and efficiency of virus replication in mouse organs. Because the VN1203/04 virus was found to be less sensitive to oseltamivir in the mouse model than the HK156/97 virus [17, 19], we evaluated whether the virulence of the 2 viruses may influence the efficacy of oseltamivir. The same stock of HK156/97 virus was used in the present study and in studies in mice that described pathogenicity and sensitivity to NAIs [17–19]. We performed a direct comparison of the growth and infectivity of these 2 viruses in embryonated chicken eggs, MDCK cells, and mice (table 3). VN1203/04 virus exhibited significantly higher viral yield, with an EID_{50} value 1.5 logs greater and an MLD_{50} value 2.4 logs greater than those of HK156/97 virus. The relative infectivity also revealed differences between the 2 viruses (table 3).

To determine whether virus replication efficiency in vivo affects the efficacy of NAIs, we studied virus replication kinetics in mouse organs at days 3, 6, and 9 after inoculation. Infectious doses of $\sim 3 EID_{50}$ and $\sim 3 MLD_{50}$ were used to compare the replication efficiency of 2 viruses in vivo. At $\sim 3 EID_{50}$, the replication kinetics of VN1203/04 virus differed significantly from those of HK156/97 virus in mouse brain and blood (table 4); the mean titers of HK156/97 virus in organs were 1.8–5.6 logs lower than titers of VN1203/04 virus. At $\sim 3 MLD_{50}$, although the lung virus titers were comparable, the brain and blood virus titers were still significantly higher in mice infected with VN1203/04 virus (table 4). Anti-HA antibodies were not detected in 4 mice that recovered from infection with VN1203/

04 virus, but anti-HA antibodies at titers of 40–80 were detected in mice that recovered from infection with HK156/97 virus. Overall, we observed a significant difference in virulence between the VN1203/04 and HK156/97 viruses in vivo.

Sequence analysis. We sequenced the NA genes of viruses isolated from mouse lungs, to identify the emergence of possible drug-resistance mutants during treatment. No amino-acid changes were identified in the conserved NA residues in the 7 viruses obtained on days 6 and 9 after inoculation from mice treated with different dosages of oseltamivir for 5 or 8 days.

Table 2. Effect of duration and dosage of oseltamivir treatment on survival of mice inoculated with VN1203/04 (H5N1) influenza virus.

Treatment duration and dose, mg/kg/day ^a	No. of survivors/total (%)	Survival, mean \pm SE, days ^b	Hazard ratio (P^c)
5 days			
0 (placebo)	0/10	9.6 \pm 0.3	1
0.1	0/10	10.7 \pm 0.6	0.69 (.41)
1	0/10	12.2 \pm 1.7	0.31 (.06)
10	5/10 (50)	20.1 \pm 1.0	0.03 (< .01)
8 days			
0 (placebo)	0/10	11.0 \pm 0.9	1
0.1	1/10 (10)	11.6 \pm 0.9	0.41 (.06)
1	6/10 (60)	12.1 \pm 0.5	0.11 (< .01)
10	8/10 (80)	13.0 \pm 0.0	0.04 (< .01)

^a Oseltamivir or PBS was administered by oral gavage twice daily, starting 4 h before inoculation of 6-week-old BALB/c mice with 5 mouse 50% lethal doses of VN1203/04 virus. Survival was observed for 24 days.

^b Estimated by the log-rank test.

^c Death hazard ratio (vs. the placebo group) was estimated by the proportional hazards model [30].

Table 3. Growth and infectivity of VN1203/04 and HK156/97 (H5N1) influenza viruses in different host systems.

H5N1 virus	Infectivity ^a			Relative infectivity ^b	
	Eggs	MDCK cells	Mice	1 MLD ₅₀ , EID ₅₀	1 MLD ₅₀ , pfu
VN1203/04	9.5 ± 0.3 ^c	9.1 ± 0.1 ^c	9.3 ± 0.3 ^d	1.6	0.6
HK156/97	8.0 ± 0.3	7.4 ± 0.2	6.9 ± 0.4	12.6	3.2

^a Virus titers were determined in eggs, MDCK cells, and mice and are expressed as log₁₀ EID₅₀/mL, log₁₀ pfu/mL, and log₁₀ mouse 50% lethal dose (MLD₅₀)/mL, correspondingly. Values are means ± SD for 2 or 3 independent determinations.

^b No. of infectious virus units (expressed in EID₅₀ and pfu) in 1 MLD₅₀ of VN1203/04 and HK156/97 viruses.

^c *P* < .05 vs. HK156/97 virus, Student's *t* test.

^d *P* < .005 vs. HK156/97 virus, Student's *t* test.

Mutations in HA may lead to NAI resistance in vitro [32, 33]. We sequenced the HA1 region of 4 viruses isolated on days 6 and 9 after inoculation from mice treated for 5 days, and we did not identify any amino-acid changes. Therefore, no NA or HA mutations that might decrease the sensitivity of the virus to oseltamivir emerged during treatment.

DISCUSSION

The mouse is a useful model for the study of the molecular basis of influenza virus virulence in mammalian species [34], and it has often been used to evaluate the pharmacodynamics and efficacy of NAIs [17, 18, 20, 31, 35, 36]. In the present study, we tried to optimize the duration and dosage of oseltamivir treatment required for the best antiviral effect, which may depend on the virulence of the H5N1 influenza virus in vivo. Twice-daily prophylaxis with NAIs (oseltamivir or peramivir) for 5 days at dosages of 1–10 mg/kg/day was shown previously in our laboratory to be effective against a 5-MLD₅₀ lethal challenge of HK156/97 virus in mice [17, 19]. However, the same regimen of oseltamivir did not provide equivalent protection against the 5-MLD₅₀ challenge of VN1203/04 virus in the present study. Lack of direct side-by-side evaluation of the oseltamivir efficacy against HK156/97 and VN1203/04 vi-

ruses in the mouse model may be a limitation of the current study, but we did make a side-by-side comparison of viral replication efficiency. A significant difference in virulence between the VN1203/04 and HK156/97 viruses was observed with regard to the viral yield and infectivity in different host systems and to replication efficiency in vivo. The higher brain and blood virus titers in mice infected with the VN1203/04 virus indicated a greater propensity toward systemic spread. Therefore, the significantly higher virulence of the VN1203/04 virus may be a factor in the different prophylactic efficacy of oseltamivir in vivo against the 2 virus strains.

The pathogenicity of H5N1 viruses in mammals has increased over time, as has been shown by the results of recent studies of isolates in mice [2]. Several features of the VN1203/04 virus may contribute to its high virulence in mice. The substitution of lysine for glutamic acid at position 627 of the PB2 protein is a determinant of host range [37], and this may contribute to the increased replication efficiency of A/HK/483/97 (H5N1) virus in mice [38, 39]. Sequence analysis has shown that VN1203/04 virus contains lysine at position 627 of PB2 [21], whereas HK156/97 virus contains glutamic acid. The higher replication efficiency of VN1203/04 virus in vivo may be related to the amino-acid difference at position 627 of PB2, although

Table 4. Replication efficiency of VN1203/04 and HK156/97 (H5N1) influenza viruses in mice.

H5N1 virus	Virus dose/ mouse		Virus titer, mean ± SD, log ₁₀ EID ₅₀ /mL ^a								
	EID ₅₀	MLD ₅₀	Lungs			Brain			Blood		
			3 dai	6 dai	9 dai	3 dai	6 dai	9 dai	3 dai	6 dai	9 dai
VN1203/04	3	3	7.2 ± 0.6	7.4 ± 0.6	5.75 ^b	2.6 ± 0.9 ^c	5.5 ± 1.0 ^c	4.5 ^b	4.3 ± 0.5	6.4 ± 0.2 ^c	NT
HK156/97	3	0.24	5.4 ± 1.0	3.6 ± 3.1	3.5 ± 3.0	0.8 ± 1.3	0.8 ± 1.3	0.8 ± 1.3	2.4 ± 2.3 ^d
	38	3	6.3 ± 0.5	6.3 ± 0.4	4.3 ± 0.9	0.8 ± 0.8	0.8 ± 0.8	1.1 ± 1.9	2.8 ± 2.4	2.4 ± 2.1	2.0 ± 1.9

NOTE. dai, days after inoculation; MLD₅₀, mouse 50% lethal dose; NT, not tested; ..., below the limit of detection (<0.75 log₁₀ EID₅₀/mL).

^a Six-week-old BALB/c mice were inoculated intranasally with virus in 50 μL of PBS. Virus titers in 3 mice were determined at 3, 6, and 9 dai.

^b Results obtained from 1 mouse.

^c *P* < .01 vs. virus titers in mice after inoculation with HK156/97 virus, analysis of variance.

^d High variation was observed in virus titers in the blood of the 3 mice (titer, 0, 2.75, and 4.5 log₁₀ EID₅₀).

other viral or host factors may also be involved. Differences in the neurotropism of the HK156/97 and VN1203/04 viruses may be another factor that affects the *in vivo* efficacy of oseltamivir. Difference in neuroinvasiveness among the H5N1/97 viruses has been reported [40], and the neurotropic H5N1 influenza virus has been shown to invade the central nervous system (CNS) through afferent fibers of the olfactory, vagal, trigeminal, and sympathetic nerves after replication in the respiratory mucosa [41]. However, only a limited amount of oseltamivir carboxylate was detected in the brains of rats that received a single 10-mg/kg dose of oseltamivir [42], which suggests a limited ability of oseltamivir to cross the blood-brain barrier. In the present study, we observed that VN1203/04 virus not only had significant higher neurotropism but also was better able to spread and to persist in various mouse organs than was HK156/97 virus. Whether applying a higher treatment dose and a longer treatment schedule might prevent lung virus replication before virus dissemination to the CNS will require further investigation. A recent report showed that H5N1 virus was isolated from the cerebrospinal fluid of a fatal human case in Vietnam and thus suggested that recent H5N1 viruses are able to invade the CNS [43], although more detailed studies need to be undertaken.

Extending oseltamivir treatment from 5 to 8 days significantly improved its efficacy. At dosages of 1 and 10 mg/kg/day, treatment for 8 days rather than 5 increased survival significantly and better inhibited residual virus replication in organs. The duration of treatment with a given dose of oseltamivir can affect the survival of mice infected with A/NWS/33 (H1N1) influenza virus, and continued daily therapy was needed to maintain the antiviral effect while the lung virus titers approached maximum levels [31]. In the present study, mice treated with 0.1 and 1 mg/kg/day of oseltamivir for 5 days had high virus titers in the lungs at day 6 after inoculation, when the treatment regimen ended; this factor may explain the 0% survival rate of these 2 groups. Mice treated for 5 days with 1 and 10 mg/kg/day of oseltamivir had increased lung virus titers at day 9 after inoculation. In particular, the group that received 10 mg/kg/day had a significantly longer mean survival, compared with the placebo group. Deaths in these treatment groups may have resulted from incomplete treatment and residual virus replication. Extending oseltamivir treatment from 5 to 8 days, the time at which the lung virus titer had begun to decrease, provided a better antiviral effect; therefore, our results support the previous observation that treatment should be continued while lung virus titers remain high [31].

The emergence of resistant mutants is one of the potential problems of antiviral treatment. In the present study, we found no amino acid substitutions at the conserved residues in NA or HA1 subunit in selected virus isolates from mouse lungs after treatment. Although extensive sequencing was not per-

formed to all the viruses isolated after treatment, our results support previous observations [32, 33, 44] that NAI-resistant mutants are not easily generated *in vivo*.

It is difficult to extrapolate results from the mouse model directly to the situation in humans. Mice that received 10 mg/kg/day of oseltamivir have been reported to achieve a plasma concentration comparable to that after a human oral dose of 75 mg twice daily, given the interspecies difference in esterase activity [45]. Our results provide initial information on neurotropic H5N1 influenza viruses that can serve as the basis for further studies on the therapeutic and prophylactic use of oseltamivir in other animal models, especially in terms of dose. It is encouraging that the new H5N1 antigenic variant VN1203/04 was sensitive to NAIs in the mouse model, despite its requiring prolonged and higher-dose oseltamivir regimens for the most beneficial protection.

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References

1. Subbarao K, Klimov A, Katz J, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **1998**; 279:393–6.
2. Chen H, Deng G, Li Z, et al. The evolution of H5N1 influenza viruses in ducks in southern China. *Proc Natl Acad Sci USA* **2004**; 101:10452–7.
3. Webster RG, Guan Y, Peiris M, et al. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. *J Virol* **2002**; 76:118–26.
4. Guan Y, Peiris JS, Lipatov AS, et al. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. *Proc Natl Acad Sci USA* **2002**; 99:8950–5.
5. Guan Y, Poon LL, Cheung CY, et al. H5N1 influenza: a protean pandemic threat. *Proc Natl Acad Sci USA* **2004**; 101:8156–61.
6. Peiris JS, Yu WC, Leung CW, et al. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* **2004**; 363:617–9.
7. Ungchusak K, Auewarakul P, Dowell SF, et al. Probable person-to-person transmission of avian influenza A (H5N1). *N Engl J Med* **2005**; 352:333–40.
8. Stephenson I, Nicholson KG, Wood JM, Zambon MC, Katz JM. Confronting the avian influenza threat: vaccine development for a potential pandemic. *Lancet Infect Dis* **2004**; 4:499–509.
9. Monto AS. The threat of an avian influenza pandemic. *N Engl J Med* **2005**; 352:323–5.
10. Monto AS, Gunn RA, Bandyk MG, King CL. Prevention of Russian influenza by amantadine. *JAMA* **1979**; 241:1003–7.
11. Smorodintsev AA, Karpuhin GI, Zlydnikov DM, et al. The prophylactic effectiveness of amantadine hydrochloride in an epidemic of Hong Kong influenza in Leningrad in 1969. *Bull World Health Organ* **1970**; 42:865–72.
12. Li KS, Guan Y, Wang J, et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* **2004**; 430:209–13.
13. Koopmans M, Wilbrink B, Conyn M, et al. Transmission of H7N9

- avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet* **2004**;363:587–93.
14. Hayden FG, Belshe R, Villanueva C, et al. Management of influenza in households: a prospective, randomized comparison of oseltamivir treatment with or without postexposure prophylaxis. *J Infect Dis* **2004**;189:440–9.
 15. Welliver R, Monto AS, Carewicz O, et al. Effectiveness of oseltamivir in preventing influenza in household contacts: a randomized controlled trial. *JAMA* **2001**;285:748–54.
 16. Nicholson KG, Wood JM, Zambon M. Influenza. *Lancet* **2003**;362:1733–45.
 17. Govorkova EA, Leneva IA, Goloubeva OG, Bush K, Webster RG. Comparison of efficacies of RWJ-270201, zanamivir, and oseltamivir against H5N1, H9N2, and other avian influenza viruses. *Antimicrob Agents Chemother* **2001**;45:2723–32.
 18. Gubareva LV, McCullers JA, Bethell RC, Webster RG. Characterization of influenza A/HongKong/156/97 (H5N1) virus in a mouse model and protective effect of zanamivir on H5N1 infection in mice. *J Infect Dis* **1998**;178:1592–6.
 19. Leneva IA, Roberts N, Govorkova EA, Goloubeva OG, Webster RG. The neuraminidase inhibitor GS4104 (oseltamivir phosphate) is efficacious against A/Hong Kong/156/97 (H5N1) and A/Hong Kong/1074/99 (H9N2) influenza viruses. *Antiviral Res* **2000**;48:101–15.
 20. Sidwell RW, Smee DF, Huffman JH, et al. Influence of virus strain, challenge dose, and time of therapy initiation on the in vivo influenza inhibitory effects of RWJ-270201. *Antiviral Res* **2001**;51:179–87.
 21. Govorkova EA, Rehg JE, Krauss S, et al. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. *J Virol* **2005**;79:2191–8.
 22. Sturm-Ramirez KM, Ellis T, Bousfield B, et al. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *J Virol* **2004**;78:4892–901.
 23. Hoffmann E, Krauss S, Perez D, Webby R, Webster RG. Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine* **2002**;20:3165–70.
 24. Matrosovich M, Matrosovich T, Carr J, Roberts NA, Klenk HD. Overexpression of the alpha-2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. *J Virol* **2003**;77:8418–25.
 25. Gubareva LV, Webster RG, Hayden FG. Detection of influenza virus resistance to neuraminidase inhibitors by an enzyme inhibition assay. *Antiviral Res* **2002**;53:47–61.
 26. Potier M, Mameli L, Belisle M, Dallaire L, Melancon SB. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-N-acetylneuraminic) substrate. *Anal Biochem* **1979**;94:287–96.
 27. Reed LJ, Muench H. A simple method for estimating fifty percent endpoints. *Am J Hyg* **1938**;27:493–7.
 28. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol* **2001**;146:2275–89.
 29. Ozaki H, Govorkova EA, Li C, Xiong X, Webster RG, Webby RJ. Generation of high-yielding influenza A viruses in African green monkey kidney (Vero) cells by reverse genetics 3. *J Virol* **2004**;78:1851–7.
 30. Cox DR. Regression models and life-tables. *J R Stat Soc B* **1972**;34:187–220.
 31. Sidwell RW, Bailey KW, Bemis PA, Wong MH, Eisenberg EJ, Huffman JH. Influence of treatment schedule and viral challenge dose on the in vivo influenza virus-inhibitory effects of the orally administered neuraminidase inhibitor GS 4104. *Antivir Chem Chemother* **1999**;10:187–93.
 32. Gubareva LV, Kaiser L, Hayden FG. Influenza virus neuraminidase inhibitors. *Lancet* **2000**;355:827–35.
 33. McKimm-Breschkin JL. Resistance of influenza viruses to neuraminidase inhibitors—a review. *Antiviral Res* **2000**;47:1–17.
 34. Katz JM, Lu X, Tumpey TM, Smith CB, Shaw MW, Subbarao K. Molecular correlates of influenza A H5N1 virus pathogenesis in mice. *J Virol* **2000**;74:10807–10.
 35. Drusano GL, Preston SL, Smee D, Bush K, Bailey K, Sidwell RW. Pharmacodynamic evaluation of RWJ-270201, a novel neuraminidase inhibitor, in a lethal murine model of influenza predicts efficacy for once-daily dosing. *Antimicrob Agents Chemother* **2001**;45:2115–8.
 36. Li W, Escarpe PA, Eisenberg EJ, et al. Identification of GS 4104 as an orally bioavailable prodrug of the influenza virus neuraminidase inhibitor GS 4071. *Antimicrob Agents Chemother* **1998**;42:647–53.
 37. Subbarao EK, London W, Murphy BR. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J Virol* **1993**;67:1761–4.
 38. Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* **2001**;293:1840–2.
 39. Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. *Virology* **2004**;320:258–66.
 40. Gao P, Watanabe S, Ito T, et al. Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. *J Virol* **1999**;73:3184–9.
 41. Park CH, Ishinaka M, Takada A, et al. The invasion routes of neurovirulent A/Hong Kong/483/97 (H5N1) influenza virus into the central nervous system after respiratory infection in mice. *Arch Virol* **2002**;147:1425–36.
 42. Sweeny DJ, Lynch G, Bidgood AM, Lew W, Wang KY, Cundy KC. Metabolism of the influenza neuraminidase inhibitor prodrug oseltamivir in the rat. *Drug Metab Dispos* **2000**;28:737–41.
 43. de Jong MD, Bach VC, Phan TQ, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N Engl J Med* **2005**;352:686–91.
 44. Zambon M, Hayden FG. Position statement: global neuraminidase inhibitor susceptibility network. *Antiviral Res* **2001**;49:147–56.
 45. Ward P, Small I, Smith J, Suter P, Dutkowski R. Oseltamivir (Tamiflu®) and its potential for use in the event of an influenza pandemic. *J Antimicrob Chemother* **2005**;55(Suppl):i5–21.