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
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, NAI may be the only effective antiviral option for the control of H5N1/04 variants in Asia. As part of pandemic preparedness, the efficacies of NAIIs 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 unknown 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 NAIIs. 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 NAIIs [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 NAIIs, and we report studies that evaluated the efficacy 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 [(3R,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid] and the prodrug oseltamivir phosphate (oseltamivir)[(ethyl[(3R,4R,5S)-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. 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 NAIIs of the same stock of H5K156/97 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 NAIIs 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 (Philippines 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 NAIIs at concentrations of 0.00005–10 μmol/L [25]. The IC_{50} 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 NAIIs (0.0001–500 μmol/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 NAIIs at concentrations of 0.0001–500 μmol/L. Virus replication was determined by measurement of hemagglutinin (HA) activity after 72 h of incubation at 37°C. The 50% effective concentration (EC_{50}) 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 μL of 10-fold serial dilutions of VN1203/04 virus in PBS. The mouse lethal dose (MLD_{50}) 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 MLD_{50} of VN1203/04 virus in 50 μL 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 log_{10} EID_{50}. For calculation of the
mean, samples with a virus titer <0.75 log<sub>10</sub> EID<sub>50</sub>/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<sub>10</sub> EID<sub>50</sub>/mL ± 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 ~3 EID<sub>50</sub> and ~3 MLD<sub>50</sub> 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<sub>10</sub> EID<sub>50</sub>/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 × 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 × 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 × PR8 virus was more sensitive than the HK156 × PR8 and PR8 viruses to oseltamivir carboxylate (mean IC<sub>50</sub>, 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<sub>50</sub> 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<sup>1.2</sup> EID<sub>50</sub> caused the deaths of all 5 infected mice. The symptoms preceding death were more pronounced at higher virus doses (virus inocula 10<sup>2.2–3.2</sup> EID<sub>50</sub>); by day 7 after inoculation, the mice had lost >25% of their body weight, were inactive, and had developed hind-limb paralysis. The MLD<sub>50</sub>/mL of the stock virus was 10<sup>3.3</sup>. We evaluated the prophylactic efficacy of 5 and 8 days of oseltamivir treatment of mice against challenge with 5 MLD<sub>50</sub> 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].

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<tr>
<th>Reverse-genetics virus</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;, nmol/L&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;, µmol/L&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>VN1203 × PR8 (H1N1)</td>
<td>0.8 ± 0.1 0.4 ± 0.1</td>
<td>0.9 ± 0.1 0.1 ± 0.1</td>
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<tr>
<td>HK156 × PR8 (H1N1)</td>
<td>0.7 ± 0.1 4.1 ± 0.2</td>
<td>0.5 ± 0.1 1.0 ± 0.1</td>
</tr>
<tr>
<td>PR8/34 (H1N1)</td>
<td>0.7 ± 0.1 4.5 ± 0.2</td>
<td>1.1 ± 0.1 4.6 ± 1.2</td>
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<sup>a</sup> NA inhibition was assayed with viruses standardized to equivalent NA activity and incubated with NAIs at concentrations of 0.0005–10 µmol/L with 2′-3′-4′-methylumbelliferyl-α-D-N-acetylneuraminic acid as substrate [25]. The IC<sub>50</sub> 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.

<sup>b</sup> 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.

Table 1. Sensitivity of reverse genetics–derived influenza viruses to neuraminidase (NA) inhibitors (NAIs) in NA-inhibition and virus-reduction assays.
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
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.
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-MLD$_{50}$ lethal challenge of HK156/97 virus in mice [17, 19]. However, the oseltamivir efficacy against HK156/97 and VN1203/04 viruses 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...
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 performed 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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