Oseltamivir Prophylactic Regimens Prevent H5N1 Influenza Morbidity and Mortality in a Ferret Model

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Background. Current oseltamivir prophylactic regimens may not be as effective against highly pathogenic H5N1 influenza viruses as they are against less pathogenic strains. An optimal regimen is urgently needed.

Methods. Ferrets were given the neuraminidase inhibitor oseltamivir orally for 10 days (5 or 10 mg/kg once daily or 2.5 or 5 mg/kg twice daily). Prophylaxis was initiated 1 day before infection, and oseltamivir was given 4 h before the ferrets were inoculated with a lethal dose of A/Vietnam/1203/04 (H5N1) influenza virus.

Results. At a dose of 5 mg/kg once daily, oseltamivir prevented death but not clinical signs of infection in ferrets; severe pathology was observed in the lungs, brain, and liver. At 10 mg/kg once daily, oseltamivir reduced clinical symptoms and systemic virus replication, but pathology was observed in the internal organs. The best results were obtained at a dose of 2.5 or 5 mg/kg given twice daily. Both regimens resulted in 100% survival and the absence of clinical symptoms, systemic virus spread, and organ pathology. Serum antibody titers were comparable across regimens and were sufficient to protect against rechallenge.

Conclusions. An increased dose of oseltamivir or twice-daily administration effectively protects ferrets against morbidity and mortality caused by H5N1 infection and does not interfere with the development of protective antibodies against subsequent H5N1 infection.

Avian influenza A H5N1 viruses are novel pathogens to the human population. Direct transmission of highly pathogenic H5N1 influenza viruses from birds to humans was first identified in 1997 [1, 2]. These viruses reemerged in humans in 2003 and have since spread throughout Eurasia, infecting and killing an increasing number of humans [3–6]. Clinical symptoms differ from those of contemporary human influenza and include pneumonia with progressive respiratory failure, gastrointestinal symptoms, and liver and renal dysfunction [7, 8]. Central nervous system involvement has also been reported, and H5N1 virus has been isolated from the cerebrospinal fluid and blood of 1 patient [9].

Neuraminidase (NA) inhibitors (orally administered oseltamivir and inhaled zanamivir) are the primary antiviral agents of choice for the treatment of influenza. Current recommendations for their use are based on information about seasonal influenza A H1N1 and H3N2 and influenza B viruses [10–12]. The recommended oseltamivir regimens for healthy adults are 75 mg once daily for 7–10 days (prophylaxis) and 75 mg twice daily for 5 days (treatment of clinical disease) [13].

There is limited information about the efficacy of oseltamivir against H5N1 infection in humans. The available clinical data show a 30% survival rate in patients treated with oseltamivir and a 20% survival rate in those not treated [14, 15]. In most cases, oseltamivir has been started late in the course of disease, although early treatment appears to be beneficial. In Vietnam, 4 of 5 patients died after treatment with oseltamivir initiated on days 5–11 of clinical symptoms [3]. In Thailand, 7 patients were treated with oseltamivir; the 2 who survived were treated within a median of 4.5 days after symptom onset, whereas the 5 who died started treatment within a median of 9 days after onset [16]. H5N1 viruses replicate for longer periods and generate higher viral loads than do seasonal influenza viruses, and...
they spread systemically [14, 15]; these factors may explain why early treatment offers a benefit.

In the absence of clinical trials in humans, animal models offer the best experimental approach to examine the efficacy of oseltamivir against H5N1 infection. In a mouse model, oseltamivir significantly reduced lung virus titers and prevented virus spread to the brain when given 4 h before inoculation with A/Hong Kong/156/97 (H5N1) [17, 18]. Protection from A/Vietnam/1203/04 (H5N1), a more pathogenic strain, required a higher dose and more prolonged treatment [19]. In a ferret model, 10 mg/kg oseltamivir once daily was effective against A/Vietnam/1203/04 (H5N1) if initiated early after inoculation, but 25 mg/kg once daily was required if treatment was delayed for 24 h [20]. Both regimens resulted in the development of protective levels of serum antibodies; however, oseltamivir treatment began after infection, and the drug was not administered prophylactically.

To prepare for a potential pandemic of H5N1 influenza virus, it is necessary to determine a prophylactic regimen that will protect those who are in close contact with infected individuals. We evaluated the effectiveness of 4 different oseltamivir prophylaxis regimens against highly pathogenic A/Vietnam/1203/04 (H5N1) influenza virus in a ferret model. We determined the optimal regimen for prevention of clinical signs, virus shedding, and death and evaluated the antibody response.

**METHODS**

**Compound.** The NA-inhibitor prodrug oseltamivir phosphate (oseltamivir) [ethyl(3R,4R,5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate] was provided by Hoffmann–La Roche.

**Viruses and cells.** The influenza A/Vietnam/1203/04 (H5N1) and A/Hong Kong/213/03 (H5N1) viruses were obtained from the World Health Organization (WHO) collaborating laboratories. Stock virus was grown in the allantoic cavities of 9-day-old embryonated chicken eggs for 32 h at 36°C, and aliquots were stored at −70 °C until used. Virus titer was determined by calculating the EID₅₀ [21]. Experiments with highly pathogenic H5N1 viruses were conducted in a biosafety level 3+ containment facility under applicable laws and guidelines. Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection and were maintained as described elsewhere [20].

**Assessment of drug efficacy.** Animal care and experimental protocols were approved by the Animal Care and Use Committee of St. Jude Children’s Research Hospital. Young adult male ferrets 4–5 months of age were obtained from Marshall’s Farms. The ferrets were seronegative to influenza A H1N1 and H5N1 and influenza B viruses but possessed hemagglutinin inhibition (HI) antibodies (1:80–1:160) to A/New York/55/04 (H3N2) virus. Prophylaxis was initiated 1 day before infection, and oseltamivir was given 4 h before virus inoculation and continued for 10 days. Oseltamivir was mixed 1:1 with sterile sugar syrup and given in 4 regimens: 5 or 10 mg/kg once daily (first experiment) or 2.5 or 5 mg/kg twice daily (12 h apart; second experiment). Control-inoculated ferrets received sterile PBS mixed 1:1 with sterile sugar syrup (placebo) on the same schedule. Ferrets were lightly anesthetized with isoflurane and inoculated intranasally with 10⁵ EID₅₀ of A/Vietnam/1203/04 (H5N1) virus in 1.0 mL of PBS. Clinical signs of infection, relative inactivity index [22], weight, and temperature (measured with a subcutaneous implantable transponder) were recorded daily. Animals that showed signs of severe disease and >25% weight loss were euthanized. Three animals per treatment group were observed for survival and clinical signs of infection, and 2 animals in each group were killed to determine virus replication in the internal organs.

**Virus titration in upper respiratory tract.** On days 3, 5, and 7 after inoculation, ferrets were anesthetized with ketamine (25 mg/kg) injected intramuscularly, and 0.5 mL of sterile PBS containing antibiotics was introduced into each nostril and collected in containers. Virus was titrated in embryonated chicken eggs and expressed as log₁₀ EID₅₀ per milliliter.

**Virus titration in organs.** Samples from the ferrets’ brains (frontal lobe and hindbrain), lungs (4 lobes tested separately), and liver were collected 6 days after inoculation. Samples were weighed and homogenized in 1 mL of sterile PBS with antibiotics, and the virus titer (log₁₀ EID₅₀ per gram) was determined in embryonated chicken eggs.

**Rechallenge with H5N1 virus.** Three weeks after inoculation, surviving ferrets were rechallenged with 10⁵ EID₅₀ of A/Vietnam/1203/04 (H5N1) virus. Clinical signs of infection, weight, and temperature were monitored daily.

**Serologic tests.** Serum samples were collected 21 and 35 days after inoculation, treated with receptor-destroying enzyme, heat inactivated at 56 °C for 30 min, and tested by HI assay with 0.5% chicken red blood cells. Virus-neutralizing titers were determined by infection of MDCK cells and expressed as the reciprocal of the highest serum dilution that neutralized 50% of 100 TCID₅₀ of virus after incubation at 37°C for 72 h [23].

**Histological analysis.** Tissues were collected at the time of necropsy, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Five-micrometer sections were stained with hematoxylin-eosin and examined by light microscopy.

**Virus sequence analysis.** Virus was isolated from nasal wash samples and lung, brain, and liver samples in embryonated chicken eggs. The HA (HA1 region) and NA genes were sequenced by reverse-transcriptase polymerase chain reaction, as described elsewhere [19], at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude.

**Statistical analysis.** Virus titers in ferret organs and nasal wash samples were compared by means of unpaired 2-tailed t test. A probability value of .05 was prospectively chosen as the cutoff to indicate that findings were not the result of chance alone.
RESULTS

Effect of oseltamivir prophylaxis on survival and clinical signs. We examined the effect of 4 oseltamivir regimens in ferrets given a lethal dose of A/Vietnam/1203/04 (H5N1) influenza virus and prophylactically treated with different oseltamivir regimens. Ten-day oral administration of oseltamivir or PBS was initiated 1 day before infection for both once- and twice-daily regimens. Animals were treated with 5 or 10 mg/kg once daily (A and C) or 2.5 or 5 mg/kg twice daily (B and D), administered 4 h before inoculation with A/Vietnam/1203/04 (H5N1) virus. Loss or gain of weight in ferrets given 5 or 10 mg/kg oseltamivir once daily or 2.5 or 5 mg/kg twice daily was calculated for each ferret as the percentage change from baseline. Data points are means ± SEs for 3 ferrets.

Figure 1. Changes in weight (A and B) and body temperature (C and D) of ferrets inoculated with 10^2 EID50 of A/Vietnam/1203/04 (H5N1) influenza virus and prophylactically treated with different oseltamivir regimens. Ten-day oral administration of oseltamivir or PBS was initiated 1 day before infection for both once- and twice-daily regimens. Animals were treated with 5 or 10 mg/kg once daily (A and C) or 2.5 or 5 mg/kg twice daily (B and D), administered 4 h before inoculation with A/Vietnam/1203/04 (H5N1) virus. Loss or gain of weight in ferrets given 5 or 10 mg/kg oseltamivir once daily or 2.5 or 5 mg/kg twice daily was calculated for each ferret as the percentage change from baseline. Data points are means ± SEs for 3 ferrets.

Effect of oseltamivir prophylaxis on virus replication in the upper respiratory tract. To assess the ability of the oseltamivir regimens to prevent virus replication in the upper respiratory tract of ferrets, we determined viral titers in nasal wash samples on days 3, 5, and 7 after inoculation (table 2). Untreated inoculated ferrets shed virus on all 3 days. All drug regimens inhibited virus replication in the upper respiratory tract on day 3 after inoculation, but some ferrets receiving 5 or 10 mg/kg once daily or 2.5 mg/kg twice daily shed virus on subsequent days. Only 1 regimen (5 mg/kg of oseltamivir twice daily) resulted in the absence of detectable virus replication on days 3, 5, and 7 after inoculation.
Effect of oseltamivir prophylaxis on virus spread to the internal organs. Virus was detected in the lungs, brains, and livers of both of 2 untreated inoculated ferrets. At a dose of 5 mg/kg once daily, virus was still isolated from the internal organs; however, titers were 2.2–2.7 log10 EID50/mL lower than those observed in controls (figure 2). At a dose of 10 mg/kg once daily, virus replication was completely inhibited in the internal organs of both animals. Prophylaxis at 2.5 or 5 mg/kg twice daily also completely inhibited virus spread to the internal organs (figure 2). Thus, 3 of the 4 oseltamivir prophylactic regimens completely inhibited virus spread to the internal organs.

Histological evaluation of tissues. Histological changes were observed in the brains, livers, and lungs of untreated inoculated animals (figure 3D–F). The brains showed meningoencephalitis, consisting of mixed inflammatory cell infiltrates with mononuclear cell dominance in the meninges and neuropil of the cerebrum, brain stem, and/or cerebellum. The livers showed mixed inflammatory cell infiltrates (often associated with biliary

Table 1. Effect of oseltamivir prophylaxis on survival and clinical signs of infection in ferrets challenged with A/Vietnam/1203/04 (H5N1) influenza virus.

<table>
<thead>
<tr>
<th>Oseltamivir regimena</th>
<th>No. dead/total no.</th>
<th>Time of death, days after infection</th>
<th>Respiratory signs</th>
<th>Neurological signs</th>
<th>Loss of appetite</th>
<th>Lethargy (RII)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Once daily</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3/3</td>
<td>5,5,6</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
<td>1.21</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>0/3</td>
<td>...</td>
<td>0/3</td>
<td>2/3</td>
<td>3/3</td>
<td>1.08</td>
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<td>10 mg/kg</td>
<td>0/3</td>
<td>...</td>
<td>0/3</td>
<td>0/3</td>
<td>2/3</td>
<td>0.16</td>
</tr>
<tr>
<td>Twice daily</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3/3</td>
<td>6,7,7</td>
<td>0/3</td>
<td>2/3</td>
<td>3/3</td>
<td>1.06</td>
</tr>
<tr>
<td>2.5 mg/kg</td>
<td>0/3</td>
<td>...</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0.06</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>0/3</td>
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<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0.0</td>
</tr>
</tbody>
</table>

a Ten-day oral administration of oseltamivir or PBS was initiated 1 day before infection for both once- and twice-daily regimens. On the day of infection, oseltamivir was given 4 h before inoculation with 10^2 EID50 of A/Vietnam/1203/04 virus.
b Clinical signs were observed for 14 days after virus inoculation. Except for lethargy, findings for clinical signs are given as no. of ferrets with sign/total no. Respiratory signs included sneezing, wheezing, and nasal discharge; neurological signs included hind-limb paresis, ataxia, torticollis, and tremors.

Table 2. Inhibition of A/Vietnam/1203/04 (H5N1) virus replication in the upper respiratory tract of ferrets prophylactically treated with oseltamivir.

<table>
<thead>
<tr>
<th>Oseltamivir regimena</th>
<th>3 days after inoculation</th>
<th>5 days after inoculation</th>
<th>7 days after inoculationc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. shedding/total no.</td>
<td>Virus titerb</td>
<td>No. shedding/total no.</td>
</tr>
<tr>
<td>Once daily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4/5</td>
<td>3.9 ± 0.6</td>
<td>5/5</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>0/5</td>
<td>&lt;0.75±EID50/mL</td>
<td>3/5</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>0/5</td>
<td>&lt;0.75±EID50/mL</td>
<td>1/5</td>
</tr>
<tr>
<td>Twice daily</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4/5</td>
<td>4.0 ± 1.7</td>
<td>5/5</td>
</tr>
<tr>
<td>2.5 mg/kg</td>
<td>0/5</td>
<td>&lt;0.75±EID50/mL</td>
<td>1/5</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>0/5</td>
<td>&lt;0.75±EID50/mL</td>
<td>0/5</td>
</tr>
</tbody>
</table>

NOTE. NA, not applicable.
a Ten-day oral administration of oseltamivir or PBS was initiated 1 day before infection for both once- and twice-daily regimens. On the day of infection, oseltamivir was given 4 h before inoculation with 10^2 EID50 of A/Vietnam/1203/04 virus.
b Titers (expressed as log10 EID50/mL) are given as means ± SD, unless only 1 animal was shedding virus.
c Two ferrets in each group were killed on day 6 after inoculation to determine virus titers in the internal organs.
d All animals in this group died before day 7.
e These titers were below the limit of detection (0.75 log10 EID50/mL).
f P < .05 for the comparison with virus titers in the control group (unpaired 2-tailed t test); for statistical analysis, virus-positive samples with a virus titer <0.75 log10 EID50/mL were assigned a value of 0.5 log10 EID50/mL.
epithelial necrosis and multiple random foci of hepatocyte necrosis) with mononuclear cell dominance in the portal tracts and parenchyma. The lungs revealed mild multifocal bronchiolar-interstitial pneumonia. Mononuclear inflammatory cell infiltrates were observed in the terminal bronchioles, in the adjacent alveoli, and around blood vessels. There was also mild necrosis, sloughing, and regeneration of the terminal bronchiolar epithelium and occasional alveolar epithelial cell hyperplasia and interstitial septal hypercellularity. When oseltamivir was administered at a dose of 5 mg/kg once daily, a significant number of lesions were observed in brain, liver, and lungs (figure 3G–3I). These lesions were decreased in number and size at a dose of 10 mg/kg once daily (figure 3J–3L). Lesions in the brain, liver, and lungs were absent or minimal when 2.5 or 5 mg/kg of oseltamivir was administered twice daily (figure 3M–3O). Histological assessment of pulmonary changes in the treated animals yielded somewhat equivocal findings because of the mildness of lung lesions in inoculated untreated controls and the occasional pulmonary mononuclear cell infiltrate in un inoculated ferrets.

**Viral serology after oseltamivir prophylaxis and inoculation.** Development of specific antibodies can provide evidence of virus infection. We titrated anti-HA and virus-neutralizing antibodies 21 days after inoculation in surviving ferrets given oseltamivir prophylaxis (figure 4). Anti-HA antibody titers to homologous H5N1 virus were low (1:10–1:40) in all animals. Because we have found previously that A/Vietnam/1203/04 (H5N1) virus tends to yield low anti-HA titers, we also included A/Hong Kong/213/03 (H5N1) virus, whose HA antigen is better suited to serologic antibody detection [20, 24]. This heterologous virus did yield higher titers of anti-HA antibody. The mean titers of virus-neutralizing antibodies to both H5N1 viruses (1:40–1:320) were comparable in all treatment groups 21 days after inoculation. We rechallenged ferrets with a lethal dose of A/Vietnam/1203/04 (H5N1) virus 21 days after inoculation and observed no clinical signs of infection. Serum collected 14 days after rechallenge (35 days after the first inoculation) showed no significant (≥4-fold) increase in anti-HA or neutralizing antibodies in any treatment group.

**Detection of resistant variants.** To monitor the emergence of oseltamivir-resistant variants during treatment, we sequenced the HA (HA1 region) and NA genes of virus isolated from nasal wash samples, lungs, brains, and livers. No amino acid–encoding changes were observed in the conserved regions of the NA gene. A single F390Y amino acid substitution was identified in the NA gene of virus isolated 6 days after inoculation from the brain of a ferret treated with 5 mg/kg once daily. This mutation has not been reported to confer resistance to oseltamivir.

**DISCUSSION**

For pandemic preparedness, it is essential to determine whether antiviral prophylaxis can prevent the morbidity and mortality caused by H5N1 influenza virus infection. We evaluated the efficacy of 4 oseltamivir prophylaxis regimens against systemically replicating A/Vietnam/1203/04 (H5N1) influenza virus in a ferret model. All regimens tested protected animals from mortality, but a dose of 10 mg/kg once daily or 2.5–5 mg/kg twice daily was required to prevent disease signs and minimize or prevent virus replication in the upper respiratory tract and internal organs.

Prevention of death is the paramount goal of antiviral treatment for human H5N1 virus infection. The current WHO recommendations for the pharmacological management of sporadic human H5N1 infection are based on available case reports [13]. Although evidence of the effectiveness of oseltamivir against H5N1 viruses in humans is limited, case reports indicate that the currently recommended antiviral regimen can improve outcomes and survival only if initiated early [5, 25].

In the absence of clinical trails in human subjects, preclinical animal studies can provide vital information. The design of the present study was based on available pharmacokinetic data and on the recommended human dosage. The lowest daily dose given to ferrets, 5 mg/kg, yields a systemic drug exposure equivalent to that of the recommended 75-mg dose of prophylactic oseltamivir in humans [26]. We observed that control animals all died, whereas those receiving oseltamivir prophylaxis survived; nevertheless, severe clinical signs were noted at this dose level. Although clinical signs were less severe in ferrets given 10 mg/kg once daily, oseltamivir prophylaxis was markedly improved by twice-daily administration of the drug, even at the lower total daily dose.

The inability of oseltamivir prophylaxis administered once daily to alleviate clinical symptoms as effectively as oseltamivir...
twice daily may be attributed to the differences in pharmacokinetics of oseltamivir carboxylate (the active metabolite of oseltamivir) given once or twice daily and the unique characteristics of H5N1 influenza viruses. The significant difference between once- and twice-daily administration may result from differences in the minimum serum concentration of oseltamivir carboxylate.

Figure 3. Photomicrographs showing histological changes in the brains, livers, and lungs of ferrets prophylactically treated with oseltamivir and inoculated with A/Vietnam/1203/04 (H5N1) virus. Oseltamivir was administered as described in the legend to figure 1. A–C, Findings in uninfected control ferrets. The brain is free of necrosis and inflammation (A). A small mononuclear cell infiltrate is present in an occasional hepatic portal tract (B) and at an occasional junction of a terminal bronchiole and alveolar duct in the lung (C). D–F, Lesions and inflammation in the brains, livers, and lungs of untreated inoculated ferrets. G–I, A significant number of lesions were observed in the brain, liver, and lungs of ferrets treated with 5 mg/kg once daily, similar to untreated ferrets. J–L, Lesions were decreased in number and size in ferrets treated with 10 mg/kg oseltamivir once daily. M–O, No necrosis associated with inflammatory infiltrates in the brains, livers, and lungs of ferrets treated with 2.5 mg/kg of oseltamivir twice daily. No histological changes were seen in the internal organs of ferrets receiving 5 mg/kg oseltamivir twice daily (data not shown). When oseltamivir was administered at a dose of 5 mg/kg once daily, a significant number of lesions were observed in brain, liver, and lungs (G–I). These lesions were decreased in no. and size at a dose of 10 mg/kg once daily (J–L). Lesions in the brain, liver, and lungs were absent or minimal when 2.5 or 5 mg/kg of oseltamivir was administered twice daily (M–O).
With equivalent total daily doses, the minimum serum concentration of oseltamivir carboxylate with twice-daily dosing would be expected to be 10 times that achieved by once-daily administration (Craig Rayner, Hoffmann–La Roche, personal communication). This difference was likely to be a key factor because of the high pathogenicity of this virus strain in ferrets.

Avian H5N1 influenza viruses differ in their pathogenicity in ferrets and can cause mild, nonlethal disease or spread systemically in animals, causing severe clinical symptoms and death. A/Vietnam/1203/04 (H5N1) influenza virus is one of the most virulent H5N1 viruses isolated thus far and shows broad tissue tropism, high replicative efficiency, and neurovirulence in the ferret model [27, 28]. In mice and ferrets, this virus has been isolated from multiple organs, including the brain, and has caused signs of neurological disease [19, 27].

In our experiments, prophylactic oseltamivir at 5 mg/kg twice daily prevented virus shedding from the upper respiratory tract, the main route of transmission of respiratory viruses. This regimen also inhibited virus replication in the lower respiratory tract and spread to internal organs. When the same dose of oseltamivir (10 mg/kg) was given once daily, shedding from the upper respiratory tract was observed; however, virus was not detected in the lower respiratory tract or in internal organs, and minor lesions were observed. Interestingly, we did not observe overt respiratory signs in the presence of viral shedding in any of the treatment groups or controls. The lack of respiratory symptoms correlates with the absence of severe lung pathology and is likely due to the low virus inoculation dose (10² EID₅₀/ferret) used in this study. A previous study in ferrets has also shown mortality in the absence of respiratory signs after inoculation with 10 or 10² EID₅₀ of A/Vietnam/1203/04 (H5N1) virus [20]. A higher virus inoculation dose of 10⁶ or 10⁷ EID₅₀ of A/Vietnam/1203/04 (H5N1) virus in ferrets resulted in dyspnea and was associated with severe pulmonary pathology [27, 28]. Nasal discharge, sneezing, and visual signs of dyspnea have also been observed in ferrets infected with H5N1 viruses isolated in Hong Kong in 1997; however, a high virus dose of 10⁷ EID₅₀ was used [29].

A/Vietnam/1203/04 (H5N1) virus is neurotropic, and controlling its spread to the brain may be essential in preventing death. In the present study, we observed 100% survival and inhibition of virus spread to the brain in ferrets given prophylactic oseltamivir at 10 mg/kg once daily or 2.5 or 5 mg/kg twice daily. In ferrets given 5 mg/kg once daily, 100% survival was observed even though virus was isolated from the brain. It is important that virus titers were decreased 2.2 log₁₀ EID₅₀/mL in the brains of animals treated with 5 mg/kg once daily, compared with those of controls. This decrease in viral titers could be a contributor to survival; however, the detrimental effects of neurotropic H5N1 influenza viruses are not clearly understood. It is unknown whether oseltamivir prevented the spread of virus to the brain or inhibited viral replication in the brain. Only a small amount of oseltamivir carboxylate has been detected in the brains of rats after a single 10 mg/kg dose of oseltamivir, although high levels were detected in the lungs [30]. The level of oseltamivir carboxylate in the brains of infected animals and its ability to inhibit viral spread and replication within the central nervous system require further investigation.

There is limited available information about the production of antibodies to influenza virus during oseltamivir treatment. The virus-inhibitory effect of oseltamivir against H1N1 virus in mice did not prevent the development of an adequate humoral immune response [31]. Similarly, antibody production was observed in ferrets inoculated with H5N1 virus and subsequently

![Figure 4](image-url)
treated with oseltamivir [20]. The present study supports the contention that oseltamivir does not interfere with serum antibody production. We observed positive HI or virus neutralization titers in each treatment group, demonstrating that oseltamivir did not prevent infection but prevented the release of virus from infected cells, limiting the infection. Interestingly, serum titers did not differ among the treatment groups, although clinical signs of infection ranged from none to severe. Lower HI titers were observed with homologous A/Vietnam/1203/04 (H5N1) virus than with A/Hong Kong/213/03 (H5N1) virus, a finding consistent with previous reports [20, 24]. Another important finding was that all ferrets were completely protected from lethal rechallenge with homologous virus. These findings indicate that oseltamivir prophylaxis did not prevent a protective immune response to the virus. Moreover, anti-HA antibody titers in ferrets treated with oseltamivir were comparable to those in ferrets vaccinated with 2 doses (7 μg of HA) of A/Vietnam/1203/04 (H5N1) vaccine, although virus-neutralizing antibody titers were lower [24].

It is important to monitor for the emergence of resistant variants during antiviral treatment. Oseltamivir-resistant H5N1 viruses with a mutation at position H274Y of the NA have been identified in patients undergoing oseltamivir treatment [5] and in a patient receiving oseltamivir prophylaxis followed by oseltamivir treatment [25]. The direct avian-human transmission of H5N1 virus containing the N294S NA mutation (known to confer oseltamivir resistance) in Egypt was recently reported [32]. Our study identified no amino acid substitutions known to confer resistance to NA inhibitors at the conserved residues in NA in virus isolates. The single NA mutation detected (F390Y) is reportedly associated with pH stability in N2 NA subtypes [33]; however, the significance of this substitution in the H5N1 subtype requires further study.

In summary, prophylaxis against lethal H5N1 influenza virus with the NA inhibitor oseltamivir effectively controls virus shedding and spread to internal organs, including the brain, in ferrets. Effective prophylaxis against highly pathogenic H5N1 strains requires higher doses or more frequent administration than might be required for seasonal influenza strains. Prophylactic oseltamivir did not prevent the development of protective immunity, even when infection was asymptomatic. This observation suggests that, in areas with a high prevalence of H5N1 viruses, oseltamivir can be used to limit the morbidity, lethality, and transmission of the viruses in an immunologically naive population. Our findings provide evidence to recommend an oseltamivir prophylaxis regimen for individuals who work with highly pathogenic H5N1 virus, perform surveillance, or respond to an H5N1 outbreak.

Acknowledgments

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