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Intramuscularly administered neuraminidase inhibitor peramivir is effective against lethal H5N1 influenza virus in mice

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ABSTRACT

The replication efficiency and multi-organ dissemination of some influenza A (H5N1) viruses requires a rapid re-evaluation of the available antiviral strategies. We assessed five regimens of the neuraminidase (NA) inhibitor peramivir in mice inoculated with H5N1 virus. The regimens differed by: (1) frequency of administration on first day (once vs twice); (2) duration of administration (1 day vs 8 days); (3) route of administration (intramuscular [IM] injection alone or followed by oral administration). In all regimens, BALB/c mice were administered 30 mg/kg peramivir IM 1 h after lethal challenge with 5 MLD_{50} of A/Vietnam/1203/04 (H5N1) influenza virus. When given only on the day of inoculation, a single IM injection produced a 33% survival rate, which increased to 55% with two injections. Eight-day regimens significantly increased survival; two IM injections followed by seven daily IM injections was the most effective regimen (100% survival; inhibition of replication in lungs and brain). When this 8-day regimen began at 24 h after inoculation, 78% of mice survived; 56% survived when treatment began at 48 hours. Anti-HA antibody titer differed with the peramivir regimen and corresponded to the severity of disease. Overall, our results demonstrate that IM administration of peramivir is effective in promoting the survival of mice infected with systemically replicating H5N1 virus.

1. Introduction

Since 1997, highly pathogenic avian influenza A (H5N1) viruses have posed a threat to human health, causing a fatality rate >60% (WHO, 2008). Although mild and even asymptomatic infections have been observed, human H5N1 virus infection is commonly associated with severe clinical disease, including pneumonia with progressive respiratory failure, gastrointestinal symptoms, hepatic and renal dysfunction, and encephalitis (Yuen et al., 1998; de Jong et al., 2005; Ungchusak et al., 2005). Systemic spread and multi-organ failure contribute to the high mortality rates observed. H5N1 virus has been isolated from the cerebrospinal fluid, feces, serum, and plasma of patients (Chutinimitkul et al., 2006; de Jong et al., 2006; Buchy et al., 2007) and from post-mortem tissues. Moreover, detection of viral RNA in intestinal tissues suggests that viral replication can occur in the gastrointestinal tract (Gu et al., 2007; Uiprasertkul et al., 2005).

Neuraminidase (NA) inhibitors which target the conserved residues of the NA active site of both influenza A and B viruses (Colman, 1994), are recommended for the treatment of H5N1 influenza virus infection (Schunemann et al., 2007). NA inhibitors approved for clinical use are orally administered oseltamivir and inhaled zanamivir, both of which are effective against potentially pandemic avian influenza strains under experimental conditions (Leneva et al., 2000; Govorkova et al., 2001; Yen et al., 2005). Information about drug efficacy in humans is limited. Treatment often starts late in the course of H5N1 virus infection, with suboptimal dosages and duration of treatment (Chotpitayasunondh et al., 2005; Beigel et al., 2005). Oseltamivir-resistant H5N1 influenza viruses with mutations at positions H274Y and N294S of the NA have been isolated from patients during (de Jong et al., 2005; Le et al., 2005) and before (Saad et al., 2007) antiviral treatment. Thus far, such isolates remain sensitive to zanamivir (Le et al., 2005; McKimm-Breschkin, 2005; Mishin et al., 2005); however, new H5N1 isolates with mutations not previously reported to be associated with resistance show decreased sensitivity to oseltamivir and zanamivir in vitro (Hurt et al., 2007; McKimm-Breschkin et al., 2007). New approaches for the control of infection with highly pathogenic influenza viruses must be explored; these may include novel

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antiviral drugs, combinations of antivirals, or optimization of the existing antiviral regimens (dosage, duration and route of administration).

Peramivir (BCX-1812, RWJ-270201), a unique cyclopentane NA inhibitor, is currently under development. Peramivir, generated by using structure-based drug design, has three chemical groups that interact with the active-site residues of the influenza virus NA protein, resulting in tight binding and a slow rate of dissociation (Babu et al., 2000; Bantia et al., 2001). Peramivir potently inhibits the NA activity of various influenza A and B viruses, in vivo and in vitro (Babu et al., 2000; Bantia et al., 2001; Drusano et al., 2001; Sidwell et al., 2001a; Smee et al., 2001). Peramivir has also shown efficacy in cell culture and in mice against influenza viruses with pandemic potential, including H5N1 and H9N2 virus subtypes (Govorkova et al., 2001). Although orally administered peramivir was effective in mice, it did not protect humans against contemporary human influenza A virus in clinical trials; likely resulting from the relatively low blood concentrations observed (Barroso et al., 2005).

Because of the low absorption of oral peramivir, injection of peramivir is being investigated. No injectable anti-influenza drug is currently approved for clinical use. Although intravenously administered zanamivir is well tolerated in humans and effectively prevents experimental influenza A infection (Calfee et al., 1999; Cass et al., 1999; Fritz et al., 1999), only the inhaled formulation of zanamivir is commercially available. Parenterally administered peramivir, now in clinical trials against seasonal influenza A infection has produced high blood levels of the drug in human volunteers and was well tolerated (Kilpatrick et al., 2007). Pre-clinical studies in mice showed that intramuscularly injected peramivir is effective against infection with contemporary influenza A viruses of H1N1 and H3N2 subtypes (Bantia et al., 2006) and with sub-lethal doses of H5N1 subtype (Yun et al., 2008); however, 100% survival was not achieved against H5N1 infection. It is unknown what regimens of parenterally administered peramivir will completely protect against H5N1 virus infection, although relatively rapid production of a high serum drug concentration could possibly offer an advantage against systemically replicating viruses.

In this study, we evaluated the efficacy of five different regimens of peramivir against infection with the highly pathogenic A/Vietnam/1203/04 (H5N1) influenza virus in a mouse model. Peramivir administered IM for 8 days prevented the deaths of mice inoculated with lethal dose of H5N1 virus and completely eliminated viral spread to internal organs.

2. Materials and methods

2.1. Neuraminidase inhibitors

Peramivir{[1S,2S,3R,4R,1′S]-3-[1′-acetylamino-2′-ethyl]butyl-4-[(aminoimino)-methyl]amino-2-hydroxycyclopentane-1-carboxylic acid; BCX-1812} and oseltamivir carboxylate, the active metabolite of oseltamivir {[3R,4R,5S]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexane-1-carboxylic acid} were synthesized by BioCryst Pharmaceuticals (Birmingham, AL). Zanamivir (4-guanidino-Neu5Ac2en) was provided by the R.W. Johnson Pharmaceutical Research Institute. Compounds were provided as lyophilized powder and were maintained at 4 °C. The compounds were dissolved in distilled water and aliquots were stored at −20 °C until used.

2.2. Viruses and cells

The H5N1 influenza viruses A/Vietnam/1203/04 and A/HongKong/213/03 were obtained from the World Health Organization collaborating laboratories. Stock viruses were grown in the allantoic cavities of 10-day-old embryonated chicken eggs for 32 h at 37 °C, and aliquots were stored at −70 °C until used. Virus titer was determined by calculating the 50% egg infectious dose (EID_{50}) per ml of virus stock (Reed and Muench, 1938). Experiments with highly pathogenic H5N1 influenza viruses were conducted in an animal biosafety level 3+ containment facility approved by the U.S. Department of Agriculture. Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in minimum essential medium (MEM) supplemented with 5% fetal bovine serum, 5 mM L-glutamine, 0.2% sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 100 μg/ml kanamycin sulfate in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. NA enzyme inhibition assay

We used a modified fluorescence-based NA enzyme inhibition assay (Potier et al., 1979). Briefly, NA inhibitors at concentrations ranging from 0.00005 μM to 10 μM were incubated with H5N1 influenza virus preparations representing a standard amount of NA activity before the addition of the substrate 2′-(4-methylumbelliferyl)-d-*N*-acetylneuraminic acid (MUNANA, Sigma, St. Louis, MO). The reaction was stopped after 1 h of incubation at 37 °C and the fluorescence of the released 4-methylumbelliferone was measured in a Fluoroskan II (Labsystems, Helsinki, Finland) spectrophotometer using excitation and emission wavelengths of 355 and 460 nm, respectively. The IC_{50} was defined as the concentration of NA inhibitor necessary to reduce NA activity by 50% relative to that in a reaction mixture containing virus but no inhibitor.

2.4. Drug susceptibility in cell culture

The drug susceptibility of A/Vietnam/1203/04 (H5N1) virus was determined by a plaque reduction assay (Hayden et al., 1980). MDCK cells were inoculated with virus diluted in MEM to yield 50–80 plaques per well and then were overlaid with infection medium [MEM with 0.3% BSA] containing NA inhibitors at concentrations of 0.0001–10 μM. Plaque size was measured after 3 days of incubation at 37 °C. At least two independent experiments were performed to determine the concentration of compound required to reduce plaque size by 50% relative to plaque size in untreated wells (EC_{50}).

2.5. Drug efficacy in vivo

Female 6–8-week-old BALB/c mice (weight, 18–20 g; Jackson Laboratories, Bar Harbor, ME) were lightly anesthetized by inhalation of isoflurane and inoculated intranasally with 5 MLD_{50} of A/Vietnam/1203/04 (H5N1) influenza virus in 50 μl of PBS. Peramivir was administered starting 1 h post-inoculation (p.i.) to groups of 10–15 BALB/c mice according to five different regimens: (1) a single intramuscular (IM) injection of 30 mg/kg [Single IM]; (2) two IM injections, each 30 mg/kg [2× IM], administered during 1 day; (3) a single IM injection of 30 mg/kg on the first day and on each of the next 7 days [Single IM + 7d IM]; (4) a single IM injection of 30 mg/kg on the first day followed by oral administration of 30 mg/kg once daily for 7 days [Single IM + 7d oral]; and (5) two IM injections of 30 mg/kg of peramivir during the first day followed by one IM injection of 30 mg/kg daily for 7 days [2× IM + 7d IM]. The design of experiments is shown in Fig. 1. All studies were conducted under applicable laws and guidelines and after approval from the St. Jude Children's Research Hospital Animal Care and Use Committee.

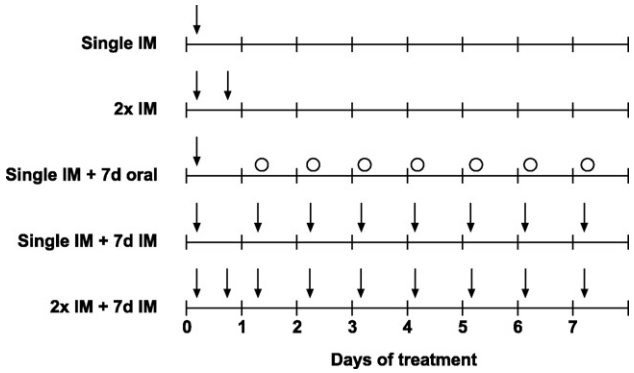

Fig. 1. Experimental design. BALB/c mice were administered peramivir by intramuscular injection (arrows) or by oral gavage (circles).

2.6. Efficacy of delayed treatment

To determine the effects of delayed treatment, we initiated the 2× IM + 7d IM regimen 24, 48, or 72 h after mice were inoculated with 5 MLD_{50} of A/Vietnam/1203/04 (H5N1) influenza virus. Control (inoculated, untreated) animals received sterile PBS on the same schedule. The groups of 13 BALB/c mice were observed daily for 21 days for clinical signs of infection and for survival. Three mice in each group were killed on day 4 p.i. to determine virus titers in the lungs as described below.

2.7. Virus titers in lungs and brain

On days 3, 6, and 9 after inoculation with A/Vietnam/1203/04 (H5N1) influenza virus, three mice in each experimental and control group were humanely killed. The brains and lungs were removed, washed thoroughly in a large volume of cold sterile PBS, homogenized, and suspended in 1 ml of PBS. The cellular debris was cleared by centrifugation at 2000 × *g* for 10 min. Supernatant was serially diluted and inoculated into 10-day-old embryonated chicken eggs. The lower limit of virus detection was 0.75 $\log_{10}$ EID_{50}/ml. For calculation of the mean, samples with a virus titer <0.75 $\log_{10}EID_{50}$/ml were assigned a value of 0. Virus titers in each organ were calculated by the method of Reed and Muench (1938) and were expressed as mean $\log_{10}$ EID_{50}/ml ± S.D.

2.8. Emergence of drug-resistant variants

The RNeasy Kit (Qiagen, Chatsworth, CA) was used to extract viral RNA from the lungs and brains of mice on days 6 and 9 p.i., and the One Step RT-PCR kit (Qiagen, Chatsworth, CA) was used according to the protocol provided. Universal primers were used for amplification of the NA and HA (HA1 region) genes (Hoffmann et al., 2001) The sequences were determined by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital by using BigDye Terminator (v. 3) chemistry and synthetic oligonucleotides. Samples were analyzed on Applied Biosystems 3700 DNA analyzers.

2.9. Anti-HA antibody response

Serum samples were collected from mice 21 days p.i., treated with receptor-destroying enzyme, heat-inactivated at 56 °C for 30 min, and tested by hemagglutination inhibition (HI) assay with 0.5% packed chicken red blood cells (CRBC).

2.10. Statistical analysis

Mean virus titers in mouse organs were compared by unpaired two-tailed *t*-test. The Kaplan-Meier method was used to estimate the probability of survival and the log-rank test to compare survival estimates of the placebo and treatment groups (Venables and Ripley, 1997). The proportional hazards model was used to determine the death hazard ratio of the treatment and placebo groups (Cox, 1972).

3. Results

3.1. Susceptibility of H5N1 virus to NA inhibitors in vitro

To compare the susceptibility of A/Vietnam/1203/04 (H5N1) influenza virus to three different NA inhibitors in vitro, we performed NA inhibition and plaque reduction assays in MDCK cells.

Table 1
Effect of peramivir regimens in mice inoculated with A/Vietnam/1203/04 (H5N1) influenza virus

Peramivir regimen[a]	Number of survivors/total (%)	Days to death, mean ± S.E.[c]	Hazard ratio (*P*-value)[d]	Mean weight change (% ± S.E.)[e]			Neurological signs[f]
				6 Days p.i.	9 Days p.i.	15 Days p.i.	
1-Day administration							
Single IM	3/9 (33)**	12.7 ± 0.7*	0.08**	−3.1 ± 1.5	−14.5 ± 2.9	−4.1 ± 1.8	5/9 (13)
2× IM[b]	5/9 (55)**	13.1 ± 0.6*	0.05**	−0.7 ± 1.1	−3.6 ± 2.0**	−2.3 ± 2.8	2/9 (13.5)
8-Day administration							
Single IM + 7d oral	6/9 (66)**	11.7 ± 0.4*	0.04**	1.8 ± 0.7*	−1.0 ± 3.0**	−3.0 ± 6.8	2/9 (11)
Single IM + 7d IM	8/9 (88)**	17.0 ± 0.0*	0.01**	3.8 ± 0.5*	3.8 ± 0.6**	1.2 ± 3.2	2/9 (15)
2× IM + 7d IM[b]	10/10 (100)**	>21*	0.00**	1.6 ± 1.2*	2.7 ± 1.5**	5.8 ± 3.8	0/10 (NA)
Control	0/10 (0)	9.2 ± 0.3	1	−6.2 ± 2.8	−19.1 ± 1.0	NA	5/10 (9)

[a] Peramivir (30 mg/kg) or PBS was administered by IM injection or oral gavage, starting 1 h after inoculation of 6-week-old BALB/c mice with 5 MLD_{50} of A/Vietnam/1203/04 (H5N1) virus. Survival was observed for 21 days.
[b] Two IM injections given 12 h apart, starting 1 h after inoculation.
[c] Estimated by the log-rank test (Venables and Ripley, 1997).
[d] Death hazard ratio (vs placebo group) was estimated by the proportional hazards model (Cox, 1972).
[e] Loss or gain of weight was calculated for each mouse as a percentage of weight on day 0 before virus inoculation. NA – not applicable: all mice died, and therefore groups cannot be statistically compared for weight change.
[f] Ataxia or hind limb paresis. Shown are the number of mice with neurological signs/total number. The median day p.i. when the signs were observed is shown in parentheses.
* $P < 0.01$.
** $P < 0.001$ compared to placebo-treated control group.

Overall, the mean IC_{50} and EC_{50} values obtained with peramivir (0.6 ± 0.2 nM and 0.3 ± 0.1 nM, respectively) were comparable to those for zanamivir (0.9 ± 0.2 nM and 0.7 ± 0.1 nM) and oseltamivir carboxylate (0.3 ± 0.1 nM and 0.5 ± 0.1 nM), demonstrating the high susceptibility of this H5N1 influenza virus to all three NA inhibitors in vitro (data not shown).

3.2. Effect of peramivir on survival and disease signs after challenge with lethal H5N1 virus

We evaluated the effect of five different regimens of peramivir on the lethality and clinical signs of A/Vietnam/1203/04 (H5N1) virus infection in mice (Fig. 1). Untreated inoculated control mice exhibited progressive weight loss with a mean day of death of 9.2. The survival rate of treated mice varied with the regimens. A single IM injection prevented death in 33% of animals, and two IM injections (2× IM) prevented death in 55% (Table 1). Minimal weight loss was observed on day 6 p.i. in mice receiving peramivir for 1 day; however, weight loss was maximal on day 9 p.i. Prolonging peramivir therapy from a 1-day to an 8-day regimen significantly lowered the risk of death: the single IM + 7d oral and single IM + 7d IM regimens prevented death in 66% and 88% of animals, respectively ($P < 0.001$). The 2× IM + 7d IM regimen had the greatest efficacy: no weight loss and 100% survival (Table 1).

Despite differences in survival among the peramivir regimens (Fig. 2), drug administration significantly delayed death in all treatment groups ($P < 0.01$). The single IM and the 2× IM treatment regimens resulted in a mean day of death of 12.7 and 13.1, respectively; however, in the single IM group mortality occurred 1 day earlier than in the 2× IM group, with the highest incidence of death on day 14 p.i. (Fig. 2A). Administration of peramivir for 8 days increased not only the likelihood of survival but also the duration of survival (Fig. 2B).

Fifty percent of control animals developed severe neurological signs (hind limb paresis and ataxia) before death. A single IM injection of peramivir did not prevent neurological complications in 5 of 9 mice (Table 1). However, the frequency of neurological signs was decreased by the 2× IM, single IM + 7d oral, and single IM + 7d IM regimens and neurological signs were inhibited by the 2× IM + 7d IM regimen.

3.3. Reduction of lung and brain virus titers by peramivir treatment

We compared the efficacy with which different peramivir regimens inhibited the replication of neurotropic A/Vietnam/1203/04 (H5N1) virus in mice. Neither single-day peramivir regimen inhibited pulmonary virus replication in inoculated mice; viral titers were comparable to those in the control group on days 3, 6, and 9 p.i. (Fig. 3A). All of the 8-day regimens significantly reduced ($P < 0.05$) virus replication in mouse lungs on days 3 and 6 p.i. (Fig. 3A). Day-9 pulmonary virus titers did not differ significantly between controls and the single IM + 7d oral treatment group but were significantly reduced ($P < 0.05$) in the single IM + 7d IM and 2× IM + 7d IM treatment groups. Notably, the 2× IM + 7d IM regimen was the only drug regimen that completely inhibited virus replication in the lungs on all days studied (limit of virus detection, 0.75 $\log_{10}$ EID_{50}/ml).

In untreated inoculated mice, H5N1 virus was detectable in the brain as early as day 3 p.i; the brain virus titer was slightly increased on day 6 p.i. and was dramatically increased (as high as 5.5 $\log_{10}EID_{50}$/ml) on day 9 p.i. Virus was undetectable in the brains of all treated mice on days 3 and 6 p.i. Both of the 1-day regimens and the single IM + 7d oral regimen significantly reduced ($P < 0.05$) brain virus titers on days 3, 6 and 9 p.i (Fig. 3B). Importantly, both of the 8-day IM regimens completely inhibited virus replication in the brains of mice (virus was not detectable on day 3, 6, or 9 p.i.). Virus replication in both the lung and brain was inhibited only in the 2× IM + 7d IM treatment group, indicating that this regimen is best for controlling systemic A/Vietnam/1203/04 (H5N1) virus spread.

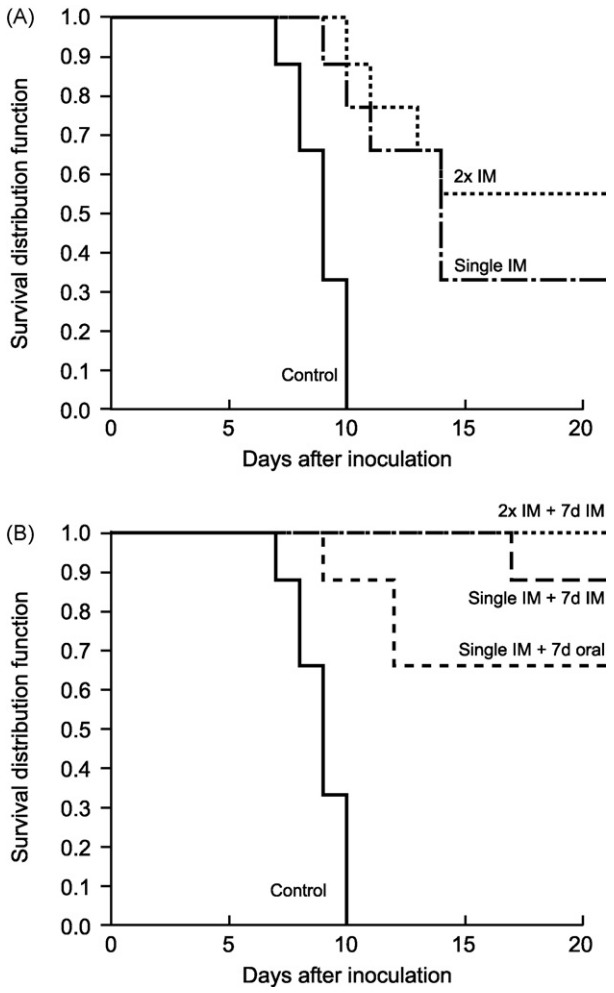

Fig. 2. Effect of 1- and 8-day peramivir regimens on mouse survival. BALB/c mice were administered peramivir for 1 day (A) or for 8 days (B). The Kaplan-Meier method was used to estimate the probability of survival, which expressed as survival distribution function. Value of 1 corresponds to 100% survival.

3.4. Emergence of peramivir-resistant variants during treatment

To monitor the emergence of resistant variants during peramivir treatment, we isolated RNA from virus detected in the lungs and brains of mice days 6 and 9 p.i. Sequence analysis of the dominant virus population identified no differences between the amino acids encoded by the NA and HA (HA1 region) genes of the wild-type A/Vietnam/1203/04 (H5N1) virus (challenge virus) and virus isolated from the organs of treated mice (data not shown).

3.5. Antibody production after peramivir treatment

To serologically confirm H5N1 virus infection and to compare the effect of the drug regimens on production of anti-HA antibodies, we collected serum 21 days p.i. for HI assay. Because A/Vietnam/1203/04 (H5N1) virus tends to induce low anti-HA titers, we included A/Hong Kong/213/03 (H5N1) virus, whose HA antigen is better suited to serologic antibody detection, in the assay. Anti-HA antibody titers to both H5N1 viruses were observed

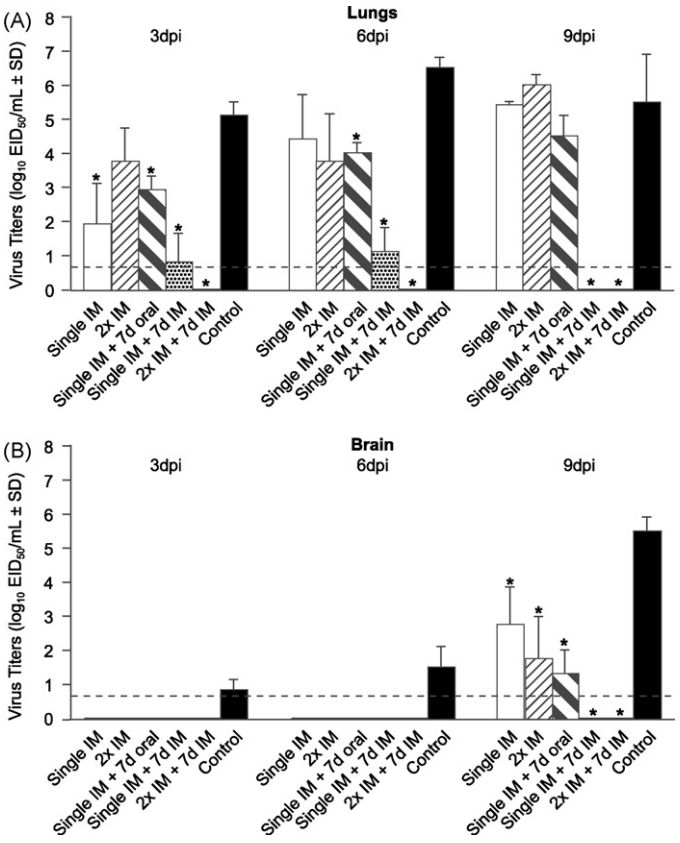

Fig. 3. Effect of peramivir regimens on virus titers in internal organs. Virus titers were determined at 3, 6, and 9 days p.i. (dpi) in the lungs (A) and brain (B). Each data point represents the mean virus titer ± S.D. ($\log_{10}$EID$_{50}$/ml) from the lungs or brains of 3 mice. Dotted line indicates minimum level of detection (0.75 $\log_{10}$EID$_{50}$/ml). * indicate $P < 0.05$.

in surviving mice given the single IM, 2× IM, and single IM + 7d oral regimens (Fig. 4). In the single IM + 7d IM treatment group, only anti-HA antibodies against A/Hong Kong/213/03 (H5N1) were detected, confirming previous observations that this heterologous virus better detects anti-HA antibodies (Hoffmann et al., 2005). Mice that underwent the 2× IM + 7d IM treatment had decreased or undetectable anti-HA serum antibodies. Overall, our data showed that the single IM and the single IM + 7d oral peramivir regimens did not protect animals from H5N1 infection, although they reduced

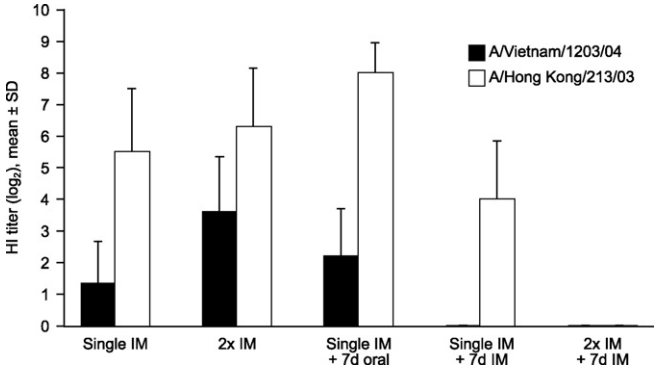

Fig. 4. Serum antibody responses in mice inoculated with A/Vietnam/1203/04 (H5N1) virus and treated with peramivir. Hemagglutination inhibition (HI) titers were tested by using A/Vietnam/1203/04 (H5N1) and A/Hong Kong/213/03 (H5N1) viruses. Drug regimens are shown as mg/kg (see Figs. 1 and 2). Sera were obtained 21 days p.i. Values are mean HI titers ($\log_2$) ± S.D.

the severity of disease. Multiple IM injections of peramivir were required to completely inhibit the spread of H5N1 viral infection.

3.6. Effect of delayed treatment with peramivir

To assess the therapeutic potential of peramivir we examined the efficacy of the drug when given during the course of established infection. Mice were inoculated with 5 MLD$_{50}$ of A/Vietnam/1203/04 (H5N1) virus and treatment with the 2× IM + 7d IM regimen was initiated 24, 48, or 72 h p.i. All untreated inoculated mice died between days 8 and 10 p.i. Treatment started either 24 or 48 h p.i. significantly increased survival (78% and 56% of animals survived, respectively) and decreased weight loss ($P < 0.01$) (Table 2). However, when treatment began 72 h p.i., mice lost weight progressively and died between days 8 and 12 p.i. Notably, initiation of treatment 24 and 48 h p.i. also reduced neurological signs. Although initiation of peramivir treatment 24 h p.i. significantly inhibited virus replication in the lungs ($P < 0.05$), lung titers did not differ significantly from those in controls when treatment began 48 or 72 h p.i. (Table 2).

4. Discussion

There is limited information about the efficacy of parenterally administered anti-influenza drugs against influenza A (H5N1) viruses. This study evaluated different regimens of intramuscularly administered peramivir in mice lethally challenged with the A/Vietnam/1203/04 (H5N1) influenza virus. We found that a single IM injection of peramivir protected 33% of animals from death, and IM administration for 8 days was required to achieve 100% survival and inhibit viral replication in the lungs and brains. Delayed IM administration also promoted survival if peramivir was given within 48 h of virus inoculation.

In the current study, A/Vietnam/1203/04 (H5N1) influenza virus was highly susceptible to peramivir in both enzymatic and cell-culture-based assays. Because virus susceptibility in vitro does not always predict results in vivo (Sidwell et al., 1998, 2001a), studies in an animal model are warranted. In the mouse animal model, we observed that the frequency and duration of peramivir administration are important factors in the adequate control of infection caused by the highly pathogenic H5N1 viruses. We found that a single IM dose of peramivir was inadequate to completely protect mice from mortality caused by the highly pathogenic A/Vietnam/1203/04 (H5N1) virus. A survival rate of 33% was achieved with a single IM dose which increased to 55% with twice daily IM injections. Against seasonal influenza strains, a single IM injection of peramivir significantly reduced weight loss and mortality in mice inoculated with mouse-adapted A/Victoria/3/75 (H3N2) virus and completely protected against lethal challenge with A/NWS/33 (H1N1) virus (Bantia et al., 2006). The difference in effectiveness of 1-day IM treatment with peramivir between seasonal influenza and the H5N1 influenza virus may be explained by the greater virulence of H5N1 viruses than of H1N1 and H3N2 viruses which affects the efficacy of NA inhibitors in experimental animal models (Sidwell and Smee, 2000; Yen et al., 2005; Ilyushina et al., 2007).

The survival rate of mice improved as the duration of peramivir administration increased from 1 day to 8 days. We observed that the IM route of peramivir administration offered greater benefit than the oral route, as the single IM + 7d oral regimen was less effective than the single IM + 7d IM and 2× IM + 7d IM regimens (lower survival rates, greater weight loss, and higher lung and brain virus titers). Recent studies also showed that IM injections of peramivir promoted survival in mice and ferrets infected with

Table 2
Effect of delayed treatment with peramivir in mice inoculated with A/Vietnam/1203/04 (H5N1) influenza virus

Initiation of treatment (hours p.i.)[a]	Number of survivors/total (%)	Days to death, mean ± S.E.[b]	Hazard ratio (*P*-value)[c]	Mean weight change (% ± S.E.)[d]			Neurological signs[e]	Viral titer in lungs[f]
				6 Days p.i.	9 Days p.i.	15 Days p.i.		
24	7/9 (78)**	11.9 ± 0.2*	0.05*	0.4 ± 0.7*	−2.3 ± 2.1**	1.0 ± 1.1	0/9 (NA)	2.5 ± 0.2*
48	5/9 (56)**	11.2 ± 0.4*	0.12*	−0.5 ± 0.6*	−3.4 ± 2.4**	−1.4 ± 3.3	0/9 (NA)	5.4 ± 0.3
72	0/8 (0)	9.2 ± 0.6	1	−1.3 ± 1.4	−6.3 ± 4.5**	NA	3/8 (9.3)	5.8 ± 0.4
Control	0/10 (0)	8.8 ± 0.3	1	−6.5 ± 2.0	−15.1 ± 1.7	NA	5/10 (9.0)	5.8 ± 0.8

[a] Two peramivir injections (each 30 mg/kg) were administered on the day of inoculation followed by one injection daily for 7 days (2× IM + 7d IM).
[b] Estimated by the log-rank test (Venables and Ripley, 1997).
[c] Death hazard ratio (vs placebo group) was estimated by the proportional hazards model (Cox, 1972).
[d] The loss or gain of weight was calculated for each mouse as a percentage of weight on day 0 before virus inoculation. NA – not applicable: all mice died and therefore groups cannot be compared statistically.
[e] Ataxia and hind limb paresis. Shown are the number of mice with neurological signs/total number. The median day p.i. when the signs were observed is shown in parentheses.
[f] Determined on day 4 p.i. Values are mean $\log_{10}$ EID_{50}/ml ± S.E. from three individual mice.
* $P < 0.01$.
** $P < 0.001$ compared to placebo-treated control group.

A/Vietnam/1203/04 (H5N1) virus (Yun et al., 2008), although low virus doses were used (9%–36% survival of control untreated animals) for the evaluation of antiviral efficacy. The comparison of bioavailability and tissue distribution of oral and IM peramivir has not been reported, but low bioavailability of orally administered peramivir may account for its lesser efficacy against a virus that spreads systemically. Although we did not directly compare the efficacy of the different NA inhibitors against lethal H5N1 virus infection in mice, we found that the single IM + 7d oral peramivir regimen was as effective as oseltamivir in preventing death from A/Vietnam/1203/04 (H5N1) virus infection. An 8-day regimen of orally administered oseltamivir 10 mg/kg/day was needed to provide an 80% survival rate in mice inoculated with 5 MLD_{50} of A/Vietnam/1203/04 (H5N1) virus (Yen et al., 2005).

The prevention of death by intramuscularly injected peramivir corresponded to the inhibition of viral replication in the lungs and inhibition of virus spread to the brain. In animal models of lethal influenza virus infection, H5N1 virus has been isolated from multiple organs, including lungs, brain, liver, spleen, and kidney (Maines et al., 2005; Yen et al., 2005; Govorkova et al., 2007). In our study, mice receiving 1-day regimens survived while viral replication in the lungs was inhibited; however, the subsequent increase of lung titers to equal those in control untreated mice was associated with death. The survival rate was 100% only when viral replication in the lungs was completely inhibited by the 2× IM + 7d IM peramivir regimen. We suggest that the spread of A/Vietnam/1203/04 (H5N1) virus to the brain contributed to the deaths of infected treated animals late in the course of infection. Neurological signs observed in 50% of untreated inoculated mice were associated with isolation of virus from the brain early during infection. In all peramivir regimens except for the single IM regimen, the frequency of neurological symptoms was significantly reduced, the survival rate was increased, and virus spread to the brain was decreased or prevented.

The emergence of resistant mutants during antiviral treatment is a major concern. NA inhibitor-resistant H5N1 viruses carrying the H274Y or N294S mutation have been identified in infected patients after oseltamivir treatment (de Jong et al., 2005; Le et al., 2005). The development of resistance to peramivir in vivo has not been described, although influenza A and B variants resistant to peramivir have emerged in vitro under drug selection pressure (Smee et al., 2001; Baz et al., 2007; Baum et al., 2003). We found no mutations in the NA or HA genes of viruses isolated from mouse lungs and brains that would reduce susceptibility to NA inhibitors. However, these studies were designed primarily to evaluate the efficacy of parentally administered peramivir in mice inoculated with H5N1 virus; issues of receptor specificity in mouse lungs and analysis of the dominant virus population may limit any conclusions we might draw about the emergence of resistance.

In previous studies, orally administered peramivir or oseltamivir did not affect cellular immune response or prevent the development of humoral immunity in mice during H1N1 influenza virus infection (Burger et al., 2000; Sidwell et al., 2001b). IM administration of peramivir did not inhibit the development of serum anti-HA antibodies to H5N1 virus in mice. However, the levels of anti-HA antibodies differed with the peramivir regimens and corresponded to the severity of disease. High titers of anti-HA antibodies were associated with peramivir regimens that did not prevent severe infection as indicated by weight loss and high pulmonary viral titers, whereas antibody titers were undetectable and pulmonary virus replication was inhibited in mice receiving peramivir IM for 8 days. Although no peramivir regimen tested completely prevented H5N1 virus infection, all of the regimens controlled virus replication and virus spread from the primary site of infection. Sufficient viral replication in the lungs and a high viral load is required to trigger an adequate immune response.

We also studied the efficacy of delayed peramivir treatment in a mouse model of lethal H5N1 virus infection. We observed 78% and 56% survival rates when peramivir was administered IM 24 or 48 h, respectively, after inoculation. Govorkova et al. (2001) reported similar survival rates when peramivir was orally administered during H5N1 infection. A higher dose of peramivir (30 mg/kg vs 10 mg/kg) was required to achieve similar efficacy against A/Vietnam/1203/04 (H5N1) virus than against A/Hong Kong/156/97 virus (H5N1) when treatment was delayed (Govorkova et al., 2001). Viral titers in the lung were significantly lower and the survival rate was higher when peramivir treatment was delayed for 24 h vs 48 or 72 h. Pulmonary virus titers in mice treated 48 and 72 h after inoculation did not differ from those in controls, although treatment continued for only 48 and 24 h, respectively, before organ collection. We can propose that earlier initiation of treatment will be the most beneficial regimen for IM peramivir, although pathogenicity of the virus can also affect the outcome of treatment and better results can be achieved with less pathogenic H5N1 influenza virus.

In summary, these studies show that IM administered peramivir is effective against systemically replicating H5N1 virus, promoting survival and inhibiting viral replication in lungs and brain. The effi-

cacy of IM peramivir against highly pathogenic H5N1 virus infection is dependent on the timing of treatment initiation, the duration of treatment and the route of drug administration. The IM administration of peramivir enhanced its antiviral effect, suggesting that this route might be successful for the treatment of human H5N1 virus infections.

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