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A single intramuscular injection of neuraminidase inhibitor peramivir demonstrates antiviral activity against novel pandemic A/California/04/2009 (H1N1) influenza virus infection in mice

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ABSTRACT

New and emerging influenza virus strains, such as the pandemic influenza A (H1N1) virus require constant vigilance for antiviral drug sensitivity and resistance. Efficacy of intramuscularly (IM) administered neuraminidase (NA) inhibitor, peramivir, was evaluated in mice infected with recently isolated pandemic A/California/04/2009 (H1N1, swine origin, mouse adapted) influenza virus. A single IM injection of peramivir (four dose groups), given 1 h prior to inoculation, significantly reduced weight loss ($p < 0.001$) and mortality ($p < 0.05$) in mice infected with LD_{90} dose of pandemic A/California/04/2009 (H1N1) influenza virus compared to vehicle group. There was 20% survival in the vehicle-treated group, whereas in the peramivir-treated groups, survival increased in a dose-dependent manner with 60, 60, 90 and 100% survivors for the 1, 3, 10, and 30 mg/kg doses, respectively. Weight loss on day 4 in the vehicle-treated group was 3.4 gm, and in the peramivir-treated groups was 2.1, 1.5, 1.8 and 1.8 g for the 1, 3, 10 and 30 mg/kg dose groups, respectively. In the treatment model, peramivir given 24 h after infection as a single IM injection at 50 mg/kg dose, showed significant protection against lethality and weight loss. There was 13% survival in the vehicle-treated group while in the peramivir-treated group at 24, 48, and 72 h post infection, survival was 100, 40, and 50%, respectively. Survival in the oseltamivir groups (10 mg/kg/d twice a day, orally for 5 days) was 90, 30 and 20% at 24, 48 and 72 h, respectively. These data demonstrate efficacy of parenterally administered peramivir against the recently isolated pandemic influenza virus in murine infection models.

1. Introduction

Influenza pandemics occur when a new strain of the influenza virus is transmitted to humans from another animal species. This starts with the virus mostly infecting animals, with a few cases where animals infect people, then moves through the stage where the virus begins to spread directly between people, and ends with a pandemic when infections from the new virus have spread worldwide (Current WHO phase of pandemic alert *World Health Organization* 2009). On June 11, 2009, a new strain of H1N1 influenza was declared to be a global pandemic by the World Health Organization (statement to the press by WHO Director-General Dr. Margaret Chan, June 11, 2009). Characterized as an influenza A virus of the H1N1 subtype, the genomic segments of the new strain were more closely related to swine virus (Novel Swine-Origin Influenza A Virus Investigators Team 2009). Antiviral compounds are the first line of defense against pandemic influenza viruses. Sequence analysis suggested that the pandemic virus is resistant to ion channel inhibitors such as amantadine and rimantadine.

The NA inhibitors represent an important advance in the management of influenza. Inhaled zanamivir and oral oseltamivir are effective in both prophylaxis and treatment of influenza A and B viruses (Hayden et al., 1997; Hayden et al., 1999; Kaiser et al., 2003). *In vitro* and *in vivo* studies demonstrated that the pandemic influenza virus was sensitive to neuraminidase inhibitors, oseltamivir and zanamivir (Itoh et al., 2009). Both oseltamivir and zanamivir were the first line of treatment for individuals infected with pandemic influenza. Peramivir was also used for pandemic 2009 Influenza A (H1N1) treatment under an Emergency IND Program (Hernandez et al., in press).

Peramivir (Fig. 1) is a specific and potent inhibitor of influenza NA and has demonstrated *in vitro* activity that is comparable to or better than oseltamivir carboxylate (the active metabolite of oseltamivir) and zanamivir (Babu et al., 2000; Bantia et al., 2001). Peramivir is active when administered intramuscularly (IM) and intravenously (IV) for both prophylaxis and treatment in mouse

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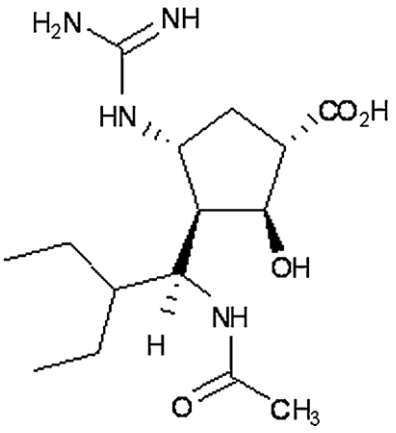

Fig. 1. Chemical structure of Peramivir.

influenza virus infection models (Bantia et al., 2006; Boltz et al., 2008; Yun et al., 2008). Intravenous administration of peramivir has been evaluated in clinical trials for seasonal influenza and to treat influenza in hospitalized patients (Kohno et al., 2009; Ison et al., 2009). The emergence of new influenza virus strains, such as the pandemic A/California/04/2009 (H1N1) influenza virus, requires constant vigilance for antiviral drug sensitivity and resistance. In this study, the efficacy of a single IM injection of peramivir given 1 h prior to or 24, 48 and 72 h after inoculation was evaluated in mice infected with the novel pandemic A/California/04/2009 (H1N1) influenza virus.

2. Materials and methods

2.1. Viruses

The influenza A virus (pandemic A/California/04/2009) used in this study was generated by Dr. Natalia A. Ilyushina, St. Jude Children's Research Hospital. This virus was adapted to replication in the lungs of BALB/c mice by 9 sequential passages through mouse lungs. Virus was plaque-purified in Madin–Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) and virus stock was further prepared in embryonated chicken eggs. Passage history: MDCK 1/Egg 1/Mouse lungs 9/MDCK 1/Egg 1. In the course of mouse lung adaptation, the pandemic A/California/04/2009 (H1N1) influenza virus has changed its antigenic specificity due to acquired mutations in the HA gene (G158E, S186P, D225G, H3 numbering), and no mutations in the NA gene of this influenza virus occurred during adaptation to BALB/c mice (Ilyushina et al., 2010).

2.2. Mice

Specific pathogen-free female BALB/c mice 6-8 weeks old (16–20 g) were obtained from Charles Rivers Laboratories (Raleigh, NC). Mice were permitted an acclimatization period of greater than 48 h (prior to virus inoculation) during which time the animals were observed for signs of disease and/or physical abnormalities. Mice were maintained on rodent diet from Harlan Teklad and tap water from the Laboratory Animal Research Center of BioCryst Pharmaceuticals, Inc. The study was conducted in accordance with the current facility's Standard Operating Procedures (SOPs). The study was conducted in compliance with the Animal Welfare Act (9 CFR Parts 1, 2 and 3).

2.3. Compounds and reagents

Peramivir, oseltamivir carboxylate and oseltamivir phosphate (oseltamivir) were synthesized by BioCryst Pharmaceuticals, Inc. (Birmingham, AL). Compounds were prepared in sterile phosphate-buffered saline for *in vivo* experiments. Calcium chloride, MES (2-(N morpholino)-ethanesulfonic acid), and MuNANA (2′-(4-methylumbelliferyl)-α-D-acetylneuraminic acid) were purchased from Sigma–Aldrich (St. Louis, MO). Isoflurane (5%) in oxygen was administered for anesthesia.

2.4. NA assay

A standard fluorimetric assay was used to measure influenza virus NA activity (Potier et al., 1979). The substrate, MuNANA, is cleaved by NA to yield a fluorescent product that can be quantified. The assay mixture contained inhibitor at various concentrations and NA enzyme (whole virus) in 32.5 mM MES buffer, 4 mM calcium chloride at pH 6.5 and incubated for 60 min at 37 °C. The reaction was started by the addition of the substrate for an incubation time of 45 min at 37 °C.

Fluorescence was recorded (excitation: 360 nm and emission: 450 nm) and substrate blanks were subtracted from the sample readings. The IC_{50} was calculated by plotting percent inhibition of NA activity vs. the inhibitor concentration. The results are reported as the average of two experiments.

2.5. General procedure for in vivo antiviral experiments

Six- to eight-week-old female BALB/c mice were anesthetized with isoflurane and exposed to 50 μL of 10-fold serial dilutions of pandemic A/California/04/2009 (H1N1) influenza virus in phosphate-buffered saline (PBS) by intranasal instillation. An approximately 90% mouse lethal dose of virus was calculated after a 21-day observation period. This virus dose consistently gave 73–100% mortality in 5 separate experiments. Groups of 10–15 mice were anesthetized with isoflurane and given an approximately 90% mouse lethal dose of virus. Drug was administered 1 h before viral inoculation. Peramivir was administered as a single IM injection at various doses ranging from 1 mg/kg to 30 mg/kg. Each infected, drug-treated group contained 10 mice and the infected, saline-treated group contained 15 mice. In the treatment model, peramivir-treated groups received one single IM injection of 50 mg/kg peramivir at 24, 48, or 72 h post virus inoculation. For comparison, three additional groups of mice were treated with oseltamivir dosed at 10 mg/kg/d orally (po) twice per day (BID) starting 24, 48, or 72 h post virus inoculation and continuing for 5 days (BID × 5). Handling animals during infection and dosing can cause stress which can impact the immune system and may alter the course of the disease. To alleviate this concern, an additional vehicle group was added that was dosed orally twice a day for comparison with the oseltamivir group. Parameters for evaluation of antiviral activity included weight loss, reduction in mortality and/or increase in mean day to death (MDD) determined through day 21.

2.6. Statistical analysis

The data were analyzed by Sigma Plot (Windows Version 11.0, SPSS, Chicago, IL) and Sigma Stat (Windows Version 3.5, Jandel Corporation, San Rafael, CA). The mean day to death was estimated as the average number of days that the mice in each group survived after viral infection. Mice that survived until the end of the study were censored. The unpaired *t*-test was used to evaluate differences in mean day to death between the treatment and the control group. One-way analysis of variance (ANOVA) was performed to evaluate differences in weight loss. Kaplan–Meier Survival analysis (log rank test) was applied to survival number differences.

3. Results

3.1. In vitro NA inhibition

Both peramivir and oseltamivir carboxylate are potent inhibitors of the NA activity of pandemic A/California/04/2009 (H1N1) influenza virus. The IC_{50} for peramivir and oseltamivir carboxylate against NA enzyme were 0.434 ± 0.07 and 1.10 ± 0.19 nM and the IC_{90} values were 2.37 ± 0.67 and 11.1 ± 1.10 nM (average of two experiments with standard error), respectively.

3.2. Influenza A mouse model

In the mouse influenza model, viral infection leads to loss of body weight and mortality, and this decrease in body weight correlates with pulmonary viral titers and pulmonary lesion scores (Johansson et al., 1993). Therefore, the efficacy of IM administered peramivir was evaluated on the basis of weight loss, mean day to death and survival rate measured for 21 days post infection.

Administration of a single IM injection of peramivir 1 h before viral inoculation was effective in reducing the weight loss and increasing the survival of pandemic A/California/04/2009 (H1N1) influenza virus infected mice. All the peramivir-treated groups demonstrated significant improvement in survival in a dose-dependent manner with the survival ranging from 60% ($p < 0.05$ vs. vehicle) in the lowest dose group (1 mg/kg) to 100% ($p < 0.001$ vs. vehicle) in the highest dose group (30 mg/kg, Fig. 2, Table 1). The percentage of survival in the vehicle-treated group was 20%.

The virus is highly virulent and mortality was observed in the vehicle group by day 5, hence day 4 was chosen to evaluate weight loss among various groups. The average baseline weights for the mice in all the groups were very similar (<0.5 g difference between various groups). The mean weight loss on day 4 was 3.4 g in the vehicle-treated group (Table 1). All the peramivir-treated groups showed significant decreases in weight loss compared to the vehicle group ($p < 0.001$ vs. vehicle).

The efficacy of a single 50 mg/kg IM injection of peramivir, administered 24, 48, or 72 h post virus infection, was evaluated in mice infected with pandemic A/California/04/2009 influenza virus; efficacy was compared with oseltamivir. As shown in Table 2 and Fig. 3A, peramivir showed significant treatment efficacy in mice infected with pandemic A/California/04/2009 (H1N1) influenza virus. When survival was assessed, 13 of the 15 mice in the vehicle-treated group died (13% survival; vehicle IM dosing). In contrast, none of the 10 mice treated with a single IM dose of 50 mg/kg peramivir, starting 24 h post infection, died during the 21-day study period (100% survival; $p < 0.001$ vs. vehicle). When peramivir treatment was delayed 48 or 72 h post infection, survival dropped to 40% and 50%, respectively.

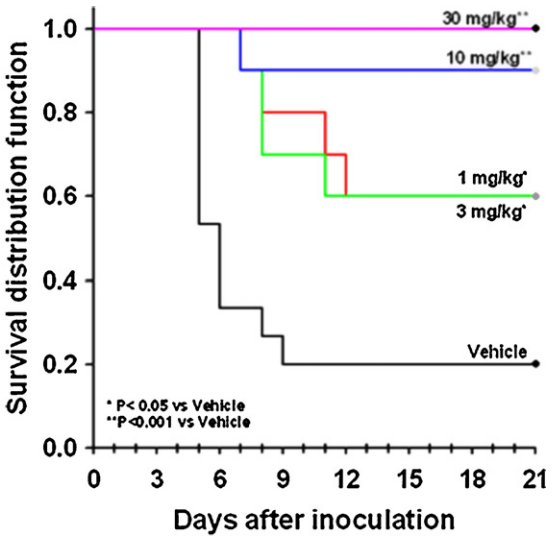

Fig. 2. Effects of peramivir on survival in mice infected with pandemic A/California/04/2009 (H1N1) influenza virus (LD_{90} dose). Peramivir was administered as a single IM injection, one hour prior to viral inoculation; $n = 10$–15 mice per group.

When body weight was evaluated, the mean weight loss on day 4 was 2.9 g in the vehicle-treated group (Table 2). In contrast, in mice treated with a single dose of peramivir 24 h post infection, the weight loss observed on day 4 was 1.7 g, which was significantly lower compared to the vehicle-treated group ($p < 0.001$). The majority of the mice from both studies and in all groups began to gain weight by day 10.

For comparison, the efficacy of oseltamivir was also evaluated in the H1N1 pandemic A/California/04/2009 influenza virus infected mice in the same study. Oseltamivir was administered po at 10 mg/kg BID for 5 days. When oseltamivir treatment was initiated 24 h post infection, survival was 90% ($p < 0.001$ vs. vehicle) and weight loss was 2.3 grams ($p < 0.01$ vs. vehicle), significantly different from the vehicle-treated mice (Table 2, Fig. 3B). Treatment was not effective with either drug when initiated 48 or 72 h post virus infection. There was no significant difference in terms of weight loss and survival between the two vehicle-treated groups (IM single injection vs. oral bid for 5 days).

4. Discussion

Peramivir is a specific and potent inhibitor of influenza NA and has demonstrated *in vitro* activity that is comparable to or better than oseltamivir carboxylate and zanamivir (Bantia et al., 2001). Because of the tight-binding and slow off-rate of peramivir from the NA enzyme compared to oseltamivir carboxylate and zanamivir,

Table 1
Effect of a single intramuscular injection of peramivir in mice infected with pandemic A/California/04/2009 (H1N1) influenza virus.[a]

Treatment	Dose (mg/kg)	Survival/total (%)	Mean day to death ± SEM	Mean weight change day 4[b] gram ± SEM (g)
Vehicle, uninfected	0	5/5 (100)	–	0.3 ± 0.2
Vehicle	0	3/15 (20)	5.8 ± 1.3	−3.4 ± 0.2
Peramivir	1	6/10* (60)	9.5 ± 1.2*	−2.1 ± 0.2**
Peramivir	3	6/10* (60)	8.5 ± 0.9*	−1.5 ± 0.3**
Peramivir	10	9/10** (90)	7.0 ± 0.0*	−1.8 ± 0.2**
Peramivir	30	10/10** (100)	–	−1.8 ± 0.2**

"–" No death occurred in these groups, therefore, no mean day to death was calculated.
[a] LD_{90} viral dose was administered to all mice (except vehicle, uninfected group) in this study.
[b] Day 4 maximum weight loss in the vehicle group with no deaths.
* $p < 0.05$; ** $p < 0.001$ vs. vehicle group.

Table 2
Effect of delayed treatment of peramivir and oseltamivir in mice infected with pandemic A/California/04/2009 (H1N1) influenza virus.[a]

Treatment	Route of administration	Treatment timepi	Survival/total (%)	Mean day to death ± SEM	Mean weight change Day4[b] grams ± SEM
Vehicle (uninfected)	IM	24 h	5/5 (100)	–	0.6 ± 0.4
Vehicle	IM single injection	24 h	2/15 (13.3)	7.8 ± 0.4	−2.9 ± 0.2
Vehicle	Oral bid × 5 days	24 h	2/15 (13.3)	7.2 ± 0.3	−3.2 ± 0.4
		24 h	10/10[c] (100)	–	−1.7 ± 0.4[c]
Peramivir 50 mg/kg	IM single injection	48 h	4/10 (40)	7.2 ± 0.5	−3.4 ± 0.5
		72 h	5/10 (50)	8.0 ± 0.4	−3.0 ± 0.5
		24 h	9/10[d] (90)	8.0 ± 0.0	−2.3 ± 0.5[e]
Oseltamivir 10mg/kg/d	Oral bid × 5 days	48 h	3/10 (30)	7.9 ± 0.6	−2.8 ± 0.6
		72 h	2/10 (20)	7.6 ± 0.6	−3.5 ± 0.6

"–" No death occurred in these groups, therefore, no mean day to death was calculated.
[a] LD_{90} viral dose was administered to all mice (except vehicle, uninfected group) in this study.
[b] Maximum weight loss in the vehicle group with no deaths; pi = post infection.
[c] $p < 0.001$ vs. vehicle (IM).
[d] $p < 0.001$.
[e] $p < 0.01$ vs. vehicle (oral).

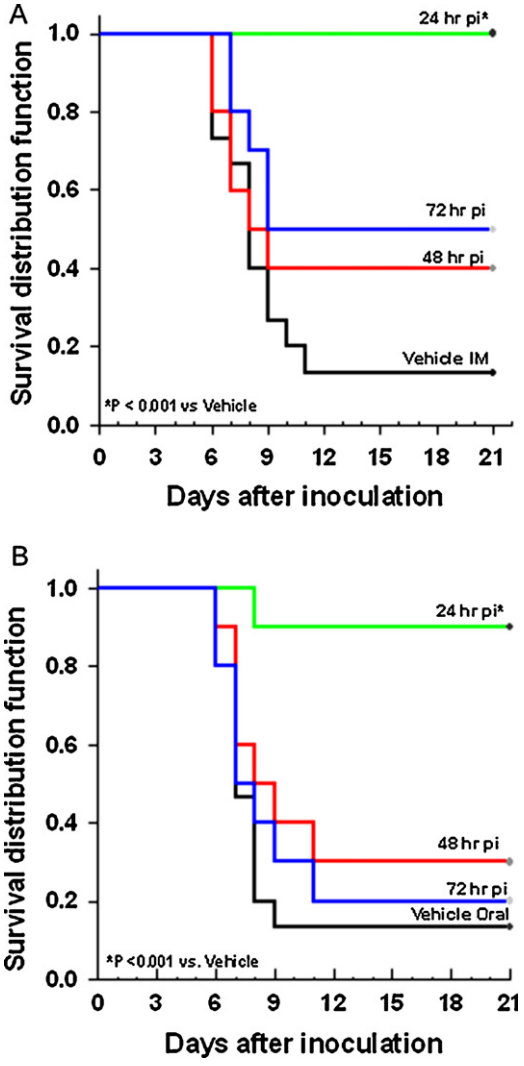

Fig. 3. Effects of peramivir and oseltamivir on survival in mice infected with pandemic A/California/04/2009 (H1N1) influenza virus. Mice were inoculated with Influenza virus (LD_{90} dose); Peramivir (A) was administered as a single IM injection (50 mg/kg) and oseltamivir (B) was administered orally BID for 5 days (10 mg/kg/d) post infection (pi) at the indicated times; $n = 10–15$ mice per group.

a single IM administration is effective in preventing lethality and weight loss in the mouse influenza models (Bantia et al., 2006). Intravenous administration of peramivir has been evaluated for seasonal influenza and in the treatment of influenza in hospitalized patients (Kohno et al., 2009; Ison et al., 2009). In April 2009 we witnessed the emergence of a novel swine-origin H1N1 influenza virus infecting the human population in Mexico and the USA (Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team et al., 2009). Since then, the novel H1N1 virus has spread worldwide and the World Health Organization (WHO) raised the pandemic alert level to phase 6 on its six-phase scale on June 11, 2009. New and emerging influenza virus strains, such as the pandemic influenza A (H1N1) virus, require constant vigilance for antiviral drug susceptibility and resistance.

Our results demonstrate that peramivir given as a single IM injection is highly effective in mice infected with the pandemic A/California/04/2009 (H1N1) influenza virus. Peramivir, administered as a single injection at a dose as low as 1 mg/kg at 1 h prior to viral inoculation, demonstrated improvement in survival and weight loss. Virus titers were not determined in this study, however. It has been shown that decrease in body weight correlates with pulmonary viral titers and pulmonary lesion scores (Johansson et al., 1993). Consistent with our observation is recently reported data on the effect of the NA inhibitors in the neuraminidase assay. The US Centers for Disease Control and Prevention (CDC, 2009) evaluated the effect of peramivir, oseltamivir carboxylate and zanamivir on the NA activity of a number of pandemic strains. The potency of peramivir against NA was significantly ($p < 0.05$) better compared to oseltamivir carboxylate and zanamivir. In addition, Tarbet et al. (2010) determined the antiviral effect, in MDCK cells, for three NA inhibitors, zanamivir, oseltamivir carboxylate, and peramivir against nine isolates of pandemic influenza A (H1N1). Peramivir was 3- to 126-fold more potent than oseltamivir carboxylate and zanamivir in inhibiting the viral growth of all the pandemic strains in MDCK cells, except for the oseltamivir resistant Hong Kong strain, HK/2369/2009.

Peramivir has been shown to be effective in various mouse influenza models given up to 60 h after virus exposure (Bantia et al., 2001, 2006; Sidwell et al., 2001). Efficacy of the drug in a mouse influenza model depends upon the virus strain, amount of virus used, strain of mice, and weight of the mice used. In the treatment model described here, efficacy of a single IM injection of peramivir was compared to oral treatment (qd × 5 days) of oseltamivir. Peramivir given as a single IM injection at 50 mg/kg dose, at 24 h after infection, completely protected mice against lethality (100% survival) and was effective in mitigating the weight

loss. Oseltamivir treatment for 5 days BID also demonstrated improvement in survival (90% survival) and weight loss when administered 24 h after virus infection.

The 50 mg/kg dose in mice is approximately 300 mg human equivalent dose (Center for Drug Evaluation and Research, 2005). Peramivir has been approved in Japan for the treatment of seasonal influenza in adults in general, as a single dose of 300 mg intravenously. For patients at high-risk, a single or multiple daily doses of 600 mg of peramivir are administered intravenously depending on the condition of the patient.

In summary, peramivir is a potent inhibitor of pandemic A/California/04/2009 (H1N1) influenza neuraminidase. Peramivir as a single intramuscular injection demonstrates efficacy in mice infected with the recently isolated pandemic A/California/04/2009 (H1N1) influenza virus.

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