Assessment of antiviral properties of Peramivir against H7N9 avian influenza virus in an experimental mouse model

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Running title: Antiviral activity of Peramivir to H7N9
The H7N9 influenza virus causes severe form of disease in humans. Neuraminidase inhibitors including oral oseltamivir and injectable peramivir are the first choices of antiviral treatment for such cases, however the clinical efficacy of these drugs is questionable. Animal experimental models are essential to understand the viral replication kinetics under the selective pressure of antiviral agents. This study, for the first time demonstrates the antiviral activity of peramivir in mouse model of H7N9 avian influenza virus infection. The data shows that repeated administration of 30mg/kg peramivir successfully eradicates virus from respiratory tract and extrapulmonary tissues during the acute response and prevents clinical signs of the disease including neuropathy and eventually protects mice against lethal H7N9 influenza infection. The early treatment with peramivir is found to be associated with better disease outcome.

Key Words: Avian influenza virus, neuraminidase inhibitors, antiviral drugs
Introduction

The H7N9 is novel avian origin influenza virus that emerged in February 2013(20). Since then, the virus sustained its presence as sporadic human cases are seen throughout the year with highest numbers typically appearing in winters following the trend of seasonal flu viruses (11, 48). Unlike other low pathogenic influenza viruses, the H7N9 causes severe human illness characterized by pneumonia that rapidly develops into acute respiratory distress syndrome (ARDS), multiple organ dysfunction (MOD) and shock (18). Up until now 619 human cases have been reported from 16 different territories or provinces in Mainland China (13, 14) while a few cases with recent travel history to China also appeared in Hong Kong (14), Taiwan (10), Malaysia and Canada (38). Of them nearly 70% required intensive care support, mechanical ventilation and approximately 34% died (41). Scientific evidences about limited airborne transmission among ferrets (52) as well as appearance of family clusters could not rule out the possibility of human-to-human transmission and raise serious global concern(40).

Due to intrinsic adamantane resistance, H7N9 influenza infections are primarily treated with neuraminidase inhibitors (NAIs) particularly oseltamivir and to some extent by intravenous administration of peramivir or zanamivir (41). Clinical data have demonstrated that the emergence of NA-R292K variants that encode NAI resistance, in few H7N9 cases during oseltamivir therapy affects on viral eradication and results in high respiratory viral loads (22). These mutants also develop NAI resistance when tested in cells but without having an effect on their replication and infectivity (21). Despite the fact that most H7N9 infected strains are sensitive to oseltamivir in cell culture, high mortality rates have been documented in H7N9
infected patients receiving oseltamivir therapy(30, 31, 34). This advocates the critical need to evaluate all available antiviral options.

Peramivir is an i.v. NAI prescribed by the National Health and Family Planning Commission for the treatment of severe H7N9 cases (12). It is a distant sialic acid analogue (cyclopentane-derivative with guanidino group and lipophilic side chain) that shares structural features with both zanamivir and oseltamivir and similarly targets influenza neuraminidase activity. Limited clinical data is available for patient compliance to this drug and so far it has not been evaluated in experimental animal models for H7N9 influenza infection. In vitro studies showed that peramivir has antiviral activity comparable to oseltamivir against H7N9 viruses (4, 9); however, rapid bioavailability of the drug through intravenous route might have an added advantage for treating patients with acute respiratory distress syndrome (ARDS) and multiple organ dysfunction (MOD). In H7N9 cases, the drug is typically administered as a follow-up to oseltamivir, at which point the virus may have accumulated mutations that confer resistance to both drugs. Peramivir has previously been used for severe pandemic H1N1 or H5N1 infected cases in Japan, United States and other parts of the world with recommended dosage of 300-600 mg i.v. daily for 5 days or till the end of viral shedding in respiratory specimens in case of immunocompromised patients (8, 25, 29, 42).

Given evidence of resistance to oseltamivir among circulating H7N9 viruses (27), we sought to evaluate the antiviral efficacy of peramivir in vivo and ascertain its suitability as a front-line therapeutic for the treatment of H7N9. Here we report antiviral activity of peramivir in H7N9 infected C57/Bl6 mice.

Materials and Methods
Isolation of H7N9 influenza virus

The virus A/Shantou/1001/2014 (H7N9) influenza virus was isolated from the lung aspirate collected from fatal case reported in The First Affiliated Hospital of Shantou University Medical College, Guangdong province of China in March 2014 and confirmed for H7N9 infection by Chinese centre of Disease Control and Prevention. The whole genome sequence of the virus is already deposited to GISAID’s EpiFlu database with identifier EPI_Isl_162618 and already reported earlier (16). Virus was cultivated, propagated and titrated in 9-10 days old embryonated chicken eggs for 72 hours at 37°C. Haemagglutination test was performed using 1% horse RBCs as described by the WHO. Virus isolation and infection procedures were performed in animal biosafety level 3 containment facilities and the ethical committee of The First Affiliated Hospital of Shantou University Medical College approved the study.

Cells and compound

Madin-Darby canine kidney (MDCK) cells were obtained from ATCC, China. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Beijing, China) supplemented with 10% heat inactivated FBS, 100 U penicillin, 100 µg/ml streptomycin and 100mM L-glutamine. Peramivir was purchased from Biocryst pharmaceutical, Shanghai, China and stock solution was prepared in 0.85% NaCl, and stored at -80°C.

Animal infection and treatment procedures

Six to eight week old female C57/Bl6 mice (Vital River, Beijing, China) were maintained on standard feed and water in Specific-Pathogen-Free (SPF) facility with controlled environmental temperature and humidity.
For this study, several sets of experiments were performed. The following protocols were used in each experiment, each group consisted of 16 mice. The animals were anesthetized with intraperitoneal (i.p.) administration of 2, 2, 2-tribromoethanol (Sigma, Steinheim, Germany) prior inoculation and experimental procedures. For survival experiments, 10 animals from each group were monitored for clinical signs, weight loss and mortality up to 14 days post infection (dpi). More than 20% loss in original body weight was considered as humane end point for this study. Animals were specifically monitored for any neurological symptoms throughout the disease course.

For the characterization of viral pathogenicity, animals were divided into four different groups (n = 16) and inoculated with 50µl of A/Shantou/1001/2014 (H7N9) virus containing 10^3, 10^4, 10^5 and 10^6 Egg Infective Dose50 (EID50) of viral particles intranasally (i.n.).

To assess antiviral activity in vivo, we administered peramivir to the animals infected with different concentrations such as 10^3, 10^4, 10^5 and 10^6 EID50 of A/Shantou/1001/2014 (H7N9) virus i.n. Briefly, 30mg/kg of peramivir was administered to the thigh muscles in final volume of 50 µl for each mouse. The treatment was given once daily from the time of infection till 8 dpi. Equal amount of 0.85% NaCl was administered in the same manner to vehicle groups.

In dose dependent experiments, 30, 15 and 3mg/kg of peramivir were administered to animals (n = 16/group) as described above. The treatment was given once daily from the time of infection till 8 dpi. The animals were infected with 10^4 EID50 of A/Shantou/1001/2014 (H7N9) virus i.n.
We next evaluated 4 different treatment regimes in mice infected with $10^4$ EID$_{50}$ of A/Shantou/1001/2014 (H7N9) virus. In first two groups, single dose of 30mg/kg of peramivir was administered to mice (n = 16/group) immediately (single dose D0) or 24 hours after inoculation (single dose D1). The next two groups were given multiple doses of peramivir initiated at the day of infection or one day later. The groups were designated as multiple doses D0-D8 and multiple doses D1-D8 respectively.

**Estimation of viral loads in body tissues**

On 3 and 6 dpi, animals from treated and untreated groups (n = 3 /group) were euthanized and body tissues such as lungs, liver, intestine, spleen, kidneys, stomach, lymph nodes, heart and brain were removed aseptically and rinsed in phosphate buffered saline (PBS). Lung tissues were homogenized in 1 ml PBS and homogenates were titrated in Madin-Darby canine kidney (MDCK) cells by TCID$_{50}$ assay using Reed and Muench’ method. Whereas viral RNA was extracted from other tissues collected aside from lung tissues using vial RNA mini kit (Qaigen, Hilden, Germany), converted into cDNA using high capacity cDNA Rt kit (Lifeteh, Foster, USA). The cDNA were then subjected to quantitative real-time PCR (qRT-PCR) targeting influenza M gene specific primers. The qRT-PCR was performed with SYBR green qPCR super Mix (Invitrogen, Cartstad,CA, USA) on MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Results were expressed as viral copy numbers/ml.

**Histology and Immunostaining**

Tissues were collected on 3 and 6 dpi from treated and untreated groups while tissues were also removed from surviving animals from peramivir treatment group and fixed with 4% buffered
formalin, processed and embedded in paraffin. Tissue sections were stained with haematoxylin and eosin (H&E) stain. Viral staining was performed using anti influenza nucleoprotein (NP) antibody (Bio X cell, West Lebanon USA).

**Statistical analyses**

Statistical analysis was performed by GraphPad Prism 6 software (GraphPad Inc.). Student’s-t test and one-way ANOVA were applied while comparing two and more than two groups respectively. Survival curves were analyzed by log-rank test. P value less than 0.05 was considered significant.

**Results**

**Characterization of A/Shantou/1001/2014 (H7N9) virus in C57/Bl6 mice**

We first characterized the pathogenicity of H7N9 influenza virus in C57/Bl6 mice. Animals exhibited severe weight loss and lethal disease following infection with $10^4$, $10^5$ and $10^6$ EID$_{50}$ of H7N9 virus with median days of death 4, 4.5 and 7 respectively. Animals infected with $10^3$ EID$_{50}$ gradually lost body weight from 6 dpi, delayed time of death and 70% mortality rate (Fig1a, b). Clinical signs included minimal physical activity, hunched posture, lethargy and ruffled fur started appearing in infected animals from 3 dpi. The virus was detected in lung tissues as high as $7.7 \log_{10}$TCID$_{50}$/ml on 3 dpi and $5.9 \log_{10}$TCID$_{50}$/ml on 6 dpi whereas viral spread to other body organs such as brain, intestine, liver, spleen, stomach, kidneys and heart was found on 3 dpi (data not shown).

Histology of lung sections revealed that lethal challenge ($10^4$ and $10^5$ EID$_{50}$) of H7N9 influenza virus induced interstitial pneumonia and marked inflammatory response in lungs. On 3 dpi,
pathology was typically characterized with the infiltration of neutrophils, mononuclear cells and
presence of multiple focalized lesions - intense in periphery and hemorrhage. Pulmonary
exudates were predominantly present in bronchial lumen while disappearance of nucleus from
bronchial epithelium suggesting bronchial necrosis was also observed (Fig2a-d). On 6 dpi, the
lesions were similar but with pre-dominant presence of lymphoid structures and involvement of
the larger portions of the lungs Typical lobular pneumonia that almost destroyed the lung
architecture combined with heavy infiltration of mononuclear cells in bronchial lumen, peri-
bronchial spaces and around blood vessels was observed. The sign of bronchial spasm was also
seen in heavily inflamed spaces. Emphysema was located at the lung periphery (Fig 2e-h).

**Effect of Peramivir on the outcome of H7N9 influenza virus disease**

We assessed the antiviral activity of peramivir in C57/bl6 mice challenged with different viral
concentrations representing high ($10^5$ and $10^4$) and low infective dose ($10^3$). Intramuscular
injection of 30mg/kg of peramivir was administered to mice once daily from the day of infection
to 8 dpi while normal saline (0.85% NaCl) was administered in the same manner to vehicle
group. Peramivir treatment saved all animals in $10^3$ group compared to 70% lethality in vehicle
(untreated) animals ($P < 0.0005$). Furthermore, these animals did not exhibit any clinical sign
and weight loss during the course of infection (fig 3a, b). Peramivir treatment also prevented
death in 80% and 20% animals challenged with $10^4$ and $10^5$ H7N9 virus. In $10^4$ group, weight
loss was observed only from 7 to 9 dpi, with maximal dip on 8 dpi which was significantly
different from vehicle group ($P < 0.001$). Milder clinical signs such as lethargy, dyspnea and
grouping were also observed during this period. Peramivir treatment significantly lowered the
risk of death ($P < 0.0001$) and the animals were able to recover toward the end of disease course
(Fig 3c-d). Although, only 20% survival benefit was noticed in heavily infected ($10^5$) animals
after peramivir treatment, these animals exhibited significant differences (P < 0.001) in weight loss from 4 to 7 dpi extending the median survival from 4.5 to 8 dpi compared to vehicle (Fig 3 e, f).

In agreement with survival curve, peramivir treatment also led to the dramatic reduction in lung virus titers regardless of viral infection dose. Approximately > 4 log_{10} reduction was observed in 10^3 and 10^4 and >2log_{10} reduction in 10^5 on 3 dpi (P < 0.0001). Complete viral eradication was seen in 10^3 and >4log_{10} reduction in viral replication was seen in other groups on 6 dpi (P < 0.0001) (Fig 3g, h). Peramivir treatment also reduced viral load in extra pulmonary tissues (A1).

Even though decreased viral replication and a survival benefit was observed, peramivir treatment did not bring about significant improvement in lung pathology during the acute phase of disease such as 3 and 6 dpi. Diffused interstitial pneumonia combined with bronchial necrosis and infiltration of mononuclear cells, comparable to control, was observed in peramivir treated animals on 6 dpi(Fig 4a,d). Histological reading of surviving animals in peramivir group revealed that by 14 dpi, most of lung architecture was devoid of inflammatory cell infiltration and inflamed areas were rarely observed toward the periphery (Fig 4b). Virus infected cells in bronchial epithelium and alveolar spaces were rarely seen when lung sections of peramivir treated animals were stained with influenza nucleoprotein (NP) antibody (Fig 4c) whereas no infected cell was found in surviving animal on 14 dpi. In contrast, vehicle mice showed infection of multiple cell types including epithelial cells from bronchi, terminal bronchioles and alveolar lining- mainly type II pneumocytes were found infected (Fig 4e,f). Infiltrating cells in heavily inflamed areas exhibited NP positive staining indicating capability of H7N9 influenza virus infecting multiple cell types (Fig 4f). Our findings clearly suggest that viral replication is directly correlated with uncontrollable H7N9 influenza induced lung pathology that lead to irreparable...
physiological damage and compromised animal health. While peramivir treatment decreases viral replication during acute phase of infection that subsequently helps the animals to resolve pathological signs and regain body weight during the recovery phase.

**Dose dependent antiviral effect of peramivir on H7N9 influenza**

To determine the therapeutic concentration of peramivir, different doses of peramivir (30, 15 and 3 mg/kg/day) were administered to separate group of C57/Bl6 mice from 0 to 8 dpi. Animals were infected with $10^4$ EID$_{50}$ of A/Shantou/1001/2014 (H7N9) virus. We found that peramivir, at all doses tested improved animal survival rate ($P < 0.0001$). Lower doses such as 15 and 3 mg/kg helped 30 and 20% animals to survive respectively with a delay in median death from 7 to 9 dpi (Fig 5b). The Areas under curve (AUCs) of animal weights from 1 to 14 dpi showed comparable improvement in animals after treatment with different doses, however significant difference in weight loss was specifically observed on 5, 6 and 7 dpi ($P < 0.001$) (Fig 5a, c). Although all doses were able to reduce viral titers in same manner on 3 dpi ($P < 0.0001$), a dose dependent effect was found on 6 dpi ($P < 0.001$) that might account for the beneficial effects on disease outcome (Fig. 5d).

**Therapeutic effect of single versus multiple doses on H7N9 influenza virus infection**

We observed that peramivir treatment from 0 to 8 dpi efficiently inhibit viral replication in lungs and protect animals from lethal disease. Therefore we next evaluated 4 different treatment regimes in infected mice. 30mg/kg of peramivir was administered immediately or 24 hours after inoculation in mice. Peramivir treatment was given as either single dose or multiple doses till 8 dpi (A2). Single dose of peramivir at the time of infection (D0 single) provided significant
improvement weight loss leading to the protection in 50% animals (P < 0.0005). In addition, single dose regime substantially lowered lung viral titer if initiated at the time of infection (P < 0.005). We further observed that 24 hours delayed treatment either in single or multiple dose regimes significantly decreased therapeutic capacity of peramivir. Only 20% animals in delayed treatment groups were able to overcome lethal virus challenge. However viral titers were significantly lower in lung samples of delayed multiple dose regime (D1 multiple) than vehicle (P < 0.005) (Fig 6).

Resolution of H7N9 associated neurological symptoms and brain virus titers after Peramivir treatment

Avian influenza viruses including H5N1 and H7N9 are known for their neurovirulent characteristic in humans and animals (19, 33, 39). In this study, we observed that A/Shantou/1001/2014 (H7N9) virus infected animals exhibited neurological symptoms such as tremors, hind limb paralysis and hunched posture. Furthermore brain tissues of 10^5 and 10^4 EID_{50} infected animals showed high viral titers on 3 dpi. In mice treated with multiple doses of peramivir, complete inhibition of viral replication in brain was observed on 3 dpi irrespective of viral infective dose (P < 0.0001). 1.5 log_{10} reduction in viral load was also found in animals treated with single dose of peramivir at the time of infection (P < 0.05). Interestingly, peramivir treatment was also helpful to resolve H7N9 induced neurological symptoms in mice (Table 1).

Histological analysis revealed the presence of brain lesions in H7N9 infected mice on 3 and 6 dpi. Animals examined on 3 dpi had severe hemorrhage in frontal cortex and mid brain. Scattered foci of inflammatory cells infiltration were also observed in these regions. The sign of neural degeneration and liquefaction were seen in cerebral cortex on 3 dpi that lead to the karyopykonosis on 6 dpi. Furthermore, the inflammation of meninges was seen with infiltrating cells, neural edema in frontal cortex and...
increased size of arachnoid space. In peramivir treated animals, sign of hemorrhage was minimal compared to untreated group, however there were foci of infiltrating cells and neural degeneration (Fig. 7).

Discussion

Here we present a mouse model of H7N9 influenza virus infection that can be used to assess therapeutic potential of antiviral drugs. We found that, A/Shantou/1001/2014 (H7N9) virus which was isolated from a fatal human case during second wave, efficiently replicated in respiratory tract, induced interstitial pneumonia, inflammatory cell infiltration in lungs and caused lethal infection in mice even at the lowest challenge dose of $10^3$ EID$_{50}$. The virus was able to disseminate to extra-pulmonary tissues indicating the efficiency of mouse model to replicate the vital properties of H7N9 infection in humans. A/Shantou/1001/2014 (H7N9) virus belongs to major phylogenetic group of H7N9 viruses that were widely distributed across China during first and second waves of H7N9 flu epidemic(16). Previous studies showed inconsistency in the pathogenicity of ancestral H7N9 strains in mice. A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9) viruses were found to be lethal with MLD$_{50}$ of $10^{3.5}$ PFU(6, 46) but no lethal infection was seen in mice infected with higher doses of A/Shanghai/2/2013 (H7N9) virus by Mok et al (37). Signature amino acid mutations specifically those in HA and polymerase complex linked with adaptation to mammalian host and viral replication efficiency have been attributed to various pathogenicity profile of H7N9 influenza viruses. For instance, Q226L mutation in HA gene, associated with increased binding to mammalian receptors, is found in some H7N9 strains(7). In addition PB2 E627K mutation, which is known to aid avian
virus adaptation to mammalian hosts is also present in H7N9 strains isolated from humans(51). A/Shantou/1001/2014 (H7N9) virus contains both of these mutations (16) like its early ancestors that explain its pathogenic potential and high replication tendency in mammalian environment.

Continuous evolution of newly emerged H7N9 influenza virus has increased the risk of infecting population at larger scale. Severe human H7N9 cases are currently being treated with NAIs. Of them, only oseltamivir has been tested for anti-H7N9 influenza activity in animals (4, 36). This study for the first time demonstrates antiviral activity of peramivir against H7N9 virus in mice. Peramivir is known to be administered intravenously particularly to the patients who developed severe influenza infections. However clinical efficacy trials were performed on intravenous as well as intramuscular administration of peramivir and the results showed their superior efficacy over oral administration on patients with complicated and uncomplicated influenza infections(47). The drug has higher affinity to bind with neuraminidase enzyme (3) while it also achieves longer plasma elimination half-life, if administered by intravenous and intramuscular routes(24). However most of animal studies were performed on intravenous route of administration except a few showed successful antiviral therapies by intramuscular route(1, 2). Considering that intramuscular injections are easily achievable and less time consuming in small animals with no inferior efficacy, a rationale was made to use in this study.

Our studies showed that repeated administration of 30mg/kg peramivir starting from the day of infection till 8 dpi is capable of protecting animals against lethal H7N9 influenza infection. This protection may be the result of successful viral eradication from lungs by 3 and 6 dpi indicating direct antiviral effects of peramivir that reduce pathology toward the end of disease course. In addition, peramivir treatment greatly affected the bio-distribution of H7N9 virus in various extra-pulmonary tissues.
The H7N9 is known to cause severe form of disease in humans with increased mortalities during second and third waves (17). Such patients require aggressive treatment, mechanical ventilation and stayed longer in hospitals (43, 49). Moreover, clinical studies have showed the tendency of H7N9 infected cases to shed virus in respiratory tract for long period of time that subsequently impact on clinical outcome of disease (26). The situation indicates that severe cases need to get longer duration of antiviral treatment. In this context, we decided to assess longer treatment regime consisted of nine consecutive doses of peramivir starting either at the time of infection or a day after in animals. The 24-hour delay in drug administration showed readily apparent differences between these two regimes. As shown by observational studies performed with pandemic H1N1 human cases (25, 45), delayed treatment of peramivir in H7N9 infected animals remained sufficient enough to eradicate virus but it resulted into moderate effect on disease process. Our further experiment assessing the effect of single and multiple dose regimes also confirmed that the time for the initiation of treatment is critical to obtain survival benefit. Single dose of peramivir administered on the day the infection provided greater clinical benefits to these animals compared to multiple dose regime initiated with a delay of 24 hours.

Neurovirulence is one of the vital properties of some influenza viruses such as H1N1 and H5N1 and this is considered as contributing factor in several neurodegenerative diseases (28, 32). Previous studies have defined that flu infection in neural cells can induce encephalitis directly (15, 23, 44). Neurovirulence of H7N9 is matter of great concern. Clinical studies reported the possible involvement of brain in severe cases of H7N9 infection (18, 35). In addition, we have previously observed H7N9 viral infection in brain tissues in ferrets but none of these animals exhibited neurological signs (53). This study confirms that H7N9 influenza is neurovirulent in mice as the virus was found in brain tissues and these animals exhibited a variety of neurological...
symptoms and brain lesions. Interestingly, single and multiple peramivir administrations were capable to eradicate virus from brain preventing neurological signs to occur. The study clearly indicates that like H5N1 infection model, clinical benefits of peramivir are not limited to localized virus infection in respiratory tract (50).

Rapid bioavailability of intravenous or intramuscular peramivir aids in superior clinical efficacy of this drug over oral administrations. For H1N1 and other influenza viruses, the drug has been tested in controlled trials of prophylaxis and treatment. These studies provide strong evidence for the direct relationship with virus eradication and earlier relief in influenza like illness (ILI) (5, 29). Our study here provides the first evidence for antiviral activity of peramivir to H7N9 viruses. Antiviral treatment contributed to resolution of clinical signs, increased survival benefit as well as prevented the occurrence of neurological symptoms in mice. This suggests that rapid assessment of the clinical efficacy of this drug is urgently required for its possible use as an option of treatment of future severe H7N9 infected individuals.

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Figure Legends

**Figure 1:** Pathogenicity of A/Shantou/1001/2014 H7N9 influenza virus in C57/Bl6 mice. Animals (n = 10/group) were infected with different viral concentrations by intranasal route and (a) weight load (b) mortality was monitored up to 14 dpi. Survival curves were found to be significantly different in each viral concentration (P < 0.0005).

**Figure 2:** Pathological changes in lung tissues infected with A/Shantou/1001/2014 H7N9 influenza virus. C57/Bl6 mice were infected with $10^4$ EID$_{50}$ of H7N9 virus and lung sections were stained with haematoxylin and eosin (H&E) stain. Lung sections at 3 dpi showed (a) interstitial pneumonia with focalized lesions at the periphery (white arrows) at original magnification 40x, (b, c) hemorrhage (black arrow) and presence of pulmonary exudates and infiltration of neutrophils and mononuclear cells in bronchial lumen (white arrows) at 100x, 200x and (d) bronchial necrosis at 200x. Tissue inflammation was intense on 6 dpi with (e) the involvement of larger portions, presence of lymphoid structures (white arrows) and sign of emphysema (black arrow) at 40x (f) heavy infiltration of inflammatory cells specifically lymphocytes in peribronchial (white arrow) and perivascular areas (black arrows) at 40x, (g) typical lobular pneumonia resulting in diffused alveolar damage at 100x and (h) sign of bronchial spasm (white arrows) at 100x.

**Figure 3:** Peramivir mediated protection of mice over lethal H7N9 challenge. Animals were infected with indicated viral concentrations and 30mg/kg of peramivir was administered...
intramuscular once daily from the time of infection till 8 dpi. Significant changes in animal body
weight (a,c,e) and lethality (b,d,f) were observed after peramivir treatment throughout the course
of infection. MDCK cells were used to titrate viral loads present in lung tissues of peramivir or
vehicle (0.85% NaCl) treated animals on (g) 3 dpi and (h) 6 dpi. Results are expressed as log_{10} of
the mean TCID_{50} /ml ± SEM in each group of mice (n = 3). * P < 0.01, ** P <0.001, *** P <
0.0001

**Figure 4:** Temporal changes on H7N9 induced lung pathology following peramivir treatment.
Haematoxylin and eosin (H&E) staining of lung sections (a) peramivir (30mg/kg, D0-D8) treated
animals minimal resolution of lung pathology compared to (d) untreated infected animals on 6
dpi. (b) Surviving animals in peramivir treated group showed resolved lung pathology evidenced
by normal lung architecture in 90% areas and localized inflammation in certain places on 14 dpi.
Lung sections stained with influenza NP antibody showed (c) minimal sign of infection in
peramivir treated animals (100x magnification) while (e) infection of type II pneumocytes,
inflammatory cells and (f) bronchial epithelium was observed in untreated infected animals at
magnification of 400x.

**Figure 5:** Dose dependent effect of peramivir on lethal H7N9 influenza challenge in mice.
Animals were infected with 10^{4} EID_{50} of A/Shantou/1001/2014 H7N9 virus and administered
with 30, 15 and 3 mg/kg/day of peramivir intramuscular from 0 to 8 dpi. Peramivir provided
protection to lethal H7N9 infection in dose dependent manner. (a) Changes in animal weight loss
and (b) lethality was observed throughout the course of infection. (c) The Areas under curve
(AUCs) of animal weights from 1 to 14 dpi showed 2 fold improvement in peramivir treated
animals (n = 10/group). (d) Dose dependent reduction of viral loads in lung homogenates of peramivir treated animals was seen on 3 and 6 dpi. Results are expressed as log_{10} of the mean TCID_{50} /ml ± SEM in each group of mice (n = 3). *** P < 0.0001, ** P < 0.001

Figure 6: Comparison of single versus multiple doses of peramivir. Animals were infected with 10^4 EID_{50} of A/Shantou/1001/2014 H7N9 virus. Single treatment regimes consisted of 30mg/kg/day of peramivir administered intramuscular at the time of infection (D0 single) or on 1 dpi (D1 single). In multiple dose regimes, similar peramivir treatment was initiated either at the time of infection (D0 multiple) or on 1 dpi (D1 multiple) till 8 dpi. (a) Changes in animal weight loss and (b) lethality was observed throughout the course of infection. (c) Reduction of viral loads in lung homogenates of peramivir treated animals was seen on 3 and 6 dpi. Results are expressed as log_{10} of the mean TCID_{50} /ml ± SEM in each group of mice (n = 3). *** P < 0.0005, ** P < 0.005

Figure 7: Pathological changes in brain tissues after A/Shantou/1001/2014 (H7N9) infection in C57/Bl6 mice. Representative sections show (A) hemorrhage (black arrow) and (B) degenerative neurons, infiltrating cells and (C, D) liquefaction of neural cells in mid brain, (E) increased size of arachnoid space and (E,F) hemorrhage in cerebral cortex. (G,H) infiltration of inflammatory cells (black arrows) and neural edema (white arrow) in frontal cortex on 3 dpi. (I,J) Karyopyknosis (black arrow) in cerebrum was on 6 dpi. (K,L) show minimal sign of hemorrhage associated with neural degeneration in H7N9 infected animals after getting treatment with 30mg/kg of peramivir on 3 dpi. Left panel shows high magnification (400X) of boxes in right panel.
Table 1: Resolution of neurological signs in H7N9 infected animals after Peramivir treatment

<table>
<thead>
<tr>
<th>Infective dose (EID^50)</th>
<th>Treatment</th>
<th>No. of animals with neurological sign/Total no. of animals</th>
<th>Viral copy numbers/ml of brain homogenates on 3dpi (n = 3/group)</th>
<th>Neurological signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^4</td>
<td>30mg/kg Peramivir, Multiple doses (D0-D8)</td>
<td>0/10</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>10^4</td>
<td>30mg/kg Peramivir, Single dose</td>
<td>0/10</td>
<td>1.52E+03</td>
<td>-</td>
</tr>
<tr>
<td>10^4</td>
<td>Vehicle</td>
<td>1/10</td>
<td>3.55E+04</td>
<td>Hind limb paralysis</td>
</tr>
<tr>
<td>10^5</td>
<td>30mg/kg Peramivir, Multiple doses (D0-D8)</td>
<td>4/10</td>
<td>0</td>
<td>Tremors, hunched posture,</td>
</tr>
<tr>
<td>10^5</td>
<td>Vehicle</td>
<td>8/10</td>
<td>1.50E+03</td>
<td>Tremors, hunched posture, minimal activity</td>
</tr>
</tbody>
</table>
EID\textsuperscript{50}: The concentration of virus that can infect 50\% of inoculated eggs, 
viral copy numbers were achieved by performing qRT-PCR on RNA extracted from brain tissue homogenates 
Dpi: day post infection