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Efficacy of a single intravenous dose of the neuraminidase inhibitor peramivir in the treatment of equine influenza

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ABSTRACT

Equine influenza A virus (EIV) of the H3N8 subtype is an important pathogen causing acute respiratory disease in horses. Peramivir is a selective inhibitor of the influenza virus neuraminidase (NA). The characteristics of peramivir are not only its capacity for parenteral administration, but also its strong affinity for NA and slow off-rate from the NA–peramivir complex, suggesting that it could lead to a prolonged inhibitory effect and thus allow a lower dosing frequency. The aims of this study were to evaluate the inhibitory efficacy of peramivir against the NA activities of EIV in vitro and the treatment efficacy of a single intravenous dose of peramivir in horses experimentally infected with EIV.

Peramivir inhibited the activities of NA from the seven contemporary EIV strains in vitro, with 50% inhibitory concentrations ranging from 0.10 to 0.20 nmol/L. Horses treated with a single IV dose of peramivir (3000 mg/600 mL/animal, 7.8–9.3 mg/kg of bodyweight) showed significantly milder clinical signs (pyrexia, nasal discharge and cough) with a shorter duration than control horses injected with normal saline. Moreover, the mean duration of virus shedding for the horses treated with peramivir was significantly shorter than for the control horses. These findings suggested that a single IV administration of peramivir had good potential for the treatment of equine influenza, and may help to limit the spread of the disease in the horse population.

Introduction

Equine influenza (EI) is caused by equine influenza A virus (EIV) and is one of the most important respiratory diseases of horses because of its high contagiousness. The virus is a member of the family *Orthmyxoviridae* of the genus influenza virus A and species influenza A virus (Webster et al., 1992). Two subtypes of EIV have been recognised, the H7N7 subtype and the H3N8 subtype (Webster et al., 1992), although the H7N7 subtype has not been isolated since 1979 and may be extinct (Webster, 1993). In contrast, the H3N8 subtype appears prevalent in many countries worldwide (Daly et al., 2011; Elton and Bryant, 2011).

Horses infected with EIV develop the acute onset of pyrexia, associated with depression and anorexia, nasal discharge and coughing (Wilson, 1993; van Maanen and Cullinane, 2002). Occasionally, EI is complicated by secondary bacterial infections, which can lead to fatal pneumonia (Wilson, 1993; van Maanen and Cullinane, 2002). Immunoprophylaxis with inactivated whole/sub-unit vaccines is widely used for preventing EI as it is for human seasonal influenza. However, many outbreaks have been reported among vaccinated horses (Powell et al., 1995; van Maanen et al., 2003; Newton et al., 2006; Martella et al., 2007; Yamanaka et al., 2008a; Barbic et al., 2009; Gildea et al., 2011), primarily because the efficacy of the inactivated vaccines is reduced by the antigenic differences between the vaccine strains and field strains (Daly et al., 2004; Paillot et al., 2006).

Peramivir is a sialic acid analogue and, along with oseltamivir and zanamivir, is a potent and selective inhibitor of influenza neuraminidase (NA) (Young et al., 2001). The viral NA is responsible for cleaving sialic acid residues on newly formed virions and plays an essential role in the release and spread of progeny virions (Webster et al., 1992). Hence, the NA inhibitors limit virus replication by competing for the enzyme-binding site on NA. In the presence of the NA inhibitors, progeny virions stay attached to the membrane of infected cells and to each other, and viral spread to infect further susceptible host cells is inhibited (Gubareva et al., 2000).

It has been shown that peramivir is at least as effective as zanamivir or oseltamivir against all nine NA subtypes of avian influenza A virus (Govorkova et al., 2001). Moreover, the advantages of peramivir include the fact that it can be administered parenterally and that it binds more tightly to viral NA than oseltamivir or

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zanamivir (Bantia et al., 2006). The parenteral administration makes it possible rapidly to obtain high drug concentrations in blood, so increasing the likelihood of drug delivery to infection sites. Peramivir for IV injection (Rapiacta, Shionogi) has been licensed for human use in Japan since 2010 and appears to be effective for human patients showing the onset of influenza-like symptoms within the previous 48 h and a positive result for a rapid antigen detection test for influenza virus (Kohno et al., 2010).

We previously reported that the oral administration (2 mg/kg bodyweight [BW]) of oseltamivir is effective for horses experimentally infected with EIV (Yamanaka et al., 2006). However, oseltamivir must be administered frequently to both horses and humans (twice daily for 5 days). Zanamivir is a dry powder which must be delivered by active inhalation (Gubareva et al., 2000), which is often impractical for horses. It would therefore be useful if a single IV administration of peramivir was effective for treating EI in horses, as it is for humans. The aim of the current study was to assess the inhibitory effects of peramivir against the NA activity of contemporary EIV strains in vitro, and the treatment efficacy of a single IV dose of peramivir in horses experimentally infected with EIV.

Materials and methods

Viruses

A total of seven EIV strains (H3N8) were used in this study. The A/equine/Avesta/1993, A/equine/La Plata/1993 and A/equine/Ibaraki/1/2007 strains have been contained in inactivated vaccines in Japan since 2009 (Yamanaka et al., 2011). A/equine/South Africa/4/2003 and A/equine/Richmond/1/2007 (Richmond07) are recommended as vaccines by the World Organization for Animal Health as representative strains currently circulating among horses in the world (OIE, 2011). At the Animal Quarantine Service of Japan (Motoshima et al., 2011), A/equine/Yokohama/aq19/2009 and A/equine/Yokohama/aq13/2010 were isolated from horses imported from Canada in 2009 and Belgium in 2010, respectively, each stock virus was propagated in 10-day old embryonated hen's eggs. The stock virus was subjected to low-speed centrifugation (1500 *g* for 10 min) to remove cell debris and then was aliquoted and stored at −80 °C until use.

NA inhibition assay

To determine the 50% inhibitory concentrations[1] for peramivir (purchased from Shionogi) against the NA activities of the seven EIV strains, NA inhibition assays were performed with the substrate (2′-(4-methylumbelliferyl)-α-D-acetylneuraminic acid, MUNANA) (Sigma–Aldrich), as previously described (Potier et al., 1979). The activity of each virus strain was first titrated by twofold serial dilutions and optimal virus dilutions for subsequent inhibition assays (i.e. those which fall in the linear portion of the enzyme activity curve) were determined graphically. For the inhibition assays, equal volumes (10 μL) of each dilution of peramivir and virus were mixed and incubated for 30 min at 37 °C while being shaken in a black 96 well flat bottom plate (Corning). The final inhibitor concentrations in the assay ranged from 0.015 to 4000 nmol/L in fourfold serial dilution. The reaction was initiated by adding 30 μL of buffer [32.5 mmol/L MES (pH 6.5), 4 mmol/L $CaCl_2$] containing 100 μmol/L MUNANA to each well. The plate was then incubated for 60 min at 37 °C. The reaction was stopped by adding 150 μL of 0.1 mol/L glycine in 25% v/v ethanol (pH 10.5). Fluorometric readings were obtained immediately with a multi-microplate reader (SH-9000, Corona electric). The excitation wavelength was 355 nm, and the emission wavelength was 460 nm. Duplicate measurements were performed in each assay. The IC_{50} was calculated by plotting the percent inhibition of NA activity versus the peramivir concentration and expressing it as a mean ± standard deviation (SD) for three independent experiments.

Evaluation of drug efficacy in horses

Six healthy 1-year old Thoroughbred horses were randomly divided into two groups. The control group received normal saline (Horses 1, 2 and 3; BW 337–366 kg) and the treated group received peramivir (Horses 4, 5 and 6; BW 323–384 kg). None of the horses showed any serological evidence of prior H3N8 virus infection or vaccination (haemagglutination inhibition titre <1:10 for antibodies to Richmond07). All the experimental procedures in this study were approved by the Animal Care Committee of the Equine Research Institute of the Japan Racing Association.

The inoculations were performed as previously described (Yamanaka et al., 2009, 2010). The horses were inoculated via the inhalation of Richmond07 ($10^{8.3}$ 50% egg infectious dose[2]/animal) with an ultrasonic nebulizer (SONICLIZ-ER305, ATOM) for 20 min on Day 0. The experiment continued for 14 days after the inoculation. Rectal temperatures (RTs) were measured and nasal swabs were taken daily. Nasal swab extracts were titrated in 10-day old embryonated hens' eggs as previously described (Yamanaka et al., 2009, 2010).

Each horse was also physically examined with a focus on nasal discharge and cough, with findings scored for nasal discharge (− Nil, + serous discharge, ++ mild mucopurulent discharge and +++ severe mucopurulent discharge) and cough (− Nil, + 2–5 times per 10 min and ++ more than six times per 10 min). Treatment was commenced once pyrexia (⩾38.9 °C) and a positive result for the rapid antigen detection test (ESPLINE influenza A and B, Fujirebio) for influenza A virus (Yamanaka et al., 2008b) occurred. The treatments consisted of normal saline (0.9% NaCl; 600 mL IV) or peramivir (3000 mg/600 mL/animal, 7.8–9.3 mg/kg BW) to the control or the peramivir groups, respectively, infused over 8–10 min via a catheter (14 G, 13 cm) placed in the left jugular vein.

Blood was collected from each horse daily to monitor the inflammatory response via the serum amyloid A (SAA) concentration. The serum was immediately separated from the blood and stored at −20 °C until use. SAA was measured using a latex agglutination method (Hobo et al., 2007) and an automated analyser (Hitachi 7020, Hitachi) using the LZ test 'EIKEN' SAA (Eiken Chemical).

Quantification of peramivir in horse plasma

Blood samples were collected into heparinised tubes from each horse of the peramivir group via a catheter placed in the right jugular vein at 0.33, 0.67, 1, 2, 4, 6, 8, 12, 24 and 36 h after treatment. Plasma was immediately separated by centrifugation and stored at −20 °C until analysed for peramivir concentration using liquid chromatography/tandem mass spectrometry (LC–MS/MS; Wiltshire et al., 2000). Peramivir was extracted from plasma with a solid phase extraction disc cartridge (Empore Mixed Phase Cation – MPC, 4 mm/1 mL) and oseltamivir carboxylate was used as the internal standard. The dry extract was reconstituted in deionised water and transferred directly to an autosampler vial for LC–MS/MS analysis. The LC–MS/MS analyses were undertaken using an API4000QTrap (AB SCIEX) triple quadrupole/linear ion trap instrument equipped with a turbo ion spray in the positive ion mode, and connected to an LC-20 system (Shimazu). The lower limit of quantification for peramivir in plasma was 5.0 ng/mL.

Statistical analysis

The mean RT and SAA concentrations were analysed with a repeated-measures analysis of variance and post hoc Bonferroni *t* tests between the groups on each day. The mean durations (days) of nasal discharge, cough and virus shedding between the groups after the administration of normal saline or peramivir were compared using an unpaired Student's *t* test. All statistical analyses were performed with SigmaPlot 11.2 (Systat Software) with a level $P < 0.05$ considered significant.

Results

In vitro NA inhibition

The IC_{50}s of the viruses tested for peramivir are presented in Table 1. The IC_{50}s for peramivir ranged from 0.10 to 0.20 nmol/L (0.03–0.07 ng/mL).

Anti-EIV efficacy in horses

The mean RTs of both groups are shown in Fig. 1. Normal saline or peramivir was administered to all the experimental horses on Day 2, following the onset of pyrexia (39.5–40.5 °C) and positive results in the rapid antigen detection test. The mean RTs of the normal saline group exhibited a bi-phasic pattern that is often seen with EI, whereas the mean RTs of the peramivir group did not show a clear second phase with an elevated RT. On Day 6, the mean RT of the peramivir group was significantly lower ($P < 0.05$) than that of the normal saline group.

Daily scores for nasal discharge and cough are shown in Table 2. All the horses of both groups began showing nasal discharge on Days 1 or 2. The horses in the normal saline group had continued nasal discharge until Day 13 or the end of the experiment (Day

[1] IC_{50}.

[2] EID_{50}.