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Short communication

Evaluation of intravenous zanamivir against experimental influenza A (H5N1) virus infection in cynomolgus macaques

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ABSTRACT

We investigated the prophylactic and therapeutic efficacy of an intravenous (IV) formulation of zanamivir in a macaque infection model for highly pathogenic influenza A (H5N1) virus. Antiviral efficacy was dose-dependent, with no reduction in viral load observed at 2 mg/kg, but a significant reduction observed at 10 mg/kg ($p = 0.039$) and at 20 mg/kg in the combined prophylactic and therapeutic groups ($p = 0.049$) with both prophylaxis (commencing 12 h before infection) and therapy (commencing 4 h after infection) showing similar reductions in viral load. Combined gross pathology and microscopic pneumonia scores in the treated animals relative to untreated controls were significantly reduced at 10 mg/kg ($p = 0.02$) and at 20 mg/kg in the prophylaxis group ($p = 0.02$), but were not significant in the treatment group ($p = 0.145$). In this new animal model for evaluation of influenza antivirals, despite variability observed between individual animals, IV zanamivir showed evidence of efficacy against highly pathogenic H5N1 virus.

Highly pathogenic avian influenza A (H5N1) viruses, which are endemic in certain bird populations, cause severe sporadic infections in humans with high mortality rates (World Health Organization). Treatment of human H5N1 with oseltamivir, a neuraminidase inhibitor (NI), has shown variable efficacy in a clinical setting and there have been two cases of resistance development reported with the acquisition of a H274Y mutation in the neuraminidase (de Jong et al., 2005). A parenterally administered anti-influenza drug may have advantages in the treatment of severe life-threatening influenza disease, but none is currently available.

Relenza (zanamivir for inhalation) is approved for treatment and prophylaxis of influenza A and B infections. In mice, intranasally applied zanamivir has shown efficacy against lethal H5N1 infections, preventing spread of virus to the brain (Gubareva et al., 1998). In addition, an intravenous (IV) formulation of zanamivir has been shown to prevent infection in volunteers experimentally infected with influenza A/Texas/36/91 (H1N1) virus (Calfee et al., 1999). IV zanamivir may be useful in treating life-threatening influenza, particularly since zanamivir is the only currently licensed NI available that is active against the H274Y oseltamivir resistant variant (Mishin et al., 2005).

Evaluation of this IV formulation against H5N1 infections is therefore warranted, but cannot be undertaken experimentally in humans due to the pathogenicity of this virus, necessitating the use of a suitable animal model. As a preliminary to this H5N1 study, we evaluated the half-life of zanamivir in different animal species, by either the IV or intraperitoneal (IP) routes. Small animal models were not appropriate due to the short zanamivir half-life observed in the mouse (IP ~ 11 min), rat (IV 16 min) and ferret (~40 min IV or IP). In contrast, the half-life of IV zanamivir in macaques was 2.7 h, which is comparable to that in humans (Churchill et al., 2007) and H5N1 infection in this species manifests itself primarily as influenza pneumonia making it a good model for these purposes (Rimmelzwaan et al., 2001). In addition, evaluation of zanamivir epithelial lining fluid levels in the respiratory tract of macaques, using bronchoalveolar lavage, showed levels to be similar to those seen in plasma (Churchill et al., 2007). The macaque model was therefore chosen to evaluate the efficacy of zanamivir given IV either prophylactically or therapeutically.

Captive-bred, influenza virus seronegative, sub-adult healthy male cynomolgus macaques (*Macaca fascicularis*) were housed in pairs in negatively pressurized glove boxes. Groups of six control or treated animals were infected intratracheally using 2.5×10^4 $CCID_{50}$ of A/Hong Kong/156/97 (H5N1) virus in 5 ml PBS.

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Animals were anaesthetised with ketamin (10 mg/kg) and domitor (0.1 mg/kg) for all procedures. On day 5 post-challenge, in order to allow consistent macroscopic inspection of the lungs and for ethical reasons animals were euthanized by intramuscular injection of anaesthetic (5 mg/kg ketamine and subsequent exsanguination) and necropsied. A complete macroscopic post-mortem examination was performed and all abnormalities were recorded. All lung lobes were inspected and lesions described.

The first experiment assessed the efficacy of prophylactically administered IV zanamivir at 2 or 10 mg/kg. Prophylaxis was initiated 12 h before infection, the second dose was given at the time of infection, and subsequent doses were given twice daily for 4 days. Doses of 1.25 ml/kg zanamivir solution (2.0 or 10 mg/ml in 0.8% sodium chloride) or saline (placebo) were administered under anaesthetic as a bolus by intravenous injection in a hind leg to achieve dosages of either 2 or 10 mg/kg of zanamivir, respectively. Samples of whole blood were collected from the femoral vein at 0 h (baseline) (1 sample) and then at 3 h (2 replicates) and 12 h (C_{trough}) (3 replicates) after the 4th and 8th dose of zanamivir because sampling was limited to 6 samples per animal. Zanamivir concentrations were determined in plasma by high pressure liquid chromatography, as described previously (Churchill et al., 2007).

During the course of the experiment, body temperatures and weights were measured as clinical signs, but no clear changes were observed in control or treated animals (data not shown). Both control and treated macaques shed virus at relatively low titers from the pharynx (data not shown) following virus challenge.

Animals were euthanized at 5 days post-infection (dpi) and necropsied. The viral load in the lungs of three of six animals of the 10 mg/kg IV zanamivir-treated group, were substantially lower (mean $\log_{10}$ lung virus titers of 4.4) compared to the lung virus titers of animals from the placebo control group or the 2 mg/kg IV zanamivir-treated group (mean $\log_{10}$ lung virus titers of 6.4 and 6.1, respectively), although there was considerable variation within each group (see Table 1). Viral loads were similar in the 2 mg/kg and placebo groups. Statistical analysis using Wilcoxon rank-sum two-sided tests were significant when the 10 mg/kg zanamivir dose was compared with the 2 mg/kg and placebo groups combined

Table 1
Cynomolgus macaque model for IV zanamivir prophylaxis against influenza A/Hong Kong/156/97 (H5N1) virus infection

Zanamivir[a]	Primate ID	Lung virus titers ($\log_{10}$ $CCID_{50}$/g tissue) [$\log_{10}$ reduction]	Gross lung pathology findings	Histology pneumonia	Pathology score[b]	Zanamivir concentration (ng/ml) after dosing	
						At 3 h	At 12 h
10 mg/kg prophylaxis	1	5.7[−0.7]	None (0)		0	2161,3279	37,69,43
	2	1.9[−4.5]	Red spots (1)		1	1581,1604	12,29,23
	3	1.9[−4.5]	None		0	574,3472	26,35,23
	4	7.0[+0.6]	Several red areas (1)	Mild (2)	3	1532,2498	25,37,46
	5	4.4[−2.0]	None		0	2216,2110	29,43,NR
	6	5.6[−0.6]	None		0	2615,6801	120,70,65
	Mean 1–6	4.4 ± 2.1[−2.0]		Total score	4		
	*p-Values	0.093 **0.039			0.02 *0.01		
2 mg/kg prophylaxis	7	6.1[−0.3]	Diffuse red areas (2)		2	255,388	NQ,NQ,NQ
	8	6.2[−0.2]	Multiple red spots/areas (2)		2	381,659	NQ,NQ,NQ
	9	6.3[−0.1]	Multiple red areas (2)	Minimal (1)	3	305,669	NQ,NQ,15
	10	5.2[−1.2]	One red spot (0)	Minimal (1)	1	500,462	NQ,18,NQ
	11	7.2[+0.8]	Multiple red areas (2)	Mild (2)	4	606,820	13,12
	12	5.8[−0.6]	Multiple red spots/areas (2)	Mild (2)	4	311,NR	NQ,NQ
	Mean 7–12	6.1 ± 0.7[−0.3]		Total score	16		
	p-Values	0.615			0.989		
Placebo	13	7.5	Multiple red spots/areas (2)	Moderate (3)	5	–	–
	14	6.5	Multiple red spots/areas (2)	Moderate (3)	5	–	–
	15	5.3	One red area (1)		1	–	–
	16	6.1	Multiple red spots/areas (2)	Mild (2)	4	–	–
	17	6.9	Multiple red spots/areas (2)		2	–	–
	18	6.0	Multiple red spots/areas (2)		2	–	–
	Mean 13–18	6.4 ± 0.1		Total score	19		

H5N1 virus titers determined by virus isolation in MDCK cells. On day 5 cranioventral, cardiodorsal, caudoventral and cardodorsal sections of the right lung from each animal were weighed and stored at −80 °C. Lung sections were pooled and homogenized with a Polytron homogenizer in 3 ml transport medium. Quadruplicate 10-fold serial dilutions of these samples were cultured on MDCK cells (Rimmelzwaan et al., 2001). Log_{10} reductions in virus titer were determined compared with the mean titer of the placebo group. A macroscopic post-mortem examination was performed on all animals which included all lung lobes (left cranial lobe with cranial and caudal segments, left caudal lobe, right cranial, middle and caudal lobes and accessory lobe) and lesions described. The left lungs were collected during autopsy, inflated with 10% neutral-buffered formalin and stored for fixation/histology and microscopic examination. Sections from the cranial segment of the cranial lobe (sectioned along length of main airway), the caudal segment of the cranial lobe (distal part transverse to main airway) and the caudal lobe (sectioned along length of main airway, including cranial edge and center of the caudal lobe with the hilar area of the lobe) were examined in detail. NQ, not quantified. NR, not recorded. Zanamivir concentrations were determined by high performance liquid chromatography. Whole blood samples were taken from the femoral vein at 0 h (baseline) (1) and then at 3 h (2) and 12 h (C_{trough}) (3) after the 4th and 8th dose of zanamivir and limited to 6 samples per animal. *Statistical analysis was performed using Wilcoxon rank-sum two sided tests. **Compared 10 mg/kg group to combined 2 mg/kg and placebo groups.

[a] IV zanamivir prophylaxis was commenced 12 h before infection, with dosages at 10 and 2 mg/kg.

[b] Cumulative gross pathology scoring of lungs and histopathological scoring of lung sections. The arbitrarily chosen values for the different characteristics are indicated between brackets in the column for gross lung pathology findings (column 4, as none/questionable = 0, mild = 1, moderate = 2) and the column for histology pneumonia (column 5, as none = 0, minimal = 1, mild = 2, moderate = 3, marked = 4, very marked = 5).

($p = 0.0394$) but not against just the placebo group ($p = 0.0931$) probably due to the small group size. Furthermore, macroscopic examination of the lungs of animals treated with 10 mg/kg IV zanamivir, revealed a lower incidence of lesions (red areas/red spots) than in animals from groups treated with either 2 mg/kg IV zanamivir or placebo (see Table 1). Pneumonia was evident in only one of six animals in the 10 mg/kg group, compared with four of six in the 2 mg/kg group and three of six in the placebo group. Pathology scores combining the macroscopic and microscopic analysis showed a significant reduction in pathology between the 10 mg/kg and placebo group ($p = 0.0195$) and between the 10 mg/kg group and the combined 2 mg/kg and placebo groups ($p = 0.0095$). Zanamivir plasma levels, determined in duplicate or triplicate depending on the volumes of blood obtained, at 3 h after dosing with 10 mg/kg were >800 times and after 12 h (C_{trough}) were >40 times higher than the IC_{50} (3 ng/ml) for A/Hong Kong 156/97 (H5N1). In contrast, for the 2 mg/kg dose by 12 h zanamivir levels were undetectable, consistent with the lack of efficacy.

The second experiment was designed to test whether prophylactic efficacy could be increased with a higher dose of zanamivir (20 mg/kg), and to test efficacy of IV zanamivir when administered therapeutically, 4 h post-challenge.

The prophylactic dosing schedule was as for the first experiment, while therapeutic dosing was initiated 4 h after infection, with follow-up treatments at 12 h after infection and twice daily for 4 days until euthanasia on 5 dpi.

On necropsy at 5 dpi, 50% of animals from both prophylactic and therapeutic groups had reduced viral load in the lungs relative to control groups (see Table 2). However, the differences in mean virus titers relative to the placebo for each individual treatment group were not significant ($p = 0.1$), but achieved significance if the two treatment groups were combined versus placebo ($p = 0.0486$). Consistent with this, only two of six and one of six animals in the prophylactic and therapeutic groups, respectively, showed some evidence of lung lesions, whereas the lungs of all animals from the control group had such lesions. Furthermore, adhesive fibrinous pleuritis was observed in three of six of the control animals (two of three of the control prophylaxis group and one of three in the control therapeutic group), but not in any of the treated animals (see Table 2). Pneumonia when present was generally less severe, mild to moderate in the treated groups, particularly with prophylaxis, where a significant reduction in pathology score was observed ($p = 0.0238$), compared with moderate to very marked in the placebo group.

Immunohistochemistry on lung tissue was undertaken in attempts to obtain more quantitative viral data to compare the histopathology analyses and virus titrations, and showed a two- to threefold mean reduction of H5N1 immunolabelled cells in the prophylactic and therapeutic groups, relative to control animals, with considerable variation within each group, but the reductions observed in the treated groups were not significantly different ($p = 0.1$) (see Table 2). In this study, there was considerable variability of viral load and gross pathology within groups of macaques, with an uneven scattering of foci of infection in the lungs. Variability in efficacy of IV zanamivir in this model may be linked to the variable infectivity of H5N1 virus

Table 2
Cynomolgus macaque model for IV zanamivir prophylaxis and therapy against influenza A/Hong Kong/156/97 (H5N1) virus infection

Zanamivir[a]	Primate ID	Lung virus titers (log_{10} $CCID_{50}$/g tissue) [log_{10} reductions]	Gross lung pathology findings	Histology pneumonia (other infection related changes[b])	Pathology score[c]	Immuno-histopathology infected cell counts*
Prophylaxis 20 mg/kg	19	2.7[−2.5]	None (0)	Not detected (0) (present)	0	203
	20	1.8[−3.4]	None	Not detected (present)	0	1
	21	5.8[+0.6]	None	Minimal (1)	1	88
	22	4.4[−0.8]	None	Not detected (present)	0	81
	23	3.7[−1.5]	Light discoloration (1)	Moderate (3)	4	69
	24	4.5[−0.7]	Red spot cranial lobe (1)	Moderate (3)	4	4
	Mean 19–24	3.8 ± 1.4[1.4]		Total score	9	Total = 446
	p-Values	0.104		*p*-Values	0.024	0.937
Treatment 20 mg/kg	25	1.1[−4.1]	None	Moderate (3)	3	46
	26	1.5[−3.7]	None	Mild (2)	2	31
	27	5.8[+0.6]	None	Moderate (3)	3	47
	28	5.7[+0.5]	Few red spots (1)	Mild (2)	3	1
	29	4.3[−0.9]	None	Moderate (3)	3	37
	30	3.3[−1.9]	None	Marked (4)	4	429
	Mean 25–30	3.6 ± 2.0[−1.6]		Total score	18	Total = 591
	p-Value	0.101		*p*-Value	0.145	0.589
Placebo	31	6.4	Pleuritis, multiple red spots (2)	Very marked (5)	7	1099
	32	6.3	Pleuritis, red spot (2) discoloration	Not detected (none)	2	0
	33	4.5	Multiple red spots (2)	Moderate (3)	5	48
	34	6.0	Multiple red spots (2)	Moderate (3)	5	83
	35	2.5	Two red spots (1)	Not detected (none)	1	5
	36	5.7	Pleuritis, red spot (2) discoloration	Marked (4)	7	101
	Mean 31–36	5.2 ± 1.5		Total score	27	Total = 1336

Methods as described in Table 1. *For quantitative immunohistology four sections of the left lung, cranial lobe, cardiac lobe, cranial edge and centre of caudal lobe, were prepared for each animal for quantitative immunohistological assessment of H5N1 positive cells, as previously described (van Riel et al., 2006). Twenty slides per animal, prepared in a standardized fashion, were examined and the total number of H5N1-immunolabelled cells per group are presented.

[a] IV zanamivir prophylaxis was commenced 12 h before infection, and treatment was commenced 4 h after infection, with a dosage at 20 mg/kg.

[b] Other infection related changes—referred to evidence of chronic lesions present possibly from a previous infection.

[c] Cumulative gross pathology scoring of lungs and histopathological scoring of lung sections. The arbitrarily chosen values for the different characteristics are indicated between brackets in the column for gross lung pathology findings (column 4, as none/questionable = 0, mild = 1, moderate = 2) and the column for histology pneumonia (column 5, as none = 0, minimal = 1, mild = 2, moderate = 3, marked = 4, very marked = 5).

and may also reflect genetic variation within the macaque population possibly relating to innate immune responses to highly pathogenic influenza infections as described by Kobasa et al. (2007). Furthermore, this variability may be consistent with that observed with H5N1 infections in man (de Jong et al., 2005).

We have demonstrated that the IV formulation of zanamivir has dose-dependent antiviral efficacy in the H5N1 macaque model. Prophylactically (10 and 20 mg/kg) treated macaques exhibited reduced gross pathology and pneumonia, and substantially lower viral titers in 50% of animals, relative to placebo-treated animals. In the therapeutic group (20 mg/kg) zanamivir showed similar efficacy in reducing viral load in 50% of animals but reductions in gross pathology and pneumonia were less marked.

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