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Short communication

Susceptibility of highly pathogenic A(H5N1) avian influenza viruses to the neuraminidase inhibitors and adamantanes

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Abstract

Since 2003, highly pathogenic A(H5N1) influenza viruses have been the cause of large-scale death in poultry and the subsequent infection and death of over 140 humans. A group of 55 influenza A(H5N1) viruses isolated from various regions of South East Asia between 2004 and 2006 were tested for their susceptibility to the anti-influenza drugs the neuraminidase inhibitors and adamantanes. The majority of strains were found to be fully sensitive to the neuraminidase inhibitors oseltamivir carboxylate, zanamivir and peramivir; however two strains demonstrated increased IC_{50} values. Sequence analysis of these strains revealed mutations in the normally highly conserved residues 116 and 117 of the N1 neuraminidase. Sequence analysis of the M2 gene showed that all of the A(H5N1) viruses from Vietnam, Malaysia and Cambodia contained mutations (L26I and S31N) associated with resistance to the adamantane drugs (rimantadine and amantadine), while strains from Indonesia were found to be a mix of both adamantane resistant (S31N) and sensitive viruses. None of the A(H5N1) viruses from Myanmar contained mutations known to confer adamantane resistance. These results support the use of neuraminidase inhibitors as the most appropriate class of antiviral drug to prevent or treat human A(H5N1) virus infections.

Keywords: Influenza; A(H5N1); NA inhibitors; Adamantanes; Drug resistance

Outbreaks of highly pathogenic avian influenza (HPAI) of various subtypes have occurred frequently in poultry over the past decade (Munster et al., 2005). Since 2003, the HPAI subtype A(H5N1) has had a devastating impact on domestic or wildbirds in many parts of South East Asia, Europe, the Middle East and parts of Africa. The virus has also been transmitted to humans, infecting more than 230 people of whom over 50% have died as a result (WHO, 2006). While the virus is not easily transmitted between humans, the concern is that further mutation or genetic reassortment may occur producing a strain which has the potential to cause a pandemic. As a result of this concern, influenza A(H5N1) vaccines are now being developed (Stephenson et al., 2006). However, many of the prototypes to date have demonstrated poor immunogenicity, requiring large doses of vaccine or the addition of adjuvants (Bresson et al., 2006; Wood, 2001). Furthermore, if a pandemic does eventuate the vaccine strain may need to be updated to ensure a good match is achieved between the emerging strain(s) and those in the vaccine. As such, an effective influenza A(H5N1) vaccine may take months before it is available for use in a pandemic situation or may only be available in limited amounts. During this interval, influenza antiviral drugs will be needed for containing early outbreaks, for the treatment of infected individuals and the prophylaxis of essential workers.

Currently there are two classes of anti-influenza drugs available for the treatment of influenza A viruses, the adamantanes and the neuraminidase (NA) inhibitors. The adamantane derivatives, amantadine and rimantadine, which are widely available and relatively inexpensive, act by blocking the M2 ion channel of the virus to inhibit the early stages of viral replication. These drugs however, have been shown to rapidly generate viral resistance in treated patients (Hayden, 2006) and are mainly prescribed for the prophylaxis of influenza. Recent studies have shown marked increases in the occurrence of adamantine

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resistant influenza viruses commonly circulating in the community, with the proportion of resistant A(H3N2) strains in the USA now being as high as 90% (Bright et al., 2006). The second and most recently developed class of influenza antiviral drugs are the NA inhibitors, which bind to the NA surface glycoprotein of newly formed virus particles and prevent their efficient release from the host cell (Gubareva, 2004). Currently two NA inhibitors, zanamivir (Relenza™) and oseltamivir (Tamiflu™) are licensed, and a third, peramivir, is being developed by BioCryst (Birmingham, USA) as an injectable formulation. Studies so far have shown that viral resistance to the NA inhibitors is considerably less common than to the adamantanes (Hayden, 2006). However, clonal analysis of viruses from oseltamivir-treated patients in Japan demonstrated evidence of resistance in up to 18% of specimens taken from young children (Kiso et al., 2004). In addition, NA inhibitor resistant viruses have also been occasionally isolated from untreated patients (Hurt et al., 2006).

As a countermeasure against a pandemic eventuating from the avian A(H5N1) outbreaks large quantities of NA inhibitors have been stockpiled for use by a number of countries (Lokuge et al., 2006; Tam et al., 2005). It is therefore essential that the level of viral sensitivity to these drugs is closely monitored to ensure that they remain effective inhibitors against these potentially pandemic strains. Here we report on both NA inhibitor and adamantane susceptibility of over 50 A(H5N1) avian influenza viruses submitted to the CSIRO Australian Animal Health Laboratories, Australia, from Vietnam, Cambodia, Malaysia, Indonesia and Myanmar between 2004 and 2006.

Under enhanced BSL3 level biosecurity containment, viruses were inoculated into the allantoic cavity of embryonated hens eggs, which were incubated at 35 °C for a maximum of 2 days, or until at least half of the embryos had died. Allantoic fluid was harvested from the eggs and the presence of virus confirmed by haemagglutination of chicken erythrocytes. To assess virus susceptibility to the adamantane anti-influenza drugs, genetic analysis of the M2 gene was conducted. RNA extraction was performed using the RNEasy kit (QIAGEN) and RT-PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Australia) according to the manufacturer's protocol. Sequencing was performed using a Big Dye III kit (Perkin-Elmer) and an ABI 310 genetic analyser (IMVS, Adelaide). Nucleotide sequences were analysed using DNASTAR V.5 (Lasergene, USA).

Analysis of the M2 genes from the A(H5N1) viruses revealed that the majority (85%) contained mutations at residues 26 and 31 (Table 1), both of which have previously been shown to play a role in adamantane resistance (Hay et al., 1985). While the Ser to Asn mutation at residue 31 is the most common mutation in human viruses resistant to the adamantanes (Bright et al., 2006), the Leu to Ile mutation at residue 26 has not been commonly seen. All of the influenza A(H5N1) viruses obtained from South and North Vietnam, Cambodia and Malaysia contained the dual L26I/S31N mutations that confer adamantane resistance. Of the six influenza A(H5N1) viruses obtained from Indonesia, four were found to have no adamantane resistance mutations, while two were found to contain the S31N resistance mutation but lacked the mutation at residue 26 (Table 1). Interestingly, no M2 mutations were found in influenza A(H5N1) viruses isolated in Myanmar, suggesting that these viruses are sensitive to the adamantanes. While levels of adamantane resistance in influenza A(H5N1) viruses isolated from Vietnam, Cambodia and Malaysia correspond well to the results described by Cheung et al. (2006), differences were observed in levels of resistance in viruses isolated from Indonesia. Thirty three percent (2/6) of the Indonesian viruses tested in this study (all isolated during 2005) were found to be adamantane resistant, whereas only 6% (2/32) of the Indonesian influenza A(H5N1) strains described previously were resistant (Cheung et al., 2006). As the isolation dates of the Indonesian viruses tested in that study were not reported, it is not clear whether the numbers of adamantane resistant influenza A(H5N1) viruses are changing over time, or if the differences in percent resistant can be explained by a sampling bias.

Table 1
Summary of adamantane resistance related M2 mutations in A(H5N1) from different regions

Region and year of virus isolation	Total (n =)	No M2 mutations	S31N	L26I/S31N
South Vietnam (2004–2005)	28	0	0	28
North Vietnam (2005)	11	0	0	11
Indonesia (2005)	6	4	2	0
Cambodia (2004)	6	0	0	6
Myanmar (2006)	2	2	0	0
Malaysia (2004)	2	0	0	2
Total	55	6 (11%)	2 (4%)	47 (85%)

The susceptibility of influenza A(H5N1) viruses to the NA inhibitor drugs oseltamivir carboxylate (the active form of oseltamivir), zanamivir and peramivir, was tested using a fluorescence-based NA enzyme inhibition assay (Hurt et al., 2004). The majority of the viruses were found to be fully sensitive to the three NA inhibitors, with IC_{50} values similar to those of commonly circulating human A(H1N1) viruses (Table 2). However, two influenza A(H5N1) strains demonstrated significantly increased IC_{50} values to at least one of the NA inhibitors (Fig. 1). Influenza A/Chicken/Indonesia/Wates/77/2005, had an oseltamivir carboxylate IC_{50} value that was 16-fold higher than the mean IC_{50} of the other viruses tested, but did not demonstrate a significant difference in zanamivir or peramivir IC_{50} compared to the mean IC_{50} from the other A(H5N1) viruses (Table 2). Sequence analysis of the NA from this virus revealed an Ile to Val substitution at position 117 (N2 numbering) and an Ile to Val substitution at position 314 (Genbank Acc# DQ884461). Both of these residues are conserved in other influenza A(H5N1) viruses, while the 117 residue is also fully conserved in all N1, N4, N5 and N8 viruses. The second strain identified to have reduced NA inhibitor sensitivity was influenza A/Chicken/Vietnam/486A/2004, which demonstrated a 63-fold increase in zanamivir IC_{50} compared to the mean IC_{50}, an 11-fold higher IC_{50} for oseltamivir carboxylate and a 4-fold higher IC_{50} for peramivir (Table 2). Sequence analysis of this strain demonstrated a single amino acid substitution of Val to Ala at

Table 2
Mean IC_{50} values (nM) ± 1 standard deviation for A(H5N1) viruses and for IC_{50} outliers A/Chicken/Indonesia/Wates/77/2005 and A/Chicken/Vietnam/486A/2004

	Zanamivir IC_{50} (nM)	Oseltamivir carboxylate IC_{50} (nM)	Peramivir IC_{50} (nM)
A(H5N1) Mean ± 1 S.D. ($n = 51$[a])	0.57 ± 0.46	0.33 ± 0.27	0.37 ± 0.26
A/Chicken/Indonesia/Wates/77/2005[b]	0.79 ± 0.19	5.38 ± 1.58	0.28 ± 0.05
A/Chicken/Vietnam/486A/2004[b]	35.69 ± 12.44	3.59 ± 1.23	1.58 ± 0.32
A(H1N1) Mean ± 1 S.D. ($n = 435$[c])	0.36 ± 0.28	0.62 ± 0.53	0.14 ± 0.04[d]

Mean IC_{50} values are also given for human A(H1N1) viruses as a comparison.
[a] Mean does not include IC_{50} values for the two outlying strains A/Chicken/Indonesia/Wates/77/2005 and A/Chicken/Vietnam/486A/2004. In addition, two strains analysed for adamantane susceptibility were not able to be included in NA inhibitor susceptibility testing due to the presence of Newcastle Disease Virus in these samples.
[b] Outlying strains tested in duplicate in three separate assays.
[c] Commonly circulating A(H1N1) human strains isolated between 2001 and 2006 from Australasia and South East Asia.
[d] $n = 27$ for A(H1N1) viruses tested with peramivir.

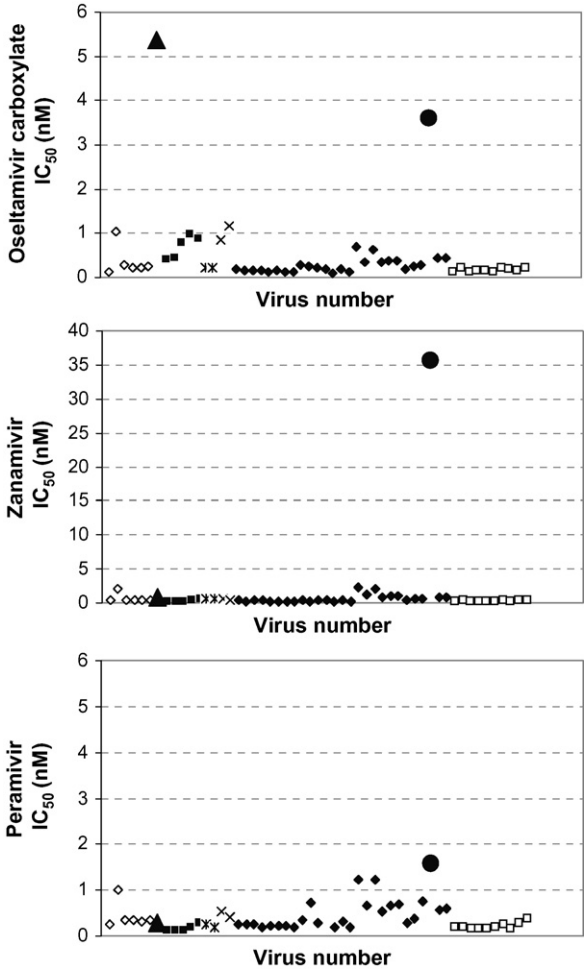

Fig. 1. Plots showing the IC_{50} range of oseltamivir carboxylate, zanamivir and peramivir for A(H5N1) viruses. IC_{50} values for outliers A/Chicken/Indonesia/Wates/77/2005 (▲) and A/Chicken/Vietnam/486A/2004 (●) are means based on three independent assays. IC_{50} values for viruses from each region are indicated as follows: Cambodia (◊), Indonesia (■), Malaysia (✖), Myanmar (×), South Vietnam (♦), North Vietnam (□). IC_{50} scale for the zanamivir figure is different.

position 116 (Genbank Acc# DQ884460), a site that appears to be fully conserved across all neuraminidase types. Although residues 116 and 117 are not believed to play either a framework or interactive role in the NA enzymatic site, they are adjacent to and as such may have an effect on Arg118, one of the three arginine residues that bind the carboxylate of the substrate sialic acid (Russell et al., 2006). These residues are currently being investigated further to fully elucidate their role in NA inhibitor resistance.

This study appears to be the first report of NA inhibitor drug sensitivity testing conducted on a large number of recent influenza A(H5N1) viruses and provides essential baseline IC_{50} values against the drugs that are most likely to be used in the event of a pandemic. While two of the 55 strains studied demonstrated increased IC_{50} values to the licensed NA inhibitors, the vast majority were sensitive to these drugs even though they were isolated around the same time, and from the same region and species as the resistant viruses. The IC_{50} values for the two resistant strains were significantly higher than the mean, however it should be noted that the reported steady-state plasma Cmin of 138 ng/ml (486 nM) oseltamivir carboxylate achieved with 75 mg bid oseltamivir dosing (Roche, 2006), exceeds the IC_{50} for A/Chicken/Indonesia/Wates/77/2005 by 90-fold and A/Chicken/Vietnam/486A/2004 by 135-fold, while levels reported for zanamivir in sputum at 6 h post-inhalation (1336 ng/ml; 4020 nM) (Peng et al., 2000) exceed the IC_{50} for the same viruses by approximately 5000-fold and 100-fold, respectively. Nevertheless, given the importance of NA inhibitors in the prevention and treatment of influenza, it is crucial to continue to monitor strains circulating in animals and man for evidence of the emergence of resistance.

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