Articles

INFLUENZA VIRUSES WITH REDUCED SENSITIVITY TO THE NEURAMINIDASE INHIBITOR DRUGS IN UNTREATED YOUNG CHILDREN

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Abstract

The neuraminidase inhibitors are a class of antiviral drugs used for both the prophylaxis and treatment of influenza infections. Clinical trials of these inhibitors detected a low level of resistant viruses from treated individuals, although a higher frequency was detected in children (5%–6%) compared to adults (1%–4%). In addition, there have been some previous reports of NA inhibitor resistant viruses being isolated from untreated individuals. Here we report on the NA inhibitor sensitivity of over 1,000 influenza isolates collected through the World Health Organization (WHO) global influenza surveillance program. Of the total number of viruses analysed, only 2 (0.2%) strains (an A(H1N1) strain and an influenza B strain) were considered to have a significant reduction in sensitivity to at least one of the neuraminidase inhibitor drugs. Interestingly, both of these strains were isolated from untreated patients in the youngest age cohort (less than 2 years). Although the influenza B strain is unlikely to be clinically resistant, the A(H1N1) virus contained the same His274Tyr neuraminidase mutation that has been observed in resistant mutants following oseltamivir treatment. Given these results it may be important to enhance neuraminidase inhibitor susceptibility testing of viruses from patients in the less than two years cohort. *Commun Dis Intell* 2008;32:57–62.

Keywords: drug resistance, influenza, neuraminidase inhibitors

Introduction

Influenza causes significant morbidity and mortality worldwide each year.[1] While the disease is generally self-limiting in healthy adults, deaths occur in infants and the elderly, usually due to life threatening complications associated with secondary bacterial infections such as pneumonia.[2] Annual vaccination is the primary option for the prevention of influenza, but efficacy relies on a good match between the strains currently circulating and those in the vaccine, and is estimated to be 60%–90% in children and healthy adults, but only 50%–60% in the elderly.[3] In Australia, influenza vaccine uptake within the elderly population (aged ≥ 65 years) is high, with states such as Victoria having an estimated 74.5% coverage in 2002.[4] In comparison, influenza vaccination of Australian children aged six months to five years is extremely low. Influenza vaccination of this age group is neither recommended nor funded by the Australian Government.

While influenza vaccination continues to be the most cost effective means of influenza prevention in the community, antiviral drugs provide an additional control measure for either the treatment or short-term prevention of influenza. Two classes of influenza antivirals exist – the adamantanes, or M2 ion channel inhibitors, and the neuraminidase (NA) inhibitors. The adamantanes, including amantadine and rimantadine, were first shown to be inhibitory for influenza A viruses in 1964,[5] and since this time have been widely used in many countries around the world, although amantadine is rarely used for influenza treatment in Australia and rimantadine is not licensed. Resistance to this class of drugs has however been identified with a high frequency, particularly in recent years where over 90% of A(H3N2) strains from North America and China were found to be resistant.[6] Adamantane resistant viruses have demonstrated no reduction in viral fitness compared to wild type viruses, and may in fact have some selective advantage given that adamantane resistance in A(H3N2) viruses is greater than 50% in Australia, even though very little of the drug is used.[7,8]

As a result of the high level of adamantane resistant viruses currently circulating, more emphasis has been placed on the role of the newer class of influenza antiviral drugs, the NA inhibitors (NAIs), in the management of influenza. This class of influenza drugs has two currently licensed products, zanamivir and oseltamivir, which are available in many countries, including Australia.[9] These inhibitors act by binding to the NA and preventing the release of newly formed virions from the host cell, disrupting further replication of the virus. Clinical studies and post marketing surveillance have shown that the frequency of NAI is significantly lower than that observed for the adamantanes.[9] Clinical

studies identified 1%–4% resistance in oseltamivir-treated adults,[10] while higher levels of resistance (5%–6%) were observed in oseltamivir treated children.[11] However more recent studies have identified levels of resistance as high as 16%–18% in oseltamivir-treated children.[12,13] Only one incident of significant resistance has been reported following zanamivir treatment and this was concerning an immunocompromised patient,[14] although this low incidence of resistance to zanamivir may be a result of the low usage of this drug compared to oseltamivir. In addition to the occurrence of NAI resistance following oseltamivir treatment, resistant strains have also been isolated from untreated individuals,[15] including one strain isolated from an 8-month old infant in Australia.[16] As NAI resistance has been more common in oseltamivir-treated children than in adults, together with our previous finding of a naturally occurring NAI resistant virus in Australia isolated from an infant, we decided to investigate further whether there was any variation in the levels of viral NAI resistance from influenza cases in different age cohorts. Given the low levels of NAI usage, it was assumed that all or nearly all of the samples that were tested in the study came from untreated patients. To achieve this, over 1,000 influenza viruses isolated between 2001 and 2006 were tested for their susceptibility to the NAI drugs and the data analysed based on the age of the patient.

Materials and methods

Viruses

All influenza viruses tested in this study were submitted to the WHO Collaborating Centre for Reference and Research on Influenza as part of the WHO global influenza surveillance program. Only specimens with patient age data available were included in the study (approximately 70% of the specimens or isolates received at the Centre). A total of 1,097 influenza viruses from Australasia (Australia 468, and New Zealand 150), South East Asia (Thailand 209, Philippines 45, Singapore 108, Vietnam 1, Cambodia 1, and Indonesia 14), South Pacific (New Caledonia 19, Solomon Islands 4, and Fiji 1), Eastern Asia (Republic of Korea 16, and Taiwan 45), and South Africa (16) were tested for their susceptibility to the NAIs. The number of viruses isolated from patients from different age groups and from different regions is shown in Table 1. The viruses tested were made up of influenza A(H1N1) (288 isolates), A(H1N2) (5 isolates), A(H3N2) (535 isolates) and influenza B strains (270 isolates). All viruses were isolated and passaged in Madin-Darby canine kidney (MDCK) cells [American Type Culture Collection (CCL-34)] maintained in DMEM Coons Basal Medium containing sodium bicarbonate (3%) with the addition of 2 mM glutamine, 1% non-essential amino acids, 0.05% $NaHCO_3$, 0.02M HEPES, 4% penicillin and streptomycin, 2 µg/ml amphotericin B and 4 µg/ml trypsin (all media were obtained from CSL Limited, Australia) and tested within a maximum of five passages from isolation.

NA inhibitors

Zanamivir was used directly from the blister packaging of Relenza (5 mg zanamivir and 20 mg lactose) (GlaxoSmithKline) as distributed through pharmacies. Oseltamivir carboxylate (GS 4071), the active form of the ethyl ester prodrug oseltamivir phosphate, was kindly provided by Dr James Smith, F Hoffmann-La Roche Ltd, Basal, Switzerland. Each of the drugs was dissolved in assay buffer and stored for up to three months as a stock solution at 4°C prior to use.

NA inhibition assay

A fluorescence-based NAI assay was used to determine the sensitivity of viruses to the NAI compounds. The assay was based on the release of the fluorescent product 4-methylumbelliferone from the substrate 2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA) as a measure of NA activity.[17] Methods followed those described previously.[18] The data were plotted as the percentage of fluorescence activity inhibited against the log NA inhibitor concentration. A logistic curve fit program (kindly provided by Dr Trevor Rae, Roche Products, Welwyn Garden City) was used to produce a curve of best fit and calculate an IC_{50} value for each virus. Known susceptible viruses and known resistant viruses were used as controls in each assay.

RT-PCR and sequencing

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 (Institute of Medical and Veterinary Science, Adelaide). Nucleotide sequences were analysed using DNASTAR V.5 (Lasergene, USA).

Results

One thousand and ninety-eight influenza viruses isolated between 2001 and 2006 were analysed for their susceptibility to the NAIs zanamivir and oseltamivir carboxylate. The data from this study demonstrated small differences in NA inhibitor susceptibility between the N1, N2 and B neuraminidases to both antiviral agents zanamivir and oseltamivir carboxylate (Table 2), similar to that

Table 1. Number of viruses isolated from patients from different age groups and regions

Region	Age group of patient			
	<2	2–17	18–50	>50
Australasia	270 (69%)	155 (39%)	129 (59%)	64 (71%)
South East Asia	108 (27%)	185 (47%)	64 (29%)	21(23%)
South Pacific	5 (1%)	12 (3%)	6 (3%)	1 (1%)
East Asia	6 (2%)	40 (10%)	14 (6%)	2 (2%)
South Africa	4 (1%)	5 (1%)	5 (2%)	2 (2%)
Total	393	397	218	90

reported previously.[18] IC_{50} values (concentration of antiviral required to inhibit NA activity by 50%) for each virus were compared based on the age of the patient, to determine both the mean NAI susceptibility and the number of viruses within that cohort that had an IC_{50} value that differed by 10-fold or greater from the mean IC_{50} value (termed an 'outlier'). Of the total number of viruses analysed, only 2 (0.2%) strains were considered to be outliers to at least one of the NAIs (Table 3). Interestingly, both of these outliers were from patients in the youngest age cohort (less than 2 years) (Table 2). However, when the IC_{50} data for the outlying strains were removed from the overall analysis (Table 2), the mean IC_{50} values were very similar between all age cohorts for all subtypes and against both NAIs.

Of the two strains with raised IC_{50} values, there was no predominance of one NA subtype, with one outlier being detected in each of the N1 and B NA subtypes. The outlying virus with the highest IC_{50} was an A(H1N1) strain designated as A/Victoria/124/2005. This virus was isolated from a 15-month-old infant who had presented to a general practitioner with cough and fever on 7 June 2005. As part of the Victorian GP sentinel influenza surveillance program,[19] a combined nose and throat swab was taken from the patient and sent in viral transport medium to the Victorian Infectious Diseases Reference Laboratory for respiratory virus identification. Following influenza A detection by RT-PCR, the specimen was forwarded to the WHO Collaborating Centre for Reference and Research on Influenza for further analysis. After a single passage in MDCK cells the viral isolate was tested for NAI susceptibility and this revealed an IC_{50} value for oseltamivir carboxylate that was approximately 900-fold higher than the mean oseltamivir carboxylate IC_{50} value for other N1 viruses. The virus however was found to be fully sensitive to zanamivir (IC_{50} = 0.4 nM). Sequence analysis of the NA gene from the virus revealed a His274Tyr mutation. Interestingly, the patient from whom the specimen was taken had not been treated with either NAI. It could not be determined if the child had contracted influenza from a drug treated individual.

The other outlier, B/Perth/211/2001, had significantly less resistance than was observed for A/Victoria/124/2005. B/Perth/211/2001, which we have reported previously,[16] demonstrated a 7-fold increase in IC_{50} to zanamivir and an 18-fold increase to oseltamivir carboxylate. This virus was isolated from an 8-month-old infant from Western Australia who had also not been treated with either zanamivir or oseltamivir. Initial sequence analysis of the virus did not reveal any NA mutations. However on further analysis the isolate was found to contain a mixed viral population, from which a proportion contained a Asp197Glu amino acid mutation in the NA gene.[16]

Discussion

The NAIs were the first class of drugs to be specifically designed for the treatment or prevention of influenza. However since their release into the market in 1999 there has been limited use of these drugs in most countries except Japan and the United States of America. In Japan, up to 18% of oseltamivir treated patients have been shown to shed resistant influenza viruses but, unlike the spread of adamantane resistant influenza viruses into populations where little drug is being used, there is little evidence of the subsequent spread of NAI resistant strains to other individuals. The results from this study found that only 0.2% of the influenza strains tested were found to have a significant reduction in sensitivity to at least one of the NAIs. While it is not possible to be definitive, it is highly likely based on the patients' records (both patients were confirmed not to have received either NAI drugs) and the low usage of NAI drugs in Australia, that these resistant strains did not arise as a result of drug selective pressure.

Although the IC_{50} values of the two strains in this study were found to be significantly higher than the other viruses tested, one of these, B/Perth/211/2001, would probably not be considered clinically significant, as the IC_{50} levels of this virus are exceeded following administration of the normal dosage of either drug.[20,21] However, the A/Victoria/124/2005 strain had an IC_{50} value (585 nM for oseltamivir carboxylate) which was higher than the reported

Table 2. NA inhibitor susceptibility of viruses taken from patients of different age cohorts

Age	N1				N2*				B			
		Mean IC_{50} ± 1 S.D. (nM)†				Mean IC_{50} ± 1 S.D. (nM)†				Mean IC_{50} ± 1 S.D. (nM)†		
	n =	Zanamivir	Oseltamivir carboxylate	Number of outliers‡	n =	Zanamivir	Oseltamivir carboxylate	Number of outliers‡	n =	Zanamivir	Oseltamivir carboxylate	Number of outliers‡
<2	60	0.37 ± 0.19	0.64 ± 0.47	1	240	1.08 ± 1.07	0.35 ± 0.27	0	93	2.05 ± 1.08	13.24 ± 11.19	1
2–17	133	0.36 ± 0.27	0.69 ± 0.53	0	152	1.09 ± 0.66	0.31 ± 0.20	0	112	1.77 ± 1.26	15.49 ± 12.05	0
18–50	77	0.34 ± 0.30	0.64 ± 0.83	0	97	0.90 ± 0.29	0.29 ± 0.24	0	44	1.72 ± 1.42	8.02 ± 7.55	0
>50	18	0.36 ± 0.27	0.68 ± 0.52	0	51	1.02 ± 0.80	0.32 ± 0.20	0	21	2.12 ± 1.63	10.08 ± 9.77	0
Total	288	0.36 ± 0.26	0.67 ± 0.62	1	540	1.02 ± 0.65	0.32 ± 0.24	0	270	1.88 ± 1.26	13.06 ± 11.23	1

* IC50 values of the five A(H1N2) viruses were not significantly different from the values of the A(H3N2) isolates.

† Mean IC50 value calculation does not include IC50 values of outliers.

‡ Outlier defined as a virus with an IC50 at least 10–fold greater than the mean of other viruses with the same NA subtype.

Table 3. NA inhibitor susceptibility of outlier influenza isolates with IC_{50} values at least 10-fold greater than the mean

Designation	Subtype	Age of patient	Zanamivir		Oseltamivir carboxylate	
			IC_{50} (nM)	Fold difference*	IC_{50} (nM)	Fold difference*
A/Victoria/124/2005	H1N1	12 months	0.4 ± 0.2	–	585.8 ± 61.2	874-fold
B/Perth/211/2001	B	8 months	13.8 ± 1.7	7-fold	233.9 ± 31.8	18-fold

* Fold-difference calculated by comparing the IC50 of the isolate with the mean IC50 of viruses with the same NA subtype.

oseltamivir carboxylate steady-state plasma Cmin of 138 ng/ml (approximately 485 nM) achieved with the normal 75 mg twice daily oseltamivir dosing.[21] This suggests that oseltamivir carboxylate treatment of the patient with this strain may not be effective. The amino acid mutation His274Tyr observed in the NA gene of A/Victoria/124/2005 has been the most commonly NA mutation detected in NAI resistant N1 viruses, which includes both the commonly circulating human A(H1N1) viruses as well as highly pathogenic A(H5N1) influenza viruses.[9] To date there have been three reported cases of oseltamivir resistant A(H5N1) viruses that have been isolated from infected Vietnamese patients undergoing oseltamivir treatment.[22,23] In all cases the resistance was due to the His274Tyr mutation in the NA gene, the same mutation that was detected in the A/Victoria/124/2005 strain in this study. It should be remembered however that the resistance mutation in the strain from Victoria appears to have occurred spontaneously in an untreated individual, unlike the A(H5N1) viruses where resistance was likely to have been generated as a result of drug selective pressure. While A(H1N1) or A(H5N1) viruses with a His274Tyr NA mutation are resistant to oseltamivir, these viruses remain fully sensitive to the other NAI, zanamivir (the zanamivir IC_{50} of A/Victoria/124/2005 was 0.4nM). As a result, zanamivir may be a better option for the treatment of patients who shed these viruses, although this drug is only licensed for use in patients aged five years or older.

Two previous studies have investigated the impact that a His274Tyr NA mutation has had on the infectivity and transmissibility of viruses in ferrets. While one study found that mutant viruses had significantly compromised fitness[24] (as is the case with many other NAI resistant mutants[9]), a second study found that transmissibility was possible, although a higher dose of the virus was necessary for infection compared to the wild type virus.[25] If the data from this second ferret study are indicative of the fitness of this virus in humans, it is therefore possible that infants may be able to facilitate the replication and transmission of NAI resistant strains better than adults due to the high titres and prolonged duration of influenza virus shed by individuals in the younger age group.[26,27] While further work is necessary to understand the risk that the two strains (with high IC_{50}) identified in this study may pose if they become widely circulating in the human population, it is of note that all of these isolates were from children aged 18 months or less. Given these results and the previous reports showing the significantly higher incidence of drug resistance in oseltamivir treated children compared to adults, it essential in the future to enhance NAI susceptibility testing in both treated and untreated individuals within this young age group.

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