Surveillance for neuraminidase inhibitor resistance in human influenza viruses from Australia

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Abstract

Two hundred and forty-five human influenza A and B viruses isolated in Australia between 1996 and 2003 were tested for their sensitivity to the NA inhibitor drugs, zanamivir and oseltamivir using a fluorescence-based neuraminidase inhibition assay. Based on mean IC_{50} values, influenza A viruses (with neuraminidase subtypes N1 and N2) were more sensitive to both the NA inhibitors than were influenza B strains. Influenza A viruses with a N1 subtype and influenza B strains both demonstrated a greater sensitivity to zanamivir than to oseltamivir carboxylate, whereas influenza A strains with a N2 subtype were more susceptible to oseltamivir carboxylate. A comparison of IC_{50} values for viruses isolated before and after the release of the NA inhibitors in Australia, found there was no significant difference in the sensitivity of strains to either neuraminidase inhibitor and none of the isolates tested showed clinically significant resistance. *Commun Dis Intell* 2003;27:542–547.

Keywords: influenza, neuraminidase inhibitors, oseltamivir carboxylate, zanamivir, Australia

Introduction

Two new antiviral agents, zanamivir and oseltamivir, have recently become available for the treatment and prophylaxis of influenza.[1,2] These compounds were designed to interfere with the activity of the neuraminidase enzyme of the influenza virus based on knowledge of the three-dimensional crystal structure of the enzyme, determined by Australian scientists, and of the chemistry of its interaction with cell surface receptors.[3]

Neuraminidase plays a crucial role in the release of new virions from the infected cell[1] and the structure of the enzyme active site is highly conserved across the known types of influenza A and B which are responsible for epidemic and, for influenza A, pandemic human disease.[4] Consequently, the neuraminidase inhibitors offer considerable promise as broadly active anti-influenza drugs. The rapid replication of influenza, which may precede onset of symptoms in the infected individual, and the difficulty in making an accurate diagnosis of influenza in time for the compounds to have maximum benefit, have limited their use for the treatment of established infection. Nevertheless, they represent an important addition to the options for prevention and treatment of influenza in situations such as institutional outbreaks, for immunocompromised individuals or where vaccines are unavailable. The NA inhibitors are likely to be of value for the treatment and prevention of influenza in the event of a future pandemic when it is expected that there will be little, if any, vaccine available initially, particularly for prophylaxis of health care workers and others providing essential services.

Influenza viruses have an exceptional capacity for adaptive change both through a high rate of mutation and their ability to undergo genetic reassortment.[5] While this is most commonly seen as antigenic variation, the rapid evolution of resistance to earlier anti-influenza drugs, the ion channel inhibitors amantadine and rimantadine,[6] illustrates the potential that these viruses have to respond to both immunological and other evolutionary pressures. Viruses resistant to the neuraminidase inhibitors can be generated *in vitro* by multiple passage in the presence of the drugs, however many of these strains demonstrate a significantly reduced infectivity in animal models.[7] *In vivo* resistance to zanamivir has only been recorded in a single persistently infected immunodeficient individual,[8] whereas viruses with reduced drug sensitivity have been recorded in 0.4 per cent of paediatric and four per cent of adult patients treated with oseltamivir.[9] To date there is no evidence of transmission and persistence of resistant viruses. Nevertheless, in view of the capricious nature of influenza and experience with development of drug resistance by

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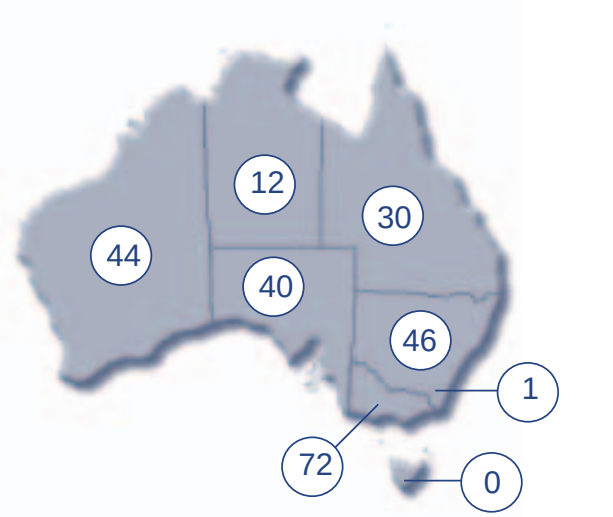
other viruses it seems prudent to maintain an active surveillance for drug sensitivity in clinical isolates. A previous study of strains collected globally between 1996 and 1999 investigated the susceptibility of viruses to the NA inhibitors.[10] However, because oseltamivir had not yet been released in 1999, and zanamivir had only just become available, the study was unable to determine if the sensitivity of influenza viruses had changed significantly as a result of the introduction of these drugs. Here we report the results of a study conducted on Australian influenza viruses isolated from 1996, prior to the release of zanamivir (1999) and oseltamivir (2001), through 2003.

Methods

Influenza isolates

Two hundred and forty-five influenza A and B viruses (Figure 1) were selected from over 4,000 strains collected through the WHO global influenza surveillance program between the years 1996 and 2003, and were tested for their sensitivity to the NA inhibitor drugs zanamivir and oseltamivir carboxylate. The majority of the viruses were isolates from hospital patients, with a smaller number derived from general practitioner surveillance. Where possible, similar numbers of each NA type (B, N1 and N2) were tested annually with similar overall numbers for each year of isolation (1996–2003) and from all geographic regions of Australia (Figure 1). Due to low numbers of influenza A(H1N1) and B viruses circulating in Australia in some years, and the absence of uniform virological surveillance this was not fully achieved. All viruses were cultured in Madin-Darby canine kidney (MDCK) cells at the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne.

Figure 1. Distribution of isolates selected for NA inhibitor drug sensitivity testing



NA inhibitor drugs

The NA inhibitors zanamivir and oseltamivir carboxylate were used in the NA inhibition assays. Zanamivir was used directly from the blister packaging of Relenza (5 mg zanamivir and 20 mg lactose) as distributed through pharmacies. Oseltamivir carboxylate (GS 4071) is the active form of the prodrug oseltamivir phosphate (tradename Tamiflu), and was kindly supplied by Professor Noel Roberts, Roche Products, Welwyn Garden City, United Kingdom.

NA inhibition assay

To determine the sensitivity of the influenza viruses to the NA inhibitor compounds a fluorescence-based NA inhibition assay was used. 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.[11] The protocol followed a previously published method,[12] except that the inhibitor/virus mix was incubated for 45 minutes rather than 30 minutes, and the MUNANA/inhibitor/virus mix was incubated for 60 minutes at 37° C with no shaking, rather than 15 minutes at 37° C with shaking. Briefly, to determine the drug concentration required to inhibit 50 per cent of the NA activity (IC_{50}), each virus was mixed with a range of concentrations of each drug (0.01 nM to 10,000 nM). Following an incubation period, the fluorescence substrate MUNANA is added to the virus/inhibitor mix and then incubated at 37° C for 60 minutes. After this time the reaction was stopped and the level of NA inhibition at different drug concentrations was quantified and an IC_{50} value for each virus calculated using a logistic curve fit program kindly provided by Dr Trevor Rae, Roche Products, Welwyn Garden City, United Kingdom. One NA inhibitor sensitive and two NA inhibitor resistant control strains were included in each assay for reference purposes. A known susceptible isolate wba-1 A(H1N9), and two known resistant strains xw-2/3 A(H1N9) (E119G mutation) and yn-1 A(H1N9) (R292K mutation) were kindly provided by Jennifer McKimm-Breschkin, CSIRO, Parkville, Australia, and used as controls in each assay for reference purposes.

Statistical analysis

Paired t-tests were used to compare the IC_{50} values of the two NA inhibitors for each virus type. To compare the sensitivity of the three NA types to the two NA inhibitors it was first necessary to adjust for year to year differences in the number of each NA type tested, by using year as a covariate. Each of the three comparisons was then analysed using a Tukey's HSD test. Changes in NA inhibitor sensitivity which can predict clinical resistance have yet to be determined, however it has been proposed that a shift of >10-fold may be an appropriate predictor.[13] In this study a more conservative approach was taken where by viruses with a >5-fold reduction in sensitivity compared to the mean, were considered outliers and investigated further.

Results

Mean IC_{50} values ± standard deviation (S.D.) of both zanamivir and oseltamivir carboxylate for influenza A viruses (grouped by NA type N1 and N2) and influenza B viruses are shown in the Table. Strains from each NA type demonstrated a different level of sensitivity to the two NA inhibitors. Generally, influenza A viruses containing a N1 NA and influenza B viruses were better inhibited by zanamivir than by oseltamivir carboxylate ($p = <0.001$), while influenza A viruses with a N2 NA type were better inhibited by oseltamivir carboxylate than by zanamivir ($p = <0.0001$) (Table).

The NA inhibitors were also shown to differentially inhibit the three NA types to different degrees. Mean IC_{50} values of zanamivir were similar for N2, and B strains, but significantly lower for N1 viruses ($p < 0.05$). While the mean IC_{50} values of oseltamivir carboxylate were similar for N1 and N2 strains, but significantly higher for influenza B viruses ($p < 0.05$) (Table).

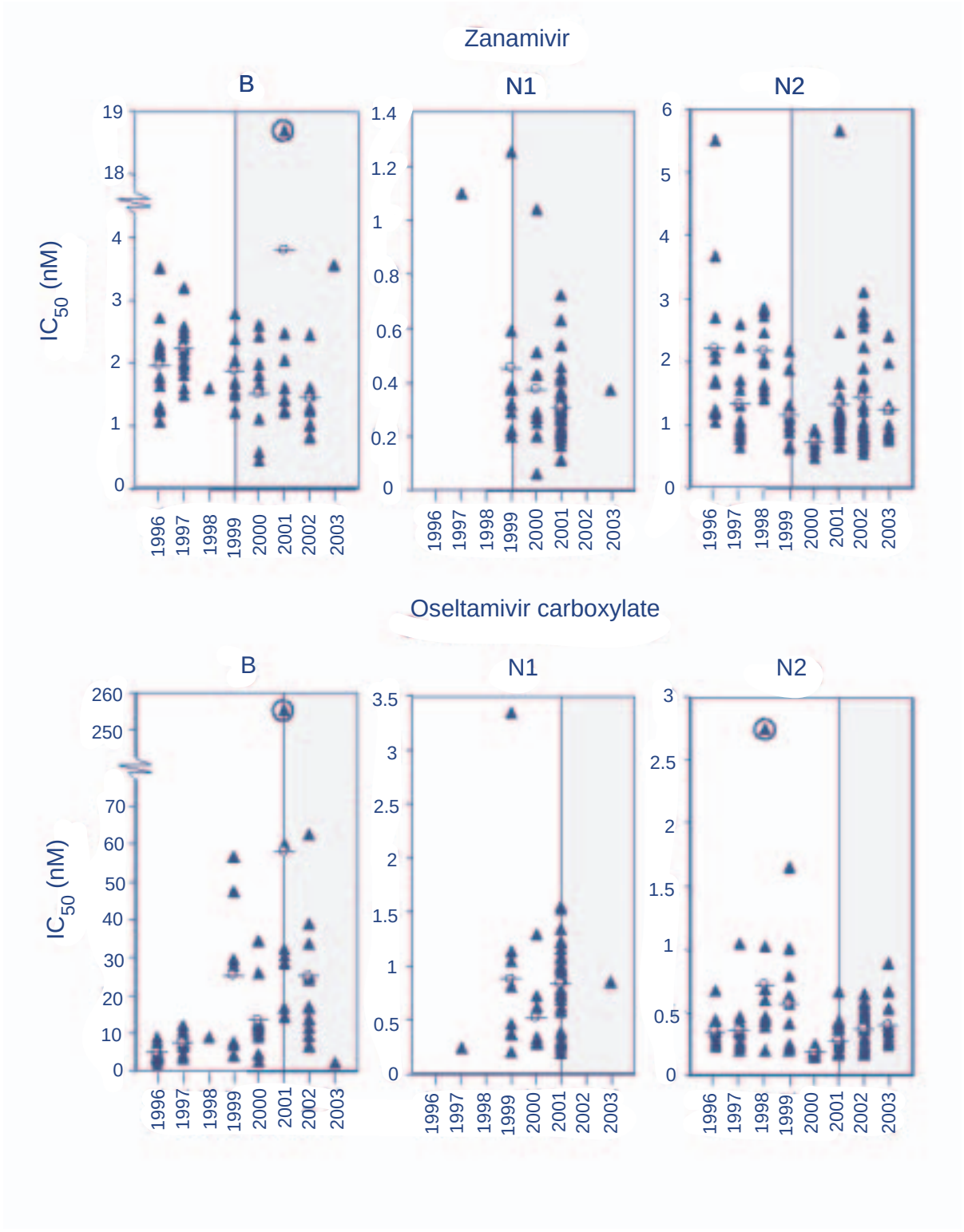
To investigate whether there has been any significant change in NA inhibitor sensitivity since the introduction of the drugs into Australia, IC_{50} values of viruses were compared based on their isolation date. Figure 2 illustrates the individual and mean IC_{50} values of viruses isolated in the years prior to and following the release of the two NA inhibitors. The mean inhibition level of Australian viruses for each drug before its introduction compared with after its introduction was not significantly different for either NA type or NA inhibitor tested ($p = <0.05$).

Of the 245 influenza viruses tested, only two demonstrated a fivefold or greater reduction in sensitivity to either of the NA inhibitors compared with the mean IC_{50} value for the relevant NA type (Figure 2). The first of these viruses was an N2 strain isolated in 1998, prior to the release of either zanamivir or oseltamivir carboxylate. This strain demonstrated an IC_{50} with oseltamivir carboxylate (2.75 nM) that was approximately sevenfold higher than the mean for all N2 viruses with this inhibitor (0.38 nM), although with zanamivir the IC_{50} for the isolate was not significantly different from the mean (1.97 nM vs Mean IC_{50} N2 = 1.39nM). The other Australian virus to demonstrate a reduction in NA inhibitor sensitivity was an influenza B virus isolated in 2001 from an eight-month-old female infant who was not undergoing NA inhibitor treatment. This strain was shown to have an oseltamivir carboxylate IC_{50} value of 256.7 nM, approximately 13-fold greater than the mean IC_{50} for type B strains (19.25 nM), and a zanamivir IC_{50} value of 18.8 nM, approximately 9-fold higher than the mean (2.05 nM). Further passages of this isolate demonstrated an equally reduced sensitivity to the NA inhibitors.

Table. Mean IC_{50} (nM) ± standard deviation of zanamivir and oseltamivir carboxylate for influenza viruses of each NA type

	B n = 65	N1 n = 55	N2 n = 125
Zanamivir	2.05 ± 2.21	0.36 ± 0.22	1.39 ± 0.86
Oseltamivir carboxylate	19.25 ± 33.22	0.81 ± 0.52	0.38 ± 0.30

Figure 2. Scatter plots of individual IC_{50} values (▲) of zanamivir and oseltamivir carboxylate for influenza viruses from each NA type/subtype and for each year of isolation between 1996 and 2003



Mean IC_{50} values (⊖) for each year are included where there were more than one virus tested for that period. Viruses with IC_{50} values that are fivefold or greater than the mean IC_{50} ▲.

Blue shaded area indicates the years following the introduction of the particular NA inhibitor.

Discussion

Due to the rapid evolution of resistance in influenza viruses following treatment with the previous class of anti-influenza drugs the M2 ion channel inhibitors, it was thought necessary, following the release of the NA inhibitors, to monitor influenza isolates for the development of resistance to zanaivir and oseltamivir carboxylate. Here we showed that Australian viruses demonstrated a high level of sensitivity to the two licensed NA inhibitors throughout the study period which were similar to those reported for strains collected globally by the Neuraminidase Inhibitor Susceptibility Network[10] and for viruses from Canada.[14] While the mean oseltamivir carboxylate IC_{50} for type B viruses from this study (19.25 nM) is higher than previously reported for strains collected globally (12.46 nM),[10] much of this difference can be explained by the inclusion of the outlying 2001 type B isolate (oseltamivir carboxylate IC_{50} of 256.7 nM) in our data set. Removal of the IC_{50} value for this virus reduced the overall mean to 15.54 nM.

Zanamivir became available for use in Australia in 1999 followed by oseltamivir in 2001. The overall IC_{50} values of Australian viruses to the two drugs has not changed significantly since their introduction, nor is there evidence of increased numbers of individual viruses with decreased sensitivity to either NA inhibitor. From 245 influenza isolates tested only two Australian viruses were identified in this study as outliers with IC_{50} values showing a 7- to 13-fold reduction in sensitivity to the NA inhibitors. These reductions in sensitivity are very low compared to resistant viruses reported in the literature. Strains isolated from patients following oseltamivir treatment have been reported to demonstrate a fold reduction in sensitivity of between 50 and >80,000 compared to the sensitive wildtype strains.[15] However, many of the viruses that have a high level of resistance to the NA inhibitors have also demonstrated a significant reduction in infectivity in mice and ferret models.[8,16,17] To date only one resistant clinical isolate has been reported following zanamivir treatment, an influenza B isolate from an immunocompromised child.[8] This isolate demonstrated a 100-fold reduction in sensitivity to zanamivir.[18]

For surveillance purposes it is important to define the reduction in sensitivity of a virus that would be expected to result in the clinical failure of the NA inhibitors. Based on the high concentrations of NA inhibitor drug that can be achieved following administration (zanamivir concentrations have been measured above 3,000 nM in sputum six hours post-inhalation)[19] even the least sensitive virus from this study should be fully inhibited by either zanamivir or oseltamivir treatment *in vivo*.

The results of this study and others[10,14] indicate that resistance to the NA inhibitors is uncommon. However, it does remain prudent to continue to conduct sensitivity testing of viruses, particularly if the use of the NA inhibitors increases in the community. For this purpose the NA inhibition method used in this study has been shown to be ideal for the surveillance for NA inhibitor resistant influenza viruses, particularly as it has been reported to be more predictive of *in vivo* susceptibility than cell-based assays.[20] Targeted surveillance directed to the isolation and testing of strains from normal or immuno-compromised individuals undergoing treatment with the NA inhibitors may allow a more focussed and thorough assessment of the potential for influenza viruses to develop clinically significant resistance to these compounds.

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