Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 62 (2004) 37–45
Antiviral Research
www.elsevier.com/locate/antiviral

Susceptibility of human influenza viruses from Australasia and South East Asia to the neuraminidase inhibitors zanamivir and oseltamivir

Aeron C. Hurt [a,*], Ian G. Barr [a], Gunter Hartel [b], Alan W. Hampson [a]

[a] *WHO Collaborating Centre for Reference and Research on Influenza, 45 Poplar Road, Parkville, Vic. 3052, Australia*
[b] *CSL Limited, Parkville, Australia*

Received 7 July 2003; accepted 20 November 2003

Abstract

Human influenza viruses isolated from Australasia (Australia and New Zealand) and South East Asia were analysed to determine their sensitivity to the NA inhibitor drugs, zanamivir and oseltamivir. A total of 532 strains isolated between 1998 and 2002 were tested using a fluorescence-based assay to measure the relative inhibition of NA activity over a range of drug concentrations. Based on median 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. For each of the neuraminidase types, IC_{50} values for viruses from Australasia and South East Asia were found to be comparable. Based on the data prior to and following the licensing of the drugs into the respective regions, the use of the NA inhibitors did not appear to have a significant impact on the susceptibility of the viruses tested to zanamivir or oseltamivir carboxylate.

Keywords: Influenza; Neuraminidase inhibitors; Oseltamivir carboxylate; Zanamivir

1. Introduction

Influenza A and B infections cause significant morbidity and mortality worldwide, with approximately 20,000 deaths annually in the USA (Simonsen et al., 1997). Two groups of antivirals have become available for the treatment of influenza infections. The first group, the M2 ion channel inhibitors amantadine and rimantadine, were found to inhibit growth of influenza over 30 years ago and still continue to be used in some countries (Ison and Hayden, 2001). These drugs act by blocking the ion channel formed by the M2 protein, consequently inhibiting viral replication (Hay, 1992). The M2 inhibitors while effective against influenza A viruses, are not effective against type B strains, and when used following infection, result in the selection of drug resistant viruses within 2–4 days of the onset of therapy in up to 30% of patients (Hayden, 1996; Belshe et al., 1988).

Between 1999–2002 a new class of anti-influenza drugs, the neuraminidase (NA) inhibitors, were introduced into clinical practice in many countries including Australia, New Zealand, USA and many parts of Europe. To date two NA inhibitors, zanamivir (Relenza®, GlaxoSmithKline) and oseltamivir (Tamiflu®, Gilead/Roche), have been licensed for use in humans. The NA inhibitors act by binding to the active site of the viral neuraminidase enzyme preventing release and spread of progeny virions from infected cells during the replication cycle (Gubareva et al., 2000). Several of the amino acids that form the NA active site are highly conserved across both influenza A and influenza B viruses (Varghese et al., 1983).

While a number of NA inhibitor resistant mutants have been generated in vitro (McKimm-Breschkin, 2000), very few clinical isolates with resistance or reduced sensitivity to the NA inhibitors have been identified in vivo (Barnett et al., 2000; Jackson et al., 2000). The only in vivo mutant that has been isolated following zanamivir treatment was an influenza B virus obtained from an immuno-compromised patient (Gubareva et al., 1998). This virus contained an R152K change in the NA gene which resulted in an approximately 50-fold reduction in sensitivity to both zanamivir and oseltamivir carboxylate (Wetherall et al., 2003). In clinical studies on virus samples from oseltamivir-treated patients with naturally acquired influenza infection, viruses with significantly reduced sensitivity were isolated from 0.4% of

* Corresponding author. Tel.: +61-3-9389-1392; fax: +61-3-9389-1881.
E-mail address: Aeron_Hurt@csl.com.au (A.C. Hurt).

0166-3542/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2003.11.008

adult and adolescent patients and in 4% of patients aged 1–12 years (Roberts, 2001). While the frequency of drug resistance has been demonstrated to be significantly less than with the M2 inhibitors, it remains possible that increased resistance may occur over time. As a result of such concerns, the global neuraminidase inhibitor susceptibility network (NISN) was established in 1999 to monitor potential development of resistance (Zambon and Hayden, 2001). Several other laboratories are also conducting analysis of influenza viruses for evidence of decreased sensitivity to the NA inhibitors.

To date there have been few reports on the susceptibility of circulating influenza strains to the NA inhibitor drugs. The NISN reported on the sensitivity of viruses isolated from 1996 to 1999 (McKimm-Breschkin et al., 2003), prior to the introduction of either NA inhibitor. A second smaller study investigated the NA inhibitor susceptibility of Canadian influenza strains isolated between 1999 and 2000, however only 65 viruses were tested (Boivin and Goyette, 2002). This current study provides sensitivity data on over 500 influenza strains isolated from throughout Australasia and South East Asia prior to and following the release of the NA inhibitor drugs.

2. Materials and methods

2.1. Viruses

A total of 532 influenza viruses, 230 from Australasia (Australia (185) and New Zealand (45)) and 302 from South East Asia (Malaysia (45), Thailand (137), Philippines (23), Singapore (59), Taiwan (25), Vietnam (1) and Indonesia (12)) were selected for NA sensitivity testing from strains collected through the WHO global influenza surveillance program between the years 1998 and 2002. Influenza A(H1N1), A(H1N2), A(H3N2) and influenza B strains were assayed (Table 1). The 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% $NaHCO_3$, 0.02 M HEPES, 4% penicillin streptomycin, 2 μg/ml fungizone and 4 μg/ml trypsin (all media were obtained from CSL Limited, Parkville, Australia). All of the viruses were tested within a maximum of five passages from isolation.

2.2. NA inhibitors

The NA inhibitor 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 Professor Noel Roberts, Roche Products, Welwyn Garden City, UK. Each of the drugs was dissolved in assay buffer and stored for up to 3 months as a stock solution at 4 °C prior to use. Based on the consistency of the IC_{50} values of control viruses used in each assay and the comparisons conducted between stored and freshly prepared solutions (results not shown), the NA inhibitor compounds were considered to be completely stable over this period of storage.

2.3. NA inhibition assay

A fluorescence-based NA inhibition assay was used to determine the sensitivity of viruses to the NA inhibitor 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 (Potier et al., 1979) using a modified version of the protocol described by Barnett et al. (2000). Initially, a NA activity assay was carried out to determine the dilution of each virus to be used in the subsequent NA inhibition assay. Two-fold dilutions of virus in assay buffer (32.5 mM MES (pH 6.5), 4 mM $CaCl_2$ with 0.1% NP-40 and 0.3 mg/ml BSA) were prepared and then mixed with an equal volume (50 μl) of MUNANA substrate (0.3 mM) and incubated at 37 °C for 60 min. The reaction was terminated by the addition of 100 μl of stop solution (0.14 M NaOH in 83% ethanol). Fluorometric quantification of 4-methylumbelliferone was determined using a Labsystems Fluoroskan II fluorometer with an excitation wavelength of 360 nm and an emission wavelength of 448 nm. The appropriate concentration of virus for use in the NA inhibition assay was determined by selecting a dilution of virus in the linear portion of the enzyme activity curve.

To determine the drug concentration required to inhibit 50% of the NA activity (IC_{50}), 50 μl of virus diluted according to the NA activity assay was mixed with varying concentrations of either inhibitor in black microtitre plates

Table 1
Number of influenza viruses tested of NI, N2, and B NA type, isolated between 1998 and 2002 from Australasia and South East Asia

		N1[a]	N2[b]	B
1998	Australasia	9	11	3
	South East Asia	15	13	18
1999	Australasia	11	17	12
	South East Asia	23	17	11
2000	Australasia	11	15	12
	South East Asia	40	12	13
2001	Australasia	38	33	13
	South East Asia	81	13	17
2002	Australasia	1	25	19
	South East Asia	6	13	10
	Total	235	169	128

[a] All N1 viruses were A(H1N1).
[b] 95% of N2 viruses were A(H3N2), 5% were A(H1N2).

(FluoroNunc plates, Nunc). The assay was conducted over a range of final reaction mixture concentrations from 0.01 to 10,000 nM for both inhibitors. The virus/inhibitor mix was incubated at room temperature for 45 min prior to the addition of MUNANA substrate (50 μl of 0.3 mM) and then incubated at 37 °C for 60 min. The reaction was terminated by the addition of 100 μl of the stop solution. 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. 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 included as controls in each assay.

2.4. Statistical analysis

IC_{50} data for the isolates grouped by neuraminidase type and geographic region (Australasian or South East Asian) were analysed for each of the years 1998 to 2002, for both inhibitors using the statistical package JMP (Version 5.0.1, © 2001 SAS Institute). Quantile boxplots and whiskers, rather than 95% confidence intervals, are displayed on the graphs to allow comparison of the distributions of the data without requiring the assumptions of normality or symmetry (McGill et al., 1978; Tukey, 1977). Quantile Boxplots facilitate comparison of the distributions of several variables by marking several quantiles of each distribution. In particular, the bottom and top of the 'box' mark the 25th and 75th percentiles (Q1 and Q3), and a line across the box marks the 50th percentile or median (or Q2). Whiskers are added to help identify potential outliers. The whiskers extend above and below the box by 1.5 times the interquartile range (IQR). The IQR is the 75th percentile minus the 25th percentile (i.e., Q3−Q1). To determine if there were significant trends in the IC_{50} values of viruses isolated between 1998 and 2003, linear regression analyses of the mean $\log_{10}$ IC_{50} values were performed.

2.5. Sequencing

Sequencing was performed on all strains that had an IC_{50} value greater than the upper quantile limit for their respective NA type and year of isolation.

3. Results

The variability of the fluorometric NA inhibition assay was first evaluated by repeated testing of 15 influenza strains (five of each NA type N1, N2 and B) within the same assay and between assays performed on six different occasions. Intra- and interassay variation with zanamivir was 14 and 13%, and with oseltamivir was 20 and 37%, respectively. Overall the combined coefficient of variance for intraassay variation was 17% and for interassay variation was 25%.

The pooled results of NA sensitivity against zanamivir and oseltamivir carboxylate for all of the viruses tested are shown by type and subtype (N1, N2 and B) in Fig. 1a. Viruses containing a N1 neuraminidase demonstrated a slightly higher level of sensitivity to zanamivir than to oseltamivir carboxylate (mean IC_{50}: 0.37 and 0.66 nM, respectively) while N2 strains were found to be more sensitive to oseltamivir carboxylate (0.31 nM versus 1.04 nM for zanamivir). Influenza B strains were considerably more sensitive to zanamivir (mean IC_{50}: 1.40 nM) than to oseltamivir carboxylate (mean IC_{50}: 14.84 nM).

In Fig. 1b the difference between the $\log_{10}$ IC_{50} for zanamivir and oseltamivir was plotted for each individual virus. A positive value indicated that a virus was more sensitive to oseltamivir carboxylate, whilst a negative result indicated a virus was more sensitive to zanamivir. The results of this analysis confirmed that the influenza B viruses and N1 subtype viruses tested were significantly ($P < 0.001$) more sensitive to zanamivir than oseltamivir carboxylate, and that N2 subtype viruses were significantly ($P < 0.001$) more sensitive to oseltamivir carboxylate than zanamivir.

For each NA type/subtype the IC_{50} data was investigated to determine if viruses isolated from either Australasia and South East Asia differed in their susceptibility to the NA inhibitor drugs (Fig. 2). The range of IC_{50} values for each NA type and inhibitor type were found to be similar for both regions. A comparison of median and lower (Q1) and upper (Q3) quartile values did however suggest some variation in sensitivity between Australasian and South East Asian type B isolates to oseltamivir carboxylate (median IC_{50} (Q1, Q3): Australia = 13.86 nM (7.29, 27.61)) versus South East Asia = 6.70 nM (3.54, 14.72)). Two antigenically and genetically distinct lineages of influenza B viruses (referred to as the B/Victoria/2/1987 and B/Yamagata/16/1988 lineages) have had different circulation patterns in Australia and South East Asia since 1983 (Rota et al., 1990; Shaw et al., 2002). Of the viruses in this study, all of the influenza B viruses isolated from Australasia between 1998 and 2001 were of the Yamagata lineage, while strains from both lineages were isolated from South East Asia during the same period. However in 2002, the predominant influenza B viruses isolated from both South East Asia and Australasia were from the Victoria lineage. Comparison of the susceptibility of viruses from the two lineages to the NA inhibitors revealed no significant differences (Table 2). This indicates that any regional or temporal variation in the influenza B strains observed in this study is unlikely to be due to the lineage (Victoria or Yamagata) differences.

Analysis was also undertaken by year of isolation for viruses originating in Australasia and South East Asia, to determine if inhibitor sensitivity had varied over the five-year period 1998–2002 (Fig. 3). Of particular interest was whether the data, over the five year period, demon-

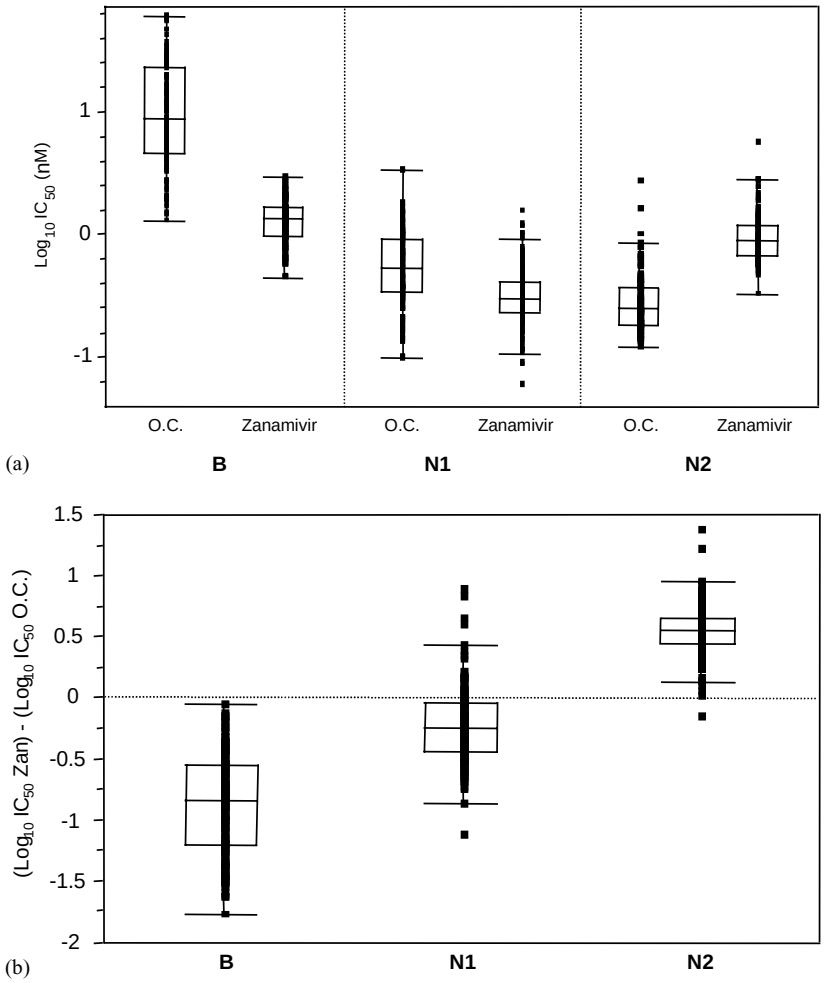

Fig. 1. (a) Quantile box plots of $\log_{10}$ IC_{50} for zanamivir and oseltamivir carboxylate by NA type for all isolates tested. Each ■ represents an individual IC_{50} result. (b) Quantile box plots of $\log_{10}$ IC_{50} for zanamivir subtracted from $\log_{10}$ IC_{50} for oseltamivir carboxylate (O.C.) by NA type. Boxes indicate 1st, 2nd and 3rd quartiles (i.e., Q1: 25th percentile, Q2: median, and Q3: 75th percentile). Whiskers are calculated as $Q1 - 1.5 \times (Q3 - Q1)$ and $Q3 + 1.5 \times (Q3 - Q1)$.

strated either a significant increase in the number of isolates with an IC_{50} value outside of the quantile range or alternatively if a significant increase in mean IC_{50} over time was evident. Only 11 of the 532 viruses tested had an IC_{50} value greater than the upper quantile limit, of these eight were Australian viruses and three were South East Asian isolates. Although only one of the 11 'outlier' strains was isolated in either 1998 or 1999, and the remainder isolated between 2000 and 2002, there is insufficient data to determine whether a significant trend exists linking an increase in the number of outlying isolates to the introduction of the NA inhibitors. The NA of each of the 11 'outlier' strains was sequenced and aligned with more than 100 strains of the same respective NA type. Sequence analysis revealed that none of the 11 viruses contained any mutations in the conserved residues of the NA head (Varghese and Colman, 1991), or had any unique amino acid changes compared to other recently or currently circulating strains. In addition there was no sequence correlation between the 'outlying' strains compared with strains that demonstrated greater sensitivity to the NA inhibitors.

Table 2
Median IC_{50} with 25th percentile (Q1) and 75th percentile (Q3) (nM) of influenza B/Victoria/2/1987 lineage and B/Yamagata/16/1988 lineage viruses for both zanamivir and oseltamivir carboxylate

	B/Victoria/2/1987 lineage viruses ($n = 22$) median (Q1, Q3)	B/Yamagata/16/1988 lineage viruses ($n = 27$) median (Q1, Q3)
Zanamivir	1.3 (1, 1.5)	1.2 (0.9, 1.5)
Oseltamivir carboxylate	24.6 (9.9, 33.8)	25.9 (11.9, 30.2)

To determine if there were any significant linear trends in sensitivity over time, the mean $\log_{10}$ IC_{50} was subjected to regression analysis against the year of isolation for viruses from Australasia and South East Asia (Fig. 4). Each of the regression analyses, except one, were found to be non-significant ($P > 0.05$), indicating that the differences between year of isolation did not exhibit a clear linear trend, but rather varied from year to year over the

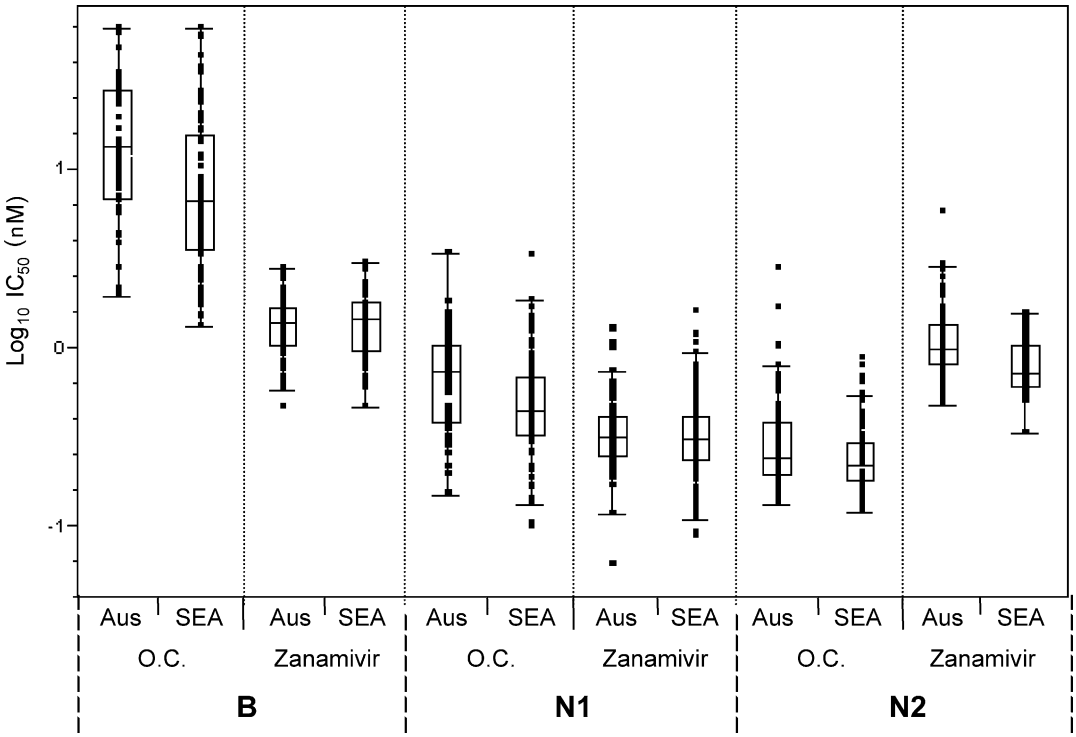

Fig. 2. Quantile box plots of zanamivir and oseltamivir carboxylate (O.C.) $\log_{10}$ IC_{50} for viruses by NA type from Australasia (Aus) and South East Asia (SEA). Boxes indicate 1st, 2nd and 3rd quartiles (i.e., Q1: 25th percentile, Q2: median, and Q3: 75th percentile). Whiskers are calculated as $Q1 - 1.5 \times (Q3 - Q1)$ and $Q3 + 1.5 \times (Q3 - Q1)$.

five year period studied. However, the mean $\log_{10}$ IC_{50} data for N2 viruses from South East Asia, with oseltamivir carboxylate, did have a significant decreasing linear trend ($P = 0.0014$) over the five-year period. This result however must be interpreted with caution, as it must be kept in mind that this set of regression analyses involved 12 hypothesis tests, which means that even if there was no biological or scientific basis for it, one significant result in 12, using an $\alpha = 0.05$, would not be unexpected. To further test the significance of the linear trend of the N2 strains from South East Asia over the five year period ($P = 0.0014$), the slope of the regression line was directly compared with the corresponding non-significant slope of N2 strains from Australasia ($P = 0.2082$). A parallelism of regression lines model applied to this data found no significant difference between the slopes of the regression lines of the two regions ($P = 0.81$), a result which would not be expected if there was truly a difference in the sensitivity of South East Asian N2 strains over the five year period.

4. Discussion

NA inhibitors have been available for the treatment of influenza infection in many countries since 1999. Due to experience with the M2 ion channel inhibitors, NISN and other independent laboratories have undertaken regular testing of isolates to determine the level of resistance to the NA inhibitors in circulating human viruses. This study demonstrates that different NA types exhibit slightly different sensitivity to the NA inhibitors, and different NA inhibitors can have different IC_{50} values for a particular NA type. The differences in the sensitivity of the three NA types to the NA inhibitors zanamivir and oseltamivir found in this study are generally in agreement with previous studies of strains collected globally by NISN (McKimm-Breschkin et al., 2003) and viruses from Canada (Boivin and Goyette, 2002). However, one difference was in the sensitivities of the two NA inhibitors to A(H1N1) isolates. In this study and the NISN study, N1 strains were slightly more susceptible to zanamivir, however the Canadian study demonstrated N1 strains to be more susceptible to oseltamivir carboxylate, although this difference may be due to the low numbers (four) of A(H1N1) viruses tested in that study (Boivin and Goyette, 2002). The reason for these minor differences in susceptibility of different NA types/subtypes to the NA inhibitors is likely to be due to minor structural differences in the NA active site which can result in different binding affinities (Boivin and Goyette, 2002).

A comparison between viruses from Australasia and South East Asia was performed to investigate whether any regional differences in NA inhibitor sensitivity existed. Although some differences in median IC_{50} values were evident, strains from both regions for each NA type demonstrated a range of values that could be attributed to minor variation in the NA of viruses within a subtype and to assay variability.

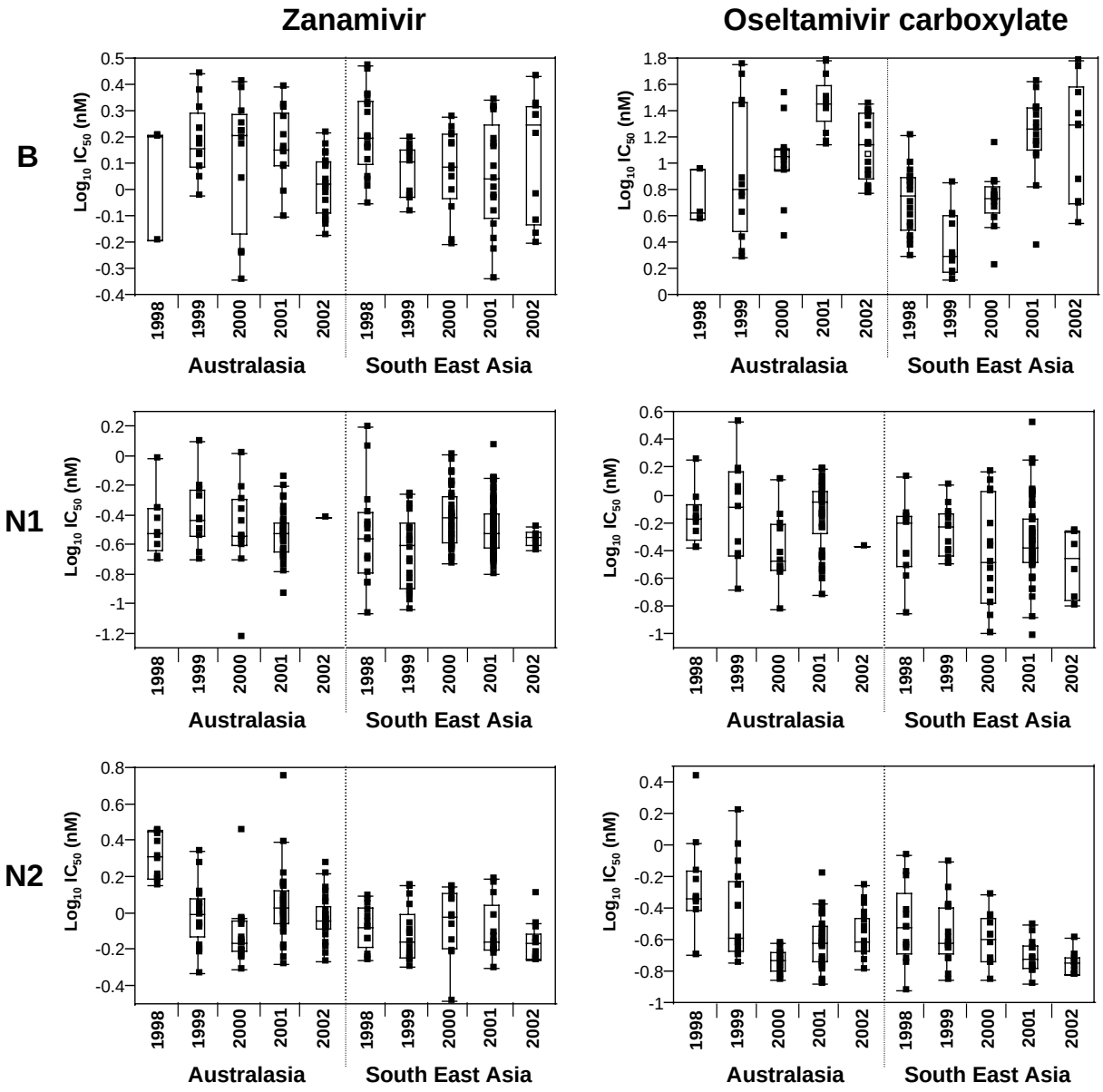

Fig. 3. Quantile box plots illustrating $\log_{10}$ IC_{50} values grouped by region and year of isolation for zanamivir and oseltamivir carboxylate by each NA type. Boxes indicate 1st, 2nd and 3rd quartiles (i.e., Q1: 25th percentile, Q2: median, and Q3: 75th percentile). Whiskers are calculated as $Q1 - 1.5 \times (Q3 - Q1)$ and $Q3 + 1.5 \times (Q3 - Q1)$.

The intra- and inter-assay variation of the fluorescence-based assay throughout these studies was 17 and 25%, respectively. This level of variability is lower than that reported for either the fluorescence or chemiluminescence-based NA inhibition assays used in other similar studies (Boivin and Goyette, 2002; Wetherall et al., 2003).

Zanamivir was first available in Australasia in 1999, however mean IC_{50} values of Australasian viruses isolated after this date do not indicate any significant decrease in sensitivity. In fact mean IC_{50} values for all NA types of Australasian viruses showed similar levels of sensitivity to viruses from South East Asia where the drug has yet to be released. Oseltamivir carboxylate was developed later than zanamivir, and was released in Australasia in 2001 and in Singapore and the Philippines in 2002. No significant increase in mean IC_{50} values for the years 2001 and 2002 were detected compared to the three previous years in N1 or N2 isolates from either region. Influenza B viruses from Australasia did however show a significant increase in mean IC_{50} in 2001, although it was lower again in 2002 demonstrating no clear trend since the introduction of the drug. Mean IC_{50} values of type B strains from South East Asia also showed an increase in 2001 and remained at a similar level in 2002. This observed increase in mean IC_{50} for South East Asian viruses isolated in 2001 can not be attributed to the use of oseltamivir carboxylate as the drug had not yet been released

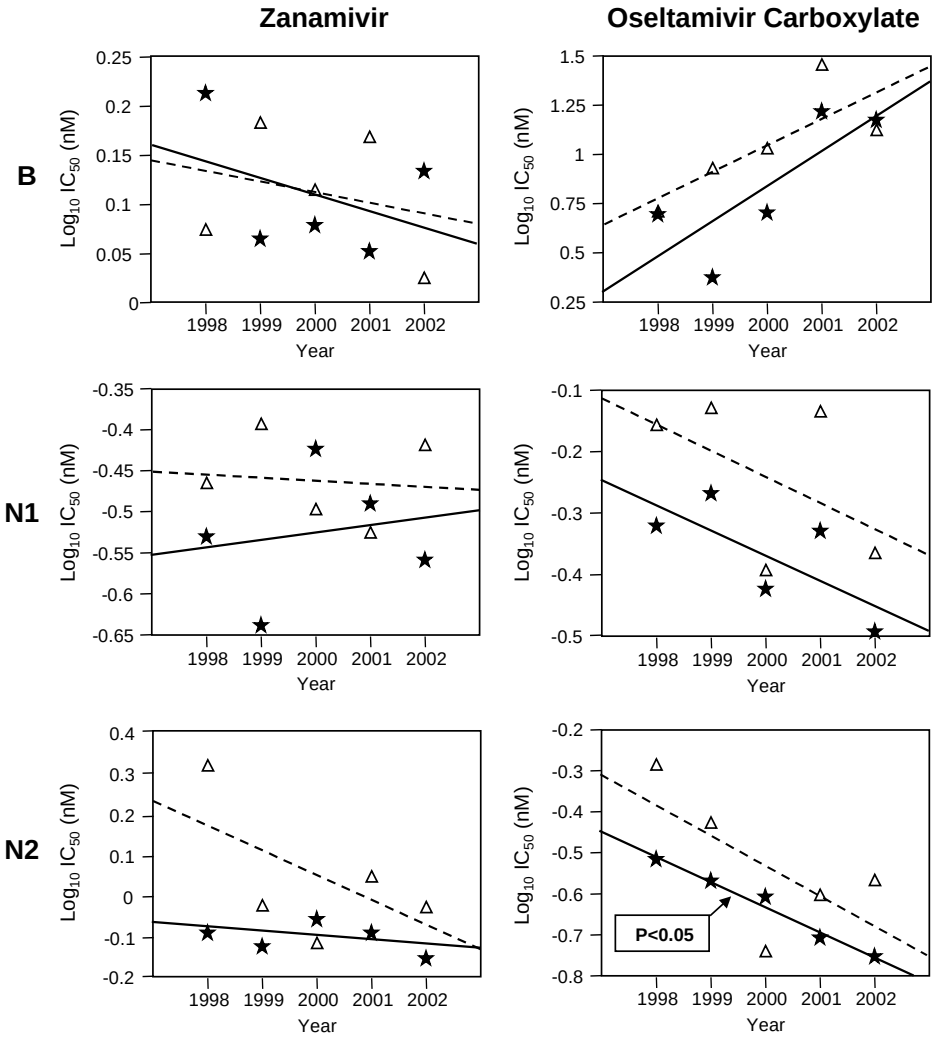

Fig. 4. Linear regression of Australasian (△) and South East Asian (★) mean $\log_{10}$ IC_{50} values for zanamivir and oseltamivir carboxylate versus year. Linear fit for Australasian data (- - -) and South Asian data (—). All regression lines were non-significant ($P > 0.05$) except for the regression of the South East Asian N2 virus IC_{50} data for oseltamivir carboxylate which was found to be significant ($P < 0.05$) (indicated on graph).

in this region. Demonstration that influenza B viruses from the B/Victoria/2/1987 and B/Yamagata/16/1988 lineage did not show any significant difference in NA inhibitor sensitivity, indicated that any variation observed within the influenza B strains in this study was not due to the antigenic or genetic lineage of the virus.

Unlike the situation with antigenic changes in the influenza virus surface antigens where new variants can arise without prejudicing transmissibility and infectivity, viruses with NA mutations which result in resistance to the NA inhibitors often demonstrate significantly reduced infectivity in mice and ferret models (Gubareva et al., 1997, 1998; Tai et al., 1998). This would suggest that further transmission of infection and selection of resistant viruses would be unlikely, and that the mean sensitivity of circulating strains may not change dramatically. Consequently, variation in the number of IC_{50}'outliers' that occurred in viruses isolated over the five year period from Australia or South East Asia was also examined in this study. It was found that very few viruses had an IC_{50} value greater than the quantile limit, and that although the majority of these strains were isolated in 2000–2002, there appeared to be no significant correlation between their occurrence and the release or use of the different NA inhibitors in the two regions.

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. Analysis of the sensitivity of resistant mutants isolated from drug-treated patients, compared to their wildtype counterparts, can give some indication of the level of fold reduction in sensitivity that might indicate truly resistant viruses isolated during surveillance. Using a similar fluorescence-based NA inhibition assay to the one used in this study, Wetherall et al. (2002) tested a type B isolate from an immuno-compromised patient undergoing zanamivir treatment, B/Memphis/20/96 (R152K mutation) (Gubareva et al., 1998) which demon-

strated a greater than 40-fold reduction in sensitivity to zanamivir ($IC_{50} \geq 250$ nM). In the same study three resistant type A viruses, isolated from patients undergoing oseltamivir treatment, all had a >100-fold reduction in sensitivity to oseltamivir carboxylate (IC_{50} values ranged from 100 to 7200 nM) (Wetherall et al., 2002). By comparison, the viruses tested in this study had a maximum of an eight-fold reduction in sensitivity compared to the respective mean for the respective NA type. Considering the high concentrations of NA inhibitor drug that can be achieved following administration (zanamivir concentrations have been measured above 3000 nM in sputum 6 h post-inhalation) (Peng et al., 2000) it is highly likely from the data generated in this study that even the least sensitive viruses (maximum IC_{50} for zanamivir = 5.7 nM; for oseltamivir carboxylate = 61.6 nM) would be fully inhibited by either zanamivir or oseltamivir treatment in vivo.

In summary, although the frequency of naturally occurring resistant strains to date has been very low, it remains prudent to continue to conduct sensitivity testing of viruses as part of a general surveillance program, particularly if the use of the NA inhibitors increases. Targeted surveillance directed to the isolation and testing of normal or immunocompromised individuals undergoing treatment with the NA inhibitors may allow a more focussed and rapid assessment of the potential for influenza viruses to develop clinically significant resistance to the NA inhibitors.

Acknowledgements

The authors would like to thank the National Influenza Centres and other laboratories in Australia, New Zealand and South East Asia that have supplied influenza isolates that were used for analysis in this paper. In addition they would like to thank Chris Durrant, Robert Shaw, and Helen Sjogren for the culture of all the influenza strains used in this study and Naomi Komadina and Fee Yee Wong for sequence analysis of the viral neuraminidases (WHO Collaborating Centre for Reference and Research on Influenza, Melbourne). The WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Commonwealth Department of Health and Ageing.

References

Barnett, J.M., Cadman, A., Gor, D., Dempsey, M., Walters, M., Candlin, A., Tisdale, M., Morley, P.J., Owens, I.J., Fenton, R.J., Lewis, A.P., Claas, E.C., Rimmelzwaan, G.F., de-Groot, R., Osterhaus, A.D., 2000. Zanamivir susceptibility monitoring and characterization of influenza virus clinical isolates obtained during phase II clinical efficacy studies. Antimicrob. Agents Chemother. 44, 78–87.

Belshe, R.B., Smith, M.H., Hall, C.B., Betts, R., Hay, A.J., 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J. Virol. 62, 1508–1512.

Boivin, G., Goyette, N., 2002. Susceptibility of recent Canadian influenza A and B virus isolates to different neuraminidase inhibitors. Antiviral Res. 54, 143–147.

Gubareva, L.V., Kaiser, L., Hayden, F.G., 2000. Influenza virus neuraminidase inhibitors. Lancet 355, 827–835.

Gubareva, L.V., Matrosovich, M.N., Brenner, M.K., Bethell, R.C., Webster, R.G., 1998. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. J. Infect. Dis. 178, 1257–1262.

Gubareva, L.V., Robinson, M.J., Bethell, R.C., Webster, R.G., 1997. Catalytic and framework mutations in the neuraminidase active site of influenza viruses that are resistant to 4-guanidino-Neu5Ac2en. J. Virol. 71, 3385–3390.

Hay, A.J., 1992. The action of adamantanamines against influenza A viruses: inhibition of the M2 ion channel protein. Sem. Virol. 3, 21–30.

Hayden, F.G., 1996. Amantadine and rimantadine: clinical aspects. In: Richman, D.D. (Ed.), Antiviral Drug Resistance. John Wiley and Sons, New York, pp. 78–92.

Ison, M.G., Hayden, F.G., 2001. Therapeutic options for the management of influenza. Curr. Opin. Pharmacol. 1, 482–490.

Jackson, H.C., Roberts, N., Wang, Z.M., Belshe, R., 2000. Management of influenza: use of new antivirals and resistance in perspective. Clin. Drug Invest. 20, 447–454.

McGill, R., Tukey, J.W., Larsen, W.A., 1978. Variations of box plots. Am. Stat. 32, 12–16.

McKimm-Breschkin, J.L., 2000. Resistance of influenza viruses to neuraminidase inhibitors—a review. Antiviral Res. 47, 1–17.

McKimm-Breschkin, J.L., Trivedi, T., Hampson, A., Hay, A., Klimov, A., Tashiro, M., Hayden, F., Zambon, M., 2003. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. Antimicrob. Agents Chemother. 47, 2264–2272.

Peng, A.W., Milleri, S., Stein, D.S., 2000. Direct measurement of the anti-influenza agent zanamivir in the respiratory tract following inhalation. Antimicrob. Agents Chemother. 44, 1974–1976.

Potier, M., Mameli, L., Belisle, M., Dallaire, L., Melancon, S.B., 1979. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-alpha-D-*N*-acetylneuraminate) substrate. Anal. Biochem. 94, 287–296.

Roberts, N.A., 2001. Treatment of influenza with neuraminidase inhibitors: virological implications. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 356, 1895–1897.

Rota, P.A., Harmon, M.W., Rota, J.S., Kendal, A.P., Nerome, K., 1990. Cocirculation of two distinct evolutionary lineages of influenza type B virus since 1983. Virology 175, 59–68.

Shaw, M.W., Xu, X., Li, Y., Normand, S., Ueki, R.T., Kunimoto, G.Y., Hall, H., Klimov, A., Cox, N.J., Subbarao, K., 2002. Reappearance and global spread of variants of influenza B/Victoria/2/87 lineage viruses in the 2000–2001 and 2001–2002 seasons. Virology 303, 1–8.

Simonsen, L., Clarke, M.J., Williamson, G.D., Stroup, D.F., Arden, N.H., Schonberger, L.B., 1997. The impact of influenza epidemics on mortality: introducing a severity index. Am. J. Pub. Health 87, 1944–1950.

Tai, C.Y., Escarpe, P.A., Sidwell, R.W., Williams, M.A., Lew, W., Wu, H., Kim, C.U., Mendel, D.B., 1998. Characterization of human influenza virus variants selected in vitro in the presence of the neuraminidase inhibitor GS 4071. Antimicrob. Agents Chemother. 42, 3234–3241.

Tukey, J.W., 1977. Exploratory Data Analysis. Addison-Wesley, Reading, MA.

Varghese, J.N., Colman, P.M., 1991. Three-dimensional structure of the neuraminidase of influenza virus A/Tokyo/3/67 at 2.2 A resolution. J. Mol. Biol. JID 2985088R 221, 473–486.

Varghese, J.N., Laver, W.G., Colman, P.M., 1983. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. Nature 303, 35–40.

Wetherall, N.T., Trivedi, T., Zeller, J., Hodges-Savola, C., McKimm-Breschkin, J.L., Zambon, M., Hayden, F.G., 2003. Evaluation of neuraminidase enzyme assays using different substrates to measure

susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. J. Clin. Microbiol. 41, 742–750.

Wetherall, N.T., Trivedi, T., Zeller, J., Hodges-Savola, C., Zambon, M., Hayden, F.G., 2002. Evaluation of neuraminidase enzyme assays using different substrates to measure the susceptibility of influenza clinical isolates to neuraminidase inhibitors. Commun. Dis. Intell.

Zambon, M., Hayden, G., 2001. Position statement: global neuraminidase inhibitor susceptibility network (on behalf of the global neuraminidase inhibitor susceptibility network). Antiviral Res. 49, 147–156.