ELSEVIER

Antiviral Research 53 (2002) 47–61

Antiviral Research

www.elsevier.com/locate/antiviral

Detection of influenza virus resistance to neuraminidase inhibitors by an enzyme inhibition assay

Larisa V. Gubareva [a,*], Robert G. Webster [b,c], Frederick G. Hayden [a,d]

[a] *Department of Internal Medicine, Division of Epidemiology and Virology, University of Virginia School of Medicine, P.O. Box 800473, Charlottesville, VA 22908-0473, USA*

[b] *Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN, USA*

[c] *Department of Pathology, University of Tennessee School of Medicine, Memphis, TN, USA*

[d] *Department of Pathology, University of Virginia School of Medicine, Charlottesville, VA, USA*

Received 3 May 2001; accepted 13 August 2001

Abstract

We previously characterized influenza viruses whose selection in the presence of neuraminidase (NA) inhibitors resulted in a substituted residue (position 119, 152, 274, or 292) in the NA active center. To identify the most favorable conditions for detecting NA inhibitor-resistant viruses we compared the results of four modifications of the NA inhibition assay utilizing a fluorogenic substrate. The IC_{50} values were highly dependent upon assay conditions, and most mutant enzymes were more sensitive to changes in assay conditions (e.g. addition of PO_4^{3-}, Ca^{2+}, DMSO, or EDTA) than wild-type enzymes or a mutant NA with an Arg292 → Lys substitution. Although the levels of resistance to zanamivir, oseltamivir carboxylate, and BCX-1812 (RWJ-270201) for each mutant varied among assays, mutants with substitutions at framework residues 119 or 274 exhibited sensitivity to at least one inhibitor. Viruses with substitutions at catalytic residues 152 or 292 were resistant to each inhibitor in all assays. Monitoring resistance in a clinical setting will require a panel of resistant viruses to ensure that assay conditions are favorable for detecting variants with substituted residues in the NA active center. Because variants selected in the presence of one NA inhibitor could be variably resistant to other inhibitors, all three inhibitors should be used in drug susceptibility testing.

Keywords: Drug resistance; Cross-resistance; Resistance monitoring; Oseltamivir; Zanamivir; RWJ-270201; BCX-1812; Neuraminidase inhibitors

1. Introduction

Until recently, only two drugs, amantadine and its derivative rimantadine, were available for the treatment of influenza infections. These drugs inhibit replication of influenza A virus during the early stage of viral infection by blocking the ion

* Corresponding author. Tel.: +1-804-243-2705; fax: +1-804-982-0384.

E-mail address: lvg9b@virginia.edu (L.V. Gubareva).

0166-3542/02/$ - see front matter
PII: S0166-3542(01)00192-9

channel formed by the M2 protein (Hay et al., 1991). Influenza B viruses lack the M2 protein and therefore are not susceptible to M2 inhibitors. Amino acid substitution(s) within the transmembrane domain of the M2 protein confers resistance to M2 inhibitors. At least five amino acid residues of M2 protein have been implicated in resistance. The involvement of another viral protein, hemagglutinin (HA), in amantadine resistance was demonstrated for certain avian influenza A viruses but not for strains circulating in humans (Steinhauer et al., 1991). Virus resistance to M2 inhibitors is usually monitored by cell culture assays, such as ELISA or assays measuring the inhibition of virus plaque formation; the results of these assays are often supplemented by sequence or restriction analysis of the *M* gene (Kimberlin et al., 1995). Resistance to amantadine or rimantadine emerges rapidly in vitro and in vivo (Hayden, 1996). The resistant strains appear to be genetically stable, cross resistant, fully pathogenic, and transmissible by close contact (Hayden, 1996). Therefore, it is important to detect and characterize the influenza mutants selected in the presence of the new anti-influenza drugs.

The enzymatic activity of viral neuraminidase (NA) is the target of a new class of anti-influenza drugs. Influenza virus NA acts extracellularly by cleaving the terminal neuraminic acid from cellular receptors recognized by HA. NA inhibitors prevent the release of virus from an infected cell and therefore limit the spread of virus to neighboring cells (Palese and Compans, 1976; Gubareva et al., 1996). Each identical subunit that makes up the influenza virus NA tetramer contains an enzyme active site formed by highly conserved amino acid residues (Colman, 1989). 'Functional' residues interact directly with the substrate, neuraminic acid, whereas 'framework' residues provide the structural support for functional residues (Colman et al., 1993).

Two NA inhibitors, zanamivir and oseltamivir, have been approved for use in humans (Gubareva et al., 2000). Zanamivir is administered by inhalation to the predominant site of virus replication, whereas oseltamivir (GS4104), the ethyl ester prodrug form of oseltamivir carboxylate (GS4071), is the first orally bioavailable NA inhibitor. The NA inhibitor BCX-1812 (Babu et al., 2000; Smee et al., 2001; Sidwell et al., 2001) is also bioavailable upon oral administration and is currently undergoing clinical evaluation.

Although resistance to NA inhibitors does not frequently occur in vivo, two mechanisms of resistance have been recognized in in vitro studies (McKimm-Breschkin, 2000). One mechanism involves a reduction in the HA-binding efficiency of receptors; the lower efficiency results in a reduction in the virus's dependence on NA activity during virus release from infected cells. Substitutions in the HA have been identified in viruses selected in the presence of NA inhibitors (McKimm-Breschkin, 2000), although sequence analysis of the *HA* gene itself is a poor predictor of the drug-related phenotype of the virus. The second mechanism of resistance involves the substitution of the conserved residues within NA; such substitutions can reduce the affinity of the active site for the inhibitor. At the present time, substitutions at one of four amino acid residues, specifically, residues 119, 152, 274, or 292 of the enzyme's active center (N2 numbering), have been identified in the mutants selected in the presence of the NA inhibitors in vitro or in vivo (Table 1).

In immunocompetent adults whose influenza infection was treated with NA inhibitors, the incidence of drug resistance emergence is very low or not observed depending on the drug tested (Barnett et al., 2000; Hayden et al., 2000a,b; Gubareva et al., 2001). The monitoring of resistance relies mainly on the results of NA enzyme assay (Tisdale, 2000). With the escalating use of NA inhibitors, routine monitoring of clinical isolates for drug resistance will require reliable and relatively simple assays. However, the currently available cell culture-based assays could not be used for detecting NA inhibitor-resistant variants selected in humans (Woods et al., 1993; Gubareva et al., 1998, 2001; Tisdale, 2000).

Several laboratories around the world have adopted the assay developed by Potier et al. (1979) to test the susceptibility of viruses to these inhibitors. Modifications of this assay employed in different laboratories have included changes in the buffer system, in the concentration of the

Table 1
Drug-resistant influenza viruses with mutant NAs selected in the presence of NA inhibitors

Type of residue	Substitution in the NA active site[a]	NA type/subtype	NA inhibitor[b]	Type of selection	Reference	Mutants used in the present study
Framework	Glu119→Gly	A/N2	Zanamivir	In vitro	Gubareva et al., 1996, 1997	Yes
		A/N9	Zanamivir	In vitro	Blick et al., 1995; Staschke et al., 1995	No
		B	Zanamivir	In vitro	Staschke et al., 1995; Barnett et al., 1999	No
	Glu119→Ala	A/N2	Zanamivir	In vitro	Gubareva et al., 1996, 1997	Yes
	Glu119→Asp	A/N2	Zanamivir	In vitro	Gubareva et al., 1996, 1997	Yes
	Glu119→Val	A/N2	Oseltamivir	In vivo	Whitley et al., 2001	No
Functional	Arg292→Lys	A/N2	Zanamivir	In vitro	Gubareva et al., 1996	Yes
		A/N9	6-Carboxamide derivative of zanamivir	In vitro	McKimm-Breschkin et al., 1998	No
		A/N2	Oseltamivir	In vitro	Tai et al., 1998	No
		A/N2	Oseltamivir	In vivo	Whitley et al., 2001	No
		A/N2	BCX-1812	In vitro	Bantia et al., 2000	No
Framework	His274→Tyr	A/N1	Oseltamivir	In vitro	Wang et al., 2000a	No
		A/N1	Oseltamivir	In vivo	Gubareva et al., 2001	Yes
Functional	Arg152→Lys	B	Zanamivir	In vivo	Gubareva et al., 1998	Yes

[a] Number indicates the location of the conserved residue in the N2 enzyme (Colman, 1989).
[b] The virus variants with mutated NAs were selected in the presence of the indicated NA inhibitor in vitro or recovered from drug-treated patients.

substrate, and in the concentration of the Ca^{2-} ions. Refinements of the methods and of the ability to interpret results of the NA inhibition assays are necessary for more reliable detection of mutants in clinical samples.

The aim of the present study was to investigate whether different NA inhibition assays would provide different estimated levels of resistance and cross-resistance for a virus with a mutated NA

2. Materials and methods

2.1. Compounds

The NA inhibitors oseltamivir carboxylate (GS4071), zanamivir (GG167), and BCX-1812 were provided by the R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ. Stock solutions of compounds diluted in distilled water were stored at −20 °C.

2.2. Viruses

Viruses were propagated in Madin-Darby canine kidney (MDCK) cells by a standard procedure before they were used in the NA assays. The zanamivir-resistant mutants and corresponding wild-type viruses were from the repository of St. Jude Children's Research Hospital, Memphis, TN (Table 1). The oseltamivir-resistant mutant was recovered from a patient who was experimentally infected with A/Texas/36/91 (H1N1) and treated with oseltamivir (Hayden et al., 1999). All viruses were stored at −70 °C before they were used in the assays.

2.3. NA enzyme activity assay

Virus in cell culture supernatants was cleared of cellular debris by centrifugation at 800 × *g* for 10 min and was used as a source of NA. We used the fluorometric assay developed by Potier et al. (1979) to measure influenza NA activity in the absence or presence of antiviral drugs. The assay measures the amount of 4-methylumbelliferone that is cleaved by the influenza virus NA from the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Sigma, St. Louis, MO). We used the four most common modifications of the assay for measurement of influenza virus NA activity. Certain steps and materials (e.g. substrate, plastic ware, standard, stop solution, and equipment) were identical for all assays.

Because of the variation among viruses, the NA activity of each virus was determined before the NA inhibition assays were performed. Substrate and serial dilutions of virus were mixed in 96-well black plates (Corning Costar, Corning, NY) and incubated at 37 °C for 30 min. Reactions were stopped by the addition of 150 μl of 0.1 M glycine buffer, pH 10.7, containing 25% ethanol. Various volumes (5, 10, 20, and 40 μl) of a 40-μM solution of free 4-methylumbelliferone in 0.9% NaCl were used in parallel to determine the linear range of detection. Fluorescence was measured by an HTS 7000 Bio Assay Reader (Perkin-Elmer, Norwalk, CT). The excitation wavelength was 365 nm, and the emission wavelength was 460 nm.

2.4. NA inhibition assay

We determined the IC_{50} values, which are the concentrations of NA inhibitors that are required to inhibit enzyme activity by 50%, by assaying the NA activity of a standard amount of virus in the presence of serial half-log dilutions (from 10 to 0.00001 μM) of NA inhibitor. After equal volumes of virus and inhibitor had been mixed and incubated at room temperature for 30 min, substrate was added, and the reaction mixture was then incubated for 1 h at 37 °C. The reaction was stopped by the addition of the stop solution (described in the preceding paragraph), and the fluorescence was measured. The relationship between the concentration of inhibitor and the percent fluorescence inhibition was determined by graphing, and the IC_{50} values were obtained by extrapolation of the data.

2.5. Modified NA inhibition assays

In the first type of NA inhibition assay (assay I), the working virus dilution was made in the buffer containing 154 mM NaCl, 16.5 mM

H_3BO_4, 1.36 mM $Na_2B_4O_7$, pH 5.9, and 6.8 mM $CaCl_2$. Five microliters of the virus dilution was mixed with 5 μl of NA inhibitor diluted in distilled water. After the mixture had been incubated at room temperature for 30 min, 10 μl of the substrate (final concentration in the reaction mixture, 1 mM in 0.1 M sodium phosphate buffer) was added. To evaluate the effect of the substrate concentration on the IC_{50} values, we also used final concentrations of substrate of 10 and 100 μM in assay I.

In the second type of modified NA assay (assay II), the virus was diluted in a solution of 33 mM MES (2-[*N*-morpholino]ethanesulfonic acid), pH 6.5, and 4 mM $CaCl_2$. Twenty microliters of the virus dilution was mixed with 20 μl of the NA inhibitor diluted in the same buffer supplemented with 12.5% (v/v) DMSO (dimethyl sulfoxide, Sigma). After the mixtures had been incubated for 30 min at 37 °C, 30 μl of substrate, which had been diluted in a solution of 33 mM MES, pH 6.5 and 4 mM $CaCl_2$ was added to the reaction. The final concentration of substrate was 100 μM, and that of DMSO was 2.5%.

In the third type of modified NA assay (assay III), the virus was diluted in a solution containing 33 mM MES, pH 6.5, and 4 mM $CaCl_2$. Twenty microliters of the virus dilution was mixed with 20 μl of NA inhibitor diluted in the same buffer and incubated for 30 min at room temperature. Substrate diluted in the same buffer was added to the mixture; the final concentration of substrate was 75 μM.

In the fourth type of modified NA assay (assay IV), the dilutions of virus and inhibitor were made in a solution of 0.1 M sodium acetate, pH 6.5, and 10 mM $CaCl_2$. The mixture was incubated at room temperature for 30 min. The substrate, which was diluted in the same buffer, was then added to the mixture of the virus and inhibitor. The final concentration of substrate was 100 μM.

2.6. EDTA and NA inhibition assays

Viruses that were grown in cell culture and not further modified were used as a source of NA activity. The NA activities of the wild-type viruses B/Memphis/20/96 and A/turkey/MN/833/80 (H4N2) and their zanamivir-resistant variants were normalized by diluting viruses in 33 mM MES, pH 6.5. Twenty microliters of the working dilution of each virus was mixed with 20 μl of the same buffer containing no ethylenediaminetetraacetic acid (EDTA, Sigma) or 0.6, 0.8, or 1.0 mM of EDTA. After the mixtures had been incubated for 30 min at 37 °C, we added substrate diluted in buffer containing the same concentration of EDTA as the virus dilutions to be assayed. The final mixtures were incubated for 1 h at 37 °C. The NA activities of virus in the buffer containing EDTA (0.6, 0.8, or 1.0 mM) were expressed as percentages of the NA activity of virus in the buffer lacking EDTA (33 mM MES, pH 6.5).

3. Results

Clear-cut thresholds for defining reduced susceptibility or resistance to NA inhibitors have not been established or validated with respect to antiviral effects in vivo. A virus is considered to have reduced susceptibility based on NA inhibition assay if the IC_{50} (50% inhibitory concentration) determined for its enzyme is consistently higher than for the wild type counterpart. Another way of expressing this difference is by determining the fold difference in IC_{50} values compared to the corresponding wild type virus.

3.1. Assessment of resistance to zanamivir in NA inhibition assays

The sensitivities of the influenza viruses and their NA inhibitor-resistant counterparts (Table 1) were tested in four NA inhibition assays (Table 2). The results indicated that there was no substantial difference in zanamivir sensitivity of the wild-type A (N1 and N2) and B viruses (< 3-fold difference in IC_{50} values). Like wild-type, the oseltamivir-selected variant with an NA containing a His274 → Tyr substitution was sensitive to zanamivir in each of the four assays (Table 3). The zanamivir-selected mutant with an Arg292 → Lys replacement in its NA exhibited a low level

of zanamivir resistance (8–12-fold greater IC_{50} than of the wild-type virus) in each of the assays; the IC_{50} values ranged from 18 to 35 nM (Table 3). The mutants with substitutions at position 119 were zanamivir-resistant, although the level of resistance indicated by the different assays varied substantially (Table 3). The IC_{50} value that was measured by assay I for the virus with the Glu119 → Gly mutation was 10-fold greater than that measured by assay III (Table 3). Over 20-fold differences existed between the results of assays I and III for the mutant whose NA contained a Glu119 → Ala substitution (417-fold difference in IC_{50} values of the mutant and wild type virus in assay I, and 20-fold difference in assay III). Over 50-fold difference existed between the results of assays I and III for the mutant whose NA contained a Glu119 → Asp replacement (3333- vs. 60-fold). The data indicate that assay I was more sensitive than assay III. The sensitivity of the assays II and IV for these mutants was intermediate between the assays I and III.

In addition, assay I detected the greatest level of resistance for the mutant with an NA possessing an Arg152 → Lys substitution (3125-fold), whereas the levels of resistance estimated in assays III and IV were substantially lower (approximately 30-fold). Therefore, assay I was approximately 100-fold more sensitive than assays III and IV in detecting resistance of viruses with mutations at NA residue 152.

3.2. Assessment of resistance to oseltamivir carboxylate in NA inhibition assays

The Glu119 → Val substitution in the N2 enzyme was detected in the virus recovered from a patient treated with oseltamivir (Whitley et al., 2001). Because this mutant enzyme exhibited an approximately 20-fold decrease in sensitivity to oseltamivir carboxylate, we tested the sensitivity of the mutants with substitutions at NA residue 119 (Glu → Gly, Ala, or Asp) to oseltamivir carboxylate; each of these mutants was originally selected in the presence of zanamivir (Table 1). The wild-type virus (N2) and the mutants whose NA contained a Glu119 → Gly substitution were sensitive to oseltamivir carboxylate in each assay (Table 4); the IC_{50} values ranged from 0.2 to 1.0 nM. Compared with wild-type virus, the mutant with the NA possessing the Glu119 → Ala substitution exhibited a 27-fold decrease in sensitivity to oseltamivir carboxylate in assay I and a 15-fold decrease in sensitivity in assay II but minimal changes (approximately three-fold) in assays III and IV. The mutant containing the Glu119 → Asp replacement exhibited a decreased level of sensitivity to oseltamivir carboxylate in assay II (approximately seven-fold greater IC_{50} values) and assay IV (nine-fold greater IC_{50} values than for the wild-type virus) but not in assay I or III. The mutant with the Arg292 → Lys substitution was highly resistant to oseltamivir carboxylate

Table 2
Modifications of the NA inhibition assay developed by Potier et al. (1979)

Assay	Buffer system	Ca^{2+}, mM	MUNANA substrate, μM	Other components	Reference
I	0.1 M sodium phosphate, pH 5.9	6.8[a]	1000	None	Gubareva et al., 1997, 1998; Mitnaul et al., 1996; Goto et al., 1997
II	0.033 M MES, pH 6.5	4	100	2.5% DMS	Relenza™: Zanamivir Laboratory Manual, 2000
III	0.033 M MES, pH 6.5	4	75	None	Bantia et al., 2000
IV	0.1 M sodium acetate	10	100	None	Blick et al., 1995; McKimm-Breschkin et al., 1998
ND[b]	0.033 M MES, pH 6.5	4	10–100	None	Woods et al., 1993; Barnett et al., 2000; Tai et al., 1998; Li et al., 1998

[a] Concentration in the buffer used to dilute virus.
[b] ND, no designation. The assays that were not assigned a roman numeral were not evaluated in the present study.

Table 3
Assessment of zanamivir susceptibility of virus variants selected in the presence of zanamivir or oseltamivir

NA type and subtype	Amino acid[a]	Inhibition of NA activity by zanamivir							
		Assay I		Assay II		Assay III		Assay IV	
		IC_{50}[b]	Fold[c]	IC_{50}	Fold	IC_{50}	Fold	IC_{50}	Fold
A/N2	Glu 119 Arg 292 (wt)[d]	3.0	1.0	2.0	1.0	2.5	1.0	3.0	1.0
	Gly 119 Arg 292	1000	333	400	200	100	40	210	70
	Ala 119 Arg 292	1250	417	200	100	50	20	150	50
	Asp 119 Arg 292	10,000	3333	700	350	150	60	350	117
	Glu 119 **Lys** 292	35	12	18	9.0	20	8.0	24	8.0
A/N1	His 274 (wt)	2.0	1.0	0.9	1.0	1.5	1.0	1.5	1.0
	Tyr 274	2.5	1.3	1.0	1.1	1.8	1.2	2.0	1.3
B	Arg 152 (wt)	3.2	1.0	3.2	1.0	3.3	1.0	3.0	1.0
	Lys 152	10,000	3125	220	69	100	30	100	33

[a] Number indicates the location of the conserved residue in the N2 enzyme (Colman, 1989). Substitutions are shown in bold font.
[b] The average value of three or more measurements is shown.
[c] The concentration of inhibitor that requires for 50% inhibition of the NA activity.
[d] wt, wild-type.

(IC_{50} values, >1000 nM) in each of the four types of assays (Table 4); this similarity in results for this mutant resembles the pattern of results reported by other groups (McKimm-Breschkin et al., 1998; Tai et al., 1998). The oseltamivir-selected mutant (His274 → Tyr) demonstrated resistance to this inhibitor in each of the four assays, and the IC_{50} values ranged from 350 to more than 1000 nM (Table 4). In each assay, the wild-type virus (N1) was sensitive to the drug (range, 0.9–2 nM).

In contrast, the sensitivity of the wild-type B virus to oseltamivir carboxylate was much lower in assays I (40 nM) and II (28 nM) than in assays III (4.3 nM) and IV (8.0 nM). Thus there was an approximately 10-fold difference in the wild-type enzyme's sensitivity depending on assay conditions. The mutant containing the Arg152 → Lys substitution was highly resistant to oseltamivir carboxylate in each assay.

3.3. Assessment of sensitivity to BCX-1812 in NA inhibition assays

BCX-1812 demonstrated potent inhibitory effects against the wild-type enzymes of N1, N2, and B viruses in each of the four assays; the IC_{50} values ranged from 0.4 to 2.0 nM (Table 5). In addition, the mutants with either a Glu119 → Gly or Glu119 → Ala substitution were sensitive to BCX-1812 in the assays (highest IC_{50} value, 2.5 nM). The mutant containing the NA Glu119 → Asp mutation showed a 7–9-fold decrease in sensitivity in assays III and IV and an approximately 20–56-fold decrease in sensitivity in assays I and II (Table 5). The mutant with Tyr274 exhibited a moderate level of resistance to BCX-1812 in assays III and IV (42- and 50-fold, respectively), but a high level of resistance (875-fold) in assay I. The mutant whose NA contained an Arg152 → Lys substitution was resistant to all NA inhibitors, including BCX-1812.

3.4. Effects of substrate concentration and EDTA on the measurement of NA activity

The concentration of the substrate used in assay I was at least 10-fold higher than that used in the three other assays. Therefore, we evaluated the effect of reduced substrate concentration on the inhibition of the mutant (Arg152 → Lys) NA activity by zanamivir in assay I. When three 10-

Table 4
Assessment of oseltamivir carboxylate susceptibility of influenza viruses selected in the presence of NA inhibitors

Virus type and NA subtype	Amino acid[a]	Inhibition of NA activity by oseltamivir carboxylate							
		Assay I		Assay II		Assay III		Assay IV	
		IC_{50}[b]	Fold[c]	IC_{50}	Fold	IC_{50}	Fold	IC_{50}	Fold
A/N2	Glu 119 Arg 292 (wt)[d]	0.9	1.0	0.2	1.0	0.4	1.0	0.3	1.0
	Gly 119 Arg 292	1.0	1.1	0.6	3.0	0.5	1.3	0.3	1.0
	Ala 119 Arg 292	24	27	3.0	15	1.1	2.8	1.0	3.3
	Asp 119 Arg 292	3.0	3.3	1.3	6.5	0.5	1.3	2.7	9.0
	Glu 119 **Lys** 292	>1000[e]	>1000	3000	15,000	3750	9375	5000	16,666
A/N1	His 274 (wt)	2.0	1.0	0.9	1.0	2.0	1.0	0.9	1.0
	Tyr 274	>1000	>500	800	890	450	225	350	390
B	Arg 152 (wt)	40	1.0	28	1.0	4.3	1.0	8.0	1.0
	Lys 152	>1000	>25	1500	54	750	174	600	75

[a] Number indicates the location of the conserved residue in the N2 enzyme (Colman, 1989).
[b] The concentration of inhibitor that reduces the NA activity by 50%.
[c] The average values for three or more measurements are shown. The ratio of the IC_{50} value for the mutant and the IC_{50} value for the wild-type virus.
[d] wt, wild-type. The wild-type virus with Glu119 also contained Arg292.
[e] The highest concentration of oseltamivir carboxylate tested in assay I was 1000 nM.

fold dilutions of substrate (final concentration equals 1000, 100, or 10 μM) were tested, the estimated IC_{50} values were 10,000, 1500, and 400 nM, respectively. Thus, when the same substrate concentration was used in all four assays (approximately 100 μM), the IC_{50} value determined for this mutant in assay I was 15-fold higher than that estimated in assay III or IV and approximately seven-fold higher than that estimated in assay II.

Next, we compared the effect of EDTA on the activity of the enzymes of the wild-type viruses and on that of the enzymes of the mutants in the absence of NA inhibitors. The depletion of Ca^{2+} by EDTA from the reaction buffer resulted in a substantial reduction in the NA activity of the mutants with substitutions at residue 119 and, especially, at residue 152 (Table 6). In contrast, the activity of the wild-type enzymes was less sensitive to the addition of EDTA. In these experiments, the signal/noise ratios for the mutant containing Arg152 → Lys substitution were 9 and 6 in the absence of EDTA and in the presence of 0.6 mM of EDTA, respectively. In contrast, the ratios signal/noise were similar (equals 9) for the wild type virus. The enzymatic activity of the mutant containing the Arg292 → Lys substitution was even higher after addition of EDTA (up to 1 mM).

In addition, we tested the zanamivir susceptibility of the mutant whose NA possessed an Arg152 → Lys substitution in the presence of EDTA. We added EDTA at a concentration at which enzyme activity was reduced but measurable (0.6 mM) (Table 6). Under this condition, the estimated IC_{50} value for the mutant was 100 nM and this was similar to those determined in assays III and IV (100 nM) and was 15-fold lower than that estimated in assay I (1500 nM; substrate concentration equals 100 μM). Therefore, partial depletion of Ca^{2+} from the reaction mixture by EDTA caused reduction of the mutant NA activity but it did not increase sensitivity of the assay compared to assay I and was similar to results of assays in which the buffer was supplemented with Ca^{2+}.

Table 5
Assessment of BCX-1812 susceptibility of influenza viruses selected in the presence of zanamivir or oseltamivir

Virus type and NA subtype	Amino acid[a]	Inhibition of NA activity by BCX-1812							
		Assay I		Assay II		Assay III		Assay IV	
		IC_{50}[b]	Fold[c]	IC_{50}	Fold	IC_{50}	Fold	IC_{50}	Fold
A/N2	Glu 119 Arg 292 (wt)[d]	1.7	1.0	0.4	1.0	1.1	1.0	1.3	1.0
	Gly 119 Arg 292	2.5	1.5	1.9	4.8	1.8	1.6	2.0	1.5
	Ala 119 Arg 292	2.0	1.2	0.9	2.3	1.6	1.5	1.3	1.0
	Asp 119 Arg 292	95	56	8.0	20	9.5	8.6	8.5	6.5
	Glu 119 **Lys** 292	30	18	16	40	30	27	27	21
A/N1	His 274	0.6	1.0	0.4	1.0	1.2	1.0	0.8	1.0
	Tyr 274	525	875	40	100	50	42	40	50
B	Arg 152	2.0	1.0	1.0	1.0	1.4	1.0	1.8	1.0
	Lys 152	>1000[e]	>500	1500	400	570	407	1000	555

[a] Number indicates the location of the conserved residue in the N2 enzyme (Colman, 1989).
[b] The concentration of inhibitor that requires for 50% inhibition of the NA activity. The average *b* values of three or measurements are shown.
[c] The ratio of the mean IC_{50} value for the mutant and the IC_{50} value for the wild-type virus.
[d] wt, wild-type.
[e] The highest concentration of BCX-1812 tested in assay I was 1000 nM.

Table 6
Effect of EDTA in the NA assays of wild-type and drug-resistant virus variants

Amino acid in wild-type or mutant NA (virus type and NA subtype)	Change (%) in NA activity[a]		
	+0.6 mM EDTA	+0.8 mM EDTA	+1.0 mM EDTA
Arg152 (B)	+14	+1	−31
Lys152 (B)	−20	−100	−100
Glu119, Arg292 (A/N2)	+4	−4	−51
Gly119 (A/N2)	−26	−50	NT[b]
Ala119 (A/N2)	−39	−41	NT
Asp119 (A/N2)	−39	−56	−76
Lys292 (A/N2)	+60	+36	+2

[a] The change (%) in NA activity was determined by comparing the enzyme activity in solutions of 33 mM MES, pH 6.5, and 0.6, 0.8, or 1.0 mM EDTA with the enzyme activity in a solution of 33 mM MES, pH 6.5, that contained no EDTA. The average values of three or more measurements are shown.

[b] NT, not tested. Substitutions are shown in bold font.

4. Discussion

The cell-based assays have been previously shown unreliable for susceptibility monitoring of viruses recovered from humans treated with the novel anti-influenza drugs, neuraminidase inhibitors (Gubareva et al., 1998, 2001; Tisdale, 2000; Zambon and Hayden, 2001). The NA inhibition assay is the primary assay for susceptibility monitoring that allows detecting virus variants with changes in the NA active site (McKimm-Breschkin, 2000). In the present study we compared the sensitivity of the four most commonly used modifications of NA inhibition assay developed by Potier et al. (1979) and demonstrated that they are not equally sensitive in detecting resistant viruses of defined genotype. In our experiments, the greatest differences in IC_{50} values of wild-type and drug-selected mutants were usually seen in assays I and II (Table 2). The mutant with NA containing replacement in the enzyme's active center Glu119 → Ala did not appear to be resistant to oseltamivir carboxylate in assays III and IV (Table 4), although this mutant did show resistance in assays I and II. The mutant with the replacement Glu119 → Asp was fully sensitive to oseltamivir carboxylate based on assays I and III, although it showed a low level of resistance in assays II and IV. Our finding that residue 119 may be involved in the development of resistance to oseltamivir has been corroborated by the isolation of a mutant with a Glu119 → Val substitution from a patient treated with an NA inhibitor (Whitley et al., 2001). The mutants with Gly or Ala at position 119 were sensitive to BCX-1812 in each assay. This result suggested that BCX-1812 could be effective against these zanamivir-resistant mutants. However, the results of assay I indicated that the mutant containing the Glu119 → Asp substitution in its NA exhibited a 60-fold increase in IC_{50} values to BCX-1812. Therefore, despite the difference in the design of the three inhibitors, treatment with any of the NA inhibitors might result in the emergence of mutants in which Glu119 is replaced. The replacement of framework residue 274 (His → Tyr) in NA (N1 type) led to a decrease in the virus's sensitivity to oseltamivir carboxylate and BCX-1812. However, this oseltamivir-selected mutant was uniformly sensitive to zanamivir in all four assays. Wang et al. (2000b) demonstrated that substitution of His274 with Gly, Ser, Asn, or Gln resulted in resistance to zanamivir but not to oseltamivir carboxylate. Overall, substitutions at framework residues, such as those at position 119 or 274, could lead to resistance to any NA inhibitor (zanamivir, oseltamivir carboxylate, or BCX-1812), but typically the mutant retains sensitivity to at least one inhibitor.

The resistance profile of the mutants with substituted functional residues in NA was different from that of variants with substituted framework residues. The Arg152 → Lys and Arg292 → Lys substitutions resulted in cross-resistance to all three NA inhibitors in each assay. Although the mutant with the Arg292 → Lys substitution was selected in the presence of zanamivir, it exhibited a high level of resistance to oseltamivir carboxylate and a substantially lower level of resistance to BCX-1812 (Tables 4 and 5). This result illustrated that some mutants (e.g. those with NA containing the Arg292 → Lys replacement) could be detected more effectively with an inhibitor (e.g. oseltamivir carboxylate) that is different from that used for treatment (e.g. zanamivir or BCX-1812). Such approach could allow early detection of minority drug-resistant species emerging in the virus population.

The replacement at position 292 is the most common substitution in the NA of NA inhibitor-selected mutants (Table 1). In contrast, the substitution at residue 152 has been detected in a single strain isolated from a patient treated with zanamivir (Table 1). We previously reported that the mutant NA with the Arg152 → Lys replacement demonstrated substantially reduced enzymatic activity in vitro and reduced infectivity and virulence in ferrets (L.V. Gubareva and R.G. Webster, unpublished data; Gubareva et al., 1998). In the present study, we also showed that the NA activity of this mutant is more sensitive to EDTA than is the wild-type enzyme (Table 6).

The mutant NA with the Glu119 → Gly substitution is reportedly more sensitive to changes in temperature, the presence of formaldehyde, and other manipulations done with the purified enzyme than is the wild-type N9 enzyme (Sahasrabudhe et al., 1998). We also reported that the mutant NAs with Asp, Ala, or Gly substitutions at residue 119 are less stable than the parental enzyme at low pH and elevated temperature (Gubareva et al., 1997). The mutations in the NA active site could alter the pH optimum of the enzyme (Lentz et al., 1987; Gubareva et al., 1997). Thus, it is possible that some components of the reaction mixture could also affect the interaction of the mutant enzyme with the inhibitor. The presence of DMSO in the buffer could be responsible for the difference between the IC_{50} values determined in assay II compared to assay III for the mutants with substitutions at position 119, although DMSO had lesser effect on the IC_{50} values estimated for BCX-1812 (Table 5).

Ca^{2+} was present in the reaction buffer in assays II, III, and IV but were absent in the buffer (0.1 M sodium phosphate buffer) of the assay I. It is known that Ca^{2+} specifically affects the interaction of substrate with NA (Chong et al., 1991). Two distinct types of Ca^{2+} binding sites have been found in the NA tetramer (Burmeister et al., 1992, 1994). The site of weak affinity is located on the four-fold axis (central Ca^{2+}), and the sites of high affinity are located near the active site (i.e. in the vicinity of Arg292) of each subunit. This central Ca^{2+} may be important in holding together the tetramer (Burmeister et al., 1992). Although each monomer has an enzyme active center, NA is enzymatically active only in the form of a tetramer (Bucher and Kilbourne, 1972). The binding of the central Ca^{2+} may promote a conformational change in the enzyme active site. Chong et al. (1991) speculated that the binding of Ca^{2+} at the subunit interface causes the two loops of the polypeptide chain of the enzyme to move and that this movement results in the reorientation of the active-site residues Glu119, Asp151, and Arg152. In this way, binding of substrates and inhibitors is optimized (Burmeister et al., 1994). It has also been demonstrated that the central Ca^{2+} can be easily removed by EDTA (Burmeister et al., 1994). Our results support this earlier finding. The activity of the mutant enzymes with substitutions at residues 119 or 152 was much more sensitive to the addition of EDTA than the activity of the wild-type NA. The mutant with the NA containing the Arg292 → Lys substitution near the high-affinity Ca^{2+} binding site was not sensitive to EDTA (up to 1 mM), and this mutant NA even exhibited increased activity after the addition of EDTA (the pH of the reaction buffer was unaltered). It is important to notice that the media in which viruses were collected contained Ca^{2+} and Mg^{2+}, and we did not perform dialysis of virus preparations before the measurement of NA activity.

Zanamivir-selected mutants with substitutions at residue 119 or 152 were more resistant to the inhibitory effect of zanamivir in buffer containing 0.1 M sodium phosphate (assay I). However, the zanamivir resistance of the mutant with the Arg152 → Lys substitution was similar when the assay was performed in buffer 33 mM MES, pH 6.5, supplemented with either 0.6 mM EDTA or 4 mM $CaCl_2$. More detailed biochemical studies are required to analyze the effect of phosphate ions and other factors on the interaction of the mutant enzymes with the substrate and inhibitors. The practical consideration is that detection of the mutant enzymes with substitutions at residues 119 or 152 could be more efficient in an NA inhibition assay that uses the phosphate buffer and a high concentration (1 mM) of substrate. Although a high concentration of the substrate could aid in the detection of the mutant NAs, it also could increase the background noise (Zambon and Hayden, 2001). A high concentration of substrate was employed in the monitoring of resistance in the clinical study with oseltamivir (Hayden et al., 2000b).

Although the active site of NA is conserved among influenza A and B viruses, there is a notable degree of variability in the features of the enzyme. The sensitivity of influenza B virus to oseltamivir carboxylate was as much as 10-fold lower in assays I and II than that of the same virus in assays III and IV. This result indicates that the assay conditions affected not only interactions between the mutant enzymes and inhibitors but also interactions between certain wild-type enzymes and inhibitors.

Monitoring virus resistance to NA inhibitors is currently based on NA inhibition assays in which the estimated IC_{50} values of viruses isolated before treatment are compared with those of viruses isolated after treatment. Such an approach poses certain difficulties in the interpretations of the results of the analysis. For example, the 8–10-fold decrease in zanamivir or BCX-1812 sensitivity of the mutant with the Arg292 → Lys substitution indicates that this variant has reduced susceptibility (McKimm-Breschkin, 2000). However, the concentration of zanamivir required to inhibit the activity of this mutant enzyme by 50% is only 20 nM (assay III). The same concentration of NA inhibitor could be equal to IC_{50} values estimated for certain wild-type enzymes, and such enzymes would not be considered resistant to zanamivir. In addition, the mutant with the Glu119 → Asp substitution exhibited a low level of resistance (approximately nine-fold) to oseltamivir carboxylate in this study and could be considered drug-resistant (Table 4). However, only 2.3 nM of this inhibitor is required to inhibit 50% of the activity of this mutant enzyme. It is important that the assay used for monitoring is sensitive enough to detect differences in the levels of inhibition of wild-type and mutant NAs; however, the impact of amino acid substitution in the NA on virus fitness, and viral drug resistance should be evaluated in experiments in vivo because it is not practical to reproduce conditions existing at the site of virus replication in the host respiratory tract in in vitro experiments. Clinical consequences of reduced susceptibility will be determined by examining antiviral effects and clinical endpoints in vivo.

The accumulated experience in detection of the mutants carrying resistance-associated mutations in the NA with the use of a fluorometric assay provide a good foundation for monitoring resistance to NA inhibitors. We have demonstrated in the present study that the use of the 0.1 M sodium phosphate buffer (assay I) affords the highest sensitivity of detection of mutated enzymes in comparison to the other buffers examined. However, the NA enzymatic activity of the mutated enzymes is often reduced in comparison to the wild type viruses, which could pose certain problems in monitoring of resistance in the clinical isolates. Thus, for the same virus preparation the ratio signal/noise was at least two-fold lower in assay I in comparison to other assays. Recently, a more sensitive NA assay that employs a chemiluminescent substrate (1,2-dioxetane derivative of sialic acid, NA-STAR) was developed (Buxton et al., 2000). It substantially increases the limit of detection of the NA activity. However, the buffer system used in the chemiluminescent assay was the same as in the fluorometric assay III which we found to be the less favorable for detection of resistant viruses. Therefore, the use of the 0.1M

phosphate buffer in the chemiluminescent NA assay may increase its sensitivity in detection of resistance in clinical settings and this possibility should be examined.

Regardless of which NA inhibition assay is being adopted for monitoring of virus susceptibility to NA inhibitors, a use of a panel of well-characterized mutants, included as positive controls, would insure adequacy of the chosen assay conditions.

Acknowledgements

This work was supported in part by grant AI-45782 (L.V.G.), AI-08831 and AI-29680 (R.G.W.) from the National Institute of Allergy and Infectious Diseases, by a grant from the R.W. Johnson Pharmaceutical Research Institute (L.V.G.), and by the American Lebanese Syrian Associated Charities (ALSAC). We thank Douglas W. Schallon from the University of Virginia for excellent technical assistance and Dr Laurie Twit from St. Jude Children's Research Hospital for editorial work.

References

Babu, Y.S., Chand, P., Bantia, S., Kotian, P., Dehghani, A., El-Kattan, Y., Lin, T.H., Hutchison, T.L., Elliott, A.J., Parker, C.D., Ananth, S.L., Horn, L.L., Laver, G.W., Montgomery, J.A., 2000. BCX-1812 (RWJ-270201): discovery of a novel, highly potent, orally active and selective influenza neuraminidase inhibitor through structure-based drug design. J. Med. Chem. 43, 3482–3486.

Bantia, S., Ananth, S.L., Horn, L.L., Parker, C.D., Gulati, U., Chand, P., Babu, Y.S., Air, G.M., 2000. Generation and characterization of a mutant of influenza A virus selected with the neuraminidase inhibitor RWJ-270201. Antivir. Res. 46, A60 (Abstract) 82.

Barnett, J.M., Cadman, A., Burrell, F.M., Madar, S.H., Lewis, A.P., Tisdale, M., Bethell, R., 1999. *In vitro* selection and characterisation of influenza B/Beijing/1/87 isolates with altered susceptibility to zanamivir. Virology 265, 286–295.

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.

Blick, T.J., Tiong, T., Sahasrabudhe, A., Varghese, J.N., Colman, P.M., Hart, G.J., Bethell, R.C., McKimm-Breschkin, J.L., 1995. Generation and characterization of an influenza virus neuraminidase variant with decreased sensitivity to the neuraminidase-specific inhibitor 4-guanidino-Neu5Ac2en. Virology 214, 475–484.

Bucher, D.J., Kilbourne, E.D., 1972. A 2 (N2) neuraminidase of the X-7 influenza virus recombinant: determination of molecular size and subunit composition of the active site. J. Virol. 10, 60–66.

Burmeister, W.P., Ruigrok, R.W., Cusack, S., 1992. The 2.2 Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. EMBO J. 11, 49–56.

Burmeister, W.P., Cusack, S., Ruigrok, R.W., 1994. Calcium is needed for the thermostability of influenza B virus neuraminidase. J. Gen. Virol. 75, 381–388.

Buxton, R.C., Edwards, B., Juo, R.R., Voyta, J.C., Tisdale, M., Bethell, R.C., 2000. Development of a sensitive chemiluminescent neuraminidase assay for the detection of influenza virus susceptibility to zanamivir. Anal. Biochem. 280, 291–300.

Chong, A.K., Pegg, M.S., von Itzstein, M., 1991. Influenza virus sialidase: effect of calcium on steady-state kinetic parameters. Biochim. Biophys. Acta 1077, 65–71.

Colman, P.M., 1989. Neuraminidase: enzyme and antigen. In: Krug, R.M. (Ed.), The Influenza Viruses. Plenum Press, New York, pp. 175–210.

Colman, P.M., Hoyne, P.A., Lawrence, M.C., 1993. Sequence and structure alignment of paramyxovirus hemagglutinin–neuraminidase with influenza virus neuraminidase. J. Virol. 67, 2972–2980.

Goto, H., Bethell, R.C., Kawaoka, Y., 1997. Mutations affecting the sensitivity of the influenza virus neuraminidase to 4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid. Virology 238, 265–272.

Gubareva, L.V., Bethell, R., Hart, G.J., Murti, K.G., Penn, C.R., Webster, R.G., 1996. Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. J. Virol. 70, 1818–1827.

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.

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

Gubareva, L.V., Kaiser, L., Matrosovich, M.N., Soo-Hoo, Y., Hayden, F.G., 2001. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. J. Infect. Dis. 183, 523–531.

Hay, A.J., Gramas, S., Bennet, M.S., 1991. Resistance of influenza A viruses to amantadine and rimantadine. In: Kumar, A. (Ed.), Advances in Molecular Biology and Targeted Treatment for AIDS. Plenum Press, New York, pp. 345–353.

Hayden, F.G., 1996. Amantadine and rimantadine—clinical aspects. In: Richman, D.D. (Ed.), Antiviral Drug Resistance. John Wiley & Sons Ltd., New York, pp. 59–77.

Hayden, F.G., Treanor, J.J., Fritz, R.S., Lobo, M., Betts, R.F., Miller, M., Kinnersley, N., Mills, R.G., Ward, P., Straus, S.E., 1999. Use of the oral neuraminidase inhibitor oseltamivir in experimental human influenza: randomized controlled trials for prevention and treatment. J. Am. Med. Assoc. 282, 1240–1246.

Hayden, F.G., Gubareva, L.V., Monto, A.S., Klein, T.C., Elliott, M.J., Hammond, J.M., Sharp, S.J., Ossi, M.J., 2000a. Inhaled zanamivir for the prevention of influenza in families. N. Engl. J. Med. 343, 1282–1289.

Hayden, F.G., Jennings, L., Robson, R., Schiff, G., Jackson, H., Rana, B., McClelland, G., Ipe, D., Roberts, N., Ward, P., 2000b. Oral oseltamivir in human experimental influenza B infection. Antivir. Ther. 5, 205–213.

Kimberlin, D.W., Spector, S.A., Hill, A.L., Biron, K.K., Hay, A.J., Mayers, D.L., Whitley, R.J., 1995. Assays for antiviral drug resistance. Antivir. Res. 26, 403–413.

Lentz, M.R., Webster, R.G., Air, G.M., 1987. Site-directed mutation of the active site of influenza neuraminidase and implications for the catalytic mechanism. Biochemistry 26, 5351–5358.

Li, W., Escarpe, P.A., Eisenberg, E.J., Cundy, K.C., Sweet, C., Jakeman, K.J., Merson, J., Lew, W., Williams, M., Zhang, L., Kim, C.U., Bischofberger, N., Chen, M.S., Mendel, D.B., 1998. Identification of GS 4104 as an orally bioavailable prodrug of the influenza virus neuraminidase inhibitor GS 4071. Antimicrob. Agents Chemother. 42, 647–653.

McKimm-Breschkin, J.L., Sahasrabudhe, A., Blick, T.J., McDonald, M., Colman, P.M., Hart, G.J., Bethell, R.C., Varghese, J.N., 1998. Mutations in a conserved residue in the influenza virus neuraminidase active site decreases sensitivity to Neu5Ac2en-derived inhibitors. J. Virol. 72, 2456–2462.

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

Mitnaul, L.J., Castrucci, M.R., Murti, K.G., Kawaoka, Y., 1996. The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology and virulence in mice but is not essential for virus replication. J. Virol. 70, 873–879.

Palese, P., Compans, R.W., 1976. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA): mechanism of action. J. Gen. Virol. 33, 159–163.

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.

Relenza™: Zanamivir Laboratory Manual. 1-1-2000.

Sahasrabudhe, A., Lawrence, L., Epa, V.C., Varghese, J.N., Colman, P.M., McKimm-Breschkin, J.L., 1998. Substrate, inhibitor or antibody stabilizes the Glu 119 Gly mutant influenza virus nueraminidase. Virology 247, 14–21.

Sidwell, R.W., Smee, D.F., Huffman, J.H., Barnard, D.L., Bailey, K.W., Morrey, J.D., Babu, Y.S., 2001. In vivo influenza virus-inhibitory effects of the cyclopentane neuraminidase inhibitor RWJ-270201. Antimicrob. Agents Chemother. 45, 749–757.

Smee, D.F., Huffman, J.H., Morrison, A.C., Barnard, D.L., Sidwell, R.W., 2001. Cyclopentane neuraminidsae inhibitors with potent in vitro anti-influenza virus activities. Antimicrob. Agents Chemother. 45, 743–748.

Staschke, K.A., Colacino, J.M., Baxter, A.J., Air, G.M., Bansal, A., Hornback, W.J., Munroe, J.E., Laver, W.G., 1995. Molecular basis for the resistance of influenza viruses to 4-guanidino-Neu5Ac2en. Virology 214, 642–646.

Steinhauer, D.A., Wharton, S.A., Skehel, J.J., Wiley, D.C., Hay, A.J., 1991. Amantadine selection of a mutant influenza virus containing an acid-stable hemagglutinin glycoprotein: evidence for virus-specific regulation of the pH of glycoprotein transport vesicles. Proc. Natl. Acad. Sci. USA 88, 11 525–11 529.

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.

Tisdale, M., 2000. Monitoring of viral susceptibility: new challenges with the develoment of influenza NA inhibitors. Rev. Med. Virol. 10, 45–55.

Wang, Z.M., Tai, C.Y., Mendel, D.B., 2000a. Characterization of an influenza A virus variant selected in vitro in the presence of the neuraminidase inhibitor GS4071. Antivir. Res. 46, A60 Abstract 80.

Wang, Z.M., Tai, C.Y., Mendel, D.B., 2000b. Studies on the mechanism by which mutations at His274 alter sensitivity of influenza A virus neuraminidase type 1 to GS4071 and zanamivir. Antivir. Res. 46, A60 (Abstract) 81.

Whitley, R.J., Hayden, F.G., Reisinger, K.S., Young, N., Dutkowski, R., Ipe, D., Mills, R.G., Ward, P., 2001. Oral oseltamivir treatment of influenza in children. Pediatr. Infect. Dis. J. 20, 127–133.

Woods, J.M., Bethell, R.C., Coates, J.A., Healy, N., Hiscox, S.A., Pearson, B.A., Ryan, D.M., Ticehurst, J., Tilling, J., Walcott, S.M., Penn, C.R., 1993. 4-Guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid is a highly effective inhibitor both of the sialidase (neuraminidase) and of growth of a wide range of influenza A and B viruses in vitro. Antimicrob. Agents Chemother. 37, 1473–1479.

Zambon, M., Hayden, F.G., 2001. Position statement: global neuraminidase inhibitor susceptibility network. Antivir. Res. 49, 147–156 (Review).