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Susceptibilities of Antiviral-Resistant Influenza Viruses to Novel Neuraminidase Inhibitors

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The susceptibilities of five zanamivir-resistant and six oseltamivir-resistant influenza viruses were assessed against four neuraminidase (NA) inhibitors, including peramivir and A-315675, by a fluorometric NA activity inhibition assay. The enzyme activity of a majority of the variants was effectively inhibited by either A-315675 or both peramivir and A-315675 (50% inhibitory concentration, <10 nM). A novel oseltamivir-resistant influenza virus B variant carrying substitution at residue 198 (Asp→Asn) (N2 numbering) retained susceptibility to peramivir and A-315675. In vivo, the Asn198 variant showed no apparent fitness impairment as judged by its recovery on day 5 from the nasal washes of ferrets coinfected with equal doses of the wild-type virus and the Asn198 variant. Based on the sequence analysis of the virus in the nasal washes, oseltamivir treatment (5 mg/kg twice daily for 5 days) did not provide growth advantage to the Asn198 variant. Nevertheless, treatment with A-315675 (prodrug A-322278) reduced the number of the animals (two of seven) shedding the Asn198 variant. These studies indicate that different patterns of susceptibility and cross-resistance between NA inhibitors may prove important if antiviral resistance to zanamivir and oseltamivir were to emerge.

Influenza A and B viruses cause significant morbidity and mortality in humans, even in otherwise healthy individuals (24). Influenza A viruses pose an especially serious threat due to their ability to produce pandemics (41). Wild and domestic birds provide a natural reservoir of influenza A viruses (38). Antigenic diversity among avian viruses represented by 16 antigenic subtypes of hemagglutinin (HA) (9) and nine subtypes of neuraminidase (NA) in various combinations poses challenges for design of effective vaccines and drugs.

Two classes of anti-influenza virus antiviral agents targeting either the M2 ion channel or the neuraminidase are currently available for influenza management and under consideration for stockpiling in the event of an influenza pandemic. However, use of the M2 blockers, amantadine and rimantadine, is limited by a lack of inhibitory effect against influenza B viruses, side effects, and a rapid emergence of antiviral resistance (18). M2 inhibitor-resistant variants are transmissible from person to person, pathogenic, and can be recovered occasionally from untreated individuals. Importantly, recent human isolates of highly virulent A/H5N1 influenza viruses are naturally resistant to these drugs (36).

The two NA inhibitors are the only drugs, besides the M2 inhibitors, that have been approved for treating influenza virus infections in humans are zanamivir and oseltamivir (13). Understandably, the molecular basis of virus resistance to NA inhibitors has been a focus of intensive studies (30). Drug escape variants selected in vitro and in vivo typically contain single substitutions at amino acid residues forming the NA active site. In clinical settings, emergence of resistance to zanamivir detected in a single immunocompromised child was associated with the substituted catalytic residue Arg152 (15) (Table 1). Oseltamivir-resistant variants have been uncommonly detected in treated adults (0.4%) (8, 14, 34), but the incidence of drug resistance has been greater (up to 18%) in oseltamivir-treated hospitalized children (27, 40). In addition, prolonged shedding of resistant variants, including those resistant to both oseltamivir and amantadine/rimantadine, has been documented in immunocompromised patients (11, 39).

The emergence of oseltamivir resistance in clinical isolates has been associated with substitutions at residue 119, 198, 274, 292, or 294 in the NA active site (14, 27, 40) (Table 1). Zanamivir has been approved by the Food and Drug Administation for treatment of uncomplicated influenza infections in patients of 7 years of age or older. The delivery of zanamivir to the respiratory tract requires an inhaling device and, the drug is not recommended for treatment of people with chronic respiratory disease. For these reasons and commercial considerations, the use of the drug has been limited. Oseltamivir is orally bioavailable; this property makes it a better candidate for stockpiling. It is imperative, therefore, to identify alternative, orally bioavailable NA inhibitors that could be active against oseltamivir-resistant viruses. Consequently, we assessed the inhibitory effects of two novel neuraminidase inhibitors, peramivir (2) and A-315675 (prodrug A-322278) (25), against oseltamivir- and zanamivir-resistant variants. We also assessed in vivo activity of A-322278 against a recently identified variant.

MATERIALS AND METHODS

Compounds. Zanamivir (GG167) was provided by GlaxoWellcome Research and Development (Stevenage, United Kingdom); oseltamivir carboxylate (GS4071) and its prodrug oseltamivir (GS4104) were provided by Roche Laboratories, Inc. (Nutley, NJ); peramivir (BCX-1812, RWJ-270201) was provided by

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TABLE 1. Susceptibilities of influenza viruses and their drug-resistant variants to neuraminidase inhibitors in the NA inhibition assay

Virus[a]	Variant[b]	Selection		Substitution in NA[c]	Susceptibility[d]							
					Zanamivir		Oseltamivir carboxylate		A-315676		Peramivir	
		Site	Drug used		IC_{50} (nM)	Fold change	IC_{50} (nM)	Fold change	IC_{50} (nM)	Fold change	IC_{50} (nM)	Fold change
A/TX/91 (H1N1)	WT				0.9		1.4		1.6		0.4	
	M1	In vivo	Oseltamivir	H274Y	1.0	1	>1,000	>700	5.5	3	40	100
A/NY/2001 (H1N1)	WT				0.8		2.0		0.6		0.3	
	M2	In vivo	Oseltamivir	H/Y274[e]	0.9	1	220	110	1.2	2	2.4	8
A/Jap/57 (H2N2)	WT				3.0		0.6		0.7		0.4	
	M3	In vivo	Oseltamivir	R292K	15	5	>1,000	>1,600	5.2	8	28	80
A/turkey/80 (H4N2)	WT				2.0		0.4		1.2		0.6	
	M4	In vitro	Zanamivir	R292K	15	8	3,000	15,000	7	6	26	43
	M5	In vitro	Zanamivir	E119G	400	200	0.6	2	1.6	1	1.5	2
	M6	In vitro	Zanamivir	E119A	200	100	3.5	9	1.4	1	0.8	1
	M7	In vitro	Zanamivir	E119D	645	323	1.8	4.5	56	46	19	33
A/TX/2002 (H3N2)	WT				2.0		0.8		1.1		1.3	
	M8	In vivo	Oseltamivir	E119V	2.5	1	105	130	1.6	1	1.5	1
A/CH/2004 (H3N2)	WT				1.0		0.3		0.5		0.4	
	M9	In vivo	Oseltamivir	E119V	2.8	3	83	277	0.4	1	0.5	1
B/Mem/96	WT				3.2		4.0		2.0		1.0	
	M10	In vivo	Zanamivir	R152K	220	70	400	100	275	150	400	400
B/Roch/2001	WT				1.6		33		0.7		1.2	
	M11	In vivo	Oseltamivir	D198N	15	9	304	9	1.5	2	5.8	4.8

[a] Full virus names: A/Texas/36/91, A/New York/02/2001, A/Japan/305/57, A/turkey/Minnesota/833/80, A/Texas/131/2002, A/Charlottesville/03/2004, B/Memphis/20/96, and B/Rochester/02/2001.

[b] WT, wild type; M, drug-selected variants.

[c] N2 amino acid numbering; residues 292 and 152 are catalytic residues and the remaining residues belong to the framework in the NA active site.

[d] Average IC_{50} values of at least three measurements are shown. Fold increase in IC_{50} value compared to that of the wild type (IC_{50}/IC_{50} of wild type).

[e] The presence of both wild-type (His274) and variant (Tyr274) residues was detected in the virus sample.

BioCryst, Inc. (Birmingham, AL); and A-315675 and its isopropyl ester prodrug (A-322278) were from Abbott Laboratories (Abbott Park, IL).

Viruses and cell cultures. The viruses used in the study were from the repository of the Respiratory Infectious Disease Study Unit at the University of Virginia. The oseltamivir-resistant variant was recovered from a volunteer who was experimentally infected with the A/Texas/36/91 (H1N1) virus and treated with oseltamivir (14, 19). The variants of the A/New York/02/2001(H1N1), the A/Texas/131/2002 (H3N2), the A/Charlottesville/03/2004 (H3N2), and the B/Rochester/02/2001 viruses were recovered from oseltamivir-treated immunocompromised patients (11, 39). The B/Memphis/20/96 strain was from an immunocompromised patient treated with zanamivir (15). The variant of the A/Japan/305/57 (H2N2) virus was recovered from an oseltamivir-treated mouse (Ison et al., submitted for publication). The zanamivir-resistant variants of the influenza A/turkey/Minnesota/833/80 (H4N2) virus were selected in MDCK cell culture in the presence of the drug (12, 16). Viruses were propagated in Madin-Darby canine kidney (MDCK) cells by a standard procedure and stored at −80°C before they were used in the NA activity inhibition assay.

NA activity inhibition assay. A fluorometric assay modified from that of Potier et al. (33) was used to measure the influenza virus NA activity as previously described in detail (17). The 50% inhibitory concentration (IC_{50}) value, which is the concentration of drug that is required to inhibit the enzyme activity by 50%, was determined by assaying the NA activity of virus in the presence of serial half-log dilutions (from 10 μM to 0.01 nM) of each NA inhibitor. After equal volumes of virus and inhibitor had been mixed and incubated at room temperature for 30 min, fluorogenic substrate (2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid; Sigma, St. Louis, MO) was added at a final concentration of 100 mM, and the reaction mixture was then incubated at 37°C for 1 h. The reaction was stopped by addition of the stop solution (150 μl of 0.5 M NaOH, pH 10.7, containing 25% ethanol), and fluorescence was measured with the use of Victor.3 1420 multilabel counter (Perkin Elmer, Boston, MA). The excitation wavelength was 365 nm, and the emission wavelength was 460 nm.

Animal model and experimental design. The experiment was carried out at the Center for Comparative Medicine at the University of Virginia accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International in accordance with the protocol approved by the Institutional Animal Care Committee. Hemagglutination inhibition (HI) assays were performed on the sera of ferrets prior to infection to establish a lack of prior immunity of the animals (HI titers $<$ 1:10). Twenty-nine young female ferrets (0.6 to 0.8 kg) obtained from Marshall Farms (North Rose, NY) were randomly divided into three groups: oseltamivir (prodrug), A-322278 (prodrug), and placebo (sterile phosphate-buffered saline). Drugs were given orally at 5 mg/kg starting 2 h before virus challenge and then treatment was continued twice daily for 5 days.

The virus inoculum was a mixture of the wild-type and oseltamivir-resistant variant (6×10^3 tissue culture infectious doses [$TCID_{50}$]/ml and 7×10^3 $TCID_{50}$/ml, respectively). Virus inoculum was administered intranasally (0.5 ml into each nostril) while animals were under light anesthesia (ketamine, 25 mg/kg, intramuscularly). Nasal washes were collected in 1 ml of sterile phosphate-buffered saline from each animal on days 1, 2, 3, 5, and 7 postchallenge. After centrifugation, the nasal washes, free of cellular debris, were used to extract viral RNA and determine the viral titers in MDCK cell culture. The limit of infectious virus detection was equal to 1.4 $log_{10}TCID_{50}$ per 1 ml of nasal wash.

Virus gene sequence analysis. Extraction of vRNA from nasal wash and MDCK cell supernatants was performed with the use of RNAeasy kit (QIAGEN, Clarita, CA) according to the manufacturer's manual. Primer U9 5′-AGCAGA AGC-3′ was used to generate cDNA with the use of the MMLV reverse transcriptase (Promega, Madison, WI). The NA gene fragment was then amplified in a standard PCR with the use of 5′-GCTCTAACCCATTATGCAG-3′ and 5′-C TTTCTTGTGTTCTTAGGATG-3′. The PCR products were purified with the use of a QIAquick PCR purification kit (QIAGEN, Clarita, CA) according to the manufacturer's instruction. Purified PCR products were sequenced with the use of *Taq* Dye terminator chemistry (Applied Biosystems, Foster City, CA) and then analyzed on an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA) at the Center of Biotechnology at the University of Virginia. Sequencher 4.0 software (Gene Codes Corporation, Ann Arbor, MI) was used for the analysis and translation of the nucleotide sequence data.

RESULTS

Comparison of drug susceptibility in the NA inhibition assay. To evaluate the usefulness of peramivir and A-322278 as alternative anti-influenza drugs, we utilized a panel of drug-

resistant variants carrying the enzymes of three antigenic (sub-)types known to cause infections in humans, A/N1, A/N2, and B. The 11 variants were either selected in cell culture in the presence of NA inhibitor ($n = 4$) or recovered after drug treatment in vivo ($n = 7$). In the NA inhibition assay, eight wild-type parental viruses were highly susceptible to all four inhibitors with IC_{50} values in a subnanomolar to a low nanomolar range with one exception (Table 1). The IC_{50} value determined for the B/Rochester/02/2001 virus against oseltamivir was approximately 10-fold higher (33 nM).

Criteria for resistance. No generally accepted criteria for antiviral resistance to NA inhibitors have been developed. In susceptibility assays, both the extent of change compared to parental virus (fold change) and the absolute drug concentration required for inhibition are relevant. Therefore, it was important, to establish the criteria of drug resistance based on the IC_{50} values assessed under the same test conditions (17). To this end, the IC_{50} values determined for zanamivir against the zanamivir-selected variants ranged from 15 nM to 645 nM (Table 1, M4 and M7), which constituted an 8-fold and a 323-fold increase compared to the IC_{50} values for the corresponding wild-type viruses. When oseltamivir carboxylate was tested against the oseltamivir-selected variants, the IC_{50} values ranged for 83 nM to >1,000 nM (Table 1, M9 and M1) and the increases in the IC_{50} values ranged from 130-fold to 15,000-fold (Table 1, M8 and M4) with one exception. The substitution 198(Asp→Asn) (Table 1, M11) caused only a ninefold increase in the IC_{50} value compared to that of the wild type. However, as noted above, the corresponding wild-type B/Rochester/02/2001 virus exhibited reduced susceptibility to oseltamivir carboxylate. Therefore, viruses were considered drug-resistant if two criteria were met: the IC_{50} was ≥15 nM and that value constituted at least an eightfold increase compared to the IC_{50} value for the corresponding wild type.

The variant (Table 1, M1) carrying the commonest substitution H274Y selected in N1 subtype viruses during clinical use of oseltamivir, retained susceptibility to A-315675 and zanamivir, however, had reduced susceptibility to peramivir. The other variant (M2) carrying the same substitution exhibited lower levels of resistance to oseltamivir carboxylate and peramivir compared to those for M1 (Table 1). The presence of both His (wild-type) and Tyr (variant) at position 274 was detected in the NA of M2, which could account for the observed difference. The oseltamivir- and zanamivir-selected variants that carry the commonest substitution 292 (Arg→Lys) in N2 subtype were susceptible to A-315675 but were resistant to peramivir (Table 1, M3 and M4). This variant (M3) also retained partial susceptibility to zanamivir. The two oseltamivir-resistant variants carrying Val at 119 (M8 and M9) in N2 subtype were fully susceptible to A-315675 and peramivir, as were the zanamivir-resistant variants carrying either Gly or Ala at 119 (Table 1, M5 and M6). The replacement of Glu with Asp, however, conferred resistance to A-315675 and peramivir, as well as to zanamivir (Table 1, M7). Oseltamivir carboxylate was effective against the zanamivir-resistant variants carrying Gly, Ala, or Asp at 119 (M5, M6, and M7, respectively), although the IC_{50} values were increased (up to 9-fold) compared to those for the wild type.

The recovery of only two drug-resistant variants of influenza B viruses from NA inhibitor-treated patients has been reported to date. In the first case, a replacement occurred at a catalytic residue Arg at 152 (M10) in the NA active site; this change was accompanied by cross-resistance to all four NA inhibitors in the current study. In contrast, the variant with the substitution (Asp→Asn) at a framework residue 198 (M11) was resistant to oseltamivir carboxylate and zanamivir but retained susceptibility to A-315675 and peramivir.

TABLE 2. Evaluation of NA inhibitor efficacy against zanamivir- and oseltamivir-selected variants in the NA inhibition assay

NA type	Variant[a]	NA mutation[b]	Susceptibility[c]			
			Zanamivir	Oseltamivir carboxylate	A-315675	Peramivir
A/N1	M1	H**274**Y	S	R	S	R
	M2	H/Y**274**	S	R	S	I[d]
A/N2	M3	R**292**K	I	R	I	R
	M4	R**292**K	R	R	I	R
	M5	E**119**G	R	S	S	S
	M6	E**119A**	R	I	S	S
	M7	E**119**D	R	S	R	R
	M8	E**119**V	S	R	S	S
	M9	E**119**V	S	R	S	S
B	M10	R**152**K	R	R	R	R
	M11	D**198**N	R	R	S	S

[a] See Table 1 for details.
[b] Position of the substituted residue in the NA is shown in bold (N2 numbering).
[c] S, sensitivity; R, resistance; I, intermediate.
[d] The presence of both the wild-type and variant sequences was detected in the sample tested.

Overall, based on the adapted criteria for resistance, the variants showed the difference in the resistance profiles against four inhibitors in the NA inhibition assay (Table 2). The variants with substitution at 292 (M3 and M4) showed intermediate resistance to A-315675 because the estimated IC_{50} values were below 15 nM. Similarly, the variant with substitution at 119 (M6) exhibited intermediate resistance to oseltamivir carboxylate. Variant M2 demonstrated intermediate resistance to peramivir although this was most likely due to the presence of the wild-type virus in the sample. Seven variants were susceptible to A-315675 and five to peramivir.

Comparison of drug susceptibility in the ferret model. Based on the results of the NA inhibition assay, the influenza B variant carrying substitution Asp198Asn retained susceptibility to peramivir and A-315765. Therefore, we hypothesized that the variant would have growth advantage over the wild-type in oseltamivir-treated animals but not in those treated with the new NA inhibitors. To test this assumption, we used the ferret model of experimental infection. Although both peramivir and A-315675 inhibited the NA activity of the D198N variant, the IC_{50} value was lower for A-315675 (M11, Table 1). In addition, both NA inhibitors oseltamivir carboxylate and A-315675 can be delivered in a form of a prodrug, and therefore in the present study, A-315675 was tested in ferrets along with oseltamivir.

Twenty-nine ferrets were divided into three groups according to the treatment: seven ferrets received placebo, 10 received oseltamivir (prodrug), and 12 received A-322278 (Table

TABLE 3. Analysis of viruses recovered from ferrets coinfected with the wild-type virus B/Rochester/02/2001 and its oseltamivir-resistant variant and treated with NA inhibitors[a]

Treatment group	No. of animals shedding virus on any day/no. in group	Detection of virus in nasal washes							
		Day 3 p.i.				Day 5 p.i.			
		Cell culture		RT-PCR		Cell culture		RT-PCR	
		No. of positive animals	Virus titer, $TCID_{50}$/ml (range)	No. of positive animals	Residue at 198 (no. of animals)	No. of positive animals	Virus titer, $TCID_{50}$/ml (range)	No. of positive animals	Residue at 198 (no. of animals)
Placebo	6/7	6/7	3.7 (1.7–5.4)	6/7	Asp (1) Asp/Asn (4) Asn (1)	5/7	3.6 (<0.7–3.9)	6/7	Asp (1) Asp/Asn (5)
Oseltamivir	7/10	7/10	3.7 (3.2–4.2)	7/10	Asp (2) Asp/Asn (4) Asn (1)	5/10	3.2 (<0.7–3.9)	7/10	Asp (2) Asp/Asn (4) Asn (1)
A-322278	9/12	8/11[b]	2.8 (1.7–4.9)	8/11	Asp (2) Asp/Asn (6)	6/11	2.4 (<0.7–3.4)	7/11	Asp (5) Asp/Asn (2)

[a] Viral RNA extracted from the nasal wash was subjected to NA gene sequence analysis. Asp at codon 198 (GAC) represents the wild-type sequence, Asn (AAC) is the oseltamivir-selected variant, and Asp/Asn indicates a mixture of nucleotides (G and A) detected at the first nucleotide of codon 198.

[b] One ferret died on day 2.

3). The NA inhibitors were given 2 h prior to challenge and then twice daily for 5 days followed the challenge. Ferrets were infected intranasally with the wild-type virus and the variant combined 1:1 (based on $TCID_{50}$) prior to inoculation. On days 1, 3, 5, and 7 postinfection, the nasal washes were collected to extract vRNA and inoculate MDCK cells.

No infectious virus was isolated from seven animals on any day. Notably, one of those animals was from the placebo-treated group, indicating that the challenge dose (10^4 $TCID_{50}$) produced infection only in 85% of the inoculated animals. One ferret from the A-322278 treatment group died accidentally on day 2 postinfection, and necropsy did not reveal any signs of pathology. The nasal washes collected from the remaining ferrets on day 3 postinfection were positive for virus presence based on the results of reverse transcription-PCR amplification of the viral NA gene and infectivity in cell culture (Table 3). The estimated average virus titer in the oseltamivir-treated animals was no different from that of the control animals, and it was eightfold lower in the A-322278-treated group. Half of the animals treated with either NA inhibitor shed infectious virus on day 5 postinfection and a slight reduction of the virus titers was observed in the other drug-treated animals. The nasal washes collected on day 7 postinfection were uniformly negative for the virus presence.

To monitor the composition of the virus population over the course of infection, the reverse transcription-PCR-amplified viral NA genes from the nasal washes were subjected to sequence analysis. In the placebo-treated group, the variant carrying a substitution at position 198 (Asp→Asn) was detected in the nasal washes recovered from five of six animals on days 3 and 5 postinfection (Table 3). In the oseltamivir-treated group, this variant was also detected in five of seven animals on days 3 and 5 postinfection, indicating that the NA substitution provided no apparent growth advantage in the ferrets treated with oseltamivir. In contrast, the Asn198 variant was detected in two of seven ferrets treated with A-322278 on day 5 postinfection (Table 3).

DISCUSSION

A set of zanamivir- and oseltamivir-resistant variants of influenza A and B viruses displayed distinct resistance profiles when assessed with four NA inhibitors (zanamivir, oseltamivir carboxylate, peramivir, and A-315675) in the NA inhibition assay. This finding is a reflection of the difference in the inhibitors' chemical structures and interactions with the amino acid residues of the NA active site (2, 25, 26, 37). The tested variants were susceptible to either peramivir or A-315675 with two exceptions; the variant carrying the substitution at the catalytic residue 152 (NA/B type) was cross-resistant to all four NA inhibitors, and the variant with the substitution at the framework residue Glu119Asp (A/N2) was resistant to both new agents.

The substitution at catalytic residue 152 is rare and thus may have little clinical and epidemiological importance. The effect of this substitution on virus resistance to zanamivir in the NA inhibition assay appears to be strain specific (23). The substitution at framework residue 119 (Glu→Asp) has emerged in vitro in the presence of zanamivir (5, 12) and A-315675 (32) and has not been detected during clinical studies to date. However, the substitution at another catalytic residue, 292 (A/N2), is rather common (34) and is known to lead to a high level of oseltamivir resistance (>10,000-fold) (Table 1). The variants carrying this substitution exhibited from 5- to 80-fold increases in IC_{50} values against the remaining NA inhibitors (Table 1, M3 and M4). However, because the actual IC_{50} values for this variant tested against A-315675 were in the nanomolar range, we consider that these variants exhibit the intermediate level of resistance.

The emergence of oseltamivir-resistant variants with a substitution at framework residue 274 is a cause of a particular concern due to recent transmissions of avian A/H5N1 viruses into the human population (28). The substitution His→Tyr conferred a >700-fold reduction in susceptibility to oseltamivir carboxylate for the variant M1 and >100-fold for the variant M2 (Table 1). The variant M1 also exhibited a 100-fold reduc-

tion in susceptibility to peramivir. This result is in agreement with the data obtained with the genetically engineered variant of the A/WSN/33 (H1N1) virus, which showed a 430-fold increase in IC_{50} against oseltamivir carboxylate and 50-fold increase against peramivir (1). The sequence analysis of variant M2 showed the presence of the wild-type NA sequence (His274) in the viral population, which could explain the lower level of resistance to oseltamivir carboxylate and peramivir. Of note, the oseltamivir-resistant variant with the His274Tyr substitution retained susceptibility to zanamivir and A-315675.

The other common substitution in oseltamivir-resistant viruses carrying the enzyme of the N2 subtype occurs at framework residue 119 (16, 30, 34). The variant with Val at 119 was resistant to oseltamivir carboxylate but retained susceptibility to the other three inhibitors (Table 2, M8 and M9). In vitro selection has determined that resistance to zanamivir was conferred by replacement of Glu with Gly, and the variant retained susceptibility to the remaining inhibitors (Table 2, M5). The replacements of Glu with Ala in the zanamivir-resistant variant also led to a ninefold increase in the IC_{50} value against oseltamivir carboxylate, yet we considered this variant to show the intermediate resistance because the actual IC_{50} value was below 15 nM (Table 1, M6). This variant was fully susceptible to peramivir and A-315675.

It was reported previously that a variant (A/N9) selected in cell culture in the presence of A-315675 acquired the substitution of Asp for Glu at 119, which led to a 355-fold reduction in drug susceptibility (32). In our study, the zanamivir-selected variant carrying this substitution in the enzyme of the N2 subtype exhibited approximately 50- and 30-fold increases in IC_{50} values against A-315675 and peramivir, respectively (Table 1, M7). The variant was oseltamivir susceptible with an IC_{50} value of 1.8 nM (fivefold increase).

The novel substitution (Asp→Asn) at residue 198 was detected in the NA of the influenza B virus recovered from an oseltamivir-treated patient (11). In the NA active site of influenza B viruses, the framework residue Asp198 interacts with the catalytic residue Arg152 (3). This substitution differs from those described previously because it occurred at semiconserved residue of the NA active site (7). In the enzymes of N7 and N9 subtypes, Asn is present at position 198, and yet oseltamivir carboxylate efficiently inhibits their activities (10). The substitution in the NA of B type was accompanied by a ninefold increase in the IC_{50} values against zanamivir and oseltamivir carboxylate (Table 1, M11). However, the estimated IC_{50} values were 15 nM and 304 nM, respectively.

It is not apparent how accurately the IC_{50} values assessed in the enzyme inhibition assay reflect actual difference in the inhibitors' potencies. For example, certain wild-type influenza B viruses showed reduced susceptibility to oseltamivir carboxylate when tested in the fluorometric NA inhibition assay but not in the chemiluminescent assay (22, 29). Substrate, buffer, and other factors can significantly influence the estimated IC_{50} values in the NA inhibition assay (17, 22). In our study, the variant carrying the substitution at 198 was resistant to oseltamivir carboxylate and zanamivir but was susceptible to peramivir and A-315675. Interestingly, a wild-type B/Perth/211/2001 virus carrying substitutions at 150 (Glu→Gly) and 199 (Ser→Asn) was recovered from an untreated person. It exhibited resistance to oseltamivir carboxylate, zanamivir, and peramivir (22); its susceptibility to A-315675 has not been tested.

Variants carrying substitutions at 292 and 274 appear to be less infectious and pathogenic in ferrets (4, 20), whereas the fitness of those with the substitution at residue 119 appeared to be less affected (6, 21). In the present study, the wild-type virus failed to outgrow the variant Asn198 in the placebo-treated animals (Table 3), and therefore we concluded that the variant virus's fitness has not been severely compromised, at least in a ferret model. This result differs from that in our previous study, where the wild-type B virus exhibited a definite growth advantage over the Lys152 variant in coinfected ferrets (15). Despite a ninefold reduction in the oseltamivir susceptibility detected in the NA inhibition assay, the variant Asn198 showed no apparent growth advantage over the wild-type virus in the animals treated with oseltamivir, whereas the variant with the substitution at residue 152 showed a definitive growth advantage over the wild-type virus in zanamivir-treated animals (15).

In the present study, treatment with oseltamivir at 5 mg/kg produced a modest inhibitory effect on influenza B virus replication in the ferret upper respiratory tract. In this respect, the results are similar to those obtained in ferrets infected with influenza A viruses (<4-fold reduction) (31, 35). However, treatment of ferrets with A-322278 (present study) and peramivir (35) at the same dose appeared to be more effective in reducing the nasal wash virus titers (8- to 18-fold and 4- to 13-fold reduction, respectively). Moreover, treatment with A-322278 led to a more rapid clearance of the Asn198 variant from the ferret upper respiratory tract. The NA inhibitor A-315765/A-322278 therefore demonstrated the ability to inhibit the enzyme activity of most oseltamivir- and zanamivir-selected variants in vitro and appears to inhibit replication of the Asn198 variant in a ferret model. Overall, these studies indicate that different patterns of susceptibility and cross-resistance between NA inhibitors may prove important if antiviral resistance to oseltamivir were to emerge.

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