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Antiviral Research 59 (2003) 13–22
Antiviral Research
www.elsevier.com/locate/antiviral

A point mutation in influenza B neuraminidase confers resistance to peramivir and loss of slow binding

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Received 13 November 2002; accepted 2 January 2003

Abstract

The influenza neuraminidase (NA) inhibitors peramivir, oseltamivir, and zanamivir are potent inhibitors of NAs from both influenza A and B strains. In general, these inhibitors are slow, tight binders of NA, exhibiting time-dependent inhibition. A mutant of influenza virus B/Yamagata/16/88 which was resistant to peramivir was generated by passage of the virus in tissue culture, in the presence of increasing concentrations (0.1–120 μM over 15 passages) of the compound. Whereas the wild type (WT) virus was inhibited by peramivir with an EC_{50} value of 0.10 μM, virus isolated at passages 3 and 15 displayed EC_{50} values of 10 and >50 μM, respectively. Passage 3 virus contained 3 hemagglutinin (HA) mutations, but no NA mutation. Passage 15 (P15R) virus contained an additional 3 HA mutations, plus the NA mutation His273Tyr. The mechanism of inhibition of WT and P15R NA by peramivir was examined in enzyme assays. The WT and P15R NAs displayed IC_{50} values of 8.4 ± 0.4 and 127 ± 16 nM, respectively, for peramivir. Peramivir inhibited the WT enzyme in a time-dependent fashion, with a K_i value of 0.066 ± 0.002 nM. In contrast, the P15R enzyme did not display the property of slow binding and was inhibited competitively with a K_i value of 4.69 ± 0.44 nM. Molecular modeling suggested that His273 was relatively distant from peramivir (>5 Å) in the NA active site, but that Tyr273 introduced a repulsive interaction between the enzyme and inhibitor, which may have been responsible for peramivir resistance.

Keywords: Neuraminidase inhibitor; Influenza virus; Drug resistance

1. Introduction

In the last several years, inhibitors of the influenza neuraminidase (NA) enzyme have been identified as potential agents for the prophylaxis and/or treatment of infections caused by influenza virus. The function of the NA enzyme is to cleave sialic acid, the cellular receptor to which the viral hemagglutinin (HA) protein binds. Inhibition of NA prevents the release of nascent viral particles from the surface of the infected cell, thereby limiting the spread of virus in the respiratory tract (Gubareva et al., 2000). In particular, zanamivir (von Itzstein et al., 1993), oseltamivir carboxylate (Kim et al., 1997), A-315675 (Kati et al., 2002), and peramivir (also designated as BCX-1812 or RWJ-270201) (Sidwell, 2002; Babu et al., 2000) specifically target the influenza NA enzyme (Fig. 1).

Previous studies by Gubareva et al. (2001b) and Bantia et al. (2001) have compared the inhibition of NA activity by peramivir with zanamivir and oseltamivir carboxylate, using IC_{50} values. For influenza A, the three compounds displayed median IC_{50} values in the low nanomolar range (0.2–2 nM). For influenza B, median IC_{50} values tended to be somewhat higher, in the range of 1–9 nM. The K_i values of peramivir, oseltamivir carboxylate, zanamivir, and A-315675 were compared in a recent study by Kati et al. (2002). Peramivir had the lowest observed K_i values for three of the six influenza NAs examined. All of these comparative studies are consistent with the observation that these four compounds, in general, are better inhibitors of the influenza A NA enzymes compared to the B enzymes.

As with any antiviral agent, the question of drug resistance is of interest. Resistance to NA inhibitors is often an interplay between mutations in two viral genes: HA, which binds to host cell sialic acid receptors, and NA, which cleaves sialic acid, releasing nascent virus from cells. The

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doi:10.1016/S0166-3542(03)00011-1

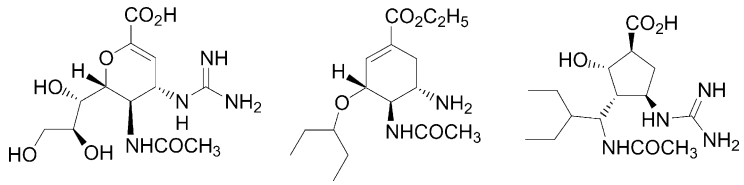

Fig. 1. Structures of the NA inhibitors used in this study.

basis for this interplay is the need to maintain an appropriate balance between HA binding to receptor, and NA-mediated destruction of the receptor. For example, a mutation in HA which decreases its binding to sialic acid may decrease viral dependence on NA activity (McKimm-Breschkin et al., 1996a; McKimm-Breschkin, 2000). Previous studies on resistance of influenza virus in tissue culture to zanamivir and oseltamivir carboxylate have demonstrated that mutations in viral HA generally arise first, followed by mutation in the NA (McKimm-Breschkin, 2000). Several laboratories have identified NA mutations that confer resistance to NA inhibitors (reviewed in McKimm-Breschkin, 2000); some of these mutations were discussed in the context of functional residues that directly interact with substrate versus framework residues that serve to provide a scaffold for the functional residues (Gubareva et al., 1997).

Most of the previous studies on resistance to NA inhibitors have focused on influenza A strains, with fewer reports on influenza B strains (McKimm-Breschkin, 2000). Despite the divergent amino acid sequences (only 20–30% homology) of the influenza A and B NA enzymes, their active site residues and three-dimensional structures are conserved (Burmeister et al., 1992; Bossart-Whitaker et al., 1993), and B NA can complement defective type A enzyme (Ghate and Air, 1999). To date, only two mutations conferring resistance to NA inhibitors have been directly isolated in B strains. The mutation Glu119Gly in the NA of B/Lee/40 was found upon selection with zanamivir in tissue culture; the mutant NA appeared to have severely reduced enzymatic activity (<1% of wild type (WT)) (Staschke et al., 1995) which was likely due to instability of the enzyme (McKimm-Breschkin et al., 1996b). This mutation was also identified in the NA of B/Beijing/1/87 upon zanamivir selection in vitro (Barnett et al., 1999). Mutation of Glu119 has also been found in influenza A NA (Gubareva et al., 1997; McKimm-Breschkin, 2000). The mutation Arg152Lys was identified in a B virus isolated from an immunocompromised child treated with zanamivir (Gubareva et al., 1998); the corresponding mutation has not been reported for an A virus.

Several mutant influenza strains resistant to the NA inhibitors oseltamivir and zanamivir have been examined for sensitivity to peramivir (Gubareva et al., 2001b). Zanamivir-resistant viruses mutated at Glu119 were susceptible to peramivir and oseltamivir carboxylate. The zanamivir-resistant Arg292Lys A/N2 virus was moderately resistant to peramivir and highly resistant to oseltamivir carboxylate. An Arg152Lys B virus resistant to zanamivir was resistant to both of the other inhibitors, and a His274Tyr A/N1 mutant selected by oseltamivir resistance was resistant to peramivir but sensitive to zanamivir. Gubareva et al. concluded that mutation in functional residues lead to variable levels of cross-resistance, but mutation of framework residues may allow susceptibility to other inhibitors.

In an effort to understand possible viral resistance to peramivir, a virus resistant to this compound in tissue culture was isolated and characterized. Previous studies identified an HA mutation (Lys189Glu) which conferred peramivir resistance in tissue culture (Smee et al., 2001), and also an HA mutation (Gly130Asp) with the NA mutation Arg292Lys (Bantia et al., 2000). Both of these studies were carried out in influenza A strains. We chose to examine resistance in an influenza B strain because of the relatively lesser amount of information available on NA inhibitor-resistant mutants of these strains. Influenza B/Yamagata/16/88 virus arose in Japan in 1988 and contained antigenic variations in HA which rendered it infectious to individuals who were vaccinated against the circulating B strain Ibaraki/2/85 (Kanegae et al., 1990). In addition, among several influenza B NAs, the NA of B/Yamagata/16/88 contained two unique amino acid changes that result in differences in orientation of other side chains (Burmeister et al., 1993). These features render this strain a particularly interesting candidate for the study of resistance to NA inhibitors.

2. Materials and methods

2.1. Compounds

Peramivir was synthesized at Johnson & Johnson Pharmaceutical Research and Development, LLC (Raritan, NJ). Zanamivir and the carboxylate form of oseltamivir (previously referred to as GS4071) were synthesized by BioCryst Pharmaceuticals (Birmingham, AL). Compounds were dissolved in distilled water (for NA assays) or in infection medium at 10 mM prior to use.

2.2. Cells and viruses

Influenza virus B/Yamagata/16/88 and Madin–Darby Canine Kidney (MDCK) cells were provided by Dr. Larisa Gubareva (University of Virginia, Charlottesville, VA).

The virus was used as a seed stock to establish working viral stocks by passage in MDCK cells which were maintained in minimum essential medium (MEM) with Earle's salts (Gibco BRL, Grand Island, NY) supplemented with glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml), HEPES buffer (10 mM, pH 7.55), and 10% calf serum. Cells infected with virus were maintained in MEM containing glutamine, penicillin and streptomycin, HEPES buffer, and 1.2% bovine serum albumin (infection medium) and trypsin (2 μg/ml, Worthington Biochemicals, Lakewood, NJ).

2.3. Selection of drug-resistant virus

Peramivir-resistant viruses were selected by passage of influenza virus B/Yamagata/16/88 (multiplicity of infection = 1 for initial infection) on MDCK cell monolayers in the presence of increasing concentrations of peramivir (0.1–120 μM over 15 passages). Putative-resistant viruses were plaque purified twice in either 1 μM (P3 virus) or 50 μM (P15 virus) peramivir and then grown to high titer stocks on MDCK cells without the addition of compound. Virus passaged without peramivir was maintained to provide WT control virus for each passage.

2.4. Plaque reduction assays

Sensitivity of viral isolates to NA inhibitors was determined using a plaque reduction assay. Isolates were first titrated on MDCK monolayers without compound to determine plaque-forming units (PFU) per milliliter of viral stocks. Virus was diluted in infection medium containing trypsin to approximately 400 PFU/ml. Confluent monolayers of MDCK cells in 12-well plates were infected with 125 μl of virus (50 PFU) for 30 min, washed with warm phosphate-buffered saline (PBS), and incubated with an agar overlay containing dilutions of compounds to be tested. Plates were incubated (35 °C in 5% CO_2) for 3 days, overlays removed, and monolayers stained with 0.1% crystal violet in 20% ethanol. Assays were performed in triplicate. Both the number and size of plaques were monitored.

2.5. Sequence analysis of NA and HA1

Viral RNA was prepared from infected tissue culture fluid, and complementary deoxyribonucleic acid (cDNA) was synthesized as described (Smee et al., 2001), using transcription primer U16 (5′ AGCAGAAGCAGAGCAT 3′) designed from the common nucleotide sequences at the 3′ noncoding region of influenza B RNA segments (Stoeckle et al., 1987). HA1 and two overlapping fragments of NA (Genbank accession numbers M58419 and 67013) were then amplified by polymerase chain reaction (PCR) and sequenced as described (Smee et al., 2001). Multiple plaque purified isolates from each passage were sequenced to look for heterogeneity in variant sequences. Sequences were analyzed using Sequencher 3.1 (Gene Codes Corp., Ann Arbor, MI) and ClustalW (Thompson et al., 1994).

2.6. Preparation of virus and hemagglutinin-neuraminidase (HANA)

Virus was prepared and HANA isolated according to a modification of the procedure of Gallagher et al. (1984). Eleven-day-old embryonated chicken eggs were inoculated with WT and resistant B/Yamagata/16/88 influenza virus at 10^{-4} and incubated at 35 °C for 60 h. The allantoic fluids were harvested and cellular debris removed by low speed centrifugation. Virus was pelleted by high speed centrifugation and purified by centrifugation on sucrose gradients. For preparation of HANA, purified virus (40 mg) was pelleted and resuspended in 1 ml of sodium acetate buffer (0.05 M sodium acetate, 2 nM NaCl, 0.2 nM EDTA, pH 7.0). An equal volume of 15% octylglucoside (1-*O*-*n*-β-D-glucopyranoside, Sigma Chemical Co., St. Louis, MO) in sodium acetate buffer was then added with mild agitation. The HANA rich supernatant was separated from the internal core proteins by centrifugation at 75,000 × *g* for 60 min. The protein concentrations of the HANA preparations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.7. NA assays

NA end-point assays were performed at 37 °C using the substrate 2′-(4 methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA) as described previously (Smee et al., 2001), using a SpectraMax Gemini XS plate reader (Molecular Devices) at excitation and emission wavelengths of 355 and 460 nm, respectively. NA activity in each of the virus or HANA preparations was titrated to select a standard amount of enzymatic activity for the determination of IC_{50} values (concentration of NA inhibitor which inhibits 50% of NA activity). For IC_{50} values, inhibitor and NA were preincubated together for 10 min prior to the addition of substrate. The molar amount of NA was calculated assuming that NA (50 kDa) was 20% by mass of total protein in the HANA preparations. The NA endpoint assay was modified to a kinetic mode for the experiments shown in Fig. 2 and Tables 3 and 5, with K_m and V_{max} determined by standard methods. Two independent HANA preparations were used for K_m and V_{max} determinations, and the average of the Lineweaver–Burk, Eadie Hofstee, and Woolf transformations was taken. To assess slow binding inhibition, reactions were initiated with addition of substrate to enzyme and inhibitor (E + I + S) which had been preincubated for 10 min as in the endpoint assay, and compared to reactions initiated with the addition of enzyme to substrate and inhibitor with no preincubation (S + I + E). Each inhibitor was used at its IC_{50} value for WT and P15R NA, respectively: peramivir, 2 and 50 nM; oseltamivir carboxylate, 10 and 60 nM; and zanamivir, 5 and 5 nM. Reaction with

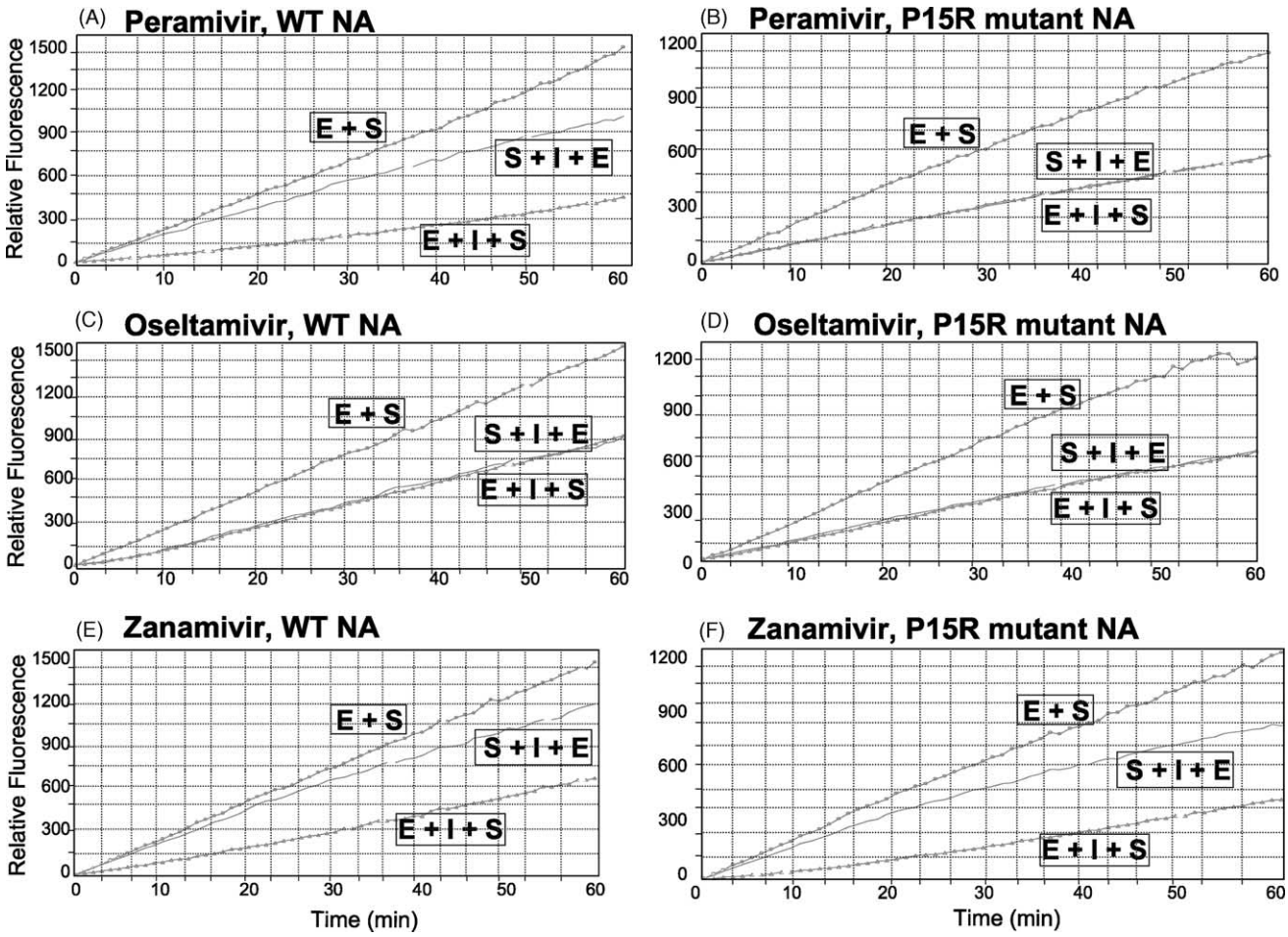

Fig. 2. Progress curves of hydrolysis of MUNANA substrate using WT or P15R HANA. Each inhibitor was used at the IC_{50} value determined in the endpoint assay: peramivir, 2 nM (A) and 50 nM (B); oseltamivir carboxylate, 10 nM (C) and 60 nM (D); zanamivir, 5 nM (E, F) and was either preincubated (10 min) with enzyme, followed by addition of substrate (E + I + S) or added together with substrate (S + I + E). The NA concentration was approximately 2 nM in each sample.

enzyme and substrate in the absence of inhibitor was used as the control (E + S). Fluorescence measurements were obtained each minute for up to 1 h, and progress curves of the hydrolysis of the substrate by HANA were generated.

The K_i value of peramivir for P15R was determined from the *x*-intercept of Dixon plots (1/rate versus inhibitor concentration) as described for other NA inhibitors (Bethell et al., 2000; Kati et al., 1998) by preincubation of HANA with peramivir (0, 10, 30, 50, 80, 100, 200 nM) for 20 min, followed by initiation of the reaction by addition of MUNANA substrate (5–100 μM). Although Lineweaver–Burk plots showed classic competitive inhibition of P15R NA by peramivir, the time-dependent inhibition of WT NA by peramivir was assessed by determination of the K_i defined in this case as the initial dissociation constant between enzyme and inhibitor (Kitz and Wilson, 1962). WT HANA was preincubated for 0–30 min with peramivir (0, 0.25, 0.5, 1, 1.5, 2 nM), and the reaction was initiated by addition of substrate (200 μM). For each concentration of peramivir, the initial rate was compared to the uninhibited control by plotting percent activity as a function of preincubation time. The rate of inactivation, *k*, was determined for each peramivir concentration. Plots of *k* as a function of peramivir concentration were generated, and the dissociation constant K_i was determined from the *x*-intercept.

2.8. Removal of peramivir from inhibited HANA samples

Equivalent enzymatic activities of WT and P15R HANA were incubated at room temperature in 200 μl 32.5 mM MES pH 6.0 and 4 mM calcium chloride, without and with peramivir (WT, 1.5 nM; P15R, 50 nM, corresponding to the IC_{50} values observed in the endpoint assay). Each of the four samples was then split into two 100 μl aliquots. One aliquot was used directly in the kinetic NA activity assay using MUNANA substrate, and the other aliquot was subjected to chromatography on a Microspin G-50 Sephadex column (Amersham, Piscataway, NJ) to remove free peramivir not bound to NA, followed by assay of the flow through fraction for NA activity. The “before” and “after” column samples

incubated without peramivir were used as controls, and activity of each sample was calculated from the rate of hydrolysis of substrate as follows: % NA activity = sample/control × 100.

2.9. Molecular modeling

The HINT program (Kellogg et al., 1991) was used to assess hydropathic interactions between pairs of atoms within the WT and P15R NA enzymes complexed with each NA inhibitor. The crystal structure of WT NA B/Beijing/1/87 complexed with zanamivir (Taylor et al., 1998) was used as the model for docking of peramivir and oseltamivir carboxylate. A model of the P15R mutant was generated by substitution of B/Yamagata His273 with Tyr (equivalent to His272 in the B/Beijing sequence).

3. Results

3.1. Isolation of virus resistant to peramivir

After passage of B/Yamagata/16/88 influenza virus in the presence of peramivir, resistant virus P3R was observed at passage 3, which displayed an EC_{50} value in plaque assay of 10 μM, compared to a WT EC_{50} value of 0.10 μM. Continued passage of the P3R virus in the presence of increasing concentrations of peramivir resulted in the selection of virus P15R at passage 15. The results of DNA sequence analysis of the HA and NA genes of the P3R and P15R viruses are shown in Table 1. The P3R virus differs from WT by three mutations in HA (Gly141Glu, Asp195Asn, Thr197Asn). The P15R virus contains the three mutations of P3R, plus an additional three mutations in HA (Thr139Asn, Arg162Met, Tyr319His). In addition to these six mutations in HA, the NA gene of the P15R virus contains the mutation His273Tyr. In the absence of peramivir, the WT and P15R viruses grew to similar titers. Plaques of P15R were cloudy in the absence and clear in the presence of peramivir, as noted for zanamivir-resistant mutants (McKimm-Breschkin et al., 1996a).

In contrast to the WT and P3R viruses, which gave unambiguous EC_{50} values using the conventional definition (the concentration of inhibitor which reduced the number of plaques by 50%), plaques of the P15R virus were reduced in size but did not disappear entirely in the presence of NA inhibitors, a phenomenon that has been observed previously for other NA mutants (McKimm-Breschkin, 2000 and references therein). For this reason, the EC_{50} values for the P15R virus are reported as the concentration of inhibitor that reduced plaque size by 50% (Table 2). For the P15R virus, the EC_{50} of peramivir is 50 μM, so that this virus is at least 500-fold less sensitive to the compound compared to the WT virus.

Table 1
Amino acid changes in HA and NA of peramivir-resistant mutants of influenza B/Yamagata/16/88

Passage	Gene	Mutation
P3R	HA	Gly141Glu
	HA	Asp195Asn
	HA	Thr197Asn
P15R	HA	Above plus
	HA	Thr139Asn
	HA	Arg162Met
	HA	Tyr319His
	NA	His273Tyr

Table 2
Sensitivity of peramivir-resistant virus to NA inhibitors (plaque reduction assay)[a]

Virus	EC_{50} (μM)		
	Peramivir	Oseltamivir carboxylate	Zanamivir
WT[b]	0.07	0.25	0.07
P15R[c]	50	25	2.5

[a] A representative of three independent experiments is shown.
[b] WT EC_{50} values are based on 50% reduction in plaque number.
[c] P15R EC_{50} values are based on 50% reduction in plaque size (see text).

The cross-resistance of the P15R virus to NA inhibitors zanamivir and oseltamivir carboxylate was also examined (Table 2). These compounds also exhibited elevated EC_{50} values for P15R compared to WT virus. Even though zanamivir inhibited plaque size by 50% at 2.5 μM, this compound, like oseltamivir carboxylate and peramivir, failed to reduce the number of plaques even at the highest concentration tested (25 μM).

3.2. Kinetic characterization of WT and P15R NA enzymes

In order to compare the sensitivity to peramivir of the NAs of the P15R and WT viruses, NA activity assays were performed. Two sources of NA were used: whole virus in the supernatants of infected MDCK cells, and HANA prepared from egg-grown virus. Since the peramivir-resistant virus was originally identified in tissue culture, it was important to test the NA from this source. Also, to have larger quantities of NA activity, the WT and P15 viruses were grown in chicken eggs for purification of HANA. Prior to inhibition studies, the NA activity in each preparation was examined in titration experiments, to determine the amount of virus or HANA needed to achieve equivalent rates of cleavage of the MUNANA substrate. For whole virus from MDCK cells, about 4–10-fold fewer PFU of P15R compared to WT virus were needed to achieve comparable cleavage of substrate. The significance of this finding is not clear—it may indicate that the P15R NA enzyme has a higher catalytic activity than the WT enzyme, and/or that perhaps more NA molecules are present on P15R virions compared to WT. This finding did not apply to the HANA purified from infected chicken eggs. Comparable amounts of WT and P15R egg HANA

Table 3
Kinetic constants for WT and P15R NA enzymes[a]

NA	K_m (μM)	V_{max} (nmol product/min/ng protein)	K_i peramivir (nM)[b]
WT	6.2 ± 0.97	23 ± 2	0.066 ± 0.002
P15R	2.5 ± 1.2	18 ± 2	4.69 ± 0.44

[a] Using HANA as the source of enzyme.
[b] The values of K_i for WT and P15 NA were determined as described in the text.

(0.5 μg/ml total protein, which corresponds to 2 nM NA enzyme) gave equivalent rates of cleavage of MUNANA or fetuin substrates, and, by gel electrophoresis, the amounts of WT and P15R NA and HA proteins present in the HANA preparations were similar (data not shown). Taken together, these data suggest that the WT and P15R NA enzymes from egg-derived HANA have similar specific activities.

The K_m and V_{max} values for the WT and P15R NAs were determined using HANA preparations (Table 3). The K_m values of WT and P15R NA were 6.2 and 2.5 μM, respectively, for MUNANA substrate. Thus, the P15R NA appears to have about a two-fold greater affinity than WT NA for this substrate. In contrast, the WT and P15R NAs displayed similar V_{max} values of 18–23 nmol product/min/ng protein.

3.3. Sensitivity of NA activity of peramivir-resistant virus to NA inhibitors

The sensitivity of the NA activity of WT and P15R to peramivir, oseltamivir carboxylate, and zanamivir was determined using both HANA and whole virus as the source of the enzyme (Table 4). In general, for all of the compounds, higher IC_{50} values (2–6-fold) were observed using whole virus compared to HANA, although the fold change in sensitivity in comparing WT with P15R was similar. For peramivir, using HANA preparations, P15R NA was about 31-fold less sensitive than WT NA based on IC_{50} values of 50 and 1.6 nM, respectively. When whole virus was used, the peramivir IC_{50} value of P15R was 16-fold higher than that of WT. In both cases, this was clear evidence for resistance at the level of NA sensitivity to the compound. For oseltamivir carboxylate, P15R displayed 2.4-fold (virus) and 6.6-fold (HANA) higher IC_{50} values than WT NA. The P15R NA displayed less resistance to oseltamivir carboxylate than to peramivir based on fold increase in IC_{50} values; however, the absolute IC_{50} values of peramivir and oseltamivir carboxylate for P15R were very similar (127 nM versus 120 nM using virus; 50 nM versus 63 nM using HANA).

In contrast, the P15R NA appeared to be slightly more sensitive to zanamivir than the WT enzyme, based on a ≤2-fold decrease in the IC_{50} value observed for both the virus and HANA P15R preparations, compared to those of WT. These data suggest that the zanamivir resistance of P15R virus observed in MDCK cells (Table 2) is due entirely to HA mutation(s).

3.4. Kinetics of inhibition of P15R and WT NA by NA inhibitors

Previous reports have indicated that the NA inhibitors oseltamivir carboxylate (Kati et al., 1998), zanamivir (Hart and Bethell, 1995), A-315675 (Kati et al., 2002), and peramivir (Baum et al., 2000) generally exhibit slow binding to the susceptible WT enzyme, although exceptions have been observed (Pegg and von Itzstein, 1994). To examine the binding of NA inhibitors to WT and P15R NA, progress curves of the hydrolysis of MUNANA substrate were generated, using WT or P15R HANA. Each inhibitor (at its respective IC_{50} value) was either preincubated (10 min) with enzyme, followed by addition of substrate (E + I + S) or added with together with substrate (S + I + E) (Fig. 2). A time lag in the achievement of the steady state rate in the non-preincubated sample (S + I + E), compared to the preincubated sample (E + I + S), is indicative of slow binding of the compound.

For peramivir, slow binding inhibition was observed for the WT enzyme (Fig. 2A) but not for the mutant P15R enzyme (Fig. 2B). In contrast, oseltamivir carboxylate did not exhibit slow binding to either the WT or P15R enzyme (Fig. 2C and D). Zanamivir was a slow binding inhibitor of both the WT and P15R enzymes (Fig. 2E and F).

Taking into consideration the observation that peramivir exhibits slow binding to the WT NA, but not to P15NA (against which the compound demonstrated classic competitive binding by standard kinetic analyses), K_i values were determined (Table 3). P15R NA has about 70-fold less

Table 4
Sensitivity of NA of WT and P15R viruses[a] and HANA[b] to NA inhibitors in the NA assay

Enzyme source	Compound					
	Peramivir		Oseltamivir carboxylate		Zanamivir	
	IC_{50} (Nm)	Fold increase (×)	IC_{50} (nM)	Fold increase (×)	IC_{50} (nM)	Fold increase (×)
Whole virus: WT	8.4 ± 0.4	1	50 ± 5.7	1	29 ± 4.5	1
Whole virus: P15R	127 ± 16	16	120 ± 5 4	2.4	14 ± 2.7	0.5
HANA: WT	1.6 ± 0.1	1	9.5 ± 0.9	1	4.7 ± 0.3	1
HANA: P15R	50 ± 1.4	31	63 ± 6.6	6.6	2.9 ± 0.1	0.6

[a] Whole virus was from tissue culture supernatants of infected MDCK cells.
[b] HANA was prepared from allantoic fluid of virus-infected chicken eggs as described in the text.

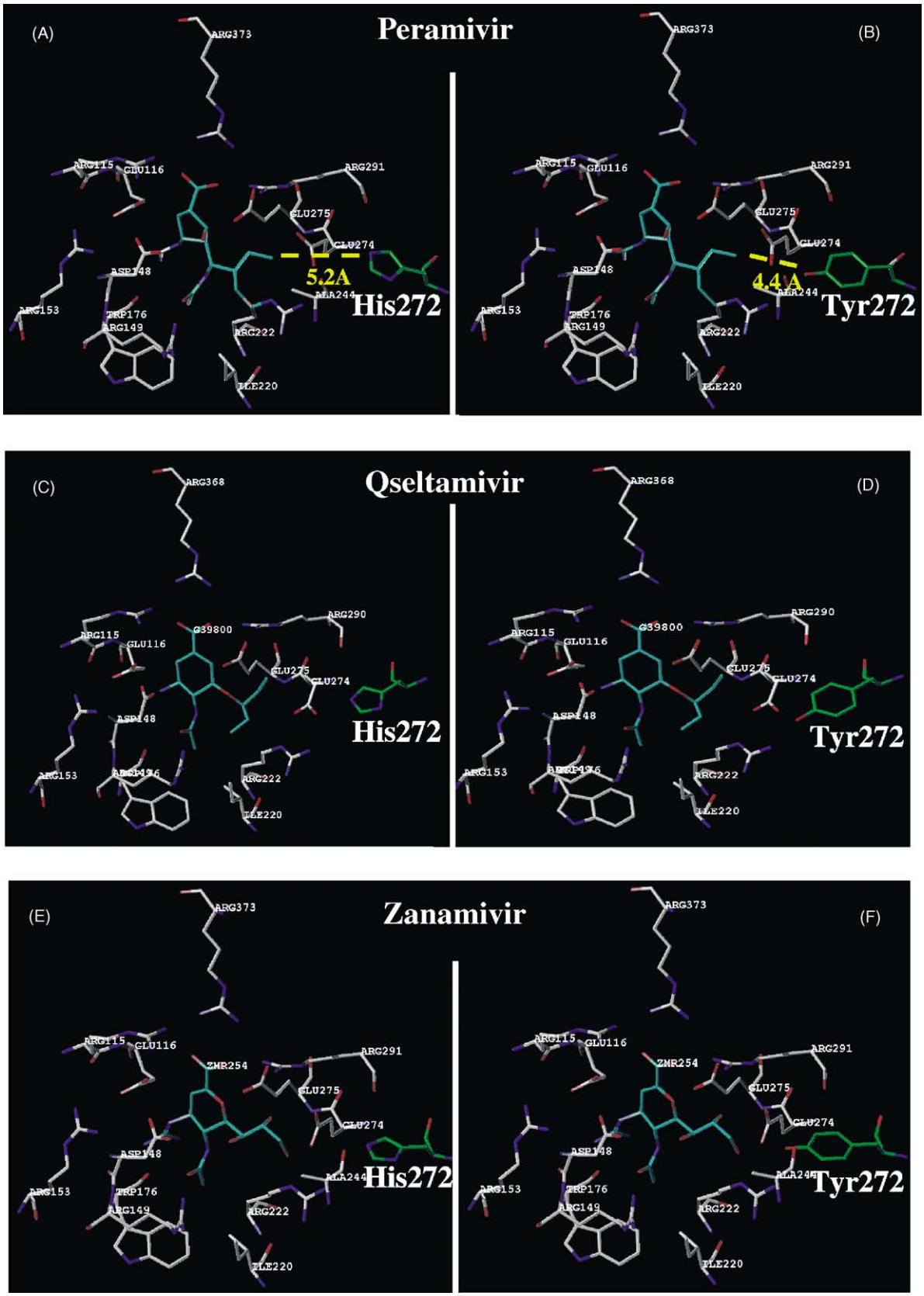

Fig. 3. Molecular modeling of influenza B NA enzymes with peramivir, zanamivir, and oseltamivir carboxylate (WT, left panels; mutant His273Tyr, right panels).

Table 5
NA activity of peramivir-treated WT and P15R HANA before and after removal of inhibitor by size exclusion chromatography[a]

HANA	NA activity (%)	
	Before	After
WT	9	17
P15R	17	83

[a] HANA was incubated with peramivir for 30 min and assayed for NA activity as described in Section 2. WT and P15R HANA was present at 0.5 μg/ml, which corresponds to approximately 2 nM NA; peramivir was present at 1.5 and 50 nM, respectively.

affinity than WT for peramivir, with K_i values of 4.69 and 0.066 nM observed, respectively.

3.5. Removal of peramivir from inhibited P15R and WT NA

It has been proposed that the slow binding phenomenon observed for NA inhibitors such as zanamivir, oseltamivir carboxylate, A-315675, and peramivir is actually due to slow dissociation of compound from the enzyme (Kati et al., 1998, 2002). Therefore, since P15R NA, unlike the WT enzyme, did not exhibit slow binding of peramivir (Fig. 2A and B), it might be possible to observe a difference in the ability to recover active enzyme upon removal of free compound. To this end, inhibited WT and P15R NA enzymes were prepared by incubation with peramivir at the IC_{50} values previously observed in the endpoint assay. After exposure to the compound, WT and P15 NA displayed 9 and 17% activity, respectively (Table 5). Inhibition >50% was observed probably due to the increased incubation time in this experiment (30 min versus 10 min for IC_{50} determinations). To remove free peramivir not bound to NA, these samples were subjected to size exclusion chromatography and were again tested for NA activity. The WT NA remained substantially inhibited (20% activity). In contrast, the P15R NA recovered most (86%) of the enzymatic activity (Table 5). These data suggest that peramivir is not as tightly bound to P15R compared to WT NA and dissociates when free compound is removed.

3.6. Molecular modeling of the active sites of WT and P15R NA with NA inhibitors

Molecular modeling studies using HINT calculations were performed, in an attempt to gain insight into how the His273Tyr mutation of P15R might change the interaction of the NA active site with each inhibitor and lead to the observed experimental results.

For the WT NA, no interactions were detected between His273 and any atoms of peramivir, oseltamivir carboxylate, or zanamivir because they are too distant (>5 Å; Fig. 3A, C and E, respectively). In contrast, in the mutant P15R NA, a repulsive hydrophobic–polar interaction (at 4.4 Å) between a carbon atom on the isopentyloxy side chains of peramivir and oseltamivir carboxylate (Fig. 1), and the hydroxyl of Tyr273 was predicted (Fig. 3B and D). This interaction may disrupt binding of these compounds to the mutant NA.

Zanamivir contains a glycerol instead of the isopentyloxy side chain present in peramivir and oseltamivir carboxylate (Fig. 1). The hydroxyl of Tyr273 in the mutant P15R enzyme appeared to introduce a small attractive, acid–base interaction with an alcohol substituent of the glycerol (Fig. 3E). In addition, no destabilizing interactions were detected between Tyr273 and zanamivir. Consequently, binding of zanamivir to NA should not be adversely impacted in the mutant, in contrast to the situation with peramivir and oseltamivir carboxylate. In fact, the attractive acid–base interaction may have led to the slightly lower IC_{50} value observed for zanamivir with P15R NA compared to WT NA.

4. Discussion

The occurrence of resistance in influenza virus exposed to NA inhibitors is of importance in assessing the efficacy of these compounds. Influenza B strains have not been as extensively studied as influenza A strains for resistance to NA inhibitors, and relatively few such studies have been performed to date with one of the newer compounds, peramivir. In this study, we report the isolation of an influenza B/Yamagata/16/88 virus resistant to peramivir which harbored mutations in HA and in NA (His273Tyr).

The His273Tyr mutation in P15R NA appeared to introduce some novel features into the enzyme, compared to its WT counterpart. P15R NA exhibited increased affinity for substrate (two-fold decrease in K_m) and decreased affinity for peramivir (500-fold increase in the IC_{50} value and 70-fold increase in the K_i value), compared to WT. Our data suggest that the enzymatic activities of the WT and His273Tyr NA enzymes are comparable, based on relative V_{max} values, in contrast to the mutations Glu119Gly, which rendered both type A and B NA less stable (McKimm-Breschkin et al., 1996b; Staschke et al., 1995), and Arg292Lys (type A) and Arg152Lys (type B) which reduced the specific activity of the enzymes (McKimm-Breschkin, 2000).

The His273 residue is a “framework” amino acid and does not directly interact with substrate (Colman et al., 1993; Burmeister et al., 1992; Finley et al., 1999). Another framework amino acid which has been shown to mutate and confer resistance to NA inhibitors in both A and B strains is Glu119. Catalytic residues which have been shown to mutate to NA inhibitor resistance include Arg152 (B strain) and Arg292 (A strain) (Gubareva et al., 1997, 2001b; McKimm-Breschkin, 2000). Arg292Lys is the only previous NA mutation reported to be selected for by resistance to peramivir (Bantia et al., 2000). This rather limited set of mutant NA residues, and the tendency of independent studies to isolate the same mutations, is probably indicative of the difficulty of simultane-

ously achieving resistance while maintaining the ability of the NA to function sufficiently to support viral replication.

The most striking feature of the His273Tyr NA in terms of kinetic analysis is that it has lost the property of time-dependent binding of peramivir seen with the WT enzyme. Molecular modeling suggested that the His273Tyr mutation introduced a repulsive interaction between NA and peramivir, consistent with the observed increase in IC_{50}, and the loss of slow binding. Kati et al. (1998), in studies with oseltamivir carboxylate, assert that slow binding is actually a consequence of slow dissociation of the inhibitor from the NA. We observed some indication of this with our WT and P15R enzymes, in that activity was recovered from inhibited P15R enzyme but not from the WT enzyme upon removal of compound by filtration, suggesting that peramivir was not as tightly bound to the mutant NA.

The two other NA inhibitors examined in this study exhibited different patterns of kinetic behavior compared to peramivir. Oseltamivir carboxylate did not exhibit slow binding, even to WT NA, despite inhibiting that enzyme. Although the NA inhibitors generally exhibit slow binding to susceptible WT enzymes, exceptions have been observed, for example, inhibition without slow binding was demonstrated for zanamivir with an unspecified influenza B NA (Pegg and von Itzstein, 1994). Mutant enzymes resistant to NA inhibitors, with concomitant loss of slow binding, have been reported previously (Gubareva et al., 1996; Blick et al., 1995; Barnett et al., 1999).

In our studies, zanamivir displayed yet another pattern of kinetics, in that both the WT and His273Tyr NA displayed slow binding. The mutant enzyme appeared to be even more susceptible than WT to zanamivir, consistent with molecular modeling which predicts that the Tyr273 substitution enhances the interaction between NA and the compound.

Despite extensive amino acid differences (70–80%) between the NA from influenza types A and B, there are 11 conserved residues that interact directly with the sialic acid substrate (Finley et al., 1999). Clearly, the overall structure in which these 11 residues reside confers important features to the enzyme, as indicated by differences in inhibition by a given compound in comparing types A to B (Bantia et al., 2001; Gubareva et al., 2001b). In particular, the function of His273 (which is present in both types A and B NA but does not directly interact with the substrate) has been discussed. In the A enzyme, His273 forms a hydrogen bond with Ser245, preventing formation of a hydrogen bond between His273 and Glu275, which does interact with substrate (Finley et al., 1999). Ser245 is absent in type B NA, suggesting that the dynamics of His273 interactions in the B and A enzymes are different. The analogous His274Tyr mutation in type A NA (N1) was isolated by oseltamivir carboxylate selection in vitro (Wang et al., 2000) or in experimentally infected human volunteers (Gubareva et al., 2001a,b; Ives et al., 2002) but has not been observed previously for the type B enzyme. The mutation in the A enzyme did not affect its K_m for the MUNANA substrate, but the K_i for oseltamivir carboxylate increased by 400-fold (Ives et al., 2002). The comparable mutation in the B enzyme in the present study resulted in a 2–3-fold increase in K_m for MUNANA, and a 76-fold increase in the K_i for peramivir. In contrast to our results, in which type B His273Tyr NA had similar IC_{50} values for peramivir and oseltamivir (Table 3), type A His274Tyr NA displayed 10-fold less resistance to peramivir than oseltamivir (IC_{50} values of 40 and 400 nM, respectively; Gubareva et al., 2001b). It is likely that the disparity in the oseltamivir carboxylate sensitivities of the mutant A and B NAs is due to the differences in the overall structures of these different enzymes. The type A His274Tyr NA retained sensitivity to zanamivir (Gubareva et al., 2001b), comparable to our results for the B enzyme. In addition, the His274Tyr mutation in A/Texas/36/91 rendered the virus less pathogenic in an animal model (Ives et al., 2002).

The slight resistance of the P15R virus to zanamivir in tissue culture is likely due to HA mutation(s), since from the NA assays, the His273Tyr mutation in the NA did not appear to confer resistance. Some of the HA mutations (Thr139Asn, Gly141Glu) appear to be at or near the sialic acid binding site (McKimm-Breschkin, 2000). The stepwise isolation of HA mutations, leading to weaker binding of HA to sialic acid and therefore a lessened dependence on NA for release of nascent virus from the surface of infected cells, followed by mutation within NA itself, has also been reported for oseltamivir carboxylate and zanamivir (McKimm-Breschkin, 2000). There are cases in which HA mutation alone conferred NA inhibitor resistance in tissue culture (e.g. McKimm-Breschkin, 2000 and references therein; Smee et al., 2001), but it has been postulated that HA mutations do not confer resistance in vivo (Barnett et al., 2000).

In summary, we have isolated a peramivir-resistant variant of an influenza B virus in vitro and have characterized its mechanism of resistance. Resistant virus isolated in vivo seems to be a rare occurrence (McKimm-Breschkin, 2000) and may not be a critical factor in the continuing evaluation of the NA inhibitors as a class for the prophylaxis and/or treatment of influenza infection in humans.

Acknowledgements

We thank Rex Pratt for helpful discussions concerning the design of kinetic experiments.

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