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Antiviral Susceptibility of Avian and Swine Influenza Virus of the N1 Neuraminidase Subtype▽

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Influenza viruses of the N1 neuraminidase (NA) subtype affecting both animals and humans caused the 2009 pandemic. Anti-influenza virus NA inhibitors are crucial early in a pandemic, when specific influenza vaccines are unavailable. Thus, it is urgent to confirm the antiviral susceptibility of the avian viruses, a potential source of a pandemic virus. We evaluated the NA inhibitor susceptibilities of viruses of the N1 subtype isolated from wild waterbirds, swine, and humans. Most avian viruses were highly or moderately susceptible to oseltamivir (50% inhibitory concentration [IC_{50}], <5.1 to 50 nM). Of 91 avian isolates, 7 (7.7%) had reduced susceptibility (IC_{50}, >50 nM) but were sensitive to the NA inhibitors zanamivir and peramivir. Oseltamivir susceptibility ranged more widely among the waterbird viruses (IC_{50}, 0.5 to 154.43 nM) than among swine and human viruses (IC_{50}, 0.33 to 2.56 nM). Swine viruses were sensitive to oseltamivir, compared to human seasonal H1N1 isolated before 2007 (mean IC_{50}, 1.4 nM). Avian viruses from 2007 to 2008 were sensitive to oseltamivir, in contrast to the emergence of resistant H1N1 in humans. Susceptibility remained high to moderate over time among influenza viruses. Sequence analysis of the outliers did not detect molecular markers of drug-resistance (e.g., H275Y NA mutation [N1 numbering]) but revealed mutations outside the NA active site. In particular, V267I, N307D, and V321I residue changes were found, and structural analyses suggest that these mutations distort hydrophobic pockets and affect residues in the NA active site. We determined that natural oseltamivir resistance among swine and wild waterbirds is rare. Minor naturally occurring variants in NA can affect antiviral susceptibility.

In little more than 1 decade, there have been three remarkable events involving the emergence and control of seasonal, prepandemic, and pandemic influenza viruses of the N1 neuraminidase (NA) subtype. The first was the emergence and transmission of a highly pathogenic H5N1 influenza virus from waterbirds to domestic poultry with inefficient transmission to humans that was detected in 1997 (10, 45). Over the next 13 years, the H5N1 virus evolved into more than 10 phylogenetically distinct hemagglutinin (HA) clades, directly or indirectly killed hundreds of millions of gallinaceous birds, and spread to many countries in Eurasia, infecting 442 people and killing 262 (43, 47, 48, 50). These highly pathogenic viruses are continuing to evolve in multiple epicenters, including China, Indonesia, and Egypt (3, 7, 30, 47, 53). The second remarkable event occurred in seasonal influenza in 2007 when resistance to the anti-influenza virus drug oseltamivir was detected in Norway in the absence of drug selection pressure (22, 49, 52). The naturally occurring oseltamivir-resistant H1N1 influenza viruses have been surprisingly fit and had spread globally in humans by early 2009 (13, 24). The third event was the emergence of the pandemic H1N1 2009 influenza virus that was antigenically distinct from the 2007-2008 seasonal H1N1 viruses (16, 40, 51). The pandemic H1N1 influenza viruses also possess the N1 NA from avian sources (8, 16). Unlike previously circulating viruses, the pandemic H1N1 viruses contained a complex of influenza virus genes of Eurasian and North American swine influenza virus origin (40) which were previously derived from reassorted genes of human, swine, and avian lineages. This novel reassortment of HA and NA genes resulted in a virus that was effectively transmitted in humans. Immunocompromised patients, children under the age of 10 years, pregnant women, and people with underlying medical conditions, including obesity, have been particularly affected (16, 25, 26, 36). Efficacious vaccines have been prepared and are being administered. At this time approximately 99% of pandemic H1N1 2009 viruses tested are sensitive to NA inhibitors (NAIs) but are resistant to the adamantanes (49).

A common feature among these influenza virus events is the possession of an N1 subtype of NA. Although each of these N1s is antigenically and phylogenetically distinct, each emerged at different times in the past from the wild waterbird reservoir. While vaccination remains the primary option for

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the prevention and control of both seasonal and pandemic influenza, anti-influenza virus drugs are being recognized as the immediate option for treatment and control, especially during the 6 months or more needed for vaccine preparation and testing (18). The inherent problem with the use of monovalent chemotherapy for the treatment of influenza is that at the onset of a pandemic influenza virus outbreak, rapid vaccine production methods or novel prophylactic vaccines cannot be introduced fast enough. A good example of this was the emergence of pandemic H1N1 2009 viruses and the lack of a novel vaccine. Oseltamivir-resistant seasonal H1N1 influenza viruses emerged in 2007 and were naturally occurring drug-resistant viruses without drug selection pressure. The surprising aspect of the emergence of the resistant seasonal virus was that the resistance appeared in Norway, where little oseltamivir has been used (22, 49). Oseltamivir-resistant strains of H5N1 and pandemic H1N1 have been detected (6, 11), but to date these resistant viruses have not spread consistently and are sensitive to zanamivir, a drug which is more closely fitted to the structure of the NA active site (5, 6, 18, 20, 21, 42).

Close monitoring of clinical isolates has detected drug-resistant influenza viruses from humans, but limited information or monitoring is available concerning the sensitivities of influenza viruses to NAIs in their natural avian reservoir or species other than humans. Our hypothesis was that influenza viruses with reduced susceptibility to anti-influenza virus drugs may exist within the natural reservoir of wild waterbirds and domestic swine. These influenza viruses with reduced susceptibility or resistance to anti-influenza virus drugs could potentially be transmitted to humans. We examined the susceptibilities of N1 NAs from wild waterbirds and domestic swine to the NAIs in a phenotypic NA enzyme inhibition assay and sequenced the NAs of the identified outliers.

MATERIALS AND METHODS

Compounds. Oseltamivir carboxylate (oseltamivir; GS4071; [3*R*,4*R*,5*S*]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexane-1 carboxylic acid) was provided by Hoffmann-La Roche (Basel, Switzerland). Zanamivir (GG167; 4-guanidino-Neu5Ac2en) was provided by GlaxoSmithKline (Research Triangle Park, NC). Peramivir ([1S,2*S*,3*R*,4*R*,1′*S*]-3-[1′-acetylamino-2′-ethyl]butyl-4-[(aminoimino)-methyl]amino-2-hydroxycyclopentane-1-carboxylic acid; BCX-1812) was provided by BioCryst Pharmaceuticals (Birmingham, AL). The compounds were dissolved in distilled water, and aliquots of stock were stored at −20°C until used.

Viruses and cells. The avian and swine influenza viruses out of 123 of the N1 NA subtype are shown in Table 1. Most isolates were from North America. Viruses obtained from avian sources were all from apparently healthy wild waterbirds and were isolated from fecal samples or from tracheal/oropharyngeal or cloacal swabs obtained from Anseriformes (ducks), *Anas platyrhynchos* (mallard), *Anas acuta* (pintail), *Anas discors* (blue-winged teal), *Anas carolinensis* (green-winged teal), *Spatula clypeata* (shoveler), *Aythya americana* (redhead), and *Aythya valisineria* (canvasback) and from Charadriiformes (shorebirds), *Arenaria interpres* (ruddy turnstone), *Larus atricilla* (laughing gull), and *Larus argentatus* (herring gull). The influenza viruses from pigs were from animals with respiratory disease. Viruses were isolated from ducks from 1983 to 2008 and from shorebirds/gulls from 1979 to 2007. Swine isolates were obtained from 2005 to 2009, and human isolates were from 1976 to 2009.

Avian viruses were obtained from the St. Jude Children's Research Hospital influenza repository, USDA-APHIS, and the University of Georgia. Swine viruses were obtained from the University of Minnesota. Human isolates were also obtained from the St. Jude Children's Research Hospital influenza virus repository and were used for comparison in this study. Stocks were made for each virus by passaging in 10-day-old embryonated chicken eggs (avian influenza viruses) or in Madin-Darby canine kidney cells (MDCK; for human and swine influenza viruses). These virus stocks were frozen at −80°C until used. MDCK cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained as described previously (54).

NA activity and NA inhibition assays. NA activity of the virus was measured in a fluorescent assay by using the fluorigenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid sodium salt hydrate (MUNANA; Sigma, St. Louis, MO) as the substrate, based on the method of Potier et al. (37). The susceptibility of viruses was tested in an NA enzyme inhibition assay (4). Fluorimetric determinations were quantified with a Fluoroskan II (Labsystems, Helsinki, Finland) or Synergy 2 (BioTek Instruments, Winooski, VT) fluorimeter using an excitation wavelength of 360 nm and an emission wavelength of 460 nm and measuring fluorescence of the released 4-methyl-umbelliferone.

Viruses were standardized to equivalent NA enzyme activity in the linear range of the curve and incubated with NA inhibitor at concentrations of 0.00005 to 100 μM. For NA inhibition assays, 10 μl of drug and 10 μl of diluted virus were mixed and preincubated for 30 min at 37°C. Next, 30 μl of 100 μM MUNANA in 325 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 6.5; Sigma, St. Louis, MO) buffer containing 10 mM $CaCl_2$ was added. After 30 min at 37°C, the reaction was stopped by addition of 150 μl of freshly prepared stop solution (25% ethanol and 12.5% glycine; Fisher Scientific, Rochester, NY) in distilled water. The concentration of NA inhibitor that reduced NA activity by 50% relative to a control mixture with no inhibitor (IC_{50}) was determined by plotting the percent inhibition of NA activity as a function of the compound concentrations calculated. IC_{50}s were calculated on a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA), and values were transferred to a GraphPad Prism (GraphPad Software, La Jolla, CA). IC_{50}s are reported as the means of three independent determinations.

Statistics. Data were heteroscedastic (Fligner-Killeen; $P < 0.0001$), and so the Kruskal-Wallis (nonparametric test, equivalent to an analysis of variance) and Dunn's multiple comparison tests were used to analyze differences in the mean IC_{50}s among the four groups (ducks, shorebirds, swine, and humans) and year of virus isolation.

Sequencing. The RNeasy kit (Qiagen, Chatsworth, CA) was used to extract viral RNA, and a one-step reverse transcription-PCR kit (Qiagen, Chatsworth, CA) and universal primers to the NA and M2 genes were used for amplification. The sequences were determined by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital by using a BigDye Terminator (version 3) cycle sequencing kit and synthetic oligonucleotides. Samples were analyzed on Applied Biosystems 3700 DNA analyzers (Foster City, CA). Analysis of amino acid residues was based on the N1 numbering system.

Three-dimensional macromolecular structural modeling and visualization. The PyMOL molecular visualization system (DeLano Scientific LLC, Palo Alto, CA) was used to produce high-quality three-dimensional images based on the crystal structure of the influenza virus NA of the N1 subtype (PDB code 3cl0) from the RCSB protein structure database. Analysis of three-dimensional structure was based on the N1 numbering system.

RESULTS

Oseltamivir susceptibility among avian species. Migratory waterfowl have been accepted as the primordial source of influenza viruses that transmit to other species, including humans. Although humans can be infected with this primordial avian source of influenza virus, little attention has been given to the sensitivity of the viruses to anti-influenza virus drugs. Influenza viruses from 62 ducks (Anseriformes), 25 shorebirds (Charadriiformes), and 4 gulls (Laridae), listed in Table 1, were examined for antiviral susceptibility in the phenotypic NA enzyme inhibition assay. Currently there is no guidance on how to scale susceptibility and analyze influenza viruses with different IC_{50}s. However, the Neuraminidase Inhibitor Susceptibility Network (NISN) provides a panel of reference viruses for the standardization of IC_{50}s, and these were used as a basis for our analysis. The two resistant viruses, A/Fukui/45/2004 (H3N2) carrying an E119V mutation and A/Mississippi/3/2001 (H1N1) carrying an H274Y NA mutation, exhibited a range of IC_{50}s for oseltamivir (48 to 413 nM; fluorescence-based NA enzyme inhibition assays). We chose an IC_{50} of >50 nM as representing reduced susceptibility. Although resistant human

TABLE 1. Susceptibility of avian influenza A viruses of N1 NA subtype to the NA inhibitor oseltamivir

Source and name of N1 NA virus	Subtype	Yr isolated	Origin	Mean IC_{50} ± SD (nM)[b]
Ducks[a]				
A/Mallard/Alberta/743/83	H9N1	1983	Canada	20.40 ± 2.69
A/Blue-Winged Teal/Alberta/212/84	H1N1	1984	Canada	9.01 ± 1.58
A/Mallard/TN/11464/85	H1N1	1985	USA	39.75 ± 10.00
A/Blue-Winged Teal/LA/B228/86	H1N1	1986	USA	5.98 ± 2.02
A/Mallard/Alberta/323/88	H2N1	1988	USA	24.88 ± 11.16
A/Mallard/Alberta/253/90	H6N1	1990	Canada	1.28 ± 0.34
A/Mallard/Alberta/107/91	H3N1	1991	USA	12.11 ± 3.60
A/Mallard/Alberta/196/92	H3N1	1992	Canada	22.75 ± 0.00
A/Pintail/Alberta/129/93	H7N1	1993	Canada	0.81 ± 0.36
A/Mallard/Alberta/5/95	H10N1	1995	Canada	1.82 ± 0.55
A/Mallard/Alberta/267/96	H1N1	1996	Canada	56.70 ± 11.82
A/Mallard/Alberta/119/98	H1N1	1998	Canada	4.35 ± 0.00
A/Mallard/Alberta/201/98	H1N1	1998	Canada	13.40 ± 0.00
A/Mallard/Alberta/34/2001	H7N1	2001	Canada	102.25 ± 3.80
A/Pintail/Alberta/210/2002	H1N1	2002	Canada	8.50 ± 10.25
A/Mallard/Alberta/130/2003	H4N1	2003	Canada	8.99 ± 6.40
A/Mallard/Alberta/88/2004	H1N1	2004	Canada	99.17 ± 61.85
A/Mallard/Alberta/226/2004	H3N1	2004	Canada	51.61 ± 14.43
A/Pintail/Alberta/68/2005	H1N1	2005	Canada	40.57 ± 8.54
A/Pintail/Alberta/69/2005	H1N1	2005	Canada	11.52 ± 3.65
A/Canvasback/Alberta/276/2005	H1N1	2005	Canada	39.30 ± 8.81
A/Mallard/PA/454069-9/2006	H5N1	2006	USA	2.33 ± 0.96
A/Mallard/MN/SG-000220/2007	H6N1	2007	USA	7.36 ± 3.96
A/Green-Winged Teal/LA/SG-00090/2007	H1N1	2007	USA	18.03 ± 8.36
A/Mallard/MN/AI07-3018/2007	H1N1	2007	USA	29.61 ± 20.09
A/Mallard/MN/AI07-3019/2007	H3N1	2007	USA	12.97 ± 2.85
A/Mallard/MN/AI07-3099/2007	H3N1	2007	USA	12.83 ± 4.18
A/Mallard/MN/AI07-3100/2007	H1N1	2007	USA	10.54 ± 2.97
A/Mallard/OH/510306-4/2007	H5N1	2007	USA	6.55 ± 2.33
A/Mallard/MI/463796-7/2007	H5N1	2007	USA	4.48 ± 1.17
A/Mallard/MN/AI07-3108/2007	H3N1	2007	USA	6.39 ± 2.10
A/Mallard/MN/AI07-3124/2007	H3N1	2007	USA	8.39 ± 2.45
A/Mallard/MN/AI07-3127/2007	H1N1	2007	USA	5.11 ± 1.89
A/Mallard/MN/AI07-3136/2007	H1N1	2007	USA	6.41 ± 3.39
A/Mallard/MN/AI07-3140/2007	H1N1	2007	USA	7.50 ± 2.36
A/Mallard/MN/AI07-3189/2007	H10N1	2007	USA	6.74 ± 4.38
A/Mallard/MN/SG-000220/2007	H6N1	2007	USA	5.69 ± 3.12
A/Mallard/MN/SG-000223/2007	H6N1	2007	USA	3.97 ± 2.68
A/Mallard/MN/SG-000170/2007	H6N1	2007	USA	12.53 ± 3.84
A/Mallard/MN/SG-000214/2007	H6N1	2007	USA	6.18 ± 1.81
A/Mallard/MN/SG-000104/2007	H6N1	2007	USA	7.62 ± 3.82
A/Mallard/MN/SG-000105/2007	H6N1	2007	USA	7.02 ± 2.56
A/Mallard/MN/SG-000121/2007	H1N1	2007	USA	12.41 ± 2.43
A/Red Headed Duck/MN/SG-000123/2007	H1N1	2007	USA	10.49 ± 2.25
A/Blue-Winged Teal/TX/SG-00087/2007	H4N1	2007	USA	6.03 ± 2.11
A/Mallard/MI/463796-7/2007	H5N1	2007	USA	4.48 ± 1.17
A/Mallard/OH/510306-4/2007	H5N1	2007	USA	6.55 ± 2.33
A/Mallard/OH/4809/2008	H1N1	2008	USA	8.58 ± 1.47
A/Mallard/MN/SG-00572/2008	H3N1	2008	USA	8.83 ± 1.96
A/Mallard/MN/SG-00627/2008	H1N1	2008	USA	6.95 ± 3.18
A/Mallard/MN/SG-00628/2008	H1N1	2008	USA	4.91 ± 1.63
A/Northern Shoveler/MN/SG-00651/2008	H1N1	2008	USA	8.80 ± 2.81
A/Northern Shoveler/MN/SG-00655/2008	H1N1	2008	USA	9.39 ± 4.22
A/Northern Shoveler/MN/SG-00661/2008	H3N1	2008	USA	8.15 ± 2.41
A/Northern Shoveler/MN/SG-00665/2008	H3N1	2008	USA	11.40 ± 4.95
A/Mallard/MN/AI08-3825/2008	H5N1	2008	USA	9.56 ± 4.80
A/Mallard/MN/AI08-4507/2008	H3N1	2008	USA	7.56 ± 4.44
A/Green-Winged Teal/AI08-4655/2008	H5N1	2008	USA	1.87 ± 0.73
A/Mallard/MN/AI08-5384/2008	H5N1	2008	USA	12.00 ± 1.14
Shorebirds/gulls[a]				
A/Gull/Kazakhstan/870/79	H1N1	1979	USSR	3.07 ± 0.91
A/Ruddy Turnstone/NJ/61/85	H11N1	1985	USA	23.97 ± 6.89
A/Herring Gull/DE/698/88	H2N1	1988	USA	10.85 ± 0.14
A/Ruddy Turnstone/DE/34/93	H2N1	1993	USA	41.13 ± 15.51
A/Ruddy Turnstone/DE/78/93	H11N1	1993	USA	10.05 ± 0.85

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TABLE 1—*Continued*

Source and name of N1 NA virus	Subtype	Yr isolated	Origin	Mean IC_{50} ± SD (nM)[b]
A/Ruddy Turnstone/DE/8/93	H2N1	1993	USA	14.22 ± 10.33
A/Laughing Gull/DE/254/93	H13N1	1993	USA	0.50 ± 0.21
A/Ruddy Turnstone/DE/81/93	H2N1	1993	USA	23.97 ± 6.89
A/Ruddy Turnstone/DE/170/94	H3N1	1994	USA	0.98 ± 0.46
A/Ruddy Turnstone/DE/185/94	H3N1	1994	USA	3.59 ± 1.41
A/Ruddy Turnstone/DE/183/94	H3N1	1994	USA	1.12 ± 0.39
A/Shorebird/DE/39/95	H3N1	1995	USA	3.23 ± 1.33
A/Shorebird/DE/288/95	H3N1	1995	USA	0.92 ± 0.33
A/Shorebird/DE/24/96	H11N1	1996	USA	35.25 ± 3.06
A/Ruddy Turnstone/DE/125/96	H12N1	1996	USA	45.82 ± 2.44
A/Shorebird/DE/111/97	H2N1	1997	USA	39.00 ± 2.84
A/Shorebird/DE/138/97	H2N1	1997	USA	2.68 ± 0.21
A/Shorebird/DE/182/97	H2N1	1997	USA	154.43 ± 38.53
A/Shorebird/DE/24/98	H2N1	1998	USA	111.07 ± 18.61
A/Shorebird/DE/95/2003	H9N1	2003	USA	67.00 ± 11.16
A/Shorebird/DE/68/2003	H9N1	2003	USA	52.85 ± 13.95
A/Laughing Gull/DE/5/2003	H9N1	2003	USA	65.80 ± 17.88
A/Shorebird/DE/65/2003	H9N1	2003	USA	5.63 ± 2.63
A/Ruddy Turnstone/NJ/AI07-69/2007	H5N1	2007	USA	5.79 ± 1.36
A/Ruddy Turnstone/NJ/AI07-72/2007	H9N1	2007	USA	6.46 ± 1.57
A/Ruddy Turnstone/NJ/AI07-283/2007	H9N1	2007	USA	5.59 ± 1.61
A/Ruddy Turnstone/NJ/AI07-296/2007	H6N1	2007	USA	7.36 ± 3.96
A/Ruddy Turnstone/NJ/AI07-699/2007	H5N1	2007	USA	4.60 ± 1.44
A/Ruddy Turnstone/NJ/AI07-839/2007	H12N1	2007	USA	6.60 ± 2.22
Swine[c]				
A/Swine/NC/38448-1/2005	H1N1	2005	USA	2.11 ± 0.91
A/Swine/KS/029170/2007	H1N1	2007	USA	0.33 ± 0.12
A/Swine/OK/038826/2007	H1N1	2007	USA	1.82 ± 0.42
A/Swine/NE/047330/2007	H1N1	2007	USA	0.90 ± 0.49
A/Swine/OH/004880/2008	H1N1	2008	USA	3.31 ± 1.52
A/Swine/NC/007270/2008	H1N1	2008	USA	1.12 ± 0.65
A/Swine/KY/012454/2008	H1N1	2008	USA	1.44 ± 0.18
A/Swine/MN/016245/2008	H1N1	2008	USA	0.66 ± 0.43
A/Swine/WI/018247-2/2008	H1N1	2008	USA	1.27 ± 0.48
A/Swine/IL/020968/2008	H1N1	2008	USA	1.90 ± 0.66
A/Swine/MO/044329/2008	H1N1	2008	USA	0.65 ± 0.25
A/Swine/MN/055403-3/2008	H1N1	2008	USA	0.79 ± 0.27
A/Swine/IA/056944/2008	H1N1	2008	USA	1.77 ± 0.79
A/Swine/IL/060530/2008	H1N1	2008	USA	0.92 ± 0.12
A/Swine/IA/003479/2009	H1N1	2009	USA	2.56 ± 1.50

[a] Influenza viruses obtained from avian sources were all from apparently healthy wild birds and were isolated from fecal samples or from tracheal/oropharyngeal or cloacal swabs. Stock viruses of avian isolates were made by passaging in 10-day-old embryonated chicken eggs. Two-letter abbreviations in virus names correspond to standard postal abbreviations for states.

[b] The NA inhibition assay used MUNANA as substrate (final concentration, 100 μM). Values are the means of at least three independent determinations.

[c] Influenza viruses from pigs were from animals with respiratory disease and were isolated from bronchial swabs, nasal swabs, or lung tissues.

viruses exhibited an IC_{50} of 2,700 nM (Table 2), none of the avian isolates tested approached this number. Thus, we classified our avian viruses as having "reduced susceptibility" instead of being "resistant." The "highly susceptible" isolates possessed IC_{50}s (<5 nM) comparable with the susceptible reference viruses from the NISN panel, A/Fukui/20/2004 (H3N2) and A/Mississippi/3/2001 (H1N1) (histidine at 275), which exhibit a range of IC_{50} values for oseltamivir of 0.2 to 3.0 nM. The higher IC_{50} cutoff was used instead of 3.0 nM because 5 nM represents a 10-fold change from the reduced susceptibility cutoff of 50 nM. Table 2 shows the levels of susceptibility among avian, swine, and human influenza viruses. Importantly, we did not detect any NAI-resistant viruses among the N1 NA avian influenza virus strains. Approximately 16% of influenza viruses isolated from ducks were highly susceptible to oseltamivir (mean IC_{50}, 0.81 to 4.91 nM), 77% were moderately susceptible (mean IC_{50}, 5.11 to 40.57 nM), and only 6.5% had reduced susceptibility (mean IC_{50}, 51.61 to 102.25 nM) (Table 2). Among combined shorebird and gull influenza isolates, 31% were highly susceptible (mean IC_{50}, 0.5 to 4.6 nM), 52% were moderately susceptible (mean IC_{50}, 5.59 to 45.82 nM), and 17% had reduced susceptibility (mean IC_{50}, 52.85 to 154.43 nM). The avian viral isolates from wild waterbirds showed similar levels of sensitivity to oseltamivir in all ranges of susceptibility, whereas human and swine influenza viruses had comparable mean IC_{50}s that were highly susceptible (Table 2). Furthermore, the variation in the waterbirds is higher than that of the mammalian samples (Fligner-Killeen test; $P < 0.0001$). In addition, within each level of susceptibility among wild waterbirds, a range in values of oseltamivir susceptibility was observed. Duck and shorebird isolates ranged mainly from ≤5 nM to <50 nM (highly susceptible to moderately susceptible), which is in contrast to the values observed for swine and human isolates. Of the avian viruses (combining ducks, shore-

TABLE 2. Susceptibilities of the avian, swine, and human influenza viruses of the N1 NA subtype to the NA inhibitor oseltamivir

Species	Susceptibility[a]	IC_{50} range (nM)[b]	No. of isolates (% of total)	Mean IC_{50} ± SD (nM)
Ducks	High	0.81–4.91	12 (18.5)	2.97 ± 1.49
	Moderate	5.11–40.57	50 (77)	12.85 ± 8.82
	Reduced	51.61–102.25	4 (6)	77.43 ± 26.99
Shorebirds/ gulls	High	0.5–4.6	9 (31)	2.80 ± 1.34
	Moderate	5.59–45.82	15 (52)	13.57 ± 9.62
	Reduced	52.85–154.43	5 (17)	92.76 ± 36.59
Swine	High	0.33–2.56	15 (100)	1.44 ± 0.81
	Moderate	NA[c]	0	NA
	Reduced	NA	0	NA
Humans	Sensitive	0.89–2.12	14 (82)	1.40 ± 0.36
	Resistant	2,588.92–2,887.63	3 (17)	2,725.1 ± 151.1

[a] Susceptibilities of influenza viruses to oseltamivir were recorded as high (mean IC_{50}, 0.5 to 5.0 nM), moderate (mean IC_{50}, 5.1 to 50.0 nM), or reduced (mean IC_{50}, 50 to ≥100 nM).

[b] The NA inhibition assay was performed with viruses standardized to equivalent NA activity and incubated with NAIs at concentrations of 0.00005 to 100 μM with MUNANA as a substrate. The IC_{50} was determined by plotting the dose-response curve of inhibition of NA activity as a function of the compound concentration. Values are from at least three independent determinations.

[c] NA, not applicable.

birds, and gulls), 19 were highly susceptible to oseltamivir (mean IC_{50}, 0.5 to 5.0 nM) and 63 were moderately susceptible (mean IC_{50}, 5.1 to 50 nM) (Table 2). Seven avian isolates (three from ducks, three from shorebirds, and one from gulls) had reduced susceptibility to oseltamivir (mean IC_{50}, >50 nM). Four avian isolates had an IC_{50} between 50 and 100 nM, and three others had an IC_{50} of >100 nM. Therefore, approximately 21% of all wild avian influenza virus isolates were highly susceptible, 69% were moderately susceptible, and only ~10% had reduced susceptibility. Overall, the avian isolates analyzed had variations in their susceptibilities to oseltamivir. The range of IC_{50}s that was observed among the avian isolates was then categorized into baseline values of susceptibility. The influenza viruses of the N1 NA subtype isolated from shorebirds had higher IC_{50}s (mean IC_{50} of 26 nM) and therefore were less susceptible to oseltamivir *in vitro* than were the susceptible human isolates. The overall mean IC_{50} for influenza viruses isolated from ducks was 16.1 nM. Figure 1 shows distribution plots that further illustrate that most of the avian influenza virus isolates fell within the range of moderate susceptibility (middle portion of the distribution graph). Ducks and shorebirds/gulls had comparable distribution patterns of antiviral susceptibility. Swine and human seasonal influenza virus isolates (except 2001 to 2008 viruses) showed a difference in their distribution pattern of antiviral susceptibility from those seen in wild waterbirds.

Oseltamivir susceptibility among human and swine influenza virus isolates. Swine and human influenza viruses had mean IC_{50}s that were much lower (1.4 nM) than those of ducks and shorebirds/gulls, which ranged from as low as 0.5 nM to as high as 154.43 nM. In addition, influenza viruses that were resistant to oseltamivir were found only in the seasonal human influenza viruses, such as A/Georgia/20/2006, whose resistance is well established (39). We determined that the mean IC_{50}s differed among the four groups analyzed (ducks, shorebirds, swine, and humans; $P < 0.0001$). Dunn's multiple comparison test of the mean IC_{50}s between the pairs of different groups revealed the following P values: (i) duck versus shorebirds, $P > 0.05$; (ii) duck versus swine, $P < 0.05$; (iii) duck versus human, $P < 0.05$; (iv) swine versus human, $P > 0.05$; (v) shorebirds versus swine, $P < 0.05$; (vi) shorebirds versus humans, $P < 0.05$. Thus, there is no significant difference in the mean IC_{50}s between ducks and shorebirds or between swine and humans, but the avian (ducks and shorebirds) values were significantly higher than the mammalian (swine and humans) isolates.

Analysis of susceptibility over time. Of particular interest was whether the data over time would reveal either a significant increase in the number of isolates with an IC_{50} outside the quantile range or, alternatively, whether a significant increase in mean IC_{50} over time would be evident. Viruses originating primarily in North America were also analyzed by year of isolation to determine whether NAI susceptibility had varied over 10-year periods between 1976 and 2009 (Fig. 2). All avian and swine influenza viruses remained susceptible to oseltamivir over time. The differences between years of isolation did not show evidence of a clear linear upward trend but remained fairly stable from year to year among the avian species over the period studied. This is quite different from the dramatic increase in the number of human influenza viruses that were resistant to oseltamivir in 2007 and 2008. Interestingly, human pandemic H1N1 influenza viruses isolated in 2009 possessed similar susceptibility to oseltamivir as the sensitive seasonal H1N1 Brisbane-like viruses from previous years (Fig. 2). The surveillance of circulating human H1N1 influenza viruses by the World Health Organization, Centers for Disease Control and Prevention, and others revealed the same trend of oseltamivir resistance among human influenza virus isolates from 2007 to 2009 (13, 22, 39). Statistical analysis revealed that there was no significant difference between isolation years within a species and their susceptibility to oseltamivir with the exception of human influenza viruses (Fig. 2). A clear upward trend

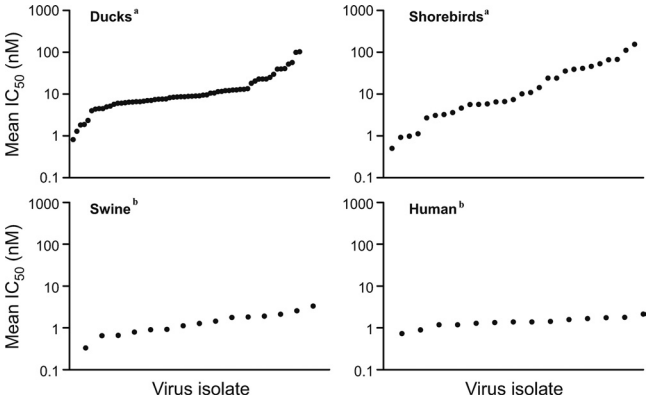

FIG. 1. Plots showing the IC_{50} (in nM) ranges of oseltamivir for avian, swine, and human influenza viruses of the N1 NA subtype. Isolates are ranked in order by the IC_{50}. Sixty-two duck, 25 shorebird, 4 gull, and 15 swine isolates and 14 human seasonal influenza viruses were analyzed. Mean IC_{50}s between the four types of hosts were analyzed using a Kruskal-Wallis test and Dunn's multiple comparison post hoc test. The groups designated with the same letter (either a or b) did not differ significantly ($P > 0.05$). The groups designated with different letters differed significantly ($P < 0.05$).

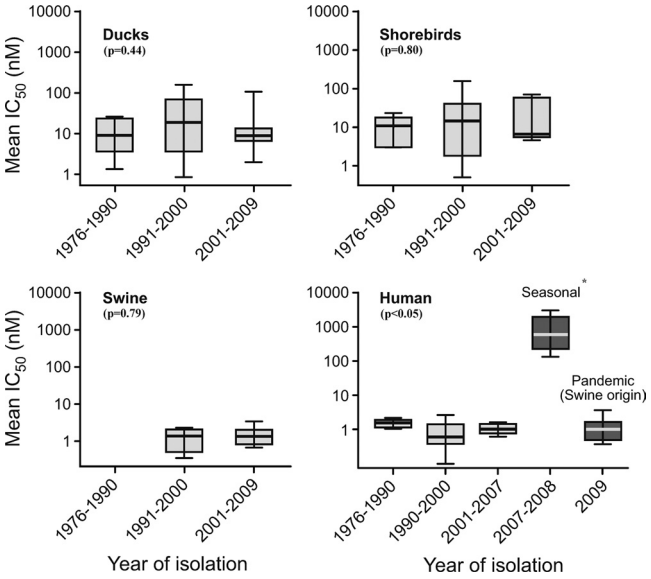

FIG. 2. Quantile box plots illustrating the $\log_{10}$ mean IC_{50}s for oseltamivir for each species from which virus was isolated. The range of isolation years for each species that virus was isolated is shown on the *x* axis. Viruses collected were all from various wild birds, mainly from the United States and Canada. Some isolates and their respective IC_{50}s are from published data (5, 21, 33 22, 28, 29). Results within a graph were analyzed using a Kruskal-Wallis test and Dunn's multiple comparison post hoc test; *P* values are included on the graphs. Data marked with an * are statistically different from data for other years within a graph.

of the mean IC_{50}s was observed among human isolates ($P = 0.0010$). This was due to seasonal influenza virus that was resistant to oseltamivir collected from 2007 to 2008, which is contrast to isolates collected from avian and swine hosts ($P > 0.05$).

Hemagglutinin subtype and oseltamivir susceptibility. All influenza A virus subtypes, H1 to H16, have been isolated from wild aquatic birds (35, 46), and their function is related to the HA/NA balance (27). In this study, avian isolates were examined by HA subtype to determine if different combinations of HA would have an effect on susceptibility to oseltamivir. We found that different combinations of HA and NA had no correlation to oseltamivir susceptibility (data not shown).

Characterization of minor outliers. Seven of the 123 viruses tested (three isolates from ducks, three from shorebirds, and one from gull) had IC_{50}s greater than any other viruses tested (Table 3). The three that had the highest IC_{50}s (>100 nM) were from A/Shorebird/DE/182/97 (H2N1), A/Shorebird/DE/24/98 (H2N1), and A/Mallard/Alberta/34/2001 (H7N1). One avian isolate with a value that was near 100 nM was A/Mallard/Alberta/88/2004 (H1N1). The A/Laughing Gull/DE/5/2003 (H9N1) and A/Shorebird/DE/95/2003 (H9N1) isolates had IC_{50}s of >50 nM but <100 nM. The NA genes of the seven avian outliers were sequenced and were aligned with the NA amino acid sequences from 188 other North American avian N1 NA isolates available in the public domain (from online databases, Influenza Sequence NCBI Influenza Virus Resource, and GenBank) to determine the consensus sequence. Sequence analysis revealed a number of NA amino acids that differed between the isolates with reduced oseltamivir susceptibility and the consensus sequence (Fig. 3 and 4). Furthermore, these mutations were also found at varying frequencies in the 188 other N1 NA sequences (Fig. 4). Significantly, none of the sequences had any of the known NA mutations, such as H275Y, that confer oseltamivir resistance (9, 34).

NA active site residues that directly interact with the substrate are the catalytic residues, and the framework residues provide a structural scaffold for the catalytic residues (9, 19). Figure 3A shows these regions within a subunit (monomer) of the NA tetramer in which these important residues are conserved. Interestingly, analysis of the residue changes observed among avian isolates with reduced oseltamivir susceptibility revealed that none of the changes was interacting directly with catalytic or framework residues (Fig. 3A). These data suggest that binding of the oseltamivir may be affected by NA amino acid residues outside the active site, and additional studies are required to test this hypothesis. One particular residue change that appeared most frequently (in approximately 8.5% [16/188] of all avian isolates) was N307D. This residue change also appeared in three of the seven avian influenza viruses with reduced susceptibility. Furthermore, V267I appeared in two of the seven of these avian influenza viruses. Residues V267 and N307 are near a hydrophobic patch (Fig. 3B). Another strain of avian influenza virus with reduced susceptibility had NA mutations K262R and V321I. Residue K262 lies on the surface of NA. In contrast, residue V321 lies within another hydrophobic

TABLE 3. Characterization of minor outliers among avian influenza viruses of the N1 NA subtype

Species	Virus strain	Subtype	Mean $IC_{50} \pm$ SD (nM)[a]			NA mutation(s)[b]
			Oseltamivir	Zanamivir	Peramivir	
Ducks	A/Mallard/Alberta/34/2001	H7N1	102.25 ± 3.80	0.48 ± 0.11	2.76 ± 2.00	N307D
	A/Mallard/Alberta/88/2004	H1N1	99.17 ± 61.85	8.72 ± 5.48	8.18 ± 2.30	K262R; V321I
	A/Mallard/TN/11464/85	H1N1	39.75 ± 10.0	6.21 ± 2.84	3.51 ± 2.65	G105S; H126N; I234M; M289V;V394I; N449S
Shorebirds/Gulls	A/Shorebird/DE/182/97	H2N1	154.43 ± 38.53	7.22 ± 4.11	4.25 ± 1.97	V267I; N307D
	A/Shorebird/DE/24/98	H2N1	111.07 ± 18.61	6.00 ± 4.86	8.67 ± 1.51	S172L; V267I; N307D
	A/Laughing Gull/DE/5/2003	H9N1	65.80 ± 17.88	3.93 ± 0.87	4.92 ± 1.57	P93L;V264A; K390R;
	A/Shorebird/DE/95/2003	H9N1	67.00 ± 11.16	5.50 ± 0.40	4.76 ± 1.38	P93L; A181S; R220G; 264A

[a] The NA inhibition assay was performed with viruses standardized to equivalent NA activity and incubated with NAIs at concentrations of 0.00005 to 100 μM with MUNANA as a substrate. The IC_{50} was determined by plotting the dose-response curve of inhibition of NA activity as a function of the compound concentration. Values are from at least three independent determinations.

[b] RNA was isolated directly from virus-containing allantoic or cultural fluids. Amino acid numbering is based on N1 NA.

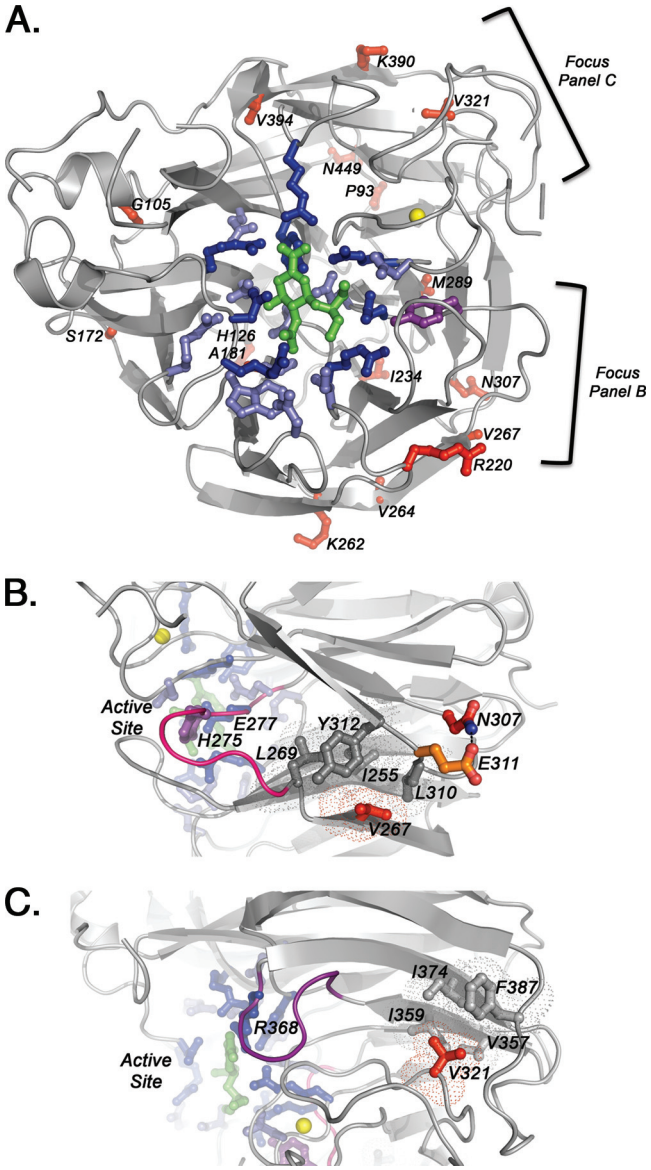

FIG. 3. Structure of the complex between N1 influenza virus NA and oseltamivir (PDB code 3cl0) and the amino acid substitutions that were found in seven avian isolates with reduced susceptibility. Amino acid analysis was based on N1 numbering system. (A) Locations of NA residue changes found in seven avian isolates with reduced susceptibility (red). Also shown are catalytic residues (dark blue and purple; 8 residues), framework residues (light blue; 11 residues), oseltamivir (green), and calcium ion (yellow). The H275Y (N1 numbering; H274Y is in N2 numbering). The NA mutation is shown in purple. (B) Two residues, V267 and N307, may be responsible for reduced susceptibility to oseltamivir. Mutation may destabilize the hydrophobic patch (dark gray; four residues), which in turn may destabilize the nearby loop (pink) containing oseltamivir-interacting residues H275 and E277. (C) Residue V321, which may be responsible for reduced susceptibility to oseltamivir. The mutation may destabilize the hydrophobic patch (light gray; four residues), which in turn may destabilize the nearby loop (purple) containing oseltamivir-interacting residue R368. All residues are labeled using N1 numbering.

patch (Fig. 3C). The three mutations (V267I, N307D, and V321I) that are potentially responsible for reduced oseltamivir susceptibility were not present in moderately or highly susceptible isolates tested in this study. Lastly, a few viral isolates had

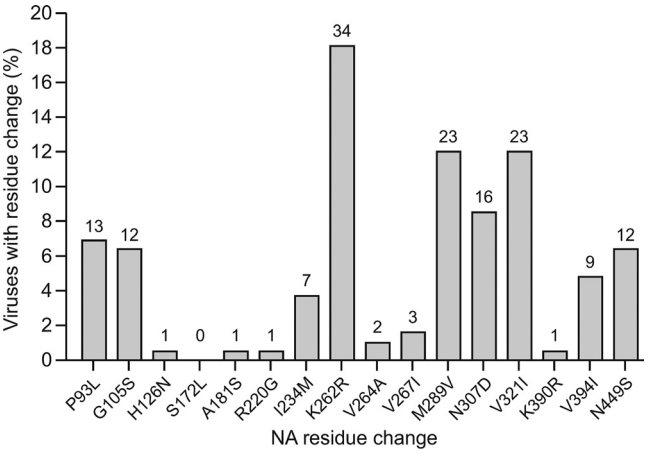

FIG. 4. Frequencies of the NA mutations identified in the seven outliers across 188 influenza viruses isolated from North American waterfowl (from the online databases, Influenza Sequence NCBI Influenza Virus Resource, GenBank). The numbers above each bar represent the number of virus isolates exhibiting the indicated mutation.

several NA mutations, making it difficult to identify the mutation that may be contributing to the reduced oseltamivir susceptibility (Fig. 4). All seven influenza viruses with reduced oseltamivir susceptibility were sensitive to other NAIs, zanamivir and peramivir (Table 3).

Sequence analysis of M2 protein. The matrix protein residues that were examined are conserved regions of the protein. These regions are shared among all avian influenza viruses and are known to confer amantadine resistance (e.g., M2 residue at positions 26, 27, 30, 31, and 34). Only the absence of known amino acid residues conferring resistance to adamantanes was analyzed in the study. Sequence alignment and analysis of the seven avian influenza virus outliers did not reveal any mutations in their M2 protein, which confers amantadine resistance (data not shown). In addition, sequence alignment and analysis of at least 10 additional avian influenza viruses of the N1 NA subtype did not reveal any residue changes at positions 26, 27, 30, 31, or 34 in the transmembrane region of the M2 protein.

DISCUSSION

There is particular importance for confirming the activity of NAIs against avian and swine influenza viruses which may infect humans and could potentially initiate a new influenza pandemic. We found that natural oseltamivir resistance among wild aquatic avian and swine influenza viruses of the N1 NA subtype is rare; most (90%) avian isolates from 1979 to 2008 were highly (21%) or moderately (69%) susceptible to oseltamivir (mean IC_{50} range, 5 to 50 nM). Out of 91 avian isolates, seven isolates (7.7%) had reduced susceptibility to oseltamivir (IC_{50}, >50 nM). However, those seven outliers were susceptible to zanamivir and peramivir. All swine influenza viruses examined (15/15) were susceptible to oseltamivir *in vitro* and had IC_{50}s of 1.4 nM, which is comparable to those of seasonal H1N1 human viruses isolated prior to 2007. We suggest that possible molecular markers of reduced oseltamivir susceptibility in avian N1 NA viruses are amino acid changes located

outside the active site (V267I, N307D, and V321I) that potentially distort hydrophobic pockets and indirectly affect the NA catalytic and framework residues. This study is the first to our knowledge to define the levels of susceptibility to NAIs among the influenza viruses isolated from wild waterbirds and swine and focus on the susceptibility of influenza viruses in their natural reservoir.

For all influenza virus subtypes there is the potential for the virus to become resistant to adamantanes or NAIs by a mutation(s), usually in response to treatment with the drug. Positive selective pressures caused by anti-influenza virus prophylaxis, such as the NAIs that are used to treat influenza virus infections in humans, do not occur among wild waterbirds or swine in nature. We hypothesize that antiviral resistance and reduced susceptibility can occur in avian influenza viruses by three different mechanisms. First, continuous evolution of influenza viruses could result in the random acquisition of specific mutations in NA that reduce NAI susceptibility or even lead to drug resistance. In addition, mutations in other viral genes could improve viral fitness and transmissibility and thus result in dissemination of these variants. A striking example of this possibility is the emergence and widespread use of oseltamivir-resistant variants with the well-established H275Y NA amino acid substitution among seasonal H1N1 influenza viruses of the A/Solomon Islands/3/2006 and A/Brisbane/2007 lineages circulating in 2007–2008 (1, 12, 29, 31, 49). It is still not well understood why oseltamivir-resistant H1N1 viruses circulating in 2007–2008 were highly fit in humans.

Second, the resistance could occur through gene reassortment with human and swine viruses carrying drug-resistant mutations. Direct transmission of whole avian influenza virus to mammals, or incorporation of avian gene segments into mammalian strains, e.g., H3N2 triple reassortants in pigs in the United States, has been documented (8, 10, 44). These viruses obtained their NA from avian viruses that do not likely have drug resistance mutations. To date, there is no evidence of drug-resistant avian influenza virus strains being transferred to humans or other species or their contribution to a reassortant event.

Finally, with low probability, resistance could occur in the avian species through drug pressure when oseltamivir is released in water or sewage. Recent studies have shown that oseltamivir carboxylate (the active metabolite of oseltamivir) is not degraded in aquatic environments and is present at detectable levels in river water and sewage discharge (17, 41). Although oseltamivir carboxylate is not orally bioavailable in humans, there is the potential that wild waterbirds and gallinaceous birds, or their excretions carrying influenza viruses, may encounter water contaminated with the drug (15). This is notable because migratory waterfowl can travel to these areas and become exposed to oseltamivir. Therefore, potential exposure of influenza viruses from wild waterbirds to oseltamivir could promote the evolution of viral resistance in nature. However, in our study, no resistance was detected among N1 NA influenza viruses isolated from wild waterbirds.

Our study revealed that duck and shorebird/gull influenza viruses have a wider range of susceptibility to oseltamivir (IC_{50} range, 0.5 to 154.43 nM) than swine or human isolates (IC_{50} range, 0.33 to 2.56 nM). The mean IC_{50} for both swine and humans is 1.4 nM, which indicates that the oseltamivir susceptibility of the swine NA is more "human-like." Oseltamivir susceptibility among wild avian and swine influenza viruses remained stable over a 10-year period except for the seven outliers. Interestingly, four out of these seven outliers were isolated in the same span of time (2001 to 2004) in which oseltamivir was put into use (1999). Other investigators have determined baseline levels of susceptibility to oseltamivir, but only among human influenza isolates after NAIs were put into use (14, 20, 23, 24, 32, 33, 39). We found small differences in the mean IC_{50}s for the inhibition of avian and swine influenza virus N1 NAs for isolates from 1979 to 2008. Similarly, it was reported for human isolates of N1 and N2 NA subtypes that susceptibility to NAIs is stable over time (23, 31). In addition, no correlation to oseltamivir susceptibility was detected with different HA/NA combinations.

Analysis of the NA sequences of the isolates that had reduced oseltamivir susceptibility revealed a number of mutations outside the catalytic and framework residues. Structural analysis suggests that these mutations may indirectly affect the stability of the active site residues and their interaction with oseltamivir. It has been documented that residues far away from an enzymatic active site can affect the enzymatic activity indirectly, through "energy channels" throughout the protein (2, 38), and this may be the case for the NA enzyme of influenza virus as well. Interestingly, a number of residue changes, such as V267I and N307D, appeared in two of three of the avian isolates with reduced oseltamivir susceptibility. One isolate carried a V321I substitution. Residues V267 and N307 are near a hydrophobic patch that may be important for stabilizing active site residues H274 and E276 (Fig. 3B). Residue V267 resides in the hydrophobic patch, and mutation to a bulkier isoleucine may distort this region. Residue N307 forms a hydrogen bond with E311, and mutation of N307 to negatively charged aspartate would likely disrupt this hydrogen bond and cause repulsion. Repulsion of E311 would disrupt hydrophobic patch residues, which again might affect the stabilization of active site residues H274 and E276. Another strain of avian influenza virus with reduced susceptibility had NA mutations K262R and V321I. Residue K262 lies on the surface of NA and is unlikely to affect oseltamivir binding upon mutation to the similar residue arginine. Taken together, these data suggest that residues outside the NA active site, such as V267I, N307D, and V321I, may distort hydrophobic pockets and indirectly affect the NA catalytic and framework residues. These residues may be new potential markers for reduced susceptibility to oseltamivir. It is also possible that two or more mutations could be acting in concert. Analysis of 188 sequences of avian influenza viruses of N1 NA subtype available in the public domain revealed that changes in the NA residues determined in our study apparently occur among the wild waterbird species but at a relatively low frequency. Thus, these NA mutations do not appear to have a selective advantage and probably would not be maintained in wild birds. It is also possible that a combination of mutations located outside the NA active site act together and is the basis for the reduced susceptibility to oseltamivir in some isolates. One report identified three NA mutations in avian H5N1 viruses (K150N, I222L, and S246N) that had decreased susceptibility to oseltamivir (4).

From our study, we can conclude that reduced susceptibility and resistance to oseltamivir can occur in influenza viruses in

the wild waterbird reservoir, but these naturally occurring variants probably have no evolutionary advantage in nature. The biological significance of reduced NA susceptibility and the role of particular mutations on oseltamivir binding warrant testing by appropriate mutagenesis and binding experiments before considering the effect of a mutation on binding affinity.

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