Antiviral Research 82 (2009) 34–41

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Different neuraminidase inhibitor susceptibilities of human H1N1, H1N2, and H3N2 influenza A viruses isolated in Germany from 2001 to 2005/2006

Katja Bauer, Martina Richter, Peter Wutzler, Michaela Schmidtke*

Institute of Virology and Antiviral Therapy, University Clinical Centre of the Friedrich Schiller University Jena, Hans Knoell Str. 2, PFD-07740 Jena, Germany

ARTICLE INFO

Article history:
Received 8 October 2008
Received in revised form 15 December 2008
Accepted 22 January 2009

Keywords:
Influenza A virus
Neuraminidase inhibitors
Oseltamivir
Zanamivir
Resistance
Evolution

ABSTRACT

In the flu season 2005/2006 amantadine-resistant human influenza A viruses (FLUAV) of subtype H3N2 circulated in Germany. This raises questions on the neuraminidase inhibitor (NAI) susceptibility of FLUAV. To get an answer, chemiluminescence-based neuraminidase inhibition assays were performed with 51 H1N1, H1N2, and H3N2 FLUAV isolated in Germany from 2001 to 2005/2006. According to the mean IC_{50} values (0.38–0.91 nM for oseltamivir and 0.76–1.13 nM for zanamivir) most H1N1 and H3N2 FLUAV were NAI-susceptible. But, about four times higher zanamivir concentrations were necessary to inhibit neuraminidase activity of H1N2 viruses. Two H1N1 isolates were less susceptible to both drugs in NA inhibition as well as virus yield reduction assays. Results from sequence analysis of viral hemagglutinin and neuraminidase genes and evolutionary analysis of N2 gene revealed (i) different subclades for N2 in H1N2 and H3N2 FLUAV that could explain the differences in zanamivir susceptibility among these viruses and (ii) specific amino acid substitutions in the neuraminidase segment of the two less NAI-susceptible H1N1 isolates. One H3N2 was isolate proved to be a mixture of a NA deletion mutant and full-length NA viruses.

1. Introduction

Influenza A viruses (FLUAV) cause significant morbidity and mortality in humans. Currently available anti-influenza virus drugs target either the viral M2 ion channel (amantadine and rimantadine) or the viral neuraminidase (oseltamivir and zanamivir). In Germany, amantadine, oseltamivir, and zanamivir are approved for treating influenza (Wutzler et al., 2004). Amantadine and oseltamivir are also licensed for prophylactic use. However, amantadine is not recommended as an alternative to neuraminidase inhibitors (NAI) in Germany because it is not effective against influenza B viruses, it frequently selects drug-resistant virus mutants and it can cause adverse effects (Hayden et al., 2005; Hayden and Hay, 1992; Shiraishi et al., 2003; Wutzler et al., 2004). A significant increase of amantadine resistance among H3N2 FLUAV circulating in Asia, Australia, North America, and Europe was noticed in recent antiviral surveillance studies (Barr et al., 2007a; Bright et al., 2005, 2006; Krumbholz et al., 2009; Saito et al., 2007). This raises further concerns about the appropriate use of adamantanamines. Moreover, the percentage of amantadine-resistant H1N1 FLUAV raised in the flu season 2005/2006 (Barr et al., 2007a; Deyde et al., 2007). 71.7% of H1N1 FLUAV in China and 44.8% in Eastern Europe were shown to harbor a resistant M2 protein. In contrast, H1N1 FLUAV isolated in Germany in the same season were amantadine-susceptible (Schmidtke et al., 2008). Some of the currently circulating human-pathogenic avian H5N1 viruses in South East Asia (Barr et al., 2007b; Cheung et al., 2006; Puthavathana et al., 2005) and other avian FLUAV subtypes (Ilyushina et al., 2005) are amantadine-resistant. Moreover, a continuous circulation of amantadine-resistant porcine FLUAV of subtypes H1N1, H1N2 as well as H3N2 between 1989 and 2005 in Germany has been demonstrated (Krumbholz et al., 2009; Schmidtke et al., 2006).

Due to the high prevalence of amantadine-resistant viruses, neuraminidase inhibitors (NAI) are the only drugs considered for antiviral therapy of influenza virus infections at the moment. These drugs exhibit antiviral activity against influenza A as well as B viruses (Moscona, 2005). They target the active site of the viral neuraminidase (NA) whose activity is essential for the release of influenza virions from host cells and for virus spread. NAI are well tolerated and antiviral resistance has been rare in previous studies (Hayden, 2006; McKimm-Breschkin et al., 2003; McKimm-Breschkin, 2000; Moscona, 2005). According to reports of the neuraminidase inhibitor susceptibility network only 0.33% of tested FLUAV exhibited reduced susceptibility to NAI during the first 3 years of their use (Monto et al., 2006). Until now, there are no reports on zanamivir-resistant FLUAV. Few cases of resistance to zanamivir are known from influenza B virus-infected children (Barr et al., 2007a; Gubareva et al., 1998; Hurt et al., 2004). As shown for zanamivir, the oseltamivir resistance rates were relatively low,

* Corresponding author. Tel.: +49 3641 9395715; fax: +49 3641 9395702.
E-mail address: michaela.schmidtke@med.uni-jena.de (M. Schmidtke).

0166-3542/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2009.01.006

varying from 0.4% to 1% in adults (Monto et al., 2006) and 4–8% in children (Roberts, 2001; Ward et al., 2005; Whitley et al., 2001). However, in a Japanese study 18% of oseltamivir-treated children harbored drug-resistant H3N2 viruses (Kiso et al., 2004).

In the present study the antiviral activity of zanamivir and oseltamivir carboxylate against German FLUAV of subtype H3N2, H1N1, and H1N2 isolated till 2006 was examined (i) to prove their efficacy for flu treatment in a situation when ion channel blockers do not act effectively and (ii) to get an insight into the natural variability of NAI susceptibility based on the evolution of viral hemagglutinin (HA) and NA genes. According to the recommendations of previously published surveillance studies, drug susceptibility was analyzed in NA inhibition assays (Zambon and Hayden, 2001; McKimm-Breschkin et al., 2003; Monto et al., 2006; Wetherall et al., 2003). Additionally, virus yield reduction assays were used to compare the NAI susceptibility of two H1N1 outliers and selected susceptible viruses in cell culture. Sequence analysis of viral HA and NA genes was performed to determine potential resistance mutations and to perform evolutionary analysis.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby canine kidney (MDCK) cells (Friedrich-Loeffler Institute, Riems, Germany) were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin. The serum-free medium applied in cell culture-based assays on confluent 3-day-old cell monolayers was formulated with 2 μg/ml trypsin and 1.2 mM bicarbonate.

The 25 H1N1 (seasons 2004/2005 and 2005/2006), 6 H1N2 (seasons 2001/2002 and 2002/2003), and 20 H3N2 human FLUAV isolates (season 2005/2006) used in this study were provided by the German reference laboratories for human influenza (Berlin and Hannover). Their season of isolation, amantadine susceptibility, and M gene sequences were published recently (Schmidtke et al., 2008). H1N1 influenza viruses A/Puerto Rico/8/34 (Institute of Virology, Philipps University Marburg, Germany), A/Bayern/7/95, and A/New Caledonia/20/99 (both Robert Koch Institute, Berlin, Germany) were included for control.

Virus stocks were prepared in MDCK cells, aliquoted and stored at −80 °C until use.

2.2. Compounds

Zanamivir (GG167) and oseltamivir carboxylate (GS4071) were kindly provided by GlaxoSmithKline (Uxbridge, UK) and Hoffmann-La Roche AG (Basel, CH), respectively. Compound stocks were prepared in water and stored at 4 °C.

2.3. Chemiluminescence-based NA inhibition assay

NA activity and enzyme inhibition were determined with the commercially available NA-Star kit (Tropix, Applied Biosystems, Darmstadt, Germany) that utilizes a 1,2-dioxetane derivative of sialic acid as the substrate (Buxton et al., 2000), as described elsewhere (Wetherall et al., 2003). To evaluate the concentration required to reduce NA enzyme activity by 50% (IC_{50}) serial 10-fold NAI concentrations in H_2O were tested 3–6 times.

2.4. Statistical analysis

Initially, box-and-whisker plots were used to identify outliers with extreme IC_{50} values as described recently (Monto et al., 2006). Briefly, the box contains 50% of the results, representing the middle two quartiles (25–75%). The length of the box represents the interquartile range (IQR). The whiskers extent to the largest and smallest values (10th and 90th percentiles) before the region containing outliers is reached. Isolates with IC_{50} values between 1.5 and 3.0 IQR from the 25th and 75th percentiles were defined as mild outliers, whereas extreme outliers have IC_{50} values more than 3.0 IQR from the 25th and 75th percentiles. After exclusion of extreme outliers, the remaining IC_{50} values were used to calculate the mean and standard deviation (S.D.) of baseline NAI susceptibility of German FLUAV (Sheu et al., 2008).

2.5. Virus yield reduction assay

Virus yield reduction assays were performed as described previously (Bauer et al., 2007). In brief, NAI-untreated and treated MDCK cells were infected with FLUAV at a multiplicity of infection (MOI) that resulted in a complete cytopathic effect in untreated virus controls 48 h after infection. Supernatants were harvested 48 h after infection and virus yields were determined by using 50% cell culture infective dose ($CCID_{50}$) assay in MDCK cells. Based on these results, the 90% inhibitory concentration (IC_{90}) of NAI was calculated and used to compare the NAI susceptibility of selected viruses in vitro.

2.6. Plaque purification

Plaque assays were performed in confluent MDCK monolayers that were inoculated with 0.5 ml of serial 10-fold diluted virus suspensions in test medium. After 1 h of virus adsorption at 37 °C, the infecting media was removed and the cell monolayers were overlaid with test medium containing 0.4% agar. Two uninfected cell controls were included in all assays. After 48–96 h of incubation at 37 °C individual plaques were picked and transferred to MDCK monolayers for plaque assay. After three rounds of plaque purification the picked plaques were transferred to MDCK monolayers grown in 60 mm tissue culture dishes. After microscopic examination of virus-induced cytopathic effect, supernatants were harvested and total RNA was isolated.

2.7. RNA isolation, reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from virus-infected MDCK cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the Manufacturer's instructions. Viral RNA encoding the surface proteins HA and NA were amplified by RT-PCR. RT was conducted with Omniscript RT (Qiagen, Hilden, Germany) by using the Uni12 primer (20 μM) as described elsewhere (Hoffmann et al., 2001) and 1.5 μg of RNA in a final reaction volume of 10 μl following the Manufacturer's instructions. PCR amplification of viral HA and NA genes was done by using MP Biomedicals molecular biology TaqDNAPol (MP Biomedicals, Heidelberg, Germany) according to the Manufacturer's protocol and HA and NA fragment specific primers (Bm-HA1, NS-890R, Ba-NA1, and Ba-NA-1413R) as described elsewhere (Hoffmann et al., 2001) in combination with primers summarized in Table 1. The PCR cycling conditions were as follows: 1 cycle of 94 °C for 5 min; 38 cycles of 94 °C for 30 s, 55 °C for 50 s, 72 °C for 1 min, and a final cycle of 72 °C for 10 min, followed by holding at 10 °C. Amplification products were purified by using QIAquick PCR purification or gel extraction kit (Qiagen, Hilden, Germany) and stored at −20 °C until sequencing.

2.8. Sequencing and analysis of RT-PCR amplicons

Sequencing was performed with fluorescent-labeled nucleotides using the GenomeLab DTCS Quick Start Kit on

Table 1
Primers used for RT-PCR and sequencing.

Gene	Primer name	Primer sequence
H1	H1-67F	5′-GGCTACCATGCCAACAACTCAACCG-3′
	H1-790F[a]	5′-CCCGGGGACACAATAATATTTGAGGC-3′
	H1-1048F	5′-GCCGGTTTCATTGAAGGGGGCTGGACTGG-3′
	H1-1346F	5′-GGACTTTGGATTTCCATGACTCC-3′
	H1-349R	5′-CCTCATAGTCGGCGAAATACCCTGGG-3′
	H1-1091R[a]	5′-CCATCCATCATTCCAGTCCACCCCCC-3′
H3	H3-383F	5′-GCCGGATTATGCCTCCCTATGGTCACTAGTTGCC-3′
	H3-792F[a]	5′-CCGGGAGACATACTTTTGATTAACAGCACAGGG-3′
	H3-282R	5′-GCCATCACACTGAGGGTCTCCC-3′
	H3-610R	5′-CCGTACCCGGGTGGTGAACCCCCC-3′
	H3-1103R[a]	5′-CCGTACCAACCGTCCACCATTCCCTCCC-3′
N1	N1-255F	5′-GGCCGGCAATTCATCTCTTTGTTCTATCAGTGGATGGGC-3′
	N1-887F[a]	5′-GGCATGGTTCAAATCGACCTTGGG-3′
	N1-461R	5′-GGACTTCTGTCCTTAACGGTCCC-3′
	N1-1171R[a]	5′-CGGTATCTGTCCATCCATTAGGATCCC-3′
N2	N2-326F	5′-CCGCTGGTGGGGACATCTGGGTGAC-3′
	N2-390F	5′-GCGATCCTGACAAGTGTTATCAATTTGCCC-3′
	N2-808F[a]	5′-GGGAGTGCYCAACATGTAGAGGAG-3′
	N2-844F[a]	5′-CCTCGATATCCTGGTGTCAGATGTGTCTGC-3′
	N2-510R	5′-ACAAACATGCAGCCATGCTTTTCC-3′
	N2-1082R[a]	5′-CCACACGTCATTTCCATCATCAAGGCCC-3′
	N2-1088R[a]	5′-CCCATCCACACGTCATTTCCATCATCAAAGGCCC-3′

[a] Primers used for PCR (each 10 μM) in combination with HA and NA fragment specific primers as described elsewhere (Hoffmann et al., 2001).

the CEQ 8000 Genetic Analysis system (both Beckman Coulter, Krefeld, Germany). The primers used for sequencing (each 5 μM) are summarized in Table 1. 30 cycles of 96 °C for 20 s, 50 °C for 20 s, 60 °C for 4 min were run.

Sequences were assembled and aligned with the computer software BioEdit version 7.0.5.3 (Hall, 1999) and CLUSTALW. The GenBank accession numbers are **FJ231761** to **FJ231862**.

2.9. Phylogenetic analysis

Molecular evolutionary genetics was conducted using MEGA version 4 software (Tamura et al., 2007). Neighbor-joining trees were calculated by using interior branch test. The reliability of the clustering was tested by 1000 iterations in the Jones–Taylor–Thornton (JTT) substitution model (Jones et al., 1992). Additional sequences of German FLUAV were obtained from the influenza virus resource data bank available at the PubMed homepage.

3. Results

3.1. Susceptibility of human FLUAV to NAI in NA inhibition assay

According to previously published surveillance studies chemiluminescence-based NA inhibition assays are appropriate to conduct assessment of NAI susceptibility of human FLUAV (Zambon and Hayden, 2001; McKimm-Breschkin et al., 2003; Monto et al., 2006; Wetherall et al., 2003). This experimental approach was used to study the oseltamivir and zanamivir susceptibility of human H1N1, H1N2, and H3N2 FLUAV isolated in Germany from 2001 to 2005/2006. Outliers were determined by statistical analysis as described recently in a publication of the neuraminidase inhibitor susceptibility network (Monto et al., 2006). Fig. 1 shows the box plots of the IC_{50} values for oseltamivir and zanamivir of the H1N1, H1N2, and H3N2 viruses, respectively. The median values represented by the horizontal lines within the boxes are 0.78, 0.36, and 0.41 nM oseltamivir and 1.02, 3.25, and 0.82 nM zanamivir for the H1N1 and H1N2 as well as H3N2 viruses, respectively. The H1N1 viruses A/Berlin/60/05 and A/Bremen/5/05 were identified as extreme and mild outliers, respectively. In accordance to Sheu et al. (2008), the extreme outlier A/Berlin/60/05 was not included in the calculation of mean IC_{50} values and standard deviation (S.D.). The mean IC_{50} values and S.D. of baseline NAI susceptibility of German FLUAV are shown in Table 2. The human reference strains were drug-sensitive. Interestingly, the zanamivir concentration necessary to inhibit the N2 activity of H1N2 viruses was about four times higher than that determined for H3N2 viruses. Compared to the mean IC_{50} values of H1N1 viruses, the outliers A/Berlin/60/05 and A/Bremen/5/05 had reduced susceptibility to both drugs (4.55 and 2.04 times for oseltamivir as well as 2.98 and 2.40 times for zanamivir, respectively (Table 3).

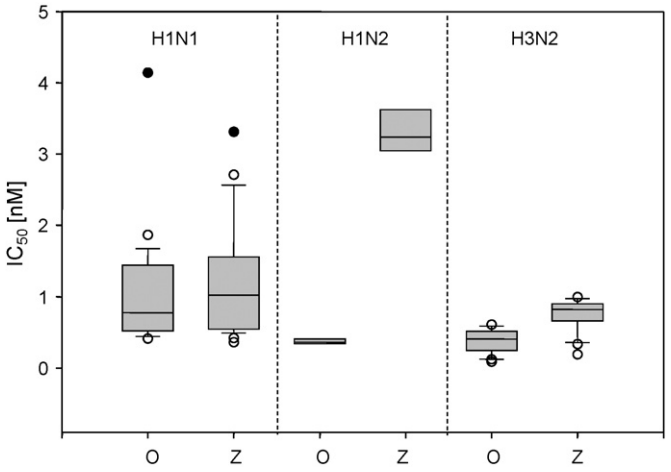

Fig. 1. Box plots of IC_{50} values [nM] for oseltamivir (O) and zanamivir (Z) of human H1N1, H1N2, and H3N2 FLUAV. Boxes represent the 25–75th percentiles and horizontal lines within the boxes represent the median values. The whiskers extend to the largest and smallest values (10th and 90th percentiles). The detected mild and extreme outliers are shown as open and closed circles, respectively.

Table 2
Baseline NAI susceptibility of German FLUAV determined in the NA activity inhibition assay with NAStar® substrate.

FLUAV	Mean IC_{50} ± S.D. (nM)	
	Oseltamivir	Zanamivir
H1N1 reference[a]	0.43 ± 0.08	0.56 ± 0.03
H1N1	0.91 ± 0.46	1.13 ± 0.64
H1N2	0.36 ± 0.06	3.34 ± 0.40
H3N2	0.38 ± 0.16	0.76 ± 0.21

[a] Strains A/Puerto Rico/8/34, A/Bayern/7/95, and A/New Caledonia/20/99.

3.2. Sequence analysis of viral HA and NA proteins

To confirm the drug susceptibility phenotype and to get an insight into the natural variability of genome segment 6 (NA) all tested FLUAV were sequenced. Moreover, genome segment 4 (HA) was sequenced because substitutions in the HA were shown to affect viral susceptibility to NAI in vitro (Abed et al., 2002;

Table 3
Characterization of H1N1 outliers with reduced susceptibility against NAI in NA activity inhibition assays.

H1N1 FLUAV	IC_{50} (nM)		Fold difference[a]		NA sequence
	Oseltamivir	Zanamivir	Oseltamivir	Zanamivir	
Berlin/60/05[b]	4.14	3.37	4.55	2.98	L134M, I266V
Bremen/5/05[c]	1.86	2.71	2.04	2.40	I38T, I106V

[a] Related to the baseline NAI susceptibility of German H1N1.
[b] Extreme outlier.
[c] Mild outlier.

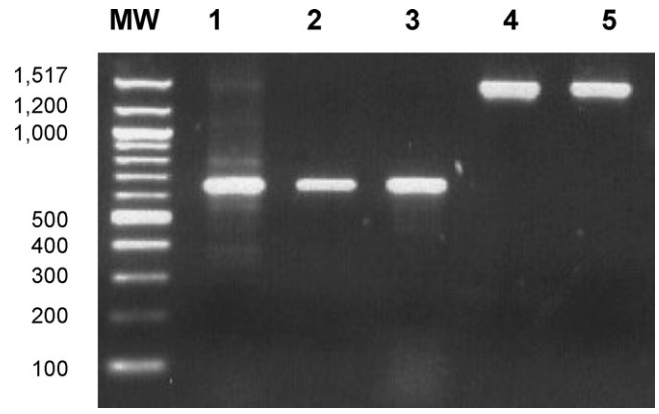

Fig. 2. Detection of full-length NA gene and NA deletion mutants in the original sample of the isolate A/Berlin/40/06 as well as in plaque-to-plaque purified viruses. Products of reverse transcriptase polymerase chain reaction amplification with primers complementary to 3′ and 5′ termini were analyzed by agarose gel electrophoresis with a 100 bp molecular marker (MW). Lane 1, original sample; lanes 2 and 3, two plaque-to-plaque purified deletions mutants, and lanes 4 and 5, two plaque-to-plaque purified variants containing the full-length NA gene.

Gubareva et al., 2000; McKimm-Breschkin, 2000; Mishin et al., 2005).

The analyzed N2 sequences were very similar within the subtype H3N2 as well as within the subtype H1N2. However, the N2 sequences of H3N2 FLUAV differ from that of H1N2 FLUAV in 13 amino acid positions (S18A, F23L, M24T, I30 V, F42C, V143G, V216G, E/D221K, T267L, I307 V, N385K, K431N, and E432Q).

With the exception of the outliers A/Berlin/60/05 and A/Bremen/5/05, a strong homology among the N1 sequences was noticeable. Sequence analysis of NA of these two isolates revealed neither amino acid exchanges in or near the enzymes active site nor any other substitutions known to confer NAI resistance. Amino acid substitutions L134 M and I266 V were found in the less NAI susceptible isolate A/Berlin/60/05. A/Bremen/5/05 differs in amino acid positions 38 and 106 (I38T and I106 V) from all other N1 isolates (Table 3).

Sequencing the H3N2 isolate A/Berlin/40/06 a NA deletion mutant (ΔNA) was detected in a mixture with full-length NA viruses. NA deletion mutants and full-length NA viruses could be separated by three rounds of plaque-to-plaque purification. To confirm the presence or absence of the ΔNA in the original sample obtained from the German Reference Center in Berlin and in the plaque-to-plaque purified samples, RT-PCR amplification of the full-length NA gene was performed. PCR products were analyzed by agarose gel electrophoresis. The results shown in Fig. 2 demonstrate the presence of the ΔNA segment in the original sample as well as in the purified mutant virus. In the original sample the PCR product of the full-length NA segment (~1400 bp) was very faint on the gels. However, the overwhelming abundance of the products synthesized from ΔNA segments is likely to be a result of the more efficient amplification of the shorter template (~550 bp) by RT and Taq polymerase. When the cDNA of three plaque-to-plaque purified full-length NA viruses and ΔNA mutants was amplified, single bands corresponding to the full-length NA segment and ΔNA segment were clearly detected (two examples of three are shown in Fig. 2). The NA deletion mutant lacked 849 nucleotides in the coding region of the enzyme.

Sequence analysis of the viral HA1 subunit revealed a strong homology among H3N2 FLUAV. There were no differences in the receptor-binding site or in glycosylation pattern.

HA1 amino acid sequences of both H1N1 and H1N2 were also very uniform. Some distinctions have been noticed in regard of the number of potential glycosylation sites. Most H1N1 and H1N2 FLUAV contain potential glycosylation sites in positions 94a, 129, and 163 (H3 numbering) that were demonstrated to influence the NAI susceptibility in vitro (Mishin et al., 2005). The H1N2 viruses A/Baden-Wuerttemberg/20/03 and A/Baden-Wuerttemberg/129/03 contain only the potential glycosylation site 163 and A/Sachsen/678/03 is not glycosylated in position 94a. The reference strain A/Bayern/7/95 contains an additional potential glycosylation site in amino acid position 271, while A/Puerto Rico/8/34 lacks all these sites.

3.3. Phylogenetic analysis

The data from NA inhibition assays showed that the H1N2 viruses exhibited an up to fourfold reduced zanamivir susceptibility compared to H3N2 viruses. German porcine H3N2 FLUAV also showed elevated IC_{50} values in NA inhibition assays (Bauer et al., 2007). To investigate whether the NA gene of H1N2 viruses might be from swine viruses and to get an insight into the evolution of the N2 gene of German human FLUAV, a phylogenetic analysis of the N2 protein based on amino acids 1–453 was performed. The 26 N2 amino acid sequences generated in this study were compared to all German N2 amino acid sequences ($n = 45$) available from the GenBank and to that of the two H3N2 reference strains A/Hong Kong/68 and A/Sydney/5/97 investigated in former studies (Bauer et al., 2007). To arrange the tree more clearly, identical sequences were combined.

The resulting N2 phylogenetic tree shown in Fig. 3 consists of two clades, one containing avian and porcine FLUAV and another one containing human H3N2 and H1N2 viruses. The latter comprises three subclades. The first subclade includes H3N2 FLUAV circulating until 2004, the second H1N2 viruses, and the third H3N2 isolates circulating since 2004. So, the N2 genes of H1N2 and H3N2 FLUAV differing in baseline susceptibility towards zanamivir belong to different subclades, but shared a common ancestor.

3.4. Susceptibility of selected human FLUAV to NAI in virus yield reduction assays

In addition to chemiluminescence-based NA inhibition assays, virus yield reduction assays were used to compare the NAI susceptibility of the H1N1 outliers A/Berlin/60/05 and A/Bremen/5/05 with two drug-sensitive H1N1 isolates (selected on the basis of NA inhibition assays) as well as the two H1N1 reference strains A/Puerto Rico/8/34 and New Caledonia/20/99 in vitro. The results are summarized in Table 4. The IC_{90} values of oseltamivir- and zanamivir-susceptible FLUAV isolates and the two reference strains were in a range from 30 to 1687 nM. Markedly reduced drug susceptibility was found for A/Berlin/60/05 and A/Bremen/5/05 that contain unique amino acid substitutions in the NA. Both viruses were >10 times less oseltamivir- and zanamivir-sensitive compared to the other isolates. So, for the virus strains analyzed in this study an agreement between the results of virus yield reduction assays and NA inhibition assays was observed.

Table 4
Comparison of NAI susceptibility of H1N1 outliers and selected NA susceptible H1N1 FLUAV in cell culture-based virus yield reduction assays.

H1N1 FLUAV	IC_{90} (nM) virus yield reduction assays	
	Oseltamivir	Zanamivir
Puerto Rico/8/34	233	250
New Caledonia/20/99	232	30
Bayern/7/95	927	1,533
Berlin/18/05	1,080	1,687
Berlin/60/05	23,728	30,120
Bremen/5/05	25,907	21,497

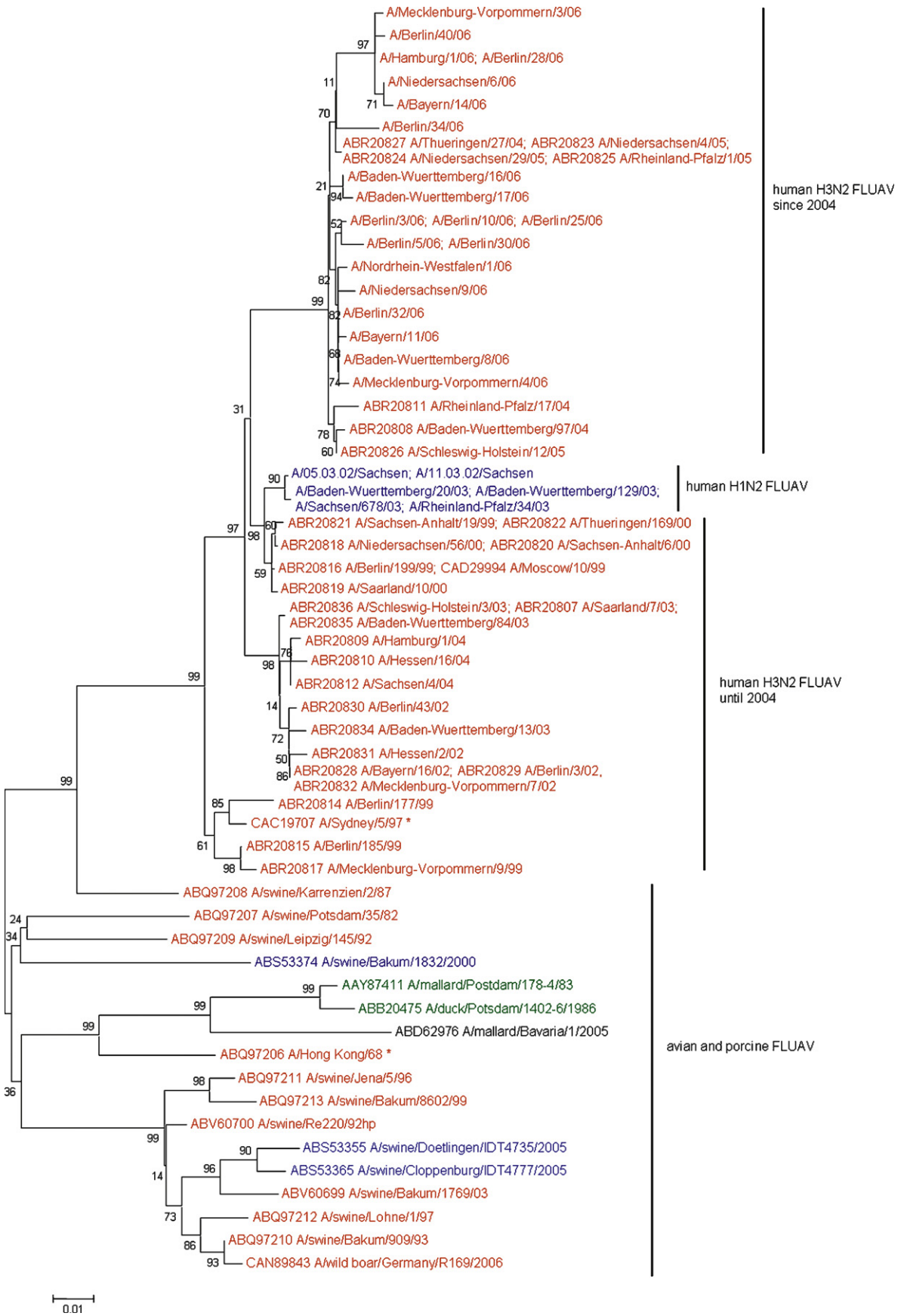

Fig. 3. N2 phylogenetic tree of German H3N2 (red), H1N2 (blue), H2N2 (green), and H5N2 (black) FLUAV. Reference strains are marked with an asterisk. The Neighbor-joining tree is based on amino acids 1–453 of the enzyme.

4. Discussion

Antivirals constitute an important option for the management of flu (Hayden, 2006; Moscona, 2005; Wutzler et al., 2004). NAI represent the drugs of choice for treatment because they inhibit both influenza A and B viruses, are well tolerated and characterized by low resistance rates. However, the natural variability of FLUAV based on point mutations may cause the emergence of drug-resistant viruses. As observed recently for amantadine (Bright et al., 2005, 2006; Deyde et al., 2007; Krumbholz et al., 2009; Schmidtke et al., 2008) and oseltamivir (Hatakeyama et al., 2007; Lackenby et al., 2008; Sheu et al., 2008; WHO, 2008) single amino acid exchanges may confer drug resistance, whereas resistant FLUAV were able to spread efficiently. There were also recent reports on changes in baseline susceptibility of FLUAV to NAI in the result of drift mutations in the NA gene (Escuret et al., 2008; McKimm-Breschkin et al., 2007; Monto et al., 2006; Rameix-Welti et al., 2006). Moreover, other species, in particular birds and pigs, are potential sources of viruses that have the potential to contribute to drug resistance in human after reassortment and transmission (Ito et al., 1998). For these reasons, continued testing of viral drug susceptibilities is important. The present study provides insights (i) into the susceptibility of human FLUAV isolated in Germany between 2001 and 2006 against oseltamivir carboxylate and zanamivir and (ii) the relationship between the NAI susceptibility of human FLUAV and the evolution of the NA genes.

NAI susceptibility is directly detectable with enzyme activity inhibition assays. Both, fluorescence- as well as chemiluminescence-based assays were applied in previous surveillance studies in conjunction with a NA sequence analysis (McKimm-Breschkin et al., 2003; Monto et al., 2006; Mungall et al., 2004; Sheu et al., 2008; Wetherall et al., 2003; Zambon and Hayden, 2001). In the present study a chemiluminescence-based assay was utilized. Most of the tested FLUAV were shown to be highly oseltamivir- as well as zanamivir-sensitive. But, two H1N1 strains were less drug-sensitive. The mean IC_{50} values determined for oseltamivir and zanamivir in the present study are in good agreement with data published elsewhere (Escuret et al., 2008; McKimm-Breschkin et al., 2003; Monto et al., 2006). Moreover, with exception of the mean zanamivir activity against H3N2 FLUAV, IC_{50} values of the present study fully correspond with the results of a worldwide surveillance study performed by the CDC (Sheu et al., 2008). According to the mean IC_{50} values, the globally selected H3N2 FLUAV (from 10/2004 to 3/2008) were generally less sensitive to zanamivir compared to H3N2 isolates collected in Germany and tested in the present study. However, the proportion of European H3N2 viruses (11 of 1233 isolates) was low in that study. Because Sheu et al. did not sequence the NA genes of susceptible viruses, there are no sequences available at the GenBank to compare the N2 sequences of H3N2 viruses with different IC_{50} values.

The results from NA inhibition assays confirm a subtype specificity in drug sensitivity of viral NA that was shown in previous drug resistance surveillance programs (Ferraris et al., 2005; Hurt et al., 2004; Monto et al., 2006). For example, the H1N1 FLUAV were just as susceptible to oseltamivir as to zanamivir. In contrast, H3N2 and H1N2 viruses are more sensitive to oseltamivir compared to zanamivir. Based on their 3-dimensional structures, NA subtypes form two genetically distinct groups (Russell et al., 2006). One group (N1 group: N1, N4, N5, and N8 subtypes) possesses a cavity in the active site closed on ligand binding. The second group (N2 group: N2, N3, N6, N7, and N9 subtypes) lacks this cavity. According to Russell et al., these differences in structure may lead to different binding affinities of NAI into the NA active site. The results of the present study and of Mungall et al. (2004) indicate that NAI susceptibility can also vary within a NA subtype as shown for N2 of H1N2 and H3N2 viruses. The human H1N2 FLUAV were 2–4 times less zanamivir-susceptible than the H3N2 viruses in NA inhibition assays. German porcine H3N2 isolates showed also elevated IC_{50} values in NA inhibition assays in a previously conducted study (Bauer et al., 2007).

The evolution of the N2 gene was studied in a phylogenetic analysis with all published German N2 amino acid sequences as well as the H3N2 isolate A/Moscow/10/99. In previous studies the origin of the NA gene of human H1N2 was traced to the NA of this H3N2 isolate (Ellis et al., 2003; Xu et al., 2002). The resulting phylogenetic tree (Fig. 3) revealed two distinct clades, one consisting of porcine and avian N2 viruses and another one composed of human H1N2 and H3N2 isolates, indicating that the N2 of the studied human and porcine viruses is different. The clade of human N2 sequences is further divided into three subclades. One subclade includes human H3N2 isolates circulating until 2004, another one consists of human H1N2 FLUAV as well as human H3N2 FLUAV including A/Moscow/10/99, and the third is composed of human H3N2 viruses circulating since 2004. This classification underlies the distinction between H1N2 and H3N2 viruses analyzed in NA inhibition assays in the present study (all isolated in 2006) and may be an explanation for their different zanamivir susceptibilities in NA inhibition assays. The results are in good agreement with a recently published phylogenetic analysis of H3N2 viruses circulating in Germany from 1998 to 2005 (Schweiger et al., 2006). Moreover, in the present as well previous studies (Ellis et al., 2003; Xu et al., 2002) the NA gene of human H1N2 was in close relationship to that of the H3N2 isolate A/Moscow/10/99. The results of an additionally performed phylogenetic analysis with NA sequences of the 6 human H1N2 generated in this study and of 54 H1N2 FLUAV isolated worldwide revealed a strong homology among this subtype (data not shown).

Interestingly, the less zanamivir-susceptible porcine H3N2 FLUAV as well as all human H1N2 FLUAV contain characteristic amino acid exchanges in positions 18, 23, 42, 307, and 432 that are also characteristic for the isolate A/Moscow/10/99. Possibly these positions may affect the drug interaction with the N2 enzyme although they do not belong to the enzyme active site.

Both outliers A/Berlin/60/05 and A/Bremen/5/05 belong to the N1 subtype. None of the known NA substitutions conferring NAI resistance were detected in these strains. However, in comparison to the other FLUAV, the extreme outlier A/Berlin/60/05 (H1N1) contained the substitutions L134 M and I266 V in the NA protein and the mild outlier A/Bremen/5/05 (H1N1) possesses I38T and I106 V. Amino acid L134 belongs to the sialic acid cavity as well as to the "150 cavity" of N1 viruses (Cheng et al., 2008; Russell et al., 2006; Zhang et al., 2008). Amino acids I106 and I266 are located near to amino acids of the active site of human N1 shown to contribute to the stabilization and destabilization of oseltamivir binding with the active site of A/PR/8/34 (Zhang et al., 2008). Because there are no experimental data, it can only be speculated that an amino acid substitution at these positions could influence the topology of at least one of these two cavities and by this manner of the compound binding site. The substitution I266V has also been found in a global surveillance study (Monto et al., 2006). This study showed that human isolates with reduced NAI susceptibility can result from drift mutations in the NA remote from the active site. Similar conclusions were drawn for H5N1 FLUAV with reduced susceptibility to oseltamivir (McKimm-Breschkin et al., 2007; Rameix-Welti et al., 2006). The clinical relevance of the newly identified NA mutations is unknown.

Interestingly, A/Berlin/40/06 (H3N2) was shown to be a mixture of full-length NA viruses and NA deletion mutants lacking ~850 nucleotides in the coding region of the NA gene. The occurrence of NA deletion mutants lacking the coding capacity for the NA active site after NAI treatment in vitro as well as in humans has been described previously (Nedyalkova et al., 2002). In general, viruses

lacking the sialidase activity can undergo multiple cycles of replication in cell culture, eggs, and mice (Hughes et al., 2000). Although sialidase activity is not absolutely required in the FLUAV life cycle, it appears to be necessary for efficient virus replication. NA deletion mutants replicated in cell culture, but showed reduced infectivity and virulence in a ferret model (Gubareva et al., 2002; Nedyalkova et al., 2002). The deletion mutant discovered in the present study remains to be elucidated in vitro and in vivo to get insights into the consequences of the deletion for sialidase activity, viral replication in vitro and virulence in vivo. Because there is no information on the background of the isolate it remains unclear how this deletion emerged.

An optimal interplay between the receptor-binding activity of the HA and the receptor-destroying activity of the NA is required for efficient virus replication in cell culture (Wagner et al., 2002). Substitutions in the HA as well as NA proteins may have an effect on the virus susceptibility to NAI in cell culture based assays (Abed et al., 2002; Gubareva et al., 2000; McKimm-Breschkin, 2000; Mishin et al., 2005). However, the relevance of changes in the HA for susceptibility in humans has not been demonstrated. The susceptibility of selected drug-sensitive FLUAV and the two H1N1 outliers was compared in virus yield reduction assays in MDCK cells. Outliers were less drug-susceptible in enzyme activity inhibition assays and contain new mutations in the NA gene. As found in an antiviral study with porcine FLUAV (Bauer et al., 2007), IC_{90} values determined in virus yield reduction assays correlated with findings in enzyme activity inhibition assays. A more than 10-fold higher oseltamivir and zanamvir concentration was necessary to inhibit the virus yield of the two H1N1 outliers by 90% compared to drug-sensitive H1N1 viruses.

In conclusion, this report further underlines the importance of consequent monitoring of NA gene evolution and influenza virus drug susceptibility. Besides sequencing, NA inhibition assays are important to discover viruses with reduced NAI susceptibility. Thereby, it seems to be very important to analyze the whole NA gene to identify not only known resistant mutations but also drift mutations out of the enzyme active site. It remains to be further analyzed whether the NA substitutions found in the less NAI-susceptible isolates are predictive of the virus drug phenotype in vivo.

Acknowledgments

This study was supported by a student grant from the Ministry of Culture and Education of Thuringia awarded to K.B. and the German Bundesministerium für Bildung und Forschung (01 KI 07142) awarded to M.S.

References

Abed, Y., Bourgault, A.M., Fenton, R.J., Morley, P.J., Gower, D., Owens, I.J., Tisdale, M., Boivin, G., 2002. Characterization of 2 influenza A(H3N2) clinical isolates with reduced susceptibility to neuraminidase inhibitors due to mutations in the hemagglutinin gene. J. Infect. Dis. 186, 1074–1080.

Barr, I.G., Hurt, A.C., Deed, N., Iannello, P., Tomasov, C., Komadina, N., 2007a. The emergence of adamantane resistance in influenza A(H1) viruses in Australia and regionally in 2006. Antiviral Res. 75, 173–176.

Barr, I.G., Hurt, A.C., Iannello, P., Tomasov, C., Deed, N., Komadina, N., 2007b. Increased adamantane resistance in influenza A(H3) viruses in Australia and neighbouring countries in 2005. Antiviral Res. 73, 112–117.

Bauer, K., Schrader, C., Suess, J., Wutzler, P., Schmidtke, M., 2007. Neuraminidase inhibitor susceptibility of porcine H3N2 influenza A viruses isolated in Germany between 1982 and 1999. Antiviral Res. 75, 219–226.

Bright, R.A., Medina, M.J., Xu, X., Perez-Oronoz, G., Wallis, T.R., Davis, X.M., Povinelli, L., Cox, N.J., Klimov, A.I., 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 366, 1175–1181.

Bright, R.A., Shay, D.K., Shu, B., Cox, N.J., Klimov, A.I., 2006. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA 295, 891–894.

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

Cheng, L.S., Amaro, R.E., Xu, D., Li, W.W., Arzberger, P.W., McCammon, J.A., 2008. Ensemble-based virtual screening reveals potential novel antiviral compounds for avian influenza neuraminidase. J. Med. Chem. 51, 3878–3894.

Cheung, C.L., Rayner, J.M., Smith, G.J., Wang, P., Naipospos, T.S., Zhang, J., Yuen, K.Y., Webster, R.G., Peiris, J.S., Guan, Y., Chen, H., 2006. Distribution of amantadine-resistant H5N1 avian influenza variants in Asia. J. Infect. Dis. 193,1626–1629.

Deyde, V.M., Xu, X., Bright, R.A., Shaw, M., Smith, C.B., Zhang, Y., Shu, Y., Gubareva, L.V., Cox, N.J., Klimov, A.I., 2007. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. J. Infect. Dis. 196, 249–257.

Ellis, J.S., Alvarez-Aguero, A., Gregory, V., Lin, Y.P., Hay, A., Zambon, M.C., 2003. Influenza AH1N2 viruses, United Kingdom, 2001–02 influenza season. Emerg. Infect. Dis. 9, 304–310.

Escuret, V., Frobert, E., Bouscambert-Duchamp, M., Sabatier, M., Grog, I., Valette, M., Lina, B., Morfin, F., Ferraris, O., 2008. Detection of human influenza A (H1N1) and B strains with reduced sensitivity to neuraminidase inhibitors. J. Clin. Virol. 41, 25–28.

Ferraris, O., Kessler, N., Lina, B., 2005. Sensitivity of influenza viruses to zanamivir and oseltamivir: a study performed on viruses circulating in France prior to the introduction of neuraminidase inhibitors in clinical practice. Antiviral Res. 68 (1), 43–48.

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

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

Gubareva, L.V., Nedyalkova, M.S., Novikov, D.V., Murti, K.G., Hoffmann, E., Hayden, F.G., 2002. A release-competent influenza A virus mutant lacking the coding capacity for the neuraminidase active site. J. Gen. Virol. 83, 2683–2692.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hatakeyama, S., Sugaya, N., Ito, M., Yamazaki, M., Ichikawa, M., Kimura, K., Kiso, M., Shimizu, H., Kawakami, C., Koike, K., Mitamura, K., Kawaoka, Y., 2007. Emergence of influenza B viruses with reduced sensitivity to neuraminidase inhibitors. JAMA 297, 1435–1442.

Hayden, F.G., 2006. Antivirals for influenza: historical perspectives and lessons learned. Antiviral Res. 71, 372–378.

Hayden, F.G., Hay, A.J., 1992. Emergence and transmission of influenza A viruses resistant to amantadine and rimantadine. Curr. Top. Microbiol. Immunol. 176, 119–130.

Hayden, F., Klimov, A., Tashiro, M., Hay, A., Monto, A., McKimm-Breschkin, J., Macken, C., Hampson, A., Webster, R.G., Amyard, M., Zambon, M., 2005. Neuraminidase inhibitor susceptibility network position statement: antiviral resistance in influenza A/H5N1 viruses. Antiviral Ther. 10, 873–877.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., Perez, D.R., 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 146, 2275–2289.

Hughes, M.T., Matrosovich, M., Rodgers, M.E., McGregor, M., Kawaoka, Y., 2000. Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice. J. Virol. 74, 5206–5212.

Hurt, A.C., Barr, I.G., Hartel, G., Hampson, A.W., 2004. Susceptibility of human influenza viruses from Australasia and South East Asia to the neuraminidase inhibitors zanamivir and oseltamivir. Antiviral Res. 62, 37–45.

Ilyushina, N.A., Govorkova, E.A., Webster, R.G., 2005. Detection of amantadine-resistant variants among avian influenza viruses isolated in North America and Asia. Virology 341, 102–106.

Ito, T., Couceiro, J.N., Kelm, S., Baum, L.G., Krauss, S., Castrucci, M.R., Donatelli, I., Kida, H., Paulson, J.C., Webster, R.G., Kawaoka, Y., 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J. Virol. 72 (9), 7367–7373.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.

Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., Hayden, F.G., Sugaya, N., Kawaoka, Y., 2004. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 364 (9436), 759–765.

Krumbholz, A., Schmidtke, M., Bergmann, S., Motzke, S., Bauer, K., Stech, J., Dürrwald, R., Wutzler, P., Zell, R., 2009. High prevalence of amantadine resistance among circulating European porcine influenza A viruses. J. Gen. Virol. [Epub ahead of print].

Lackenby, A., Hungnes, O., Dudman, S.G., Meijer, A., Paget, W.J., Hay, A.J., Zambon, M.C., 2008. Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. Euro. Surveill. 13 (5), pii: 8026.

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

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

McKimm-Breschkin, J.L., Selleck, P.W., Usman, T.B., Johnson, M.A., 2007. Reduced sensitivity of influenza A (H5N1) to oseltamivir. Emerg. Infect. Dis. 13, 1354–1357.

Mishin, V.P., Novikov, D., Hayden, F.G., Gubareva, L.V., 2005. Effect of hemagglutinin glycosylation on influenza virus susceptibility to neuraminidase inhibitors. J. Virol. 79, 12416–12424.

Monto, A.S., McKimm-Breschkin, J.L., Macken, C., Hampson, A.W., Hay, A., Klimov, A., Tashiro, M., Webster, R.G., Aymard, M., Hayden, F.G., Zambon, M., 2006. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob. Agents Chemother. 50, 2395–2402.

Moscona, A., 2005. Neuraminidase inhibitors for influenza. N. Engl. J. Med. 353, 1363–1373.

Mungall, B.A., Xu, X., Klimov, A., 2004. Surveillance of influenza isolates for susceptibility to neuraminidase inhibitors during the 2000–2002 influenza seasons. Virus Res. 103, 195–197.

Nedyalkova, M.S., Hayden, F.G., Webster, R.G., Gubareva, L.V., 2002. Accumulation of defective neuraminidase (NA) genes by influenza A viruses in the presence of NA inhibitors as a marker of reduced dependence on NA. J. Infect. Dis. 185 (5), 591–598.

Puthavathana, P., Auewarakul, P., Charoenying, P.C., Sangsiriwut, K., Pooruk, P., Boonnak, K., Khanyok, R., Thawachsupa, P., Kijphati, R., Sawanpanyalert, P., 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J. Gen. Virol. 86, 423–433.

Rameix-Welti, M.A., Agou, F., Buchy, P., Mardy, S., Aubin, J.T., Véron, M., van der, Werf##S., Naffakh, N., 2006. Natural variation can significantly alter the sensitivity of influenza A (H5N1) viruses to oseltamivir. Antimicrob. Agents Chemother. 50, 3809–3815.

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

Russell, R.J., Haire, L.F., Stevens, D.J., Collins, P.J., Lin, Y.P., Blackburn, G.M., Hay, A.J., Gamblin, S.J., Skehel, J.J., 2006. The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature 443, 45–49.

Saito, R., Li, D., Suzuki, Y., Sato, I., Masaki, H., Nishimura, H., Kawashima, T., Shirahige, Y., Shimomura, C., Asoh, N., Degawa, S., Ishikawa, H., Sato, M., Shobugawa, Y., Suzuki, H., 2007. High prevalence of amantadine-resistance influenza A (H3N2) in six prefectures, Japan, in the 2005–2006 season. J. Med. Virol. 79, 1569–1576.

Schmidtke, M., Zell, R., Bauer, K., Krumbholz, A., Schrader, C., Suess, J., Wutzler, P., 2006. Amantadine resistance among porcine H1N1, H1N2, and H3N2 influenza A viruses isolated in Germany between 1981 and 2001. Intervirology 49, 286–293.

Schmidtke, M., Bauer, K., Ludwig, N., Wutzler, P., 2008. Emergence and phylogenetic relationships of amantadine-resistant human H3N2 influenza A viruses in Germany in the season 2005/2006. Int. J. Antimicrob. Agents 32, 192–195.

Schweiger, B., Bruns, L., Meixenberger, K., 2006. Reassortment between human A(H3N2) viruses is an important evolutionary mechanism. Vaccine 24, 6683–6690.

Sheu, T.G., Deyde, V.M., Okomo-Adhiambo, M., Garten, R.J., Xu, X., Bright, R.A., Butler, E.N., Wallis, T.R., Klimov, A.I., Gubareva, L.V., 2008. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob. Agents Chemother. 52, 3284–3292.

Shiraishi, K., Mitamura, K., Sakai-Tagawa, Y., Goto, H., Sugaya, N., Kawaoka, Y., 2003. High frequency of resistant viruses harboring different mutations in amantadine-treated children with influenza. J. Infect. Dis. 188, 57–61.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24 (8), 1596–1599.

Wagner, R., Matrosovich, M., Klenk, H.D., 2002. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev. Med. Virol. 12, 159–166.

Ward, P., Small, I., Smith, J., Suter, P., Dutkowski, R., 2005. Oseltamivir (Tamiflu) and its potential for use in the event of an influenza pandemic. J. Antimicrob. Chemother. 55 (Suppl. 1), i5–i21.

Wetherall, N.T., Trivedi, T., Zeller, J., Hodges-Savola, C., McKimm-Breschkin, J.L., Zambon, M., Hayden, F.G., 2003. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. J. Clin. Microbiol. 41, 742–750.

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

WHO, 2008. Influenza A(H1N1) virus resistance to oseltamivir—last quarter 2007 to 4 April 2008. http://wwwwhoint/csr/disease/influenza/H1N1ResistanceWeb20080403pdf.

Wutzler, P., Kossow, K.D., Lode, H., Ruf, B.R., Scholz, H., Vogel, G.E., 2004. Antiviral treatment and prophylaxis of influenza in primary care: German recommendations. J. Clin. Virol. 31, 84–91.

Xu, X., Smith, C.B., Mungall, B.A., Lindstrom, S.E., Hall, H.E., Subbarao, K., Cox, N.J., Klimov, A., 2002. Intercontinental circulation of human influenza A(H1N2) reassortant viruses during the 2001–2002 influenza season. J. Infect. Dis. 186, 1490–1493.

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

Zhang, Q., Yang, J., Liang, K., Feng, L., Li, S., Wan, J., Xu, X., Yang, G., Liu, D., Yang, S., 2008. Binding interaction analysis of the active site and its inhibitors for neuraminidase (N1 subtype) of human influenza virus by the integration of molecular docking, FMO calculation and 3D-QSAR CoMFA modeling. J. Chem. Inf. Model. 48, 1802–1812.