

Neuraminidase inhibitor susceptibility of porcine H3N2 influenza A viruses isolated in Germany between 1982 and 1999

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Abstract

As an intermediate host of avian and human influenza A viruses (FLUAV) pigs may play a potential role in interspecies virus transmission and reassortment of viral genes including those conferring antiviral drug resistance. Porcine FLUAV isolated in Germany between 1989 and 2001 contains mutations in the M2 gene inducing amantadine resistance. No data exist on neuraminidase inhibitor (NAI) susceptibility of these porcine FLUAV. We studied the antiviral activity of NAI against seven selected H3N2 FLUAV isolated from pigs in Germany between 1982 and 1999. All isolates were susceptible towards oseltamivir and zanamivir in neuraminidase enzyme-inhibition assays. Both compounds inhibited virus spreading and reduced the virus yields and plaque size at low concentrations. Higher concentrations were necessary to reduce the plaque number. Two isolates that differed in glycosylation pattern of viral hemagglutinin (HA) showed markedly reduced drug susceptibility in cell culture-based assays.

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1. Introduction

Influenza A viruses (FLUAV) cause significant morbidity and mortality in humans in epidemics or even pandemics, and are also responsible for enzootic outbreaks. Wild birds provide a natural reservoir of all known FLUAV subtypes (H1–16, N1–9) (Fouchier et al., 2005). The subtypes H1N1, H1N2, and H3N2 were co-circulating in the human European population until 2006 (Paget et al., 2006). The same subtypes were isolated from pigs in Germany (Schrader and Suess, 2003, 2004). Pigs are susceptible to infection by FLUAV of human and avian origin because their tracheal cells possess cell surface receptors for both viruses. That is the reason why pigs are discussed as a mixing vessel for genetic reassortants with the potential for emergence of novel human viruses (Ito et al., 1998). Genetic reassortment between avian and human FLUAV was observed in Italian pigs

(Castrucci et al., 1993). Reassorted porcine strains were also transmitted to humans (Claas et al., 1994; Gregory et al., 2001). Fortunately, subsequent person-to-person spread was limited so far, and these viruses did not spread in the human population.

The currently available anti-influenza virus drugs target either the viral M2 ion channel or the viral neuraminidase (NA). The use of M2 ion channel blockers, amantadine and rimantadine, is limited by the lack of an inhibitory effect on influenza B viruses as well as the rapid emergence of antiviral resistance during therapy (Hayden et al., 2005; Hayden and Hay, 1992; Shiraishi et al., 2003). A significant increase of amantadine resistance among H3N2 FLUAV was noted in North America and some East Asian countries since the flu season 2003–2004 (Bright et al., 2005, 2006). Some of the currently circulating human pathogenic H5N1 viruses in Taiwan and Vietnam are also resistant to amantadine (Puthavathana et al., 2005). Moreover, some avian and porcine FLUAV were shown to harbour a resistant M2 protein (Ilyushina et al., 2005; Schmidtke et al., 2006). This raises doubts that ion channel inhibitors will not act in epidemic and pandemic situations.

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Because of its conserved catalytic site, the viral NA is another target for anti-influenza therapy. In contrast to ion channel inhibitors, the neuraminidase inhibitors (NAIs) zanamivir and oseltamivir exhibit antiviral activity against influenza A as well as B viruses (Moscona, 2005; Wutzler et al., 2004). Both drugs interact directly with the catalytic site of the NA and prevent enzyme activity. One of the advantages of NAIs is the rare emergence of antiviral resistance (McKimm-Breschkin, 2000; Moscona, 2005). After oseltamivir treatment resistant variants were found in <1% of adults and in up to 8% of children and immunocompromised patients (Ferraris et al., 2005; McKimm-Breschkin et al., 2003). In a Japanese study 18% of oseltamivir-treated children (lower NAI dosage) harboured drug-resistant H3N2 viruses (Kiso et al., 2004). Potential pandemic H5N1 and H9N2 strains were NAI-sensitive until now (Govorkova et al., 2001; Yuen and Wong, 2005). However, there is a recent report of treatment failure and isolation of drug-resistant H5N1 virus from a patient treated with oseltamivir in Vietnam (de Jong et al., 2005).

Despite the fact that pigs may play a potential role in interspecies virus transmission and reassortment of viral genes including those important for antiviral therapy, there are no data on NAI susceptibility of porcine influenza viruses. To fill this gap, the NAI susceptibility of selected porcine FLUAV of subtype H3N2 isolated in Germany between 1982 and 1999 was examined in enzyme- and cell culture-based inhibition assays and by sequencing of the viral HA and NA genes. Additionally, agglutinating properties of these viruses were compared.

2. Materials and methods

2.1. Cell line 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 µg/ml streptomycin. The serum-free medium applied in cell culture-based assays on confluent 3-day-old MDCK cell monolayers was formulated with 2 µg/ml trypsin and 1.2 mM bicarbonate.

The seven H3N2 influenza A viruses examined in the present study (A/swine/Potsdam/35/82, A/swine/Karrenzien/2/87, A/swine/Leipzig/145/92, A/swine/Bakum/909/93, A/swine/Jena/5/96, A/swine/Lohne/1/97, and A/swine/Bakum/8602/99) were selected from 16 porcine FLUAV isolated in embryonated hen's eggs in Germany between 1982 and 1999 before the introduction of NAIs. Selection was based on a comparative analysis of HA and NA gene sequences which revealed a high sequence homology and demonstrate that the isolates belong to four different genetic clusters (Schrader and Suess, 2004). Influenza viruses A/Hong Kong/68 (Schaper & Brümmer, Salzgitter, Germany) and A/Sydney/5/97 (Chiron Behring, Marburg, 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 F. Hoffmann-La Roche AG (Basel, CH), respectively.

2.3. Chemiluminescence-based NA enzyme-inhibition assay

NA enzyme activity and drug inhibition assays were evaluated using a 1,2-dioxetane derivative of sialic acid (NA-Star, Tropix, Applied Biosystems, Darmstadt, Germany) as the substrate (Buxton et al., 2000), as described elsewhere (Wetherall et al., 2003). Chemiluminescence was read using a MLX Microtiter plate luminometer (Dynex Technologies, Chantilly, VA). At first, NA activity of FLUAV was titrated by serial two-fold dilutions of the virus. To evaluate NA inhibition, serial 10-fold NAI concentrations ranging from 10^{-7} to 10 µg/ml in H₂O were tested in duplicate and two untreated virus controls were included. The IC₅₀ were calculated from two independent assays.

2.4. Plaque-reduction assay

Plaque-reduction assays were done as described previously (Schmidtke et al., 2006). In brief, serial two-fold NAI concentrations were tested in duplicate. Two uninfected untreated cell controls as well as three infected untreated virus controls were included in all assays. After 48–96 h of incubation at 37 °C, plates were fixed and stained with a crystal violet formalin solution, the number of virus-induced plaques was counted, plaque diameter determined, and the compound-induced plaque reduction calculated. The concentration required to reduce the plaque number by 50% (IC₅₀) was calculated from the mean dose–response curves of at least three independent assays.

2.5. Virus yield-reduction assay

Cell monolayers grown in 96-well plates were infected with virus at a multiplicity of infection (MOI) of 0.001 (A/swine/Bakum/909/93 and A/Sydney/5/97), or 0.1 pfu per cell (all other isolates). After 1 h of virus adsorption at 37 °C cells were washed three times with EMEM and then cultured in test medium containing serial 10-fold dilutions of NAI. The final concentrations of the inhibitors ranged from 10^{-4} to 10 µg/ml. Each concentration was tested in triplicate. Three untreated cell- and virus controls were included. Supernatants were harvested 48 h (A/swine/Bakum/909/93 and A/Sydney/5/97) or 14 h after infection (all other viruses). Virus yields were determined by using 50% cell culture infective dose (CCID₅₀) assay in MDCK cells.

2.6. Inhibition of virus spread

Cell monolayers grown in 16-well chamber slides (Nunc, Wiesbaden, Germany) were infected with virus at a MOI of 0.001 (A/swine/Bakum/909/93 and A/Sydney/5/97) or 0.0001 pfu per cell (all other viruses). After 1 h of virus

adsorption at 37 °C, cells were washed three times with EMEM and then cultured in test medium without or with 1 µg/ml of the respective NAI. After 48 h of incubation, cells were fixed with ice-cold methanol. Drug effects on virus spreading were examined by immunostaining of viral nucleoprotein with monoclonal mouse antibody specific for the influenza A nucleoprotein (Acris Antibodies, Hiddenhausen, Germany) and DAKO detection kit APAAP Mouse (Dako, Hamburg, Germany) according to the Manufacturer's instructions.

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

Virus stocks were directly used to infect MDCK cells grown in 60 mm Petri dishes to avoid adaptation mutations. Additionally, viruses were picked from individual plaques grown in the presence of NAI (1–25 µg/ml) and used to infect MDCK cells. Total RNA was extracted from infected 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), T-RACE2 primer 5'-CCGATCGCTCGAGAATAGCCCTTTTTTTTTTTTTTTTTTTT-3' (20 µM), and 3 µg of RNA in a final reaction volume of 20 µl following the Manufacturer's instructions.

PCR amplification was done by using Qbiogene molecular biology TaqDNApol (Qbiogene, Heidelberg, Germany) according to the Manufacturer's protocol. Primers (each 10 µM) utilised for PCR are summarised in Table 1. 5'-RACE was done using the universal 5'-primer 5'-RGCRAAAGCAGG-3'. 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 incubation at 10 °C. Amplification products were subjected to agarose gel electrophoresis and extracted by using QIAquick gel extraction

kit (Qiagen, Hilden, Germany). Purified products were stored at –20 °C.

2.8. Sequencing and analysis of RT-PCR amplicons

Sequencing was performed with fluorescent-labelled nucleotides (DYEnamic ET terminator kit, Amersham Biosciences, Freiburg, Germany) on an ABI Prism 310 Genetic Analyser (Applied Biosystems, Darmstadt, Germany). Sequencing primers included those used in PCR each 5 µM. Twenty-five cycles of 95 °C for 20 s, 50 °C for 15 s, 60 °C for 1 min were run. The GenBank accession nos. are EF409245–EF409258. Sequence analysis was performed by using DNASTAR software. Amino acid alignment and sequence comparisons were made by using MegAlign and CLUSTAL W algorithm.

2.9. Hemagglutination assay

Serial two-fold virus dilutions (maximum 10⁵ TCID₅₀/50 µl) in 0.9% NaCl were incubated with 50 µl of a 1% suspension of chicken erythrocytes (C-C-pro, Oberdorla, Germany) in a 96-well U-shaped plate (Greiner, Frickenhausen, Germany) at either 4 °C or 37 °C for 1 h. The HA titers were calculated as the reciprocal value of the highest dilution that caused agglutination of chicken erythrocytes.

3. Results

3.1. Susceptibility of porcine H3N2 FLUAV to NAI in NA enzyme-inhibition assay

Oseltamivir and zanamivir susceptibility of porcine H3N2 FLUAV isolated in Germany between 1982 and 1999 was determined in enzyme- and cell culture-based inhibition assays. To avoid cell culture adaptation, virus strains were passaged only once in MDCK cells to get a virus stock. Like the human reference strains A/Hong Kong/68 and A/Sydney/5/97, all tested porcine viruses were susceptible against both tested NAIs in NA enzyme-inhibition assays (Table 2). The mean 50% inhibitory concentration (IC₅₀) of zanamivir was 0.0044 ± 0.0019 µg/ml and that of oseltamivir 0.0009 ± 0.0007 µg/ml.

3.2. Susceptibility of porcine H3N2 FLUAV to NAI in cell culture-based assays

Based on the experience of previous studies that demonstrated strong differences regarding the effect of NAI on plaque number and size (Gubareva et al., 2001; McKimm-Breschkin et al., 1998), the IC₅₀ concentrations concerning reduction of plaque number as well as plaque size were determined. The results are summarised in Table 2. With exception of influenza virus A/swine/Potsdam/35/82 (IC₅₀ = 0.17 µg/ml), the plaque number of porcine FLUAV was reduced by 50% applying 0.05 ± 0.06 µg/ml of oseltamivir. Reduction in plaque size was achieved by using 1.5–26-fold lower concentrations of oseltamivir (0.003–0.013 µg/ml). The IC₅₀ concentrations determined for the human reference strain influenza A/Hong

Table 1
Primers used for RT-PCR

Gene	Primer name	Primer sequence	
HA	HAH3-1F	5'-AGCAAAAGCAGGGGATAATTCTAT-3'	
	HAH3F	5'-AGCAARGCTTTCAGCAACTG-3'	
	HAH3-820F	5'-CACTGGGAAAAGCTCAATAATGAG-3'	
	HAH3-1063F	5'-GAGGCATATTCGGTGAATAGCAGG-3'	
	HAH3-1627F	5'-GGATTCCTTTGCCATATCATGC-3'	
	HAH3R	5'-AAGGYTTGTCATTGGGTATGCT-3'	
	HAH3-462R	5'-GCTTCCACCATTCTGGGTAAACCC-3'	
	HAH3-1724R	5'-GCAAAATGTTGCACCTAATGTTGCC-3'	
	NA	NAN2F	5'-TGGGTGTTCCATTTCATTTGGG-3'
		NAN2-63F	5'-CCCAGCGAACAACCAAGTAATGCC-3'
NAN2-792F		5'-CAGATGTATTGTCAGGGACAACACTGG-3'	
NAN2-820F		5'-GGCTCTAATAGGCCCGTCGTAGAC-3'	
NAN2-1151F		5'-CTGGTATTTCTCTGTTGAGGGC-3'	
NAN2R		5'-CTGTCGTTTTTCTGGGTGTGTC-3'	
NAN2-185R		5'-CTGTATTCACCTAATTTGGGGC-3'	
NAN2-510R		5'-ACAAACATGCAGCCATGCTTTTCC-3'	
NAN2-539R		5'-GCAGTTGCATTTTATCATTCCAG-3'	
NAN2-911R		5'-CTGGGTGTGTCGCCAACAAGCCC-3'	
NAN2-1311R		5'-GGCATGAGATTGATGTCGCC-3'	

Table 2
Agglutination of chicken erythrocytes by porcine H3N2 FLUAV and their susceptibilities to NAIs in MDCK cells

	HAU		IC ₅₀ oseltamivir carboxylate (µg/ml)				IC ₅₀ zanamivir (µg/ml)			
	4 °C	37 °C	Plaque-reduction assay		Virus yield-reduction	NA inhibition	Plaque-reduction assay		Virus yield-reduction	NA inhibition
			Plaque no.	Plaque size ^a			Plaque no.	Plaque size		
A/Hong Kong/68 ^b	16	16	0.06	0.001	0.001	0.0007	0.02	0.006	0.001	0.0014
A/Sydney/5/97 ^b	8	4–8	n.d.	n.d.	0.001	0.0008	n.d.	n.d.	0.001	0.0057
A/swine/Potsdam/35/82	32	32	0.17	0.025	0.032	0.0008	>25	1.6	0.048	0.0066
A/swine/Karrenzien/2/87	32	16	0.02	≤0.003	0.004	0.0005	1.4	0.8	0.007	0.0018
A/swine/Leipzig/145/92	8	8	0.01	0.013	0.009	0.0007	>25	0.8	0.007	0.0019
A/swine/Bakum/909/93	2	2	n.d.	n.d.	0.006	0.0024	n.d.	n.d.	0.020	0.0064
A/swine/Jena/5/96	32	32	0.08	≤0.003	0.001	0.0007	15.6	0.1	0.059	0.0044
A/swine/Lohne/1/97	32	16	0.02	0.013	0.001	0.0007	20	3.1	0.009	0.0050
A/swine/Bakum/8602/99	8	8	0.01	≤0.003	0.004	0.0006	>25	0.8	0.009	0.0044

n.d.: not determined; did not produce countable plaques under agar.

^a Drug concentration that resulted in a noticeable reduction in plaque size.

^b Human reference strain.

Kong/68 were 0.06 and 0.001 µg/ml of oseltamivir for reduction in plaque number and plaque size, respectively. To visualise the different drug effect on plaque number and plaque size, the photographs of representative plaque-reduction assays with A/swine/Potsdam/35/82 are shown in Fig. 1. In comparison to oseltamivir, the tested porcine H3N2 isolates were clearly less susceptible to zanamivir than the human reference strain A/Hong Kong/68 in plaque-reduction assay. Whereas 0.02 µg/ml of zanamivir reduced the plaque number of influenza virus A/Hong Kong/68 by 50%, 1.4 to >25 µg/ml of zanamivir had to be added to provide the same results with porcine FLUAV.

With the exception of A/swine/Potsdam/35/82 (oseltamivir and zanamivir) and A/swine/Jena/5/96 (zanamivir) no differences in NAI susceptibility were observed between porcine and human strains tested in virus yield-reduction assays (Table 2).

Immunostaining for expression of influenza A virus nucleoprotein was applied to examine drug effects on virus spreading. The experimental conditions allowed multi-step growth cycles that resulted in nearly 100% infected cell monolayers in untreated virus controls. The effect of NAI treatment (1 µg/ml) on virus spreading in MDCK cells is shown in Fig. 2. Replication of the two human reference strains as well as porcine FLUAV was restricted to the initial foci of infection upon oseltamivir as well as zanamivir exposure. In contrast, viral nucleoprotein was detected in nearly all cells in untreated virus controls.

3.3. Differences in hemagglutination

The ability of porcine viruses to agglutinate chicken erythrocytes was determined at 4 and 37 °C to evaluate the influence of elevated temperature on viral binding properties (Gubareva et al., 2001). Viruses with reduced binding affinity lead to hemagglutination at 4 °C but often fail to agglutinate chicken erythrocytes at elevated temperatures (37 °C). All porcine FLUAV agglutinate chicken erythrocytes, even at elevated temperatures. Differences ≤ two-fold are in a range of this assay error (Table 2).

3.4. Viral amino acid substitutions in HA and NA

According to previous publications, substitutions in HA as well as NA proteins may affect viral susceptibility to NAIs (Abed et al., 2002; Gubareva et al., 2000, 2001; McKimm-Breschkin, 2000). Therefore, the complete influenza A genome segment 4 (HA) and segment 6 (NA) were sequenced. No differences could be found between amino acid sequences of viruses isolated from single plaques under drug pressure (sequence data are not shown) and those of virus stocks.

The substitution R249K was detected in the NA gene of influenza A/swine/Bakum/909/93, A/swine/Jena/5/96, A/swine/Lohne/1/97, and A/swine/Bakum/8602/99. There were neither amino acid exchanges in or near the NA enzyme active site nor other NA substitutions that have been described previously. Influenza virus A/Sydney/5/97 as well as porcine viruses

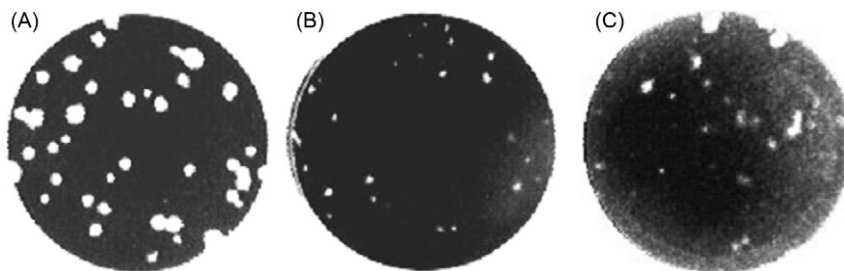


Fig. 1. Different influence of NAI on virus plaque size and number in plaque-reduction assays performed with influenza virus A/swine/Potsdam/35/82. Untreated, infected MDCK cells are shown in (A). Treatment with 0.1 µg/ml oseltamivir (B) and 0.8 µg/ml zanamivir (C) results in a shape reduction of plaque size, whereas the plaque number remained nearly unchanged.

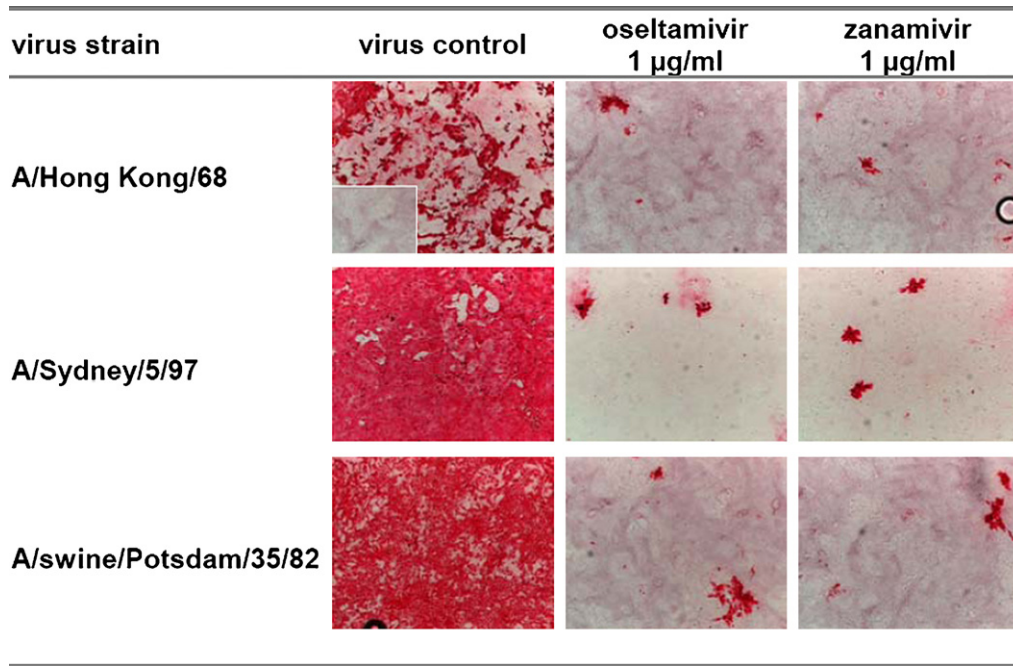


Fig. 2. Example for the effect of NAI treatment on virus spreading in MDCK cells. Immunostaining for expression of the viral nucleoprotein was used to visualise influenza virus A/swine/Potsdam/35/82-infected cells grown without (virus control) and with oseltamivir and zanamivir (magnification: 100 \times).

Table 3
HA1 and NA glycosylation patterns of H3N2 FLUAV

	HA1 amino acid position														NA amino acid position									
	8	22	38	45	63	81	122	126	133	144	146	165	246	285	61	69	70	86	146	200	234	329	402	
A/Hong Kong/68	+	+	+	-	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	-	+		
A/Sydney/5/97 ^a	+	+	+	-	+	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+		
A/swine/Potsdam/35/82 ^b	-	+	+	-	-	+	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	+		
A/swine/Karrenzien/2/87	+	+	+	-	+	-	-	+	-	-	-	+	-	+	+	-	+	+	+	+	+	-		
A/swine/Leipzig/145/92	+	+	+	-	+	-	+	+	-	-	-	+	-	+	+	-	+	+	+	+	+	-		
A/swine/Bakum/909/93 ^c	+	+	+	+	+	-	-	+	-	+	-	+	-	+	+	-	+	+	+	+	+	-		
A/swine/Jena/5/96	-	+	+	+	+	-	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	-		
A/swine/Lohne/1/97	+	+	+	+	+	-	-	+	-	+	-	+	-	+	+	-	+	+	+	+	+	-		
A/swine/Bakum/8602/99	+	+	+	+	+	-	-	+	-	+	-	+	-	+	+	-	+	+	+	+	+	-		

^a GenBank accession nos. AJ311466 (HA) and AJ291403 (NA).

^b GenBank accession no. AJ252132 (HA).

^c GenBank accession no. AJ252130 (HA).

do not contain a glycosylation site in amino acid 69 as was found in A/Hong Kong/68 (Table 3). A glycosylation site in position 402 is missing in influenza virus A/swine/Karrenzien/2/87. A/Sydney/5/97 has an additional glycosylation site at amino acid 329.

Different glycosylation patterns were also detected in the HA1 subunit of porcine FLUAV (Table 3). The number of glycosylation sites in the HA1 of porcine viruses increased from five to nine between 1982 and 1999. In contrast to the human reference strains and all other porcine isolates, influenza viruses A/swine/Potsdam/35/82 and A/swine/Jena/5/96 lack the glycosylation site in amino acid position 8. All porcine viruses differ from both human reference strains in position 155 and from A/Hong Kong/68 in position 226 (T/H155Y and Q226L). According to previous publications, these amino acids are located in the receptor-

binding site or on its left edge, respectively (Nobusawa et al., 1991). On the right edge of the HA receptor-binding site A/swine/Karrenzien/2/87, A/swine/Bakum/909/93, A/swine/Jena/5/96, A/swine/Lohne/1/97, and A/swine/Bakum/8602/99 possess the amino acid substitution N137S, whereas A/Sydney/5/97 possesses N137Y. Moreover, R132Q as well as S165N were detected in all isolates and N199S in all except for A/swine/Potsdam/35/82.

4. Discussion

The highly conserved catalytic site of the viral NA is the target for zanamivir and oseltamivir. The interaction of these antiviral drugs with the catalytic site blocks enzyme activity and prevents virus release and transmission. Therefore, results from NA enzyme-inhibition assays directly indicate the susceptibility

of the enzyme against inhibitors. In chemiluminescence-based NA enzyme-inhibition assays all tested porcine FLUAV isolates were NAI-susceptible. Like the human reference strain A/Sydney/5/97 but not A/Hong Kong/68, porcine FLUAV were two to seven times more susceptible towards oseltamivir than zanamivir. Such differences in enzyme inhibitory activity of both NAIs were also detected for avian and human N2 viruses (Abed et al., 2002; Govorkova et al., 2001; McKimm-Breschkin et al., 2003). The tested porcine H3N2 viruses were less zanamivir-susceptible than the previously published human and avian N2 isolates (Abed et al., 2002; Govorkova et al., 2001; McKimm-Breschkin et al., 2003; Wetherall et al., 2003) and influenza virus A/Hong Kong/68. However, their IC₅₀ values correspond to that of the second human reference strain A/Sydney/5/97.

Because no data on NAI susceptibility of porcine FLUAV were available until now and in view of the widely different results obtained with the various techniques in other studies, the anti-influenza A virus activity of NAI was also comparatively studied in cell culture-based assays. Using FACS analysis, it was proved that the membrane of MDCK cells used in our laboratory contains large amounts of 2.3 as well as 2.6 sialic acid (K. Bauer, unpublished data). All porcine FLUAV multiply well in these cells. Both NAIs effectively inhibited virus spreading, reduced virus yields and plaque size of porcine FLUAV at nanomolar concentrations. But, much higher drug concentrations were necessary to achieve reduction in plaque number. The results obtained with porcine FLUAV are in agreement with previous studies demonstrating strong differences between the effect of NAIs on plaque number and size for human FLUAV (Gubareva et al., 2001; McKimm-Breschkin et al., 1996, 1998). Porcine isolates exhibit a relatively wide range of IC₅₀ values when tested in plaque-reduction assay in MDCK cells. Such a wide range of IC₅₀ values has also been shown for human influenza viruses when tested in MDCK cells. According to a review of Tisdale (2000) one explanation for reduced antiviral activity of NAIs in cell culture-based assays may be that the target enzyme acts extracellularly. Inhibition of the virus is a direct result of inhibition of the viral NA in the extracellular fluid during virus release. Because cells used in cell-based assays have lost their apical polarity, viruses may also spread directly from cell to cell without the need of NA activity. So, NAI can not act. Another explanation may be the species-dependent substrate specificity of HA as well as NA of FLUAV (Matrosovich et al., 2003; Wagner et al., 2002). 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. Sialic acids in cell culture systems and on target cells in vivo may differ. These differences can disturb the balance between the antagonistic HA and NA functions and affect the therapeutic efficacy of NAIs in cell culture. The results of plaque-reduction assays of the present study indicate that this cell culture-based assay is unsuitable for monitoring the NAI sensitivity of porcine FLUAV. In contrast, results of virus yield-reduction assays are in good agreement with results of NA enzyme-inhibition assays for most of the isolates. The influenza viruses A/swine/Potsdam/35/82 and A/swine/Jena/5/96 possess a markedly reduced NAI susceptibility in virus yield-reduction assays.

According to previous publications obtained with avian and human FLUAV (Abed et al., 2002; Blick et al., 1998; Gubareva et al., 1996, 2000, 2001; McKimm-Breschkin, 2000; Staschke et al., 1995) reduced susceptibility to NAI in vitro may result (i) from amino acid substitutions in the viral NA or (ii) from reduction of the virus dependence on NA activity for release due to changes in the HA. The analysis of amino acid sequences of porcine FLUAV revealed several substitutions in the NA and HA. Among them were also substitutions that were suggested to affect NAI susceptibility e.g. R132Q, T155Y, S165N, N199S, and Q226L in the HA (Bantia et al., 1998; McKimm-Breschkin, 2000; McKimm-Breschkin et al., 1998). However, neither T155Y nor Q226L in the HA impaired NAI susceptibility. Moreover, HA amino acid substitutions implicated in NAI resistance were found in all isolates (R132Q and S165N) or all except one (N199S; not in A/swine/Potsdam/35/82). Furthermore, additional glycosylation sites of the viral HA can lead to NAI resistance of human FLUAV in cell culture (Mishin et al., 2005). Possibly, the increasing number of glycosylation sites in porcine FLUAV isolated in Germany between 1982 and 1999 is associated with reduced zanamivir susceptibility in plaque-reduction assays. However, differences in glycosylation pattern did not have an influence on agglutination of chicken erythrocytes by porcine FLUAV at 4 and 37 °C. The glycosylation at amino acid position 144 in A/swine/Bakum/909/93, A/swine/Jena/5/96, A/swine/Lohne/1/97, and A/swine/Bakum/8602/99 is close to the receptor-binding site and was reported to be important in antigenic drift of porcine and human H3N2 viruses (Abe et al., 2004; de Jong et al., 1999).

Genotyping further revealed unique substitution N8K in A/swine/Potsdam/35/82 and A/swine/Jena/5/96 and K83T in A/Hong Kong/68 and A/swine/Potsdam/35/82. Whereas N8K abolishes one potential glycosylation site, K83T adds one in position 81. The glycosylation site at amino acid 8 of human H3 FLUAV is strictly conserved (Abe et al., 2004). There are only three human FLUAV in the GenBank having the substitution N8K (accession nos. AAT6472, AAT6473, AAT6474). The two studied porcine virus isolates lacking this glycosylation site exhibit a lower oseltamivir and/or zanamivir susceptibility in virus yield-reduction assays.

In summary, the present study compares the NAI susceptibility of porcine FLUAV of subtype H3N2 in enzyme- as well as cell culture-based assays. The results suggest that plaque-reduction assay is unsuitable to monitor NAI sensitivity of porcine FLUAV. A good antiviral efficacy of NAI was demonstrated in NA enzyme-inhibition and virus yield-reduction assays. Two porcine isolates lacking a glycosylation site in HA exhibited a reduced NAI sensitivity in virus yield-reduction assay. It remains to investigate whether these results are predictive for the virus drug phenotype in vivo.

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