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Anti-influenza A virus activity of uridine derivatives of 2-deoxy sugars

Ewelina Krol [a,*], Ilona Wandzik [b], Beata Gromadzka [a], Dawid Nidzworski [a], Malgorzata Rychlowska [c], Marta Matlacz [a], Jolanta Tyborowska [a], Boguslaw Szewczyk [a]

[a] Department of Recombinant Vaccines, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Kladki 24, 80-822 Gdansk, Poland
[b] Department of Organic Chemistry, Bioorganic Chemistry and Biotechnology, Faculty of Chemistry, Silesian University of Technology, Krzywoustego 8, 44-101 Gliwice, Poland
[c] Department of Virus Molecular Biology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Kladki 24, 80-822 Gdansk, Poland

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ABSTRACT

Influenza viruses are important pathogens that cause respiratory infections in humans and animals. Apart from vaccinations, antiviral drugs play a significant role in controlling spread of the disease. Influenza A virus contains two membrane glycoproteins on the external part of viral envelope: hemagglutinin (HA) and neuraminidase (NA), which are crucial for productive infection in target cells. In the present work, two derivatives of tunicamycin – uridine derivatives of 2-deoxy sugars (designated IW3 and IW7), which target the glycan processing steps during maturation of viral glycoproteins, were assayed for their ability to inhibit influenza A virus infection *in vitro*. Using the cytopathic effect (CPE) inhibition assay and viral plaque reduction assay we showed, that both IW3 and IW7 inhibitors exerted significant inhibitory effect on influenza A virus infection in MDCK cells without significant toxicity for the cells. Moreover, tested compounds selectively suppressed viral protein expression in a dose-dependent manner, suggesting that the mechanism of their antiviral activity may be similar to this shown previously for other viruses. We have also excluded the possibility that both inhibitors act at the replication step of virus life cycle. Using real-time PCR assay it was shown that IW3 and IW7 did not change the level of viral RNA in infected MDCK cells after a single round of infection. Therefore, inhibition of influenza A virus infection by uridine derivatives of 2-deoxy sugars, acting as glycosylation inhibitors, is a promising alternative approach for the development of new anti-influenza A therapy.

1. Introduction

Each year influenza viruses cause severe respiratory illness in humans and animals with high morbidity and mortality rates worldwide (Miller et al., 2009). Vaccines and antiviral drugs are major options to control new infections. Although it is obvious that vaccination is the best way of prevention, the composition of vaccines needs to be updated seasonally. Moreover, variable effectiveness of vaccination is the result of antigenic shift and antigenic drift of the viral genome (Nichol and Treanor, 2006). Due to the fact, that vaccines are not effective against rapidly emerging mutant viruses, antivirals are still needed to prevent transmission of novel influenza strains.

Currently, there are two major classes of anti-influenza drugs approved by the FDA for clinical use: M2 ion-channel inhibitors (amantadine and rimantadine) (Hayden, 1997; Wang et al., 1993) and neuraminidase (NA) inhibitors (oseltamivir and zanamivir) (Monto et al., 1999; Nicholson et al., 2000). M2 ion-channel inhibitors prevent viral uncoating and are effective only against type A viruses (Luscher-Mattli, 2000). Moreover, the widespread resistance of viral strains to both amantadine and rimantadine limits the use of this class of drugs for influenza treatment (Bright et al., 2005, 2006; Deyde et al., 2007). Neuraminidase inhibitors specifically bind to the conserved active site of the enzyme, thereby preventing the release of newly synthesized influenza A and B viruses from infected cells (Garten et al., 2009). Resistance to neuraminidase inhibitors has been shown to be associated with mutations in NA gene occurring rapidly during treatment (Ferraris and Lina, 2008; Thorlund et al., 2011). The increase of oseltamivir-resistant seasonal influenza A (H1N1) viruses has been observed in USA, growing from less than 0.5% in 2006–2007 to 99% in 2008–2009 (Hurt et al., 2011; Renaud et al., 2011). Oseltamivir-resistant H1N1 viruses were also isolated in Europe and other

Abbreviations: CC_{50}, concentration of the compound required to reduce cell viability by 50%; CPE, cytopathic effect; CSFV, classical swine fever virus; Ct, cycle threshold; ER, endoplasmic reticulum; HA, hemagglutinin; IC_{50}, concentration of the compound required to reduce virus plaque formation by 50%; MDCK, Madin–Darby canine kidney cells; MOI, multiplicity of infection; NA, neuraminidase; Sf9, *Spodoptera frugiperda* insect cell line; S.D., standard deviations; SI, selectivity index; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

* Corresponding author. Tel.: +48 58 523 63 36; fax: +48 58 305 73 12.
E-mail addresses: ewelina@biotech.ug.gda.pl (E. Krol), ilona.wandzik@polsl.pl (I. Wandzik), g.beata@biotech.ug.gda.pl (B. Gromadzka), dawid.nidzworski@biotech.ug.gda.pl (D. Nidzworski), ggordon@biotech.ug.gda.pl (M. Rychlowska), mmatlacz@biotech.ug.gda.pl (M. Matlacz), jtybor@biotech.ug.gda.pl (J. Tyborowska), szewczyk@biotech.ug.gda.pl (B. Szewczyk).

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countries and are now widely circulating (Hauge et al., 2009; Meijer et al., 2009; Tamura et al., 2009). Due to the continuous threat of influenza resistant strains, there is an urgent need to search for new potential antiviral drugs directed toward different viral or cellular targets.

Two membrane glycoproteins of influenza virus: hemagglutinin (HA) and neuraminidase play a crucial role in the replication and infectivity of the virus and are likely to be good targets for new antiviral strategy. HA is a homotrimeric class I transmembrane glycoprotein responsible for viral entry by interactions with sialic acid-containing host cell receptors and plays a role in membrane fusion (Wiley and Skehel, 1987). NA is anchored in the viral envelope as a homotetramer with type II membrane topology and acts as a receptor-degrading enzyme by catalyzing the removal of sialic acids from viral and cellular components at late stages of infection, allowing for the release of progeny virions (Colman, 1998). Both NA and HA are highly glycosylated with 4 and 3–9 potential N-glycosylation sites, respectively (Schulze, 1997; Ward et al., 1983). Number and type of the oligosaccharides attached to HA strongly depends on the virus subtype and strain (Inkster et al., 1993; Matrosovich et al., 1999). It has been reported that N-linked oligosaccharides attached to the stalk region of HA are highly conserved, while those in other regions of the molecule vary considerably in structure and number among different influenza viruses (Wagner et al., 2000).

N-glycosylation influences not only the correct folding and stability of many viral glycoproteins, but also has vital effects on their biological functions such as receptor binding, membrane fusion, and penetration into host cells. Lack or removal of N-oligosaccharides very often leads to aggregation and protein retention in the endoplasmic reticulum. Misfolded proteins are usually translocated back to the cytosol for proteasome degradation (Parodi, 2000; Trombetta, 2003). The nucleoside antibiotic tunicamycin is a potent inhibitor of the first steps of N-glycosylation with strong antibacterial, antitumor and antiviral activity (Duksin and Mahoney, 1982; Elbein, 1987). However, the therapeutic use of tunicamycin has been limited due to its toxicity in animals (Bourke and Carrigan, 1993; Kohsaka et al., 1985).

We have previously shown that two derivatives of tunicamycin, compounds IW3 and IW7 (uridine derivatives of 2-deoxy sugars), efficiently inhibited propagation of classical swine fever virus (CSFV), demonstrating significantly lower cytotoxicity than tunicamycin *in vitro* (Krol et al., 2010). The observed antiviral effect of IW3 and IW7 was attributed to the reduced yield of CSFV glycoproteins caused by the N-glycosylation inhibition at the late stage of glycan modification process (cis- or very early in medial-Golgi) characteristic for mammalian cells.

In this study we present the new data on the *in vitro* antiviral activity of previously identified compounds against influenza A virus. We have demonstrated that IW3 and IW7 compounds inhibit influenza virus by different mechanism than currently used anti-influenza drugs. These results suggest that uridine derivatives of 2-deoxy sugars may potentially complement other anti-influenza therapeutic agents either in individual or multidrug strategies.

2. Materials and methods

2.1. Cells and viruses

Madin–Darby canine kidney cells (MDCK) were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) (Sigma–Aldrich, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 0.2% bovine serum albumin, 25 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, at 37 °C under 5% CO_2.

The avian influenza virus A/ostrich/Denmark/725/96 (H5N2) was kindly provided by Department of Poultry Diseases, National Veterinary Research Institute, Pulawy, Poland. The human influenza virus (isolate 32U of the pandemic human influenza A/H1N1 virus) from the collection of the Department of Recombinant Vaccines, University of Gdansk, Poland was also used in this study. All influenza A viruses were propagated in MDCK cells in the presence of 2 μg/ml TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone) – trypsin (Sigma–Aldrich, USA). Viral stocks were stored at −70 °C and titrated by plaque assay before use.

2.2. Antivirals

The synthesis of uridine derivatives of 2-deoxy sugars (designated by us IW3 and IW7) was reported elsewhere (Wandzik and Bieg, 2007). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C until future use. Tunicamycin was purchased from Sigma–Aldrich, USA. Stock solutions were made in DMSO.

2.3. Antibodies

Mouse monoclonal anti-influenza A virus M1 antibody was purchased from Abcam (Cambridge, UK). Rabbit monospecific polyclonal serum raised against a His-tagged truncated form of HA protein of A/England/195/2009 (H1N1) strain (anti-sHA) was obtained by rabbit immunization with baculovirus produced HA purified by affinity chromatography. Anti-β-actin, anti-mouse and anti-rabbit alkaline phosphatase (AP)-conjugated secondary antibodies as well as anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa–Cruz Biotechnology (Santa Cruz, CA, USA).

2.4. Cell viability assay

To determine cell viability, CellTiter 96 AQ_{ueous} non-radioactive cell proliferation assay (MTS) (Promega, USA) was performed. MDCK cells were grown in 96-well plates for 24 h followed with medium change and future culturing in the presence of different concentrations of tested compounds (in triplicate) for two days. Cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) for 3 h at 37 °C and the absorbance at 490 nm was read using a microplate reader. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

2.5. Cytopathic effect (CPE) inhibition assay

MDCK cells were seeded in 6-well plates, incubated overnight and infected with influenza virus A/ostrich/Denmark/725/96 (H5N2) for 1 h at 37 °C. The inoculum was removed by washing with serum-free medium, and the cell monolayers were overlaid with fresh serum-free medium containing 2 μg/ml TPCK-trypsin and increasing amounts of inhibitors. After 24 h of incubation, CPE was evaluated after immunostaining with a monoclonal antibody specific for M1 protein as described in plaque reduction assay.

2.6. Plaque reduction assay

The plaque reduction assay was performed as previously described (Matrosovich et al., 2006). In brief, MDCK cell monolayers in 12-well plates were infected with influenza A virus for 1 h at 37 °C. The inoculum was removed by washing with serum-free medium, and the cell monolayers were overlaid with fresh serum-free medium containing 1.2% Avicel (FMC BioPolymer,

USA), 2 μg/ml TPCK-trypsin and increasing amounts of inhibitors. 3 days post infection, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and virus-infected cells were visualized by immunostaining with anti-M1 antibody (diluted 1:1000 in PBS, 1% Tween 20, 5% FBS). Anti-mouse horseradish peroxidase (HRP)-conjugated antibody was used as a secondary antibody (diluted 1:1000 in PBS containing 1% Tween 20 and 5% FBS). Plaques were detected using the Vector Nova-Red kit (Vector Laboratories Ltd., UK) and counted. IC_{50} was calculated as the compound concentration reducing the number of plaques by 50% compared to untreated infected cells.

2.7. SDS–PAGE and Western blot analysis

MDCK cell monolayers grown in 12-wells plates were infected with influenza A virus at an MOI of 1 for 1 h at 37 °C. Unbound virus was removed by washing with serum-free medium. Fresh serum-free medium containing 2 μg/ml TPCK-trypsin and different concentrations of inhibitors was added and incubation was carried out for 48 h. Cell lysis was performed at 4 °C for 1 h in TNET buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100), proteins were separated by SDS–PAGE under reducing conditions and transferred to PVDF membranes. Rabbit polyclonal serum anti-sHA (1:500 dilution) and anti-β-actin antibody (1:1000 dilution) were used as primary antibodies. Anti-rabbit or anti-mouse alkaline phosphatase (AP)-conjugated antibodies were used as secondary antibodies (diluted 1:2000). Nitrotetrazolium blue (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used as substrates for alkaline phosphatase (AP).

2.8. Viral RNA isolation and cDNA synthesis

MDCK cells cultured in 12-wells plates were infected with influenza A virus at an MOI of 0.01 or 0.1 for 1 h. The inoculum was removed and the cells were washed with serum-free medium. Fresh serum-free medium containing 2 μg/ml TPCK-trypsin and inhibitor at the concentrations indicated was added and incubation was carried out for 8 h for cells infected with influenza A virus at an MOI of 0.1 and 24 or 48 h for cells infected with the virus at an MOI of 0.01. At 8, 24 or 48 h p.i., the supernatants were harvested, centrifuged at 5000*g* for 5 min and used for viral RNA purification. Viral RNA was also extracted from influenza A virus-infected cells. Total intracellular and secreted influenza A virus RNA was purified using the Total RNA Mini purification kit (A&A Biotechnology S.C., Poland) according to the manufacturer's instructions. RNA extracted from MDCK cells and culture medium supernatants were reverse transcribed to cDNA by RT-PCR using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according the manufacturer's instructions. The reaction mixture containing: viral RNA, random hexamer primers and water was incubated for 10 min at 65 °C. Subsequently, the reaction buffer, RNase inhibitor [20U], dNTPs [1 mM each], DTT [5 mM] and reverse transcriptase [10U] were added and incubated for 10 min at 29 °C then 60 min at 48 °C, followed by 5 min incubation at 85 °C and chilled on ice.

2.9. Real-time PCR assay

The assay was based on SYBR Green dye technology. Real-time PCR was performed using the LightCycler 2.0™ (Roche Diagnostics, Mannheim, Germany). A 10 μl reaction mixture contained: 1 μl 10 × LC DNA Master SYBR Green I buffer (Roche Diagnostics, Mannheim, Germany), 6.0 μl H_2O, 1.0 μl $MgCl_2$ (25 mM), 0.5 μl 5 μM forward primer (5′-AGATGAGTCTTCTAACCGAGGTCG-3′), 0.5 μl 5 μM reverse primer (5′-TGCAAAAACATCTTCAAGTCTCTG-3′) (for amplification of a 102-bp conserved region of matrix protein (M1) gene) and 1 μl of cDNA. A negative control lacking cDNA was also prepared. The samples were initially denatured at 95 °C for 10 min., followed by 45 cycles of: 95 °C (denaturation) for 10 s, 50 °C (annealing) for 10 s, and 72 °C (extension) for 14 s. To confirm the specificity of the amplified product, the melting curve analysis step was included. The internal temperature of the LightCycler was rapidly increased to 95 °C, then decreased at 0.1 °C/s to 65 °C, and the sample was incubated for 15 s. The fluorescence at 530 nm was measured continuously. The melting peaks were generated using LightCycler software by plotting the first negative derivative of the fluorescence over the temperature versus the temperature ($-dF/dT$).

3. Results

3.1. Inhibition of influenza A virus propagation in MDCK cells by uridine derivatives of 2-deoxy sugars

Previously, we have reported that tunicamycin derivatives, uridine derivatives of 2-deoxy sugars (named IW3 and IW7) show an antiviral effect against classical swine fever virus *in vitro* (Krol et al., 2010). Based upon the activity of IW3 and IW7 affecting CSFV glycoproteins, the inhibitors were evaluated for their ability to inhibit influenza A virus propagation in MDCK cell culture.

In a first set of experiments, the cytotoxicity of the compounds was measured using MTS assay. We determined that IW3 and IW7 reduced viability of MDCK cells with the CC_{50} values, corresponding to a 50% cytotoxic effect after 48 h of inhibitor treatment, at 530 and 88 μg/ml, respectively (Table 1). No morphological alternations, loss of cell viability or modification of cell multiplication rates could be observed in cells treated with IW3 or IW7 with selected working doses of each compound.

The antiviral property of IW3 and IW7 against influenza A virus was investigated using CPE inhibition assay and plaque reduction assay. MDCK cells were infected with influenza virus A/ostrich/Denmark/725/96 (H5N2) and then treated with varying amounts of IW3 or IW7, or left untreated (positive control) after removal of the virus inoculum. Infection with influenza A/ostrich/Denmark/725/96 virus normally results in a severe CPE. A reduction in virus-induced CPE after 24 h incubation indicated antiviral activity of the tested compounds. The CPE was significantly reduced when 120 μg/ml of IW3 and 60 μg/ml of IW7 were added to MDCK infected cells, which suggest that both inhibitors can protect cells from virus-induced cell death (Fig. 1).

To further characterize the antiviral activity of IW3 and IW7 inhibitors on influenza virus propagation, the plaque reduction assay was performed. MDCK cells were infected with influenza virus A/ostrich/Denmark/725/96 (H5N2) at an MOI of 0.01, and incubated with overlay medium supplemented with different concentrations of tested compounds or left untreated. 3 days p.i., cells were immunostained using a monoclonal antibody specific for M1 protein to measure the extent of influenza A virus infection. The example of the effect of IW3 inhibitor on influenza A virus plaque formation is shown in Fig. 2.

Table 1
Cytotoxic properties and anti-influenza A virus activities of IW3 and IW7 in MDCK cells.

Compound	CC_{50} (μg/ml)[a]	IC_{50} (μg/ml)[b]	SI[c]
IW3	530	60 ± 1.20	8.83
IW7	88	45 ± 1.17	1.96

[a] Concentration required to reduce cell viability by 50%.
[b] Concentration required to reduce virus plaque formation by 50%. Expressed as the mean ± S.D. from three independent experiments.
[c] In vitro selectivity index (CC_{50}/IC_{50}).

The average size and number of plaques in both IW3 and IW7-treated cells were markedly reduced in a dose-dependent manner, demonstrating that tested compounds exhibited antiviral properties against influenza A virus. The IC_{50} values for inhibition of virus propagation for IW3 and IW7 were 60 and 45 μg/ml, respectively. The selectivity indexes (SIs), defined as the CC_{50}/IC_{50} ratio, are summarized in Table 1.

3.2. Inhibitory effect of IW3 and IW7 on viral HA and NA glycoprotein synthesis in MDCK cells

We have previously shown that IW3 and IW7 inhibitors effectively blocked CSFV propagation by affecting the synthesis of viral glycoproteins (Krol et al., 2010). To confirm that the antiviral activity of these compounds is similar against influenza A virus, Western blot analysis after SDS–PAGE in reducing conditions was performed. MDCK cell were infected with isolate 32U of the pandemic human influenza A/H1N1 virus and were treated with decreasing amounts of either IW3 or IW7 inhibitor. The total proteins accumulated in MDCK influenza A virus-infected cells (represented by HA) were analysed by Western blotting using rabbit polyclonal serum anti-sHA, which recognize the HA1 subunit of HA glycoprotein. The amount of influenza virus HA produced in both IW3 (Fig. 3A) and IW7 (Fig. 3B) treated cells was reduced in a dose-dependent manner comparing with untreated cells. However, as shown in Fig. 3, much more significant effect of reduction in the amount of viral HA protein was observed in IW3-treated cells. Furthermore, after treatment with the highest doses of IW3 (150–75 μg/ml) and IW7 (50 μg/ml), the bands representing the mature form of HA protein were nearly undetectable, and it was not possible to detect the unglycosylated or underglycosylated forms of HA protein. We hypothesize that viral glycoproteins were not detectable probably due to quick degradation process of incorrectly matured proteins. Similar results were obtained for CSFV, but not for pseudorabies virus (PRV) where underglycosylated forms were stable and could be easily detected (Krol et al., 2010). Our results indicated that the reduction in virus production, monitored by number and size of plaques in plaque reduction assay obtained in the previous experiment (Fig. 2) can be attributed to the inhibition of viral glycoprotein accumulation. Similar results were obtained for NA glycoprotein (data not shown).

3.3. Effect of IW3 and IW7 on RNA synthesis in MDCK infected cells

Higher concentrations of IW3 and IW7 led to the complete loss of detectable viral glycoproteins when examined by Western blot analysis, therefore in the next experimental step we wanted to determine whether this correlates with the decrease in viral RNA production. The level of avian influenza A/ostrich/Denmark/725/96 (H5N2) virus RNA was analysed by real-time PCR using SYBR Green dye technology.

Monolayers of MDCK cells were infected with influenza A virus and incubated with inhibitor concentrations resulting in the lack of detectable glycoproteins in the previous experiment or left untreated. To identify the inhibitory effect of uridine derivatives of 2-deoxy sugars on influenza virus replication, the synthesis of matrix protein mRNA was analyzed. Total RNA extraction was carried out 8, 24 or 48 h post infection, and the levels of intracellular and secreted mRNA were measured in controls, IW3- or IW7-treated cells and culture medium.

MOI equal to 0.1 and a short infection period (8 h) were employed to estimate the direct effect of IW3 and IW7 on virus replication during a single round of infection. Ct values for positive control (without inhibitors) as well as for IW3 or IW7 treated samples were very similar (data not shown). The expected 102-bp PCR product, corresponding to the fragment of a conserved region of

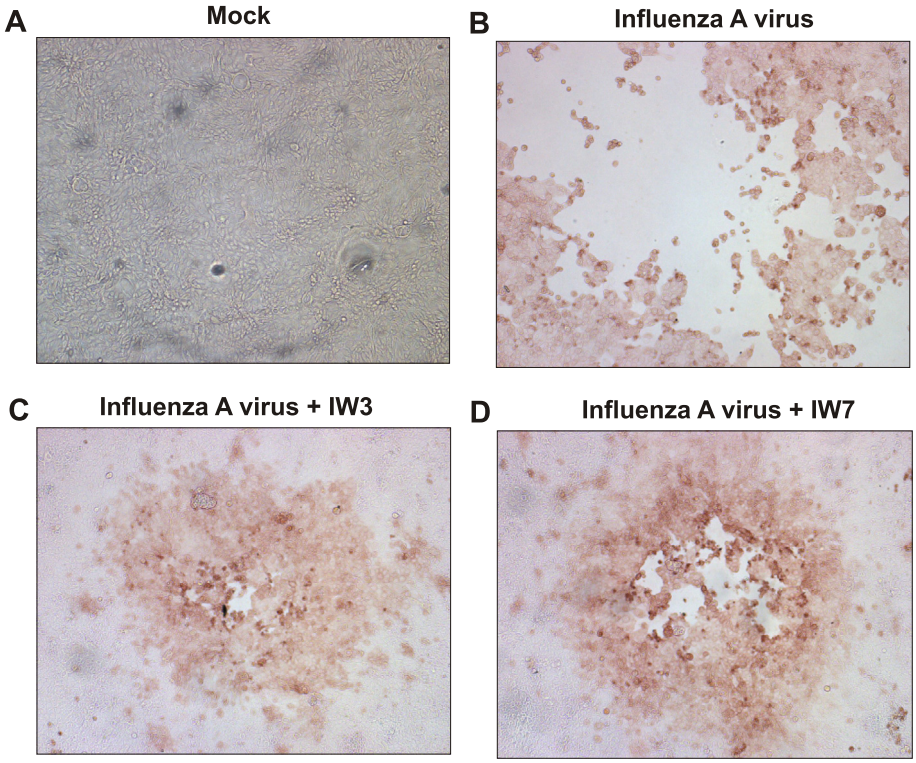

Fig. 1. IW3 and IW7 compounds inhibit virus-induced CPE in MDCK cells. MDCK cells were mock infected (A) or infected with influenza A virus A/ostrich/Denmark/725/96 (H5N2) (B–D). At 1 h p.i., cells were treated with 120 μg/ml of IW3 (C), 60 μg/ml of IW7 (D) or left untreated (positive control – B). 24 h p.i., the inhibition of virus replication by tested compounds was evaluated by immunostaining using a monoclonal antibody specific for M1 protein.

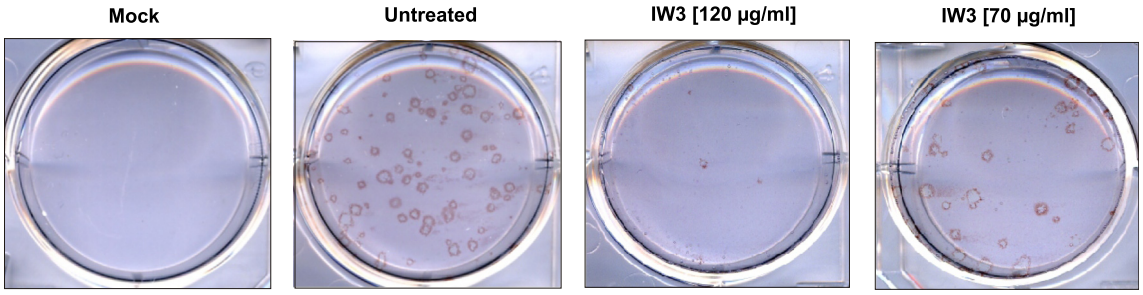

Fig. 2. Effect of IW3 inhibitor on influenza A virus plaque formation. MDCK cells were infected with influenza virus A/ostrich/Denmark/725/96 (H5N2) or mock infected. At 1 h p.i., the unbound virus was removed and the cells were incubated with overlay medium supplemented with 120 or 70 µg/ml of IW3 inhibitor. 3 days p.i., cells were fixed and immunostained using a monoclonal antibody specific for M1 protein to detect influenza A virus plaques.

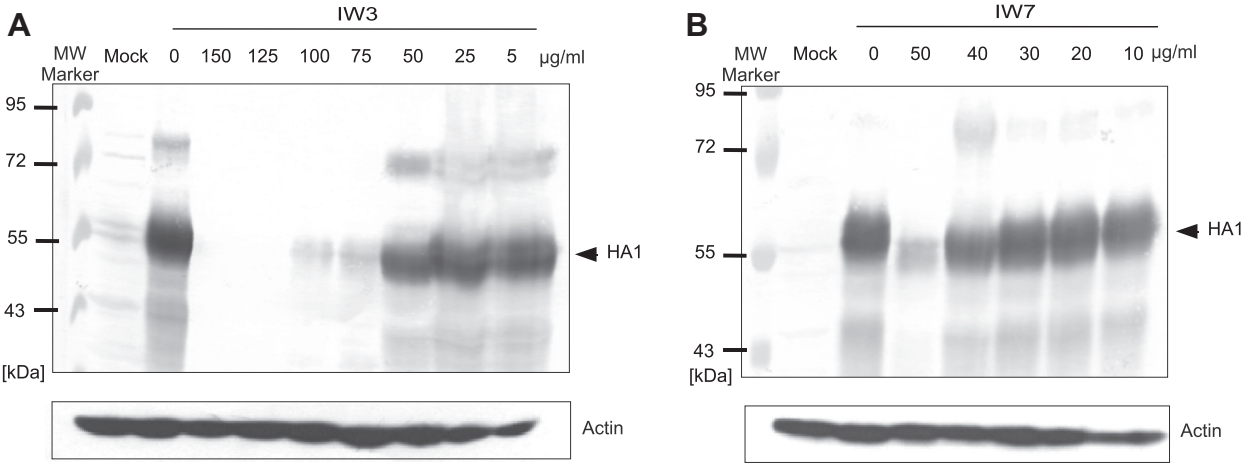

Fig. 3. Effect of uridine derivatives of 2-deoxy sugars on the synthesis of viral HA glycoprotein. Influenza A virus (isolate 32U of the pandemic human influenza A/H1N1 virus)-infected MDCK cells were treated with various concentrations of IW3 (0–150 µg/ml) (A) or IW7 (0–50 µg/ml) (B). At 48 h p.i., cells were lysed and proteins were separated by SDS–PAGE (10% polyacrylamide) under reducing conditions. Western blot analysis was performed using the specific rabbit polyclonal serum anti-sHA and anti-β-actin monoclonal antibodies. Positions of HA1 subunit of HA protein are marked with arrows. MW marker – molecular weight marker.

matrix protein (M1) gene, was detected at the same level in all tested samples. These results indicate that viral RNA accumulation is not affected by treatment with both inhibitors after a single round of infection and that both compounds exert its antiviral effect at the other than RNA synthesis level of virus life cycle.

To study whether tested compounds influence the secretion of viral particles, we used culture medium from influenza virus infected cells as described above to extract RNA for amplification. Interestingly, significant change in M1 mRNA level was observed in culture medium after IW3 and IW7 treatment when compared to untreated cells. Ct value for positive control (medium from untreated cells) was approximately 37. Treatment with the highest doses of both inhibitors reduced the viral RNA almost completely (Table 2A, and B), suggesting that the secretion of viral particles is inhibited by both compounds after single round of infection.

This finding was additionally confirmed by another experiment in which media from untreated (positive control) and IW3 or IW7 treated cells after 8 h p.i. were used to infect fresh monolayers of MDCK cells. The relative infectivity of P1 supernatants was determined at 3 days p.i., by plaque assay. Calculated titers of P1 progeny viruses were strongly reduced, confirming the results obtained from real-time PCR that IW3 and IW7 strongly inhibit the secretion of the virus (Fig. 4).

It has been reported that a single replication cycle of the influenza A virus takes approximately 8 h. Therefore, to study the effect of the inhibitors not only on the first round of viral replication but also on the accumulation and infectivity of viral particles in subsequent replication cycles, infection with low MOI of 0.01 was performed and 24 or 48 h inhibitor treatment periods were studied. Real-time PCR assays after 24 or 48 h treatment with both inhibitors indicated that viral RNA accumulation in influenza A virus infected cells is significantly reduced in a dose-dependent manner. Ct value for untreated cells was approximately 17. After IW3 treatment with 150 and 100 µg/ml Ct values were increased to 40.2 and 27.3, respectively for 24 h which corresponds to about 99.9% reduction in viral RNA in infected cells (Table 2A). For 48 h Ct values were increased to 28.7 and 22, respectively, which corresponds to the similar RNA level reduction. Similar results were observed for IW7 inhibitor. Approximately, 99% reduction of viral RNA was observed after treatment with 60 µg/ml of IW7 (Ct value was increased to 23.5 for 24 h and 22.8 for 48 h) (Table 2B).

Furthermore, a significant decrease of the M1 mRNA levels were also observed in culture medium of IW3 or IW7-treated cells compared to untreated cells. Ct value for positive control (medium from untreated cells) was approximately 21. 24 or 48 h treatment with the highest doses of both inhibitors reduced the viral RNA up to 99% (Table 2A, and B).

Taken together, these data indicate that due to the changes in viral proteins glycosylation status, IW3 or IW7 interfere with the assembly and/or secretion of the mature virions after single round

Table 2
SYBR Green real-time PCR detection of viral cDNA after 8, 24 or 48 h treatment with IW3 (A) and IW7 (B).

	8 h Incubation			24 h Incubation						48 h Incubation					
	Culture medium			MDCK cells			Culture medium			MDCK cells			Culture medium		
	Ct	ΔCt	% RNA decrease (%)	Ct	ΔCt	% RNA decrease (%)	Ct	ΔCt	% RNA decrease (%)	Ct	ΔCt	% RNA decrease (%)	Ct	ΔCt	% RNA decrease (%)
A															
IW 3 (μg/ml)															
0	37.0 ± 0.1	0.0	0	17.0 ± 0.2	0	0	21.0 ± 0.1	0	0	16.7 ± 0.1	0	0	20.3 ± 0.2	0	0
150	43.3 ± 0.2	6.3	99	40.2 ± 0.2	23.2	99.9	33.4 ± 0.1	12.4	99.9	28.7 ± 0.2	12	99.9	32.1 ± 0.1	11.8	99.9
100	40.1 ± 0.1	3.2	88	27.3 ± 0.2	10.3	99.9	31.8 ± 0.2	10.8	99.9	22.0 ± 0.1	5.3	97.5	25.9 ± 0.2	5.6	98.0
50	37.0 ± 0.2	0.0	0	17.0 ± 0.2	0	0	21.0 ± 0.2	0	0	16.7 ± 0.2	0	0	20.3 ± 0.1	0	0
Neg. control	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B															
IW 7 (μg/ml)															
0	37.0 ± 0.2	0.0	0	17.0 ± 0.1	0	0	20.8 ± 0.2	0	0	16.7 ± 0.1	0	0	20.3 ± 0.1	0	0
60	43.2 ± 0.2	6.2	99	23.5 ± 0.2	6.5	99	27.2 ± 0.1	6.4	99	22.8 ± 0.2	6.1	98.5	26.5 ± 0.2	6.2	98.6
50	40.2 ± 0.1	3.1	89	17.8 ± 0.1	0.8	43	21.6 ± 0.2	0.8	43	17.6 ± 0.2	0.9	46	21.0 ± 0.1	0.7	40
40	37.7 ± 0.2	0.7	39	17.0 ± 0.2	0	0	20.8 ± 0.2	0	0	16.7 ± 0.2	0	0	20.3 ± 0.2	0	0
Neg. control	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Ct (cycle threshold) values are expressed as the mean ± S.D. from three independent experiments.
ΔCt were calculated as a subtraction of Ct values of treated and untreated cells.

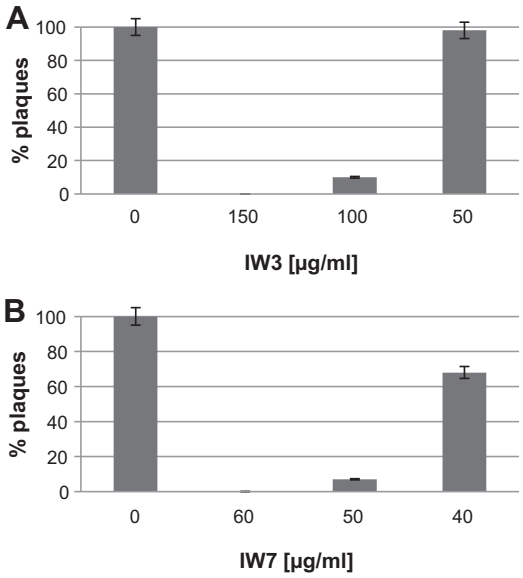

Fig. 4. Effect of IW3 (A) and IW7 (B) on relative amount of infectious influenza A virus production after 8 h incubation period. MDCK cells were infected with influenza virus A/ostrich/Denmark/725/96 (H5N2). At 1 h p.i., the unbound virus was removed and the cells were treated with IW3, IW7 or left untreated (positive control). After 8 h incubation, the collected media were used to infect fresh monolayers of MDCK cells. The amount of infectious virus in all tested samples was determined by plaque assay. Data represent means from three independent experiments ± S.D.

of infection which results further in the reduction of virus RNA in both infected cells and culture medium during secondary infections.

4. Discussion

The limited efficacy of current anti-influenza virus treatment and severe drug resistance among circulating influenza A strains motivate the search for alternative antiviral compounds with different target sites. Two surface glycoproteins of influenza A virus, hemagglutinin (HA) and neuraminidase (NA), essential for the viral infectious cycle appear to be good candidates for new antiviral strategies. Several reports have shown that the arrest or alterations of the glycosylation processes of viral proteins by different inhibitors usually result in antiviral effects (Asano, 2003; Chapel et al., 2007; Durantel et al., 2001; Lazar et al., 2007).

In our previous work we have demonstrated that tunicamycin derivatives – uridine derivatives of 2-deoxy sugars (named IW3 and IW7) exhibit a significant antiviral activity against classical swine fever virus with the mechanism of action similar to tunicamycin, but reduced cellular toxicity (Krol et al., 2010). In this study, the antiviral activity of IW3 and IW7 against influenza A virus was tested to evaluate their potential use as new anti-influenza compounds. The results strongly indicate that uridine derivatives of 2-deoxy sugars are active against influenza A virus H5N2 and H1N1 strains in cell culture at non-toxic concentrations. Our results show that IW3 and IW7 protect MDCK cells from cell death induced by influenza A virus (Fig. 1). IW3 and IW7 inhibit influenza A virus propagation with IC_{50} values of 60 ± 1.20 and 45 ± 1.17 μg/ml, respectively as observed in plaque reduction assay (Fig. 2). Our studies have confirmed that the mechanism underlying the antiviral activity of uridine derivatives of 2-deoxy sugars against influenza virus is the same as it was shown for CSFV. We have examined the effect of IW3 and IW7 on the synthesis of viral proteins indicating that the reduction in virus production can be attributed to the inhibition of viral glycoprotein accumulation in virus infected cells. For both IW3 and IW7, a dose-dependent decrease of intracellular HA and NA glycoproteins was observed (Fig. 3). Interestingly, we were not able to detect underglycosylated species of HA or NA glycoproteins suggesting that such polypeptides are rapidly degraded in host cells. It has been shown that the proteins that do not pass the ER quality control are transported back to the cytosol for proteosome degradation in a process known as ER-associated degradation (Plemper and Wolf, 1999). Tunicamycin has been shown to cause a complete misfolding of influenza virus HA, while the protein structure was only partly affected by another glucosidase inhibitor, castanospermine (Hebert et al., 1996; Hurtley et al., 1989). When the glycosylation process is blocked by tunicamycin, the misfolded HA is not transported to the plasma membrane, but retained in the ER prior to degradation. We hypothesize that uridine derivatives of 2-deoxy sugars which are structurally related to tunicamycin may have similar effect on the synthesis of influenza A virus glycoproteins. Hemagglutinin and neuraminidase without native oligosaccharide side chains due to IW3 and IW7 treatment, are most probably degraded by cellular protease(s) and cannot be detected in infected cells. The lack of

detection of unglycosylated polypeptides was also observed in our previous study on CSFV E2 and E^{rns} glycoproteins after IW3 and IW7 treatment (Krol et al., 2010). We have previously shown that uridine derivatives of 2-deoxy sugars inhibit the glycosylation process at a later step of glycan assembly than tunicamycin. Similarly in this work we did not observe any influence of both compounds on the early ER-dependent glycosylation steps studied in insect cells using constructed recombinant baculovirus vectors expressing influenza HA and NA proteins (data not shown). Our current results confirmed that IW3 and IW7 inhibitors act rather on late Golgi-dependent steps of glycosylation process, as the effect of both compounds on influenza virus glycoproteins accumulation could only be detected in mammalian cells (Fig. 3).

To corroborate the mechanism of anti-influenza virus activity, real-time PCR method was used to test the *in vitro* effect of the drugs on viral replication. No changes in the intracellular RNA level was detected upon short treatment (8 h) with IW3 and IW7, suggesting that both compounds do not affect virus replication during a single round of infection. However, analogous experiments using RNA extracted from the culture medium showed significant reduction in viral RNA indicating that the virus release from the inhibitor-treated cells is most probably impaired due to changes in glycosylation status of viral proteins. This was further confirmed by comparing the infectious virus titers from the same experiment, where significant loss of progeny viruses secreted from infected cells was observed for both compounds (Fig. 4). Finally, we have shown that treatment with IW3 and IW7 for 24 or 48 h p.i., allowing for secondary infections, resulted in the reduction of both secreted and intracellular viral RNA levels, indicating the loss of infectious virus progeny in sequential infection cycles affecting also long-term intracellular accumulation of viral RNA.

The importance of N-glycosylation and N-glycan processing of viral envelope proteins in influenza virus life cycle has been reported previously in many studies (de Vries et al., 2010; Hebert et al., 1997;Ohuchi et al., 1997a, b; Saito and Yamaguchi 2000; Wagner et al., 2000, 2002; Wu et al., 2009). These analyses showed that not only the presence of N-linked glycans is necessary for HA and NA folding, but their number and location are also crucial. Additionally, the receptor-binding specificity of influenza A virus HA was shown to be affected not only by the presence or absence of sialylated glycans, but also by length of glycan chains (de Vries et al., 2010). Roberts and co-workers found that two out of three of the conserved stem domain glycans are required for the correct maturation and transport of HA protein from the fowl plaque virus strain (Roberts et al., 1993). Moreover, it was shown that stem glycans are important for the maintenance of the metastable conformation of HA required for fusion activity (Ohuchi et al., 1997a). Stem glycans of HA were also demonstrated to be important determinants of efficient influenza virus replication (Wagner et al., 2002). Additionally, the importance of tip glycans of HA for virus growth in cell culture was also confirmed (Wagner et al., 2000). Growth of HA mutants lacking either one or both tip N-glycans was significantly restricted, resulting in limited cell-to-cell spread.

Glycosylation was also shown to be crucial for NA-catalyzed removal of sialic acid both from the host cells and viral particles, thus facilitating the release of progeny virus from infected cells and preventing them from self-aggregation. Correct glycosylation is required for the folding of native NA subunits that can further oligomerize into a highly active tetramer (Saito et al., 1995; Wu et al., 2009). Saito and Yamaguchi showed that the inhibition of N-glycosylation impairs cell surface transport of the NA glycoprotein. Consequently, a smaller amount of mature NA reaches the host cell surface, which is the limiting factor for the virus release (Saito and Yamaguchi, 2000). In the light of the presented evidence, our results showing impaired secretion of viral particles from infected cells upon treatment with our two new glycosylation inhibitors are in agreement with the literature data.

In conclusion, the results presented in this paper provide evidence for the efficacy of uridine derivatives of 2-deoxy sugars in inhibiting influenza A virus propagation *in vitro*. The results of the current and previous studies demonstrated that the mechanism of antiviral activity of both IW3 and IW7 inhibitors is related to impaired maturation of viral glycoproteins by blocking the late step of N-glycosylation process. These findings support our idea that targeting glycan composition may be a promising therapeutic option for controlling viral infection and IW3 as well as IW7 inhibitor might be the candidates for use as anti-influenza agents in combination therapy with existing drugs. For this purpose, new analogues of current compounds should be synthesized in an attempt to improve the antiviral activity, and to further reduce the toxicity. Additional study on animal models will be also crucial to address the issue of the systemic toxicity, stability and effectiveness of new compounds.

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References

Asano, N., 2003. Glycosidase inhibitors: update and perspectives on practical use. Glycobiology 13, 93R–104R.
Bourke, C.A., Carrigan, M.J., 1993. Experimental tunicamycin toxicity in cattle, sheep and pigs. Aust. Vet. J. 70, 188–189.
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.
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.
Colman, P., 1998. Structure and function of the neuraminidase. In: Nicholson, K.G., Webster, R.G., Hay, A.J. (Eds.), Textbook of influenza. Blackwell Science, London, England, pp. 65–73.
Chapel, C., Garcia, C., Bartosch, B., Roingeard, P., Zitzmann, N., Cosset, F.-L., Dubuisson, J., Dwek, R.A., Trepo, C., Zoulim, F., Durantel, D., 2007. Reduction of the infectivity of hepatitis C virus pseudoparticles by incorporation of misfolded glycoproteins induced by glucosidase inhibitors. J. Gen. Virol. 88, 1133–1143.
de Vries, R.P., de Vries, E., Bosch, B.J., de Groot, R.J., Rottier, P.J.M., de Haan, C.A.M., 2010. The influenza A virus hemagglutinin glycosylation state effects receptor-binding specifity. Virology 403, 17–25.
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.
Duksin, D., Mahoney, W.C., 1982. Relationship of the structure and biological activity of the natural homologues of tunicamycin. J. Biol. Chem. 257, 3105–3109.
Durantel, D., Branza-Nichita, N., Carrouee-Durantel, S., Butters, T.D., Dwek, R.A., Zitzmann, N., 2001. Study of the mechanism of antiviral action of iminosugar derivatives against bovine viral diarrhea virus. J. Virol. 75, 8987–8998.
Elbein, A.D., 1987. Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. Annu. Rev. Biochem. 56, 497–534.
Ferraris, O., Lina, B., 2008. Mutations of neuraminidase implicated in neuraminidase inhibitors resistance. J. Clin. Virol. 41, 13–19.
Garten, R.J., Davis, C.T., Russell, C.A., Shu, B., Lindstrom, S., Balish, A., Sessions, W.M., Xu, X., Skepner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C.B., Emery, S.L., Hillman, M.J., Rivailler, P., Smagala, J., de Graaf, M., Burke, D.F., Fouchier, R.A., Pappas, C., Alpuche-Aranda, C.M., Lopez-Gatell, H., Olivera, H., Lopez, I., Myers, C.A., Faix, D., Blair, P.J., Yu, C., Keene, K.M., Dotson Jr., P.D., Boxrud, D., Sambol, A.R., Abid, S.H., St George, K., Bannerman, T., Moore,

A.L., Stringer, D.J., Blevins, P., Demmler-Harrison, G.J., Ginsberg, M., Kriner, P., Waterman, S., Smole, S., Guevara, H.F., Belongia, E.A., Clark, P.A., Beatrice, S.T., Donis, R., Katz, J., Finelli, L., Bridges, C.B., Shaw, M., Jernigan, D.B., Uyeki, T.M., Smith, D.J., Klimov, A.I., Cox, N.J., 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 325, 197–201.

Hauge, S.H., Dudman, S., Borgen, K., Lackenby, A., Hungnes, O., 2009. Oseltamivir-resistant influenza viruses A (H1N1), Norway, 2007–08. Emerg. Infect. Dis. 15, 155–162.

Hayden, F.G., 1997. Antivirals for pandemic influenza. J. Infect. Dis. 176, S56–S61.

Hebert, D.N., Zhang, J.-X., Chen, W., Foellmer, B., Helenius, A., 1997. The number and location of glycans on influenza hemagglutinin determine folding and association with calnexin and calreticulin. J. Cell Biol. 139, 613–623.

Hebert, D.N., Foellmer, B., Helenius, A., 1996. Calnexin and calreticulin promote folding, delay oligomerization, and suppress degradation of influenza hemagglutinin in microsomes. EMBO J. 15, 2961–2968.

Hurt, A.C., Deng, Y.M., Ernest, J., Caldwell, N., Leang, L., Iannello, P., Komadina, N., Shaw, R., Smith, D., Dwyer, D.E., Tramontana, A.R., Lin, R.T., Freeman, K., Kelso, A., Barr, I.G., 2011. Oseltamivir-resistant influenza viruses circulating during the first year of the influenza A(H1N1) 2009 pandemic in the Asia-Pacific region, March 2009 to March 2010. Eurosurveillance 16, pii: 19770.

Hurtley, S.M., Bole, D.G., Hoover-Litty, H., Helenius, A., Copeland, C.S., 1989. Interactions of misfolded influenza hemagglutinin with binding protein (BiP). J. Cell Biol. 108, 2117–2126.

Inkster, M.D., Hinshaw, V.S., Schulze, I.T., 1993. The hemagglutinins of duck and human H1 influenza viruses differ in sequence conservation and in glycosylation. J. Virol. 67 (12), 7436–7443.

Kohsaka, S., Mita, K., Matsuyama, M., Mizuno, M., Tsukuda, Y., 1985. Impaired development of rat cerebellum induced by neonatal injection of the glycoprotein synthesis inhibitor, tunicamycin. J. Neurochem. 44, 406–410.

Krol, E., Wandzik, I., Szeja, W., Grynkiewicz, G., Szewczyk, B., 2010. In vitro antiviral activity of some uridine derivatives of 2-deoxy sugars against classical swine fever virus. Antiviral Res. 86, 154–162.

Lazar, C., Durantel, D., Macovei, A., Zitzmann, N., Zoulim, F., Dwek, R.A., Branza-Nichita, N., 2007. Treatment of hepatitis B virus-infected cells with -glucosidase inhibitors results in production of virions with altered molecular composition and infectivity. Antiviral Res. 76, 30–37.

Luscher-Mattli, M., 2000. Influenza chemotherapy: a review of the present state of art and of new drugs in development. Arch. Virol. 145, 2233–2248.

Matrosovich, M., Matrasovich, T., Garten, W., Klenk, H.D., 2006. New low-viscosity overlay medium for viral plaque assays. Virol. J. 3, 63.

Matrosovich, M., Zhou, N., Kawaoka, Y., Webster, R., 1999. The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J. Virol. 73 (2), 1146–1155.

Meijer, A., Lackenby, A., Hungnes, O., Lina, B., van-der-Werf, S., Schweiger, B., Opp, M., Paget, J., van-de-Kassteele, J., Hay, A., Zambon, M., 2009. Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007–08 season. Emerg. Infect. Dis. 15, 552–560.

Miller, M., Viboud, C., Simonsen, L., Olson, D.R., Russell, C., 2009. Mortality and morbidity burden associated with A/H1N1 pdm influenza virus: Who is likely to be infected, experience clinical symptoms, or die from the H1N1 pdm 2009 pandemic virus? PLoSCurr. 1, RRN1013.

Monto, A.S., Fleming, D.M., Henry, D., de Groot, R., Makela, M., Klein, T., Elliott, M., Keene, O.N., Man, C.Y., 1999. Efficacy and safety of the neuraminidase inhibitor zanamivirin the treatment of influenza A and B virus infections. J. Infect. Dis. 180, 254–261.

Nichol, K.L., Treanor, J.J., 2006. Vaccines for seasonal and pandemic influenza. J. Infect. Dis. 194, S111–S118.

Nicholson, K.G., Aoki, F.Y., Osterhaus, A.D., Trottier, S., Carewicz, O., Mercier, C.H., Rode, A., Kinnersley, N., Ward, P., 2000. Efficacy and safety of oseltamivir in treatment of acute influenza: a randomised controlled trial. Neuraminidase Inhibitor Flu Treatment Investigator Group. Lancet 355, 1845–1850.

Ohuchi, R., Ohuchi, M., Garten, W., Klenk, H.-D., 1997a. Oligosaccharides in the stem region maintain the influenza virus hemagglutinin in the metastable form required for fusion activity. J. Virol. 71, 3719–3725.

Ohuchi, M., Ohuchi, R., Feldmann, A., Klenk, H.-D., 1997b. Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. J. Virol. 71, 8377–8384.

Parodi, A.J., 2000. Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. Biochem. J. 348, 1–13.

Plemper, R.K., Wolf, D.H., 1999. Retrograde protein translocation: eradication of secretory proteins in health and disease. Trends Biochem. Sci. 24, 266–270.

Renaud, C., Kuypers, J., Englund, J.A., 2011. Emerging oseltamivir resistance in seasonal and pandemic influenza A/H1N1. J. Clin. Virol. 52, 70–78.

Roberts, P.C., Garten, W., Klenk, H.-D., 1993. Role of conserved glycosylation sites in maturation and transport of influenza A virus hemagglutinin. J. Virol. 67, 3048–3060.

Saito, T., Yamaguchi, I., 2000. Effect of glycosylation and glucose trimming inhibitors on the influenza A virus glycoproteins. J. Vet. Med. Sci. 62, 575–581.

Saito, T., Taylor, G., Webster, R.G., 1995. Steps in maturation of influenza A virus neuraminidase. J. Virol. 69, 5011–5017.

Schulze, I.T., 1997. Effects of glycosylation on the properties and functions of influenza virus hemagglutinin. J. Infect. Dis. 176 (Suppl. 1), S24–S28.

Tamura, D., Mitamura, K., Yamazaki, M., Fujino, M., Nirasawa, M., Kimura, K., Kiso, M., Shimizu, H., Kawakami, C., Hiroi, S., Takahashi, K., Hata, M., Minagawa, H., Kimura, Y., Keneda, S., Sugita, S., Horimoto, T., Sugaya, N., Kawaoka, Y., 2009. Oseltamivir-resistant influenza a viruses circulating in Japan. J. Clin. Microbiol. 47, 1424–1427.

Thorlund, K., Awad, T., Boivin, G., Thabane, L., 2011. Systematic review of influenza resistance to the neuraminidase inhibitors. BMC Infect. Dis. 11, 134.

Trombetta, E.S., 2003. The contribution of N-glycans and their processing in the endoplasmic reticulum to glycoprotein biosynthesis. Glycobiology 13, 77R–91R.

Wagner, R., Heuer, D., Wolff, T., Herwig, A., Klenk, H.-D., 2002. N-glycans attached to the stem domain of haemagglutinin efficiently regulate influenza A virus replication. J. Gen. Virol. 83, 601–609.

Wagner, R., Wolff, T., Herwig, A., Pleschka, S., Klenk, H.-D., 2000. Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. J. Virol. 74, 6316–6323.

Wandzik, I., Bieg, T., 2007. Adducts of uridine and glycals as potential substrates for glycosyltransferases. Bioorg. Chem. 35, 401–416.

Wang, C., Takeuchi, K., Pinto, L.H., Lamb, R.A., 1993. Ion channel activity of influenza A virus M2 protein: characterization of the amantadine block. J. Virol. 67, 5585–5594.

Ward, C.W., Murray, J.M., Roxburgh, C.M., Jackson, D.C., 1983. Chemical and antigenic characterisation of the carbohydrate side chains of an Asian (N2) influenza virus neuraminidase. Virology 126, 370–375.

Wiley, D.C., Skehel, J.J., 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. 56, 365–394.

Wu, Z.L., Ethen, C., Hickey, G.E., Jiang, W., 2009. Active 1918 pandemic flu viral neuraminidase has distinct N-glycan profile and is resistant to trypsin digestion. Biochem. Biophys. Res. Commun. 379, 749–753.