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In vitro antiviral activity of some uridine derivatives of 2-deoxy sugars against classical swine fever virus

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ABSTRACT

Classical swine fever virus glycoproteins: E2, E^{rns} (E0) and E1 are detected on the external part of viral particles and play a major role in the initial stages of viral infection. They form heterodimeric and homodimeric complexes needed to effectively infect host cells. Some glycosylation inhibitors, such as tunicamycin, which act at the early stages of glycan chain processing, can influence, not only glycosylation, but also the stability of E2 and E^{rns} glycoproteins, effectively inhibiting the formation of glycoprotein complexes and virus yield. In our study we tested two of newly designed uridine derivatives of 2-deoxy sugars, IW3 and IW7 mimicking part of tunicamycin. We showed that inhibitors effectively arrest viral growth with IC_{50} of 9 and 7 μg/ml respectively, without significant toxicity for mammalian cells. Moreover, IW3 and IW7 reduced the formation of viral glycoproteins E2 and E^{rns} in a dose-dependent manner. These compounds were further studied in order to elucidate the molecular mechanism of the antiviral effect using mammalian SK6 and insect Sf9 cell lines. We found that they inhibit N-glycosylation process of viral proteins at the late stage of glycan modification characteristic for mammalian cells. Due to the observed antiviral effect accompanied by low cytotoxicity, these inhibitors are potential candidates for anti-pestivirus therapy.

1. Introduction

Classical swine fever virus (CSFV), together with bovine viral diarrhea virus (BVDV) and border disease virus (BDV), belong to the Pestivirus genus in the Flaviviridae family (Becher et al., 2003). CSFV is the causative agent of a highly contagious, economically damaging disease of swine and wild boars (Laevens et al., 1999; Edwards et al., 2000). Several vaccines against CSF are currently available (Greiser-Wilke and Moennig, 2004; Beer et al., 2007). Although vaccines guarantee high protection rates, the inability to differentiate vaccinated from infected pigs, resulted in a ban of vaccination in European Union (EU) (van Oirschot, 2003; Ganges et al., 2008). The non-vaccination policy is now based on the culling of infected animals or those in contact with infected herds (Ganges et al., 2008). Due to the ethical as well as economical considerations, a more acceptable control strategy is required (van Oirschot, 2003). The use of antiviral agents could be a good alternative to prevent transmission of the virus in case of an outbreak.

CSFV is a small, enveloped positive-stranded RNA virus. The CSFV genome contains a single open reading frame coding for a polyprotein of approximately 4000 amino acids, which is processed co- and posttranslationally by host and viral proteases into structural and non-structural proteins. Structural protein region, located in the N-terminal part of the polyprotein, is composed of nucleocapsid protein (C) and three envelope glycoproteins: E^{rns} (E0), E1 and E2. In virions and in infected cells, glycoproteins form disulfide-linked homo- and heterodimers: an E^{rns} homodimer with a size of about 97 kDa, an E1–E2 heterodimer with a size of 75 kDa, and an E2 homodimer with a size of about 100 kDa (Rümenapf et al., 1993). Two envelope proteins: E^{rns} and E2 were reported to induce neutralizing antibodies (Weiland et al., 1990, 1992; König et al., 1995). E^{rns} (E0) is a virus-associated protein but it is also secreted from virus-infected cells as a soluble protein (Rümenapf et al., 1993; Hausmann et al., 2004). Moreover, E^{rns} possesses an

Abbreviations: Asn, asparagine; BDV, border disease virus; BVDV, bovine viral diarrhea virus; CC_{50}, concentration of the compound required to reduce cell viability by 50%; CSF, classical swine fever; CSFV, classical swine fever virus; DMSO, dimethyl sulfoxide; E^{rns} (E0), E2, E1, CSFV envelope proteins; Endo H, endoglycosidase H; ER, endoplasmic reticulum; EU, European Union; FBS, fetal bovine serum; GTs, glycosyltransferases; IC_{50}, concentration of the compound required to reduce virus plaque formation by 50%; IPMA, immunoperoxidase monolayer assay; MOI, multiplicity of infection; NR, neutral red; PBS, phosphate-buffered saline; PNGase F, peptide:N-glycosidase F; PRV, pseudorabies virus; PVDF, Polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Ser, serine; Sf9, *Spodoptera frugiperda* insect cell line; S.I., selectivity index; SK6, swine kidney cells; Thr, threonine; Xaa, any amino acid.

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unusual ribonuclease activity with specificity for uridine residues, and is classified as a member of RNase T2 family (Schneider et al., 1993; Meyers et al., 1999; Langedijk et al., 2002). E2 glycoprotein is essential for virus attachment and entry into target cells as well as for cell tropism (Reimann et al., 2004; Wang et al., 2004). It is the major neutralizing antigen during CSFV infection; due to this fact the recombinant E2 was successfully used for vaccination of animals (van Rijn et al., 1999). Both E^{rns} and E2 glycoproteins are highly glycosylated with 9 and 6 potential glycosylation sites, respectively (Moormann et al., 1990; Montesino et al., 2008).

N-glycosylation is one of the major types of protein modifications, which influences not only the correct folding and stability of many glycoproteins, but also has vital effects on functions such as receptor binding, membrane fusion, and penetration into host cells (Wang et al., 2004; Pande et al., 2005). The 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 growth of sugar chains on a variety of biomacromolecules including glycolipids and glycoproteins is mediated by a large group of enzymes called glycosyltransferases (GTs). These enzymes catalyze the transfer of a sugar moiety from an activated donor sugar (usually UDP-sugar) onto saccharide and non-saccharide acceptors (Breton et al., 2006). One of the major events in N-glycosylation of polypeptide chains is the transfer of an oligosaccharide precursor from a lipid intermediate to an Asn residue (consensus sequence Asn-Xaa-Ser/Thr) in a nascent protein (Duvet et al., 2002). This step can be blocked by the antibiotic tunicamycin (Duksin and Mahoney, 1982; Elbein, 1987). Tunicamycin is in fact a mixture of related compounds consisting of uridine conjugated to the dialdose tunicamine which is linked to a family of fatty acids varying in the degree of unsaturation, length and branching.

Tunicamycin has been often used in in vitro studies, however the severe neurological side effects in vivo prevented the use of this antibiotic as a therapeutic agent (Kohsaka et al., 1985; Bourke and Carrigan, 1993). We have previously shown that the addition of very low amounts of tunicamycin to the CSFV-infected cells led, not only to the deglycosylation of E2 glycoprotein, but also to the complete disappearance of the E2 polypeptide, which resulted in the effective arrest of the spread of the virus (Tyborowska et al., 2007). The unique effect of tunicamycin on E2 glycoprotein prompted our studies on the examination of the effect of a wide array of synthetic compounds mimicking tunicamycin structure (or a part of this structure) on the yield of CSFV.

In this study we demonstrate the in vitro antiviral activity of two novel inhibitors, uridine derivatives of 2-deoxy sugars—IW3 and IW7. We further report their potential mechanism of action using mammalian and insect cell lines thus indicating, that IW3 and IW7 inhibitors are potential candidates in future anti-pestiviral therapy.

2. Materials and methods

2.1. Cells and viruses

Swine kidney cells (SK6) were cultured in Minimum Essential Medium Eagle (E-MEM) (Sigma–Aldrich), supplemented with 8% fetal bovine serum (FBS), at 37 °C under 5% CO_2. The insect cell line *Spodoptera frugiperda* (Sf9) was grown in HyQ-SFX medium (HyClone) at 27 °C.

Classical swine fever virus Cellpest strain (Björklund et al., 1998) and pseudorabies virus (PRV) NIA-3 strain were obtained from the National Veterinary Institute in Pulawy, Poland.

Autographa californica nuclear polyhedrosis virus (AcNPV) recombinants expressing CSFV E^{rns} or E2 glycoproteins were generated using Bac-to-Bac baculovirus expression system of Invitrogen (Luckow et al., 1993) as described by Tyborowska et al. (2007).

2.2. Glycosylation inhibitors

The synthesis of uridine derivatives of 2-deoxy sugars (designated by us IW3 and IW7) was described elsewhere (Wandzik and Bieg, 2007). Stock solutions of IW3 and IW7 were prepared by dissolving the reagent in dimethyl sulfoxide (DMSO). Tunicamycin was purchased from Sigma–Aldrich. Stock solutions were made in DMSO.

2.3. Serological reagents

Monoclonal antibody (MAb V3 39.5) anti-E2 was purchased from Cedi Diagnostic B.V., The Netherlands. Rabbit monospecific polyclonal serum anti-E^{rns} CSFV and anti-gE PRV were obtained by immunizing rabbits with purified glycoproteins according to Szewczyk and Summers, 1998. Anti-β-actin, anti-mouse and anti-rabbit alkaline phosphatase (AP)-conjugated secondary antibodies as well as anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa-Cruz Biotechnology, U.S.A.

2.4. Cell viability assay

To determine SK6 cell viability, either a neutral red (NR) cytotoxicity assay or CellTiter 96 AQ_{ueous} non-radioactive cell proliferation assay (MTS) (Promega) was performed. For neutral red (NR) cytotoxicity test SK6 cells were grown on 12-well plates and incubated in the presence of different concentrations of inhibitors (in triplicate) for 2 days. The cells were incubated with 0.005% Neutral Red Medium for 3 h at 37 °C. The medium was removed, cells were washed with phosphate-buffered saline and the dye was extracted from viable cells using ethanol/acetic acid solution. After shaking, 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%.

SK6 cell viability was also measured using CellTiter 96 AQ_{ueous} non-radioactive cell proliferation assay (MTS) (Promega). SK6 cells were grown and incubated on 96-well plates at 37 °C in the presence of different concentrations of inhibitors (in triplicate) for 2 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. 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%.

To determine the number of viable cells for insect Sf9 cell line, dye-exclusion method with trypan blue was performed. Sf9 cells were grown and incubated on 12-well plates with various concentrations of inhibitors (in triplicate) for 2 days at 27 °C. 0.4% trypan blue solution was added to the suspension of cells, and the number of living and dead cells were counted using an hemocytometer. The cytotoxic concentration 50% (CC_{50}) was calculated as the concentration of inhibitor that reduces cell viability by 50%.

2.5. Plaque reduction and virus yield assay

SK6 cell monolayers in 12-well plates were infected with CSFV for 1 h at 37 °C. After adsorption, unbound virus was removed by washing with serum-free medium and then fresh medium containing increasing amounts of inhibitors was added. After 2 or 3 days, the medium containing secreted virus was collected and centrifuged for virus yield assay. For plaque-reduction assay cells were washed with phosphate-buffered saline (PBS), fixed with

40% acetone in 0.5 × PBS, dried and immunoperoxidase monolayer assay (IPMA) was performed to detect CSFV plaques. Rabbit polyclonal serum anti-E^{rns} diluted 1:800 in PBS containing 1% Tween 20 and 5% FBS was used as primary antibody. Anti-rabbit horseradish peroxidase (HRP)-conjugated antibody was used as secondary antibody (diluted 1:1000 in PBS containing 1% Tween 20 and 5% FBS). CSFV plaques were detected using H_2O_2/AEC (3-amino-9-ethylcarbazole) and counted. IC_{50} was calculated as the concentration at which the number of plaques was reduced by 50% compared to untreated infected control cells.

To determine virus yield, different dilutions of collected medium, obtained as described in a previous paragraph, were used to infect fresh monolayer of SK6 cells in 12-well plates. After 2 or 3 days, the cells were fixed and plaques after IPMA assay were counted as described above.

2.6. SDS-PAGE and Western blot analysis

SK6 cells were grown in E-MEM medium with 2% FBS in 12-wells plates and infected with CSFV (MOI of 0.01, 0.1 or 1 depending on the experiment) or PRV (MOI of 1). After 1 h, the inoculum was removed and the cells were washed with serum-free medium. Fresh medium containing different concentrations of inhibitors was added and incubation was carried out for 24 h for PRV and 48 h for CSFV. Cells were lysed with lysis buffer (20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100) and proteins were separated by SDS-PAGE under reducing or non-reducing conditions and transferred to PVDF membranes. Monoclonal anti-E2 CSFV (MAb V3 39.5; 1:2000 dilution), anti-β-actin (1:1000 dilution), rabbit polyclonal serum anti-E^{rns} CSFV (1:250 dilution) or rabbit polyclonal serum anti-gE PRV (1:200 dilution) were used as primary antibodies. Anti-rabbit alkaline phosphatase (AP)-conjugated antibodies or anti-mouse peroxidase (HRP)-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). In some experiments, antigen–antibody complexes were detected using SuperSignal West Pico Substrate system (Pierce).

Sf9 cells were grown in HyQ medium in 12-wells plates and infected with recombinant baculoviruses expressing E^{rns} or E2 (MOI of 1). Two hours later, the inoculum was removed and replaced with fresh medium containing inhibitors at specified concentrations. At 48 h post-infection, cells were lysed and proteins were separated by SDS-PAGE under non-reducing conditions, and then transferred to PVDF membranes. Monoclonal anti-E2 CSFV antibodies (diluted 1:1000) and rabbit polyclonal serum anti-E^{rns} CSFV (diluted 1:250) were used as primary antibodies. Anti-mouse and anti-rabbit alkaline phosphatase (AP)-conjugated antibodies were used as secondary antibodies (diluted 1:2000). Antigen–antibody complexes were detected using NBT and BCIP as substrates.

2.7. Endo H and PNGase F treatment

Cell lysates (20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100) were boiled for 10 minutes in Glycoprotein Denaturing Buffer (0.5% SDS, 40 mM DTT) (reducing conditions) or left untreated (non-reducing conditions). For endoglycosidase H (Endo H) digestion, sodium citrate (pH 5.5) was added to a final concentration of 50 mM and samples were incubated for 1–5 h at 37 °C with or without Endo H (New England Biolabs). For peptide:N-glycosidase F (PNGase F) treatment, sodium phosphate (pH 7.5) and NP-40 were added to a final concentration of 50 mM and 1%, respectively and samples were incubated for 1–5 h at 37 °C with or without PNGase F (New England Biolabs). The treated samples were separated by SDS-PAGE under reducing or non-reducing conditions and analyzed by immunoblotting.

3. Results

3.1. Antiviral activity of uridine derivatives of 2-deoxy sugars

Tunicamycin prevents N-glycosylation of proteins and has a potent antiviral activity in vitro. However, due to its toxicity, tunicamycin cannot be used as a therapeutic agent. Previously, we reported that tunicamycin had antiviral properties against CSFV in vitro (Tyborowska et al., 2007). Thirty similar compounds mimicking tunicamycin or part of its structure were synthesized and evaluated for their ability to inhibit the propagation of CSF virus. Two uridine derivatives of 2-deoxy sugars—IW3 and IW7 (Fig. 1) were identified as the most selective inhibitors.

The non-cytotoxic concentrations of the compounds were determined using MTS assay and NR cytotoxicity assay. Over 95% of SK6 cells survived the treatment with 20 μg/ml of IW3 and 10 μg/ml of IW7 without apparent changes in cell morphology. The 50% cytotoxic concentrations (CC_{50} values) corresponding to a 50% cytotoxic effect after 48 h of IW3 and IW7 treatment were 86 and 16 μg/ml, respectively (Table 1).

To check the inhibitory effect of the uridine derivatives of 2-deoxy sugars, cells were infected with CSFV at a low MOI of 0.0001 to visualize single plaques. The virus was allowed to adsorb to cells for 1–2 h, and the inoculum was removed and replaced with fresh medium (positive control) or medium containing varying amounts of IW3 or IW7. Incubation was carried out for 2 days and then plaque-reduction assay was performed. CSFV replication is restricted to the cell cytoplasm and does not result in cytopathic effect (Laude, 1987), therefore it was not possible to observe directly the foci of viral growth, which normally, in case of cytopathogenic viruses, are visible as viral plaques. Due to this fact, to visualize the foci caused by CSFV (pseudoplaques), we have usually performed immunoperoxidase monolayer assay (IPMA) which detects the areas of maximum concentration of glycoproteins of the viral outer layer. The immunochemical reagent used in this assay was rabbit polyclonal serum anti-E^{rns}. The typical IPMA reactions for IW3 inhibitor are shown in Fig. 2. The pseudoplaques in infected cells indicate the presence of the virus (Fig. 2B). In IW3 (Fig. 2C–F) as well as in IW7 treated cells (data not shown) the virus propagation decreased in a dose-dependent manner evidenced by the reduction in the number and size of pseudoplaques. The IC_{50} values for IW3 and IW7 were 9 ± 0.15 and 7 ± 0.11 μg/ml, respectively. Both CC_{50} and IC_{50} values were used to calculate the in vitro selectivity index (CC_{50}/IC_{50}) (Table 1).

3.2. Uridine derivatives of 2-deoxy sugars act on the postadsorption step of the CSFV life cycle

To define the stage at which IW3 and IW7 affect the viral life cycle, we performed a series of experiments according to Wu et al. (2002). Inhibitors (IW3 or IW7) were added to the SK6 cells at different time points before, during and after virus adsorption

Table 1
Cytotoxic features and anti-CSFV activities of IW3 and IW7 in SK6 cells.

Compounds	CC_{50} (μg/ml)[a]	IC_{50} (μg/ml)[b]	S.I.[c]
IW3	86	9 ± 0.15	9.5
IW7	16	7 ± 0.11	2.3

[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}).

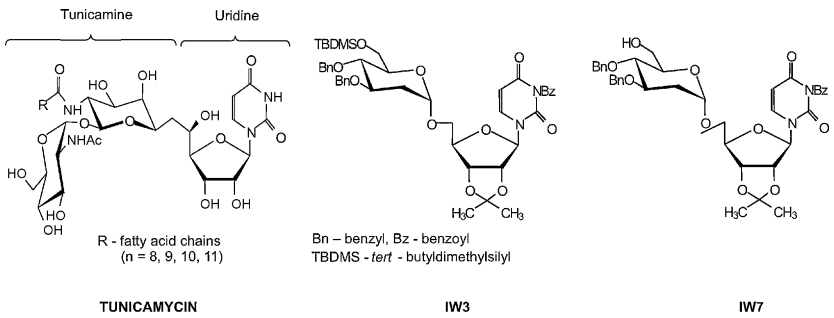

Fig. 1. Chemical structures of tunicamycin and the uridine derivatives of 2-deoxy sugars (IW3 and IW7).

according to the scheme: (I) overnight before virus adsorption, (II) during virus adsorption for 1 h or (III) post-virus adsorption for 3 days. After incubation for the required time in case of I and II, the inhibitors were washed away with fresh medium and cells were grown for 3 days. The amount of infectious virus present in the culture medium was determined by yield assay. IW3 and IW7 inhibitors strongly reduced the propagation of CSFV only when they were added to the cells after, not before or during virus adsorption (Fig. 3). These results suggest that the inhibitors act predominantly on the intracellular steps of the viral replication cycle.

3.3. The influence of uridine derivatives of 2-deoxy sugars on CSFV E^{rns} and E2 glycoprotein synthesis in SK6 cells

IW3 and IW7 inhibitors effectively blocked virus propagation. Therefore, to analyze the effect of IW3 and IW7 on viral glycoproteins synthesis and oligomerization, we performed Western blot analysis after SDS-PAGE in both reducing and non-reducing conditions. E2 glycoprotein in SK6-infected cells is usually found as a homodimer or a heterodimer with E1, while E^{rns} is present only as a homodimer. N-glycan chains have a significant effect on protein folding and in consequence on dimerization process. SK6 cells were infected with CSFV at an MOI of 0.01 for 1 h, and then the medium was changed for fresh medium with decreasing amounts of either IW3 or IW7 inhibitor. IW3 (Fig. 4A, B) and IW7 (Fig. 4C, D) reduced the level of viral E2 glycoprotein synthesis in a dose-dependent manner compared to the untreated samples. After treatment with IW3 (from 50–20 μg/ml) and IW7 (15–10 μg/ml) it was not possible to detect the unglycosylated or underglycosylated forms of E2 viral glycoprotein under both reducing (Fig. 4A, C) and non-reducing (Fig. 4B, D) conditions, indicating that viral glycoprotein synthesis is affected by the presence of both inhibitors. These results confirmed the reduction in viral yield after IW3 and IW7 treatment obtained in a previous experiment (Fig. 3). Similar results were obtained for E^{rns} glycoprotein (data not shown).

The glycoproteins synthesized in cell cultures without the addition of inhibitors and deglycosylated with PNGase F and Endo H were detected in Western blot using the same antibody (Fig. 4E). We concluded that the lack of signal after IW3 and IW7 treatment was not due to the loss of the epitope encompassing glycan chains but by the complete lack of immunoreactive proteins.

3.4. Effect of IW3 and IW7 inhibitors on the synthesis of E2 glycoprotein at different multiplicity of infection

Previous experiments were performed using a low MOI of 0.01. To check the effect of both IW3 and IW7 inhibitors on the synthesis of the viral envelope glycoproteins we increased the MOI in order to obtain a higher level of viral protein expression.

SK6 cells were infected with CSFV at an MOI of 0.01, 0.1 or 1. After virus adsorption, varying amounts of tunicamycin (0.5 and 0.25 μg/ml), IW3 (50 and 20 μg/ml) and IW7 (15 μg/ml) were added to the cells and incubated for 2 days. Tunicamycin, which prevents protein glycosylation, was used as a control. Treatment with 50 μg/ml of IW3 and 15 μg/ml of IW7 inhibitor completely arrested E2 glycoprotein synthesis after infection with CSFV at MOI of 0.01 and 0.1 (Fig. 5). The inhibitors also reduced the level of E2 when an MOI of 1 was tested.

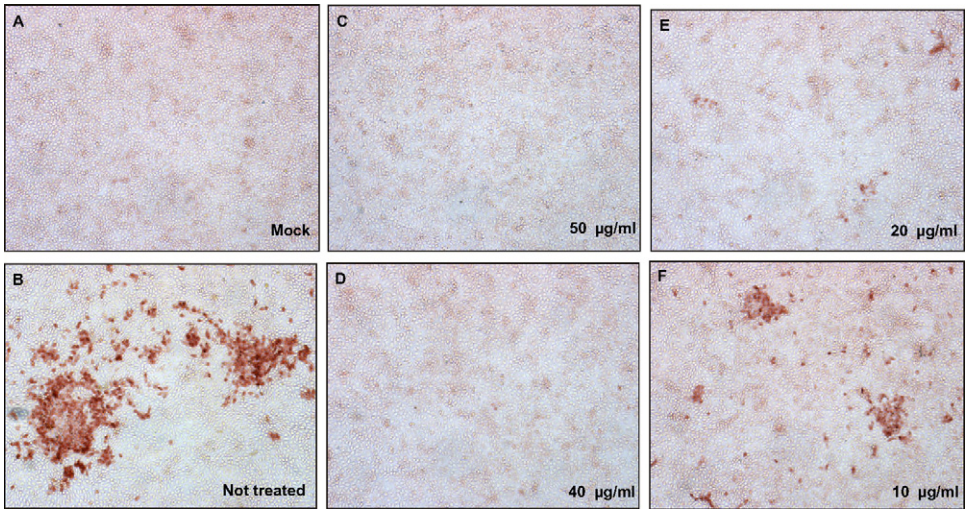

Fig. 2. Immunoperoxidase monolayer assay (IPMA) of CSFV-infected SK6 cells after IW3 treatment. SK6 cells were mock infected (A) or infected with CSFV at an MOI of 0.0001 (B–F). At 2 h p.i., cells were treated with different doses of IW3 (C—50 μg/ml, D—40 μg/ml, E—20 μg/ml, F—10 μg/ml) or left untreated (positive control—B). Two days post-infection, cells were fixed and IPMA was performed using rabbit polyclonal serum anti-E^{rns} to detect CSFV pseudoplaques.

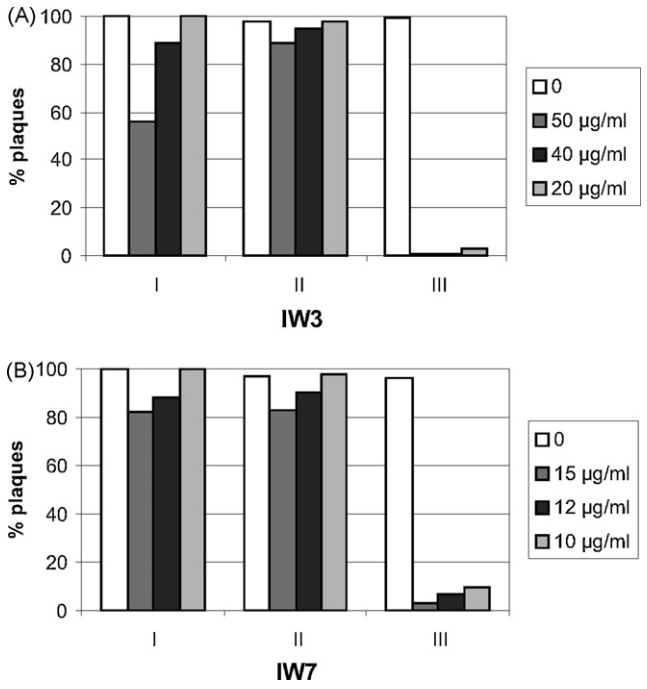

Fig. 3. Uridine derivatives of 2-deoxy sugars act on the post-adsorption step of the CSF virus life cycle. Inhibitor (IW3—A or IW7—B) was added to SK6 cells overnight (O/N) before virus adsorption (I), during virus adsorption for 1 h (II), or post-virus adsorption (III) for 72 h. CSFV was added at an MOI of 0.005 and the infected culture was incubated for 3 days. The culture supernatants were then harvested for virus yield assay.

3.5. Detection of glycosylated forms of PRV gE glycoprotein after IW3 and IW7 treatment

The confirmation of the hypothesis that uridine derivatives of 2-deoxy sugars may change the glycosylation pattern of glycoproteins, called for experiments with some other viruses for whom the effect of tunicamycin was established. For these viruses, glycan-free polypeptides are relatively stable and can be detected by classical protein detection methods (Sabara et al., 1982; Wang et al., 1996; Yoshitake et al., 1997; Nishikawa et al., 1999). For this experiment we chose CSFV-unrelated virus—pseudorabies virus (PRV), a pig virus belonging to Herpesviridae family, very distant from Flaviviridae, but infecting swine kidney (SK6) cells.

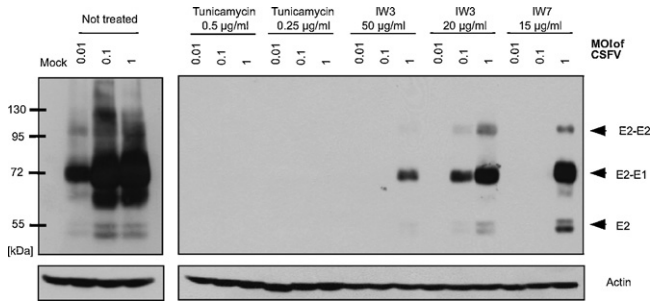

Fig. 5. Synthesis of E2 glycoprotein in the presence of IW3 and IW7 inhibitors at different MOI of CSFV. SK6 cells infected with CSFV at an MOI of 0.01, 0.1 or 1 were treated with various doses of tunicamycin, IW3 or IW7 inhibitor, or left untreated (positive control). Two days post-infection cells were lysed and separated by 10% SDS-PAGE under non-reducing conditions. Proteins were analyzed by Western blot with anti-E2 (V3 39.5) and anti-β-actin monoclonal antibodies. Positions of E2/E2 and E2/E1 complexes and E2 monomer are marked with arrows.

Western blotting under reducing conditions was used to determine the effect of IW3 and IW7 inhibitors on the PRV gE glycoprotein synthesis in SK6 cells. Both IW3 (50 µg/ml) and IW7 (15 µg/ml) inhibitors reduced the molecular mass of PRV gE glycoprotein (Fig. 6), which suggests that the tested inhibitors are true glycosylation inhibitors. However, this reduction in molecular mass is not identical to that caused by tunicamycin which blocks the first steps of core oligosaccharide attachment to a polypeptide chain. These data demonstrate that the inhibition occurs on the later steps of maturation of the growing glycan chains.

3.6. Analysis of N-glycosylation of PRV gE glycoprotein after IW3 and IW7 treatment

To examine whether the forms of PRV glycoprotein gE after IW3 or IW7 treatment still possess high-mannose glycan chains, we performed digestion of glycoproteins from PRV-infected SK6 cells with endoglycosidase H (Endo H). Endo H is a highly specific endoglycosidase, which cleaves asparagine-linked mannose

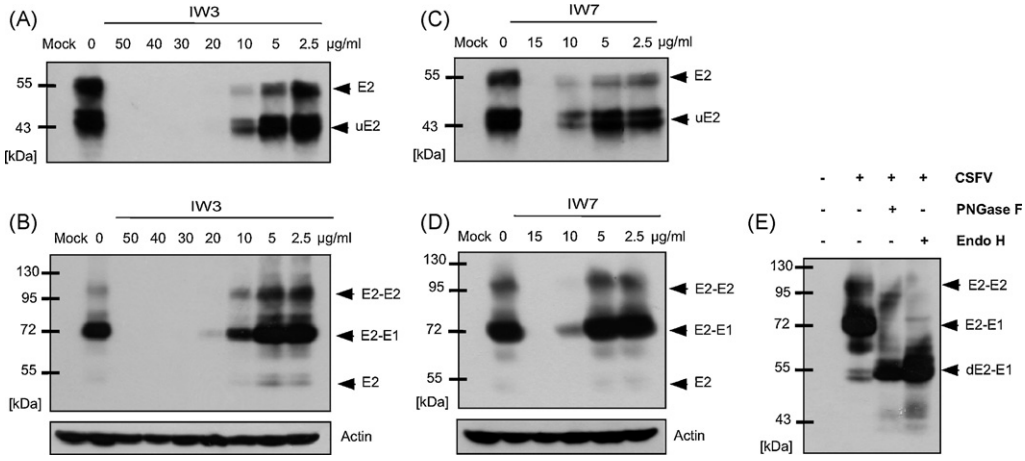

Fig. 4. Effect of uridine derivatives of 2-deoxy sugars on the synthesis of viral E2 glycoprotein. CSFV-infected SK6 cells were treated with various concentrations of IW3 (0–50 µg/ml) (A, B) or IW7 (0–15 µg/ml) (C, D). At 48 h p.i., cells were lysed and proteins were separated by SDS-PAGE (10% polyacrylamide) under reducing (A, C) or non-reducing (B, D) conditions. Western blot analysis was performed using the specific anti-E2 (V3 39.5) and anti-β-actin monoclonal antibodies. Positions of E2/E2 and E2/E1 complexes, E2 and underglycosylated E2 monomers are marked with arrows. Effect of deglycosylating enzymes is shown in panel (E). At 48 h p.i., CSFV-infected SK6 cells were lysed and digested with peptide:N-glycosidase F (PNGase F), endoglycosidase H (Endo H), or left untreated. Samples were separated by SDS-PAGE (10% polyacrylamide) under non-reducing conditions. Western blot analysis was performed using the anti-E2 (V3 39.5) monoclonal antibody. Positions of E2/E2, E2/E1 and deglycosylated E2/E1 complexes are marked with arrows.

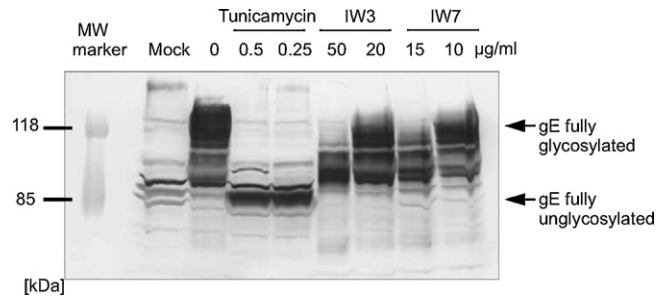

Fig. 6. Detection of under-glycosylated forms of PRV gE glycoprotein after IW3 and IW7 treatment. SK6 cells were mock infected or infected with PRV at an MOI of 1 for 24 h in the absence or presence of tunicamycin, IW3 or IW7. Cells were lysed and proteins were separated by SDS-PAGE (10% polyacrylamide) under reducing conditions. Western blot analysis was performed using the specific anti-gE rabbit serum. MW marker—molecular weight marker.

rich oligosaccharides, but not matured glycans which are trimmed within the Golgi to complex structures. We used tunicamycin treatment as a control for gE unglycosylated form. This analysis showed that the forms of gE after IW3 (50 μg/ml) and IW7 (15 μg/ml) treatment are sensitive to Endo H digestion indicating that the N-glycan chains are predominantly of a high-mannose type (Fig. 7). However, the digestion patterns generated by Endo H and tunicamycin treatment were not identical. The gE form after digestion with Endo H was of a lower-molecular-mass than the gE form treated with tunicamycin.

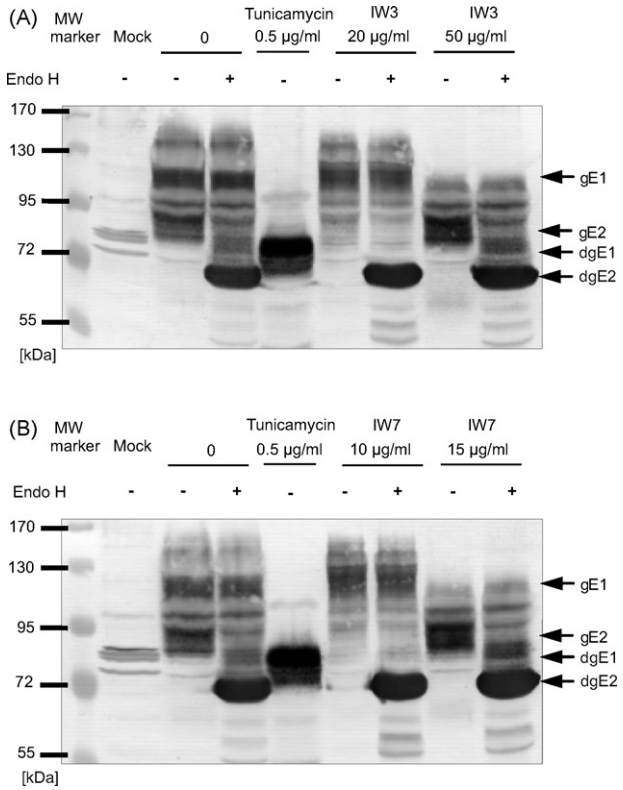

Fig. 7. Biochemical analysis of N-glycan chains on PRV gE glycoprotein after IW3 and IW7 treatment. SK6 cells were mock infected or infected with PRV at an MOI of 1 for 24 h in the absence or presence of tunicamycin, IW3 (A) or IW7 (B). Cell lysates were treated with endoglycosidase H (Endo H) (+) or left untreated (−). Samples were separated by SDS-PAGE (10% polyacrylamide) under reducing conditions. Western blot analysis was performed using the specific anti-gE rabbit serum. gE isoforms are marked as follows: gE1—fully glycosylated form of gE; gE2—partially glycosylated form of gE after IW3 or IW7 treatment; dgE1—deglycosylated form of gE after tunicamycin treatment; dgE2—deglycosylated form of gE after Endo H treatment. MW marker—molecular weight marker.

3.7. Uridine derivatives of 2-deoxy sugars do not change glycosylation pattern of recombinant CSFV E^{rns} and E2 in insect Sf9 cells

To get better insight into the mechanism of action of uridine derivatives of 2-deoxy sugars we performed experiment which were aimed at the explanation whether the inhibitors act on the late, Golgi-dependent, stages of glycosylation (which are unique for mammalian cells), or on earlier steps taking place in the endoplasmic reticulum (ER) as is the case of insect cells (Fig. 8A). To this end, we have checked the effect of IW3 and IW7 on CSFV recombinant glycoproteins expressed in insect cells. In these cells, the glycosylation is terminated at the stage of addition of mannoses in the ER. To resolve experimentally whether the glycosylation in insect cells is affected, Sf9 cells were infected with recombinant baculoviruses containing genes coding for E2 or E^{rns} glycoproteins. Two hours post-infection, cells were treated with a decreasing concentration of IW3 (40, 30 and 20 μg/ml) or IW7 (17, 15 and 10 μg/ml) for 48 h. At the concentrations used, no toxicity was observed in Sf9 cells, as determined by the trypan blue method. The CC_{50} of IW3 and IW7 was 68 and 26 μg/ml, respectively. As a control we used tunicamycin (2 and 1 μg/ml) which blocks the early stages of N-glycosylation and as it was shown previously it led to the complete disappearance of E2 and E^{rns} glycoproteins in soluble (cytoplasmic) fraction expressed in insect cells (Tyborowska et al., 2007). As we could not detect changes in the glycosylation pattern of both E2 (Fig. 8B) and E^{rns} (Fig. 8C) glycoproteins produced in the presence of IW3 and IW7 inhibitors, we concluded that both inhibitors act specifically only on glycan chains processed in mammalian cells in Golgi-dependent processes.

4. Discussion

In this report, we demonstrate that two novel uridine derivatives of 2-deoxy sugars—IW3 and IW7, synthesized by us, possess a significant antiviral activity against classical swine fever virus and exhibit low toxicity for swine kidney cells. Treatment of CSFV-infected SK6 cells with IW3 and IW7 results in a dose-dependent reduction in the amount of infectious virus (yield assay) as well as in the synthesis of envelope glycoprotein E2. These compounds are derivatives of tunicamycin and similarly to this antibiotic affect protein glycosylation, but they are significantly less toxic.

N-glycosylation and N-glycan processing of viral envelope proteins play a vital role in correct folding, stabilization of conformations, intracellular trafficking as well as in conferring resistance to protein degradation and recognition by the immune system (Parodi, 2000; Trombetta, 2003; Scanlan et al., 2007). Therefore it is not surprising that the arrest or alterations of the glycosylation process of viral proteins by different inhibitors usually result in antiviral effects (Durantel et al., 2001; Asano, 2003; Chapel et al., 2007; Lazar et al., 2007).

The important role of outer envelope glycoproteins and their N-glycan chains in the maturation and survival of CSFV in mammalian cells was reported previously (Wang et al., 2004; Pande et al., 2005). As it was shown, the CSF virus mutants missing partial or complete E2 gene were unable to generate viable virus (van Gennip et al., 2002). Also though, a single mutations of putative glycosylation sites of E2 (Risatti et al., 2007) and E^{rns} (Sainz et al., 2008) did not affect in vitro and in vivo replication, the multiple site mutations rendered non-viable viruses. Similar effect of N-glycosylation inhibition on the growth of related BVDV was also described (Pande et al., 2005). Our observations described in this report confirm the fact that after inhibition of glycosylation, the production of viral progeny is no longer possible.

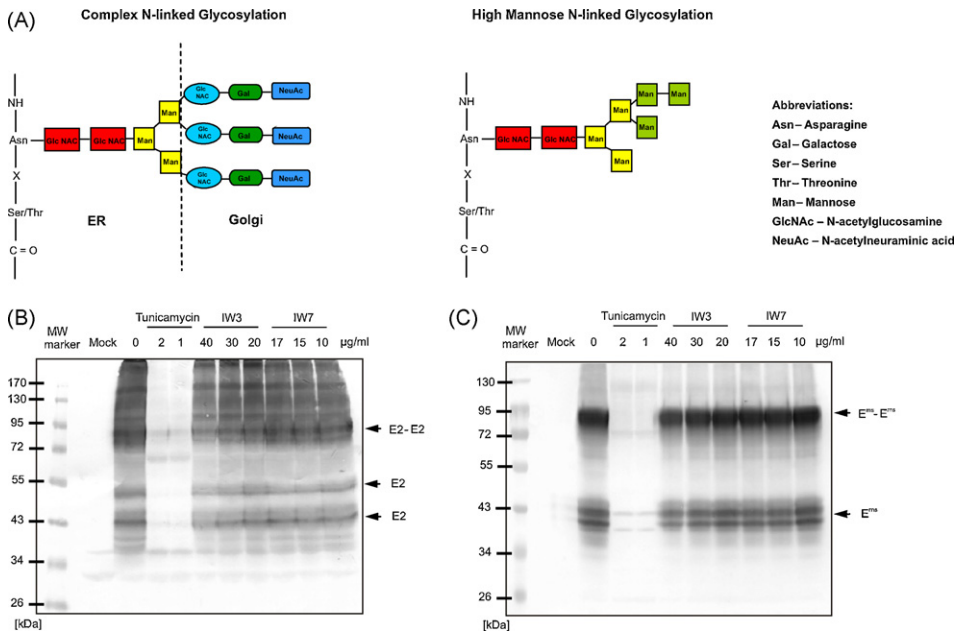

Fig. 8. The influence of uridine derivatives of 2-deoxy sugars on E2 and E^{rns} glycoprotein synthesis in insect Sf9 cells. Schematic representation of typical structures of complex N-glycans (mammalian cells) and high-mannose N-glycans (insect cells) (A). Sf9 cells were infected with recombinant baculovirus expressing CSFV E2 (B) or E^{rns} (C) glycoprotein at an MOI of 1 or mock infected. At 2 h p.i., cells were treated with tunicamycin, IW3 or IW7 inhibitor or left untreated. At 48 h p.i., cells were lysed and proteins were separated by SDS-PAGE under non-reducing conditions. Western blot analysis was performed using monoclonal anti-E2 antibody (V3 39.5) (B) or rabbit polyclonal serum anti-E^{rns} (C). Positions of E2/E2 and E^{rns}/E^{rns} complexes and E^{rns} and E2 monomers (fully and low-glycosylated) are marked with arrows. The bands above E2/E2 dimers represent higher molecular aggregates of E2 which are formed in non-reducing conditions. MW marker—molecular weight marker.

The arrest of glycosylation at the very early stage of transfer of 14-residue oligosaccharide core unit to the nascent glycan-free polypeptide backbone is frequently used in viral research laboratories for the determination of the molecular mass of polypeptide chains of glycoproteins. Most often it is performed by adding antibiotic tunicamycin to the cell culture. Tunicamycin, though very toxic to vertebrate cells, does not kill them quickly and allows for the accumulation of glycan-free polypeptides in infected cells as it was shown for many viruses such as rotavirus (Sabara et al., 1982), hepatitis delta virus (Wang et al., 1996), canine herpesvirus (Nishikawa et al., 1999) and bovine herpesvirus-1 (Yoshitake et al., 1997). In a similar experiment performed for CSFV E2 and E^{rns} glycoproteins, we could not detect deglycosylated polypeptide chains by Western blotting even when high MOIs of CSFV were used (Tyborowska et al., 2007). This was a strong indication that glycoproteins of CSFV differ from the outer envelope glycoproteins of other viruses in one, very important property—the removal of their glycan chains leads to very fast, complete disappearance of outer envelope polypeptides. This characteristic, differential effect of tunicamycin on CSFV glycoproteins, and hence on viral growth inhibition, convinced us to continue the investigations on the effect of N-glycosylation inhibitors in a much thorough way.

Fig. 8A represents exemplary structures of N-glycan chains present in mammalian and insect cells. Mammalian glycoproteins contain highly branched structures which are composed of mannose, galactose, N-acetylglucosamine and sialic acids. N-glycosylation in insect cells is simpler; it is dependent on the protein and the host cell line but it is generally of the high-mannose type with additional fucose residues often attached to the core N-acetyl glucosamine (Altmann and März, 1995; Jarvis and Finn, 1995). Tunicamycin acts on the first step of glycosylation process specifically blocking the attachment of the whole glycan chain. We have expected that its structural analogs synthesized by us would act on the same stage. However, neither IW3 nor IW7 acts on the attachment step (Fig. 6), though the net effect of both inhibitors and tunicamycin on CSFV glycoproteins is the same—the disappearance of glycoproteins bands on Western blots (Fig. 4) and drastic drop in viral yield (Fig. 3) even when high MOIs of CSFV were used (Fig. 5).

The possibility that the unglycosylated E2 glycoprotein is not detected by V3 39.5 anti-E2 antibody, was excluded by deglycosylation with PNGase F and Endo H treatment (Fig. 4E). However, the reason for the lack of unglycosylated forms of E2 and E^{rns} is not clear. This may be due to the fact that the tested inhibitors, structurally related to tunicamycin, may exert a similar function. Tunicamycin is known as an endoplasmic reticulum (ER) stress inducer that triggers the accumulation of unfolded proteins in ER (Schröder, 2008). The proteins that do not pass the ER quality control are transported back to the cytosol, where they are degraded by the proteosome system in a process known as ER-associated degradation (Plemper and Wolf, 1999). This effect was also shown for HCV (Pavio et al., 2001). By real-time RT-PCR for IW3 inhibitor (data not shown) we have excluded the possibility that the reduction of viral progeny yield is related to the inhibition of replication of viral genetic material.

Using Pseudorabies virus we confirmed that N-glycosylation of glycoproteins in the presence of IW3 or IW7 is inhibited but it occurs at later stages than the step where tunicamycin is involved because we observed the accumulation of molecules of larger molecular mass than glycan-free polypeptide chains (Fig. 6). Our results indicate that N-glycans of glycoprotein gE of PRV synthesized in the presence of IW3 and IW7 are Endo H sensitive, therefore they represent high-mannose type of oligosaccharides (detectable and often functional for most of viruses but rapidly degraded for CSFV) (Fig. 7). However, we noticed that the gE form after digestion with Endo H was of a lower-molecular-mass than the gE form after tunicamycin treatment, which was used as a control for gE unglycosylated form. The observed difference in the electrophoretic mobilities between the gE proteins deglycosylated enzymatically by Endo H and synthesized unglycosylated in the presence of tunicamycin may be due to differences in the protein folding possibly caused by initial presence (Endo H) or absence (tunicamycin) of glycan chains.

The results obtained for CSFV glycoproteins in insect cell culture suggest that IW3 and IW7 act on Golgi-dependent process of glycosylation (Fig. 8B, C). The conversion of high-mannose glycans to complex forms occurs in the medial and trans-Golgi compartments of Golgi apparatus (Kornfeld and Kornfeld, 1985) therefore we can conclude that both IW3 and IW7 inhibitors act on glycosylation steps, which occur in cis-Golgi or very early in medial-Golgi compartments. This hypothesis requires experimental verification by cleavage of glycans from the polypeptide backbone and glycan structural analysis.

In summary, the results presented in this paper provide evidence for the efficacy of N-glycosylation inhibitors mimicking parts of tunicamycin structure in arresting CSFV viral growth. Although both IW3 and IW7 inhibitors are active against CSFV in vitro, further experiments are needed for testing whether these compounds can be used as safe antiviral agents. It is also of vital importance to check whether the results described in this article can be extrapolated to other members of Flaviviridae family, with the special emphasis on human hepatitis C virus.

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