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Synthesis and antiviral activity of a novel glycosyl sulfoxide against classical swine fever virus

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ABSTRACT

A novel compound—2″,3″,4″,6″-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2′,3′,6′-tri-*O*-acetyl-1-thio-β-D-glucopyranosyl-(5-nitro-2-pyridyl) sulfoxide—designated GP6 was synthesized and assayed for cytotoxicity and in vitro antiviral properties against classical swine fever virus (CSFV) in this study. We showed that the examined compound effectively arrested CSFV growth in swine kidney cells (SK6) at a 50% inhibitory concentration (IC_{50}) of 5 ± 0.12 μg/ml without significant toxicity for mammalian cells. Moreover, GP6 reduced the viral E2 and E^{rns} glycoproteins expression in a dose-dependent manner. We have excluded the possibility that the inhibitor acts at the replication step of virus life cycle as assessed by monitoring of RNA level in cells and culture medium of SK6 cells after single round of infection as a function of GP6 treatment. Using recombinant E^{rns} and E2 proteins of classical swine fever virus produced in baculovirus expression system we have demonstrated that GP6 did not influence glycoprotein production and maturation in insect cells. In contrast to mammalian glycosylation pathway, insect cells support only the ER-dependent early steps of this process. Therefore, we concluded that the late steps of glycosylation process are probably the main targets of GP6. Due to the observed antiviral effect accompanied by low cytotoxicity, this inhibitor represents potential candidate for the development of antiviral agents for anti-flavivirus therapy. Further experiments are needed for investigating whether this compound can be used as a safe antiviral agent against other viruses from unrelated groups.

1. Introduction

Classical swine fever virus (CSFV), belongs to the family of Flaviviridae, genus Pestivirus.[1] CSFV is the causative agent of classical swine fever (CSF) and can cause an acute, highly infectious and economically damaging disease in swine and wild boars.[2,3] Several vaccines against CSF have been developed. These are live attenuated vaccines, subunit marker vaccines or DNA vaccines. Each of these vaccines have some disadvantages. Vaccination with conventional vaccines does not allow for discrimination between vaccinated and infected pigs and is banned in the European Union, others are less potent and more expensive.[4–6] CSF is present in many parts of the world, however since the 1980s, it is eradicated from the domestic pig population in most countries of the European Union. Nevertheless, the virus is still circulating in some populations of wild boars and the pig population is continuously at risk for virus introduction. Present outbreaks are controlled by culling of infected animals or those in contact with infected herds and the restriction of animal movements which are very costly and ethically questionable. Therefore, new strategies have to be implemented to control CSF. Few drugs against CSFV infections have been developed recently.[7–9] The use of antiviral agents could be a good control strategy to prevent transmission of the virus in case of an outbreak.

CSFV is an enveloped virus with a single-stranded positive sense RNA genome of 12.5 kb that contains a single open reading frame

Abbreviations: CC_{50}, concentration of the compound required to reduce cell viability by 50%; CSF, classical swine fever; CSFV, classical swine fever virus; Ct, cycle threshold; ER, endoplasmic reticulum; GTs, glycosyltransferases; IC_{50}, concentration of the compound required to reduce virus plaque formation by 50%; IPMA, immunoperoxidase monolayer assay; MOI, multiplicity of infection; PRV, pseudorabies virus; Sf9, *Spodoptera frugiperda* insect cell line; SD, standard deviations; S.I., selectivity index; SK6, swine kidney cells.

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encoding a polyprotein of approximately 4000 amino acids. The polyprotein is processed into mature structural and non-structural proteins by host and viral proteases. Structural components of the virion include the capsid (C) protein and three envelope glycoproteins: E^{rns}, E1 and E2.[10] E^{rns} and E2 glycoproteins are present in virions as disulfide-linked homodimers: an E^{rns} homodimer with a size of about 97 kDa and an E2 homodimer with a size of about 100 kDa. E2 is also present as a heterodimer with E1 (75 kDa).[11] Both E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain and a C-terminal anchor.[10] E^{rns} lacks a typical membrane anchor. Apart from being a virion protein, E^{rns} is secreted from virus-infected cells as a soluble protein.[11,12] Moreover, E^{rns} has RNase activity, which is the unique feature for a viral surface protein, and is classified as a member of RNase T2 family.[13,14] E2 glycoprotein is essential for virus attachment and entry into target cells as well as for cell tropism.[15,16] It is the major immunodominant protein inducing the production of neutralizing antibodies and protection against lethal challenge.[17] Both E^{rns} and E2 glycoproteins are highly glycosylated with 7 and 6 glycosylation sites, respectively.[18,19]

N-glycans of enveloped virus glycoproteins have been shown to be important not only for the correct folding and stability, but also for various functions such as host cell receptor binding, membrane fusion, penetration into host cells.[16,20] Lack of N-glycan chains can lead to protein misfolding causing aggregation and protein retention in the endoplasmic reticulum or proteasome degradation.[21,22] It has been observed that changes in the glycosylation patterns of viral glycoproteins can influence infectivity, virulence and host immune response.

Glycosyltransferases (GTs) are a large class of enzymes involved in the biosynthesis of oligosaccharides, polysaccharides and glycoconjugates, such as glycoproteins and glycolipids.[23] GTs catalyze glycosidic bond formation by the transfer of a saccharide, typically a monosaccharide, from an activated nucleotide sugar donor to an acceptor substrate.[24] GTs are involved in many fundamental biological processes and modulation of their activities by efficient inhibitors has potential for the control of certain cellular functions. Since the 3D structures of several GTs have been proposed, a large number of potent inhibitors have been identified. Structures of these compounds are based on analogies between donor substrates, acceptor substrates as well as transition state.

Glycosyl sulfoxides have been found to possess a wide application in the field of complex oligosaccharides synthesis as powerful glycosyl donors.[25] Many chiral sulfoxides were reported to exhibit broad spectrum biological activities including inhibition of many different enzymes.[26–28] According to the literature phenyl sulfinyl β-D-galactopyranosides were cleaved in the diastereoselective manner in the presence of β-galactosidase from *Escherichia coli*. It could be explained in such a way that only one diastereoisomer orients its sulfinyl oxygen toward the activating amino acid of enzyme whilst second diastereoisomer behaves as inhibitor.[29] On the other hand, heterocyclic sulfoxides and sulfones proved to be 10–100 fold more potent inhibitor of fatty acid amide hydrolase (FAAH) than corresponding sulfides.[28] Also polyhydroxylated cyclohexenyl sulfoxides demonstrated weak abilities to the inhibition of α-D-glucosidase from Brewers yeast and β-D-glucosidase from sweet almonds.[27]

On the basis of these data we decided to focus our attention on sulfoxides derivatives of 5-nitro-2-pyridyl 1-thioglycosides as potential GTs inhibitors. Most of GTs are usually metal ion dependent, where manganese is the most typical metal found in active sites.[30,31] We expected, that heteroaryl sulfoxides, due to the presence of free electron pairs on sulfinyl oxygen and heterocyclic nitrogen, could favourably interact with Mn^{2+} similarly to natural GTs substrates.

Therefore, a number of glycosyl sufoxides containing different sugar moieties (D-glucose, D-galactose or lactose with ester or ether protecting groups) and varying aromatic substituents at sulfur atom were synthesized in order to evaluate their potential biological activity. Here, we describe the synthesis and the in vitro antiviral evaluation of a lead compound—designated GP6, which was found to have significant antiviral activity and the lowest toxicity out of all synthesized compounds. We further investigated the potential mechanism of action by which GP6 exerts its anti-CSFV activity. Further experiments are needed for investigating whether this compound can be used as a safe antiviral agent against other viruses from Flaviviridae family and other viral families.

2. Materials and methods

2.1. Chemistry

2.1.1. General methods

Melting points were determined on a SRS OptiMelt melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO P2000 polarimeter using sodium lamp (589 nm) at room temperature. Mass spectra were recorded with a WATERS LCT Premier XE system (high resolution mass spectrometer with TOF analyzer) using electrospray-ionization (ESI) technique. FT-IR spectra were recorded using ATR method with a Thermo Scientific NICOLET 6700 spectrophotometer. UV–vis spectra were recorded with a JASCO V-650 spectrophotometer with the use of 1.0 cm quartz cell and methyl alcohol as a solvent. NMR spectra were recorded on an Agilent spectrometer (400 MHz). Deuterochloroform 98.8% (code 209561000) of isotopic purity (ACROS) with 0.03 v/v% TMS as internal standard was used as a solvent. Column chromatography was performed on silica gel 60 (Merck; 70–230 mesh, code 1.07734.5000) column. All compounds were routinely checked by TLC by using aluminium-baked silica gel plates (Merck TLC Silica gel 60 F_{254}, code 1.05554.0001). Developed plates were visualized by UV light and by charring after spraying with 10% H_2SO_4 in EtOH. Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of solutions after reactions, extractions and column chromatography involved the use of rotary evaporator (Heidolph, Germany) operating at a reduced pressure (ca. 40 Torr). Organic solutions were dried over anhydrous magnesium sulfate (POCH Gliwice, Poland).

2.1.2. 2″,3″,4″,6″-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2′,3′,6′-tri-*O*-acetyl-1-thio-β-D-glucopyranosyl-(5-nitro-2-pyridyl) sulfoxide GP6

To a solution of (5-nitro-2-pyridyl) per-*O*-acetyl-1-thio-β-D-lactoside (1.4 mmol) in a dry dichloromethane (30 mL) cooled down to 0 °C the *m*-CPBA (1.4 mmol) was added. The reaction mixture was stirred at 0 °C and was monitored by TLC. The reaction was stopped without full conversion of 1-thioglycoside in order to limiting creating the sulfone. The reaction mixture was diluted with CH_2Cl_2 and washed with water (3 × 15 mL). The organic layer was dried over anhydrous $MgSO_4$. The solid was filtered off and the filtrate was concentrated to give crude product which was purified by a column chromatography (toluene/AcOEt 20:1 to 2:1 [v/v]) to give diastereoisomeric mixture of sulfoxides GP6 (yield 59% or 68% when calculated on utilized 1-thioglycoside). The CPBA oxidation of the sulfide substrate gave approximately equal amounts of epimeric sulfoxides and no attempt was made to control its stereoselectivity. Small amount (approximately 15 mg) of each diastereoisomer was isolated by repeated column chromatography and characterized separately.

Less polar, dextrorotatory sulfoxide had: mp 93–98 °C (with decomposition); $[\alpha]_D^{20} = 7.5^\circ$ ($CHCl_3$, $c = 0.4$); IR (ATR method) ν 1743 (C=O), 1361 (NO_2), 1213 (C–O), 1043 (S=O) cm^{-1}; UV–vis (MeOH) λ_{max} 204.8, 237.5 and 291 nm; ^1H NMR ($CDCl_3$, 400 MHz)

δ 1.96, 2.02, 2.04, 2.05, 2.06, 2.08, 2.14 (7s, 21H, CH_3CO), 3.74 (m, 1H, H5′), 3.79.(dd∼t, J = 9.8 Hz, 1H, H4′), 3.86 (m, 1H, H5″), 4.03–4.16 (m, 3H, H6′a, H6″a, H6″b), 4.48 (d, J = 7.8 Hz, 1H, H1″), 4.54 (dd, J = 1.6, 12.5 Hz, 1H, H6′b), 4.96 (dd, J = 3.5, 10.5 Hz, 1H, H3″), 5.03 (d, J = 9.8 Hz, 1H, H1′), 5.09 (dd, J = 7.8, 10.5 Hz, 1H, H2″), 5.22 (dd∼t, J = 8.6 Hz, 1H, H3′), 5.34 (d, J = 3.5 Hz, 1H, H4″), 5.48 (dd∼t, J = 9.8 Hz, 1H, H2′), 8.11 (d, J = 8.6 Hz, 1H, $H3_{pyr}$), 8.71 (dd, J = 2.4, 8.6 Hz, 1H, $H4_{pyr}$), 9.47 (d, J = 2.4 Hz, 1H, $H6_{pyr}$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.43, 20.46, 20.57, 20.59, 20.67, 20.69 (CH_3CO), 60.74, 61.42 (C6′, C6″), 66.09, 66.56, 69.06, 70.75, 70.85, 74.00, 75.08, 77.74 (C2′, C2″, C3′, C3″, C4′, C4″, C5′, C5″), 94.38 (C1′), 101.04 (C1″), 121.41, 132.65, 144.52, 144.86, 168.13 ($C2_{pyr}$, $C3_{pyr}$, $C4_{pyr}$, $C5_{pyr}$, $C6_{pyr}$), 169.05, 169.30, 169.96, 170.02, 170.04, 170.07, 170.35 (C═O).

The sulfoxide eluting second had: mp 91–96 °C (with decomposition); $[\alpha]_D^{20} = -59.3^\circ$ ($CHCl_3$, c = 0.5); IR (ATR method) ν 1742 (C═O), 1366 (NO_2), 1211 (C–O), 1043 (S═O) cm^{-1}; UV–vis (MeOH) λ_{max} 204.2, 238.8 and 287 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.88, 1.95, 1.97, 2.08, 2.09, 2.14, 2.15 (7s, 21H, CH_3CO), 3.45 (ddd, J = 1.8, 5.5, 9.8 Hz, 1H, H5′), 3.78.(dd∼t, J = 9.6 Hz, 1H, H4′), 3.82 (dd, J = 3.8, 12.3 Hz, 1H, H6′a), 3.68 (m, 1H, H5″), 4.04–4.18 (m, 2H, H6″a, H6″b), 4.27 (dd, J = 1.8, 12.2 Hz, 1H, H6′b), 4.47 (d, J = 7.8 Hz, 1H, H1″), 4.61 (d, J = 10.1 Hz, 1H, H1′), 4.95 (dd, J = 3.4, 10.4 Hz, 1H, H3″), 5.07 (dd, J = 7.8, 10.4 Hz, 1H, H2″), 5.30–5.40 (m, 2H, H-4″, H3′), 5.47 (dd∼t, J = 9.5 Hz, 1H, H-2′), 8.18 (d, J = 8.6 Hz, 1H, $H3_{pyr}$), 8.63 (dd, J = 2.4, J = 8.6 Hz, 1H, $H4_{pyr}$), 9.41 (d, J = 2.4 Hz, 1H, $H6_{pyr}$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.42, 20.47, 20.55, 20.59, 20.69 (CH_3CO), 60.88, 60.95 (C6′, C6″), 66.62, 66.81, 69.06, 70.81, 70.83, 73.52, 75.41, 77.59 (C2′, C2″, C3′, C3″, C4′, C4″, C5′, C5″), 90.75 (C1′), 101.02 (C1″), 122.92, 132.56, 144.55, 144.72, 167.86 ($C2_{pyr}$, $C3_{pyr}$, $C4_{pyr}$, $C5_{pyr}$, $C6_{pyr}$), 168.92, 169.08, 169.57, 169.83, 169.96, 170.03, 170.32 (C═O); HR-MS (ESI): $[M+H]^+$ calcd for $C_{31}H_{38}N_2O_{20}S$: 791.1811, found 791.1817; $[M+Na]^+$ calcd for $C_{31}H_{38}N_2O_{20}SNa$: 813.1631, found 813.1638.

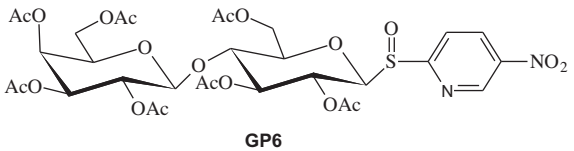
2.2. Biological evaluation

2.2.1. Antiviral compounds

The glycosyl sulfoxide (Fig. 1–designated by us GP6) was synthesized as described in this communication. Stock solutions of GP6 were prepared by dissolving the reagent 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.

2.2.2. Cells and viruses

Swine kidney cells (SK6) were cultured in Eagle's Minimum Essential Medium (E-MEM) (Sigma–Aldrich, USA), 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, USA) at 27 °C. Classical swine fever virus Cellpest strain[32] 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[33] as described previously.[34]



GP6

Figure 1. Chemical structure of GP6.

2.2.3. Cell viability assay

SK6 cell viability was measured by CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay (MTS) (Promega, USA) as described previously.[35] 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 according to the procedure described previously.[35] The cytotoxic concentration 50% (CC_{50}) was calculated as the concentration of inhibitor that reduces cell viability by 50%.

2.2.4. Evaluation of antiviral activity against CSFV

Antiviral activity was evaluated in plaque reduction assay by methods reported previously.[35] Briefly, SK6 cell monolayers in 6 or 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. Next fresh medium containing increasing amounts of inhibitor was added. After 2 or 3 days, the medium containing secreted virus was collected, centrifuged at low speed to remove cellular debris and used 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 (Santa-Cruz Biotechnology, USA) 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 3 days, the cells were fixed and plaques after IPMA assay were counted as described above.

2.2.5. 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 at an MOI of 0.01 or PRV at an 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 inhibitor was added and incubation was carried out for 24 h for PRV and 48 h for CSFV. 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) 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; Cedi Diagnostic B.V., The Netherlands; 1:2000 dilution), anti-β-actin (Santa-Cruz Biotechnology, USA; 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 (Santa-Cruz Biotechnology, USA) 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, USA).

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 inhibitor 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 antibody (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.

2.2.6. Viral RNA isolation and cDNA synthesis

SK6 cells cultured in 12-wells plates were infected with CSFV 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 medium containing inhibitor at the concentrations indicated was added and incubation was carried out for 12 h for cells infected with CSFV at an MOI of 0.1 and 48 h for cells infected with CSFV at an MOI of 0.01. At 12 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 CSFV-infected cells. Total intracellular and secreted CSFV RNA was purified using the Total RNA Mini purification kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. RNA extracted from SK6 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 [20 U], dNTPs [1 mM each], DTT [5 mM] and reverse transcriptase [10 U] 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.

2.2.7. 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), 4.5 μl H_20, 1.5 μl $MgCl_2$ (25 mM), 1 μl 5 μM forward primer (5′—AGTACAGGACAGTCGTCAGTAGTTCG—3′), 1 μl 5 μM reverse primer (5′—CAACTCCATGTGCCATGTACAGCAG—3′), which amplify a 220-bp region of 5′NCR region[36,37] 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, 54 °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.

3. Results

3.1. Synthesis

(5-Nitro-2-pyridyl) per-*O*-acetyl-1-thio-β-D-lactoside was synthesized as reported in literature[38] (Scheme 1). For oxidation of 1-thioglycoside to sulfoxide the procedure described in literature was adapted.[39–41] In contrast to the reaction conditions described in these papers (very low temperatures from −78 °C to −30 °C), the oxidation reaction were carried out at 0 °C in our experiment (Scheme 2).

3.2. Biological activity

3.2.1. Antiviral activity of newly developed glycosyl sulfoxide

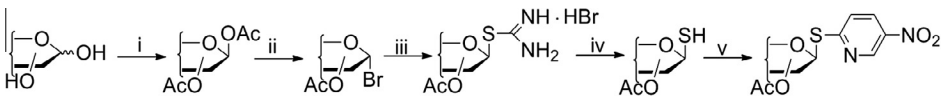
In the first set of experiments SK6 cell viability after GP6 treatment was measured using MTS assay. This test as well as microscopical examination demonstrated that tested compound produced a dose-dependent toxic effect. We determined that GP6 reduced viability of SK6 cells with the CC_{50} value of 15 μg/ml, corresponding to a 50% cytotoxic effect after 48 h of inhibitor treatment. The morphology and multiplication rates of SK6 cells untreated and treated with selected working doses of GP6 were similar.

To further characterize the in vitro antiviral property of GP6, the effect of the drug on virus propagation was evaluated using plaque reduction assay in SK6 cells. The majority of isolates of CSFV do not exhibit cytopathogenecity in tissue culture, therefore it is not possible to observe directly the foci of viral growth.[42] Due to the lack of cytopathic effect, accurate virus propagation measurement was based on the visualization of the foci caused by CSFV (pseudoplaques) using immunoperoxidase monolayer assay (IPMA) which detects the areas of maximum concentration of glycoproteins of the viral outer layer. Cells were infected with CSFV at a low MOI of 0.0001 to visualize single plaques. After virus adsorption for 1 h, cells were washed, replenished with fresh medium (positive control) or medium containing varying amounts of GP6 (0–12 μg/ml), and incubated for 2 days. The extent of CSFV infection was measured using rabbit polyclonal serum anti-E^{rns} to detect viral antigen. The typical IPMA reactions for GP6 inhibitor are shown in Figure 2. In non-infected cells, the viral antigen was undetectable (Fig. 2A), the pseudoplaques observed in infected cells indicate the presence of the virus (Fig. 2B). 10 μg/ml of GP6 nearly completely blocked pseudoplaque formation induced by CSFV. GP6 decreased CSFV propagation in SK6 cells in a dose-dependent manner evidenced by the reduction in the number and size of pseudoplaques, suggesting that the drug inhibited proliferation and spread of the virus (Fig. 2C–F). The IC_{50} value for inhibition of virus propagation, corresponding to GP6 concentration required to reduce virus plaque formation by 50% in SK6 cells, was 5 ± 0.12 μg/ml. The selectivity index (SI), defined as the CC_{50}/IC_{50} ratio, was 3.

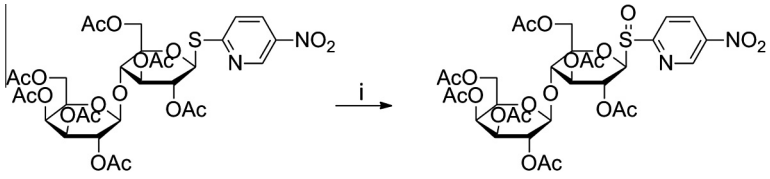
Additionally, the amount of infectious virus released into the culture medium after 72 h GP6 treatment was determined by yield assay, where serial dilutions of media from untreated (positive control) and GP6 treated cells were used to infect fresh monolayers of SK6 cells. The relative infectivity of P1 supernatants was determined at 3 days p.i. by IPMA assay. As shown in Figure 3, virus titer was dose-dependently inhibited by GP6 compound. In particular, calculated titer of progeny virus was markedly reduced by 6 μg/ml of GP6 (67% vs control), while the concentration of 12 μg/ml was able to nearly completely inhibit the infectivity of the virus, which confirmed the results of the previous experiment.

3.2.2. Inhibitory effect of GP6 on viral E^{rns} and E2 glycoprotein synthesis in SK6 cells

To understand the mechanism underlying GP6 inhibition of CSF virus spread and production, we investigated the effect of GP6 on



Scheme 1. Synthetic procedure for the synthesis of (5-nitro-2-pyridyl) per-*O*-acetyl-1-thio-β-D-lactoside. Reagents and conditions: (i) acetic acid, sodium acetate, boiling temp; (ii) 33% HBr/AcOH, 0 °C; (iii) thiourea, acetone, boiling temp; (iv) $K_2S_2O_5$, $CHCl_3$, boiling temp; (v) 2-chloro-5-nitropyridine, K_2CO_3, acetone, room temp.



Scheme 2. Oxidation procedure for the synthesis of GP6. Reagents and conditions: (i) *m*-CPBA, CH_2Cl_2, 0 °C.

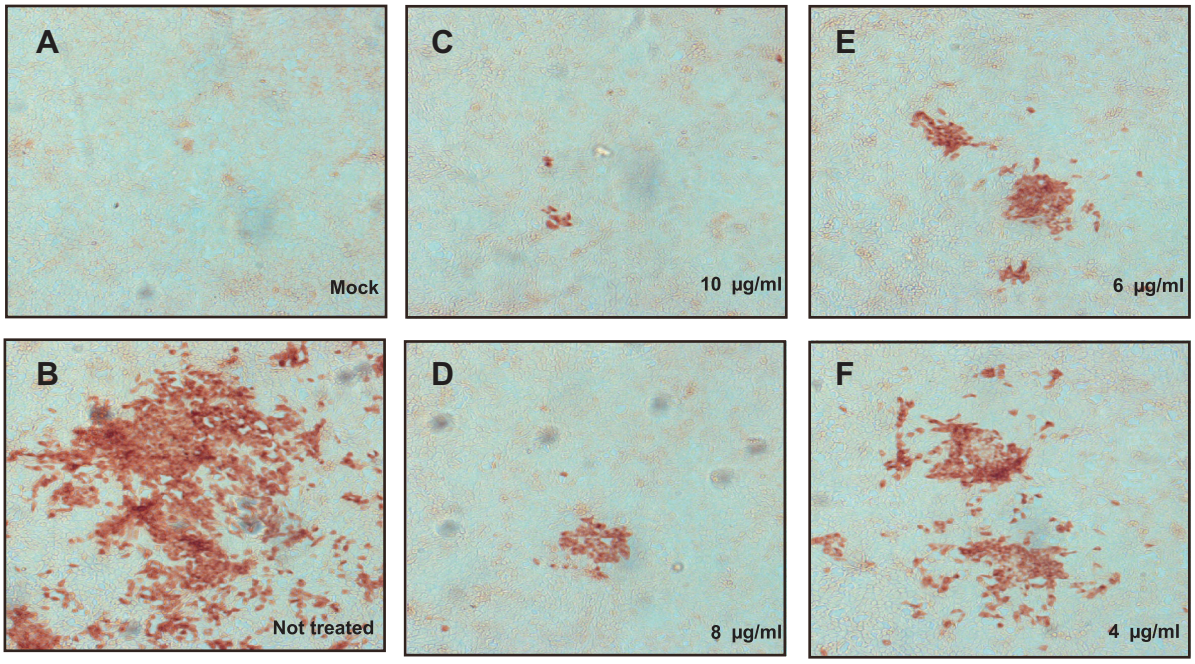


Figure 2. Effect of GP6 inhibitor on CSFV pseudoplaque formation in SK6 cells. SK6 cells were mock infected (A) or infected with CSFV at an MOI of 0.0001 (B–F). At 1 h p.i., cells were treated with different doses of GP6 (C—10 μg/ml, D—8 μg/ml, E—6 μg/ml, F—4 μg/ml) or left untreated (positive control—B). Two days post infection, cells were fixed and IPMA was performed using rabbit polyclonal serum anti-E^{rns} to detect CSFV pseudoplaques.

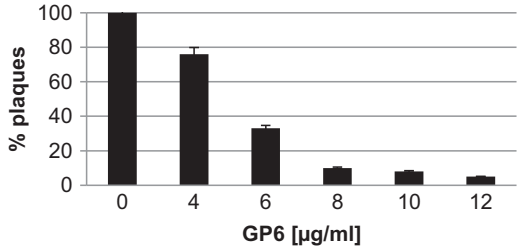


Figure 3. Effect of GP6 inhibitor on the viral yield. SK6 cells were infected with CSFV at an MOI of 0.005. At 1 h p.i., cells were treated with different doses of GP6 (12–4 μg/ml) or left untreated (positive control). Three days post infection, the collected culture supernatants were harvested for virus yield assay. The amount of infectious virus in all tested samples was determined by IPMA assay. Data represent means from three independent experiments ± SD.

glycoprotein synthesis and oligomerization by SDS–PAGE in both reducing and non-reducing conditions, followed by Western blotting. E2 glycoprotein in SK6-infected cells is usually found as a homodimer (100 kDa) or a heterodimer with E1 (75 kDa), while E^{rns} is present only as a homodimer (97 kDa). N-glycan chains have a significant effect on protein folding and in consequence on dimerization process. Lysates of SK6 CSFV-infected cells (MOI of 0.01) in the presence or absence of GP6 were analyzed for E2 and E^{rns} glycoprotein using MAb V3 39.5 and rabbit monospecific polyclonal serum, respectively. Two bands, corresponding to E2 and underglycosylated E2 monomer in reducing conditions and three bands, corresponding to E2/E2, E2/E1 complexes and E2 monomer in non-reducing conditions, were identified in the untreated, control samples (Fig. 4A and B). The GP6 treatment resulted in a dose-dependent reduction in the amount of E2 glycoprotein compared to the untreated control samples. Furthermore, after treatment with the highest doses of GP6 (from 12–10 μg/ml), when the bands representing E2–E2 homodimer, E2–E1 heterodimer and E2 monomer were nearly undetectable, it was not possible to detect the unglycosylated or underglycosylated forms of E2 viral glycoprotein under both reducing (Fig. 4A) and non-reducing (Fig. 4B) conditions. Also the ratio between E2–E2 homodimer, E2–E1 heterodimer and E2 monomer after GP6 treatment was not changed. These observations indicate that either viral glycoprotein accumulation within infected cells is affected by the presence of inhibitor or it is the consequence of inhibition of infectious virus particles production which affects the total protein level in cell lysates after 48 h. These results are in agreement with the reduction in virus production as assessed by monitoring the number and size of pseudoplaques after GP6 treatment by plaque reduction assay and virus yield assay, obtained in the previous experiments (Figs. 2 and 3).

Lack of glycans in a nascent peptide chain often causes the loss of its immunodominant epitopes, what can result in weaker reactivity of antibodies with unglycosylated product. The glycoproteins synthesized in cell cultures without the addition of inhibitor and deglycosylated with PNGase F and Endo H were detected in Western blot using the same antibody (Fig. 4C), suggesting that

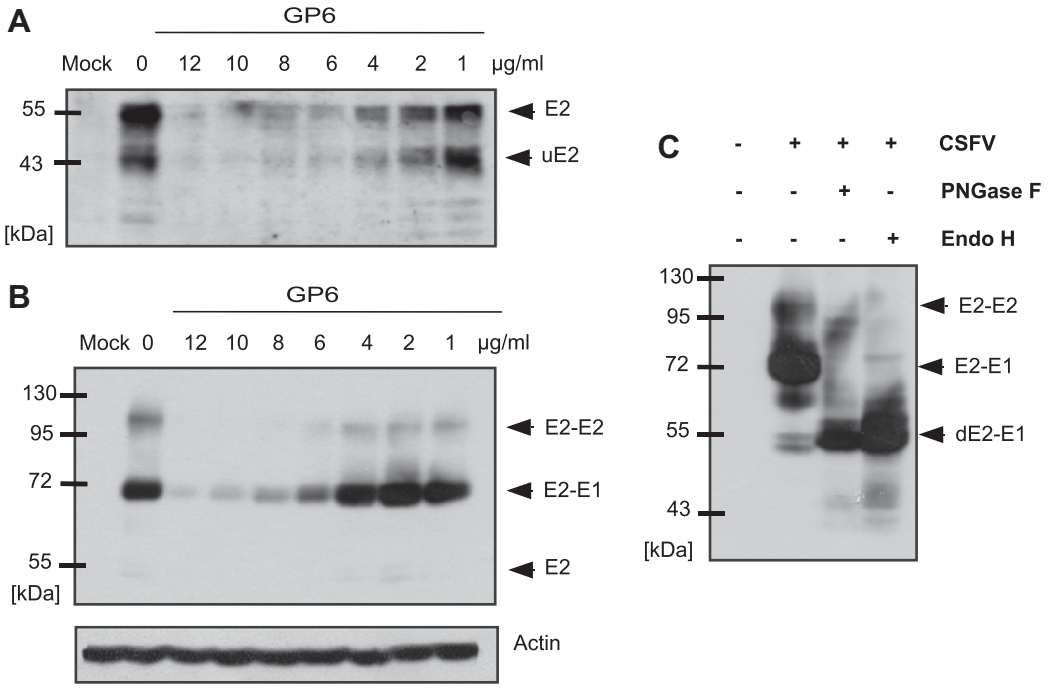


Figure 4. Effect of GP6 inhibitor on the synthesis of viral E2 glycoprotein. CSFV-infected SK6 cells were treated with various concentrations of GP6 (0–12 μg/ml) (A and B). At 48 h p.i., cells were lysed and proteins were separated by SDS–PAGE (10% polyacrylamide) under reducing (A) or non-reducing (B) 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 (uE2) monomers are marked with arrows. The effect of deglycosylating enzymes is shown in panel (C). 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 (dE2–E1) complexes are marked with arrows.

the disappearance of E2 and E^{rns} in the presence of GP6 was not related to the lack of reactivity with specific antibodies.

3.2.3. GP6 does not affect the replication of CSFV RNA

Due to the fact that high concentrations of GP6 lead to the complete loss of detectable glycoproteins when examined by Western blot analysis, in the next experimental step we wanted to determine whether this is related to the decrease in viral RNA production. Detection of CSFV RNA and its replication was performed by real-time PCR method using SYBR Green dye technology.

Monolayers of SK6 cells were infected with CSFV and left untreated, or incubated with high concentrations of GP6 (12 and 10 μg/ml), which resulted in the complete arrest of glycoprotein accumulation in the previous experiment. High MOI (0.1) and a short infection period (12 h) were employed to estimate the direct effect of GP6 on virus replication during a single round of infection. Viral RNA was extracted from CSFV-infected cells and real-time PCR was performed. The expected 220-bp PCR product, corresponding to the fragment of a highly conserved 5′NCR region, was detected on the same level in all tested samples. These results indicate that viral RNA accumulation is not affected by treatment with both doses of GP6 in infected cells after a single round of infection (data not shown). Ct values for positive control (without GP6 treatment) and GP6 treated samples were approximately 28.7 (Table 1). These results suggest that GP6 exerts its antiviral effect at the other than RNA synthesis level of virus life cycle.

To check whether GP6 inhibitor prevents the secretion of viral particles into the medium, we used culture medium from CSFV-infected cells as described above to extract RNA for amplification. Interestingly, no change in RNA level was observed in culture medium either incubated or not incubated with GP6, suggesting that the secretion of viral particles is not inhibited by tested compound.

Although GP6 inhibitor does not prevent viral RNA synthesis in CSFV-infected cells and secretion into the culture medium after single round of infection, previous experiments showed that GP6 treatment affects virus production and spread after more than 12 h. This observation raised the possibility that CSFV virions which arised during single cycle growth were secreted but they were either less, or non-infectious in subsequent replication cycles. This hypothesis was checked using real-time PCR method to determine the amount of viral RNA in SK6 cells and the culture medium of cells after infection with a low MOI of CSFV (0.01) and 48 h inhibitor treatment period to allow multi-cycle replication. Real-time PCR assays after 48 h indicated that 12 and 10 μg/ml of GP6 significantly reduced the RNA level in both cells and culture medium. Ct values for positive controls, untreated cells and culture medium, were 21.9 and 26.7, respectively. After GP6 treatment with 12 μg/ml Ct values were increased to 25.2 ± 0.2 and 29.8 ± 0.1 for cells and culture medium, respectively which corresponds to about 90% and 88% reduction in viral RNA in cells and culture medium, respectively. Additionally, 71–73% reduction of viral RNA was observed after treatment with 10 μg/ml of GP6 (Ct values for cells and medium were 23.7 ± 0.2 and 28.6 ± 0.2, respectively) (Table 1).

Taken together, these data suggest that virus particles after GP6 treatment are produced but either they are less or non-infectious most probably due to the changes in glycosylation status of viral proteins and they are not able to infect new cells, which results in the reduction of virus RNA in both infected cells and culture medium of infected cells during secondary infections.

3.2.4. GP6 inhibitor do not change glycosylation pattern of recombinant CSFV E^{rns} and E2 in insect Sf9 cells

The examination of RNA level after treatment with the inhibitor showed that CSFV particles formed in the presence of GP6 are probably less infectious. To verify this hypothesis and get better insight into the mechanism of action of GP6 we performed an experiment using baculovirus expression system to test the influence of this

Table 1
SYBR Green real-time PCR detection of viral cDNA after 12 and 48 h treatment with GP6

GP 6	12 h Incubation						48 h Incubation					
	SK6 cells			Culture medium			SK6 cells			Culture medium		
	Ct	ΔCt	% RNA decrease	Ct	ΔCt	% RNA decrease	Ct	ΔCt	% RNA decrease	Ct	ΔCt	% RNA decrease
0 μg/ml	28.7	0.0	0	34.2	0.0	0	21.9	0	0	26.7	0	0
10 μg/ml	28.7 ± 0.2	0.0	0	34.2 ± 0.1	0.0	0	23.7 ± 0.2	1.8	71	28.6 ± 0.2	1.9	73
12 μg/ml	28.7 ± 0.2	0.0	0	34.2 ± 0.2	0.0	0	25.2 ± 0.2	3.3	90	29.8 ± 0.1	3.1	88
Neg. control	—	—	—	—	—	—	—	—	—	—	—	—

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

compound on the early steps of glycosylation process taking place in the endoplasmic reticulum (ER). N-glycosylation process in insect cells is much simpler than in mammalian cells and it is generally of a high-mannose type. It was shown previously that the antibiotic—tunicamycin led to complete disappearance of E2 and E^{rns} glycoproteins in soluble (cytoplasmic) fraction expressed in insect cells.[34] For this purpose insect cells infected with recombinant baculoviruses containing genes coding for E2 or E^{rns} glycoproteins were grown for 48 h in the presence of decreasing concentration of GP6 (12, 10 and 8 μg/ml) and tunicamycin (2 and 1 μg/ml) as a control. At the concentrations used, no toxicity was observed in Sf9 cells, as determined by trypan blue method.

No changes in molecular weight were observed for both E2 (Fig. 5A) and E^{rns} (Fig. 5B) glycoproteins produced in insect cells in the presence of GP6 inhibitor suggesting that glycosylation pattern was not affected. As expected, in control experiment tunicamycin treatment resulted in the complete disappearance of both proteins. Therefore, we concluded that GP6 inhibitor is not active on early ER-dependent steps of glycosylation process.

3.2.5. The influence of GP6 inhibitor on PRV gE glycoprotein synthesis

Unglycosylated forms of E2 and E^{rns} glycoproteins after GP6 treatment were not detected in CSFV-infected cells (Fig. 4). It was previously reported that CSFV glycoproteins lacking N-glycans after tunicamycin treatment are very unstable and very quickly degraded.[34] To check the hypothesis that GP6 may change the glycosylation pattern of glycoproteins acting on the late, Golgi-dependent, stages of glycosylation (which are unique only for mammalian cells) we performed the experiment using pseudorabies virus (PRV). PRV is a pig virus belonging to Herpesviridae family, very distant from Flaviviridae, but infecting swine kidney (SK6) cells. For herpesviruses, glycan-free polypeptides are relatively stable and can be detected by classical protein detection methods.[43,44]

We investigated the impact of GP6 inhibitor on PRV gE glycoprotein synthesis in SK6 cells in SDS–PAGE under reducing conditions followed by Western blotting. Tunicamycin treatment was used as a control for unglycosylated form of gE glycoprotein. The band corresponding to fully glycosylated form of gE glycoprotein was detected in both untreated and GP6 treated samples. Treatment with GP6 did not result in visible reduction of the molecular mass of gE protein (Fig. 6), which suggests that the tested inhibitor is probably acting on the very late step of glycosylation and the difference in molecular mass of treated and untreated gE glycoprotein is too small to be detected by this method. At the present stage we have no direct proof of this hypothesis and further studies are in progress to better characterize the molecular mechanism underlying the GP6 anti-CSFV activity.

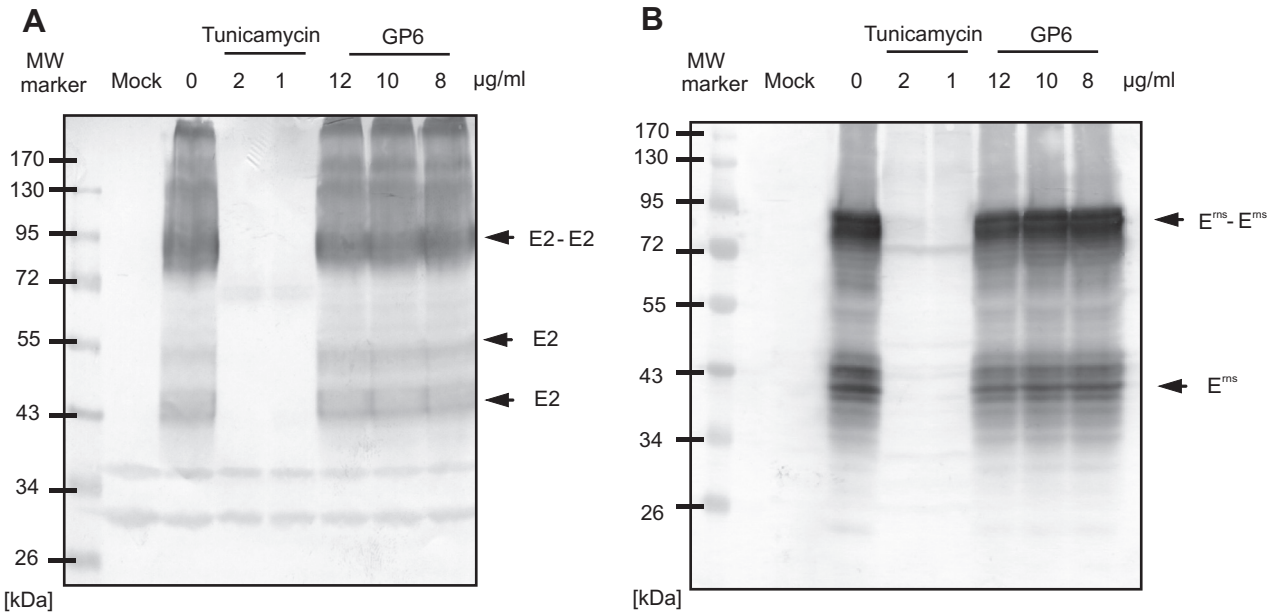


Figure 5. The influence of GP6 inhibitor on CSFV E2 and E^{rns} glycoprotein synthesis in insect Sf9 cells. Sf9 cells were infected with recombinant baculovirus expressing CSFV E2 (A) or E^{rns} (B) glycoprotein at an MOI of 1 or mock infected. At 2 h p.i., cells were treated with tunicamycin or GP6 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) (A) or rabbit polyclonal serum anti-E^{rns} (B). 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.

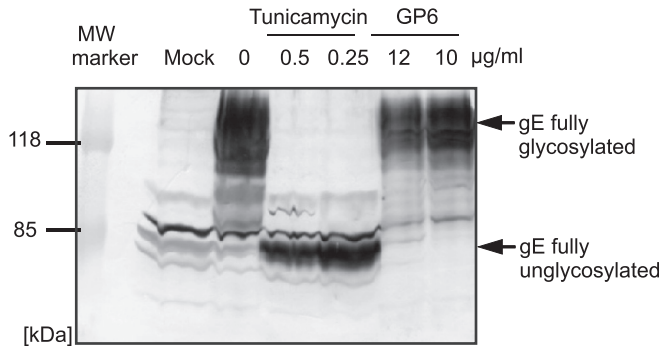


Figure 6. Analysis of PRV gE glycoprotein expressed in GP6-treated SK6 cells. SK6 cells were mock infected or infected with PRV at an MOI of 1 for 24 h in the absence or presence of tunicamycin or GP6. Cells were lysed and proteins were separated by SDS–PAGE (10% polyacrylamide) under reducing conditions. Western blot analysis was performed using the specific anti-gE rabbit serum. MW marker—molecular weight marker.

4. Discussion

Due to continuous risk for virus introduction from CSF-endemic countries or wildlife reservoirs into pig population in European Union, the use of antiviral agents has been proposed as an acceptable control strategy in case of an outbreak.[45] In this report we have demonstrated the synthesis and antiviral activity of the glycosyl sulfoxide—compound GP6. This compound inhibited classical swine fever virus propagation in a dose-dependent manner without inducing any cytotoxic effect (Fig. 2). The antiviral activity of GP6 inhibitor is probably due to modifications in glycosylation process.

Several reports have shown that the arrest or alterations of the glycosylation process of viral proteins by different inhibitors usually result in antiviral effects.[46–50] The use of well-known glycosylation inhibitor—tunicamycin as an antiviral agent against different viruses such as rotavirus,[51] hepatitis delta virus,[52] canine herpesvirus[44] and bovine herpesvirus-1[43] has been reported. For these viruses glycan-free polypeptides in infected cells are accumulated and can be detected by classical protein detection methods. It was reported that blocking N-glycosylation of CSFV E2 and E^{rns} glycoproteins with tunicamycin results in the disappearance of both glycoproteins synthesized in SK6 cells.[34] The possible explanation for the loss of CSFV glycoproteins is sorting out of the deglycosylated proteins to rapid degradation. It was shown that 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.[53]

We analyzed the synthesis and dimer formation of CSFV envelope glycoproteins after GP6 treatment. The E1–E2 dimer is the major component of mature virions and is thought to play a central role in virus infection. Any conformational changes in the subunits of the complex could affect the receptor binding. The GP6 treatment resulted in a dose-dependent reduction of E2 and E^{rns} glycoprotein synthesis (Fig. 4). We were not able to detect unglycosylated or less glycosylated forms of E2 or E^{rns} in CSFV-infected cells. Accordingly, the lack of glycoproteins may be due to a rapid degradation of misfolded glycoproteins as it was shown for tunicamycin.[54] The specific reduction in viral glycoprotein level might contribute to the arrest of viral infection.

Using real-time PCR method we demonstrated the effect of GP6 on virus replication and secretion during a single round of infection. Our results show that GP6 does not reduce the RNA level in both cases indicating that replication of the virus is not affected by GP6 (Table 1). The presence of RNA in medium of infected cells after a single round of CSFV life cycle shows that the polyprotein formation and processing is not blocked. However, the same experiment after 48 h showed that treatment with GP6 leads to the reduction in the amount of viral RNA extracted from the cells and medium. The lower amount of virus particles in infected cells and in medium after 48 h indicate that viral particles produced after one round of replication cycle are less or not-infectious as the result of the incorporation of non-functional glycoprotein complexes and they are not able to infect other cells due to the inhibition of receptor binding.

The inhibition of N-glycosylation processes may be the cause of reduced infectivity of secreted viral particles. The importance of N-glycosylation and N-glycan processing of viral envelope proteins in CSFV life cycle has been reported previously.[16,20] As it was shown, the CSF virus mutants missing partial or complete E2 gene were unable to generate viable virus.[55] Also, though a single mutations of putative glycosylation sites of E2[56] and E^{rns}[57] did not affect in vitro and in vivo replication, the multiple site mutations rendered non-viable viruses. In previous studies, it has been observed that changes in the glycosylation patterns of CSFV envelope proteins affected virus virulence and viability.[56,58,59] Moreover, it has been shown that glycosylation plays a major role in the immunogenicity of CSFV envelope proteins, most likely an effect linked to the correct conformation of E^{rns}, E1 and E2 proteins.[60]

Using baculovirus recombinants expressing full-length forms of E^{rns} and E2 glycoproteins of CSFV we have excluded the influence of GP6 on first steps of glycosylation process taking place in insect cells (Fig. 5). Furthermore, we used Pseudorabies virus, for which glycan-free polypeptides are stable, to check whether the late steps of polypeptides N-glycosylation process in the presence of GP6 is arrested. However, the patterns of glycosylation of glycoprotein gE for GP6-treated and untreated samples were the same (Fig. 6). These results suggest that GP6 affects the very late steps of glycosylation process.

In eukaryotes, most of the glycosylation reactions that generate the diversity of oligosaccharide structures of eukaryotic cells occur in the Golgi apparatus. The medial and trans Golgi apparatus is the cellular site of action for many glycosyltansferases that decide this branching patterns of N-glycans. GlcNAc-transferases I and II as well as fucosyltransferases are present in medial Golgi, while glycosyltansferases such as galactosyltransferases or sialyltransferases regulate the synthesis of diverse structures in trans Golgi elements. The results obtained during the presented study suggest that one of these enzymes may be the target for GP6 compound. Additional studies evaluating the precise mechanism of action are underway. Furthermore, the interaction of the compound with the indicated enzyme will be confirmed by molecular modeling to predict GP6 binding mode.

In conclusion, we report here on antiviral activity of GP6 inhibitor in arresting CSFV viral growth. This compound can serve as the basis for further modification in the search for more potent candidates for anti-CSFV therapy. Further experiments are needed for testing whether this compound can be used as a safe antiviral agent against other flaviviruses or viruses from other families.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.03.027.

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