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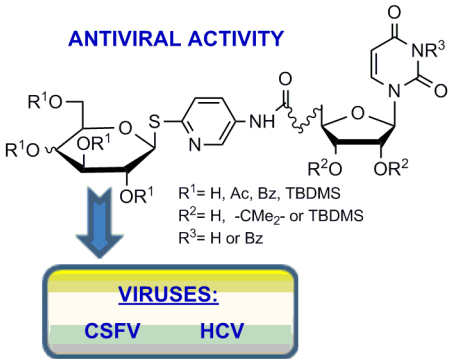

Novel thioglycosyl analogs of glycosyltransferase substrates as antiviral compounds against Classical Swine Fever Virus and Hepatitis C Virus

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ABSTRACT:

Hepatitis C virus (HCV) and classical swine fever virus (CSFV) are important pathogens for which new therapeutic approaches are in high demand. Herein, we report the synthesis of newly designed thioglycosyl analogs of glycosyltransferase substrates which were evaluated using cell-based assays for cytotoxicity and antiviral activity against both viruses. The antiviral activity of synthesized compounds against CSFV and HCV was confirmed using pseudo-plaque reduction assays where a significant arrest of viral growth was observed in the presence of selected compounds. We showed that compounds **13** and **14** exerted the most significant inhibitory effect on *in vitro* CSFV and HCV infections in the series. Glycoconjugates **13** and **14** not only inhibited both viral propagation with IC_{50} values in low micromolar range, but efficiently suppressed the production of viral proteins in a dose-dependent manner. In addition, studies using *in vitro* HCV infection and replication models have shown that both compounds are able to significantly reduce viral genomic replication. We demonstrated that compounds **13** and **14** showed a strong inhibition, up to 90% of replication which inscribe them in the promising alternative approach for the development of new anti-CSFV and anti-HCV drugs.

KEYWORDS:

hepatitis C virus, classical swine fever virus, antivirals, glycoconjugates, analogs of glycosyltransferase substrates

ABBREVIATIONS:

Ac, acetyl; Bz, benzoyl; BAIB, [bis(acetoxy)-iodo]benzene; CSFV, classical swine fever virus; DAAs, direct-acting antivirals; DMTMM, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; DMSO, dimethyl sulfoxide; GTs, glycosyltransferases; HCV, hepatitis C virus; TBDMS, *tert*-butyldimethylsilyl; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy

1. Introduction

The *Flaviviridae* family comprises many viruses associated with human and animal diseases of great impact on public health. The most well-known members of this family are important human pathogens including hepatitis C virus (HCV), dengue fever virus, yellow fever virus as well as viruses of veterinary importance causing economically important diseases like bovine viral diarrhea virus and classical swine fever virus (CSFV). In spite of effective vaccines and therapies for some of these pathogens, viral mutations, drug resistance or viral re-emergence generate problems for global control and eradication.

HCV, a member of the *Hepacivirus* genus and CSFV, a *Pestivirus* member, show high degree of homology in genomic organization, replication and protein function. They are enveloped viruses with single-stranded RNA genomes encoding a single polyprotein that is cleaved into different structural and non-structural proteins by host and viral proteases. Due to the similarity of CSFV and HCV, the former was frequently used as a surrogate model to study the role of envelope glycoproteins of HCV [1,2].

CSFV causes an acute, highly infectious and economically damaging disease in swine and wild boars in many countries [3,4]. Classical swine fever (CSF) is a major threat to

commercial pig production worldwide [5]. CSF outbreaks among wild boars present a constant threat for domestic pigs [6]. There is no treatment for classical swine fever, other than supportive care. Several vaccines against CSFV are currently available, however the most efficient vaccines are live attenuated ones developed over 50 years ago, which do not allow for differentiating between infected and vaccinated animals [7]. The virus is still widely circulating in endemic regions, therefore, the use of antiviral drugs could be a good control strategy to prevent transmission of the virus in case of an outbreak.

HCV is a major cause of liver diseases in human, like chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. So far, there is no vaccine available [8]. It is estimated that around 180 million people are chronically infected worldwide, which makes the virus one of the most serious burdens to public health. Since the introduction of PEGylated interferon alpha and Ribavirin as the standard drugs for clinical therapy of HCV in 1990, significant efforts have been made to discover new and more effective treatments. New potent HCV inhibitors belonging to direct-acting antivirals (DAAs) such as Boceprevir, Telaprevir, Sofosbuvir, Simeprevir, Daclatasvir, and Ledipasvir have been developed in recent years [9]. These DAAs directly target enzymatic functions of viral proteins such as NS3/4A proteases, NS5A or NS5B polymerase. New formulations based on combination of the new agents and the traditional HCV inhibitors as well as multiple DAAs and interferon-free therapies have improved the rates of sustained virologic response (SVR) and greatly reduced the side effects [10]. However, although the combination therapy based on Boceprevir or Telaprevir (inhibitors of NS3/4A protease) together with Ribavirin and PEGylated interferon alpha improves antiviral response, it is limited to hepatitis C genotype 1. Introduced in 2013 Sofosbuvir (NS5B polymerase inhibitor) is the first drug used in combination with Ribavirin in interferon-free therapy, however it is effective only for genotype 2 and 3 HCV patients. In

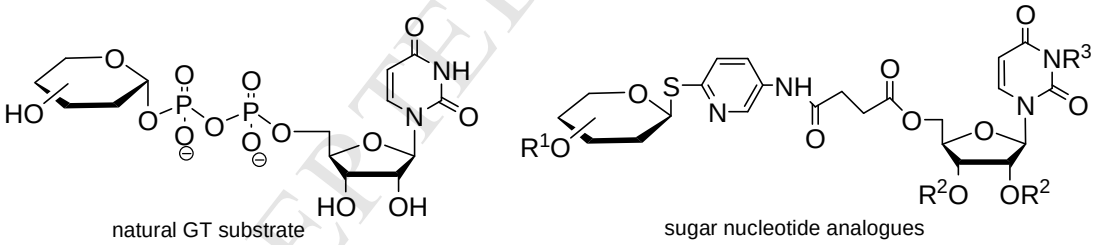
2013 another drug Simeprevir (protease inhibitor) has been approved by FDA in interferon-free regimen. Combination therapy containing two other NS5A inhibitors, Daclatasvir and Ledipasvir, show high success rates (even over 90%) depending on viral genotype [11]. Although a significant progress in anti-HCV therapy has been undoubtedly attained, many limitations still exist. Monotherapy with DAAs are associated with rapid emergence of drug-resistant viral mutants, therefore clinical application of DAAs is mostly limited to combination regimens which is associated with additional side effects, drug-drug interactions, high costs and availability. Thus, new therapeutic strategies consisting of new HCV inhibitors targeting different stages of HCV life cycle, with increased effectiveness and wider availability are still needed to overcome these limitations.

Proteins protruding from the viral envelope of many viruses are usually highly glycosylated. Protein glycosylation is carried out by a family of glycosyltransferases (GTs), which catalyze the transfer of a sugar moiety from an activated nucleotide sugar donor to a hydroxyl or an amino groups of acceptor substrate [12,13]. GTs are involved in many fundamental biological processes and modulation of their activities by efficient inhibitors is a potential way for the control of certain cellular functions. A large number of potent natural as well as synthesized GT inhibitors based on 3D structures of several GTs have been identified. Most of synthesized GT inhibitors belong to donor substrate analogues, acceptor substrate analogues or bisubstrate analogues.

In case of metal-dependent GTs, the pyrophosphate moiety of the donor-type substrate interacts with bivalent cations such as Mn^{2+} or Mg^{2+} with coordination of two aspartate residues within a DXD motif in the enzyme active site. Numerous analogues of nucleosidediphosphate (NDP) sugars with modifications of the diphosphate bridge have been described. Some of them were obtained by varying the pyrophosphate moiety on phosphonate

[14,15], methylenediphosphonate [16] or ethyl phosphonophosphate [17]. However, the main drawback of such compounds is their anionic character, which precludes their entry into cells through the phospholipid bilayer. Due to this fact, the promising strategy for *in vivo* biological applications is the preparation of GT inhibitors containing a neutral diphosphate surrogate [18,19].

Previously, a new kind of sugar nucleotide analogues, which were designed to act as GT inhibitors were reported [20]. When compared to the natural GT substrates, the anomeric oxygen atom was replaced in these structures by the sulfur in order to increase their resistance to enzymatic hydrolysis. Another change in the structure was the replacement of pyrophosphate bridge with a succinic linkage. The choice of such linker was based on the ability to coordinate divalent metal ions (**Scheme 1**) [21]. Based on the foregoing assumptions, glycoconjugates **8**–**10** where aminopyridyl 1-thioglycosides (derivatives of D-glucose **5** or D-galactose **6**) were connected to selectively protected uridine with succinic spacer **1** or **2** through the amide bond were prepared [20].



Scheme 1. Natural GT substrates and sugar nucleotide analogues.

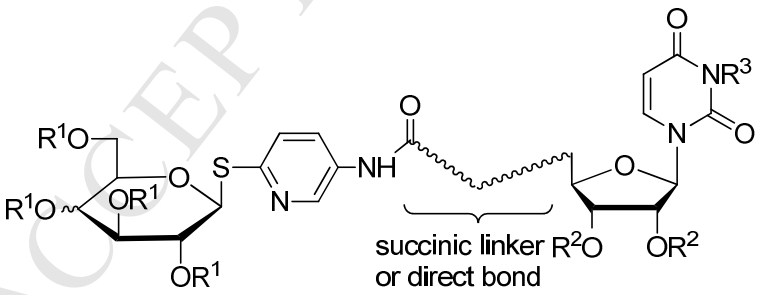
In this paper, we described the synthesis and evaluation of antiviral activity of novel thioglycosyl analogs of glycosyltransferase substrates against two viruses belonging to the *Flaviviridae* family – CSFV and HCV in cell culture system. To investigate the effect of the presence of the succinic linker on glycoconjugates biological activity, a series of new glycoconjugates (**11-17**) were synthesized based on the same structural fragments as in case

of glycoconjugates **8-10** but omitting the succinic linker. For the propose of this study, glycoconjugates **8-10** were additionally prepared using improved method using microwave irradiation. Two promising compounds with novel properties were chosen for further development as lead hits. The broad spectrum activity exhibited by selected compounds against these two viruses suggests their suitability as potential inhibitors against infections caused by other viruses from the *Flaviviridae* family.

2. Results and discussion

2.1. Chemistry

Our preliminary studies on biological activity of compounds **8**–**10** indicated that some of them were able to inhibit cell proliferation of the classical swine fever virus. These results encouraged us to synthesize other glycoconjugates in which succinic fragment has been omitted and *N*-(6-mercaptopyridyn-3-yl)formamide part was the only substitute of diphosphate bridge (**Scheme 2**).



R^1: acetyl, benzoyl, TBDMS or H, R^2: isopropylidene, TBDMS or H, R^3: H or benzoyl

Scheme 2. General structure of tested glycoconjugates used in these studies.

The effect of the presence and the type of protecting groups in both parts of glycoconjugate: in the sugar ring and in the uridine moiety was also examined. As protecting groups in the sugar ring acetyl, benzoyl and TBDMS groups were selected. Ribose in uridine moiety was protected using the isopropylidene group or more hydrophobic TBDMS groups. Ester type protecting groups were selected due to the possibility of their hydrolysis by enzymes present within the cells. These groups only increased the hydrophobicity of glycoconjugates and allowed them to penetrate into the cell. In turn, protecting groups in the uridine part were chosen not only to allow the regioselective synthesis of glycoconjugates but also to improve the hydrophobicity of the products and stability within the cell.

A new group of glycoconjugates, potential glycosyltransferases inhibitors, were synthesized using improved, compared to the previously described, method of condensation. The substrates used in condensation reactions were uridine derivatives containing carboxyl group **1**–**4** and (5-amino-2-pyridyl) β-D-1-thioglycosides derivatives of D-glucose and D-galactose **5**–**7** (**Fig. 1**).

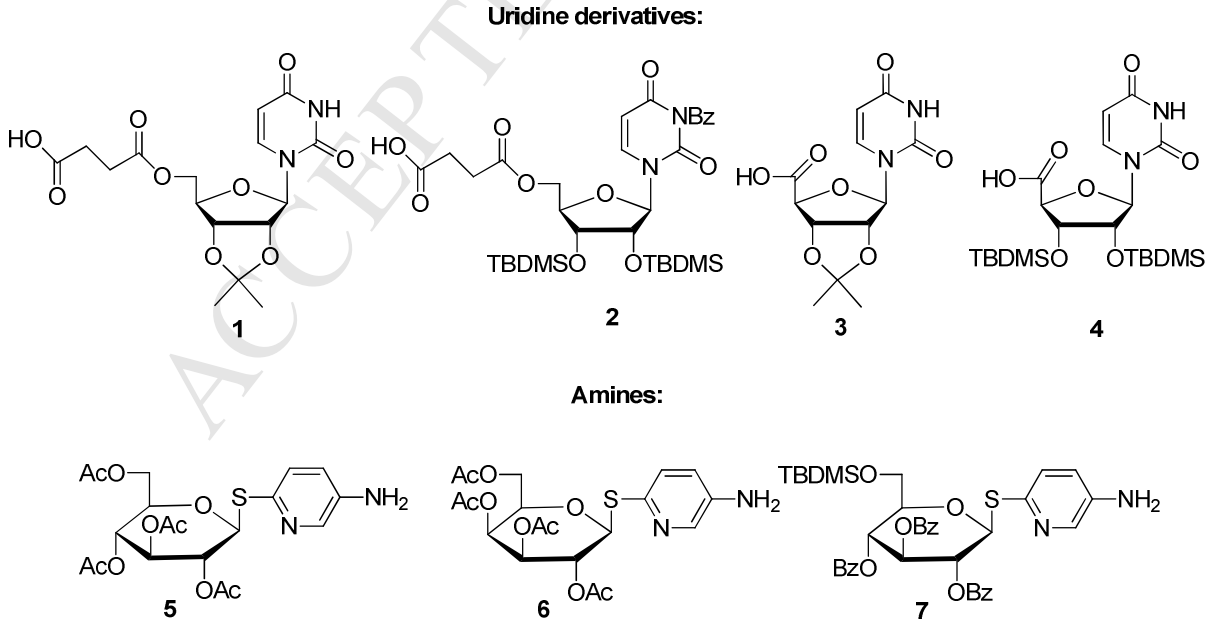
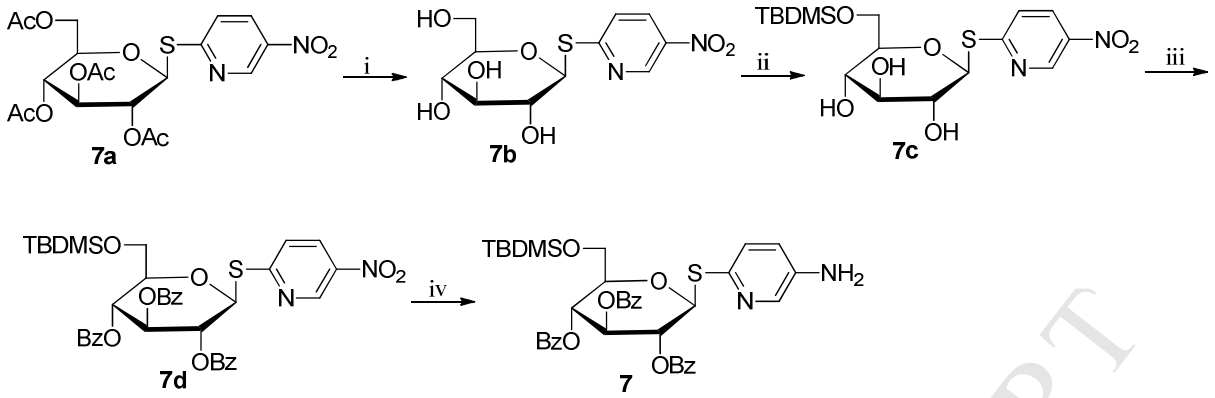


Figure 1. Structure of substrates **1**-**7**.

The synthesis of uridine derivatives **1**–**2** [20] and 2',3'-*O*-isopropylideneuridine-5'-carboxylic acid **3** [22] was described earlier. 2',3'-Di-*O*-*tert*-butyldimethylsilyluridine-5'-carboxylic acid **4** was synthesized by oxidation of the corresponding 2',3'-di-*O*-*tert*-butyldimethylsilyluridine **4a** with TEMPO/BAIB system in CH_3CN-water 1:1 solvents system. ^{1}H NMR and ^{13}C NMR spectra confirmed the structure of obtained compound.

A second group of structural components of glycoconjugates was (5-amino-2-pyridyl) β-D-1-thioglycosides **5**–**7**. A simple and efficient synthesis of per-*O*-acetylated (5-amino-2-pyridyl) β-D-1-thioglycosides **5** and **6** was described a few years ago [23]. (5-Amino-2-pyridyl) 2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl-1-thio-β-D-glucopyranoside **7** was synthesized in a four-step procedure (**Scheme 3**) consisting of de-*O*-acetylation of (5-nitro-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **7a**, selective silylation of 6-OH group in compound **7b**, benzoylation of the other three OH groups in the compound **7c** and reduction of the nitro group in aglycon of compound **7d**. The nitro group reduction procedure with zinc powder/acetic acid system in CH_2Cl_2 was previously successfully used for the preparation of derivatives **5** and **6**. The reaction proceeded at room temperature. It is worth noting that desilylation reaction at C-6 position was not observed under the reaction conditions despite acidic reaction medium. This method allows to obtain the compound **7** in good overall yield for the four step (50%).



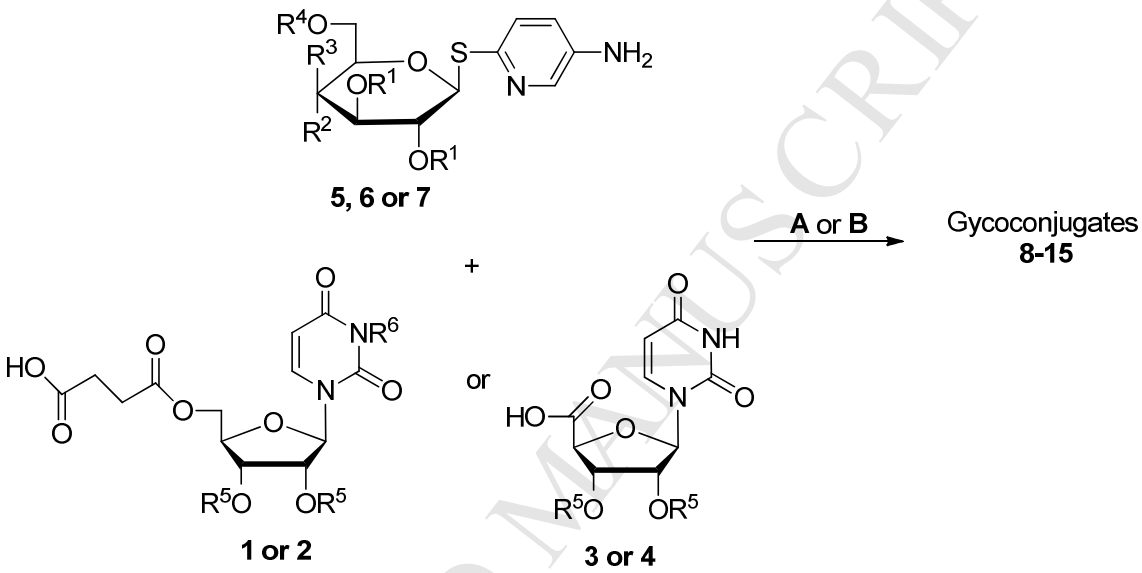
Reagents and Conditions: (i) MeONa, MeOH, r.t., 1 h; (ii) TBDMSCl, imidazole, DMF, r.t., 0.5 h; (iii) BzCl, pyridine/CH_2Cl_2, 0 °C to r.t., 24 h; (iv) Zn powder, AcOH/CH_2Cl_2, r.t., 0.5 h.

Scheme 3. Preparation of compound **7**.

For amine and uridine derivatives, the next step was to carry out the condensation reaction. The simple approach for the amide bond formation by mixing an amine and carboxylic acid results in formation of a stable salt. Therefore, activation of carboxylic acid is necessary [24]. Numerous condensing agents for efficient formation of an amide bond have been described, of which the most common are carbodiimide derivatives (generally DCC) [25] alone or with additives such as DMAP or HOBt [26]. Another method for amide bond formation utilizes an ethyl chloroformate generation of mixed anhydride [27]. Effective way to synthesize amides in the presence of 2-chloro-4,6-disubstituted-1,3,5-triazines and tertiary amines (e.g., *N*-methylmorpholine) has been published by Kaminski and co-workers [28]. These methods were previously used to form uridine glycoconjugates **8**–**10** [20]. The best results were obtained using DMTMM (4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride) as a condensing agent. The only drawback of this method was a long reaction time (up to two days). In the currently described experiments, in order to avoid long reaction time microwave irradiation was used. For condensation of the amine group in sugar derivatives **5**–**7** with carboxylic group in uridine derivatives **1**–**4** (**Scheme 4**), DMTMM

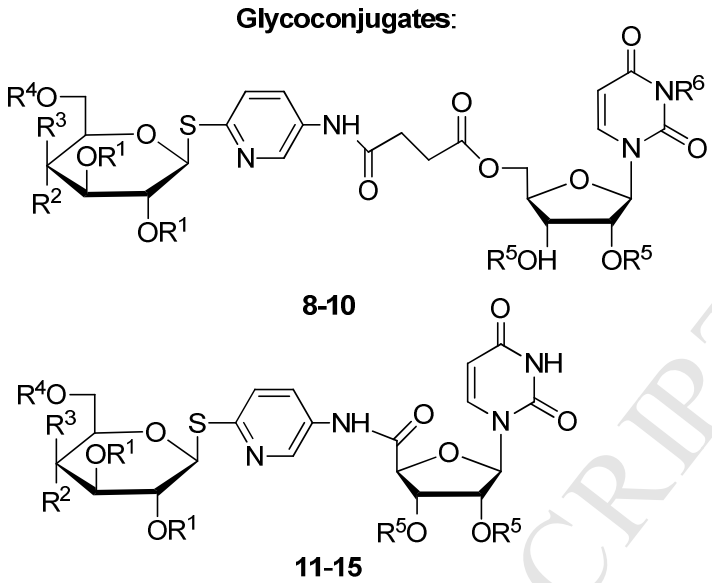
was used in combination with microwave irradiation (Standard Program, 50°C) (**Table 1**, Procedure B). Parallel condensation reactions using DMTMM at room temperature on a magnetic stirrer were conducted (**Table 1**, Procedure A). As results of these reactions, glycoconjugates **8**–**15** were obtained. The yields of the respective products are presented in **Table 1**.

A



Reagents and Conditions: (**A**) DMTMM, THF, r.t.; (**B**) DMTMM, THF, 50 °C, MW

B



8: R^1=R^4=Ac, R^2=OAc, R^3=H, R^5=-CMe_2-, R^6=H
9: R^1=R^4=Ac, R^2=H, R^3=OAc, R^5=-CMe_2-, R^6=H
10: R^1=R^4=Ac, R^2=OAc, R^3=H, R^5=TBDMS, R^6=Bz
11: R^1=R^4=Ac, R^2=OAc, R^3=H, R^5=-CMe_2-, R^6=H
12: R^1=R^4=Ac, R^2=H, R^3=OAc, R^5=-CMe_2-, R^6=H
13: R^1=R^4=Ac, R^2=OAc, R^3=H, R^5=TBDMS, R^6=H
14: R^1=R^4=Ac, R^2=H, R^3=OAc, R^5=TBDMS, R^6=H
15: R^1=Bz, R^2=OBz, R^3=H, R^4=R^5=TBDMS, R^6=H

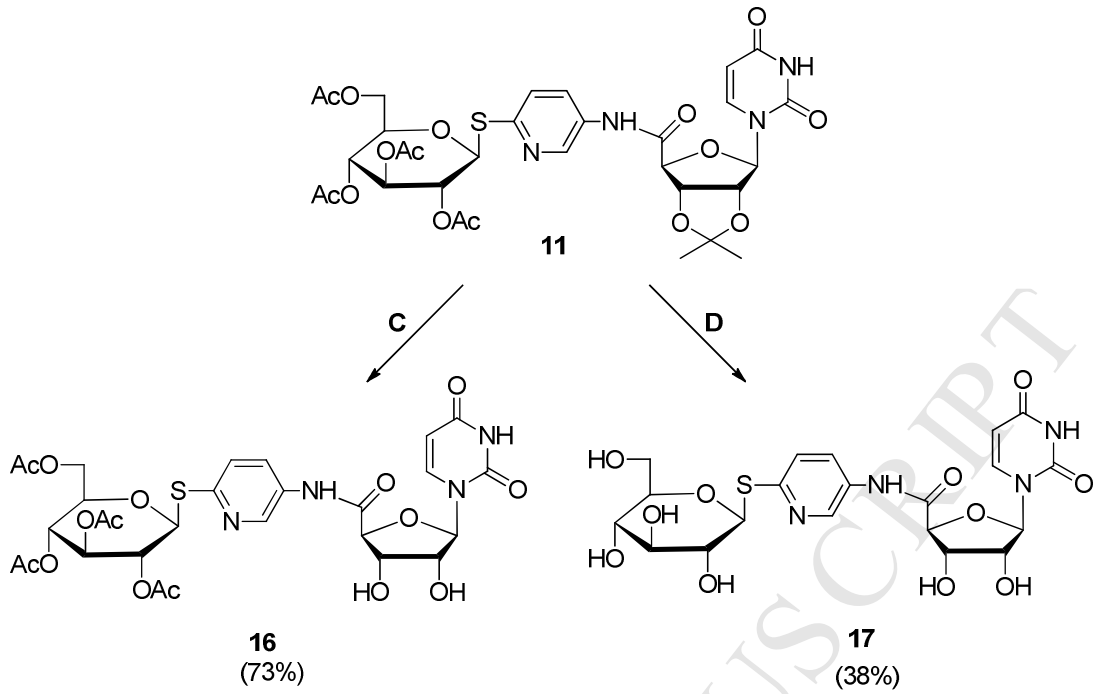
Scheme 4. A. Synthesis of glycoconjugates **8**-**15**. B. Structures of compounds **8**-**15**.

Table 1. Yields of glycoconjugates **8**-**15**.

Entry	Substrate 1 Amine	Substrate 2 Uridine deriv.	Product	Procedure	Reaction time [h]	Yield [%]
1	**5**	**1**	**8**	**A**	48	53
2	**5**	**1**	**8**	**B**	3	56
3	**6**	**1**	**9**	**A**	24	39
4	**6**	**1**	**9**	**B**	4	41
5	**5**	**2**	**10**	**A**	48	51
6	**5**	**2**	**10**	**B**	4	58

7	**5**	**3**	**11**	**A**	96	38
8	**5**	**3**	**11**	**B**	2	49
9	**6**	**3**	**12**	**A**	96	29
10	**6**	**3**	**12**	**B**	2	33
11	**5**	**4**	**13**	**A**	96	32
12	**5**	**4**	**13**	**B**	2	53
13	**6**	**4**	**14**	**A**	96	43
14	**6**	**4**	**14**	**B**	2	61
15	**7**	**4**	**15**	**A**	120	52
16	**7**	**4**	**15**	**B**	4	54

Both condensation procedures yielded the desired glycoconjugates. It should be noted, that the use of microwave irradiation not only allowed for the shortening of the reaction time but also led to higher yield of products. This is particularly evident in the case of glycoconjugates **13** and **14** containing uridine part protected with TBDMS groups. Most likely, bulky TBDMS group in uridine derivative **4** causes a reduction of its reactivity in condensation reaction. It is worth noting that the introduction of large protective groups to the amine derivative **7** also decreased its reactivity. In the case of the reaction between amine **7** and uridine derivative **4**, the reaction time for the standard procedure B (2 hours) was insufficient. TLC analysis of the reaction mixture showed the presence of unreacted substrates and therefore the reaction was continued for additional 2 hours.



Reagents and Conditions:(**C**) MeOH/H_2O, Amberlyst 15, 70 ^oC, 2 h; (**D**) 1. MeOH, NaOMe, r.t., 1 h, 2. MeOH/H_2O, Amberlyst 15, 70 ^oC, 2 h.

Scheme 5. Synthesis of glycoconjugates **16** and **17**.

The final step of glycoconjugate synthesis was the hydrolysis of the protecting groups. Compound **11** was selected as the substrate to provide selectively or fully deprotected glycoconjugates (**Scheme 5**). The hydrolysis of the isopropylidene group from the uridine part is facilitated in acidic conditions. In this case an acidic ion exchange resin Amberlyst-15 in MeOH/H_2O system was used for deprotection to give glycoconjugate **16** (73% yield). Complete removal of the protecting groups from glycoconjugate **11** was conducted in two steps. The first step was the methanolysis of acetyl groups in the sugar part by the action of sodium methoxide (NaOMe) in methanol. It should be noted that the same reagent was used in the neutralization step of acetyl removal and in the reaction of isopropylidene group removal. To explore fully the advantages of this convenient reagent, Amberlyst-15 was employed in the two-step, one-pot deprotection of derivative **11** to give glycoconjugate **17** at a moderate yield (38%).

All glycoconjugates were purified by column chromatography and their structures were determined by NMR spectra. The obtained compounds were evaluated for their inhibitory properties against classical swine fever virus and hepatitis C virus.

2.2. *In vitro* antiviral activity.

In primary screening we evaluated the *in vitro* antiviral activities of all synthesized glycoconjugates using classical swine fever virus and hepatitis C virus. The measurements included the cytotoxic concentration CC_{50}, which was defined as the compound concentration that reduced cell viability by 50% measured using MTS assay for SK6 cells and MTT assay for Huh-7.5 cells, the 50% inhibitory concentration (IC_{50}), which was defined as the compound concentration that reduced virus plaque formation by 50% and the selectivity index SI (the ratio of CC_{50} to IC_{50}). Due to the lack of cytopathic effect caused by both CSFV and HCV viruses, accurate virus propagation measurement was based on visualization of the foci caused by the virus (pseudo-plaques) using immunoperoxidase monolayer assay (IPMA) which detects the areas of maximum concentrations of viral glycoproteins. The results of these experiments are summarized in **Table 2.**

Table 2. Inhibitory effects of all synthesized compounds on CSFV and HCV replication in SK6 and Huh-7.5 cells.

Compound	*CSFV*			*HCV*		
	CC_{50} (μM)[a]	IC_{50} (μM)[b]	SI[c]	CC_{50}(μM)[a]	IC_{50} (μM)[b]	SI[c]
8	207	103 ± 2	2	328	231 ± 3.9	1.4
9	194	49 ± 1.18	4	340	213 ± 2.8	1.6
10	45	13 ± 0.6	3.5	71	45 ± 1.15	1.6
11	>380	>325	>1.2	271	244 ± 3.4	1.1

12	>353	>299	>1.2	312	271 ± 2.4	1.2
13	86	3 ± 0.1	28.7	135	7 ± 0.7	19.3
14	151	6 ± 0.4	25.2	173	7 ± 0.4	24.7
15	186	97 ± 2.15	1.9	>270	>236	>1.1
16	350	320	1.1	402	373	1.1
17	380	370	1	420	402	1.05
SOFOSBUVIR	ND	ND	ND	31	0.26	119.23

[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}).

ND: not determined

In the *in vitro* antiviral screening of the compounds, cytotoxicity analysis and antiviral assay using CSFV were performed according to the procedures previously reported [29,30]. MTS-based cell proliferation assay (CellTiter 96 AQueous nonradioactive cell proliferation assay) was carried out in order to determine the maximum concentrations of glycoconjugates that could be used without causing a significant effect on cell viability. The toxicity analysis of the compounds in SK6 cells demonstrated that tested compounds produced a dose-dependent toxic effect, however no obvious cytotoxicity was observed with selected working doses of the compounds in further experiments. Moreover, there was no cytotoxicity observed in cells treated with the highest concentrations of the solvent (DMSO) used in the screening.

Anti-CSFV activity of all compounds was determined by pseudo-plaque reduction assay where SK6 cells were infected with low MOI of the virus to visualize single plaques and incubated for 48 h with varying amounts of tested glycoconjugates. Among ten

synthesized compounds, some compounds were found to inhibit CSFV replication as evidenced by immunoperoxidase monalayer assay using rabbit polyclonal anti-E^{rns} serum to detect the viral antigen. These compounds had IC_{50} values < 50 µM showing selectivity indexes (SI), defined as the CC_{50}/IC_{50} ratio, from 3.5 to 28.7. Compound **13** exhibited the most potent antiviral activity from all synthesized compounds, with an IC_{50} value of 3 µM and low cytotoxicity (CC_{50} of 86 µM), resulting in a selectivity index of 28.7 (**Table 2**). Compound **14** also showed a very good activity against CSFV virus and had an IC_{50} value of 6 µM with similar to **13** selectivity index of 25.2. The typical IPMA reaction corresponding to the dose-dependent inhibition of the hit compound **13** on CSFV infectivity is shown in **Fig. 2A.** This compound caused more than 90% inhibition of CSFV infection at 9 µM concentration, which was evaluated by reduction in the average size and number of pseudo-plaques compared to the control.

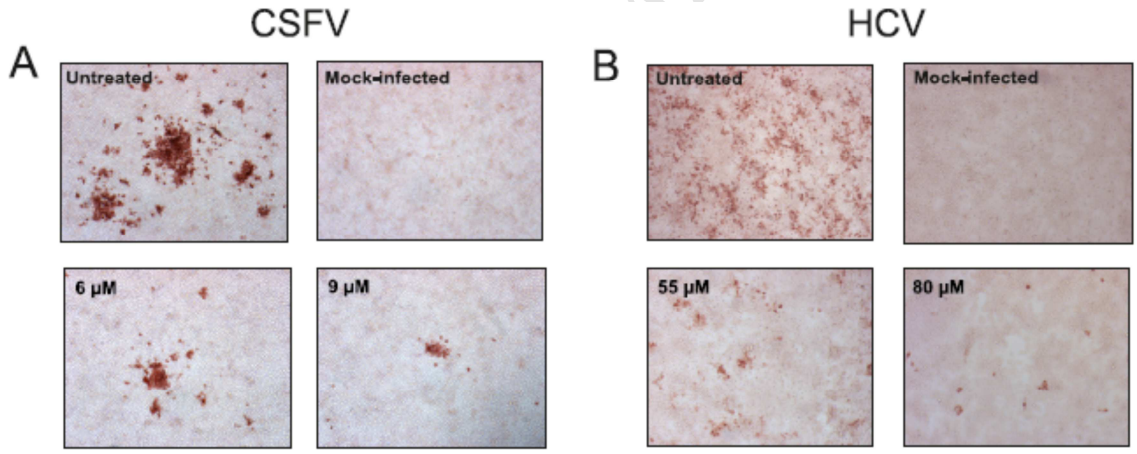


Fig. 2. Dose-dependent effect of compound 13 on CSFV (A) and HCV (B) pseudo-plaque formation in SK6 and Huh-7.5 cells, respectively. SK6 and Huh-7.5 cells were mock infected or infected with CSFV at an MOI of 0.001 (A) or with HCV at an MOI of 0.1 (B). At 2-3 h p.i. cells were treated with various concentrations of compound **13** or left untreated (positive control). Two or three days post infection, cells were fixed and IPMA was

performed using rabbit polyclonal anti-E^{rns} serum (A) or anti-HCV Core antibody (B) to detect CSFV and HCV pseudo-plaques, respectively.

The analysis of anti-CSFV activity of all synthesized compounds revealed that the sugar moiety type (D-glucose or D-galactose) does not significantly affect the biological activity. In all the examined structures, amidopyridyl aglycon is linked with the sugar unit by thioglycosyl linkage. In case of compounds **8**–**10** this heteroaromatic part is connected to uridine derivative through succinic linker. Compounds **8** and **9**, in which positions 2'-OH and 3'-OH in ribose were protected using isopropylidene group, showed low activity against CSFV virus with IC_{50} of 103 and 49 μM, respectively. Replacement of isopropylidene protecting group with TBDMS groups and introduction of additional benzoyl protection at *N*–3 position in uracil ring in compound **10** resulted in slight improvement in anti-CSFV activity as compared to the parent compound **8**. However, compound **10** had a higher toxicity to the virus host cells resulting in moderate *in vitro* selectivity index of 3.5. Significant loss of activity was observed for compounds **11** and **12** in which succinic linker was omitted and aminopyridyl 1-thioglycoside was directly connected to the uridine part. These structural modifications reduced the lipophilicity of compounds. Such a situation occurred when hydroxyl groups in ribose were protected with an isopropylidene group. Both compounds did not show any inhibition against CSFV in antiviral assay showing very low S.I. about 1. Changing the isopropylidene protective group in compounds **11** and **12** to TBDMS groups yielded respectively compounds **13** and **14**, which had a slightly higher toxicity to virus host cells however showed definitely higher activity. The increase of lipophilicity of compounds had beneficial effect on their antiviral activity. At this point it is worth mentioning that earlier observed increased toxicity of the compound **10** may be related to the fact that it may well act as a benzoylation agent. This observation was supported by the fact that compounds **13** and **14**, which lack the benzoyl group at the *N*–3 imide, do not show the increase in toxicity.

These two compounds exhibited the most potent antiviral activity in the series with IC_{50} values of 3 and 6 μM, respectively and significant selectivity indexes of 28.7 and 25.2. In case of compound **15,** which showed no activity in the antiviral screening, acetyl protecting groups in D-glucose part were replaced with benzoyl groups (positions 2-OH, 3-OH and 4-OH in sugar part) and with TBDMS group (position 6-OH in sugar part). In this case, all protective groups make compound **15** less toxic, however they are probably too bulky. These data suggest that TBDMS group, especially inserted in the uridine moiety, may be important for antiviral activity. Further experiments with the compound **16** showed, that the removal of protective groups from the positions 2–OH and 3–OH in the uridine moiety leads to a loss of antiviral activity. This is another proof of the importance of TBDMS groups in uridine part. The complete removal of protective groups in compound **17** did not influence substantially the results of antiviral activity with respect to compound **16**. It was also shown that the acetyl protective groups in sugar part affected the increase of glycoconjugate hydrophobicity but inside cells most likely they undergo enzymatic hydrolysis.

All synthesized compounds were also evaluated for anti-HCV activity in HCV cell culture system measured by HCVcc pseudo-plaque reduction assay (HCVcc, Jc1/JFH genotype 2a) in human hepatoma Huh-7.5 cells, with Sofosbuvir, an inhibitor of the HCV NS5B RNA-dependent RNA polymerase, as a positive control [31,32]. This system allows for complete replication of HCV including *in vitro* production and secretion of HCVcc. Infected cells were treated with increasing concentrations of the compounds, DMSO or Sofosbuvir and IPMA assay using the specific anti-HCV Core antibody was performed. All the compounds were used below their CC_{50} values as determined by MTT assay on Huh-7.5 cells after 48 h (**Table 2**). The analysis showed that two compounds (**13** and **14**) inhibited the Jc1/JFH virus propagation in a dose-dependent manner in Huh-7.5 cells and showed IC_{50} values of 7 μM

which led to selectivity index of 19.3 and 24.7, respectively. These results were in agreement with the experiments using CSFV indicating that synthetic compounds which showed the inhibitory effect against CSFV were also active against hepatitis C virus. Although, compound **13** showed poorer activity against HCV infection compared to its activity against CSFV, compound **14** enhanced its potency. It can be concluded that these two compounds effectively inhibit both flaviviruses. As shown in **Fig. 2B** the treatment with compound **13** resulted in a dose-dependent inhibition of viral replication, as monitored by IPMA, where significant reduction in the size as well as number of infected foci among the drug treated cells as compared to the untreated infected cells was observed. Compound **13** showed more than 90% inhibition of HCV infection at a concentration of 80 µM.

2.3. Inhibitory properties of selected compounds against CSFV and HCV viral glycoprotein synthesis.

In the light of promising activity of **13** and **14**, the action of these two compounds on proliferation of CSFV and HCV was more thoroughly examined. The compounds were synthesized as glycosyltransferase inhibitors, the enzymes involved in the glycosylation process. Therefore, the effect of selected compounds on the glycoprotein synthesis of both viruses was investigated using Western blot analysis under nonreducing and reducing conditions. The levels of CSFV E2 protein in SK6 infected cells and HCV E1 protein in Huh-7.5 infected cells treated with compounds **13** and **14** were measured by Western blot analysis using rabbit monospecific polyclonal serum anti-E2 CSFV and MAb anti-E1 HCV antibody, respectively. These two proteins are highly glycosylated containing 6 and 5 glycosylation sites, respectively.

Both analysis showed that the level of synthesis of structural proteins for CSFV- and HCV-infected cells was downregulated upon treatment with these compounds. Compounds **13** and **14** reduced CSFV E2 and HCV E1 protein production in a dose-dependent manner relative to the positive DMSO-treated control, confirming that these two selected compounds exerted an antiviral action against both CSFV (**Fig. 3**) and HCV (**Fig. 4**). The extent of reduction in expression of CSFV E2 and HCV E1 protein treated with both compounds was consistent with the results obtained in pseudo-plaques reduction assays.

Compound **13** completely inhibited CSFV E2 (**Fig. 3A**) and HCV E1 (**Fig. 4A**) protein synthesis at concentrations of 10 µM and 75 µM, respectively. Similarly, treatment with 15 µM for CSFV (**Fig. 3B**) and 75 µM for HCV (**Fig. 4B**) of compound **14** resulted in a complete arrest of CSFV E2 and HCV E1 glycoproteins synthesis compared to the untreated control samples. However, no unglycosylated or underglycosylated forms of CSFV E2 and HCV E1 proteins were detected after treatment with all, even the highest doses of both compounds. The CSFV and HCV 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 antibodies, suggesting that the disappearance of glycoproteins in the presence of inhibitor **13** and **14** was not related to the lack of reactivity with specific antibodies [29].

The consistent synthesis of internal control (housekeeping gene - β-actin) in all samples indicated no effect of tested concentrations of the compounds on the level of host cellular proteins. These results demonstrate that compounds **13** and **14** inhibit protein synthesis without cytoxicity.

The obtained results suggest that either the less glycosylated viral glycoproteins are not detectable due to quick degradation process of incorrectly matured proteins or they are not

produced due to other mechanism of action of synthesized compounds which can be associated with another step of the viral life cycle.

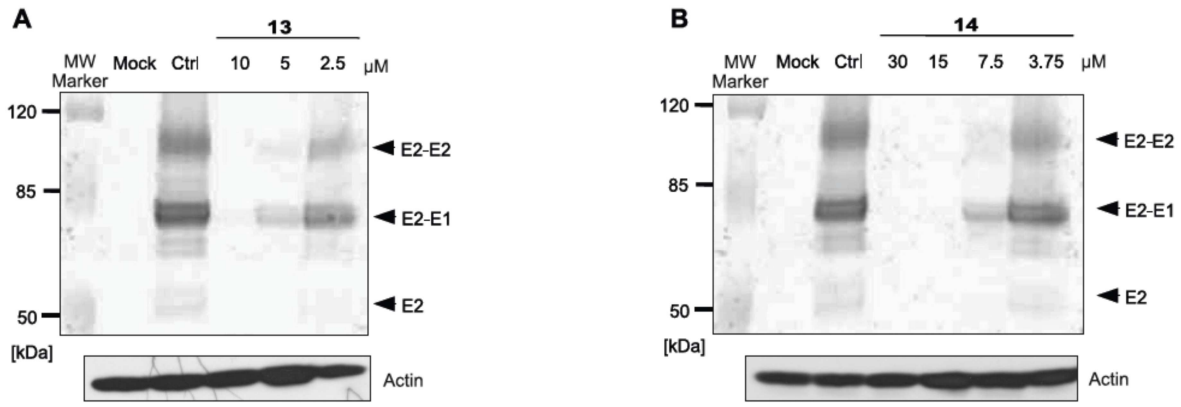


Fig. 3. Effect of compounds 13 and 14 on the E2 CSFV protein accumulation.

CSFV-infected SK6 cells were treated with varying concentrations of compound **13** (A) or **14** (B). At 48 h p.i., cells were lysed and 20 µg protein was analysed by Western blot with CSFV anti-E2 and anti-actin antibodies after SDS-PAGE under non-reducing conditions.

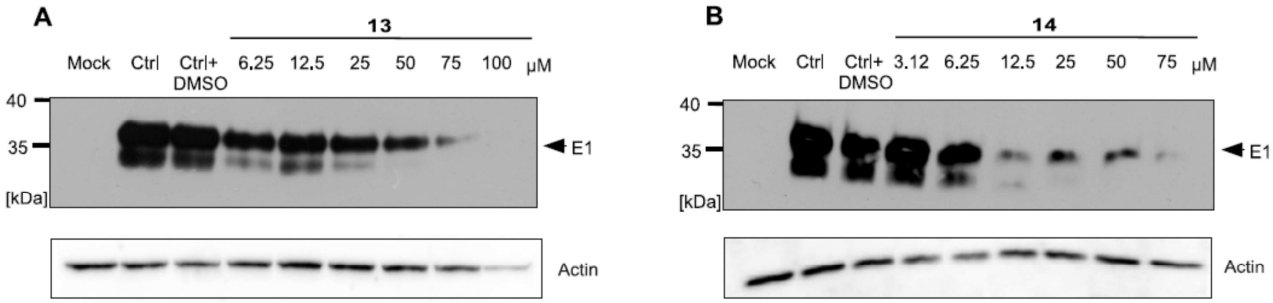


Fig. 4. Effect of compounds 13 and 14 on the E1 HCV protein accumulation.

HCV-infected Huh-7.5 cells were treated with varying concentrations of compound **13** (A) or **14** (B). At 72 h p.i., cells were lysed and 20 µg protein was analysed by Western blot with HCV E1 and anti-actin antibodies after SDS-PAGE under reducing conditions.

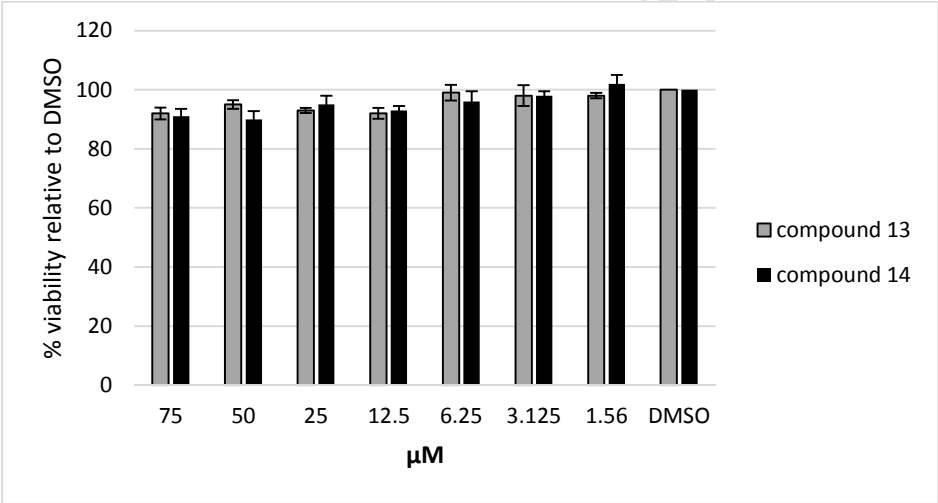
2.4. Inhibitory properties of 13 and 14 against HCV depending on the timing of infection and the exposure to compounds.

To further elucidate the mechanism of action and to assess the antiviral effect of the most active compounds (**13** and **14**) on HCV infection, they were tested in the three different models of infection according to Magri et al., 2016 using human hepatoma cell line Huh7-J20 generated to facilitate *in vitro* studies of HCV infection and replication [33]. This cell line stably expresses enhanced green fluorescent protein (EGFP) fused in-frame to secretory alkaline phosphatase (SEAP) via a recognition sequence of the viral NS3/4A serine protease as a reporter system [34]. The SEAP reporter is released from the fusion protein and secreted into the extracellular culture medium following cleavage by viral NS3/4A protease, produced during viral replication. The level of SEAP activity in the culture medium directly correlates with the level of intracellular viral RNA replication. In Model 1, Huh7-J20 cells were pre-treated for 1 h and infected with cell culture infectious HCV (HCVcc) genotype 2a strain JFH-1 in the presence of different concentrations of the compound or DMSO as a control for 3 h. The cells were then washed and fresh medium without the inhibitor was added for 72 h. This model was used to investigate a possible effect on HCV entry. In Model 2, Huh7-J20 cells were pre-treated for 1 h, infected with JFH-1 HCVcc for 3 h in the presence of various **13** and **14** concentrations or DMSO and then fresh medium with compound or DMSO was added for further 72 h. This model was used to evaluate the effect of inhibitors on full viral life cycle. In Model 3, Huh7-J20 cells were first infected for 3 h with the virus and then the fresh medium containing various concentrations of the inhibitor or DMSO was added in order to determine the effect of drugs on post-viral entry (e.g. RNA replication and/or virus

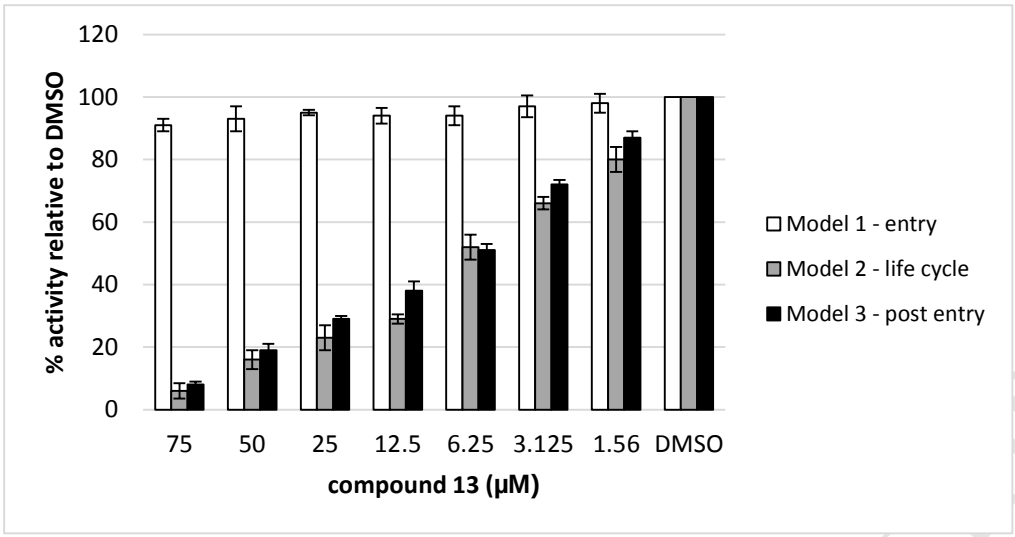
assembly). All of the doses of both compounds used in the experiments exerted no or little effect on Huh7-J20 cell viability as determined by MTT assay (**Fig. 5A**).

The results showed that none of the tested compounds showed inhibitory effect on virus entry (Model 1). Both compounds showed a strong anti-HCV activity on both the full life cycle (Model 2) or post-entry model (Model 3) observed by a significant reduction in the SEAP level, which suggest that they can affect virus genome replication. The results are summarized in **Fig. 5.** The same modes of action were obtained for Sobosbuvir used as a positive control, which is a licensed drug targeting viral replication (Supplementary Information).

A.



B.



C.

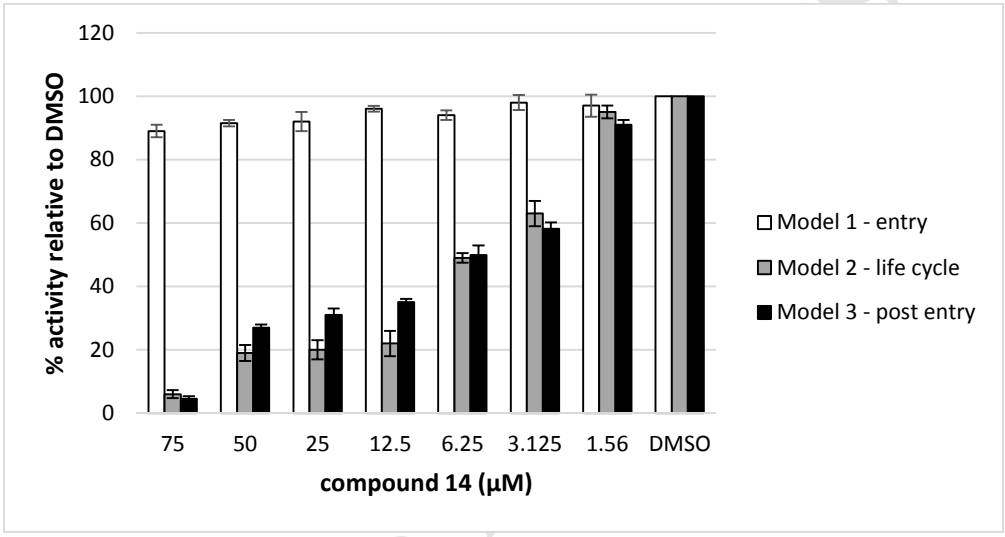


Fig. 5. Huh7-J20 cell viability (A) and antiviral activity of compounds 13 (B) and 14 (C) on HCV infection. (A) Cell viability analysis after treatment with different doses of compound **13** and **14** for 72 h without viral infection. The values of compound-treated cells are expressed as percentage relative to DMSO-treated cells expressed as 100% (control). (B, C) Huh7-J20 cells were pre-treated for 1 h and infected with cell culture infectious HCV in the presence of different concentrations of **13** (B), **14** (C) or DMSO as a control for 3 h. Then, the inoculum was removed and fresh medium without compound was added for 72 h (Model 1, white bars). Huh7-J20 cells were pre-treated for 1 h, infected with JFH-1 for 3 h in the

presence of various concentrations of **13** (B), **14** (C) or DMSO and then incubated for 72 h with fresh medium with inhibitor or DMSO (Model 2, grey bars). Huh7-J20 cells were infected for 3 h with JFH-1 and then treated with various concentrations of **13** (B), **14** (C) or DMSO for 72 h (Model 3, black bars). All inhibitory effects were determined by measuring SEAP assay performed on infected cell medium. Errors bars represent the SD of the means for 3 experiments.

2.5. The inhibitory effect of selected compounds on HCV replication.

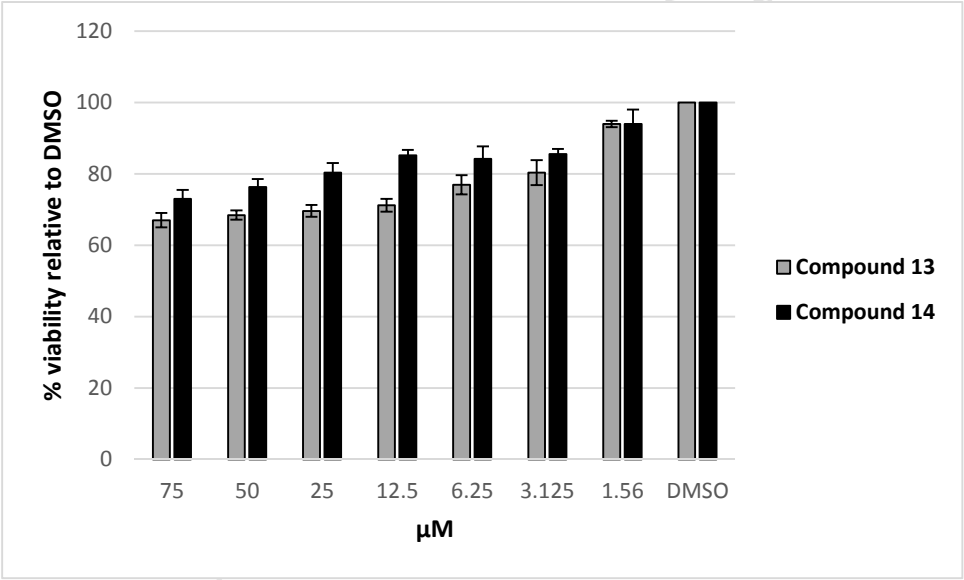
Due to the fact that previous experiments indicated that both compounds could interfere with viral replication, to confirm this hypothesis, both compounds were tested for the ability to inhibit HCV replication using Huh7-J17 stable cell line, which constitutively harbors the subgenomic HCV RNA. This is the puromycin-resistant cell line expressing monocistronic replicon encoding non-structural proteins, structural core protein and firefly luciferase as a reporter gene, the levels of which directly correlate with virus RNA replication [35].

These cells were plated on 96-well plates together with the various concentrations of both drugs or Sofosbuvir as a positive control, incubated for 72 h and inhibition of HCV RNA replication was estimated by measuring luciferase activity according to the protocol described in Materials and Methods. Huh7-J17 cell viability was measured by MTT assay (**Fig. 6A**).

The obtained results confirmed the previous observation that both compounds highly inhibited viral RNA replication (**Fig. 6B**). The highest doses of both compounds were able to block the replication nearly in 90%. Half maximal inhibitory concentration (IC_{50}) values for

compound **13** and **14** were 4.005 µM and 3.741 µM, respectively. The half maximal cytotoxic concentration (CC_{50}) values were 185 µM for **13** and 245 µM for **14**. Sofosbuvir displayed an IC_{50} of 0.028 µM and CC_{50} of 23.5 µM in our assay. All the data obtained using Huh7-J17 cells confirmed the hypothesis previously observed in HCVcc system that compounds **13** and **14** are able to inhibit HCV replication.

A.



B.

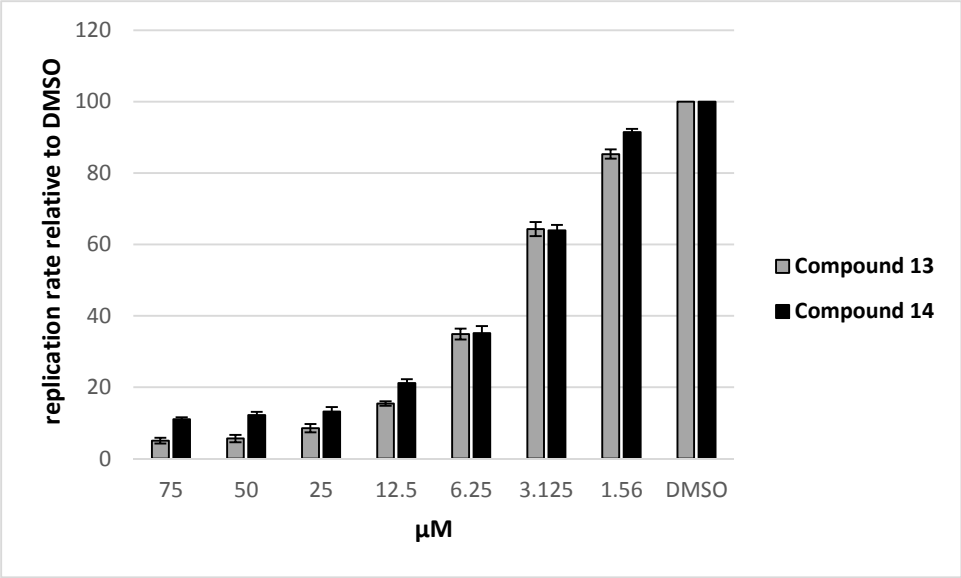


Fig. 6. Inhibitory effect of compounds 13 and 14 on viral replication. Huh7-J17 cells were seeded in the presence of various concentrations of **13** and **14**. After 72 h, cell viability was determined using MTT assay (A) or cells were lysed and luciferase levels were measured (B). The values of drug-treated cells are expressed as percentage relative to DMSO-treated cells expressed as 100% (control). Errors bars represent the SD of the means for 3 experiments.

Additionally, the effect of compounds **13** and **14** against viral RNA production was also determined using RT-PCR for NS5B gene performed on viral RNA isolated from Huh-7.5 HCV-infected cells treated with various concentrations of both compounds. As shown in **Fig. 7** treatment with these compounds resulted in a significant dose-dependent reduction in the amount of viral RNA level compared to untreated infected cells.

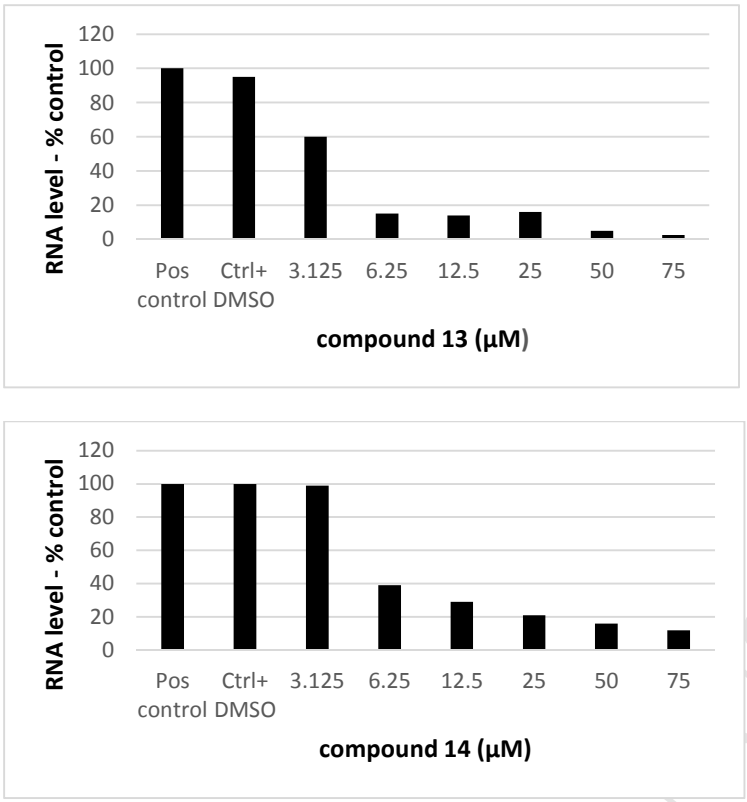


Fig. 7. Inhibition of HCV replication in the presence of compounds 13 and 14.

Huh-7.5 cells were infected with HCV for 4h, washed with PBS and fresh media was added with different concentrations of compounds. Total RNA was isolated after 48 h and subjected to RT-PCR for HCV NS5B gene to detect the level of viral RNA. The bar graphs present the densitometry analysis of NS5B level detected in infected Huh-7 cells treated with different doses of compounds expressed as a percentage of NS5B detected in the control.

3. Conclusions

In this study, we describe the synthesis of glycoconjugates and the evaluation of their antiviral activity against two viruses: HCV and CSFV. In these compounds (5-amino-2-pyridyl) 1-thioglycosides are connected via an amide bond with a uridine derivative with or without succinic linker. Our experiments showed that succinic linker omission and direct connection of acetylated (5-amino-2-pyridyl) 1-thioglycosides and 2',3'-di-*O*-*tert*-

butyldimethylsilyluridine 5'-carboxylic acid through an amide bond is important for antiviral activity, as evidenced by compounds **13** and **14**. Changing of the protecting groups in both part of glycoconjugates reduced the inhibitory effect of these glycoconjugates (compounds **11**, **12** and **15**). Also partial or full deprotection of glycoconjugates significantly impairs their activity (compounds **16** and **17**).

Four out of ten synthesized compounds were shown to inhibit either CSFV or HCV replication in cell culture system, two of which inhibited both viruses. The most promising results were obtained for glycoconjugates **13** and **14**, which showed high antiviral activity and acceptable selectivity indexes in both antiviral activity assays. The IC_{50} values of these two compounds were in low micromolar range and they were found to be non-toxic at concentrations up to 150-245 μM, depending on cell type, which confirmed their antiviral potential. We showed that both compounds were at least 5 times less toxic than Sofosbuvir. These two compounds inhibited both CSFV and HCV viral propagation as well as CSFV E2 and HCV E1 protein synthesis at low concentrations. Using cell-based assays involving three different models of infection we showed that compounds **13** and **14** do not affect viral entry, but both compounds significantly inhibited viral genome replication process by up to 90%. Further studies using Huh7-J17 replicon cell line confirmed the hypothesis that both compounds target mainly viral genome replication.

Overall, our data suggest that compounds **13** and **14** can be potentially developed as anti-CSFV and/or anti-HCV compounds. Moreover, these compounds have the very good potential to be lead compounds on the way to novel next generation compounds that can be used as potential inhibitors against other viruses. Therefore, the antiviral activities of these two compounds against other emerging and neglected RNA viruses belonging to the flavivirus genus such as dengue virus, zika virus and tick-borne encephalitis virus will be examined in the near future.

4. Experimental

4.1. Synthesis

Microwave reactions were carried out in a Discover® BenchMate^{TM} (CEM) microwave equipped with 10 mL vessels using a standard program at 50 °C (max. pressure 1.5 Bar, average potency 20 W). NMR spectra were recorded for solutions in $CDCl_3$ or DMSO-d_6 with TMS as the internal standards using Varian spectrometers at a frequency of 300 MHz and 600 MHz or an Agilent spectrometer at a frequency of 400 MHz. NMR solvents were purchased from ACROS Organics (Geel, Belgium). Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in Hz. The ^{1}H-NMR and ^{13}C-NMR signals of some compounds were assigned using COSY, HMQC and HMBC experiments. Optical rotations were measured with a JASCO P-2000 polarimeter using a sodium lamp (589.3 nm) at room temperature. Melting point measurements were performed on a Stanford Research Systems OptiMelt (MPA 100). Mass spectra were measured in the positive mode with a Mariner (Perspective Biosystem) detector using electrospray-ionization (ESI) technique. Reactions were monitored by TLC on precoated plates of silica gel 60 F254 (Merck Millipore). TLC plates were inspected under UV light (λ = 254 nm) or charring after spraying with 10% sulfuric acid in ethanol. Crude products were purified using column chromatography performed on Silica Gel 60 (70-230 mesh, Fluka) developed using toluene/EtOAc or $CHCl_3$/MeOH solvent systems. All evaporations were performed on a rotary evaporator under diminished pressure at 50°C. Purity of tested compounds **8**-**17** was determined using HPLC-MS/MS. All tested compounds had a purity at least 95%.

2',3'-*O*-Isopropylidene-uridine **1a** [36], succinic acid mono-2',3'-*O*-isopropylidene-uridin-5'yl ester **1**, succinic acid mono-3-*N*-benzoyl-2',3'-di-*O*-*tert*-butyldimethylsilyl-uridin-5'-yl

ester **2** [20], 2',3'-di-*O*-*tert*-butyldimethylsilyl-uridine **2a** [37], 3-*N*-benzoyl-2',3'-di-*O*-*tert*-butyldimethylsilyl-uridine **2b** [20], 2',3'-*O*-isopropylideneuridine-5'-carboxylic acid **3** [22], (5-amino-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside **5**, (5-amino-2-pyridyl) 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside **6** [23], (5-nitro-2-pyridyl) 2,3,4-tri-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl-1-thio-β-D-glucopyranoside **7d** [38], 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) [39] were prepared according to the respective published procedures. All the chemicals that were used were purchased from Sigma–Aldrich, Fluka and ACROS Organics and were used without purification.

4.1.1. Synthesis of glycoconjugates 8-15

Procedure A. To a solution of amine **5**, **6** or **7** (0.25 mmol) and uridine derivative **1**–**4** (0.25 mmol) in dry THF (5 mL) with addition of MeOH (0.3 mL), a previously obtained DMTMM (0.07 g, 0.25 mmol) and *N*-methylmorpholine (0.016 mL, 0.12 mmol) were added. The mixture was stirred at room temperature for 24 to 96 h (appropriate reaction times are given in Table 1). The reaction progress was monitored on TLC in two alternative eluents – $CHCl_3$:MeOH (10:1) or toluene:AcOEt (1:1). After completion, reaction mixtures were concentrated to give crude products **8–15** which were purified directly by column chromatography with an appropriate solvent system as indicated.

Procedure B. The appropriate amine **5, 6** or **7** (0.25 mmol) and uridine derivative **1**–**4** (0.25 mmol) were dissolved in dry THF (5 mL). The DMTMM (0.07 g, 0.25 mmol) and *N*-methylmorpholine (0.016 mL, 0.12 mmol) were added to this mixture. The resulting mixture was microwaved in a reactor set for 2 h. The reaction progress was monitored on TLC in two alternative eluents – $CHCl_3$:MeOH (10:1) or toluene:AcOEt (1:1). Then the solvent was evaporated and the residue was dissolved in CH_2Cl_2 (30 mL), washed two times with brine

(5 mL), dried with $MgSO_4$, the adsorbent was filtered off and the filtrate was concentrated. Crude products were purified by column chromatography with an appropriate solvent system as indicated to give products **8–15**.

4.1.1.1. Glycoconjugate **8**.

Starting from amine **5** (0.114 g) and uridine derivative **1** (0.096 g) according to **Procedure A**, glycoconjugate **8** was obtained after column chromatography (toluene : AcOEt; gradient: 10:1 to 1:1, then $CHCl_3$: MeOH 100:1 to 20:1) as a white solid (0.109 g, 53%), according to **Procedure B** (0.115 g, 56%). Physicochemical parameters and the spectral data of the obtained product **8** were consistent with the literature [20].

4.1.1.2. Glycoconjugate **9**.

Starting from amine **6** (0.114 g) and uridine derivative **1** (0.096 g) according to **Procedure A**, glycoconjugate **9** was obtained after column chromatography (toluene : AcOEt; gradient: 10:1 to 1:1, then $CHCl_3$: MeOH 100:1 to 20:1) as a white solid (0.081 g, 39%), according to **Procedure B** (0.085 g, 41%). Physicochemical parameters and the spectral data of the obtained product **9** were consistent with the literature [20].

4.1.1.3. Glycoconjugate **10**.

Starting from amine **5** (0.114 g) and uridine derivative **2** (0.169 g) according to **Procedure A**, glycoconjugate **10** was obtained after column chromatography (toluene : AcOEt; gradient: 10:1 to 1:1, then $CHCl_3$: MeOH 100:1 to 60:1) as a white solid (0.142 g, 51%), according to **Procedure B** (0.161 g, 58%). Physicochemical parameters and the spectral data of the obtained product **10** were consistent with the literature [20].

4.1.1.4. Glycoconjugate **11**.

Starting from amine **5** (0.114 g) and uridine derivative **3** (0.075 g) according to **Procedure A**, glycoconjugate **11** was obtained after column chromatography (toluene : AcOEt; gradient: 10:1 to 1:1, then $CHCl_3$: MeOH 100:1 to 20:1) as a white solid (0.070 g, 38%), according to **Procedure B** (0.090 g, 49%): m.p. 159–160 °C; $[\alpha]_D^{20}$ -46.5 (c 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.37, 1.59 (2s, 6H, $(CH_3)_2C$), 2.01, 2.02, 2.03, 2.04 (4s, 12H, CH_3CO), 3.87 (ddd, 1H, *J* = 2.4 Hz, *J* = 4.4 Hz, *J* = 10.0 Hz, H-5_{glu}), 4.08 (dd, 1H, *J* = 2.4 Hz, *J* = 12.4 Hz, H-$6a_{glu}$), 4.25 (dd, 1H, *J* = 4.4 Hz, *J* = 12.4 Hz, H-$6b_{glu}$), 4.72 (d, 1H, *J* = 2.4 Hz, H-$4'_{ur}$), 5.15 (dd, 1H, *J* = 9.6 Hz, *J* = 10.0 Hz, H-4_{glu}), 5.18 (dd, 1H, *J* = 9.4 Hz, *J* = 10.2 Hz, H-2_{glu}), 5.26 (dd, 1H, *J* = 2.4 Hz, *J* = 6.6 Hz, H-$3'_{ur}$), 5.29 (dd, 1H, *J* = 2.2 Hz, *J* = 6.6 Hz, H-$2'_{ur}$), 5.36 (dd, 1H, *J* = 9.4 Hz, *J* = 9.6 Hz, H-3_{glu}), 5.49 (d, 1H, *J* = 2.2 Hz, H-$1'_{ur}$), 5.68 (d, 1H, *J* = 10.2 Hz, H-1_{glu}), 5.78 (d, 1H, *J* = 8.3 Hz, H-5_{ur}), 7.20 (d, 1H, *J* = 8.6 Hz, H-3_{pyr}), 7.28 (d, 1H, *J* = 8.1 Hz, H-6_{ur}), 7.97 (dd, 1H, *J* = 2.4 Hz, *J* = 8.6 Hz, H-4_{pyr}), 8.49 (d, 1H, *J* = 2.4 Hz, H-6_{pyr}), 8.67 (s, 1H, NH), 9.50 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.61, 20.69, 20.74 ($\underline{C}H_3CO$), 24.99, 26.94 (($\underline{C}H_3)_2C$), 62.01 (C-6_{glu}), 68.39 (C-4_{glu}), 69.65 (C-2_{glu}), 74.05 (C-3_{glu}), 75.76 (C-5_{glu}), 82.39 (C-1_{glu}), 82.76 (C-$3'_{ur}$), 83.66 (C-$2'_{ur}$), 87.54 (C-$4'_{ur}$), 99.56 (C-$1'_{ur}$), 103.21 (C-5_{ur}), 114.65 ($(CH_3)_2\underline{C}$), 123.85 (C-3_{pyr}), 128.44 (C-4_{pyr}), 132.24 (C-2_{pyr}), 141.45 (C-6_{pyr}), 144.07 (C-6_{ur}), 150.07 (C-5_{pyr}), 150.61 (C-2_{ur}), 162.84 (C-4_{ur}), 167.88, 169.49, 169.57, 170.20, 170.86 (CO); ESI-HRMS: calcd for $C_{31}H_{36}N_4O_{15}SNa$ ($[M+Na]^+$): m/z 759.1790; found: m/z 759.1818.

4.1.1.5. Glycoconjugate **12**.

Starting from amine **6** (0.114 g) and uridine derivative **3** (0.075 g) according to **Procedure A**, glycoconjugate **12** was obtained after column chromatography (toluene : AcOEt; gradient: 10:1 to 1:1, then $CHCl_3$: MeOH 100:1 to 20:1) as a white solid (0.053 g, 29%), according to **Procedure B** (0.061 g, 33%): m.p. 161–163 °C; $[\alpha]_D^{20}$ -40.4 (c 1.0,

$CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.37, 1.59 (2s, 6H, $(CH_3)_2C$), 1.99, 2.00, 2.02, 2.17 (4s, 12H, CH_3CO), 4.04–4.16 (m, 3H, H-5_{gal}, $6a_{gal}$, H-$6b_{gal}$), 4.73 (d, 1H, *J* = 2.4 Hz, H-4'$_{ur}$), 5.18 (dd, 1H, *J* = 3.4 Hz, *J* = 9.8 Hz, H-3_{gal}), 5.23 (dd, 1H, *J* = 2.4 Hz, *J* = 6.6 Hz, H-3'$_{ur}$), 5.27 (dd, 1H, *J* = 2.3 Hz, *J* = 6.6 Hz, H-2'$_{ur}$), 5.38 (dd, 1H, *J* = 9.8 Hz, *J* = 10.6 Hz, H-2_{gal}), 5.48 (d, 1H, *J* = 3.4 Hz, H-4_{gal}), 5.52 (d, 1H, *J* = 2.3 Hz, H-1'$_{ur}$), 5.63 (d, 1H, *J* = 10.6 Hz, H-1_{gal}), 5.77 (d, 1H, *J* = 8.2 Hz, H-5_{ur}), 7.22 (d, 1H, *J* = 8.6 Hz, H-3_{pyr}), 7.31 (d, 1H, *J* = 8.2 Hz, H-6_{ur}), 8.00 (dd, 1H, *J* = 2.7 Hz, *J* = 8.6 Hz, H-4_{pyr}), 8.51 (d, 1H, *J* = 2.4 Hz, H-6_{pyr}), 8.75 (s, 1H, NH), 9.60 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.55, 20.64, 20.73 ($\underline{C}H_3CO$), 24.97, 26.91 (($\underline{C}H_3)_2C$), 61.26 (C-6_{gal}), 66.91, 67.30 (C-4_{gal}, C-2_{gal}), 72.01 (C-3_{gal}), 74.38 (C-5_{gal}), 82.61(C-2'$_{ur}$), 82.83 (C-1_{gal}), 83.51 (C-3'ur), 87.24 (C-4'$_{ur}$), 99.29 (C-1'$_{ur}$), 103.18 (C-5_{ur}), 114.66 ($(CH_3)_2\underline{C}$), 123.69 (C-3_{pyr}), 128.39 (C-4_{pyr}), 132.18 (C-2_{pyr}), 141.39 (C-6_{pyr}), 143.93 (C-6_{ur}), 150.24 (C-5_{pyr}), 150.56 (C-2_{ur}), 162.85 (C-4_{ur}), 167.79, 169.67, 170.01, 170.24, 170.54 (CO); ESI-HRMS: calcd for $C_{31}H_{36}N_4O_{15}SNa$ ($[M+Na]^+$): m/z 759.1790; found: m/z 759.1801.

4.1.1.6. Glycoconjugate **13**.

Starting from amine **5** (0.114 g) and uridine derivative **4** (0.122 g) according to **Procedure A**, glycoconjugate **13** was obtained after column chromatography (toluene : AcOEt; gradient: 10:1 to 1:1, then $CHCl_3$: MeOH 100:1 to 60:1) as a white solid (0.074 g, 32%), according to **Procedure B** (0.122 g, 53%): m.p. 137–139 °C; $[\alpha]_D^{20}$ -66.2 (c 0.4, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ -0.05, 0.03, 0.15, 0.24 (4s, 12H, CH_3Si), 0.85, 0.96 (2s, 18H, $(CH_3)_3CSi$), 2.03, 2.04, 2.05 (3s, 12H, CH_3CO), 3.88 (ddd, 1H, *J* = 2.2 Hz, *J* = 4.6 Hz, *J* = 10.0 Hz, H-5_{glu}), 4.11 (dd, 1H, *J* = 2.2 Hz, *J* = 12.4 Hz, H-$6a_{glu}$), 4.28 (dd, 1H, *J* = 4.6 Hz, *J* = 12.4 Hz, H-$6b_{glu}$), 4.33 (d, 1H, *J* = 4.4 Hz, H-3'$_{ur}$), 4.56 (s, 1H, H-4'$_{ur}$), 4.89 (dd, 1H, *J* = 4.4 Hz, *J* = 8.1 Hz, H-2'$_{ur}$), 5.16 (dd, 1H, *J* = 9.4 Hz, *J* = 10.0 Hz, H-4_{glu}), 5.18 (dd,

1H, *J* = 9.3 Hz, *J* = 10.3 Hz, H-2_{glu}), 5.28 (d, 1H, *J* = 8.1 Hz, H-1'$_{ur}$), 5.36 (dd, 1H, *J* = 9.3 Hz, *J* = 9.4 Hz, H-3_{glu}), 5.70 (d, 1H, *J* = 10.3 Hz, H-1_{glu}), 5.88 (d, 1H, *J* = 8.0 Hz, H-5_{ur}), 7.23 (d, 1H, *J* = 8.6 Hz, H-3_{pyr}), 7.28 (d, 1H, *J* = 8.0 Hz, H-6_{ur}), 8.15 (dd, 1H, *J* = 2.6 Hz, *J* = 8.6 Hz, H-4_{pyr}), 8.76 (d, 1H, *J* = 2.6 Hz, H-6_{pyr}), 9.69 (s, 1H, NH), 10.00 (s, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ -5.35, -4.86, -4.75, -4.60 (CH_3Si), 17.73, 17.90 (($CH_3)_3\underline{C}$), 20.51, 20.58, 20.62, 21.34 ($\underline{C}H_3CO$), 25.57, 25.68 (($\underline{C}H_3)_3CSi$), 61.91 (C-6_{glu}), 68.22 (C-4_{glu}), 69.50 (C-2_{glu}, C-2'$_{ur}$), 74.02 (C-3_{glu}), 74.88 (C-3'$_{ur}$), 75.74 (C-5_{glu}), 82.24 (C-1_{glu}), 86.10 (C-4'$_{ur}$), 96.97 (C-1'$_{ur}$), 103.01 (C-5_{ur}), 123.51 (C-3_{pyr}), 127.66 (C-4_{pyr}), 132.61 (C-2_{pyr}), 141.23 (C-6_{pyr}), 145.49 (C-6_{ur}), 149.62 (C-5_{pyr}), 150.77 (C-2_{ur}), 162.88 (C-4_{ur}), 167.72, 169.36, 169.44, 170.07, 170.67 (CO); ESI-HRMS: calcd for $C_{40}H_{60}N_4O_{15}SSi_2Na$ ($[M+Na]^+$): m/z 947.3207; found: m/z 947.3175.

4.1.1.7. Glycoconjugate **14**.

Starting from amine **6** (0.114 g) and uridine derivative **4** (0.122 g) according to **Procedure A**, glycoconjugate **14** was obtained after column chromatography (toluene : AcOEt; gradient: 10:1 to 1:1, then $CHCl_3$: MeOH 100:1 to 60:1) as a white solid (0.099 g, 43%), according to **Procedure B** (0.141 g, 61%): m.p. 144–145 °C; $[\alpha]_D^{20}$ -35.7 (c 0.8, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ -0.06, 0.03, 0.15, 0.24 (4s, 12H, CH_3Si), 0.84, 0.96 (2s, 18H, $(CH_3)_3CSi$), 2.00, 2.01, 2.04, 2.17 (4s, 12H, CH_3CO), 4.05–4.18 (m, 3H, H-5_{gal}, $6a_{gal}$, H-$6b_{gal}$), 4.32 (d, 1H, *J* = 4.5 Hz, H-3'$_{ur}$), 4.53 (s, 1H, H-4'$_{ur}$), 4.88 (dd, 1H, *J* = 4.5 Hz, *J* = 8.3 Hz, H-2'$_{ur}$), 5.18 (dd, 1H, *J* = 3.4 Hz, *J* = 9.9 Hz, H-3_{gal}), 5.26 (d, 1H, *J* = 8.3 Hz, H-1'$_{ur}$), 5.38 (dd, 1H, *J* = 9.9 Hz, *J* = 10.2 Hz, H-2_{gal}), 5.48 (d, 1H, *J* = 3.4 Hz, H-4_{gal}), 5.74 (d, 1H, *J* = 10.2 Hz, H-1_{gal}), 5.82 (d, 1H, *J* = 8.2 Hz, H-5_{ur}), 7.22 (d, 1H, *J* = 8.6 Hz, H-3_{pyr}), 7.31 (d, 1H, *J* = 8.2 Hz, H-6_{ur}), 8.28 (dd, 1H, *J* = 2.4 Hz, *J* = 8.6 Hz, H-4_{pyr}), 8.65 (s, 1H, NH), 8.76 (d, 1H, *J* = 2.4 Hz, H-6_{pyr}), 10.12 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$): δ -5.23, -

4.71, -4.60, -4.42 (CH_3Si), 17.88, 18.06 (($CH_3)_3\underline{C}$), 20.62, 20.73, 20.82 ($\underline{C}H_3CO$), 25.71, 25.82 (($\underline{C}H_3)_3CSi$), 61.28 (C-6_{gal}), 66.95, 67.34 (C-4_{gal}, C-2_{gal}), 69.47 (C-2'$_{ur}$), 72.05 (C-3_{gal}), 74.60 (C-5_{gal}), 75.05 (C-3'ur), 82.72 (C-1_{gal}), 86.35 (C-4'$_{ur}$), 97.31 (C-1'$_{ur}$), 103.35 (C-5_{ur}), 124.34 (C-3_{pyr}), 128.62 (C-4_{pyr}), 133.16 (C-2_{pyr}), 140.20 (C-6_{pyr}), 145.40 (C-6_{ur}), 149.86 (C-5_{pyr}), 150.95 (C-2_{ur}), 161.69(C-4_{ur}), 168.01, 169.79, 169.97, 170.25, 170.44 (CO); ESI-HRMS: calcd for $C_{40}H_{60}N_4O_{15}SSi_2Na$ ($[M+Na]^+$): m/z 947.3207; found: m/z 947.3160.

4.1.1.8. Glycoconjugate **15**.

Starting from amine **7** (0.178 g) and uridine derivative **4** (0.122 g) according to **Procedure A**, glycoconjugate **15** was obtained after column chromatography (toluene : AcOEt; gradient: 10:1 to 1:1) as a white solid (0.153 g, 52%), according to **Procedure B** (0.160 g, 54%): m.p. 123–125 °C; $[\alpha]_D^{20}$ 26.2 (c 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ --0.10, -0.05, -0.3, 0.03, 0.15, 0.24 (6s, 18H, CH_3Si), 0.79, 0.84, 0.96 (3s, 27H, $(CH_3)_3CSi$), 3.80 (dd, 1H, *J* = 5.1 Hz, *J* = 11.6 Hz, H-$6a_{glu}$), 3.84 (dd, 1H, *J* = 2.1 Hz, *J* = 11.6 Hz, H-$6b_{glu}$), 4.07 (ddd, 1H, *J* = 2.1 Hz, *J* = 5.1 Hz, *J* = 9.9 Hz, H-5_{glu}), 4.32 (d, 1H, *J* = 4.5 Hz, H-4'$_{ur}$), 4.53 (s, 1H, H-1'$_{ur}$), 4.88 (dd, 1H, *J* = 4.5 Hz, *J* = 8.2 Hz, H-3'$_{ur}$), 5.25 (d, 1H, *J* = 8.2 Hz, H-2'$_{ur}$), 5.57 (dd, 1H, *J* = 9.7 Hz, *J* = 9.9 Hz, H-4_{glu}), 5.64 (dd, 1H, *J* = 9.5 Hz, *J* = 10.2 Hz, H-2_{glu}), 5.82 (dd, 1H, *J* = 2.0Hz, *J* = 8.1 Hz, H-5_{ur}), 5.97 (dd, 1H, *J* = 9.5 Hz, *J* = 9.7 Hz, H-3_{glu}), 6.06 (d, 1H, *J* = 10.2 Hz, H-1_{glu}), 7.14-7.56 (m, 11H, H_{Ph}, H-3_{pyr}, H-6_{ur}), 7.78-7.98 (m, 6H, H_{Ph}), 8.10 (dd, 1H, *J* = 2.5 Hz, *J* = 8.7 Hz, H-4_{pyr}), 8.75 (d, 1H, *J* = 2.6 Hz, H-6_{pyr}), 8.95 (bs, 1H, NH), 9.97 (s, 1H, NH); ^{13}C NMR (75 MHz, $CDCl_3$): δ -5.17, -5.16, -5.01, -4.51, -4.38, -4.22 (CH_3Si), 18.07, 18.26, 18.50 (($CH_3)_3\underline{C}$), 25.92, 26.03, 26.04 (($\underline{C}H_3)_3CSi$), 63.26 (C-6_{glu}), 63.26 (C-6_{glu}), 69.71 (C-2'$_{ur}$), 69.92 (C-4_{glu}), 70.81 (C-2_{glu},), 74.89 (C-3_{glu}), 75.24 (C-3'$_{ur}$), 79.75 (C-5_{glu}), 82.77 (C-1_{glu}), 86.53 (C-4'$_{ur}$), 97.38 (C-1'$_{ur}$), 103.34 (C-5_{ur}), 123.75 (C-3_{pyr}), 128.50, 128.62, 129.23, 129.28, 129.36, 129.43, 129.98, 130.04, 130.16 (C_{Ph}, C-4_{pyr}),

132.73 (C-2_{pyr}), 133.36, 133.43, 133.52 (C_{Ph}), 141.50 (C-6_{pyr}), 145.79 (C-6_{ur}), 150.56 (C-5_{pyr}), 150.93 (C-2_{ur}), 157.90 (C-4_{ur}), 162.60, 165.40, 165.57, 166.09, 167.93 (CO); ESI-HRMS: calcd for $C_{59}H_{78}N_4O_{14}SSi_3Na$ ($[M+Na]^+$): m/z 1205.4441; found: m/z 1205.4434.

4.1.2. Deprotection of glycoconjugate 13

Procedure C. Glycoconjugate **11** (50 mg, 0.070 mmol) was dissolved in MeOH (2.5 mL). To obtained solution H_2O was added (2.5 mL) and then Amberlyst-15 was added up until pH=3. Reaction was carried out for 2 h at 70 °C. Reaction mixture was filtered, neutralized with aqueous ammonia solution and concentrated *in vacuo* with silica gel and purified by column chromatography ($CHCl_3$: MeOH; gradient: 60:1 to 10:1) to give **16** as white solid (34 mg, 73%): m.p. 141−143 °C; $[\alpha]_D^{20}$ -44.3 (c 1.0, MeOH); ^{1}H NMR (600 MHz, CD_3OD): δ 1.98, 2.018, 2.019, 2.021 (4s, 12H, CH_3CO), 3.99 (ddd, 1H, *J* = 2.3 Hz, *J* = 5.3 Hz, *J* = 10.0 Hz, H-5_{glu}), 4.11 (dd, 1H, *J* = 2.3 Hz, *J* = 12.3 Hz, H-$6a_{glu}$), 4.26 (dd, 1H, *J* = 5.3 Hz, *J* = 12.3 Hz, H-$6b_{glu}$), 4.35 (dd, 1H, *J* = 2.9 Hz, *J* = 4.7 Hz, H-3'$_{ur}$), 4.55 (d, 1H, *J* = 2.9 Hz, H-4'$_{ur}$), 4.59 (dd, 1H, *J* = 4.7 Hz, *J* = 5.9 Hz, H-2'$_{ur}$), 5.07 (dd, 1H, *J* = 9.7 Hz, *J* = 10.0 Hz, H-4_{glu}), 5.09 (dd, 1H, *J* = 9.4 Hz, *J* = 10.0 Hz, H-2_{glu}), 5.37 (dd, 1H, *J* = 9.3 Hz, *J* = 9.7 Hz, H-3_{glu}), 5.59 (d, 1H, *J* = 10.0 Hz, H-1_{glu}), 5.77 (d, 1H, *J* = 8.0 Hz, H-5_{ur}), 5.79 (d, 1H, *J* = 5.9 Hz, H-1'$_{ur}$), 7.41 (d, 1H, *J* = 8.6 Hz, H-3_{pyr}), 7.88 (s, 1H, NH), 8.06 (d, 1H, *J* = 8.0 Hz, H-6_{ur}), 8.10 (dd, 1H, *J* = 2.6 Hz, *J* = 8.6 Hz, H-4_{pyr}), 8.75 (d, 1H, *J* = 2.6 Hz, H-6_{pyr}), ^{13}C NMR (150 MHz, CD_3OD): δ 20.64, 20.66, 20.69, 20.77 ($\underline{C}H_3CO$), 63.37 (C-6_{glu}), 69.82 (C-4_{glu}), 71.20, 73.30, 74.83, 75.41, 77.01, 83.85, 85.70 (C-2_{glu}, C-2'$_{ur}$, C-3_{glu}, C-3'$_{ur}$, C-5_{glu}, C-4'$_{ur}$, C-1_{glu}), 94.38 (C-1'$_{ur}$), 103.26 (C-5_{ur}), 125.28 (C-3_{pyr}), 129.95 (C-4_{pyr}), 134.67 (C-2_{pyr}), 142.62 (C-6_{pyr}), 145.06 (C-6_{ur}), 151.74 (C-5_{pyr}), 152.82 (C-2_{ur}), 166.04 (C-4_{ur}), 170.99, 171.15, 171.34, 171.66 (CO), 172.37 (NHCO); ESI-HRMS: calcd for $C_{28}H_{32}N_4O_{15}SNa$ ($[M+Na]^+$): m/z 719.1483; found: m/z 719.1471.

Procedure D. Glycoconjugate **11** (120 mg, 0.160 mmol) was dissolved in MeOH (5.5 mL) then 1 M solution of NaOMe (150 µL, 0.150 mmol) was added. Reaction was mixed for 1 h, then H_2O (5.5 mL) and Amberlyst 15 were added up until pH=3. Reaction was continued for 2 h at 70 °C. Reaction mixture was filtered, neutralized with aqueous ammonia solution and concentrated *in vacuo* with silica gel and purified by column chromatography ($CHCl_3$: MeOH; gradient: 7:1 to 1:1 and only MeOH) to give **17** as white solid (29 mg, 38%) : m.p. 162−163 °C; $[\alpha]_D^{20}$ -20.8 (c 0.5, H_2O); ^{1}H NMR (400 MHz, CD_3OD): δ 3.14 (dd, 1H, *J* = 7.8 Hz, *J* = 9.0 Hz, H-2_{glu}), 3.35 (dd, 1H, *J* = 7.4 Hz, *J* = 8.6 Hz, H-4_{glu}), 3.46 (dd, 1H, *J* = 7.8 Hz, *J* = 8.6 Hz, H-3_{glu}), 3.75-3.89 (m, 3H, H-5_{glu}, H-$6a_{glu}$, H-$6b_{glu}$), 4.34 (dd, 1H, *J* = 2.8 Hz, *J* = 5.0 Hz, H-3'$_{ur}$), 4.54 (d, 1H, *J* = 2.8 Hz, H-4'$_{ur}$), 4.58 (dd, 1H, *J* = 5.0 Hz, *J* = 6.3 Hz, H-2'$_{ur}$), 5.11 (d, 1H, *J* = 9.0 Hz, H-1_{glu}), 5.77 (d, 1H, *J* = 8.2 Hz, H-5_{ur}), 5.80 (d, 1H, *J* = 6.3 Hz, H-1'$_{ur}$), 7.49 (d, 1H, *J* = 8.6 Hz, H-3_{pyr}), 7.89 (s, 1H, NH), 8.06 (dd, 1H, *J* = 2.7 Hz, *J* = 8.6 Hz, H-4_{pyr}), 8.07 (d, 1H, *J* = 8.2 Hz, H-6_{ur}), 8.71 (d, 1H, *J* = 2.7 Hz, H-6_{pyr}), ^{13}C NMR (100 MHz, CD_3OD): δ 62.75 (C-6_{glu}), 71.28, 71.86, 73.83, 79.68 (C-2_{glu}, C-3_{glu}, C-4_{glu}, C-5_{glu},), 76.29, 78.08, 85.57, 86.79 (C-2'$_{ur}$, C-3'$_{ur}$, C-4'$_{ur}$, C-1_{glu}), 98.18 (C-1'$_{ur}$), 103.14 (C-5_{ur}), 125.16 (C-3_{pyr}), 130.03 (C-4_{pyr}), 134.16 (C-2_{pyr}), 142.22 (C-6_{pyr}), 144.95 (C-6_{ur}), 152.74 (C-5_{pyr}), 153.66 (C-2_{ur}), 166.03 (C-4_{ur}), 170.89 (NHCO); ESI-HRMS: calcd for $C_{20}H_{24}N_4O_{11}SNa$ ($[M+Na]^+$): m/z 551.1060; found: m/z 551.1061.

4.2. Biological assays

4.2.1. Antiviral compounds

The stocks solutions of compounds synthesized as described in this communication were prepared by dissolving the reagents in dimethyl sulfoxide (DMSO) and stored in -20°C until future use. Sofosbuvir was purchased from Selleckchem (Munich, Germany).

4.2.2. 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) (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), at 37°C under 5% CO_2. Human hepatoma cells Huh-7.5 were grown in Dulbecco's Modified Eagle Medium (DMEM)(Sigma-Aldrich), supplemented with 10% fetal bovine serum, 0.5mM GlutaMax (Invitrogen) and 100U/ml penicillin/ml and 100 µg/ml streptomycin at 37°C under 5% humidified CO_2. Another variants of human hepatoma cell line, Huh7-J20, which is stably transformed with secretory alkaline phosphatase (SEAP) reporter system under HCV promoter [34] and the replicon Huh7-J17, which stably expresses viral RNA [35] (kindly provided by Dr Arvind Patel (MRC, University of Glasgow Centre for Virus Research, University of Glasgow, UK)) were used for high throughput screening of antivirals. These cells were also maintained in the same media like Huh-7.5 cells with addition of puromycin (2 µg/ml) and NEAA (0.5 ml / 50ml).

Classical swine fever virus Cellpest strain [40] was obtained from the National Veterinary Institute in Pulawy, Poland. The viral stocks were obtained by inoculating monolayers of SK6 cells in 75 cm^2 tissue culture flasks. The infection was allowed to proceed for 96 h. Viral stocks were stored at -70°C and titrated before use.

Hepatitis C virus was produced in Huh-7.5 cell culture as previously described [41,42]. In brief, pFK-Jc1 plasmid containing full length chimeric clone of HCV genotype 2a kindly provided by Dr Ralf Bartenschlager (University of Heidelberg, Germany) was linearized by

Mlu I. Additionally, pUC-JFH-1/AM7+1 plasmid kindly provided by Dr A. Patel was linearized by Xba I. Both plasmids were purified using Clean-up kit following manufacturer's instructions (Qiagen). Full length HCV RNA was synthesized using the TranscriptAid T7 High Yield Transcription Kit (Thermo Fischer Scientific) using manufacturer's protocol. In-vitro transcribed genomic Jc1/JFH RNA purified using RNeasy Mini Kit (Qiagen) was delivered to overnight grown Huh-7.5 cells ($10x10^6$) by electroporation in a 4mm cuvette at 270V and 500 µF on gene pulsar (BioRad). Infectious HCV particles (HCVcc) were recovered 72 h postelectroporation, and the virus-containing supernatant was filtered through 0.45 µm pore membrane. Aliquots of supernatant were stored at -80^{o}C for further use. $TCID_{50}$ was determined by the Hierholzer & Killington method [43] using plaque reduction assay as described below.

4.2.3. Cell viability assays

SK6 cell viability was measured by CellTiter 96 AQueous nonradioactive cell proliferation assay (MTS) (Promega, USA) as described previously [29]. Cytotoxicity of the compounds on Huh-7.5, Huh7-J20 and Huh7-J17 cells were analysed by MTT assay using standard protocol [44]. The cytotoxic concentration 50% (CC_{50}) values for each cell type calculated as the compound concentration required to reduce cell viability by 50% was determined using CalcuSyn software (Biosoft) from a dose response curve.

4.2.4. Evaluation of antiviral activity against CSFV

Antiviral activity was evaluated by a pseudo-plaque reduction assay as reported previously [29]. Briefly, 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. Next, fresh medium containing increasing amounts of inhibitor was added. After 2

days, cells were washed with phosphate buffered saline (PBS), fixed with 40% acetone in 0,5 x 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. IC50 was calculated as the concentration at which the number of plaques were reduced by 50% compared to untreated infected control cells.

4.2.5. HCVcc infection

Human hepatoma cells, Huh-7.5 cells or Huh7-J20 cells, were seeded in a 12-well or 96-well plates (1 $x10^5$ or 1.2 x 10^4 cells/well, respectively) and after overnight incubation virus supernatant was added to the cells at an MOI of 0.1. After 3-6h of infection cells were washed twice with PBS and fresh media was added to the cells along with different concentrations of the compounds, Sofosbuvir, as a positive control or DMSO as solvent control. After 48-72h of incubation, total protein or supernatant was isolated and subjected to western blotting, HCVcc pseudo-plaque reduction assay, SEAP reporter assay or RT-PCR respectively, to evaluate the antiviral activity.

4.2.6. HCVcc pseudo-plaque reduction assay

Huh-7.5 cells (1.5 $x10^4$ cells/well) were seeded in a 96-well plate and incubated at 37°C. Next day, HCVcc containing supernatant at MOI of 0.1 was added and the cells were incubated for 3h. Subsequently, the virus was removed and fresh medium containing different concentrations of compounds or Sofosbuvir was added. Infection was allowed to proceed for

72 h, and then immunohistochemistry (IPMA assay to detect pseudo-plaques) was performed. Incubation media was removed and cells were washed once with PBS and fixed in chilled methanol for 30 min. After washing, cells were permeabilized in 0.5% TritonX100 in PBS for 5 min followed by another wash with PBS and incubation with HCV anti-core (Hep C cAg antibody (C7-50); Santa Cruz Biotechnology, USA; 1:300 dilution). After 1h incubation at room temperature, cells were washed and incubated with anti-mouse HRP labelled secondary antibody (1:1000 dilution) for 1h. HCV positive plaques were detected using Vector Nova Red kit (Vector Laboratories Ltd, UK) as per manufacturer's protocol. Plaques were observed by microscopy and IC_{50} was calculated as the concentration at which the number of plaques was reduced by 50% compared to untreated infected control cells.

4.2.7. SDS-PAGE and Western Blotting

SK6 cells and Huh-7.5 cells grown in 12 and 24-wells plates were infected with CSFV (MOI of 0.01) or HCV (MOI of 0.1), respectively. After 1-6 h, medium 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 48 h. Cells infected with CSFV were lysed with TNET buffer (20mM Tris–HCl (pH 7.4), 1mM EDTA, 150mM NaCl, 1% Triton X-100) and Huh-7.5 cells infected with HCV were lysed in RIPA buffer (20mM Tris-Cl (pH 7.4), 1mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and freshly prepared protease inhibitor cocktail from Roche Diagnostic). Proteins quantified by Bradford reagent (Santa Cruz USA) were separated by SDS-PAGE under non-reducing or reducing conditions and transferred to PVDF membranes. Rabbit polyclonal serum anti-E2 CSFV (1:200 dilution), monoclonal anti-E1 HCV (1:2000 dilution) or anti-actin (1:1000 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) on the X-ray films (Fuji, Japan) or Chemdoc XRS+ (BioRad) and analysed.

4.2.8. SEAP reporter assay

For screening of antiviral compounds, Huh7-J20 reporter cells ($1.3x10^4$ cells /well) were grown in a 96-well tissue culture plate and incubated overnight at 37°C. Antiviral activity of compounds was analysed using three different infection models according to Magri et al., 2016 [33]. In Model 1, Huh7-J20 cells were pre-treated with various concentrations of compounds for 1 h and then infected in the presence of the compounds. After 3 h the inoculum was replaced with fresh medium without inhibitor and incubated for 72 h. In Model 2, the cells were pre-treated with the compounds, infected with the virus in the presence of the inhibitors which were also present through the whole course of infection for 72 h. In Model 3, cells were infected and the compounds were added only after infection for 72 h. The levels of virus infectivity and replication were determined by measuring the secreted alkaline phosphatase (SEAP) activity using Phospha-Light kit (Applied Biosystems) using manufacturer's protocol with minor modifications. In brief, 50 µl of culture supernatant was mixed with equal volume of assay buffer and incubated at room temp for 7 min and then 50 µl of freshly prepared chemiluminescent substrate was added to the reaction mix, incubated for 45 min in dark and the plate was read in a Luminometer.

4.2.9. Antiviral screening using replicon cell line

The replicon cells Huh7-J17, stably expressing viral RNA, were seeded in 96-well plates together with various concentrations of compounds, DMSO or Sobosbuvir for 72 h. Inhibition of viral RNA replication was determined by measuring luciferase activity in lysed cells using the Bright-Glo Luciferase Assay system (Promega, UK) according manufacturer's protocol. The IC_{50} values were determined using the GraphPad Prism software.

4.2.10. RNA inhibition (RT-PCR)

Huh-7.5 cells, infected with HCV at MOI of 1:0.1, were grown in 12 well plate ($0.1x10^6$ cells/well) in presence or absence of drug molecules or DMSO solvent control. After 48 h of incubation, total RNA was isolated from Huh7.5 cells using Tri Reagent (MRC USA) following manufacturer's protocol. Total RNA was dissolved in DNAse/RNAse free water and 1µg total RNA was subjected to cDNA synthesis using NS5B gene specific reverse primer in a final volume of 10µl with MuLV-RT (Thermo). PCR for HCV NS5B gene was conducted in 20µl final reaction volume using 2µl of cDNA and RUN-Taq Polymerase (A&A Biotechnology, Poland). Samples were amplified using the following parameters: 2min at 94^oC followed by 30 cycles at 94^oC, 62^oC and 72^oC , 30 sec each, and a final extension step of 2 min at 72^oC. A 213 bp sequence from HCV NS5B gene was amplified using forward primer- 5'ACA TCA AGT CCG TGT GGA AGG-3' and reverse primer- 5'GCT CCC ATT ACC GCC TGA GGA AGC3'. RT-PCR for actin was used as an internal control. For Actin PCR, a 232 bp region was amplified using forward primer 5'-GCG GGA AAT CGT GCG TGA CAT T-3' and reverse primer 5'-GAT GGA GTT GAA GGT AGT TTC GTG-3'. The PCR products were resolved on 2% agarose gel, images were captured and analyzed by densitometry using BioRad Quantity One software.

SUPPLEMENTARY MATERIAL

Supplementary experimental and analytical data related to this article can be found in Supplementary Information.

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4. Type of pyrophosphate-mimicking linker greatly influence on biological activity.