Journal Pre-proof

Anti-norovirus activity of C7-modified 4-amino-pyrrolo[2,1-f][1,2,4]triazine *C*-nucleosides

Qingfeng Li, Elisabetta Groaz, Joana Rocha-Pereira, Johan Neyts, Piet Herdewijn

PII: S0223-5234(20)30165-3

DOI: https://doi.org/10.1016/j.ejmech.2020.112198

Reference: EJMECH 112198

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 20 November 2019

Revised Date: 24 December 2019

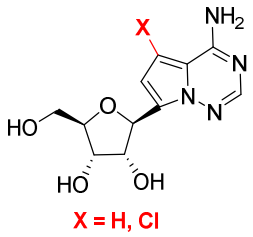
Accepted Date: 28 February 2020

Please cite this article as: Q. Li, E. Groaz, J. Rocha-Pereira, J. Neyts, P. Herdewijn, Anti-norovirus activity of C7-modified 4-amino-pyrrolo[2,1-f][1,2,4]triazine *C*-nucleosides, *European Journal of Medicinal Chemistry* (2020), doi: https://doi.org/10.1016/j.ejmech.2020.112198.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Masson SAS.

Journal Pre-proof



X	Virus	Cell lines	EC_{50} (µM)
H	MNV	RAW 264.7 cells	0.007
H	HuNoV	HGT-1 cells	0.015
Cl	MNV	RAW 264.7 cells	0.007

Journal Pre-proof

Journal Pre-proof

Anti-norovirus activity of C7-modified 4-amino-pyrrolo[2,1-f][1,2,4]triazine *C*-nucleosides

Qingfeng Li,[a] Elisabetta Groaz,[a,*] Joana Rocha-Pereira,[b] Johan Neyts,[b] and Piet Herdewijn[a,*]

[a] KU Leuven, Rega Institute for Medical Research, Medicinal Chemistry, Herestraat 49 - Box 1041, Leuven 3000, Belgium

[b] KU Leuven, Rega Institute for Medical Research, Department of Microbiology and Immunology, Laboratory of Virology and Chemotherapy, Herestraat 49 - Box 1041, Leuven 3000, Leuven, Belgium

ABSTRACT

Synthetic nucleoside analogues characterized by a C-C anomeric linkage form a family of promising therapeutics against infectious and malignant diseases. Herein, *C*-nucleosides comprising structural variations at the sugar and nucleobase moieties were examined for their ability to inhibit both murine and human norovirus RNA-dependent RNA polymerase (RdRp). We have found that the combination of 4-amino-pyrrolo[2,1-f][1,2,4]triazine and its 7-halogenated congeners with either a D-ribose or 2'-*C*-methyl-D-ribose unit resulted in analogues with good antiviral activity against murine norovirus (MNV), albeit coupled with a significant cytotoxicity. Among this series, 4-aza-7,9-dideazaadenosine notably retained a strong antiviral effect in a human norovirus (HuNoV) replicon assay with an EC_{50} = 0.015 μM. This study demonstrates that *C*-nucleosides can be used as viable starting scaffolds for further optimization towards the development of nucleoside-based inhibitors of norovirus replication.

Keywords: C-Nucleosides, Structure-activity relationship, Norovirus, Antiviral activity

1. Introduction

Noroviruses are positive-sense single-stranded RNA (+ssRNA) viruses responsible for large outbreaks of acute gastroenteritis around the world.[1,2] Typically, symptoms including vomiting, diarrhea, abdominal cramps, and nausea may last about two or three days in healthy adults. However, this infection can be prolonged and severe (even life-threatening) in young children, elderly and immunocompromised persons, who can develop chronic grastroenteritis.[3,4] The current therapy for norovirus infections relies on electrolyte replenishment for dehydrated individuals along with measures for outbreak control and prevention that are restricted to the often inefficient use of antiseptics and hand-sanitizers. Despite the clear need for medical intervention, no approved vaccine or small molecule antiviral treatment is currently available. This is partly due to the fact that only recently human norovirus (HuNoV) was successfully cultivated in vitro.[5,6] Hence, there is an urgency to develop therapeutic agents that can either directly inhibit norovirus RNA replication or interfere with the function of structural and non-structural proteins encoded by the norovirus genome.[7,8] Selected examples of molecules recognized as inhibitors of the activity of

norovirus RNA-dependent RNA polymerase (RdRp) are given in Fig.1. From a structural standpoint, they include both nucleosides and non-nucleoside compounds.[9,10]

Several small heterocycles exhibiting anti-norovirus inhibitory activity in the micromolar range, such as the phenylthiazole (NIC02) and triazole (NIC10) derivatives shown in Fig. 1,[11] were identified as potential scaffolds for further drug development. On the other hand, ribavirin was one of the first nucleosides found to effectively inhibit norovirus replication (EC_{50} = 43 μM).[12] 2'-*C*-Methyl-cytidine (2CM-C) was initially developed as a HCV polymerase inhibitor, but later proven to inhibit also murine norovirus (MNV) polymerase (EC_{50} = 2 μM)[13] and HuNoV replication in the human B cell BJAB cell line (EC_{50} = 0.3 μM).[14] Both 2CM-C and its fluorinated analogue 2'-fluoro-2'-*C*-methyl-cytidine (2'-F-2'-CM-C) displayed comparable antiviral activity against both MNV and HuNoV in cell based assays.[10] Among non-nucleoside anti-norovirus agents, it is worth mentioning polyanionic naphthalene analogues such as suramin[15,16] and NF023.[16]

In recent years, much effort has been devoted to the chemical synthesis and biological evaluation of *C*-nucleoside analogues as potential antiviral agents.[17,18] In particular, the coupling of 4-amino-pyrrolo[2,1-f][1,2,4]triazine (or 4-aza-7,9-dideazaadenine) to various sugar moieties has delivered modified *C*-nucleosides with a broad-spectrum activity against viruses belonging to the *Flaviviridae* (HCV), *Orthomyxoviridae*, *Paramyxoviridae*, and *Coronaviridae* families. In an early study, 2'-*C*-methyl-4-aza-7,9-dideazaadenosine (Fig. 2, **1**) was identified as a selective HCV polymerase inhibitor in cell cultures (EC_{50} = 1.98 μM).[19] Base-modified derivatives of **1** have also shown promising anti-HCV properties in vitro. Specifically, the 7-carboamido analogue **2** exhibited a remarkable 40-fold improvement in potency relative to the parent compound **1**, while the 7-fluoro pyrrolotriazine analogue **3** showed good anti-HCV activity (EC_{50} = 3.1 μM) without concomitant cytotoxicity (CC_{50} >100 μM).[20] We too became interested in these analogues and synthesized ribose containing *C*-nucleosides **4**-**7** (Fig. 2) with either a hydrogen atom or halogen group at the 7-position of the nucleobase.[21]

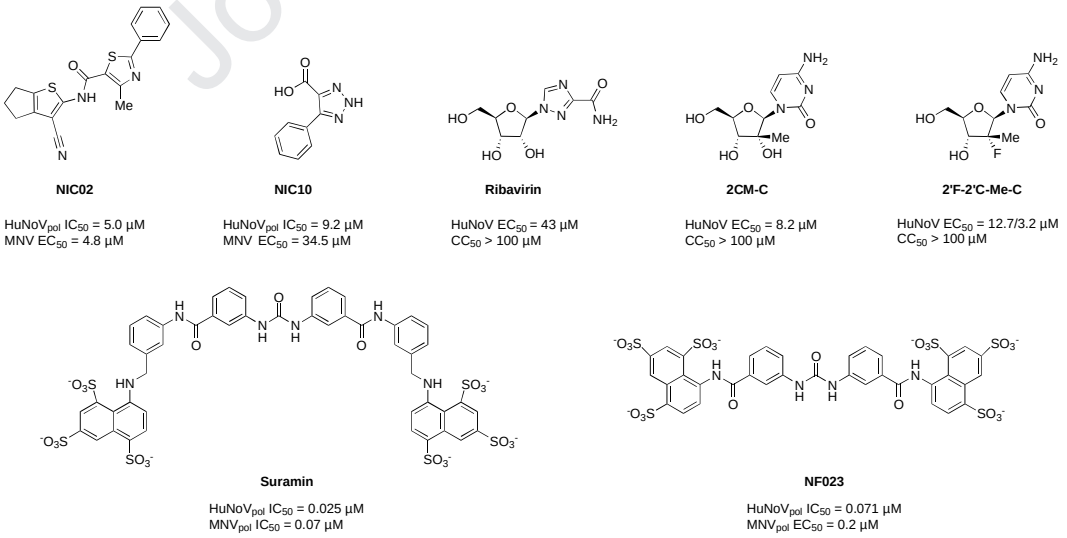


Fig. 1. Nucleoside and non-nucleoside inhibitors of norovirus replication.

Given the broad-spectrum activity of pyrrolotriazine containing *C*-nucleosides against RNA viruses, we compared a series of sugar- and base-modified *C*-nucleosides as potential

norovirus inhibitors. Our planned structure-activity relationship study comprehended three types of sugar modifications, i.e., D-ribose, 2'-*C*-methyl-D-ribose, and L-ribose, in combination with the presence of H, Cl, Br, or I atoms at the 7 position of the pyrrolotriazine scaffold (Fig. 2). In addition, *N*-nucleoside analogue **8** featuring 7-deaza-7-chloroadenine as nucleobase was also included in our study. The synthesis of L-ribose type of analogues such as **10** and **11** was motivated by the generally lower toxicity of nucleoside antivirals bearing a L-sugar moiety compared to their D-counterparts, as demonstrated by the clinical use of lamivudine (L-3TC), clevudine, and telbivudine (L-dT).[22]

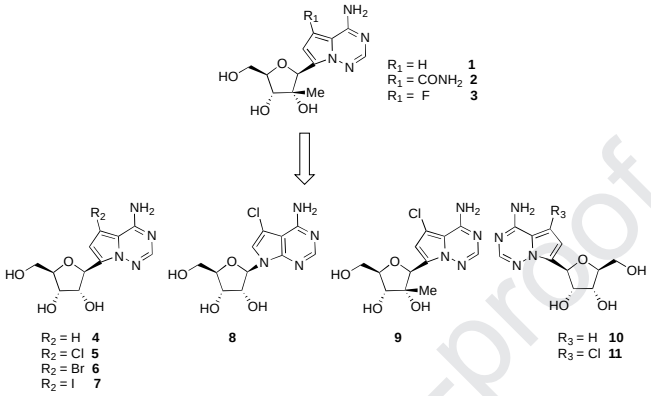
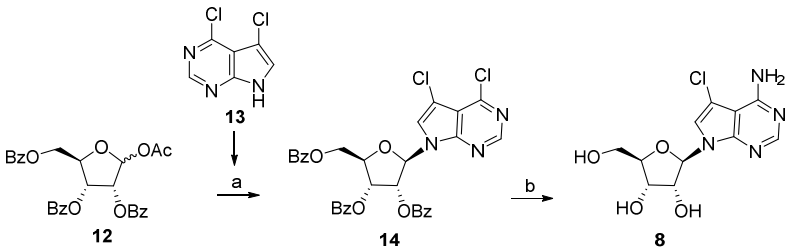


Fig. 2. Known *C*-nucleosides **1-7** and additional structural analogues (**8-11**) investigated in this study.

2. Results and discussion

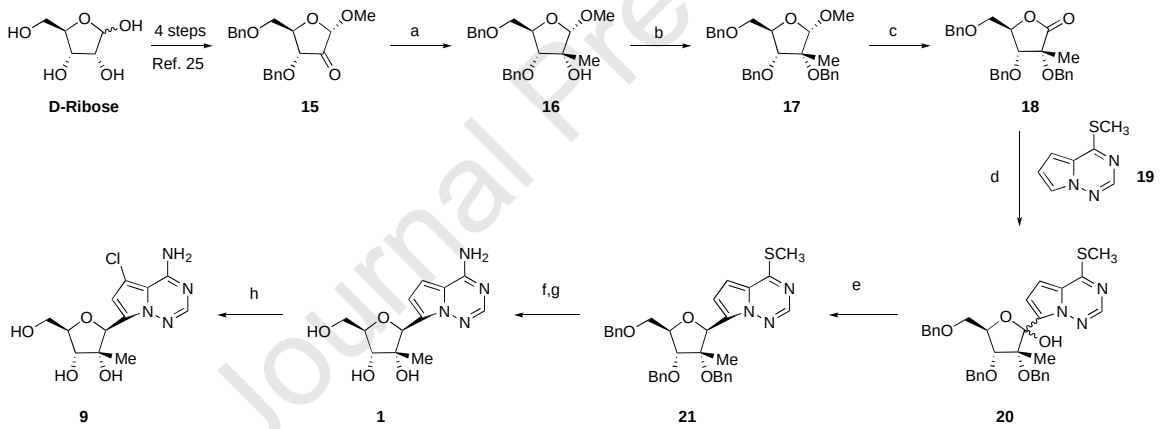
2.1. Chemistry

Suitable synthetic procedures for the preparation of compounds **4-7** were previously described.[21] Compound **8** was synthesized according to a literature method reported by Seela *et al*.[23] As shown in Scheme 1, nucleobase **13** was first silylated under standard conditions [bis(trimethylsilyl)acetamide (BSA) in anhydrous MeCN] at room temperature, and then reacted with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-*β*-D-ribofuranose **12** to afford glycosylated intermediate **13** with a 1'-*β*-configuration. Compound **14** was then converted into 7-chloro tubercidin derivative **8** upon treatment with methanolic ammonia in a sealed tube (120 °C, 24 h).



Scheme 1. Synthesis of 7-chloro tubercidin analogue **8**. Reagents and conditions*:* (a) BSA, TMSOTf, MeCN, 50 °C, 1 h, 70%; (b) 7 M NH_3 in MeOH, 120 °C, 24 h, 84%.

4-Amino-pyrrolo[2,1-f][1,2,4]triazine containing *C*-nucleoside analogue **1** was previously obtained using a linear synthetic route by building the bicyclic heterocyclic nucleobase at the 1-position of a 2-methyl modified sugar moiety.[24] However, the 3,5-bis-dichlorobenzyl protected 2-*C*-Me-riboside used as starting material is not easily available. Moreover, in the initial stage of the nucleobase construction an anomeric mixture of pyrrolo nucleoside intermediates was obtained, which needed to be further separated. Therefore, it was deemed preferable to follow a convergent synthetic approach to obtain this compound or its analogues such as compound **9**. As shown in Scheme 2, 3,5-di-*O*-benzyl-2-keto-1-α-*O*-methyl-D-ribofuranose **15** was synthesized in a 43% overall yield over four steps starting from D-ribose and by employing a similar procedure as that reported by Li *et al*.[25] Subsequent stereospecific addition of a 2'-methyl group at the *β*-face of the ribofuranose moiety was accomplished by reacting **15** with methylmagnesium bromide in THF to give di-*O*-benzyl-2-*C*-*β*-methoxyl-1-α-*O*-methyl-D-ribofuranose **16** in 70% yield. At this stage of the synthesis, the [*S*]- and [*R*]-configuration of 1-C and 2-C, respectively, were confirmed by 2D NMR analysis based on the NOE interactions between H-1 and H-3, 2-*C*-Me as well as OMe with H-4 (see Supporting Information).

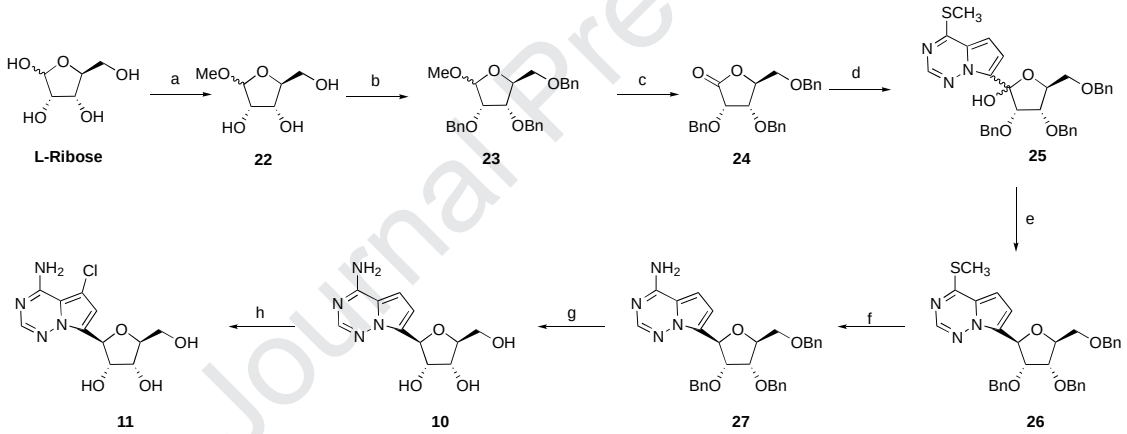


Scheme 2. Synthesis of 2'-*C*-methyl-4-aza-7-chloro-7,9-dideazaadenosine *C*-nucleoside **9**. Reagents and conditions*:* (a) CH_3MgBr, THF, 3 h, 92%; (b) NaH, BnBr, TBAI, THF, 70 °C, on, 73%; (c) (i) AcOH, 1 N HCl in H_2O, 80 °C, 4 h; (ii) DMSO, Ac_2O, rt, on, 76% over two steps; (d) Lithium diisopropylamide (LDA), THF, -78 °C, 3 h, 32%; (e) $BF_3.OEt_2$, Et_3SiH, DCM, 0 °C, 40 min, 71%; (f) 7 M NH_3 in MeOH, 100 °C, 24 h, 59%; (g) $Pd(OH)_2/C$, H_2, EtOH:cyclohexene = 4:1, rt, 24 h, 80%; (h) *N*-chlorosuccinimide (NCS), DMF, 0 °C to rt, 2 days, 32%.

Subsequently, compound **16** underwent a benzylation reaction to afford fully protected sugar analogue **17**. This step was found to proceed sluggishly at room temperature, however, a satisfactory 73% yield could be obtained when the reaction was performed at elevated temperature (70 °C). Hydrolysis of compound **17** by treatment with 1 N HCl, followed by oxidation of the resulting 1-OH group in the presence of DMSO/Ac_2O[26] afforded lactone **18** in 76% yield over two steps. For the *C*-nucleoside construction, we employed a similar synthetic strategy as that previously used for the synthesis of **4**.[21] The lithium salt of thiomethyl substituted heterocycle **19** was coupled with protected ribonolactone **18**, affording

nucleoside lactol **20** as a mixture of anomers. Anomeric reduction of hemiacetal **20** using triethylsilane and boron trifluoride etherate afforded stereoselectively *C*-nucleoside **21** as a *β*-anomer. Next, amine replacement of the thiomethyl group furnished a benzyl protected *C*-nucleoside intermediate, which underwent *O*-debenzylation upon hydrogenolysis catalyzed by palladium hydroxide on carbon ($Pd(OH)_2/C$) affording *C*-nucleoside **1**. Chlorine was then readily introduced at the 7-position of the nucleobase by treatment with *N*-chlorosuccinimide to provide **9** in 32% yield.

The same general strategy was used to synthesize L-*C*-nucleosides **10** and **11**, as illustrated in Scheme 3. In this case, the synthetic route started from L-ribose, which was first reacted with methanol under Fischer glycosylation conditions to give **23** in 91% yield. A subsequent perbenzylation reaction yielded methyl-2,3,5-tri-*O*-benzyl-*β*/*α*-L-riboside **24**, which was then subjected to acid hydrolysis and oxidation in the presence of $DMSO/Ac_2O$[26] to afford lactone **24** in 50% yield over two steps. Compound **25** was then reacted with its aglycone coupling partner under the conditions described above to provide **26** as a mixture of anomers in 60% yield. Upon reduction, only *β*-anomer **27** was isolated in 70% yield. Subsequent protecting group manipulation led to **10**, which smoothly underwent further chlorination at the nucleobase to afford **11**.

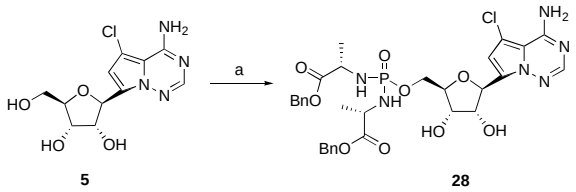


Scheme 3. Synthesis of L-sugar modified 7-chloro pyrrolotriazine nucleosides **10** and **11**. Reagents and conditions: (a) conc. H_2SO_4, MeOH, rt, 5 h, 91%; (b) NaH, BnBr, THF, 0 °C to rt, on, 75%; (c) (i) AcOH, 1 N HCl in H_2O, 80 °C, 4 h; (ii) DMSO, Ac_2O, rt, on, 60% over two steps; (d) **19**, Lithium diisopropylamide (LDA), THF, -78 °C, 3 h, 60%; (e) $BF_3.OEt_2$, Et_3SiH, DCM, 0 °C, 40 min, 70%; (f) 7 M NH_3 in MeOH, 100 °C, 24 h, 80%; (g) $Pd(OH)_2/C$, H_2, CH_3COOH, rt, 48 h, 80%; (h) *N*-Chlorosuccinimide (NCS), DMF, 0 °C to rt, 2 days, 25%.

In addition, encouraged by previous literature examples,[27,28] a selected prodrug of compound **5**, which emerged as the most active congener against MNV but did not retain its activity against HuNoV (see below), was also prepared (Scheme 4). Benzyl esters are commonly used as prodrug moieties to improve the potency of nucleoside prodrugs due to the fact that they are good substrates for the esterase involved in phosphoramidate conversion.[27-28] In this study, bisphosphoramide **28** was specifically proposed in order to avoid the introduction of a chiral phosphorus atom in the parent compound.

Journal Pre-proof

Journal Pre-proof



Scheme 4. Synthesis of bisphosphoramide prodrug **28**. Reagents and conditions*:* (a) L-alanine benzyl ester hydrochloride, $POCl_3$, $NaHCO_3$, $PO(OMe)_3$, 0 °C to rt, 11%.

2.2. Antiviral activity

At first, compounds **1**, **4-11**, and **28** were screened for their ability to inhibit the in vitro replication of genogroup V (GV) MNV, which is a surrogate for the HuNoV. To this aim, a cytopathic effect (CPE)-based assay was performed in the murine macrophage cell line RAW 264.7 with a readout at 72 h post-infection. All compounds were evaluated by performing dose response experiments at 8 different concentrations in order to determine their EC_{50} values. The related cytotoxicity (CC_{50}) was also determined in parallel in uninfected cells. All results obtained from these tests are summarized in Table 1.

Compound **4** along with its halogenated derivatives **5-6** and 2'-*β*-Me-*C*-nucleoside **1** exhibited good to potent inhibitory activity against norovirus, yet also displayed substantial cytotoxicity. In particular, 2'-*β*-Me *C*-nucleoside **1** inhibited MNV replication with an EC_{50} of ~11 µM and CC_{50} of ~24 µM. Compound **4** bearing an unmodified pyrrolo[2,1-f][triazin-4-amino]adenine base and its 7-chlorinated analogue **5** emerged as the most potent compounds with a similar EC_{50} of 0.007 µM. The cytotoxic effect increased upon replacement of the 7-H (**4**) with a Cl atom (**5**), as demonstrated by the corresponding decrease in CC_{50} from 0.14 to 0.01 along with a diminishing SI. Bromo and iodo analogues **6** and **7** were slightly less active and cytotoxic with an EC_{50} of 0.024 and 0.035 µM and CC_{50} of 0.45 and 0.78 µM, respectively.

In a previous study, cellular toxicity against specific immortalized cell lines was also observed for compounds **4-7**.[21] On the other hand, the *N*-nucleoside congener **8** and the base chlorinated derivative of 2'-*β*-Me *C*-nucleoside **1** (compound **9**) were found to be devoid of any significant antiviral activity, while exhibiting a CC_{50} of 2.53 and 4.78 µM, respectively. It should be noted that the 7-chloro modified 2'-*β*-Me *C*-nucleoside **9** was more toxic than **1**.

No antiviral activity or cytotoxicity was observed at the highest concentration tested for compounds **10** and **11** featuring a L-sugar moiety. Surprisingly, the introduction of a prodrug moiety at the 5'-position of compound **5** led to the complete loss of both antiviral and cytotoxic effects, as shown in Table 1 for compound **28** (entry 10). This is unusual as generally phosphoramidate prodrugs are more potent than the parent drugs; however, a similar case was recently reported[29] and could most likely be justified by the fact that the prodrug cannot be readily cleaved to release the parent nucleoside inside the cell.

Table 1

In vitro inhibitory activity of compounds **1**, **4-11**, and **28** against murine norovirus (MNV).

Entry	Compound	EC_{50} (µM)[a]	CC_{50} (µM)[b]	SI

1	**1**	11.1	23.7	2
2	**4**	0.007	0.14	20
3	**5**	0.007	>0.01	>1
4	**6**	0.024	0.45	19
5	**7**	0.035	0.78	22
6	**8**	>2[c]	2.53	–
7	**9**	>4[c]	4.78	–
8	**10**	>100[c]	>100[c]	–
9	**11**	>100[c]	>100[c]	–
10	**28**	>10[c]	>10	–

[a]EC_{50} is the effective concentration to inhibit the replication of the virus by 50%; [b]CC_{50} is the cytotoxic concentration that reduces the number of viable cells by 50%. [c]EC_{50}> indicates that no antiviral activity was observed at the highest concentration tested.

Furthermore, compounds **4-7**, which emerged as the most active of this series in the MNV assay, were also evaluated against HuNoV using a human gastric tumor-1 (HGT-1) cell line that stably expresses the genome of genogroup I (GI.1) HuNoV. This HuNoV GI.1 replicon-bearing cell line is the standardly used model for studying the antiviral effect of small molecules.[30] In this system, the intracellular GI.1 virus replicon RNA was quantified by quantitative reverse transcription-PCR [qRT-PCR] using *β*-actin mRNA as reference gene.[31] Since the gene encoding for the major capsid protein is replaced by a neomycin resistance gene, no new virus particles are produced, however the non-structural proteins are expressed and the replication of the genomic RNA can be analyzed.[30,32]

As shown in Table 2, it can be seen that compound **4** reduced the levels of HuNoV virus RNA with an EC_{50} of 0.015 µM. However, at concentrations higher than the EC_{50}, a toxic effect was observed on the host cells. Compound **5-7** did not show activity to reduce level of HuNoV virus RNA but displayed cytotoxicity with a CC_{50} ranges from 3.1 µM to 6.3 µM.

The differences in EC_{50} values between the antiviral assays used in this study could be due to the fact that the corresponding viruses belong to two different norovirus genogroups. Despite the great similarities between the RdRps of GI and GV noroviruses, at the amino acid level they are ~70% alike. Another factor could be attributed to the technical details of the assays that use very different readouts.

Table 2

In vitro inhibitory activity of compounds **4-7** against human norovirus (HuNoV).

Entry	Compound	EC_{50} (µM)[a]	CC_{50} (µM)[b]
1	**4**	0.015	0.031
2	**5**	>10	4.7
3	**6**	>10	3.1
4	**7**	>10	6.3

[a]EC_{50} is defined as the compound concentration that resulted in a 50% reduction of the relative HuNoV replicon RNA levels (when compared to a housekeeping gene, *β*-actin). In addition, a potential toxic effect of the compounds in host cells was scored microscopically at the same time point (72 hours pi). [b]CC_{50} is defined as the compound concentration that reduced the number of cells with a normal morphology by 50%.

3. Conclusions

In summary, a series of *C*-nucleoside derivatives obtained by combining diversely modified sugar and nucleobase moieties were evaluated for antiviral activity against both murine and human norovirus. Synthetically, all compounds were prepared using a direct coupling method based on the nucleophilic addition of a heterocyclic base to suitably protected modified furanolactone intermediates. A prodrug (compound **28**) was also prepared in the expectation that the delivery of the monophosphate of the parent modified nucleoside to the cell could lead to enhanced biological activity. Among these compounds, 4-aza-7,9-dideazaadenosine, its 7-halogenated analogue as well as a 2'-*β*-Me-*C*-nucleoside bearing 4-aza-7,9-dideazaadenine displayed good inhibitory activity in the MVN assay with EC_{50} values ranging from 0.007 to 11 µM. Despite the activity being coupled with significant cytotoxicity, it was interesting to note that 4-aza-7,9-dideazaadenosine retained its antiviral activity also in a HuNoV GI.1 replicon system. Owing to its dual activity against both murine and human norovirus, compound **4** could function as reference structure to further elaborate the structure-activity relationship of *C*-nucleosides as selective anti-norovirus agents.

4. Experimental section

4.1. General information

All reagents and solvents were purchased from commercial sources and used as obtained. Moisture sensitive reactions were carried out using oven-dried glassware under a nitrogen or argon atmosphere. ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were recorded on a Bruker Avance 300 or 600 MHz spectrometer using tetramethylsilane as internal standard or referenced to the residual solvent signal. The following abbreviations were used to indicate multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad), and dd (doublet of doublets). Coupling constants are expressed in hertz (Hz). High-resolution mass spectra (HRMS) were obtained on a quadrupole orthogonal acceleration time-of-flight mass spectrometer (Synapt G2 HDMS, Waters, Milford, MA). Samples were infused at 3 µL/min and spectra were obtained in positive ionization mode with a resolution of 15000 (fwhm) using leucine enkephalin as lock mass. Pre-coated aluminum sheets (254 nm) were used for thin layer chromatography (TLC), and spots were visualized with UV light. All intermediate products were purified by silica gel column chromatography on silica gel (40-60 µ, 60 Å). Final compounds were purified by preparative RP-HPLC (C18 Phenomenex Gemini column, 110 Å, 10 µm, 21.2 mm × 250 mm) using a linear gradient of CH_3CN or MeOH and H_2O.

A detailed account of the synthesis and characterization of compounds **4-7** was previously reported.[21]

*4.1.1. 7-Deaza-7-chloroadenosine (**8**).* A solution of compound **14**[23] (1.0 g, 1.58 mmol) in 7 N

methanolic NH_3 (60 mL) was stirred for 24 h at 120 °C. After removal of all the volatiles in vacuo, the crude residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 10:1, v/v) to afford title compound **8** as a white solid (400 mg, 84% yield). ^{1}H NMR (300 MHz, CD_3OD) δ 8.08 (s, 1H, H_2), 7.56 (s, 1H, H_8), 6.06 (d, $J_{1',2'}$ = 6.0 Hz, 1H, $H_{1'}$), 4.32 (dd, $J_{2',3'}$ = 4.9 Hz, $J_{2',1'}$ = 6.0 Hz, 1H, $H_{2'}$), 4.06 (dd, $J_{3',4'}$ = 3.4 Hz, $J_{3',2'}$ = 4.9 Hz, 1H, $H_{3'}$), 3.88 (ddd, $J_{4',3'}$ = 3.4 Hz, $J_{4',5'}$ = 3.8, $J_{4',5''}$ = 3.7 Hz, 1H, $H_{4'}$), 3.63-3.47 (2 × dd, J_{gem} = 11.8 Hz, $J_{5',4'}$ = 3.8 Hz, $J_{5'',4'}$ = 3.7 Hz, 2H, $H_{5'}$, $H_{5''}$); ^{13}C{^{1}H} NMR (75 MHz, CD_3OD) δ 156.8 (C-2), 152.7 (C-4), 149, 3 (C-5), 119.2 (C-6), 102.7 (C-7), 100.0 (C-8), 86.9 (C-1'), 85.2 (C-4'), 73.9 (C-2'), 70.5 (C-3'), 61.5 (C-5'); HRMS (ESI-TOF) m/z: calcd for $C_{11}H_{13}Cl_1N_4O_5$ ([M+Na]$^+$), 301.0704, found 301.0703.

*4.1.2. Methyl 3,5-di-O-benzyl-2-C-β-methyl-α-D-ribofuranoside (**16**).* To a solution of methyl 3,5-di-*O*-benzyl-2-keto-α-D-ribofuranoside **15**[25] (8.0 g, 23.4 mmol) in dry THF (50.0 mL) at 0 ºC was added methylmagnesium bromide (3.0 M in THF, 19.5 mL, 58.4 mmol) under an argon atmosphere. After stirring at room temperature for 4 h, the reaction mixture was quenched with saturated aq. NH_4Cl. It was then extracted with dichloromethane, and the organic layer was washed with brine. After removal of all the volatiles in vacuo, the residue was purified by silica gel column chromatography (heptane/EtOAc, 2:1, v/v) to give compound **16** as an oil (7.8 g, 92% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.33-7.25 (m, 10H, Ar-*H*), 4.76 (d, *J* = 12.0 Hz, 1H, OCH_2Ph), 4.50-4.41 (m, 4H, H_1, OCH_2Ph), 4.12 (ddd, $J_{4,3}$ = 4.2 Hz, $J_{4,5a}$ = 4.2 Hz, $J_{4,5b}$ = 3.2, 1H, H_4), 3.51-3.43 (2 × dd, J_{gem} = 11.3 Hz, $J_{5b,4}$ = 3.2 Hz, $J_{5a,4}$ = 4.2 Hz, 2H, H_{5a}, H_{5b}), 3.41 (s, 3H, OCH_3), 3.39 (s, 1H, OH), 3.33 (d, $J_{3,4}$ = 4.1 Hz, 1H, H_3); ^{13}C{^{1}H} NMR (150 MHz, $CDCl_3$) δ 138.2, 128.7, 128.2, 128.1, 127.9, 107.8, 82.6, 81.9, 76.9, 73.7, 73.3, 70.2, 55.5, 24.9; HRMS (ESI-TOF) m/z: calcd for $C_{21}H_{26}O_5$ ([M+Na]$^+$), 381.1678, found 381.1670.

*4.1.3. Methyl 2,3,5-di-O-benzyl-2-C-β-methyl-α-D-ribofuranoside (**17**).* Sodium hydride (60%, 17 g, 87.0 mmol) was added to a solution of **16** (7.8 g, 21.8 mmol) in dry THF (100 mL) at room temperature. After hydrogen generation ceased, benzyl bromide (7.8 mL, 65.3 mmol) and tetrabutylammonium iodide (241 mg, 0.653 mmol) were added. After stirring overnight at 70 ºC, the reaction mixture was slowly poured into cold saturated aq. ammonium chloride (100 mL) at 0 ºC. The mixture was then extracted with EtOAc (3 × 200 mL), and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting crude residue was purified by silica gel column chromatography (gradient heptane/EtOAc, 20:1 to 5:1, v/v) to afford **17** as an oil (7.2 g, 73%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43 (d, *J* = 7.0 Hz, 2H, Ar-*H*), 7.35-7.21 (m, 13H, Ar-*H*), 4.79 (d, *J* = 12.0 Hz, 1H, OCH_2Ph), 4.69 (s, 1H, H_1), 4.63-4.42 (m, 4H, H_3 and OCH_2Ph), 4.27 (ddd, $J_{4,5a}$ = 4.5 Hz, $J_{4,5b}$ = 3.6 Hz, $J_{4,3}$ = 4.2 Hz, 1H, H_4), 3.58-3.50 (2 × dd, J_{gem} = 13.2 Hz, $J_{5b,4}$ = 3.6 Hz, $J_{5a,4}$ = 4.5 Hz, 2H, H_{5a}, H_{5b}), 3.45 (s, 3H, OCH_3), 1.33 (s, 3H, CH_3); ^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$) δ 139.7, 138.8, 138.3, 128.7, 128.6, 128.5, 128.0, 127.9, 127.6, 127.5, 107.7, 82.5, 82.1, 82.0, 73.8, 73.0, 70.2, 66.8, 55.6, 22.9; HRMS (ESI-TOF) m/z: calcd for $C_{28}H_{32}O_5$ ([M+Na]$^+$), 471.2147, found 471.2135.

*4.1.4. 2,3,5-Tri-O-benzyl-2-C-β-methyl-D-ribono-1,4-lactone (**18**).* To a solution of **17** (7.2 g, 16.1 mmol) in AcOH (50 mL) was added 1 N aq. HCl (12.5 mL), and the mixture was stirred for 4 h at 80 °C. After removal of all the volatiles in vacuo, the residue was redissolved in EtOAc. The organic layer was washed with saturated aq. $NaHCO_3$ and brine, dried over

Na_2SO_4, filtered, and evaporated under reduced pressure. The resulting residue was dissolved in DMSO (25 mL), and then Ac_2O (15 mL) was slowly added at room temperature. The mixture was stirred at room temperature overnight. It was then extracted with EtOAc (3 × 200 mL), and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (heptane/EtOAc, 5:1, v/v) to afford **18** as an oil (5.3 g, 76%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40-7.22 (m, 15H, Ar-*H*), 4.81-4.45 (m, 4H, H_3, H_4 and OCH_2Ph), 4.02 (d, *J* = 7.4 Hz, 1H, OCH_2Ph), 3.76-3.53 (2 × dd, J_{gem} = 11.6 Hz, $J_{5b,4}$ = 2.3 Hz, $J_{5a,4}$ = 3.7 Hz, 2H, $H_{5a,}$ H_{5b}); ^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$) δ 173.7, 138.3, 137.9, 137.7, 128.9, 128.8, 128.7, 128.5, 128.2, 128.0, 80.7, 80.4, 77.7, 73.8, 73.6, 67.8, 20.3; HRMS (ESI-TOF) m/z: calcd for $C_{27}H_{28}O_5$ ([M+Na]$^+$), 455.1834, found 455.1825.

*4.1.5. 2',3',5'-O-Tribenzyl-1'-hydroxy-2'-C-methyl-6-methylthio-4-aza-7,9-dideazadenosine (**20**).* To a solution of 4-(methylthio)pyrrolo[2,1-f][1,2,4]triazine **19** (1.2 g, 7.26 mmol) in THF (20 mL) was added lithium diisopropylamide (2 M in THF, 5.4 mL, 10.9 mmol) at -78 °C under an argon atmosphere. The resulting mixture was stirred for 30 min at -78 °C. Then, a solution of compound **18** (3.2 g, 7.40 mmol) in THF (10 mL) was added at -78 °C and the resulting reaction mixture was stirred for 3 h at -78 °C. The reaction was quenched by adding saturated aq. NH_4Cl and then extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The crude residue was purified by silica gel silica gel column chromatography (heptane/ EtOAc = 5:1, v/v) to give the title compound as an oil (1.2 g, 32% yield). HRMS (ESI-TOF) m/z: calcd for $C_{33}H_{35}N_3O_5S_1$ ([M+H]$^+$), 598.2376, found 598.2339.

*4.1.6. 2',3',5'-O-Tribenzyl-2'-C-methyl-6-methylthio-4-aza-7,9-dideazadenosine (**21**).* To a solution of compound **20** (1.3 g, 2.17 mmol) in dichloromethane (50 mL) was added triethylsilane (1.01 g, 8.70 mmol) and trifluoroborane (552.0 mg, 4.35 mmol) at 0 °C under an argon atmosphere. The resulting solution was stirred for 40 min at 0 °C and then quenched with saturated aq. Na_2CO_3. The mixture was extracted with ethyl acetate. The organic layer was washed with water, brine, dried over Na_2SO_4, and concentrated in vacuo. The crude residue was purified by silica gel column chromatography (heptane/EtOAc = 10:1, v/v) to give the title compound as an oil (900 mg, 71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17 (s, 1H, H_2), 7.47-7.20 (m, 15H, Ar-*H*), 7.04 (d, *J* = 4.5 Hz, 1H, H_7), 6.70 (d, *J* = 4.5 Hz, H_8), 5.92 (s, 1H, $H_{1'}$), 4.85 (q, *J* = 11.5 Hz, 2H, OCH_2Ph), 4.71-4.39 (m, 5H, $H_{3'}$, $H_{4'}$ and OCH_2Ph), 4.08 (d, *J* = 8.3 Hz, 1H, OCH_2Ph), 3.94-3.68 (2 × dd, J_{gem} = 11.2 Hz, $J_{5',4}$ = 2.2 Hz, $J_{5'',4}$ = 3.4 Hz, 2H, $H_{5',}$ $H_{5''}$), 2.62 (s, 3H, SCH_3), 1.00 (s, 3H, CH_3); ^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$) δ 164.6, 145.3, 140.0, 138.5, 128.7, 128.6, 128.5, 128.2, 128.1, 128.0, 127.5, 127.4, 121.9, 112.6, 102.5, 85.3, 82.3, 79.6, 78.1, 73.8, 69.4, 66.1, 17.7, 11.7; HRMS (ESI-TOF) m/z: calcd for $C_{34}H_{35}N_3O_4S_1$ ([M+H]$^+$), 582.2427, found 582.2441.

*4.1.7. 2'-C-Methyl-4-aza-7,9-dideazaadenosine (**1**).* A solution of compound **21** (900 mg, 2.11 mmol) in 7 N methanolic ammonia (60 mL) was stirred for 24 h at 100 °C. After removal of all the volatiles under reduced pressure, the remaining crude residue was purified by silica gel column chromatography (heptane/EtOAc = 5:1, v/v) to afford the debenzylated product as an oil (500 mg, 59% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 (s, 1H, H_2), 7.45-7.22 (m, 15H, Ar-*H*), 6.87 (d, *J* = 4.5 Hz, H_7), 6.52 (d, *J* = 4.5 Hz, H_8), 6.14 (br, 2H, NH_2), 5.90 (s, 1H, $H_{1'}$), 4.85 (q, *J* = 12.0 Hz, 2H, OCH_2Ph), 4.70-4.37 (m, 5H, $H_{4',}$ $H_{3'}$, and OCH_2Ph), 4.07 (d, *J* = 8.5

Hz, 1H, O*C*H$_2$Ph), 3.92-3.69 (2 × dd, J_{gem} = 10.8 Hz, $J_{5',4}$ = 2.2 Hz, $J_{5'',4}$ = 3.4 Hz, 2H, $H_{5'}$, $H_{5''}$), 1.04 (s, 3H, C*H*$_3$); ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$) δ 155.6, 146.9, 140.0, 138.5, 131.1, 128.7, 128.6, 128.5, 128.3, 128.1, 127.9, 127.8, 127.5, 127.4, 114.3, 110.9, 100.9, 85.2, 82.7, 79.5, 78.3, 73.7, 69.6, 66.0, 17.9; HRMS (ESI-TOF) m/z: calcd for $C_{33}H_{34}N_4O_4$ ([M+H]$^+$), 551.2658, found 551.2661. Such intermediate (500 mg, 0.908 mmol) was dissolved in cyclohexene/ethanol = 4/1 (10 mL), followed by the addition of Pd(OH)$_2$ (10%) (500 mg). The reaction mixture was stirred under a hydrogen atmosphere at 85 °C for 12 h, cooled, and then filtered through a pad of Celite. After evaporation under reduced pressure, the remaining crude residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 10:1, v/v) affording compound **1** as a white solid (200 mg, 80% yield). ^{1}H NMR (300 MHz, CD$_3$OD) δ 7.79 (s, 1H, H_2), 6.88 (d, *J* = 4.5 Hz, H_7), 6.77 (d, *J* = 4.5 Hz, H_8), 5.57 (s, 1H, $H_{1'}$), 4.02-3.78 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{5''}$), 3.64 (s, 1H, $H_{3'}$), 0.94 (s, 3H, C*H*$_3$); ^{13}C{^{1}H} NMR (75 MHz, CD$_3$OD) δ 155.3 (C-2), 146.0 (C-5), 129.9 (C-6), 113.8 (C-9), 109.65 (C-7), 101.27 (C-8), 81.9 (C-1'), 78.8 (C-4'), 74.4 (C-2'), 69.6 (C-3'), 61.2 (C-5'), 19.6 (2-*C*-CH$_3$); HRMS (ESI-TOF) m/z: calcd for $C_{12}H_{16}N_4O_4$ ([M+H]$^+$), 281.1250, found 281.1243.

4.1.8. 2'-C-Methyl-7-chloro-4-aza-7,9-dideazaadenosine (9). A solution of compound **1** (85 mg, 0.303 mmol) in anhydrous DMF (5 mL) was cooled to 0 °C. *N*-Chlorosuccinimide (32 mg, 0.242 mmol) was added and the reaction mixture was stirred for 2 days at room temperature. After evaporation under reduced pressure, the crude residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 10:1, v/v) furnishing the title compound as a white solid (30 mg, 32% yield). ^{1}H NMR (300 MHz, CD$_3$OD) δ 7.74 (s, 1H, H_2), 6.83 (s, 1H, H_8), 5.53 (s, 1H, $H_{1'}$), 4.00-3.75 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{5''}$), 3.64 (s, 1H, $H_{3'}$), 0.96 (s, 3H, C*H*$_3$); ^{13}C{^{1}H} NMR (75 MHz, CD$_3$OD) δ 154.9 (C-2), 146.9 (C-5), 130.0 (C-6), 109.5 (C-9), 104.6 (C-8), 81.9 (C-7), 80.6 (C-1'), 78.8 (C-4'), 74.0 (C-2'), 69.8 (C-3'), 60.8 (C-5'), 19.4 (2-*C*-CH$_3$); HRMS (ESI-TOF) m/z: calcd for $C_{12}H_{15}Cl_1N_4O_4$ ([M+H]$^+$), 315.0860, found 315.0869.

*4.1.9. Methyl α/β-L-ribofuranoside (**22**).* A solution of L-ribose (5.0 g, 33.3 mmol) in anhydrous methanol (100 mL) at 0 °C was treated with concentrated sulfuric acid (0.6 mL). The reaction mixture was stirred at room temperature for 4.5 h. The solution was then neutralized with solid Na_2CO_3 to pH > 7. The solid was filtered and washed with methanol (3 × 10 mL). After evaporation of the filtrate, methyl *α*/*β*-L-ribofuranoside **22** was obtained as an oil (5.0 g, 91% yield). ^{1}H NMR analysis showed a ratio of *α*-**22**/*β*-**22** ~1/4. Data for α: ^{13}C{^{1}H} NMR (75 MHz, CD$_3$OD) δ 102.9 (C-1), 85.3 (C-4), 71.1 (C-2), 69.4 (C-3), 61.6 (C-5), 53.9 (O*C*H$_3$); HRMS: calcd for $C_6H_{12}O_5$ ([M+Na]$^+$), 187.0582, found 187.0576. Data for *β*-**22**: ^{1}H NMR (300 MHz, CD$_3$OD) δ 4.78 (s, 1H, H_1), 4.06 (d, $J_{2,3}$ = 4.5 Hz, 1H, H_2), 3.98 (dd, $J_{4,5b}$ = 3.4 Hz, $J_{4,5a}$ = 6.1 Hz, 1H, H_4), 3.92 (d, $J_{3,2}$ = 4.5 Hz, H_3), 3.74-3.54 (2 × dd, J_{gem} = 11.8 Hz, $J_{5a,4}$ = 6.1 Hz, $J_{5b,4}$ = 3.6 Hz, 2H, H_{5a}, H_{5b}), 3.36 (s, 3H, OC*H*$_3$); ^{13}C{^{1}H} NMR (75 MHz, CD$_3$OD) δ 108.1, 83.0, 74.4, 71.0, 63.3, 53.8.

4.1.10. Methyl 2,3,5-tri-O-benzyl-α/β-L-ribofuranoside (23). Sodium hydride (60%, 13.0 g, 152.3 mmol) was added to a solution of methyl *α*/*β*-L-ribofuranoside **22** (5.0 g, 30.5 mmol) in dry DMF (100 mL) at room temperature under an argon atmosphere. After hydrogen generation ceased, benzyl bromide (19.0 mL, 121.8 mmol) was added, and the mixture was stirred at 0 °C to rt for 24 h. It was then slowly poured into cold saturated aq. NH_4Cl (100 mL) at 0 °C, and further diluted and extracted with EtOAc (3 × 200 mL). The combined organic

layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (gradient heptane/EtOAc, 20:1 to 5:1, v/v) to afford *α*-**23**/*β*-**23** (10.0 g, 75% yield). Data for *β*: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.18-7.99 (m, 15H, Ar-*H*), 5.74 (s, 1H, H_1), 5.47-5.17 (m, 7H, H_2, 3 × OC*H*$_2$Ph), 4.86 (m, 1H, H_3), 4.66 (m, 1H, H_4), 4.44-4.32 (m, 2H, H_{5a}, H_{5b}), 4.09 (s, 3H, OC*H*$_3$); ^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$) δ 138.8, 138.4, 138.3, 128.8, 128.4, 128.3, 128.2, 128.0, 127.9, 106.8, 80.9, 80.2, 78.9, 73.5, 72.8, 72.7, 71.8, 55.4; HRMS (ESI-TOF) m/z: calcd for $C_{27}H_{30}O_5$ ([M+Na]$^+$), 457.1991, found 457.1978.

*4.1.11. 2,3,5-Tri-O-benzyl-L-ribono-1,4-lactone (**24**).* To a solution of **23** (10.0 g, 23.0 mmol) in AcOH (100 mL) was added 1 N aq. HCl (25 mL), and the mixture was stirred for 4 h at 80 °C. After removal of all the volatiles under reduced pressure, the residue was dissolved in EtOAc and washed with aq. saturated $NaHCO_3$ and brine. The organic layer was then dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The resulting residue was dissolved in DMSO (55 mL), then Ac_2O (33 mL) was slowly added at room temperature. The mixture was stirred at room temperature overnight, evaporated under reduced pressure, and the residue was diluted and extracted with EtOAc (3 × 200 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified by silica gel column chromatography (heptane/EtOAc, 5:1, v/v) to afford **24** as an oil (6.0 g, 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36-7.21 (m, 13H, Ar-*H*), 7.15-7.08 (m, 2H, Ar-*H*), 4.87 (d, *J* = 12.0 Hz, 1H, OC*H*$_2$Ph), 4.67 (t, *J* = 12.3 Hz, 1H, OC*H*$_2$Ph), 4.66-4.29 (m, 6H, H_2, OC*H*$_2$Ph), 4.07 (d, $J_{3,2}$ = 5.2 Hz, 1H, H_3), 3.53 (m, 2H, H_{5a}, H_{5b}); ^{13}C{^{1}H} NMR (75 MHz, $CDCl_3$) δ 174.2, 137.7, 137.6, 137.4, 128.9, 128.9, 128.6, 128.5, 128.4, 128.3, 127.9, 82.2, 75.8, 74.3, 73.9, 73.1, 72.70, 69.2; HRMS (ESI-TOF) m/z: calcd for $C_{26}H_{26}O_5$ ([M+Na]$^+$), 441.1678, found 441.1669.

*4.1.12. 2',3',5'-O-Tribenzyl-1'-hydroxy-6-methylthio-4-aza-7,9-dideaza-L-adenosine (**25**).* To a solution of 4-(methylthio)pyrrolo[2,1-f][1,2,4]triazine **19** (800 mg, 4.78 mmol) in dry THF (20 mL) was added lithium diisopropylamide (2 M in THF, 3.6 mL, 7.17 mmol) at -78 °C under an argon atmosphere, and the resulting mixture was stirred for 30 min at this temperature. Then, a solution of compound **24** (3.0 g, 7.17 mmol) in dry THF (10 mL) was added and the resulting reaction mixture was stirred for further 3 h at -78 °C. The reaction was quenched with saturated aq. NH_4Cl and then extracted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The crude residue was purified by silica gel column chromatography (heptane/EtOAc = 5:1, v/v) to give the title compound as a yellow oil (1.6 g, 60% yield). HRMS (ESI-TOF) m/z: calcd for $C_{33}H_{35}N_3O_5S_1$ ([M+H]$^+$), 584.2219, found 584.2186.

*4.1.13. 2',3',5'-O-Tribenzyl-6-methylthio-4-aza-7,9-dideaza-L-adenosine (**26**).* To a solution of compound **25** (1.60 g, 2.74 mmol) in dichloromethane (50 mL) was added triethylsilane (1.27 g, 11.0 mmol) and trifluoroborane (778.1 mg, 5.48 mmol) at 0 °C under an argon atmosphere. The resulting solution was stirred for 40 min at 0 °C and then quenched with a saturated aq. Na_2CO_3 solution. The mixture was extracted with ethyl acetate, and the organic layer was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was purified by silica gel column chromatography (heptane/EtOAc = 10:1, v/v) to give the title compound as an oil (1.2 g, 80% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17 (s, 1H, H_2), 7.32-7.20 (m, 15H, Ar-*H*), 6.80 (d, *J* = 4.6 Hz, 1H, H_7), 6.66 (d, *J* =

4.6 Hz, 1H, H_8), 5.71 (d, $J_{1',2'}$ = 4.2 Hz, 1H, $H_{1'}$), 4.72-4.37 (m, 7H, 3 × OC*H*$_2$Ph, $H_{2'}$), 4.25 (m, 1H, $H_{3'}$), 4.12 (m, $H_{4'}$), 3.81-3.60 (2 × dd, $J_{5'',4'}$ = 3.3 Hz, $J_{5'',4'}$ = 3.9 Hz, J_{gem} = 10.8 Hz, 2H, $H_{5'}$, $H_{5''}$), 2.59 (s, 3H, SC*H*$_3$); $^{13}C\{^1H\}$ NMR (75 MHz, $CDCl_3$) δ 164.8, 145.4, 138.6, 138.2, 129.6, 128.7, 128.6, 128.4, 128.2, 128.1, 127.9, 122.6, 112.6, 102.2, 81.0, 79.4, 77.6, 76.3, 73.7, 72.4, 72.0, 70.0, 11.7; HRMS (ESI-TOF) m/z: calcd for $C_{33}H_{33}N_3O_4S_1$ ([M+H]$^+$), 568.2270, found 568.2258.

*4.1.14. 2',3',5'-O-Tribenzyl-4-aza-7,9-dideaza-L-adenosine (**27**).* A solution of compound **26** (1.2 g, 2.11 mmol) in 7 N methanolic ammonia (60 mL) was stirred for 24 h at 100 °C. After removal of all the volatiles in vacuo, the crude residue was purified by silica gel column chromatography (heptane/EtOAc = 5:1, v/v) to afford the title compound as a yellow oil (1.0 g, 80% yield). ^1H NMR (300 MHz, $CDCl_3$) δ 7.86 (s, 1H, H_2), 7.41-7.19 (m, 15H, Ar-*H*), 6.60 (d, *J* = 4.6 Hz, 1H, H_7), 6.49 (d, *J* = 4.6 Hz, 1H, H_8), 6.16 (br, 2H, N*H*$_2$), 5.68 (d, $J_{1',2'}$ = 4.2 Hz, 1H, H_1'), 4.74-4.35 (m, 7H, 3 × OC*H*$_2$Ph, H_2'), 4.25 (m, 1H, H_3'), 4.12 (m, 1H, H_4'), 3.81-3.60 (m, 2H, $H_{5'}$, $H_{5''}$); $^{13}C\{^1H\}$ NMR (75 MHz, $CDCl_3$) δ 155.7, 147.2, 138.5, 138.2, 129.8, 128.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 1278; HRMS (ESI-TOF) m/z: calcd for $C_{32}H_{32}N_4O_4$ ([M+H]$^+$), 537.2502, found 537.2512.

*4.1.15. 4-Aza-7,9-dideaza-L-adenosine (**10**).* To a solution of compound **27** (1.0 g, 1.86 mmol) in AcOH (10 mL) was added $Pd(OH)_2$ (10%) (1.0 g) and the reaction mixture was stirred under a hydrogen atmosphere at room temperature for 48 h. It was then filtered through a pad of Celite, and the filtrate was concentrated under reduced pressure. The resulting crude residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 8:1, v/v) to afford the title compound as a white solid (400 mg, 80% yield). ^1H NMR (300 MHz, CD_3OD) δ 7.78 (s, 1H, H_2), 6.88 (d, *J* = 4.6 Hz, 1H, H_7), 6.76 (d, *J* = 4.6 Hz, 1H, H_8), 5.25 (d, $J_{1',2'}$ = 6.3 Hz, 1H, $H_{1'}$), 4.53 (dd, $J_{2',1'}$ = 6.3 Hz, $J_{2',3'}$ = 5.4, 1H, $H_{2'}$), 4.20 (dd, $J_{3',4'}$ = 3.9 Hz, $J_{3',2'}$ = 5.4 Hz, 1H, $H_{3'}$), 4.06 (ddd, $J_{4',5''}$ = 3.2 Hz, $J_{4',5'}$ = 3.9 Hz, $J_{4',3'}$ = 3.9 Hz, 1H, $H_{4'}$), 3.85-3.67 (2 × dd, $J_{5'',4'}$ = 3.2 Hz, $J_{5',4'}$ = 3.9 Hz, J_{gem} = 12.1 Hz, 2H, $H_{5'}$, $H_{5''}$); $^{13}C\{^1H\}$ NMR (75 MHz, CD_3OD-d_4) δ 155.6 (C-2), 146.2 (C-5), 128.3 (C-6), 114.9 (C-9), 110.4 (C-7), 100.9 (C-8), 84.7 (C-1'), 76.9 (C-4'), 73.2 (C-2'), 71.6 (C-3'), 62.1 (C-5'); HRMS (ESI-TOF) m/z: calcd for $C_{11}H_{14}N_4O_5$ ([M+H]$^+$), 267.1093, found 267.1091.

*4.1.16. 7-Chloro-4-aza-7,9-dideaza-L-adenosine (**11**).* A solution of compound **10** (92 mg, 0.345 mmol) in anhydrous DMF (6 mL) was cooled to 0 °C. *N*-Chlorosuccinimide (51 mg, 0.345 mmol) was added and the reaction mixture was stirred for 2 days at room temperature. The reaction mixture was concentrated and the crude residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 10:1, v/v) furnishing the title compound as a white solid (30 mg, 29% yield). ^1H NMR (300 MHz, DMSO-d_6) δ 7.85 (s, 1H), 6.83 (s, 1H), 5.12 (d, $J_{1',2'}$ = 6.0 Hz, 1H, $H_{1'}$), 4.16 (dd, $J_{2',1'}$ = 6.1 Hz, $J_{2',3'}$ = 5.6 Hz, 1H, $H_{2'}$), 3.94 (dd, $J_{3',2'}$ = 5.6 Hz, $J_{3',4'}$= 4.6 Hz, $H_{3'}$), 3.78 (ddd, $J_{4',3'}$ = 4.6 Hz, $J_{4',5''}$ = 3.8 Hz, $J_{4',5'}$ = 4.1 Hz, $H_{4'}$), 3.60-3.41 (2xdd, $J_{5',4'}$ = 4.1 Hz, $J_{5'',4'}$ = 3.8 Hz, J_{gem} = 11.3 Hz, $H_{5'}$, $H_{5''}$); $^{13}C\{^1H\}$ NMR (75 MHz, DMSO-d_6) δ 155.0 (C-2), 148.1 (C-5), 129.5 (C-6), 110.5 (C-9), 109.8 (C-8), 103.1 (C-7), 84.6 (C-1'), 75.0 (C-4'), 74.2 (C-2'), 71.1 (C-3'), 61.8 (C-5'); HRMS (ESI-TOF) m/z: calcd for $C_{17}H_{13}Cl_1N_4O_4$ ([M+H]$^+$), 301.0704, found 301.0703.

*4.1.17. 7-Chloro-5'-bis(l-alanine benzyl ester)phosphate-4-aza-7,9-dideazaadenosine (**28**).* To a suspension of compound **5** (50.0 mg, 0.16 mmol) and solid sodium bicarbonate (50 mg) in trimethyl phosphate (1.5 mL) at 0 °C was added $POCl_3$ (117 mg, 0.78 mmol). The mixture

was stirred at 0 °C for 3 h, and a solution of L-alanine benzyl ester hydrochloride (900 mg, 4.16 mmol) was then added. The reaction mixture was stirred at 0 °C for 0.5 h, then triethylamine (0.1 mL) was added and stirred at room temperature for 0.5 h. The reaction mixture was diluted with ethyl acetate (10 mL), washed with water (10 mL), brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified first by silica gel column chromatography (CH_2Cl_2/MeOH = 10:1, v/v) and then reverse phase HPLC (5-60% MeCN in water) to afford compound **28** as a white foam (12 mg, 11%). ^{1}H NMR (300 MHz, CD_3OD) δ 7.78 (s, 1H, H_2), 7.38-7.28 (m, 10H, Ar-*H*), 6.77 (s, 1H, H_8), 5.32 (d, $J_{1',2'}$ = 5.4 Hz, 1H, $H_{1'}$), 5.16-5.05 (m, 4H, 2 × OCH_2Ph), 4.34 (dd, $J_{2',1'}$ = 5.4 Hz, 1H, $J_{2',3'}$ = 4.8 Hz, $H_{2'}$), 4.20-3.84 (m, 6H, 2 × C*H*(CH_3)N*H*), $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{5''}$), 1.29 (d, *J* = 7.2 Hz, 3H, CH_3), 1.25 (d, *J* = 7.2 Hz, 3H, CH_3); ^{13}C{^{1}H} NMR (300 MHz, CD_3OD) δ 177.3, 177.2 (q, *J* = 4.8 Hz, *C*O), 156.6, 148.8, 137.2 (d, *J* = 5.5 Hz), 130.0, 129.5, 129.2, 112.2, 111.1, 106.0, 83.6, 83.5 (d, *J* = 7.4 Hz), 77.8, 75.3, 72.3, 67.8, 67.9 (d, *J* = 3.7 Hz), 66.5, 66.4 (d, *J* = 5.7 Hz), 51.0, 20.7 (d, *J* = 5.9 Hz), 20.5 (d, *J* = 6.3 Hz); ^{31}P NMR (121 MHz, CD_3OD) δ = 13.70; HRMS (ESI-TOF) m/z: calcd for $C_{31}H_{36}Cl_1N_6O_9P_1$ ([M+H]$^+$), 703.2048, found 703.2062.

4.2. Biological assays

4.2.1. Cells, viruses, compounds

Murine norovirus (strain MNV-1.CW1) was propagated in RAW 264.7 cells grown in DMEM supplemented with 10 or 2% FBS, 2 mM L-glutamine, 20 mM HEPES, 0.075 g/L sodium bicarbonate, 1 mM sodium pyruvate, 100 U penicillin/mL, 100 μg/mL streptomycin (Thermo Fisher, Gent, Belgium) at 37 °C in a humidified atmosphere of 5% CO_2.

For *in vitro* assays, a stock solution of each compound was prepared either in dimethyl sulfoxide (DMSO, VWR Chemicals, Haasrode, Belgium) or MilliQ water.

4.2.2. Antiviral assay for murine norovirus (MNV)

The antiviral activity of the synthesized compounds was initially determined using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-based cytopathic effect (CPE) reduction assay. RAW 264.7 cells (1 × 10^4 cells/well) were seeded in a 96-well plate and infected with MNV (MOI of 0.001) in the presence (or absence) of a dilution series of compounds (0.023–50 or 100 μg/mL). Cells were incubated for 3 days, i.e., until complete CPE was observed in infected untreated cells. Then, a MTS-phenazinemethosulfate (MTS/PMS) stock solution [(2 mg/mL MTS (Promega, Leiden, The Netherlands) and 46 g/mL PMS (Sigma–Aldrich, Bornem, Belgium) in PBS at pH 6–6.5)] was diluted 1/20 in MEM (Thermo Fisher, Gent, Belgium) and 75 μL were added to each well. After 2 h, the optical density (OD) was read at 498 nm. The %CPE reduction was calculated as $[(OD_{treated})_{MNV} - OD_{VC}]/[OD_{CC} - OD_{VC}] \times 100$, where OD_{CC} represents the OD of the uninfected untreated cells, while OD_{VC} and $(OD_{treated})_{MNV}$ represent the OD of infected untreated cells and virus-infected cells treated with a compound concentration, respectively. The 50% effective concentration (EC_{50}) was defined as the compound concentration that protected 50% of the cells from virus-induced CPE.

4.2.3. Antiviral assay for human norovirus (HuNoV)

The *in vitro* antiviral activity of the compounds was evaluated based on the determination of

intracellular GI.1 virus replicon RNA by quantitative reverse transcription-PCR [qRT-PCR]) using *β*-actin mRNA as reference (housekeeping) gene. HGT-1 cells (5,000/well) were seeded into the wells of 96-well plates in complete DMEM without G418. After 24 h of incubation, serial dilutions of compounds were added. Cells were further incubated for 72 h, after which cell culture supernatant was removed and monolayers were washed with phosphate-buffered saline (PBS). Cell monolayers were collected for RNA load quantification by qRT-PCR. To determine the relative levels of GI.1 virus replicon RNA, *β*-actin was used as a normalizer and ratios were calculated by the Pfaffl method.[29] The GI.1 virus replicon/*β*-actin ratio was calculated as follows: Ratio = $E_{\text{GI.1}}{}^{\Delta \text{CT, GI.1 (CC - TC)}}/E_{\beta\text{-actin}}{}^{\Delta \text{CT, }\beta\text{-actin (CC - TC)}}$, where $E_{\text{GI.1}}$ and $E_{\beta\text{-actin}}$ represent the amplification efficiencies ($E = 10^{-1/\text{slope}}$) of the GI.1 virus replicon and *β*-actin qRT-PCRs, respectively, ΔC_T, GI.1 virus (CC - TC) is the C_T (threshold cycle) of untreated control cells (CC) minus the C_T of cells treated with a compound concentration (TC) obtained with GI.1 virus replicon primers and probe; and ΔC_T, *β*-actin (CC - TC) is the C_T of untreated control cells (CC) minus the C_T of cells treated with a compound concentration (TC) obtained with *β*-actin primers and probe. Efficiency values ($E_{\text{GI.1}}$ and $E_{\beta\text{-actin}}$) were determined for each qRT-PCR. The 50% effective concentration (EC_{50}) was defined as the compound concentrations that resulted in 50% reductions of the relative GI.1 virus replicon RNA levels.

4.2.4. Cytotoxicity assay

The cytotoxicity of the compounds was evaluated by the MTS-method, by exposing uninfected cells to the same concentrations of compounds for 3 days. The % cell viability was calculated as $(OD_{treated}/OD_{CC}) \times 100$, where OD_{CC} is the OD of uninfected untreated cells and OD treated are uninfected cells treated with compound. The CC_{50} was defined as the compound concentration that reduces the number of viable cells by 50%. The selectivity index (SI) was calculated as CC_{50}/EC_{50}.

AUTHOR INFORMATION

Corresponding Author

* *E-mail addresses*: Piet.Herdewijn@kuleuven.be (P. Herdewijn); E-mail: elisabetta.groaz@kuleuven.be (E. Groaz).

Notes

The authors declare no competing financial interest.

Acknowledgments

Q.L. thanks the China Scholarship Council (CSC) for funding (grant 201506890014). We thank Jasper Rymenants and Lindsey Bervoets (KU Leuven) for excellent technical assistance. We are grateful to Ian Goodfellow (University of Cambridge) for kindly providing the HuNoV GI replicon.

Appendix A. Supplementary data

Supplementary data related to this article was uploaded.

References

Journal Pre-proof

(1) Patel, M. M.; Hall, A. J.; Vinje, J.; Parashara, U. D. Noroviruses: A Comprehensive Review. *J. Clin. Virol.* **2009**, *44*, 1-8.

(2) Glass, R. I.; Parashar, U. D.; Estes, M. K. CURRENT CONCEPTS Norovirus Gastroenteritis. *New Engl. J. Med.* **2009**, *361*, 1776-1785.

(3) Payne, D. C.; Vinje, J.; Szilagyi, P. G.; Edwards, K. M.; Staat, M. A.; Weinberg, G. A.; Hall, C. B.; Chappell, J.; Bernstein, D. I.; Curns, A. T.; Wikswo, M.; Shirley, S. H.; Hall, A. J.; Lopman, B.; Parashar, U. D. Norovirus and Medically Attended Gastroenteritis in U.S. Children. *New Engl. J. Med.* **2013**, *368*, 1121-1130.

(4) Bok, K.; Green, K. Y. Norovirus Gastroenteritis in Immunocompromised Patients. *New Engl. J. Med.* **2012**, *367*, 2126-2132.

(5) Duizer, E.; Schwab, K. J.; Neill, F. H.; Atmar, R. L.; Koopmans, M. P. G.; Estes, M. K. Laboratory Efforts To Cultivate Noroviruses. *J. Gen. Virol.* **2004**, *85*, 79-87.

(6) Lay, M. K.; Atmar, R. L.; Guix, S.; Bharadwaj, U.; He, H.; Neill, F. H.; Sastry, K. J.; Yao, Q. Z.; Estes, M. K. Norwalk Virus Does Not Replicate in Human Macrophages or Dendritic Cells Derived from the Peripheral Blood of Susceptible Humans. *Virology* **2010**, *406*, 1-11.

(7) Bassetto, M.; Van Dycke, J.; Neyts, J.; Brancale, A.; Rocha-Pereira, J. Targeting the Viral Polymerase of Diarrhea-Causing Viruses as a Strategy to Develop a Single Broad-Spectrum Antiviral Therapy. *Viruses* **2019**, *11*, doi:10.3390/v11020173.

(8) Weerasekara, S.; Prior, A. M.; Hua, D. H. Current Tools for Norovirus Drug Discovery. *Expert Opin. Drug Dis.* **2016**, *11*, 529-541.

(9) Netzler, N. E.; Tuipulotu, D. E.; White, P. A. Norovirus Antivirals: Where Are We Now? *Med. Res. Rev.* **2019**, *39*, 860-886.

(10) Costantini, V. P.; Whitaker, T.; Barclay, L.; Lee, D.; McBrayer, T. R.; Schinazi, R. F.; Vinje, J. Antiviral Activity of Nucleoside Analogues Against Norovirus. *Antivir. Ther.* **2012**, *17*, 981-991.

(11) Eltahla, A. A.; Lim, K. L.; Eden, J. S.; Kelly, A. G.; Mackenzie, J. M.; White, P. A. Nonnucleoside Inhibitors of Norovirus RNA Polymerase: Scaffolds for Rational Drug Design. *Antimicrob. Agents Chemother.* **2014**, *58*, 3115-3123.

(12) Chang, K. O.; George, D. W. Interferons and Ribavirin Effectively Inhibit Norwalk Virus Replication in Replicon-Bearing Cells. *J. Virol.* **2007**, *81*, 12111-12118.

(13) Rocha-Pereira, J.; Jochmans, D.; Dallmeier, K.; Leyssen, P.; Cunha, R.; Costa, I.; Nascimento, M. S. J.; Neyts, J. Inhibition of Norovirus Replication by the Nucleoside Analogue 2 '-C-Methylcytidine. *Biochem. Biophys. Res. Commun.* **2012**, *427*, 796-800.

(14) Kolawole, A. O.; Rocha-Pereira, J.; Elftman, M. D.; Neyts, J.; Wobus, C. E. Inhibition of Human Norovirus by a Viral Polymerase Inhibitor in the B Cell Culture System and in the Mouse Model. *Antivir. Res.* **2016**, *132*, 46-49.

(15) Mastrangelo, E.; Mazzitelli, S.; Fabbri, J.; Rohayem, J.; Ruokolainen, J.; Nykanen, A.; Milani, M.; Pezzullo, M.; Nastruzzi, C.; Bolognesi, M. Delivery of Suramin as an Antiviral Agent through Liposomal Systems. *Chemmedchem* **2014**, *9*, 933-939.

(16) Mastrangelo, E.; Pezzullo, M.; Tarantino, D.; Petazzi, R.; Germani, F.; Kramer, D.; Robel, I.; Rohayem, J.; Bolognesi, M.; Milani, M. Structure-Based Inhibition of Norovirus RNA-Dependent RNA Polymerases. *J. Mol. Biol.* **2012**, *419*, 198-210.

(17) De Clercq, E. C-Nucleosides To Be Revisited. *J. Med. Chem.* **2016**, *59*, 2301-2311.

(18) Temburnikar, K.; Seley-Radtke, K. L. Recent Advances in Synthetic Approaches for Medicinal Chemistry of C-Nucleosides. *Beilstein J. Org. Chem.* **2018**, *14*, 772-785.

Journal Pre-proof

Journal Pre-proof

(19) Cho, A.; Zhang, L. J.; Xu, J.; Babusis, D.; Butler, T.; Lee, R.; Saunders, O. L.; Wang, T.; Parrish, J.; Perry, J.; Feng, J. Y.; Ray, A. S.; Kim, C. U. Synthesis and Characterization of 2 '-C-Me Branched C-Nucleosides as HCV Polymerase Inhibitors. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4127-4132.

(20) Draffan, A. G.; Frey, B.; Fraser, B. H.; Pool, B.; Gannon, C.; Tyndall, E. M.; Cianci, J.; Harding, M.; Lilly, M.; Hufton, R.; Halim, R.; Jahangiri, S.; Bond, S.; Jeynes, T. P.; Nguyen, V. T. T.; Wirth, V.; Luttick, A.; Tilmanis, D.; Pryor, M.; Porter, K.; Morton, C. J.; Lin, B.; Duan, J. M.; Bethell, R. C.; Kukolj, G.; Simoneau, B.; Tucker, S. P. Derivatives of imidazotriazine and pyrrolotriazine C-nucleosides as potential new anti-HCV agents. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 4984-4988.

(21) Li, Q. F.; Lescrinier, E.; Groaz, E.; Persoons, L.; Daelemans, D.; Herdewijn, P.; De Jonghe, S. Synthesis and Biological Evaluation of Pyrrolo[2,1-f][1,2,4]triazine C-Nucleosides with a Ribose, 2-Deoxyribose, and 2,3-Dideoxyribose Sugar Moiety. *Chemmedchem* **2018**, *13*, 97-104.

(22) Mathe, C.; Gosselin, G. L-Nucleoside Enantiomers as Antivirals Drugs: A Mini-Review. *Antivir. Res.* **2006**, *71*, 276-281.

(23) Seela, F.; Ming, X. 7-Functionalized 7-Deazapurine Beta-D and Beta-L-Ribonucleosides Related to Tubercidin and 7-Deazainosine: Glycosylation of Pyrrolo[2,3-D] Pyrimidines with 1-O-Acetyl-2,3,5-Tri-O-Benzoyl-Beta-D or Beta-L-Ribofuranose. *Tetrahedron* **2007**, *63*, 9850-9861.

(24) Draffan, A. G.; Frey, B.; Pool, B.; Gannon, C.; Tyndall, E. M.; Lilly, M.; Francom, P.; Hufton, R.; Halim, R.; Jahangiri, S.; Bond, S.; Nguyen, V. T. T.; Jeynes, T. P.; Wirth, V.; Luttick, A.; Tilmanis, D.; Thomas, J. D.; Pryor, M.; Porter, K.; Morton, C. J.; Lin, B.; Duan, J. M.; Kukolj, G.; Simoneau, B.; McKercher, G.; Lagace, L.; Amad, M.; Bethell, R. C.; Tucker, S. P. Discovery and Synthesis of C-Nucleosides as Potential New Anti-HCV Agents. *ACS Med. Chem. Lett.* **2014**, *5*, 679-684.

(25) Li, N. S.; Lu, J.; Piccirilli, J. A. Efficient Synthesis of Methyl 3,5-Di-O-Benzyl-Alpha-D-Ribofuranoside and Application to the Synthesis of 2'-C-Beta-Alkoxymethyluridines. *Org. Lett.* **2007**, *9*, 3009-3012.

(26) Albright, J. D.; Goldman, L. Dimethyl Sulfoxide-Acid Anhydride Mixtures . New Reagents for Oxidation of Alcohols. *J. Am. Chem. Soc.* **1965**, *87*, 4214-4216.

(27) Clark, J. L.; Hollecker, L.; Mason, J. C.; Stuyver, L. J.; Tharnish, P. M.; Lostia, S.; McBrayer, T. R.; Schinazi, R. F.; Watanabe, K. A.; Otto, M. J.; Furman, P. A.; Stec, W. J.; Patterson, S. E.; Pankiewiez, K. W. Design, synthesis, and antiviral activity of 2 '-deoxy-2 '-fluoro-2 '-C-methylcytidine, a potent inhibitor of hepatitis C virus replication. *J. Med. Chem.* **2005**, *48*, 5504-5508.

(28) Sofia, M. J.; Bao, D.; Chang, W.; Du, J. F.; Nagarathnam, D.; Rachakonda, S.; Reddy, P. G.; Ross, B. S.; Wang, P. Y.; Zhang, H. R.; Bansal, S.; Espiritu, C.; Keilman, M.; Lam, A. M.; Steuer, H. M. M.; Niu, C. R.; Otto, M. J.; Furman, P. A. Discovery of a beta-D-2 '-Deoxy-2 '-alpha-fluoro-2 '-beta-C-methyluridine Nucleotide Prodrug (PSI-7977) for the Treatment of Hepatitis C Virus. *J. Med. Chem.* **2010**, *53*, 7202-7218.

(29) Pfaffl, M. W. A New Mathematical Model for Relative Quantification in Real-Time RT-PCR. *Nucleic Acids Res.* **2001**, *29*.

Journal Pre-proof

Journal Pre-proof

- *C*-Nucleosides comprising structural variations at the sugar and nucleobase were prepared.
- Their ability to inhibit both murine and human norovirus was examined.
- Compounds **4** and **5** resulted in good antiviral activity against murine norovirus.
- Compound **4** retained its antiviral effect in a human replicon assay (EC_{50} = 0.015 μM).
- Phosphonodiamidate prodrug **28** was devoid of antiviral activity.

Journal Pre-proof

Journal Pre-proof

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Journal Pre-p