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Article

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Synthesis and Anti-HCV Activity of Sugar-Modified Guanosine Analogues: Discovery of AL-611 as HCV NS5B Polymerase Inhibitor for the Treatment of Chronic Hepatitis C

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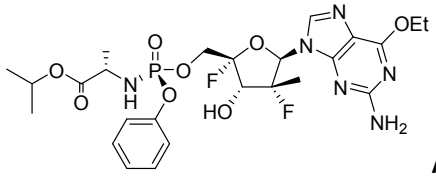
Abstract: Chronic hepatitis C (CHC) is a major liver disease caused by the hepatitis C virus. The current standard of care for CHC can achieve cure rates above 95%, however the drugs in current use are administered for a period of 8-16 weeks. A combination of safe and effective drugs with a shorter treatment period is highly desirable. We report synthesis and biological evaluation of a series of 2',3'- and 2',4'-substituted guanosine nucleotide analogues. Their triphosphates exhibited potent inhibition of the HCV NS5B polymerase with IC_{50} as low as 0.13 μM. In the HCV replicon assay, the phosphoramidate prodrugs of these analogues demonstrated excellent activity with EC_{50} values as low as 5 nM. A lead compound **AL-611** showed high levels of the NTP in vitro in primary human hepatocytes and in vivo in dog liver following oral administration.



AL-611

EC_{50} = 0.005 μM, CC_{50} > 100 μM

INTRODUCTION

Chronic hepatitis C (CHC) is a major liver disease caused by the hepatitis C virus. The standard of care for hepatitis C has changed rapidly with the advent of new therapies and other developments. Combinations of sofosbuvir/velpatasvir or sofosbuvir/ledipasvir are part of the preferred regimens in the AASLD guidelines[1-3] and can achieve cure rates above 95%. Therapy with these direct antiviral agents can cure persons infected with HCV and the treatment period is typically 12 weeks. Drugs in chronic use may develop resistance and show more adverse effects with time. Therefore, a combination of safe and effective drugs with a shorter treatment period is highly desirable. A shorter treatment therapy is expected to increase both patient compliance and treatment accessibility to patients infected by HCV. Nucleoside analogues have excellent records as antiviral drugs[4]. Their triphosphate metabolites may selectively inhibit viral targets, particularly viral polymerases, effectively preventing viral replication.[5] The well-known HCV drug sofosbuvir, a uridine analogue, is widely used in HCV therapies.[2, 6] Another nucleoside drug-candidate BMS094 (INX-189), which is a prodrug of 2'-*C*-methylguanosine **1**, is also well known for its very potent activity against HCV infection.[7] However, the drug was halted in a clinical trial owing to its severe toxicity.[8] It was reported two more guanosine prodrugs of β-D-2′-deoxy-2′-α-fluoro-2′-β-*C*-methylguanosine **2**: PSI-661[9], a phosphoramidate nucleotide, and PSI-938[10-11], a cyclic phosphate nucleotide. Both compounds are metabolized to the same active 5′-triphosphate of **2**. Despite the potent clinical anti-HCV activity, development of these compounds was discontinued, with PSI-938 halted due to hepatic toxicity observed with extended dosing in the clinic. Recently a new phosphoroamidate prodrugs AT-527 was reported.[12] Its active metabolite is also 5′-triphosphate of **2**. AT-527 demonstrated potent, pan-genotypic activity against hepatitis C virus and is now in clinical development. Incorporation of the guanosine nucleoside triphosphate into host RNA by human mitochondrial RNA polymerase was at least partially responsible for the toxicity.[13] In fact, the HCV nucleosides that are either an approved drug (such as sofosbuvir) or were successful in clinical trials (such as AL-335[14-15] or uprifosbuvir[16]) are pyrimidine analogues. In order to obtain guanosine derivatives with a good safety profile and high antiviral potency, we explored a variety of sugar-modified guanosine analogues. One of our approaches was to increase the complexity of the sugar moiety to limit off-target effects caused by interactions with host polymerases and therefore increase the chance to identify a safe nucleoside analogue. Towards this goal, we modified the sugar moiety by combining substituents at 2'-, 3'-, and 4'-positions. In this

article, we report the synthesis, HCV NS5B inhibition, host polymerase inhibition, and HCV replicon activity of a series of guanosine analogues **4-10** having 2',3'- and 2',4'-disubstituted sugar moieties (Figure 1). For the lead prodrug **AL-611** (**42**) the in vivo and in vitro NTP formation, replicon activity, and cytotoxicity are also presented.

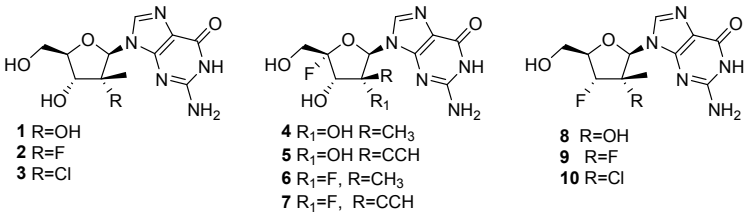


Figure 1. Sugar modified guanosine analogues.

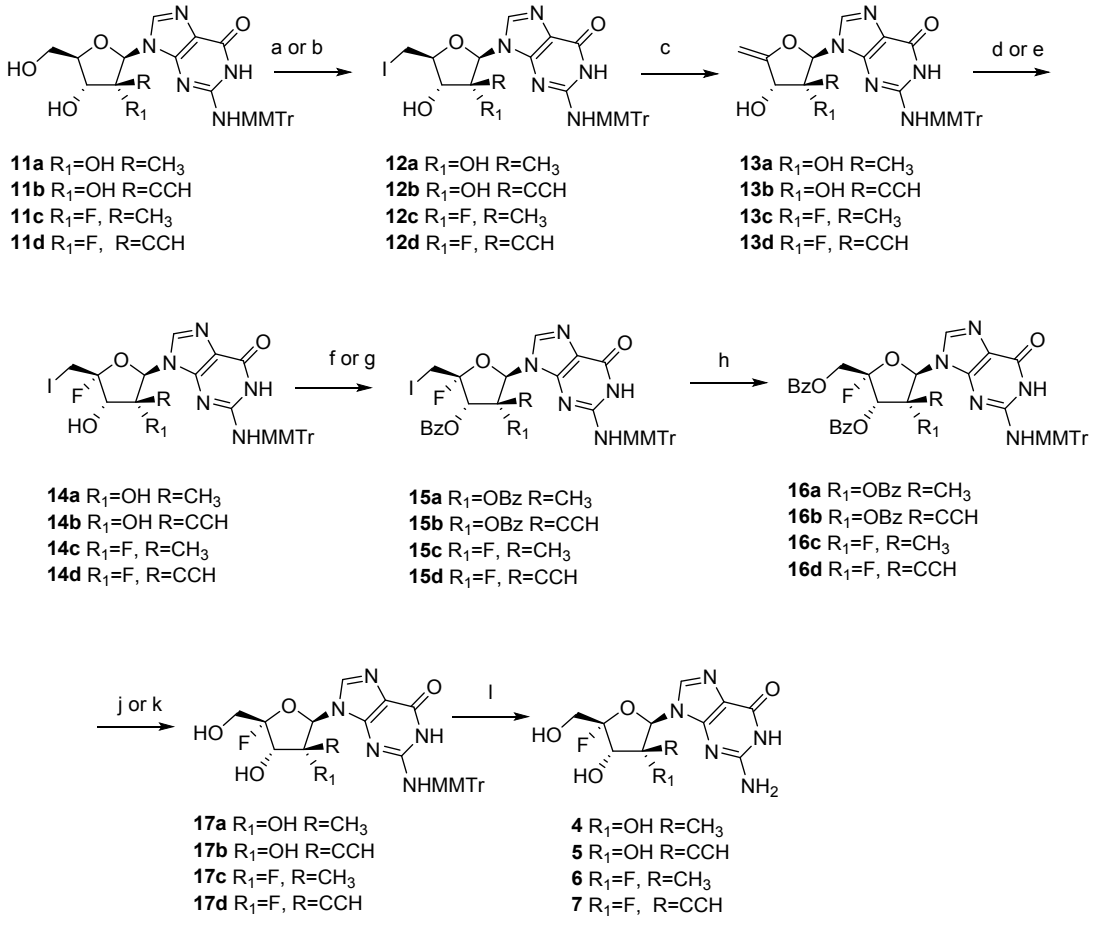
RESULTS AND DISCUSSION

Chemistry

Synthesis of compounds **4-7** is shown in Scheme 1. Starting nucleosides were prepared according to known procedures: **11a**,[17] **11b,**[18] **11c,**[17,19] and **11d**.[20] N^2-monomethoxytrytilated guanosines **11a-d** were subjected to direct iodination or were converted to the alkyl iodides via a tosyl intermediate. Treatment of 5'-iodo nucleosides **12a-d** with DBU then afforded olefins **13a-d**. Stereoselective introduction of 4'-fluorine on a nucleoside using iodine and silver fluoride was first described by Moffatt.[21] This method results in one isomer with 4'-fluorine on alpha phase of the ribose and was used for preparation 4'-fluoro pyridine[22] and pyrimidine[23] nucleosides. We used this approach for synthesis of compounds **14a,b,d** from **13a,b,d.** Subsequent benzoylation and silica gel chromatography gave **15a,b,d** as single isomers. Treatment of **13c** with NIS and triethylamine trihydrofluoride resulted in mixture of stereoisomers, which were separated by HPLC. The D-ribo isomer **14c** was benzoylated to yield **15c.** Treatment of **15a-d** with sodium benzoate at elevated temperature produced the perbenzoylated products **16a-d**. The target nucleosides **4-7** were obtained via global deprotection using ammonia or butylamine followed by HCl/dioxane. Configuration at C-4' for **4-7** was confirmed by 2D NMR enhancement spectroscopy (NOESY), interaction between H-8 and H-5',5" demonstrated that the 4'-F is on the alpha face of the ribose.

The synthesis of compounds **9** and **10** is shown in Scheme 2. Starting 3-fluoro-*O*-methylriboside **18** was prepared as described earlier.[24] Condensation of **18** with 6-chloro-2-aminopurine under Vorbruggen conditions gave nucleoside **19** with good yield. Monomethoxytrityl protection of the heterocyclic base and subsequent debenzoylation resulted in nucleoside **20**. TEMPO-mediated oxidation of the 2'-alcohol to the ketone provided intermediate **21** with high yield. Grignard methylation of ketone **21** followed by fluorination with DAST afforded 2',3'-dideoxy-2',3'-difluoronucleoside **22** as a single isomer, which was transformed to guanosine **9** under standard conditions.

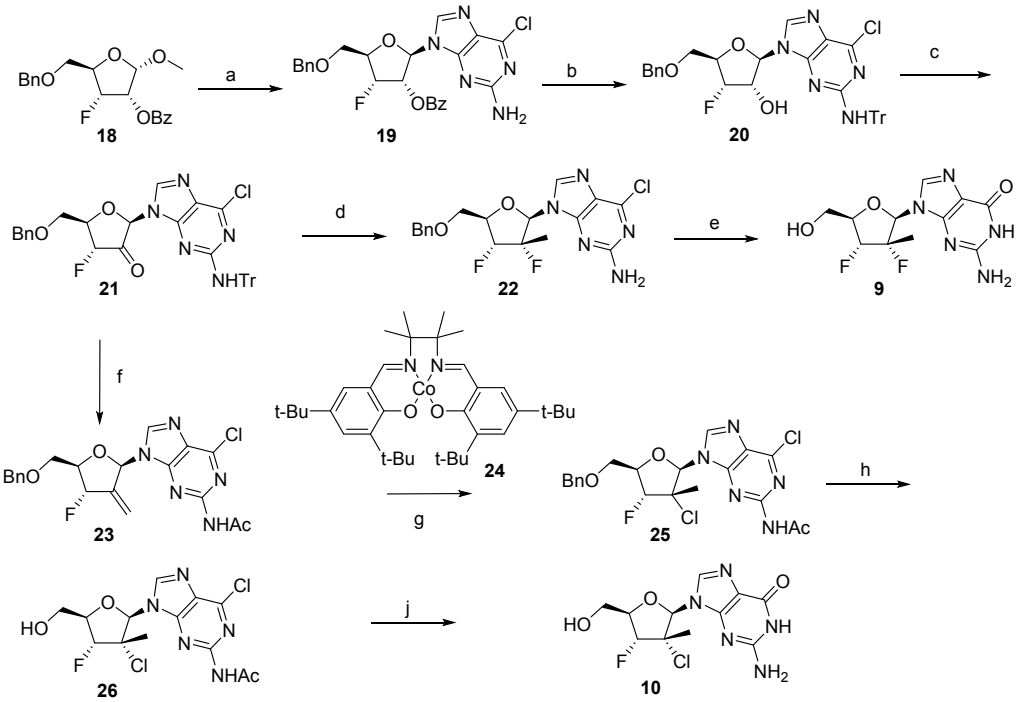
Scheme 1



Reagents and conditions: (a) i.TsCl, pyridine, 0 °C, 1 h; ii. NaI, acetone, reflux, overnight, 41% for **12a**, 52% for **12b**, 41% for **12c**; (b) PPh_3, imidazole, I_2, THF, rt, overnight, 90% for **12d**; (c) DBU, THF, 60 °C, overnight, 60% for **13a**, 62% for **13b**, 67% for **13c**, 83% for **13d**; (d) AgF, I_2, DCM, rt, 3 h; (e) $NEt_3 \cdot 3HF$, NIS, CH_3CN, rt, 2 h, 16% for **14c**; (f) BzCl, DMAP, Et_3N, DCM, rt, overnight, 81% for **15c**; (g) BzCl,

pyridine; (h) HMPA, NaOBz, 15-crown-5, 48h, 60 °C, yield for 3 steps 15% for **16a** from **13a**, 6% for **16b**, 11% for **16d**; (j) NH_3/MeOH, 49% for 2 steps for **17c**; (k) n-butylamine, rt, 80% for **17a**, 86% for **17b**, 75% for **17d**; (l) HCl/dioxane, CH_3CN, 80% for **4**, 73% for **5**, 81% for **6**, 36% for **7** (for 2 steps).

Scheme 2



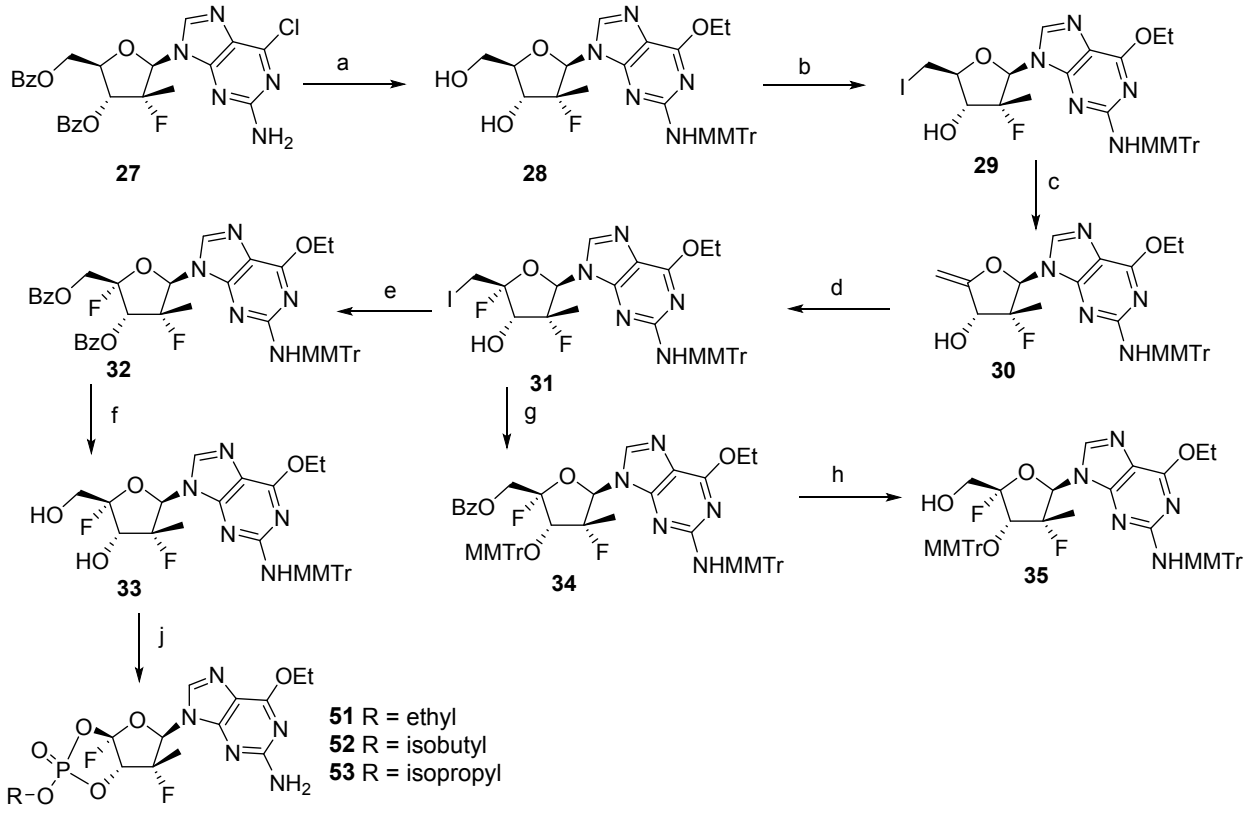
Reagents and conditions: (a) BSA, TMSOTf, CH_3CN, 80 °C, 16 h, 72%; (b) i. TrCl, Et_3N, DMAP, DMF, 45 °C, 24 h, 81%; ii. NH_3/MeOH, rt, 4h, 78%; (c) i. TEMPO, $PhI(OAc)_2$, AcOH, DCM, rt, 16 h, 100%; (d) i. CH_3MgBr, ether, 2 h, -30 °C, 20%; ii. DAST, DCM, rt, 2 h, 22%; (e) i. NaOMe, 2-mercaptoethanol, 12 h, 65 °C; ii. BCl_3, DCM, -75 °C, 0.5 h, 55% for 2 steps; (f) i. CH_3Ph_3PBr, LiHMDS, THF, rt, 4 h, 34%; ii. AcOH, MeOH, 50 °C, 5 h, 60%; iii. AcCl, pyridine, DCM, rt, 2 h, 65%; (g) TsCl, $PhSiH_3$, EtOH, 30 °C, 2.5 h, 29%; (h) BCl_3, DCM, -30 °C, 3 h; (j) NaOMe, 2-mercaptoethanol, 12 h, 60 °C, 26% for 2 steps.

Ketone **21** could be converted to the exocyclic alkene **23** by treatment with potassium salt of methylphosphonium bromide followed by subsequent detritylation and acetylation of the purine base. Compound **23** was a subjected to a hydrochlorination reaction[25] with TsCl in the presence of Co catalyst[26] **24** to yield the desired 2'-Cl guanosine **25** as a single isomer. Exclusive formation of the desired alpha-isomer of the 2'-chloride was most likely due to the steric congestion of the beta face of the ribose. Debenzylation of **25** with BCl_3 afforded **26** which was treated with NaOMe and thioethanol to provide target guanosine **10**. Stereoconfiguration at C-2' for both **9** and **10** was

proven by NMR. In both cases, NOE interaction between H-8 and 2'-CH_3 demonstrated the methyl to be on the □-face of the nucleoside.

Nucleosides **1**,[27] **2**,[28] **3**,[18] and **8**[19] were synthesized according published procedures. All nucleosides **1-10** in Figure 1 were converted to their 5'-*O*-triphosphates for testing in viral and human polymerase assays.

Scheme 3

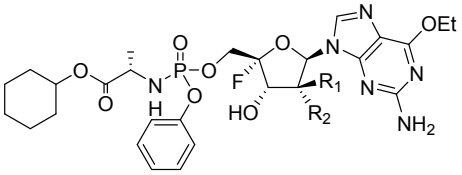


Reagents and conditions: (a) i. MMTrCl, $AgNO_3$, collidine, DCM, rt, overnight, 84%; ii. NaOEt, EtOH, rt, 1 h, 98%; (b) I_2, PPh_3, imidazole, THF, rt, 5 h, 75%; (c) DBU, THF, 75 °C, 3 h, 37%; (d) $NEt_3{\cdot}3HF$, NIS, DCM, 0 °C, 2 h, 60%; (e) i. BzCl, Py, rt, overnight; ii. NaOBz, DMF, 15-crown-5, 100 °C, 48 h, 45% (for 2 steps); (f) *n*-butylamine, rt, overnight, 85%; (g) i. MMTrCl, $AgNO_3$, collidine, DCM, rt, overnight, 82%; ii. NaOBz, DMF, 15-crown-5, 95 °C, 72 h, 65%; (h) *n*-butylamine, rt, overnight, 56%; (j) i. tert-BuMgCl, alkyl phosphorodichloridate, THF, rt, 2-3 h, 12-32%; ii. HCl/dioxane, rt, 1 h, 95%.

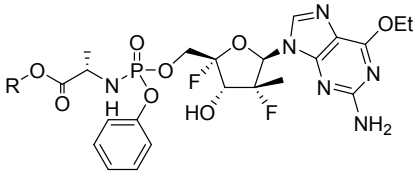
Guanosine analogues were shown to be weakly active in the HCV replicon assay. The weak potency is a result of poor phosphorylation at some stage of nucleoside phosphorylation cascade.[29]

For testing in cell-based assays, nucleosides were converted to monophosphate prodrugs to overcome the most discriminating the first phosphorylation step. Protection of the guanosine was necessary to avoid functional group incompatibility during prodrug synthesis. The 6-OEt group has been shown to be a viable guanosine prodrug motif. [9, 30]

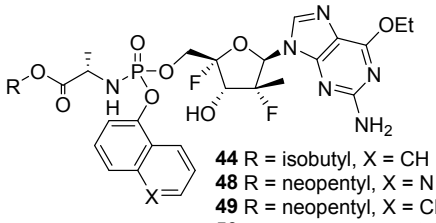
Synthesis of the protected 6-OEt nucleosides **33** and **35** is shown in Scheme 3. The amino group of the nucleoside **27**[6] was monomethoxytritylated and obtained product was treated with NaOEt to afford **28**. Fluorination of **28** was achieved using the same synthetic strategy as shown in Scheme 1, via formation of olefin **30**. Iodofluorination with triethylamine trihydrofluoride and NIS provided nucleoside **31** as a single isomer in a good yield. Nucleophilic substitution of the iodo moiety with OBz, together with simple protecting groups manipulations yielded **33** and **35** as protected starting materials for the synthesis of prodrugs of 2',4'-difluoro-2'-*C*-methyl-guanosine **6**. Nucleoside **33** was converted to cyclic phosphates **51-53**. Nucleoside **35** was a starting material for preparation of phosphoroamidate prodrugs **39-50** (Figure 2). Protected 3'-O,N^2-bismonomethoxytrityl-O^6-ethyl analogues of nucleosides **4**, **5**, and **7** were synthesized in a similar way and the synthesis thereof described previously.[19] These compounds were converted to 5'-phosphoroamidate prodrugs **36-38** (Figure 2) using standard conditions.[31] All prodrugs were isolated as mixtures of diastereomers.



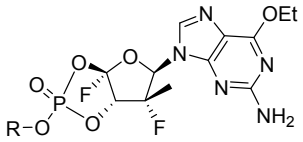
36 R_1 = CCH, R_2 = OH
37 R_1 = CH_3, R_2 = OH
38 R_1 = CCH, R_2 = F



39 R = cyclohexyl **45** R = isobutyl
40 R = isopropyl **46** R = cyclopentyl
43 R = neopentyl **47** R = pentan-3-yl



44 R = isobutyl, X = CH
48 R = neopentyl, X = N
49 R = neopentyl, X = CH
50 R = isopropyl, X = CH

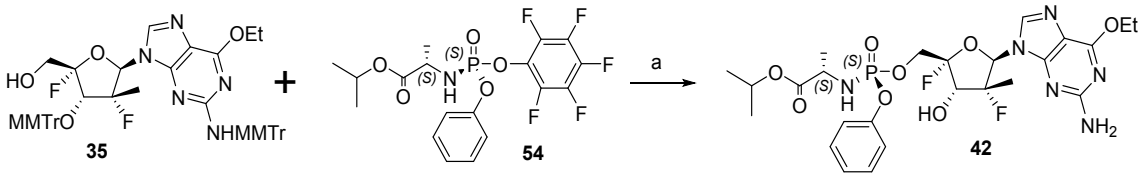


51 R = ethyl
52 R = isobutyl
53 R = isopropyl

Figure 2. Structure of synthesized prodrugs.

Among these mixtures was a pair of P-isomeric prodrugs **41** and **42 (AL-611)**, which were separated from diastereomeric mixture **40** by RP HPLC. For assignment of the stereochemistry at the P-atom of **41** and **42 (AL-611)**, an alternative synthesis was performed. Nucleoside **35** was treated with *S*p-diastereomer of the phosphoramidating reagent reported by Ross et al.[32] Reaction with the 5'-hydroxyl group of **35** through selective nucleophilic displacement of pentafluorophenol resulted in *S*p-diastereomer identical to prodrug **42** by HPLC and ^{1}H-NMR (Scheme 4). Therefore **42 (AL-611)** was assigned as *S*p- and **41** as *R*p-diastereomer.

Scheme 4



Reagents and conditions: (a) i. *tert*-BuMgCl, THF, 5 ^{o}C, 1 h; ii. HCl/ CH_3CN

Cyclophosphate prodrugs of guanosine analogues are known to demonstrate anti-HCV activity when tested in vitro and in vivo.[10-11] We synthesized cyclophosphates **51-53** (Figure 2) in one step by treatment of nucleoside **33** with the corresponding phosphodichloridate in presence of *tert*-BuMgCl. Subsequent deprotection and isolation by silica chromatography yielded cyclophosphate prodrugs **51-53** as mixture of isomers in moderate yields (Scheme 3).

X-Ray Crystal Structure of Compound 42

The absolute stereochemisty of compound **42** (**AL-611)** was assigned by small-molecule X-ray crystallography, from a difraction data set with resolution of 0.81 Å (Figure 3). The chiralities of all stereocenters were confirmed as follows: P1 ***(S)***; C1 ***(R)***; C2 ***(R)***; C3 ***(S)***; C4 ***(S)***; C21 ***(S)***. Atom

numbers of chiral centers are shown in Figure 3. Compound **42** is, unambiguosly, the ***S***p isomer.

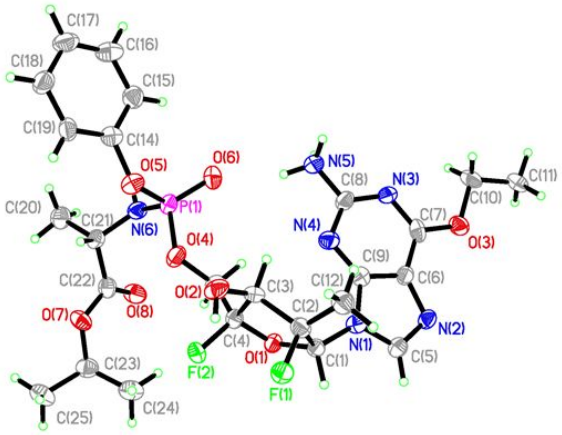
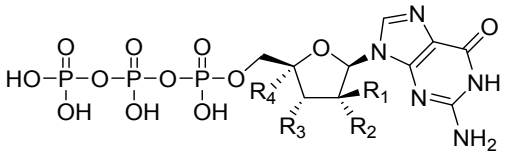


Figure 3. X-ray crystal structure of compound **42**

Inhibition of HCV NS5B Polymerase

To determine the inhibitory effect of nucleosides on HCV NS5B polymerase, the 5'-triphosphates of nucleosides **1-9** shown in Figure 1 were tested in an HCV polymerase assay. As shown in Table 1, most of the NTPs demonstrated good inhibitory activity against HCV NS5B polymerase with IC_{50} as low as 0.064 μM. Comparison of IC_{50} values of 2'-*C*-methyl-GTP (**1-TP**) with its 4'-fluorinated analogue (**4-TP**) demonstrated increased potency for the 4'-fluorinated nucleotide (0.42 μM and 0.14 μM, respectively). 2'-Fluoro-2'-*C*-methyl-GTP (**2-TP**) and its 4'-fluorinated analogues (**6-TP**) demonstrated similar inhibition (0.099 μM and 0.16 μM, respectively). These data suggest that fluorination at the 4'-position does not alter inhibitory properties of compounds on NS5B. The replacement of the 2'-methyl (**4-TP** and **6-TP**) with a 2'-ethynyl group (**5-TP** and **7-TP**) appeared well tolerated with IC_{50} values of 0.13-0.16 μM. Compound **3-TP** which has a 2'-α-chloro-2'-*C*-methyl substitution exhibited potent inhibition with IC_{50} value of 0.064 μM despite containing the larger Cl substituent. When fluorine was introduced at the 3'-position of **8-TP, 9-TP**, and **10-TP**, measured inhibitory effects were lower than of the corresponding 3'-OH compounds **1-TP**, **2-TP**, and **3-TP**, suggesting 3'-fluorination is not beneficial for inhibition of NS5B.

Table 1. Inhibition of HCV Polymerase NS5B by Guanosine Triphosphates.[a]



NTP	R_1	R_2	R_3	R_4	IC_{50} [μM]
1-TP	CH_3	OH	OH	H	0.42
2-TP	CH_3	F	OH	H	0.099
3-TP	CH_3	Cl	OH	H	0.064
4-TP	CH_3	OH	OH	F	0.14
5-TP	CCH	OH	OH	F	0.13
6-TP	CH_3	F	OH	F	0.16
7-TP	CCH	F	OH	F	0.15
8-TP	CH_3	OH	F	H	0.63
9-TP	CH_3	F	F	H	0.31
10-TP	CH_3	Cl	F	H	0.76

[a] IC_{50} indicates a concentration at which the activity of HCV NS5B polymerase is inhibited by 50%. Each IC_{50} value is an average of $n \geq 2$ determinations.

To unravel the mechanism of action of 4'-fluorinated GTPs, **6-TP** was tested in a chain termination assay. We measured the incorporation of single nucleotides with fixed NS5B enzyme and RNA concentrations, using a polyacrylamide gel-based method, as previously described.[33] We isolated the stable NS5B-RNA in the elongation mode and incubated this elongation complex with various combinations of nucleotides. As shown in Figure 4, when natural GTP is missing, the 10-mer elongation complex cannot be further elongated (lane 1 and 2). When only GTP was added to the reaction, we observed the formation of the 11-mer product corresponding to single guanosine

monophosphate (GMP) incorporation (lane 3). When GTP, UTP, and ATP were added together to the reaction, we observed an expected 20-mer full-length RNA product (lane 4). Similarly, substituting natural GTP by 3'dGTP and **6-TP** supported the formation of an 11-mer product (lanes 5 and 7, respectively) but stopped further elongation to the full-length 20-mer product in the presence of UTP and ATP (lanes 6 and 8, respectively). Therefore, we concluded that incorporation of **6-TP** resulted in immediate chain termination.

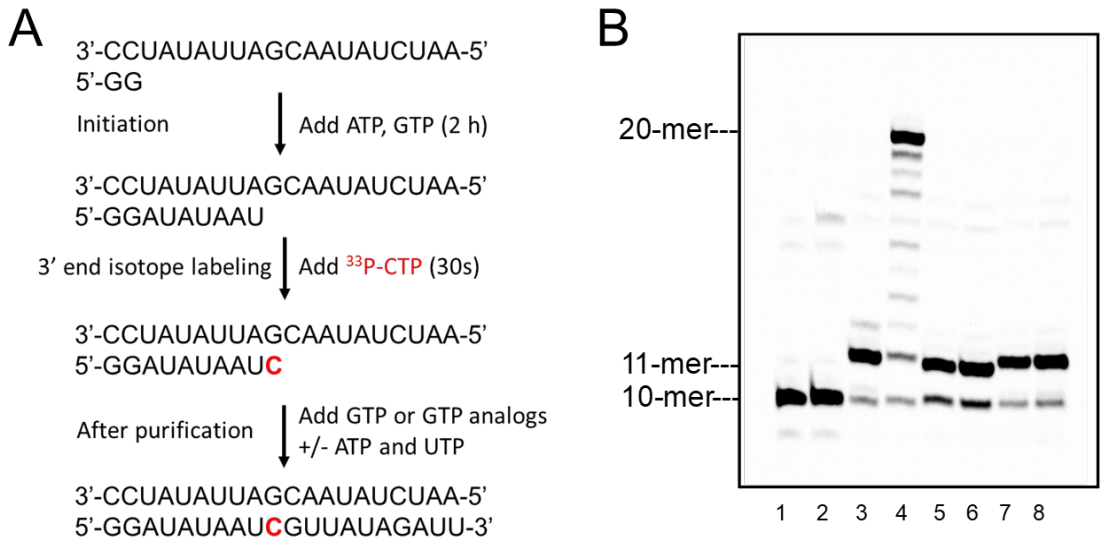


Figure 4. Primer extension and chain termination with 6-TP. A. reaction scheme as described in Methods. B. reaction products shown on a sequencing gel. Lane 1, No NTP; Lane 2, 10 μM ATP + 10 μM UTP ; Lane 3, 10 μM GTP; Lane 4, 10 μM GTP + 10 μM ATP + 10 μM UTP; Lane 5, 100 μM 3'dGTP; Lane 6, 100 μM 3'dGTP + 10 μM ATP + 10 μM UTP; Lane 7, 100 μM **6-TP**; Lane 8, 100 μM **6-TP** + 10 μM ATP + 10 μM UTP.

Inhibition of Human Polymerases

Host polymerases have been generally regarded as the primary off-targets for nucleoside analogues.[34] It was reported association between HCV nucleoside inhibitors that interact with human mitochondrial DNA (hPolγ) and RNA (HMRP) polymerases and the observation of adverse events.[13, 35] For assessment of the selectivity between viral and human polymerase inhibitions,

selected NTPs from Table 1 were tested for inhibition of human DNA polymerases α, β, and γ as well as human RNA pol II.[36] The results are listed in Table 2. None of the NTPs tested inhibited human DNA pol-β and human RNA pol II. However, **2-TP** [9, 12] and **6-TP** showed appreciable inhibition of human DNA pol-α with 79% and 73% inhibition, respectively, at 100 μM. The inhibition by **2-TP** and **6-TP** might be caused by the replacement of 2'-OH with fluorine. Human DNA pol-α tolerated 2'-α-F nucleotide to a certain degree. Inhibition of human DNA pol-α by **2-TP** and **6-TP** and inhibition of human DNA pol-γ by **8-TP** should be noted. Inhibition of human DNA pol-γ by **8-TP** was higher (81%), **2-TP** and **6-TP** didn't demonstrate any significant inhibition of hPolγ.

Table 2. Inhibition of Human Polymerases by Nucleoside Triphosphates

NTP	hPol-α		hPol-β		hPol-γ		hRNA pol II
	% inhibition @ 100 uM	Standard deviation	% inhibition @ 100 uM	Standard deviation	% inhibition @ 100 uM	Standard deviation	IC_{50} [μM]
1-Tp	3.6	8.1	15.3	11.6	7.1	9.4	>100
2-Tp	78.7	2.0	14.3	12.6	24.6	8.7	>100
3-Tp	36.0	4.2	21.1	5.3	38.4	2.8	>100
4-TP	36.0	2.9	20.9	10.8	37.1	1.4	>100
6-TP	73.2	0.6	16.3	14.9	29.8	4.7	>100
8-TP	16.8	11.6	8.4	11.4	81.0	0.6	>100

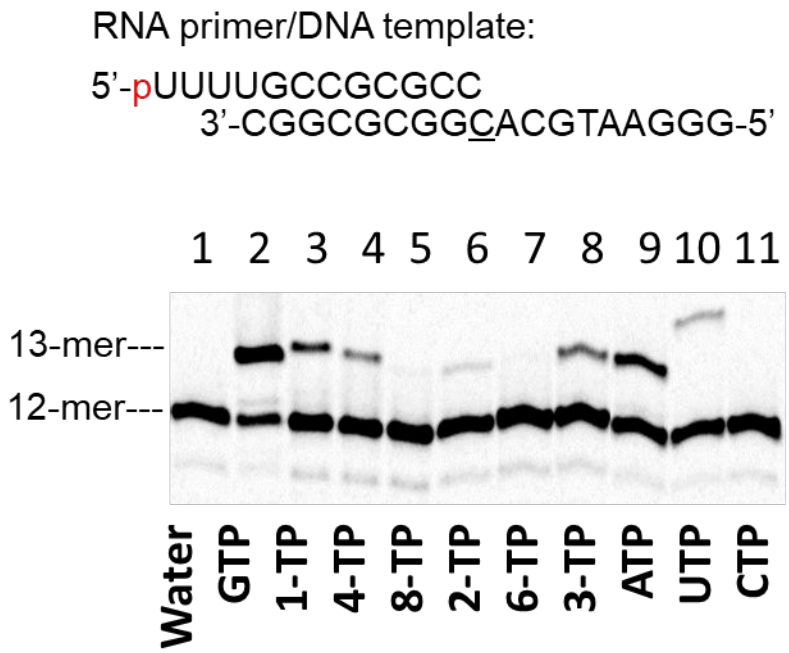


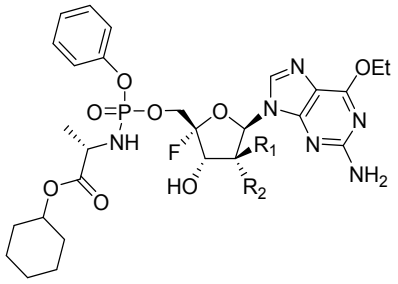
Figure 5. Incorporation of nucleotide by human mitochondrial RNA polymerase. Top, the RNA primer and the DNA template used in this assay. Bottom, nucleotide incorporation products shown on a sequencing gel. 100 μM nucleotide was used in each assay.

Six NTPs were tested in an assay investigating nucleotide incorporation by human mitochondrial RNA polymerase (HMRP) (Figure 5). **1-TP** (TP of INX-189's parent nucleoside) was found to be incorporated significantly, which may have contributed to INX-189's toxicity. The 4'-F analogue **4-TP** was able to reduce the incorporation, but the incorporation was still appreciable. When the 2'-OH was replaced by 2'-F, **2-TP** [9, 12] could be incorporated, but at a much lesser extent compared to **1-TP**. After introducing 4'-F to **2-TP**, the resulting **6-TP** was no longer the substrate of HMRP, as shown in Figure 5. Replacing of 2'-OH with Cl resulted in **3-TP** which was significantly incorporated into the RNA, similar to **1-TP**. These results suggest that fluorine modification at the 2' and 4' positions disfavored the incorporation by HMRP. **8-TP** (3'-F-2'-C-Me GTP) was not a substrate of HMRP. Overall, the **6-TP** tested did not show any alarming inhibition of the human mitochondrial polymerases and low inhibition of DNA pol-α. However, the actual toxicity liability cannot be assessed until tested in animal studies and in human trials eventually. Based on the promising viral and human polymerase inhibition results, the nucleotide prodrugs of the 4'-F analogue **4-7** were evaluated in cell based assays against HCV.

HCV Replicon Activity

NTPs of 4'-fluorinated nucleosides **4-7** demonstrated similar inhibition of HCV polymerase NS5B. To compare their in vitro activity in an HCV subgenomic replicon system[37] we synthesized compounds **36-39,** phosphoramidate prodrugs of nucleosides **4**, **5**, **7**, and **6**.[38] As shown in Table 3 2'-ethynyl derivatives **36** and **38** demonstrated low antiviral activity and high cell toxicity. Prodrugs **37** and **39** of 2'-methyl nucleosides **4** and **6**, respectively, demonstrated comparable replicon activity and low toxicity. Considering that **6-TP** exibited good inhibition of viral polymerase and was not a substrate of HMRP we selected 2',4'-difluoro-2'-C-methyl scaffold for prodrug development.

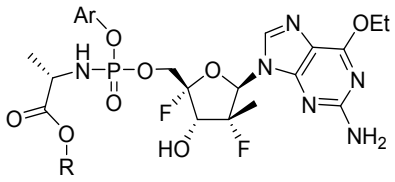
Table 3. HCV Replicon Activity of Phosphoroamidate Prodrugs of Nucleosides 4,5,6, and 7



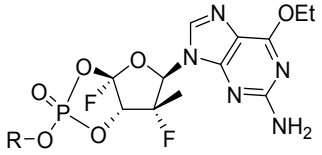
Prodrug	Parent nucleoside	R_1	R_2	EC_{50} [μM]	CC_{50} [μM]
36	**5**	CCH	OH	0.27	16.41
37	**4**	CH_3	OH	0.035	>100
38	**7**	CCH	F	1.0	3.26
39	**6**	CH_3	F	0.066	>100

EC_{50} indicates a concentration at which HCV replicon is inhibited by 50%. Each EC_{50} value is an average of ≥2 independent determinations. CC_{50} indicates a concentration of 50% cell viability.

Table 4. HCV Replicon Activity of 2',4'-Difluoro-2'-C-Methylguanosine Phosphoroamidate Prodrugs



39-50



51-53

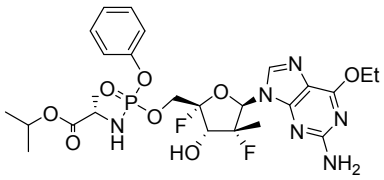
Prodrug	P-chirality	Ar	R	EC_{50} [μM]	CC_{50} [μM]	SI
INX-189	achiral			0.348	16.42	47.1
39	achiral	phenyl	cyclohexyl	0.066	>100	>1500
40(41+42)	achiral	phenyl	isopropyl	0.014	>100	> 7000
41	chiral, *R*p	phenyl	isopropyl	0.057	>100	> 1700
42	chiral *S*p	phenyl	isopropyl	0.005	>100	>20000
43	achiral	phenyl	neopentyl	0.19	>100	>500
44	achiral	naphthalen-1-yl	isobutyl	0.017	56.9	3000
45	achiral	phenyl	isobutyl	0.042	>100	>2000
46	achiral	phenyl	cyclopentyl	0.034	92.9	2700
47	achiral	phenyl	pentan-3-yl	0.23	72.5	315
48	achiral	quinoline-5-yl	neopentyl	0.12	>100	>800
49	achiral	naphthalen-1-yl	neopentyl	0.06	39.6	660
50	achiral	naphthalen-1-yl	isopropyl	0.02	80.5	4000
51	achiral	N/A	ethyl	0.31	>100	322
52	achiral	N/A	isobutyl	1.34	>100	74

53	achiral	N/A	isopropyl	0.03	>100	3000

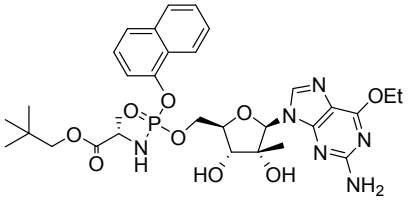
Several phosphoramidate prodrugs of nucleoside **6** shown in Table 4 were synthesized and tested, INX-189 was used as a positive control. Compounds **39, 40**, and **43-50** were tested as mixtures of diastereomers and showed activities ranging from 0.014 to 0.2 μM. Naphthalenyl-containing prodrugs **44**, **49**, and **50** were very potent (0.02-0.06 μM) but also showed significant cytotoxicity (56.9-80.5 μM). Increased lipophilicity of the ester moiety of **43**, **47**, and **48** also led to high activity in replicon assay. Prodrug **40** was selected for further investigation based on high selectivity index (SI>7000) and taking into consideration that its phosphate moiety was proven to be not toxic.[39] Cyclophosphate prodrugs **51-53** did not show superior properties compared with phosphoramidates.

Two stereoisomers of **40** were separated and **41** and **42** were tested for replicon activity. *S*p-isomer **42** was 10 times more active than corresponding *R*p-isomer **41** (0.005 and 0.057 μM, respectively) with SI>20000. This *S*p-isomer **42 (AL-611)** was selected for further evaluation.

Table 5. Eight Day Cytotoxicity (CC_{50} μM) of Compound 40 in Six Cell Lines



40



INX-189

	Cytotoxic Concentration Causing 50% Inhibition (CC_{50}) μM					
Compound/ Cell Line	Huh-7	HepG2	A549	HeLa	U937	MT4
40	> 100	> 100	> 100	>100	10	92
INX-189	0.35 ± 0.2	1.5 ± 0.07	7.8 ± 0.6	13.4 ± 1.7	0.17 ± 0.04	0.96 ± 0.2

Prodrug **40** did not show any appreciable cytotoxicity in Huh-7 cells. To assess the potential cytotoxicity under more stringent conditions, this prodrug was tested in multiple cell lines for 8

days. The well-known toxic compound INX-189 was used as a control. Compound **40** demonstrated no measurable dose-dependent decreases in cell viability (CC_{50} > 100 μM) across most cell lines except for U937 and MT-4 cells where the concentration causing 50% cellular 10 and 92 μM, respectively. In all cases, the cytotoxicity of compound **40** was significantly lower than that observed for INX-189 (Table 5).

In Vivo and in Vitro NTP Formation

NTP levels in the target tissue are important for in vivo efficacy of an antiviral nucleoside polymerase inhibitor. In vitro NTP formation in appropriate cell lines may provide the first assessment for predicting the in vivo NTP concentration. To understand difference in the extent of in vitro activation of the individual diastereomers **41** and **42** and the isomeric mixture **40** the in vitro NTP levels were measured in fresh primary human hepatocytes as the most relevant cell line. As shown in Table 6, high levels of **6-TP** were formed in human hepatocytes following an incubation of **40, 41, and 42** at 50 μM extracellular concentration for 24 hours.

Table 6. NTP Formation in Vitro and in Vivo

Compound	Human Hepatocytes (pmol/million cells) at 24 h		Dog liver NTP (μM) at 4h
	NMP	NTP	
40	89	271	11.2
41	67.1	174	6.33
42	105	321	22.3

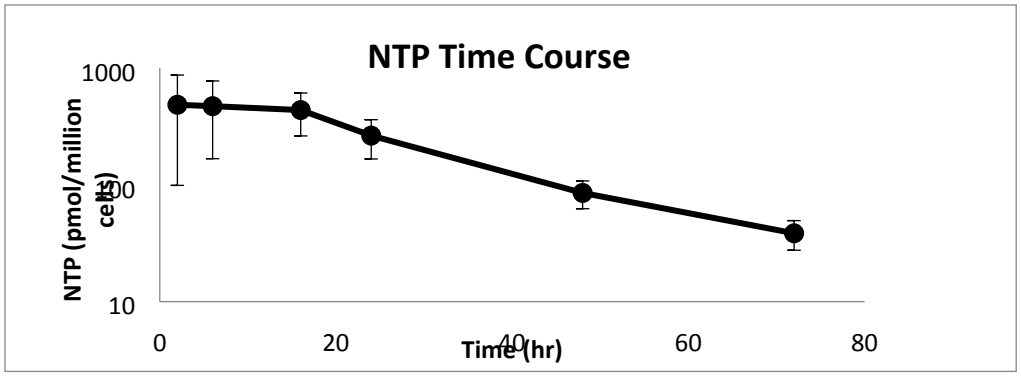


Figure 6. Time course of intracellular NTP in primary human hepatocytes following an incubation with **40** at 10 μM for 2-72 h.

The experiment presented in Figure 6 was conducted to determine time-course and steady state of NTP formation. In human hepatocytes, the steady state (equilibrium between NTP formation and degradation) was typically reached between 2 and 16 hours (Figure 6). The decrease in NTP formation beyond 24-48 hours is likely due to decline in function of fresh hepatocytes, a known phenomenon. Intracellular concentrations of **6-TP** were measured at multiple dose concentrations to determine dose-linearity of the NTP formation. **6-TP** Intracellular concentrations increased dose proportionally between 0.5-10 μM of **40** incubation concentrations (Figure 7) but was not observed at >10 μM. At incubation concentrations beyond 10 μM the NTP levels increased in less than dose proportional manner.

Following a 24-hr incubation of **40** (~1:1 mixture of **41** and **42**) in human hepatocytes, high concentrations of NTP were formed. Once formed and after the removal of **40** from the incubation media, NTP levels were maintained for 6 hours before degradation (Figure 8). The mean intracellular half-life of the NTP was estimated as 11.2 ± 0.7 hours (n = 3) in human hepatocytes.

In vivo NTP formation was evaluated in beagle dogs following a single oral administration of **40**, **41**, and **42** at 9.69 mg/kg (5 mg/kg parent nucleoside equivalent dose). As shown in Table 6, liver NTP concentrations were 11.2, 6.3, and 22.3 μM at 4 hours post dose of **40**, **41**, and **42**, respectively.

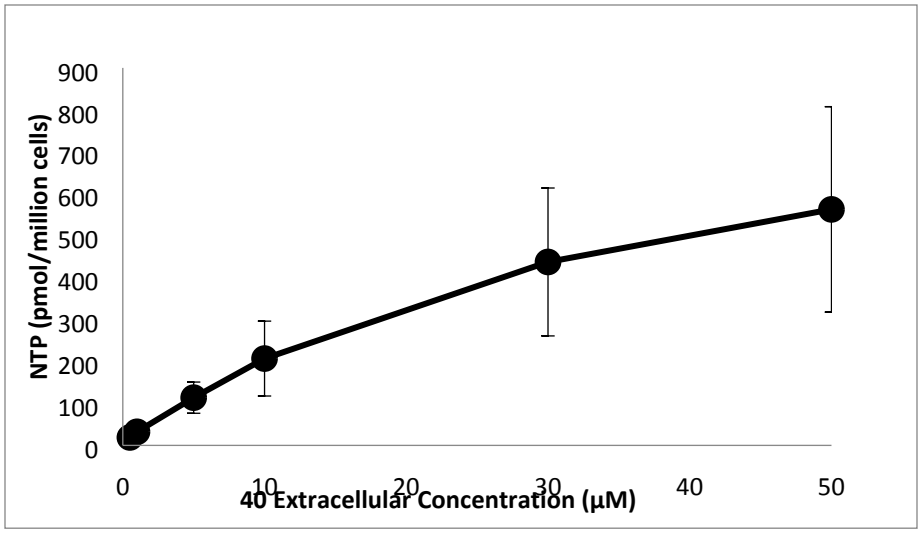


Figure 7. Intracellular NTP concentration as a function of extracellular concentration of compound **40** in primary human hepatocytes. Data were collected from hepatocytes from three different human donors and expressed as mean ± standard deviation. Each experiment was conducted with 24 h of incubation of **40** with hepatocytes.

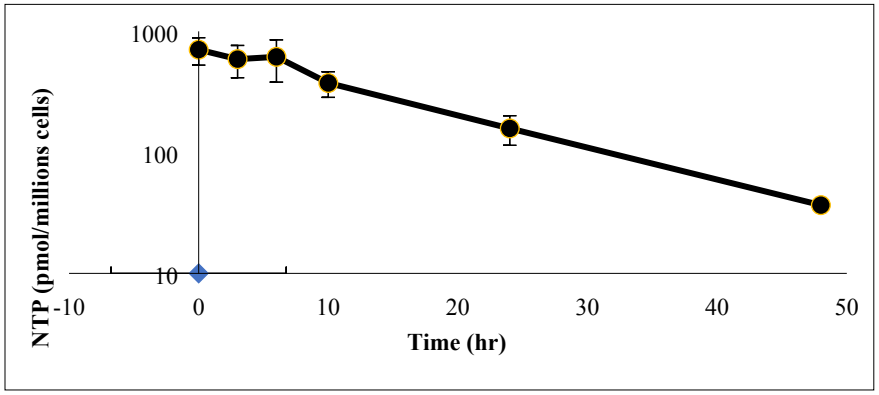


Figure 8. Intracellular NTP concentration as a function of time in primary human hepatocytes following an initial 24 h incubation with **40** at 50 μM.

CONCLUSION

We demonstrated that guanosine nucleoside with properly modified ribose moiety can exhibit potent HCV replicon activity and acceptable safety properties. 5'-Triphosphates of 4'-fluorinated guanosine derivatives demonstrated potent inhibition in the HCV polymerase NS5B assay with

IC_{50} values as low as 0.13 μM. The 5'-triphosphate of 2',4'-difluoro-2'-*C*-methyl-guanosine **(6-TP)** exhibited IC_{50} 0.16 μM and was confirmed to be a chain terminator in a chain elongation assay using the HCV NS5B polymerase and showed no inhibition of human DNA polymerases β and γ as well as human RNA pol II while inhibition of human DNA polymerases α was low. Phosphoramidate prodrug of this nucleoside exhibited very potent HCV subgenomic replicon activity with EC_{50} values as low as 5 nM.

The *S*p-diastereomer prodrug **42** (**AL-611**) was selected for preclinical toxicology studies based on the biochemical potency of **6-TP** combined with its good in vitro safety profile, corresponding in vivo NTP formation, and cell-based replicon potency. Compound was selected as drug candidate but given the effectiveness of already approved therapies for HCV a decision was taken to discontinue further development.

EXPERIMENTAL SECTION

All commercially obtained solvents and reagents were used as received. All solvents used for chemical reactions were anhydrous grade, unless specifically indicated. Structures of the target compounds in this work were assigned by use of NMR and MS spectroscopy. The purities of parent nucleosides and prodrugs were >95% as determined on an Agilent 1200 HPLC, Synergy 4-μm Hydro RP 80 Å 150 × 4. 6 mm, flow 1.5 mL/min. using 0.5% HCOOH in water and 0.5% HCOOH in CH_3CN as mobile phase or 50 mM TEAA in water and 50 mM TEAA in CH_3CN as mobile phase. The purities of triphosphates were >95% determined on the same column using linear gradient of CH_3CN from 0% to 25 % in 50 mM triethylammonium acetate buffer (pH 7.5). ^1H-, ^{19}F- and ^{13}C-NMR spectra were recorded on a Bruker Advance III (400 MHz) or a Varian 400MR (400 MHz) NMR spectrometer. Chemical shifts are reported in parts per million (ppm, δ) using residual solvent line as an internal reference. Splitting patterns are reported as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), or broad singlet (br s). Coupling constants (J) are reported in hertz (Hz). Mass spectrometric analyses for nucleosides were performed on an Agilent 1200 HPLC with Agilent 6110/6140/1956C MSD mass spectrometer using ESI as ionization. Work-up procedures for most of chemical reactions are the same or similar, therefore, unless specifically indicated, the work-up refers to the following procedure: the reaction mixture at 0 °C

is quenched with water, diluted with EtOAc or dichloromethane, washed with 5% sodium bicarbonate and then with brine, dried over anhydrous sodium sulfate, filtered, and concentrated to dryness. Purification on silica gel refers to a flash chromatography on a silica gel column.

5'-Deoxy-5'-iodo-2'-*C*-methyl-N^2-(methoxytrityl)guanosine (12a). TsCl (3.43g, 17.5 mmol) was added to the cold solution of **11a** (5 g, 8.8 mmol) in pyridine (15 mL). The reaction mixture was stirred for 1h at 0 ^oC and quenched with water. After usual work-up and without further purification 5'- tosyl intermediate (6.3 g) was dissolved in acetone (60 mL) and treated with NaI (13.2 g, 88 mmol) overnight at reflux temperature. Reaction mixture was quenched with aq. $Na_2S_2O_3$. After usual work-up and column chromatography (MeOH in DCM from 1% to 6%) nucleoside **12a** (2.4 g, 41%) was obtained as a white solid. MS, *m/z* 680.3 $(M + 1)^+$. ^1H NMR (CD_3OD): δ 8.14 (s, 1H), 7.18-7.35 (m, 12H), 6.83-6.85 (m, 2H), 5.52 (s, 1H), 3.86-3.96 (m, 3H), 3.74 (s, 3H), 3.70-3.76 (m, 1H), 0.53 (s, 3H).

5'-Deoxy-5'-iodo-2'-*C*-ethynyl-N^2-(methoxytrityl)guanosine (12b). Compound **12b** (4 g, 52%) as a white solid was synthesized from 6.3 g of **11b** the same way as **12a**. MS, *m/z* 690.3 $(M + 1)^+$.

5'-Deoxy-5'-iodo-2'-fluoro-2'-*C*-methyl-N^2-(methoxytrityl)guanosine (12c). Compound **12c** (2.4 g, 41%) as a white solid was synthesized from 5.0 g of **11c** the same way as **12a**. MS, *m/z* 682.4 $(M + 1)^+$. ^1H-NMR ($CDCl_3$): δ 7.16-7.59 (m, 13H), 6.71-6.73 (m, 2H), 5.56 (d, J = 18.8 Hz, 1H), 3.25-4.07 (m, 7H), 0.64 (d, J = 22.4 Hz, 3H).

5'-Deoxy-5'-iodo-2'-fluoro-2'-*C*-ethynyl-N^2-(methoxytrityl)guanosine (12d). To a solution of compound **11d** (3.1 g, 5.3 mmol) in dry THF (80 mL) at 0 ^oC ware added PPh_3 (2.8 g, 10.6 mmol) and imidazole (720 mg, 10.6 mmol) and stirred for 30 min. To this mixture was added the solution of I_2 (2.0 g, 8.0 mmol) in dry THF (10 mL) at 0 ^oC and stirred at rt for 18 h. The reaction was quenched with sat. aq. Na_2SO_3 (10 mL) and extracted with DCM (2 × 100 mL). The organic layer was dried (Na_2SO_4) and filtered. Evaporated residue was purified by silica gel chromatography (MeOH in DCM from 1% to 4%) to give **12d** (3.6 g, 90%) as a yellow solid. MS, *m/z* 692 $(M + 1)^+$.

4',5'-Didehydro-5'-deoxy-2'-*C*-methyl -N^2-(methoxytrityl)guanosine (13a). To the solution of iodide **12a** (11.5 g, 16.9 mmol) in dry THF (120 mL) was added DBU (12.87 g, 84.7 mmol), and

heated overnight at 60 °C. The reaction was quenched with saturated NH_4Cl solution. Usual work-up and chromatography (MeOH in DCM from 1% to 5%) yielded compound **13a** (5.5 g, 60%) as a white solid. MS, *m/z* 552.3 $(M + 1)^+$. ^1H NMR (CD_3OD): δ 7.47 (s, 1H), 7.22-7.34 (m, 12H), 6.83-6.85 (m, 2H), 5.39 (s, 1H), 4.47 (s, 1H), 4.46 (s,1H), 4.24 (s,1H), 3.76 (s, 3H), 0.63 (s, 3H).

4',5'-Didehydro-5'-deoxy-2'-*C*-ethynyl -N^2-(methoxytrityl)guanosine (13b). Compound **13b** (2.0 g, 62%) as a white solid was synthesized from 4.0 g of **12b** in the same manner as **13a**. MS, *m/z* 602.2 $(M + 1)^+$. ^1H NMR (CD_3OD): δ 7.65 (s, 1H), 7.18-7.34 (m, 12H), 6.82-6.86 (m, 2H), 5.52 (s, 1H), 4.70 (t, *J* = 1.8 Hz, 1H), 4.46 (t, *J* = 2.2 Hz, 1H), 4.27 (t, *J* = 1.8 Hz, 1H), 3.76 (s, 3H), 2.80 (s, 1H).

4',5'-Didehydro-2',5'-dideoxy-2'-fluoro-2'-*C*-methyl-N^2-(methoxytrityl)guanosine (13c). Compound **13c** (1.9 g, 67%) was obtained from **12c** using the same procedure as for the compound **13a**. MS, *m/z* 554.4 $(M + 1)^+$. ^1H-NMR ($CDCl_3$): δ 7.12-7.32 (m, 13H), 6.71-6.73 (m, 2H), 5.56 (d, *J* = 14.8 Hz, 1H), 4.40-4.62 (m, 3H), 3.71 (s, 3H), 0.75 (d, *J* = 22.4 Hz, 3H).

4',5'-Didehydro-2',5'-dideoxy-2'-fluoro-2'-*C*-ethynyl-N^2-(methoxytrityl)guanosine (13d). Compound **13d** (3.0 g, 83%) was obtained as a yellow solid from 4.4 g of **12d** using the same procedure as for the compound **13a**. MS, *m/z* 564 $(M + 1)^+$.

2'5'-Dideoxy-2',4'-difluoro-5'-iodo-2'-*C*-methyl-3'-*O*-benzoyl-N^2-(methoxytrityl)guanosine (15c). To the ice-cold solution of **13c** (3.0 g, 5.4 mmol) in CH_3CN (20 mL) was added $Et_3N{\cdot}3HF$ (0.65 g, 4.1 mmol) followed by NIS (1.53 g, 6.78 mmol). The reaction mixture was kept at rt for 2 h. After usual work-up C-4' stereoisomers were separated by RP-HPLC (Waters X-bridge column 150 × 25 mm, 5 µm; gradient of 0.1% NH_4CO_3 in water and in CH_3CN from 0% to 70%). To the solution of desired R-isomer **14c** (0.6 g, 0.9 mmol) in pyridine (10 mL) was added BzCl (145 mg, 1 mmol) and kept at rt for 3 h. After usual work-up and chromatography (25% to 50% EtOAc in hexane) was compound **15c** (0.55 g, 13% for two steps) isolated as a white solid. ^1H-NMR ($CDCl_3$): δ 8.08 (s, 1H), 7.10-7.97 (m, 17H), 6.70-6.72 (m, 2H), 5.86 (d, *J* = 17.2 Hz, 1H), 3.49-3.68 (m, 6H), 0.84 (d, *J* = 22.4 Hz, 3H).

4'-Fluoro-2',3',5'-tri-*O*-benzoyl-2'-*C*-methyl-N^2-(methoxytrityl)guanosine (16a). To an ice-cold solution of **13a** (2 g, 3.6 mmol) in DCM (80 mL) was added AgF (2.5 g, 20 mmol) and

solution of I_2 (2 g, 8 mmol) in DCM (80 mL). The reaction mixture was stirred at rt for 3 h. Reaction mixture was quenched with solution of $Na_2S_2O_3$ and $NaHCO_3$. After usual work-up the crude 4'-fluorinated nucleoside **14a** (1.7 g) was dissolved in DCM (250 mL). DMAP (1.1 g, 9.6 mmol) was added, followed by Et_3N (1.4 mL, 9.6 mmol) and BzCl (0.8 mL, 7.2 mmol). The reaction mixture was kept at rt overnight. After usual work-up and chromatography (3% MeOH in DCM) benzoate **15a** (1.5 g, 2 mmol) was isolated. It was dissolved in HMPA (40 mL), NaOBz (7.2 g, 20 mmol) and 15-crown-5 (4.1 g, 20 mmol) were added and the mixture was stirred for 48 h at 60 ^oC. Usual work-up and chromatography (50% EtOAc in hexane) yielded 480 mg of **16a** (15% yield for 3 steps). MS, *m/z* 900.4 $(M + 1)^+$. ^{1}H-NMR (CD_3OD): δ 7.81-7.95 (m, 7H), 7.60-7.66 (m, 8H), 7.39-7.51 (m, 2H), 7.19-7.31 (m, 13H), 6.36 (s, 1H), 5.95 (d, *J* = 18 Hz, 1H), 4.50-4.70 (m, 2H), 3.72 (s, 3H), 1.41 (s, 3H).

4'-Fluoro-2',3',5'-tri-*O*-benzoyl-2'-*C*-ethynyl-N^2-(methoxytrityl)guanosine (16b). Compound **16b** (180 mg) was synthesized in 6% yield (for 3 steps) from **13b** by the sequence of transformations described above for **16a**. MS, *m/z* 910.4 $(M + 1)^+$. ^{1}H NMR (CD_3OD): δ 7.83-7.93 (m, 7H), 7.19-7.83 (m, 21H), 6.76-6.78 (m, 2H), 6.36 (s, 1H), 5.92-5.97 (m, 1H), 4.50-4.70 (m,2H), 3.72 (s, 3H), 1.40 (s, 3H).

2'-Deoxy-2',4'-difluoro-2',3',5'-tri-*O*-benzoyl-2'-*C*-ethynyl-N^2-(methoxytrityl)guanosine (16d). Compound **16d** was synthesized from **13d** by the sequence of transformations described for **16a**. The yield was 430 mg, 11% for 3 steps. MS, *m/z* 808 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$): δ 6.80-7.78 (m, 27H), 6.22 (d, *J* = 14.8 Hz, 1H), 4.30 (t, *J* = 9.2 Hz, 1H), 3.70 (s, 3H), 2.42 (d, *J* = 5.6 Hz, 1H).

4'-Fluoro-2'-*C*-methyl-N^2-(methoxytrityl)guanosine (17a). The mixture of protected nucleoside **16a** (400 mg, 0.44 mmol) and n-butylamine (1.5 mL) was kept overnight at rt and then evaporated. The residue was purified by chromatography (MeOH in DCM from 4% to 15%) to yield 206 mg (80%) of the target **17a**. MS, *m/z* 586 $(M+1)^+$. ^{1}H-NMR (CD_3OD): δ 8.01(s, 1H), 7.24-7.34 (m, 12H) 6.84-6.86 (m, 2H), 5.54 (s, 1H), 4.09 (d, *J* = 24.0 Hz, 1H), 3.76 (s, 3H), 3.64-3.73 (m, 2H), 0.59 (s, 3H). ^{19}F-NMR (CD_3OD) δ -123.24.

4'-Fluoro-2'-*C*-ethynyl-N^2-(methoxytrityl)guanosine (17b). Compound **17b** (100 mg, 86%) was obtained from **16b** using the same procedure as for the compound **17a**. MS, m/z 598 (M +

1)$^+$. ^{1}H-NMR (CD_3OD): δ 7.89 (s, 1H), 7.18-7.35 (m, 12H), 6.83-6.87 (m, 2H), 5.64 (s, 1H), 4.45-4.58 (m, 1H), 3.76 (s, 3H), 3.62-3.70 (m, 2H), 2.69 (s, 1H).

2'-Deoxy-2',4'-difluoro-2'-*C*-methyl-*N*^{2}-(methoxytrityl)guanosine (17c). Solution of **15c** (0.65 g, 0.8 mmol), NaOBz (1.15 g, 8 mmol), and 15-crown-5 (1.77 g, 8 mmol) in DMF (10 mL) was stirred for 48 h at 100 ^{o}C. Usual work-up and chromatography (15% to 50% EtOAc in hexane) yielded 500 mg of **16c**. Obtained nucleoside was treated with NH_3/MeOH (50 mL) for 18 h at rt. Solvent was evaporated and the residue was purified by chromatography (MeOH in DCM from 1% to 4%) to give **17c** as a white solid. Yield: 231 mg (49% for 2 steps). MS, *m/z* 590.3 (M + 1)$^+$. ^{1}H-NMR (CD_3OD): δ 7.93 (s, 1H), 7.22-7.31 (m, 12H), 6.83-6.85 (m, 2H), 5.65 (d, *J* = 16.0 Hz, 1H), 4.21-4.30 (m, 1H), 3.71-3.77 (m, 5H), 0.75 (d, *J* = 24.0 Hz, 3H). ^{19}F-NMR (CD_3OD): δ -125.1, -162.4.

4'-Fluoro-2'-*C*-methylguanosine (4). A solution of **17a** (261 mg, 0.44 mmol) in CH_3CN (0.5 mL) and 4N HCl/dioxane (0.05 mL) was kept at rt for 45 min and then evaporated. The crude residue was treated with CH_3CN, precipitate was filtered, washed with CH_3CN, and dried to yield **4** (112 mg, 80%). MS, *m/z* 314.6 (M -1)$^-$. ^{1}H-NMR (CD_3OD): δ 9.00 (s, 1H), 6.23 (s, 1H), 4.44 (d, *J* = 19.5 Hz, 1H), 3.75-3.81 (m, 2H), 1.05 (s, 3H). ^{19}F-NMR (CD_3OD): δ -123.40.

4'-Fluoro-2'-*C*-ethynylylguanosine (5). Compound **5** was synthesized as described for **4** starting from **17b**. Yield: 104 mg (73%). MS, *m/z* 326.6 (M + 1)$^+$. ^{1}H-NMR (CD_3OD): δ 7.96 (s, 1H), 6.29 (s, 1H), 4.75 (d, *J* = 18.4 Hz, 1H), 3.79 (m, 2H), 2.80 (s, 1H). ^{19}F (DMSO-d_6): δ -124.24.

2'-Deoxy-2',4'-difluoro-2'-*C*-methylguanosine (6). Compound **6** was synthesized as described for **4** starting from **17c**. Yield: 70 mg (81%). MS, *m/z* 317.6 (M-1)$^-$. ^{1}H-NMR (DMSO-d_6): δ 11.08 (s, 1H), 8.16 (s, 1H), 6.87 (br, 2H), 6.28 (d, *J* = 1.6 Hz, 1H), 4.41 (d, *J* = 2.2 Hz, 1H), 3.70-3.63 (m, 2H), 1.05 (d, *J* = 2.3 Hz, 3H). ^{19}F-NMR (DMSO-d_6): δ -122.0, -158.1; ^{13}C (DMSO-d_6): δ 156.6, 154.0. 150.7, 134.3, 116.6, 116.6 (d, *J* = 233 Hz), 98.6(d, *J* = 184 Hz), 88.8 (d, *J* = 43 Hz), 71.3, 59.4 (d, *J* = 40 Hz), 17.2 (d, *J* = 25 Hz).

2'-Deoxy-2',4'-difluoro-2'-*C*-ethynylguanosine (7). The mixture of protected nucleoside **16d** (430 mg, 0.47 mmol) and n-butylamine (1.5 mL) was kept overnight at rt and then evaporated yielding **17d**. The solution of crude **17d** in CH_3CN (0.5 mL) and 4N HCl/dioxane (0.05 mL) was

kept at rt for 45 min and then evaporated. The crude residue was treated with CH_3CN, precipitate was filtered, washed with CH_3CN, and dried to yield **7** (60 mg, 36%). MS, *m/z* 326.5 $(M-1)^-$. ^{1}H-NMR (DMSO-d_6): δ 10.75 (s, 1H), 7.79 (s, 1H), 6.60 (s, 2H), 6.36 (d, *J* = 8.0 Hz, 1H), 5.71 (br, 1H), 4.8 (br, 1H), 3.69-3.62 (m, 2H), 3.00-2.70 (m, 1H). ^{19}F-NMR (DMSO-d6): *δ* -122.0, -158.1. ^{13}C (DMSO-d6): *δ* 156.5, 153.9, 150.9, 134.2, 116.4, 116.3 (d, *J* = 235 Hz), 93.2 (d, *J* = 186 Hz), 88.1 (d, *J* = 42 Hz), 82.7 (d, *J* = 8 Hz), 75.2 (d, *J* = 31 Hz), 73.5 (d, *J* = 20 Hz), 59.4 (d, *J* = 39 Hz).

9-(2- *O*-Benzoyl-5-*O*-benzyl-3-deoxy-3-fluoro-β-D-ribofuranosyl)-6-chloro-2-aminopurine (19). To a solution of 6-chloro-9*H*-purin-2-amine (9.4 g, 55.4 mmol) in CH_3CN (100 mL) was added *N,O*-bis(trimethylsilyl)acetamide (33.8 g, 166.2 mmol). The resulting solution was stirred for 2 h at 80 °C. To this reaction mixture was added a solution of compound **18** (10 g, 27.8 mmol) in CH_3CN (100 mL), and trimethylsilyl trifluoromethanesulfonate (25 g, 112.6 mmol) at 0 °C. The resulting solution was let to react overnight at 80 °C. Usual work-up and chromatography (50% EtOAc in hexanes) yielded nucleoside **19** as a white solid (10 g, 72%). MS, *m/z* 498 $(M+1)^+$. ^{1}H-NMR ($CDCl_3$): δ 8.2-8.50 (m, 3H), 7.54-7.61 (m, 1H), 7.33-7.51 (m, 7H), 6.46 (d, *J* = 7.6 Hz, 1H), 5.96-6.04 (m, 1H), 5.58-5.59 (m, 1H), 5.36-5.46 (m, 2H), 4.57-4.71 (m, 3H), 3.71-3.84 (m, 2H). ^{19}F-NMR ($CDCl_3$): δ -197.52.

9-(5-*O*-Benzyl-3-deoxy-3-fluoro-β-D-ribofuranosyl)-6-chloro-2-(tritylamino)purine (20). To a solution of nucleoside **19** (3 g, 6 mmol) in DMF (50 mL) was added triethylamine (3.4 mL, 24.0 mmol), DMAP (298 mg, 2.4 mmol), and chlorotriphenylmethane (5.5 g, 20 mmol). The resulting solution was stirred for 24 h at 45 °C. The reaction was then quenched with MeOH (20 mL) and concentrated under vacuum. The residue was purified by column chromatography (35% EtOAc in hexanes) to get 3.6 g (81%) of N-tritylated nucleoside as a white solid. MS, *m/z* 740 $(M+1)^+$. Obtained nucleoside (3.0 g, 3.9 mmol) in methanolic ammonia (60 mL) was stirred for 4 h at rt. Evaporated residue was purified by column chromatography (50% EtOAc in hexanes) to yield compound **20** (2.0 g 78%) as a white solid MS, *m/z* 636.3 $(M+1)^+$.

9-(5-*O*-Benzyl-3-deoxy-3-fluoro-2-oxo-β-D-ribofuranosyl)-6-chloro-2-(tritylamino)purine (21). To a solution of compound **20** (2.0 g, 3.0 mmol) in DCM (20 mL) was added iodobenzene diacetate (1.3 g, 4.0 mmol) and acetic acid (2 mL). It was followed by dropwise addition of a

solution of TEMPO (0.15 g, 0.9 mmol) in DCM (5 mL) at 0 °C. The resulting solution was stirred for 16 h at rt then diluted with DCM (40 mL) and quenched with solution of $Na_2S_2O_3$. The organic layer was washed with brine, dried (Na_2SO_4) and concentrated. The crude product was crystallized from DCM /heptane (1:1 v/v) to yield ketone **21** (2.0 g, 100%) as a red solid. MS, *m/z* 652.9 $(M+1+H_2O)^+$.

9-(5-*O*-Benzyl-2,3-dideoxy-2,3-difluoro-2-*C*-methyl-β-D-ribofuranosyl)-6-chloro-2-aminopurine (22). To a solution of methylmagnesium bromide (3M in ethyl ether, 10.5 mL) was added a solution of compound **21** (1.0 g, 1.6 mmol) in THF (7 mL) dropwise with stirring at -78 °C. The resulting solution was stirred for 2 h at -30 °C, and then quenched with sat. aq. NH_4Cl (20 mL). Usual work-up and column chromatography purification (50% EtOAc in hexanes) yielded 206 mg (20%) of 2'-methylated nucleoside as a yellow oil. MS, *m/z* 650 $(M+1)^+$. DAST (102 mg, 0.63 mmol) was added dropwise to the solution of obtained nucleoside in DCM (10 mL) at -78°C. The resulting solution was stirred for 2 h at rt, then quenched with aq. $NaHCO_3$. Usual work-up and column chromatography purification (25% EtOAc in hexanes) yielded 2'-fluorinated nucleoside (45 mg, 22%) as a white solid. MS, *m/z* 652 $(M+1)^+$. A mixture of obtained nucleoside in MeOH (0.34 mL) was treated with AcOH (0.30 mL) for 12 h at 50 °C. Solvent was concentrated, and the residue purified by silica gel chromatography (50% EtOAc in hexanes) to yield compound **22 (**29 mg, 98%) as a yellow oil. MS, *m/z* 410 $(M+1)^+$. ^{1}H-NMR ($CDCl_3$): δ 8.32 (s, 1H), 7.28-7.41 (m, 5H), 6.20 (d, *J* = 16.8 Hz, 1H), 5.52 (s, 2H), 5.15-5.46 (m, 1H), 4.63-4.71 (m, 2H), 4.43-4.49 (m, 1H), 3.96-3.99 (m, 1H), 3.76-3.82 (m, 1H), 1.22-1.34 (m, 3H). ^{19}F-NMR ($CDCl_3$): δ -163.92, -221.81.

2', 3'-Dideoxy-2',3'-difluoro-2'-*C*-methylguanosine (9). To a solution of compound **22** (25 mg, 0.06 mmol) in MeOH (2 mL) was added 2-mercaptoethanol (23.8 mg, 0.30 mmol) and sodium methylate (16.5 mg, 0.31 mmol). The resulting solution was stirred for 12 h at 65 °C. The pH value was adjusted to 7 with AcOH and the solution was concentrated. Residue was purified by silica gel chromatography (10% MeOH in DCM) to yield 5-benzylated guanosine analogue (29 mg) as a white solid. MS, *m/z* 392 $(M+H)^+$. A solution of obtained compound in DCM (0.5 mL) was cooled down to -78 °C. BCl_3 (1 M in heptane, 1 mL) was added and the reaction mixture was left for 0.5 h at the same temperature. Reaction was quenched with MeOH and the mixture concentrated. The crude product was purified by Prep-HPLC (XBridge BEH Phenyl OBD Prep

column, 19 × 150 mm; using 10-25% CH_3CN in 10 mmol aq. NH_4HCO_3 for elution. Difluoronucleoside **9** (10 mg, 55%) was isolated as a white solid. MS, *m/z* 302.1 $(M+1)^+$. ^{1}H-NMR ($CDCl_3$): δ 8.06 (s, 1H), 6.12-6.18 (m, 1H), 5.33-5.53 (m, 1H), 4.30 (m, 1H), 4.01-4.05 (m, 1H), 3.87-3.91 (m, 1H), 1.20 (d, *J* = 22.4 Hz, 3H), ^{19}F-NMR ($CDCl_3$): δ -163.03, -222.52. ^{13}C (DMSO-d_6): δ 156.7, 154.0, 150.5, 135.5, 116.8, 99.2 (dd, *J* = 183 Hz, 13 Hz), 89.9 (dd, *J* = 194 Hz, 15 Hz), 87.3 (dd, *J* = 38 Hz, 4 Hz), 59.6, 16.4 (d, *J* = 25 Hz).

9-(5-*O*-Benzyl-2,3-dideoxy-3-fluoro-2-*C*-methylene-β-D-ribofuranosyl)-6-chloro-2-(acetylamino)purine (23). To a solution of methyltriphenylphosphonium bromide (28.2 g, 78.3 mmol) in THF (400 mL) under inert atmosphere was added lithium bis(trimethylsilyl)amide (79 mL, 1 M in THF) dropwise with stirring at 0 °C. The resulting solution was stirred for 2 h at rt and then cooled to 0 °C. THF solution of ketone **21** (20 g, 31.5 mmol) was added. The resulting mixture was left for 4 h at rt. Reaction was quenched with sat. aq. NH_4Cl. Usual work-up and column chromatography purification (30% EtOAc in hexanes) yielded 2'-methylene derivative (6.8 g, 34%) as a yellow solid. MS, *m/z* 632 *(*$M+H)^+$. Methanolic solution of this nucleoside was treated with AcOH (51 ml) for 16 h at 50 °C. Solvents were evaporated and detritylated nucleoside was purified by silica gel chromatography (30% EtOAc in hexanes) to get 2.5 g (60%) of white solid. Obtained nucleoside was dissolved in pyridine (50 mL) and DCM (100 mL). AcCl (1.45 g, 18.5 mmol) was added dropwise and the reaction mixture was kept at rt for 1 h. Reaction was quenched with MeOH and solvents were evaporated. Product was purified by silica gel chromatography (50% EtOAcin hexanes) to yield nucleoside **23** (1.8 g, 65%) as a white solid. MS, *m/z* 432 $(M+1)^+$.

9-(5-*O*-Benzyl-2-chloro-2,3-dideoxy-3-fluoro-2-*C*-methyl-β-D-ribofuranosyl)-6-chloro-2-(acetylamino)purine (25). To a solution of compound **23** (570 mg, 1.32 mmol) in dioxane (15 mL) under inert atmosphere was added reagent **24** (24 mg, 0.04 mmol) and 4-toluene sulfonyl chloride (5.0 g, 26.23 mmol). The resulting solution was stirred for 1.5 h at 30 °C followed by dropwise addition of a solution of phenylsilane (143 mg, 1.32 mmol) in EtOH (10 mL). The resulting solution was kept for an additional 1 h at 30 °C. Solvents were evaporated and product was purified by silica gel chromatography (25% EtOAc in hexanes) to get nucleoside **25** (180 mg, 29%) as a yellow solid. MS, *m/z* 468.1 $(M+1)^+$.

9-(2,3-Dideoxy-2-chloro-3-fluoro-2-*C*-methyl-β-D-ribofuranosyl)-6-chloro-2-(acetylamino)purine (26). To a solution of compound **25** (60 mg, 0.13 mmol) in DCM (1.5 mL) under inert atmosphere was added borontrichloride (1.2 mL, 1 M in hexane) dropwise at -78 °C. The resulting solution was stirred for 2 h at -78 °C and 3 h at -30 °C. The reaction was then quenched by the addition of MeOH/DCM (1.5 mL, 1/1). The resulting mixture was concentrated under reduced pressure to dryness to yield 45 mg of compound **26** which was used without purification for further reaction. MS, *m/z* 378.2 $(M+1)^+$.

2',3'-Dideoxy-2'-chloro-3'-fluoro-2'-*C*-methylguanosine (10). To a solution of nucleoside **26** (200 mg, 0.53 mmol) in MeOH (20 mL) was added 2-mercaptoethanol (206 mg, 2.64 mmol) and NaOMe (476 mg, 30% in MeOH). The resulting solution was stirred for 3 h at 60 °C. The pH value of the solution was adjusted to 7 with acetic acid. The resulting mixture was concentrated and purified by Prep-HPLC (SunFire C18 OBD Prep Column, 19 × 100 mm; 15-35% MeCN in water). Guanosine **10** (44 mg, 26%) was isolated as a white solid. MS, *m/z* 318.1 $(M+1)^+$. ^{1}H-NMR (CD_3OD): δ 8.16 (s, 1H), 6.33 (s, 1H), 5.46-5.25 (m, 1H), 4.28-4.37 (m, 1H), 3.94-4.03 (m, 1H), 3.78-3.88 (m, 1H), 1.40 (s, 3H). ^{19}F-NMR (CD_3OD): δ -208.26. ^{13}C (DMSO-d6): δ 156.7, 154.0, 150.7, 135.2, 116.5, 91.0 (d, J = 196 Hz), 89.8 (d, J = 4 Hz), 80.6 (d, J = 22 Hz), 73.9 (d, J = 14 Hz), 59.2, 22.3.

9-(2-Deoxy-2-fluoro-2-*C*-methyl-β-D-ribofuranosyl)-6-ethoxy-2-(methoxytritylamino)-purine (28). To a solution of compound **27** (12.5 g, 23.8 mmol) in DCM (50 mL) were added $AgNO_3$ (8.1 g, 47.6 mmol), collidine (5.8 g, 47.6 mmol) and MMTrCl (11 g, 35.6 mmol). Reaction mixture was kept at rt overnight, quenched with MeOH and filtered. Solvents were evaporated and the residue purified by column chromatography (5% MeOH in DCM) to get 16 g (84%) of N-tritylated nucleoside. A solution of this product in EtOH (150 mL) was treated with NaOEt (50 mL, 2N in ethanol) at 0 °C. Resulting reaction mixture was kept at rt for 1 h. The pH was then adjusted to 7 with conc. aq. NH_4Cl. Usual work-up and column chromatography purification (5% MeOH in DCM) afforded 10.9 g (98%) of nucleoside **28**. MS, *m/z* 600.5 $(M+1)^+$. ^{1}H-NMR (DMSO-d_6): δ 8.13 (s, 1H), 7.51 (s, 1H), 7.15-7.30 (m 13H), 6.80 (m, 2H), 5.61 (d, J = 7.2 Hz, 1H), 5.25 (t, J = 5.0 Hz, 1H), 4.05 (br, 2H), 3.80 (m, 2H), 3.68 (s, 3H), 3.61 (m, 1H) 1.15 (br, 3H), 0.75 (br, 3H).

9-(2,5-Dideoxy-2-fluoro-2-*C*-methyl-5-iodo-β-D-ribofuranosyl)-6-ethoxy-2-(methoxytritylamino)purine (29). Nucleoside **28** (10 g, 16.5 mmol), triphenylphosphine (5.4 g, 20.6 mmol) and imidazole (1.6 g, 24.7 mmol) were suspended in THF (30 mL) and the resulting mixture cooled to 10 ^{o}C. Solution of iodine (5 g, 20 mmol) in THF (20 mL) was added and the reaction mixture was stirred at rt for 5 h. The reaction was quenched with aq. $Na_2S_2O_3$ Usual work-up and purification by column chromatography (2% MeOH in DCM) yielded nucleoside **29** (8.9 g, 75%) as a yellow solid. MS, *m/z* 710.2 $(M+H)^+$.

9-(4,5-Didehydro-2,5-dideoxy-2-fluoro-2-*C*-methyl-β-D-ribofuranosyl)-6-ethoxy-2-(methoxytritylamino)purine (30). To a solution of compound **29** (8 g, 11.3 mmol) in THF (40 mL) was added DBU (2.5 mL, 17 mmol) and the mixture was heated at 70-75 ^{0}C for 3 h. Reaction was quenched with 1M solution of citric acid. Usual work-up and purification by column chromatography (20% EtOH in hexane) yielded nucleoside **30** (2.4 g, 35%) as a white solid. MS, *m/z* 582.4 $(M+1)^+$. ^{1}H-NMR ($CDCl_3$): δ 7.42 (s, 1H), 7.19-7.29 (m 12H), 6.78 (m, 2H), 6.30 (s, 1H), 5.8 (br, 1H), 4.6, 4.5, 4.3 (3 br, 4H), 3.77 (m, 2H), 3.68 (s, 3H), 3.61 (m, 1H) 1.35 (br, 3H), 0.75 (br, 3H).

9-(2,5-Dideoxy-2,4-difluoro-5-iodo-2-*C*-methyl-β-D-ribofuranosyl)-6-ethoxy-2-(methoxytritylamino)purine (31). To a stirred cold (0 ^{o}C) solution of nucleoside **30** (4.8 g, 8.2 mmol) in DCM (50 mL) was added triethylamine trihydrofluoride (2 mL, 12.6 mmol) followed by addition of NIS (2.7 g, 12 mmol). Reaction was allowed to proceed for 2h at the same temperature and quenched with saturated solutions of $NaHCO_3$ and $Na_2S_2O_3$ (1:1). Usual work-up and purification by column chromatography (20% EtOH in hexane) afforded 3.5 g (60%) of nucleoside **31.** MS, *m/z* 728.1 $(M+1)^+$.

9-(3,5-Di-*O*-benzoyl-2-deoxy-2,4-difluoro-2-*C*-methyl-β-D-ribofuranosyl)-6-ethoxy-2-(methoxytritylamino)purine (32). To a solution of nucleoside **31** (2.1 g, 2.9 mmol) in pyridine (40 mL) was added BzCl (464 mg, 3.3 mmol) and the reaction mixture was kept at rt overnight. Usual work-up and chromatography on silica gel (20% EtOAc in hexane) yielded 2g of 3'-*O*-benzoylated product. To a solution of this compound in DMF (60 mL) were added NaOBz (3.45 g, 24 mmol) and 15-crown-5 (5.31 g, 24 mmol) and the reaction mixture was stirred at 100 ^{o}C for

48 h. Solvent was evaporated. Usual work-up and column chromatography purification (20% EtOH in hexane) yielded 1.1 g (45% for 2 steps) of nucleoside **32.** MS, *m/z* 826.2 $(M+H)^+$.

9-(2-Deoxy-2,4-difluoro-2-*C*-methyl-β-D-ribofuranosyl)-6-ethoxy-2-(methoxytritylamino)purine (33). A solution of **32** (1 g, 1.2 mmol) in n-butylamine (5 mL) was kept at rt overnight. Butylamine was evaporated and the residue was purified by column chromatography (10% MeOH in DCM) to get 0.62 g (85%) of nucleoside **33.** ^{1}H-NMR (DMSO-d_6): δ 7.74 (br, 1H), 7.54 (s, 1H), 7.14-7.27 (m 12H), 6.79 (m, 2H), 5.90 (br, 1H), 5.62 (br, 1H), 4.36 (br, 1H), 4.02 (br, 1H), 3.99 (q, *J* = 7.2 Hz, 2H,), 3.67 (s, 3H), 3.62 (m, 2H) 1.14 (t, *J* = 7.2 Hz, 3H), 1.0 (br, 3H). MS, *m/z* 618.1 $(M+1)^+$.

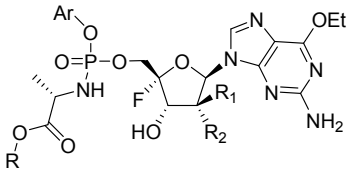
9-(5-*O*-Benzoyl-2-deoxy-2,4-difluoro-2-*C*-methyl-3-*O*-methoxytrityl-β-D-ribofuranosyl)-6-ethoxy-2(methoxytritylamino)purine (34). To a solution of **32** (10.0 g, 13.7 mmol) in DCM (100 mL) were added collidine (2.0 g, 16.5 mmol), MMTrCl (8.49 g, 27.5 mmol), and $AgNO_3$ (4.67 g, 27.5 mmol). The mixture was stirred at rt overnight. The solids were filtered off and the filtrate was diluted with solution of $NaHCO_3$. Organic layer was separated, washed with brine, and dried. The evaporated residue was purified by silica gel chromatography (20% EtOAc in hexane) to afford bistritylated nucleoside (11.7 g, 82%). MS, *m/z* 1000.2 $(M+1)^+$. To a solution of this compound (10.0 g, 10.0 mmol) in DMF (200 mL) were added 15-crown-5 (22 g, 100.0 mmol) and NaOBz (14.0 g, 100.0 mmol). The mixture was stirred for 72 h at 95 ^oC. After usual work-up and chromatography (10% EtOAc in hexane) nucleoside **34** (6.4g, 65%) was isolated as a white solid. MS, *m/z* 994.2 $(M+1)^+$.

9-(2-Deoxy-2,4-difluoro-3-*O*-methoxytrityl-2-*C*-methyl-β-D-ribofuranosyl)-6-ethoxy-2-(methoxytritylamino)purine (35). A solution of **34** (6.0 g, 6.0 mmol) in n-butylamine (50 mL) was kept at rt overnight. Butylamine was evaporated and the residue was purified by column chromatography (20% EtOAc in hexane) to yield **35** (3.7 g, 56%) as off-white foam. MS, *m/z* 890.2 $(M+1)^+$. ^{1}H-NMR ($CDCl_3$): δ 6.80-7.60 (m, 29H), 6.24 (d, *J* =18.3 Hz, 1H), 5.02 (s, 1H), 4.52-4.78 (m, 3H), 3.3.74-3.83 (m, 7H), 3.41-3.45 (m, 1H), 1.44-1.49 (m, 3H), 0.61 (d, *J* = 21.9 Hz, 3H). ^{19}F-NMR ($CDCl_3$): *δ* -120.16, 152.08.

General Method for Preparation of Phosphoramidates

Dry nucleosides **35** (445 mg, 0.5 mmol) was dissolved in the mixture of dry CH_3CN (4.5 mL) and N-methylimidazole (0.5 mL). The appropriate phosphorochloridate[40] (2.0 mmol) was added and the reaction mixture was kept overnight at rt or heated up to 70 °C for 2-10 h. Disappearance of starting nucleoside was controlled by LCMS. After the reaction was completed it was diluted with EtOAc (30 mL). Organic phase was washed with 10% citric acid, brine, dried (Na_2SO_4) and reduced to dryness. The residue was dissolved in CH_3CN (5 mL) and 4 N HCl in dioxane (0.5 mL) was added. The mixture was kept for 1 h at ambient temperature and evaporated. The residue was purified on silica gel with gradient of MeOH in DCM from 2% to 15%. Prodrugs **40**, **43-50** were obtained as mixtures of *R*p and *S*p isomers with total yield 50-85%.

Table 7. MS and ^{31}P- and ^{19}F-NMR (CD_3OD) of Nucleoside Phosphoramidate Prodrugs



Prodrug	Ar	R	R_1	R_2	^{31}P δ [ppm]	^{19}F δ [ppm]	MS $[M+1]^+$
36	phenyl	cyclohexyl	CCH	OH	3.96; 4.02	-122.35	663.20
37	phenyl	cyclohexyl	CH_3	OH	4.00; 4.11	-121.02	653.3
38	phenyl	cyclohexyl	CCH	F	3.94; 4.04	-122.9; -155.9	665.15
39	phenyl	cyclohexyl	CH_3	F	3.94, 4.09	-123.5; -156.8	655.2
40	phenyl	isopropyl	CH_3	F	3.98; 4.13	-123.2; -158.6	615.2
43	phenyl	neopentyl	CH_3	F	3.90; 4.08	-123.2; -158.5	6.43.2
44	naphthalen-1-yl	isobutyl	CH_3	F	4.28; 4.43	-123.2; -158.5	679.2
45	phenyl	isobutyl	CH_3	F	3.90; 4.06	-123.2; -158.6	629.2

46	phenyl	cyclopentyl	CH_3	F	3.99; 4.11	-123.2; -158.5	641.2
47	phenyl	pentan-3-yl	CH_3	F	4.00; 4.07	-123.1; -158.6	643.2
48	quinoline-5-yl	neopentyl	CH_3	F	4.15; 4.55	-123.5; -158.0	694.1
49	naphthalen-1-yl	neopentyl	CH_3	F	4.28; 4.39	-123.1; -158.8	693.2
50	naphthalen-1-yl	isopropyl	CH_3	F	4.48; 4.36	-123.5; -158.0	665.1

Separation of *S*p- (42) and *R*p-(41) Isomers from Isomeric Mixture 40

Mixture **40** was separated by RP HPLC on Synergy 4-µM Hydro-RP 80Å (250 × 30 mm) column. A linear gradient of CH_3CN in water from 40% to 70% in 26 min with flow rate 24 mL/min was used. Two compounds were eluted. The corresponding fractions were combined and concentrated. Analytical HPLC (Synergi 150 × 4.6mm, 4µm Hydro-RP 80 Å column, 50 mM TEAA in water and 50 mM in CH_3CN, flow rate 2 mL/min) was performed with gradient from 40% to 60% B over 10 min.

Compound **41** has retention time on analytical HPLC 7.77 min. MS, *m/z* 615.3 $(M+1)^+$. ^{1}H-NMR (DMSO-d_6): δ 7.92 (s, 1H), 7.11-7.30 (m, 5H); 6.61 (s, 1H); 6.45 (d, 1H, J = 18 Hz), 5.99-6.05 (m, 2H); 4.74-4.84 (m, 2H), 4.30-4.50 (m, 4H); 3.70-3.80 (m, 1H), 1.32 (t, *J* = 7.0 Hz, 3H), 1.15-1.18 (m, 6H), 1.06-1.09 (m, 9H). ^{31}P-NMR (DMSO-d_6): δ 4.08. ^{19}F-NMR (DMSO-d_6): δ -154.8, -190.3. ^{13}C-NMR (DMSO-d6): δ 172.6, 160.6, 160.1, 153.0, 150.5, 138.0, 129.5, 124.6, 120.1, 116.1 (d, *J* = 10 Hz), 113.8.0, 99.0 (br), 74.0 (br), 68.0, 65.3 (br), 61.7, 49.9, 21.3, 19.6, 17.0 (d, *J* = 25 Hz), 14.4.

Compound **42 (AL-611)** has retention time on analytical HPLC 7.52 min. MS, *m/z* 615.2 $(M+1)^+$. ^{1}H-NMR (DMSO-d_6): δ 7.94 (s, 1H), 7.15-7.38 (m, 5H); 6.61 (s, 2H); 6.44 (d, 1H, *J* = 18 Hz), 5.98-6.05 (m, 2H); 4.71-4.80 (m, 2H), 4.38-4.58 (m, 3H); 4.19-4.30 (m, 1H); 3.65-3.79 (m, 1H), 1.32 (t, *J* = 7.0 Hz, 3H), 1.05-1.15 (m, 12H). ^{31}P-NMR (DMSO-d_6): δ 4.24. ^{19}F-NMR (DMSO-

d_6): δ -154.8, -191.2. ^{13}C-NMR (DMSO-d_6): δ 172.9, 161.0, 160.4, 151.0, 150.9, 138.5 (br), 130.0, 125.0, 120.6, 120.5, 114.5 (br), 114.5 (br), 98.5(br), 73.8, 68.3, 62.1, 50.1, 21.7, 20.6, 17 (d, *J* = 25 Hz), 14.9.

Stereoselective Synthesis of Prodrug 42 (AL-611).

Nucleoside **35** (890 mg, 1 mmol) and phosphoramidate **54** [32] (815 mg, 1.8 mmol) were dissolved in THF (15 mL) and cooled to 5 °C. *tert*-BuMgCl (1.5 mL, 1.0 M solution in THF) was added dropwise and the reaction mixture was stirred for 1 h at the same temperature. Reaction was quenched with aq. NH_4Cl. Usual work-up was followed by column chromatography purification (20% to 60% EtOAc in hexanes). The corresponding fractions were concentrated and treated with the mixture of CH_3CN and 36% HCl (10 mL, 9:1 v/v) for 40 min. at ambient temperature. Solvents were evaporated, the residue was purified on silica gel with gradient of MeOH in DCM from 2% to 10% to obtain 367 mg (60%) of **42**.

General Method for Preparation of 3',5'-Cyclophosphate Prodrugs 51-53.

Table 8. MS and ^{31}P NMR of 3',5'-Cyclophosphate Prodrugs

Compound	Yield (for 2 steps)	^{31}P δ [ppm]	^{19}F δ [ppm]	Solvent	MS $(M+1)^+$
51	30%	-6.92, -7.92	-121.8; -158.3 -122.3; -159.9	CD_3OD	436.3
52	10%	-6.69, -7.92	-123.6; -158.9	DMSO-d_6	464.0
53	21%	-7.13, -9.03	-123.1; -158.5	DMSO-d_6	450.0

Nucleoside **33** (180 mg, 0.3 mmol) and *tert*-BuMgCl (1.2 mL, 1.0 M solution in THF) in dry THF (5 mL) were treated with alkyl phosphorodichloridate (0.45 mmol). Usual work-up was followed by column chromatography purification (2-5% MeOH in DCM). Protected prodrug was dissolved

in CH_3CN (5 mL), HCl in dioxane was added (0.5 mL), the mixture kept for 1h at ambient temperature and evaporated. The residue was purified on silica gel with gradient of MeOH in DCM from 2% to 10%. Yields, ^{31}P, ^{19}F-NMR, and MS are reported in Table 8.

General Method for Preparation of Nucleoside 5'-Triphosphates

Table 9. MS and ^{31}P NMR (D_2O) of Nucleoside 5'-Triphosphates

NTP	^{31}P, δ [ppm]	MS $[M-1]^-$	Purity %[a]
2-TP	-9.59(d), -11.39(d), -21.06(t)	536.1	100
3-TP	-11.05(d), -11.48(d), -23.36(t)	537.4	100
4-TP	-10.88(d), -11.58(d), -23.24(t)	554.0	100
5-TP	-10.98(d), -11.92(d), -23.25(t)	554.0	97
6-TP	-11.05 (br), -12.02(d), -23.25(t)	564.0	100
7-TP	-10.97(d), -11.92(d), -21.19(t)	556.2	100
8-TP	-10.98(d), -11.99(d), -23.18(t)	566.0	99
9-TP	-10.51(d), -11.41(d), -23.13(t)	540.0	100
10-TP	-10.53(d), -11.56(d), -23.30(t)	556.4	100

[a] Analytical HPLC was performed on Synergy 4 μM Hydro RP 80 Å 150 × 6 mm, flow 1.5 mL/min. A linear gradient of CH_3CN from 0% to 25 % in 50 mM triethylammonium acetate buffer (pH 7.5) was used for elution.

Dry nucleoside (0.05 mmol) was dissolved in $PO(OMe)_3$ (0.7 mL). N-Methylimidazole (0.009 mL, 0.11 mmol) was added followed by $POCl_3$ (0.009 mL, 0.11 mmol), and the mixture was kept at rt for 20-40 mins. The reaction was controlled by LCMS and monitored by the appearance of corresponding nucleoside 5'-monophosphate. After completion of the reaction, tetrabutylammonium salt of pyrophosphate (150 mg) was added, followed by DMF (0.5 mL) to get a homogeneous solution. After 1.5 h at rt, the reaction was diluted with water (10 mL) and loaded on HiLoad 16/10 with Q Sepharose High Performance column. Separation was done in a linear gradient of NaCl from 0 to 1N in 50mM TRIS-buffer (pH7.5). Triphosphate was eluted with 75-80% B. Corresponding fractions were concentrated. Desalting was achieved by RP HPLC on Synergy 4-μM Hydro-RP column. A linear gradient of CH_3CN from 0 to 30% in 50 mM triethylammonium acetate buffer (pH 7.5) was used for elution. The corresponding fractions were combined, concentrated and lyophilized 3 times to remove excess of buffer. ^{31}P-NMR, MS and purity presented in Table 9.

HCV RNA Polymerase Inhibition Assay

The HCV polymerase RdRp activity was measured as the incorporation of radiolabeled ribonucleotide monophosphates into acid-insoluble RNA products using HCV NS5B and complementary internal ribosome entry site (cIRES)-derived RNA templates, as described previously.[41] HCV polymerase reaction mixtures contained 50 nM cIRES template, 1 μM tritiated CTP, 1 μM ATP, 1 μM GTP, 0.5 μM UTP, 40 mM Tris-HCl (pH 8.0), 20 mM NaCl, 3 mM DTT, 4 mM $MgCl_2$, serial diluted inhibitor, and 100 nM NS5B enzyme. Reaction mixtures were incubated for 2 h at 30 °C and stopped by RNA precipitation with the addition of equal volumes of 20% (w/v) trichloroacetic acid. MicroscintTM 20 (PerkinElmer, Waltham, MA) was added to the acid-insoluble RNA products and read on a MicroBeta Trilux (PerkinElmer, Waltham, MA).

Human DNA and RNA Polymerase Assays

The enzymatic activity of human DNA and RNA polymerases was measured as previously described.[36] Briefly, activated calf thymus DNA was used as substrate for the DdDp activity of DNA polymerase α, β, and γ, in a buffer containing 50 mM Tris HCl (pH 8.0), 60 mM KCl, 5 mM

$MgCl_2$, 4 mM dithiothreitol (DTT), 2 μM dCTP, 2 μM dATP, 2 μM dGTP, and 2.5 μCi 3H dTTP. The DNA product was precipitated in 20% (w/v) trichloroacetic acid. After the addition of 50 μL of MicroscintTM 20 (PerkinElmer, Waltham, MA), the precipitated high molecular weight DNA products were measured in a Trilux Microbeta microplate scintillation reader (PerkinElmer, Waltham, MA). The RNA polymerase II mediated in vitro transcription was performed with commercially available HeLa cell nuclear extract (Promega; Madison). RNA products were resolved by electrophoresis on 6% Novex TBE Urea polyacrylamide gels (Invitrogen; Carlsbad, CA). The DdRp assay with the human mitochondrial RNA polymerase was performed under single turnover conditions where enzyme concentration was in excess of the primer/template (Jin Z, Antiviral Research 2017). Therefore, the ^{33}P-RNA/DNA primer/template (RNA: 5′-UUUUGCCGCGCC-3′ and DNA 3′-CGGCGCGGCACGTAAGGG-5′) was used at a concentration of 100 nM, together with 320 nM enzyme. Each NTP (natural or analogues) was tested at 100 μM; ATP, UTP and CTP were used to quantify the extent of mis-incorporation of an incorrect nucleotide opposite templating CMP. RNA products were resolved by electrophoresis on 22.5% TBE Urea polyacrylamide sequencing gels. Quantification of the radiolabeled band was performed using a TYPHOON PhosphorImager and the ImageQuant 5.2 software (GE Life Sciences).

HCV RNA Polymerase Chain Termination Assay

The preparation of the NS5B elongation complex (EC) was performed by an extension and pause reaction as previously described.[41] In summary, a 50 μL extension and pause reaction containing 6.5 μM NS5B, 20 μM pGG, 20 μM RNA template (3' – CCUAUAUUAGCAAUAUCUAA – 5'), 25 μM ATP and 12.5 μM GTP in the optimized reaction buffer (40 mM Tris-Cl, pH 7.0, 40 mM NaCl, 5 mM DTT, and 2 mM $MgCl_2$) was run for 2 h at 30 ˚C to generate a 9-mer, followed by the addition of 20 μM CTP containing 13 nM ^{33}P radiolabeled CTP, and incubated for 30s at 30 ˚C to generate a 10-mer. All the active EC was precipitated out during the reaction and isolated by centrifugation at 16,000-g for 5 min at room temperature using a bench top centrifuge. After centrifugation, the supernatant was removed and the remaining pellet was washed twice by additional resuspension in the wash buffer (40 mM Tris-Cl, pH 7.0, 20 mM NaCl, 5 mM DTT and

2 mM $MgCl_2$) and centrifugation to remove residual contaminants. The final pellet containing the EC was resuspended and solubilized in 500 μL of a physiologically relevant reaction buffer (40 mM Tris-Cl, pH 7.4, 5 mM DTT, 2 mM $MgCl_2$, 150 mM NaCl) to serve as the isolated EC for this study. Elongation reactions contained 5 μL of the 10-mer EC and 100 μM each final concentration of NTPs in a buffer containing 40 mM Tris-Cl, pH 7.4, 5 mM DTT, 2 mM $MgCl_2$ and 150 mM NaCl. Reactions were incubated at 30 ºC for 1 minute and quenched with 20 μL of a gel loading buffer containing 90% formamide, 50mM EDTA, 0.025% (w/v) bromophenol blue and 0.025% (w/v) xylene cyanol FF and heated at 95 ºC for 5 minutes. The samples were run on a 22.5% polyacrylamide gel (8M urea) and exposed to a phosphorimager screen. The EC and subsequent incorporation products were quantified using a TYPHOON PhosphorImager and Image Quant TL software (GE Life Sciences).

HCV Replicon Assay

Huh-7 cells harboring an autonomously replicating, subgenomic HCV replicon of the Con1 strain were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and nonessential amino acids (JRH Biosciences, Lenexa, KS), plus 0.25 mg/ml G418 (Invitrogen, Carlsbad, CA). The Huh-7 HCV replicon cell line was obtained from R. Bartenschlager's Lab (University Heidelberg, Heidelberg, Germany) and was maintained and cultured in-house at Janssen BioPharma. The subgenomic HCV replicon encodes a neomycin phosphotransferase, which allows selective growth of HCV replicon-containing Huh-7cells over HCV replicon-negative Huh-7 cells in the presence of G418. The compound concentrations at which the HCV RNA level in the replicon cells is reduced by 50% (EC_{50}) or by 90% (EC_{90}) or the cell viability is reduced by 50% (CC_{50}), were determined in HCV Con1 subgenomic replicon cells using 4-parameter curve fitting (SoftMax Pro) as described previously. Briefly, the replicon cells were incubated with compounds diluted in DMEM containing 2% FBS Briefly, the replicon cells were incubated with compounds diluted in DMEM containing2% FBS and 0.5% DMSO (without G418) at 37 °C. Total cellular RNA was extracted using an RNeasy-96 kit (QIAGEN, Valencia, CA), and the copy number of the HCV RNA was determined in a quantitative, real-time, multiplex reverse transcription-PCR (QRT-PCR, or Taqman) assay. The

cytotoxicity of compounds in the HCV replicon cells was measured under the same experimental settings After 3 days of treatment, viability was determined with the CellTiter-Blue cell viability assay solution (Promega) in a Victor3 V 1420 multilabel counter (PerkinElmer), and 50% cytotoxic concentration (CC_{50}) values were determined using the Microsoft Excel and XLFit 4.1 software.

8 Days Cytotoxicity

Long-term cytotoxicity assays were performed with Huh-7, HepG2, A549, HeLa, MT4 and U935 cells (1×10^5 cells/well) in collagen I-coated 96-well plates that were maintained for 8 days in the recommended medium without drug to ensure terminal differentiation prior to drug exposure. The cells were treated with compounds for 8 days and were refed every other day to ensure proper nutrition and exposure to the drug. At the end of treatment, cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). CC_{50} values were determined using XLFit 4.1 software.

NTP Formation in Vitro

The in vitro NTP formation experiments were conducted in primary human hepatocytes. The primary human hepatocytes from male and female donors of various ethnic backgrounds were purchased from Corning Life Science (Tewksbury, MA), BioIVT (Hicksville, NY), and Thermo Fisher Scientific (Waltham, MA). Primary human hepatocytes were seeded at 1.5 - 2 million cells/well in 6-well plates and maintained in Williams E. medium containing Life Tech's proprietary supplement cocktail. The plated human hepatocytes in 3 mL of medium per well were acclimated overnight (> 18 hours) in a cell culture incubator at 37 ºC, 5% CO_2. Addition of a test article standard solution into each well achieved the target final incubation (extracellular) concentration. The cells were continually incubated at 37 ºC, 5% CO_2 for the designated time. At the end of incubation, cells were washed twice with 700 µL of cold 0.9% sodium chloride solution before lysis with the addition of 700 µL of methanol/water (70/30, v/v). The cell lysate was transferred into an Eppendorf tube and stored at -20 ºC for at least 3 hours before analysis. After vortexing and centrifuging, the supernatant was dried and reconstituted with 500 µL of 1 mM ammonium phosphate solution. An internal standard (N^6-benzoyladenosine) was added to a 50-µL aliquot of the supernatant before LC-MS/MS analysis for the NTP concentration. The NTP

calibration standards for the analysis was constructed by spiking the NTP standard solutions into control cellular samples of human hepatocytes, which were incubated without the test article and treated in the same manner as study samples. Quantification of the NTP was conducted using Analyst® software on a Sciex® API 3200 or API 5000 tandem triple quadrupole mass spectrometer coupled to a Shimadzu® LC-20AD HPLC system with a Leap Technologies HTC PAL autosampler. The HPLC column was a Phenomenex Gemini C18 (50 × 2 mm, 3-µm particle size) column employed a step gradient for chromatographic separation coupled with negative ion MS/MS detection of the NTP. Mobile phase A consisted of 3mM ammonium formate and 10 mM dimethyl hexylamine (DMHA) in water and mobile phase B consisted of 3 mM ammonium formate and 10 mM DMHA in 1:1 acetonitrile/water.

To obtain the intracellular half-life of the NTP, the primary human hepatocytes were initially incubated with the test article at 50 µM for 24 hours at 37 °C, 5% CO_2 before the incubation media containing the test article was replaced with blank incubation media. The cells were placed back into the incubator after collection of the 0-hour timepoint. Further timepoints for the NTP stability were collected at 3, 6, 10, 24, and 48 hours. The half-life was calculated based on the assumption that the disappearance of NTP followed first-order kinetics. Thus,

$$C_t = C_0 \bullet e^{-\ln 2 / t_{1/2} \bullet t}$$

Where C_t represents the intracellular concentration of NTP at time t, C_0 represents the concentration of NTP at time zero extrapolated to time zero based on the selected terminal phase, t is the time, and $t_{1/2}$ represents half-life of NTP.

NTP Formation in Vivo

The in vivo pharmacokinetic studies were conducted at Wuxi AppTec. The studies conformed to International regulations and guidelines regarding animal care and welfare and the study protocols were reviewed and approved by Wuxi AppTec's Institutional Animal Care and Use Committee (IACUC) prior to the study initiation. Male non-naïve beagle dogs (n=2 per time point) were fasted overnight before administered orally with the test article. The test article was formulated as a solution in 40% PEG400 in water and administered at 9.69 mg/kg (5 mg/kg parent nucleoside

equivalent dose). For liver tissue collection, animals were anesthetized with pentobarbital. A piece of liver (at least 1 g) was removed and immediately flash-frozen into liquid nitrogen to prevent any ex vivo degradation of the NTP. The frozen tissue was homogenized in an extraction solution in a sample tube set in a dry ice/ethanol bath to maintain cold temperature. The supernatant of the liver extracts was subjected to LC/MS/MS analysis for the concentrations of the NTP. The NTP calibration standards for the analysis was constructed by spiking the NTP working standard solutions into blank liver homogenate. The ACQUITY UPLC system used a Phenomenex Gemini C18 column (50 x 4.6 mm, 5 μm) with a step gradient for chromatographic separation. It was coupled with an AB-Sciex® API 4000 mass spectrometer operated in the negative ion mode for NTP detection.

X-Ray Crystallography Methods

Single colorless needle-shaped crystals of compound **42** (**Al-611**) generated during compound purification were used. A suitable crystal (0.21 mm × 0.10 mm × 0.05 mm) was selected and mounted on a nylon loop with paratone oil on a Bruker APEX-II CCD diffractometer. The crystal was kept at T = 173(2) K during data collection. Using Olex2[42] the structure was solved with the ShelXT[43] structure solution program, using the Direct Methods solution method. The model was refined with version 2014/6 of XL[44] using Least Squares minimization.

Crystal Data: C25H34F2N6O8P, Mr = 615.55, orthorhombic, P212121, a = 7.8919(3) Å, b = 15.7818(6) Å, c = 22.8797(10) Å, α = β = γ = 90°, V = 2849.6(2) Å3, T = 173(2) K, Z = 4, Z' = 1, μ(CuK_α) = 1.490, 17819 reflections measured, 5505 unique (R_{int} = 0.2423) which were used in all calculations. The final wR_2 was 0.1612 (all data) and R_1 was 0.0747 (I > 2(I)).

Data were measured using □ and ϕ scans of 1.00° per frame for 45.00 s using CuK_α radiation (sealed tube, 40 kV, 30 mA). The total number of runs and images was based on the strategy calculation from the program COSMO (BRUKER, V1.61, 2009).[45] The achieved resolution was Θ = 72.153 (0.81 Å). Cell parameters were retrieved using the SAINT (Bruker, V8.34A, 2013)[46] software and refined using SAINT on 1173 reflections, 7 of the observed reflections. Data reduction was performed using the SAINT software which corrects for Lorentz polarization. The final completeness was 99.70 out to 72.153 in Θ. The absorption coefficient (μ) of this material was 1.490 and the minimum and maximum transmissions were 0.5937 and 0.7536.

The structure was solved in the space group P212121 by Direct Methods using the ShelXT[35] structure solution program and refined by Least Squares using version 2014/6 of XL[36]. All non-hydrogen atoms were refined anisotropically. Hydrogen atom positions were calculated geometrically and refined using the riding model. Structure refined by least squares method on F2, ShelXL-97, incorporated in Olex2. All H atoms were placed in calculated positions and refined using a riding model. The Flack parameter was refined to 0.00(7). Determination of absolute structure using Bayesian statistics on Bijvoet differences using the Olex2 results in 0.02(6). Note: The Flack parameter is used to determine chirality of the crystal studied, the value should be near 0, a value of 1 means that the stereochemistry is incorrect, and the model should be inverted. A value of 0.5 means that the crystal consists of a racemic mixture of the two enantiomers.

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ABBREVIATIONS

NTP, nucleoside 5'-triphosphate; TEMPO**, 2,2,6,6-Tetramethylpiperidine 1-oxyl; DAST,** Diethylaminosulfur trifluoride;
NIS, N-Iodosuccinimide; HMPA, Hexamethylphosphoric acid triamide;
BSA, *N*,*O*-Bis(trimethylsilyl) acetamide;
DBU, 2,3,4,6,7,8,9,10-Octahydropyrimidol[1,2-*a*]azepine; DMAP, 4-(Dimethylamino)pyridine, TrCl, **Triphenylchloromethane; MMTrCl, 4-Methoxytriphenylchloromethane.**

Supporting Information

Molecular formula strings (CSV)

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