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Synthesis and Evaluation of 2,6-Modified Purine 2′-*C*-Methyl Ribonucleosides as Inhibitors of HCV Replication

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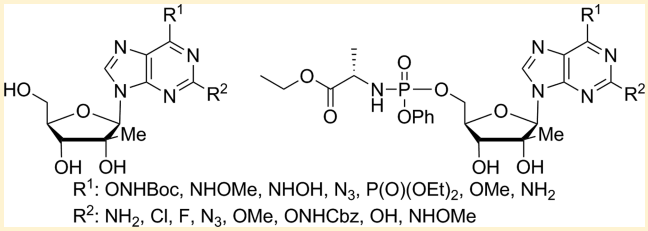
Supporting Information

ABSTRACT: A variety of 2,6-modified purine 2′-*C*-methyl-ribonucleosides and their phosphoramidate prodrugs were synthesized and evaluated for inhibition of HCV RNA replication in Huh-7 cells and for cytotoxicity in various cell lines. Cellular pharmacology and HCV polymerase incorporation studies on the most potent and selective compound are reported.



KEYWORDS: *HCV, antiviral, phosphoramidate prodrug, purine, nucleoside*

Hepatitis C virus (HCV) is a global health problem affecting an estimated 170 million individuals worldwide, and it is a leading cause of liver cirrhosis and hepatocellular carcinoma.[1,2] Several curative options are now available for HCV infections, but they all require at least two direct acting antiviral agents to result in cure rates of 90 to 100%. Nucleoside inhibitors of HCV NS5B polymerase are favored since they generally have a high genetic barrier to drug resistance and are pangenotypic activity.[3] Sofosbuvir (PSI/GS-7977) **1**,[4] a 2′-deoxy-2′-*α*-fluoro-2′-*β*-*C*-methyl nucleoside monophosphate prodrug, was approved by the FDA in December 2013 as a safe and effective anti-HCV agent (Figure 1).[5] IDX-184 **2** and BMS-986094 (INX-189) **3**, two related nucleoside prodrugs, precursors of the same active 2′-*β*-*C*-methyl guanosine triphosphate, also showed high potency *in vitro* and promising results in early clinical studies, but their development was terminated after low effectiveness in humans for IDX-184, and severe cardiac effects observed during a phase 2b study with BMS-986094. Based on the potential of these 2′-*C*-methyl nucleosides, we present, herein, the synthesis of new 2,6-modified purine 2′-*C*-methyl ribonucleosides that may offer potential alternatives to BMS-986094 and IDX-184 or maybe used in combination with Sofosbuvir.

In the design of 6-position modifications, we strived to maintain groups that retained hydrogen bond accepting characteristics as is present in the 6-position of natural guanosine. The key to the synthesis of 2-amino, 6-modified 2′-*C*-methyl nucleosides was intermediate **5** that was prepared by known methods.[6] Targeted 6-N_3 and 6-NH-O-substituted purines **10**−**12** were easily prepared from debenzoylated nucleoside **6** by reaction with NaN_3, methoxyamine, and hydroxylamine (Scheme 1). Interestingly, attempts to prepare the 6-ONH_2 compound **9** from *N*-Boc-hydroxylamino and

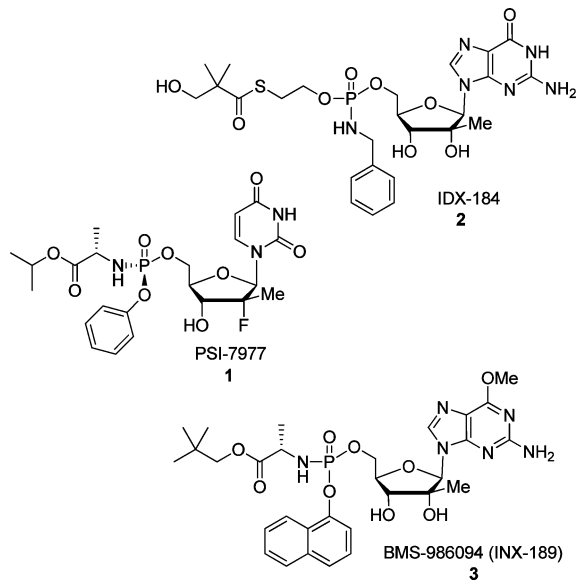


Figure 1. Selected clinical anti-HCV nucleoside analogues.

N-(benzyloxycarbonyl)hydroxylamino derivatives **7** and **8** were unsuccessful as all attempts to deprotect intermediates **7** and **8**, using acidity (compound **7**) or transition metal catalyzed hydrogenation (compound **8**), lead exclusively to the formation of 2′-*C*-methyl guanosine.

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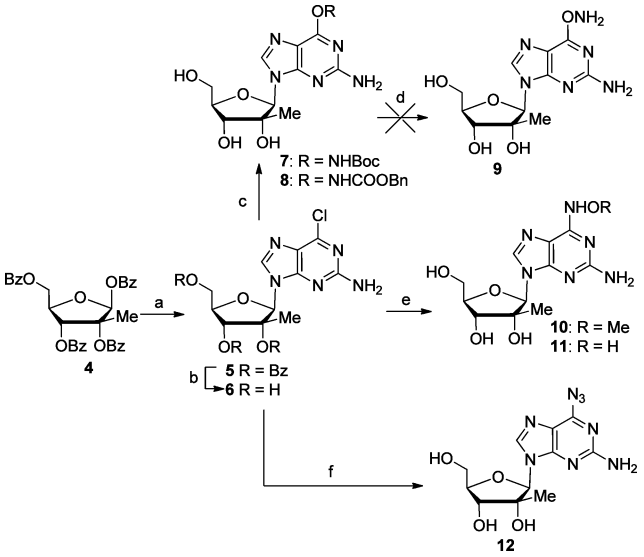
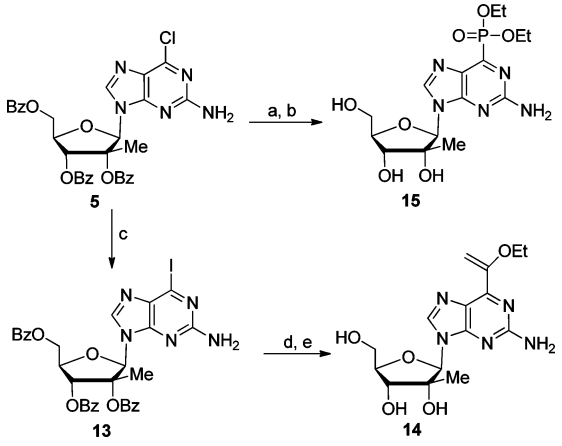
Scheme 1[a]



[a]Reagents and conditions: (a) 2-amino-6-chloropurine, DBU, TMSOTf, −40 to 80 °C, 5 h, 92%; (b) sat. NH_3/MeOH, rt, overnight, 90%; (c) for **7**, HONHBoc, NaH, THF, rt, 3 h, 69%; for **8**, HONHCbz, NaH, THF, rt, 3 h, 88%; (d) for **7**, 80% TFA, H_2O, rt, overnight; for **8**, H_2, Pd/C 10%, MeOH, rt, overnight; (e) for **10**, $MeONH_2$, Et_3N, EtOH/H_2O, 65 °C, 24 h, 79%; for **11**, NH_2OH, EtOH/H_2O, 35 °C, 24 h, 51%; (f) NaN_3, DMF, 95 °C, 2 h, 62%.

Other, more uncommon functionalities such as a phosphonate and an ethoxyvinyl group were also introduced at the 6-position (Scheme 2). Thus, 6-diethylphosphonate derivative **15** was

Scheme 2[a]



[a]Reagents and conditions: (a) $P(OEt)_3$, 130 °C, overnight, 78%; (b) sat. NH_3/EtOH, rt, 4 d, 44%; (c) TMSI, CH_2Cl_2, rt, 9 h, 63%; (d) tributyl (1-ethoxyvinyl) stannane, $Pd(PhP_3)_2Cl_2$, THF, 80 °C, 24 h, 54%; (e) Cat. NaOMe, CH_3OH, rt, 12 h, 79%.

prepared by reaction of 6-chloropurine nucleoside **5** with triethyl phosphite (Scheme 2) at 130 °C and subsequent deprotection in a saturated solution of ammonia in ethanol. Compound **5** was also reacted with TMSI to generate the more reactive iodo intermediate **13**, which was coupled with tributyl(1-ethoxyvinyl)stannane under palladium catalyzed Stille coupling conditions. Final deprotection using a catalytic amount of sodium methoxide in methanol afforded final compound **14**.

With the knowledge that the 2-position of adenosine is not involved in the hydrogen bonding of base pairing while the 2-amino group of guanosine is, we next turned our attention to the synthesis of 2-modified purine nucleosides. 2-Hydroxylamino-, 2-fluoro-, 2-methoxy-, and 2-azido-purine nucleosides were targeted as they potentially offer a variety of steric, electronic, and hydrogen bonding interactions, which may enhance recognition by HCV NS5B polymerase in their 5′-triphospate forms. The key tribenzoylated 2,6-dichloropurine-2′-Me nucleoside **16** was prepared in a manner similar to **6** (Scheme 1) with 2,6-dichloropurine, TMSOTf, and DBU. Treatment of the 2,6-dichloropurine nucleoside **16** with a saturated solution of ammonia in methanol lead to concomitant formation of 6-amino compound **17** and 6-methoxy compound **18** (Scheme 3). This reaction was preformed at room temperature to avoid displacement of the 2-chloro group and appeared to proceed predominantly by first formation of the 6-methoxy compound, which is then converted to the 6-amino via ammonia displacement. The benzoyl group deprotection occurred quite rapidly and was followed by a slow partial conversion of the 6-methoxy nucleoside **18** to the 6-amino nucleoside **17** over the three-day reaction. With **17** and **18** in hand, our initial goal was to prepare the corresponding 2-O-NH_2 derivatives. While the reaction of **17** and **18** with *N*-Cbz hydroxylamine worked well, the subsequent palladium catalyzed hydrogenation of the CBz group generated only 2-hydroxy purine products **22** and **25**. However, 2,6-dimethoxypurine **23** and 6-amino-2-methoxy purine derivative **26** were successfully synthesized by simple treatment of compounds **16** and **17** with sodium methoxide. 2-Azidopurine **20** was prepared in two steps by the reaction of 2-chloro purine derivative **17** with hydrazine hydrate followed by treatment of the resulting hydrazine compound **19** with sodium nitrite in acetic acid.[7,8] As expected, ^{1}H NMR showed that compound **20** exists as an equilibrium of azido (**20a**) and 1-*N*-tetrazole (**20b**) tautomeric forms.[9−11] It is worth noting that a two-step sequence was used because direct treatment of **17** with NaN_3 failed to provide 2-azido nucleoside **20**.

Vorbruggen-type coupling between tetrabenzoylated sugar **4** and 2-fluoro-6-aminopurine in the presence of TMSOTf and DBU afforded compound **27** in 80% (Scheme 3). Deprotection of the three benzyl groups using a saturated solution of ammonia in methanol gave access to 2-fluoro-6-aminopurine nucleoside **28**, which was subsequently treated with *O*-methyl hydroxylamine to give the desired 2-*N*-methoxylamine purine derivative **29**.

It has been now well established that nucleoside analogues are oftentimes unable to be intracellularly metabolized to their corresponding nucleoside triphosphates. Therefore, in order to overcome the often rate-limiting first phosphorylation step and improve the antiviral activity of our nucleoside analogues, we prepared their corresponding monophosphate McGuigan-type prodrugs.[12] The synthesis of phosphoramidates **31**−**46** was performed following the Uchiyama procedure by reacting the nucleosides **10**−**12**, **14**, **15**, **17**, **18**, **20**, **22**, **23**, **25**, **26**, **28**, and **29** with chlorophosphoramidate **30**[13] in the presence of *N*-methylimidazole (Scheme 4).[14,15] It is noteworthy that the use of acetonitrile as a cosolvent improved the solubility of certain nucleosides leading to better overall yields. Attempts to prepare the 2-aminooxypurine nucleoside prodrugs by Cbz removal of compounds **38** and **40** were not successful and instead afforded the isoguanosine derivatives **39** and **41**.

The nucleosides and phosphoramidate prodrugs were evaluated for inhibition of HCV RNA replication in Huh7

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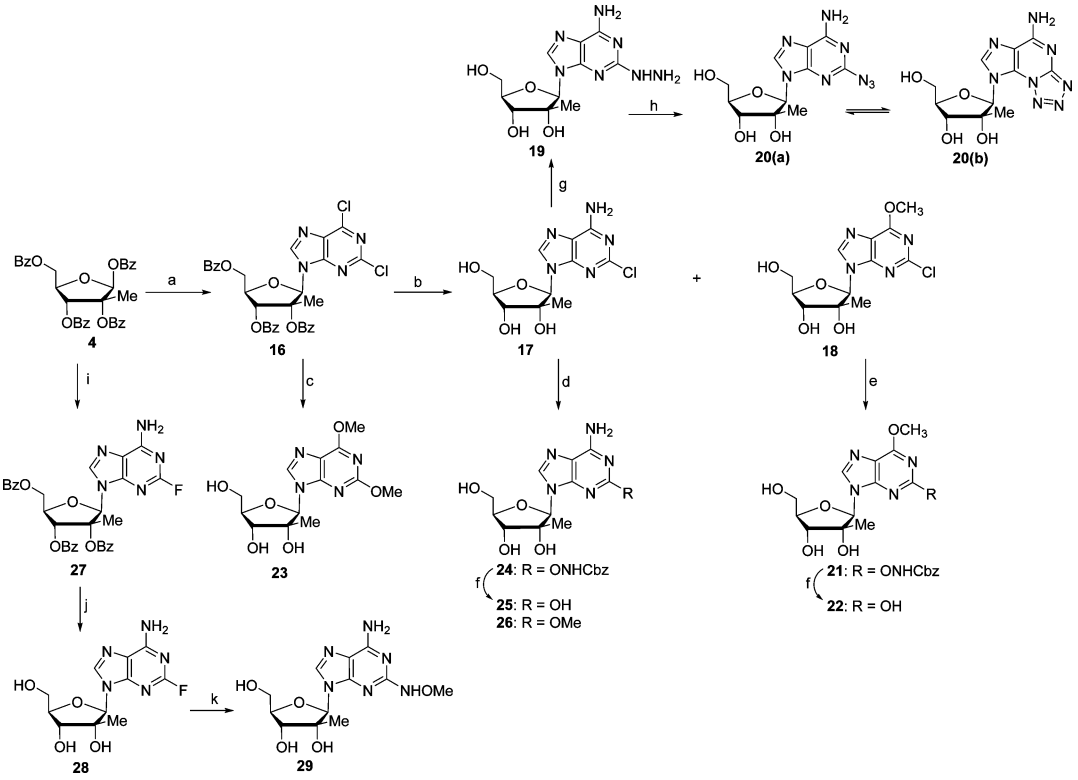
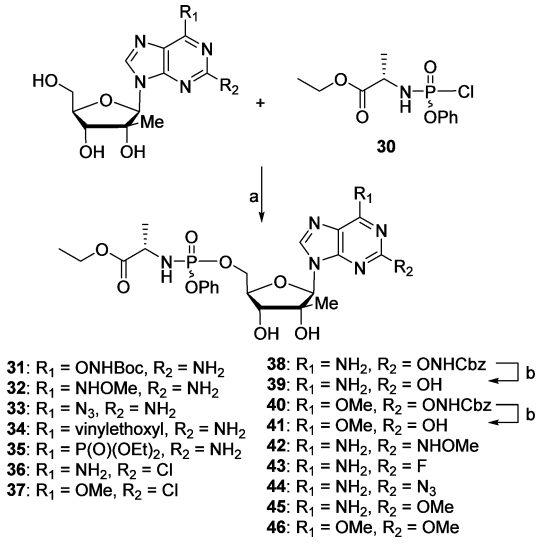
Scheme 3[a]



[a]Reagents and conditions: (a) 2,6-dichloropurine, DBU, TMSOTf, −40 to 80 °C, 4 h, 80%; (b) sat. NH_3/MeOH, rt, 3 days; for **17**, 46%; for **18**, 21%; (c) K_2CO_3, MeOH, rt, 24 h, 87%; (d) for **24**, HONHCbz, NaH, THF, 50 °C, 24 h, 81%; for **26**, MeONa, MeOH, 65 °C, 24 h, 92%; (e) HONHCbz, NaH, THF, 50 °C, 24 h, 78%; (f) Pd/C, H_2, MeOH, rt, 15 h; for 22, 83%; for 25, 97%; (g) NH_2NH_2, $MeOCH_2CH_2OH$, 110 °C, 5 h, 40%; (h) $NaNO_2$, HOAc, 1 h, 77%; (i) 2-fluoroadenine, DBU, TMSOTf, −40 to 65 °C, 5 h; (j) sat. NH_3/MeOH, rt, 2 d, 72% for two steps; (k) $MeONH_2$, Et_3N, EtOH/H_2O, 110 °C, 15 h, 66%.

Scheme 4[a]



[a]Reagents and conditions: (a) NMI, THF/CH_3CN, rt, 2−3 h; (b) Pd/C, H_2, MeOH, 15 h.

cells using a subgenomic HCV replicon system.[16] Cytotoxicity in Huh7 cells was determined simultaneously with anti-HCV activity by extraction and amplification of both HCV RNA and cellular rRNA (rRNA).[17] In addition cytotoxicity was determined in primary human peripheral blood mononuclear (PBM) cells, human lymphoblastoid CEM, and African Green monkey Vero cells (Table 1).[18,19] In an initial set of compounds, unusual 6-modifications such as introduction of a phosphonate ester or an ethoxy vinyl group were counterproductive and lead to inactive nucleosides and monophosphate prodrugs **14**, **15**, **34**, and **35**. Similarly, purine derivatives **25** (2,6-diMeO), **22** (2-OH, 6-NH_2), **23** (2-OH, 6-MeO), and **29** (2-NHOMe, 6-NH_2) and their corresponding phosphoramidate prodrugs **39**, **41**, **46**, and **42** showed to be inactive against HCV up to 10 μM. In contrast, 6-substituted purines derivatives **7** (−ONHBoc), **8** (−ONHCbz), and **11** (−NHOH) displayed EC_{50} values against HCV of 0.9, 2.4, and 0.3 μM, respectively, without apparent toxicity up to 10 μM in Huh7 cells and up to 100 μM in human PBM, CEM, and Vero cells. Preparation of **31**, the phosphoramidate prodrug of 6-NHBoc substituted compound **7**, even decreased the EC_{90} of **7** by a factor of 10 (8.4 compared to 0.9 μM for **7**). However, it has been well established that 6-modified nucleosides can be substrate of deaminases,[20] and therefore, we studied the fate of such compounds intracellularly. Thus, compound **7** and its prodrug **31** were incubated in Huh7 cells at 50 μM for 4 h at 37 °C. The cells were washed with phosphate-buffered saline and the intracellular metabolites were extracted with 70% ice-cold methanol in water and identified by LC−MS/MS. In this particular case, the only NTP metabolite observed *in vitro* was a 2′-β-*C*-methylguanosine-5′-triphosphate, a known inhibitor of HCV NS5B polymerase.[3]

Interestingly, compounds **10** (2-NH_2 6-NHOMe), **12** (2-NH_2, 6-N_3), **17** (2-Cl, 6-NH_2), **18** (2-Cl, 6-OMe), **24** (2-ONHCbz,

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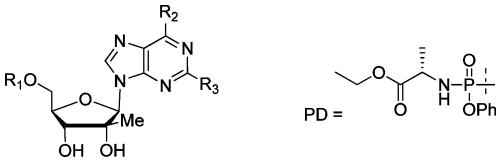
Table 1. *In Vitro* Anti-HCV Activity and Cytotoxicity of Nucleosides and Phosphoramidate Prodrugs[a]



Cmpd	R_1	R_2	R_3	Anti-HCV activity (μM)[a]		rRNA (μM)	Cytotoxicity, CC_{50} (μM)		
				EC_{50}	EC_{90}	CC_{50}	PBM	CEM	Vero
7	H	ONHBoc	NH_2	0.9	8.4	> 10	> 100	> 100	> 100
31	PD	ONHBoc	NH_2	0.3	0.9	> 10	> 100	39 ± 4.1	> 100
8	H	ONHCbz	NH_2	2.4	8.0	> 10	88 ± 4.4	> 100	> 100
10	H	NHOMe	NH_2	> 10	> 10	> 10	> 100	> 100	> 100
32	PD	NHOMe	NH_2	0.3	1.0	> 10	> 100	32 ± 17	> 100
11	H	NHOH	NH_2	1.9	5.5	> 10	> 100	> 100	> 100
12	H	N_3	NH_2	> 10	> 10	> 10	> 100	> 100	> 100
33	PD	N_3	NH_2	2.4	7.7	> 10	> 100	> 100	> 100
14	H		NH_2	> 10	> 10	> 10	> 100	> 100	> 100
34	PD		NH_2	> 10	> 10	> 10	> 100	> 100	> 100
15	H	P(O)(OEt)	NH_2	> 10	> 10	> 10	> 100	> 100	> 100
35	PD	P(O)(OEt)	NH_2	> 10	> 10	> 10	> 100	> 100	> 100
17	H	NH_2	Cl	> 10	> 10	> 10	> 100	> 100	> 100
36	PD	NH_2	Cl	1.7	5.3	> 10	> 100	> 100	> 100
18	H	OMe	Cl	> 10	> 10	> 10	> 100	> 100	> 100
37	PD	OMe	Cl	6.3	9.9	> 10	> 100	> 100	> 100
23	H	OMe	OMe	> 10	> 10	> 10	> 100	> 100	> 100
46	PD	OMe	OMe	> 10	> 10	> 10	> 100	> 100	> 100
24	H	NH_2	ONHCbz	> 10	> 10	> 10	> 100	> 100	> 100
38	PD	NH_2	ONHCbz	4.8	10	> 33	> 100	> 100	> 100
26	H	NH_2	OMe	> 10	> 10	> 10	> 100	> 100	> 100
45	PD	NH_2	OMe	1.0	2.8	> 10	> 100	> 100	> 100
25	H	NH_2	OH	> 10	> 10	> 10	> 100	> 100	> 100
39	PD	NH_2	OH	> 10	> 10	> 10	> 100	> 100	> 100
21	H	OMe	ONHCbz	> 10	> 10	> 10	> 100	> 100	> 100
40	PD	OMe	ONHCbz	4.1	9.2	> 33	> 100	24 ± 5.8	> 100
22	H	OMe	OH	> 10	> 10	> 10	> 100	> 100	> 100
41	PD	OMe	OH	> 10	> 10	> 10	> 100	> 100	> 100
20	H	NH_2	N_3	> 10	> 10	> 10	> 100	> 100	> 100
44	PD	NH_2	N_3	2.3	3.0	> 10	> 100	> 100	> 100
28	H	NH_2	F	> 10	> 10	> 10	> 100	> 100	> 100
43	PD	NH_2	F	2.7	8.3	> 10	> 100	> 100	> 100
29	H	NH_2	NHOMe	> 10	> 10	> 10	> 100	> 100	> 100
42	PD	NH_2	NHOMe	> 10	> 10	> 10	> 100	> 100	18 ± 3.0
IDX-184	NA	OH	NH_2	0.3	0.9	> 100	> 100	> 100	> 100
BMS-986094	NA	OMe	NH_2	0.02	0.04	0.8	4.5 ± 3.0	8.0 ± 6.3	14 ± 3.9

[a]The HCV replicon data is run in triplicate and the interwell variability must be less than $\pm 0.5\Delta C_t$. NA: not available.

6-NH_2), **26** (2-OMe, 6-NH_2), **21** (2-ONHCBz, 6-OMe), **20** (2-N_3, 6-NH_2), and **28** (2-F, 6-NH_2) did not show any activity against HCV in the replicon system when tested up to 10 μM, while their corresponding phosphoramidate prodrugs **32**, **33**, **36**, **37**, **38**, **45**, **40**, **44**, and **43** revealed their potency and exhibited median effective concentrations (EC_{50}) between 0.3 and 6.3 μM

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(Table 1). As with compounds **7** and **31**, a cellular pharmacology study of some of the most potent inhibitors **45** (2-OMe, 6-NH_2, 1 μM), **44** (2-N_3, 6-NH_2, 2.3 μM), and **43** (2-F, 6-NH_2, 2.7 μM) was undertaken and intracellular levels of nucleoside-MP, -DP, and -TP formed in Huh7 cells were quantified. All phosphoramidate derivatives **45**, **44**, and **43** produced high levels of their corresponding NTP along with considerable amounts of NMP and NDP derivatives. No other NTP was observed, which implies that **45**, **44**, and **43** NTPs are responsible for the anti-HCV activity observed in the replicon system and that 2-position modification of purines, unlike 6-modification, are quite stable *in vitro*.

As a first step toward understanding the difference of antiviral activity observed between inactive nucleoside **28** and its phosphoramidate prodrug **43** (EC_{50} = 2.7 μM), a comparative pharmacology study in Huh7 cells using the LC−MS/MS method described above was performed. While both compounds were found to deliver **28**-TP, prodrug **43** produces ∼20 times more 5′-triphosphate than **28**. This discrepancy directly correlates with the anti-HCV activity of these two compounds. Interestingly, we also noted that levels of metabolites produced by **23** were very low in Huh7 cells, even for the nucleoside itself. This seems to imply that, not only can the monophosphate prodrug **43** bypass the first phosphorylation step, but it also allows for a better intracellular penetration of the compound.

To further characterize compound **43**, we decided to study the *in vitro* incorporation of **28**-TP (active metabolite of phosphoramidate **43**) by HCV NS5B polymerase (see Figure 2 in the Supporting Information section). RNA synthesis by HCV NS5B polymerase was monitored in the presence of increasing concentrations of **28**-TP up to 100 μM. Incorporation of **28**-TP was marked by the appearance of pausing sites opposite uridine residues at positions +11, +13, and +15 of the 20mer RNA template, confirming that **28**-TP behaves as an A analogue. Finally, as expected, increased incorporation of **28**-TP correlated with inhibition of full-length 20mer RNA product formation (K_i = 32 ± 0.07 μM). Finally, since off-target effects can be an issue with nucleoside analogues, we assessed the selectivity of our compound by testing **28**-TP against host RNA polymerase II and human mitochondrial RNA polymerase (POLRMT). At concentrations up to 100 μM, **28**-TP did not inhibit host RNA polymerase II (while α-amanitin, used as positive control, had an IC_{50} of 2.5 ± 1.7 nM) and was not significantly incorporated by POLRMT (11% incorporation as normalized to ATP).[21]

Despite the fact that base modifications are often not accepted by polymerases and can lead to undesired toxicity, we identified several substitutions to the purine base that allow their NTP to be recognized by HCV polymerase. Thus, we discovered phosphoramidates **32**, **33**, **36**, **37**, **38**, **45**, **40**, **44**, and **43**, which displayed EC_{50} values in the low micromolar range against HCV without apparent toxicity in Huh7, PBM, CEM, and Vero cells up to 100 μM. Phosphoramidate **43** (2-F, 6-NH_2), one of the most potent compounds, was further characterized. Interestingly, we found that **43**, unlike its corresponding nucleoside **28**, produced high levels of **28**-TP in Huh7 cells. The **28**-TP was shown to be a substrate of HCV NS5B polymerase and was incorporated as an adenosine analogue. Further preclinical profiling of compound **43** and exploration of its prodrug portion is in progress. We thus envisage comparing this compound or a related prodrug to Sofosbuvir, INX-189, and IDX-184 in different cellular and animal assays and evaluate the potential therapeutic benefit of such 2-position modification in purine nucleosides and nucleotides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00402.

Biological assays and complete experimental section with full characterization of all new compounds (PDF)

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Notes

The authors declare the following competing financial interest: Dr. Schinazi is the Chairman and a major shareholder of CoCrystal Pharma, Inc. Emory received no funding from CoCrystal Pharma, Inc. to perform this work and vice versa.

■ ABBREVIATIONS

RNA, ribo nucleic acid; HCV, hepatitis C; FDA, Food and Drug Administration; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; TMSOTf, trimethylsilyl trifluoromethanesulfonate; Boc, *tert*-Butyloxycarbonyl; CBz, benzyloxy carbamate; TFA, trifluoroacetic acid; TMSI, trimethylsilyl iodide; HOAc, acetic acid; NMI, 1-methylimidazole; LC, liquid chromatography; MS, mass spectrometry; NTP, nucleoside triphosphate; NMP, nucleoside monophosphate; NDP, nucleoside diphosphate; TP, triphosphate

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