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2′-Deoxy-2′-α-fluoro-2′-β-C-methyl 3′,5′-cyclic phosphate nucleotide prodrug analogs as inhibitors of HCV NS5B polymerase: Discovery of PSI-352938

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ABSTRACT

A series of novel 2′-deoxy-2′-α-fluoro-2′-β-C-methyl 3′,5′-cyclic phosphate nucleotide prodrug analogs were synthesized and evaluated for their in vitro anti-HCV activity and safety. These prodrugs demonstrated a 10–100-fold greater potency than the parent nucleoside in a cell-based replicon assay due to higher cellular triphosphate levels. Our structure–activity relationship (SAR) studies provided compounds that gave high levels of active triphosphate in rat liver when administered orally to rats. These studies ultimately led to the selection of the clinical development candidate **24a** (PSI-352938).

An estimated 180 million people worldwide are infected with the hepatitis C virus (HCV).[1] The current standard of care (SOC) for HCV infection, the combination of pegylated interferon-α and ribavirin, provides less than 50% response rates among patients infected with the most prevalent genotype 1 virus.[2] There is an urgent medical need for more effective and well tolerated anti-HCV agents to treat HCV infections across all genotypes.[3] We have shown that nucleoside/tide analogs with 2′-α-F-2′-β-C-methyl substitution represent an important class of HCV NS5B polymerase inhibitors with broad genotype coverage.[4] Although several nucleoside classes that include NM-283[5] (**1**), RG7128 (**3**), and R1626[6] (**4**) have been in development as inhibitors of HCV, only RG7128 is still in development and is currently in Phase 2b clinical trials (Fig. 1). RG7128 is a 3′,5′-diisobutyrate ester prodrug of the nucleoside PSI-6130 (**2**).[4a–c,7] In a 4-week combination study with SOC, RG7128 demonstrated efficacy in genotype 1, 2, and 3 patients,[8] the first direct-acting antiviral to show pan-genotype coverage in the clinic.[9]

Since RG7128 has demonstrated positive clinical attributes, we were interested in identifying second generation agents with improved potency, better resistance profile, enhanced pharmacokinetic properties to support QD dosing and with the potential for generating high concentrations of the active triphosphate in the liver. In our search for second generation nucleosides with unique characteristics, we investigated purine derivatives of the 2′-α-F-2′-β-C-methyl class of nucleosides.[4d] The guanosine analog **5** (Fig. 2) was shown to be weakly active in the HCV replicon assay (EC_{90} = 69 μM), but its triphosphate **6** was found to be a potent inhibitor of the HCV NS5B polymerase (IC_{50} = 5.94 μM). These results indicated that weak potency of the parent nucleoside **5** could be a result of poor phosphorylation at some stage of the nucleoside phosphorylation cascade. Nucleoside analogs need to be phosphorylated to their corresponding active triphosphate by host cellular kinases before they can bind to the HCV NS5B polymerase

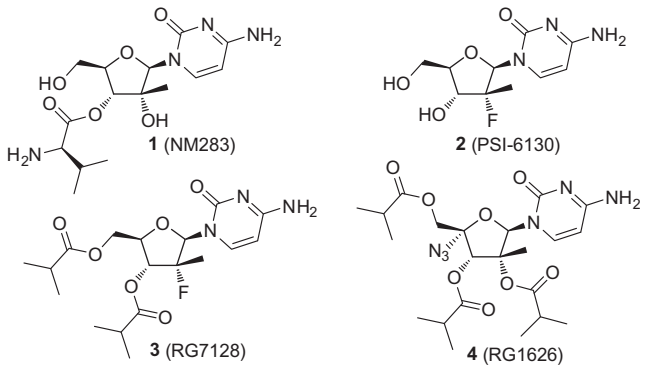

Figure 1. Nucleoside inhibitors of HCV.

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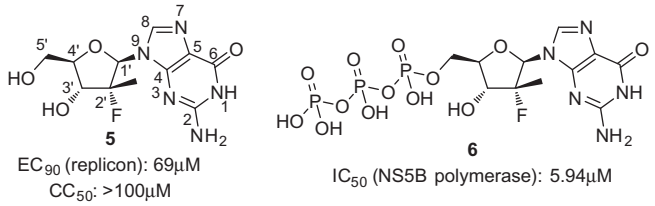
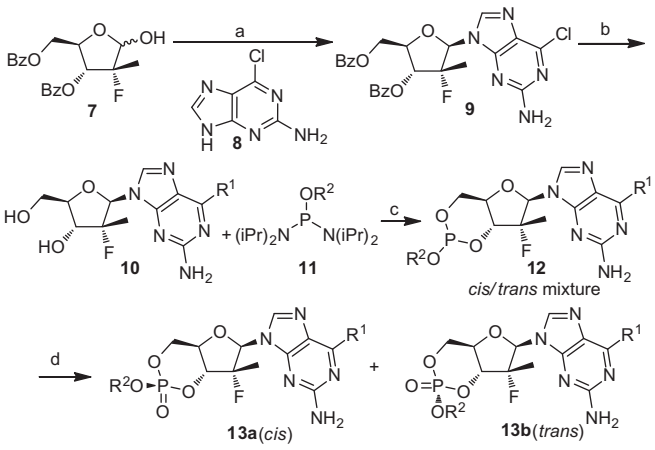
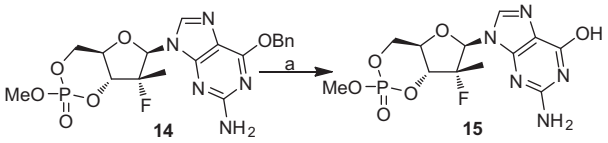


Figure 2. 2′-Deoxy-2′-α-F-2′-β-*C*-methyl guanosine triphosphate **6** as an inhibitor of HCV NS5B polymerase.



Scheme 1. Synthesis of cyclic phosphate prodrugs. Reagents and conditions: (a) DEAD, PPh_3, THF, rt, 16 h; (b) NaH, ROH (6-alkoxy derivatives) or NHR′R″ (6-alkylamino derivatives); (c) 0.5 M tetrazole in CH_3CN or 4,5-dicyanoimidazole, CH_3CN, rt, 3 h; (d) 77% mCPBA, DCM, rt, 30 min or 1 M tBuOOH in DCM, CH_3CN, rt, 20 h.



Scheme 2. Preparation of guanosine methyl cyclic phosphate **15**. Reagents and conditions: (a) 10% Pd/C, MeOH, H_2 (balloon), rt, 30 min, 98%.

and inhibit HCV RNA synthesis. Therefore, to produce the active triphosphate a nucleoside analog must be a substrate for each of the kinases in the phosphorylation cascade where the first phosphorylation step is the most discriminating.[10] Neither the active triphosphate nor the mono- or diphosphate species can be considered as possible drug candidates because of their instability and poor cellular permeability. Nucleoside cyclic phosphate prodrugs have been shown in vitro to be precursors of the nucleoside monophosphate with improved cellular permeability.[11] We therefore decided to investigate cyclic phosphate prodrugs of the 2′-deoxy-2′-α-F-2′-β-*C*-methyl nucleoside derivatives. When 3′,5′-cyclic phosphate derivatives of 2′-deoxy-2′-α-F-2′-β-*C*-methyl uridine and cytidine nucleosides were prepared, the resulting prodrugs did provide compounds that were 10–100-fold more potent than the parent nucleoside, but they did not offer any potency advantage over the corresponding phosphoramidate prodrugs already in clinical development.[12] However, unlike the uridine and cytidine analogs, 2′-deoxy-2′-α-F-2′-β-*C*-methyl purine cyclic phosphate analogs provided both the desired potency and resistance profiles in our initial cell-based replicon assay. Here, we report the synthesis, SAR, and preclinical in vitro and in vivo rat PK data supporting the nomination of a 2′-deoxy-2′-α-F-2′-β-C-methyl purine nucleoside cyclic phosphate prodrug to preclinical development status.

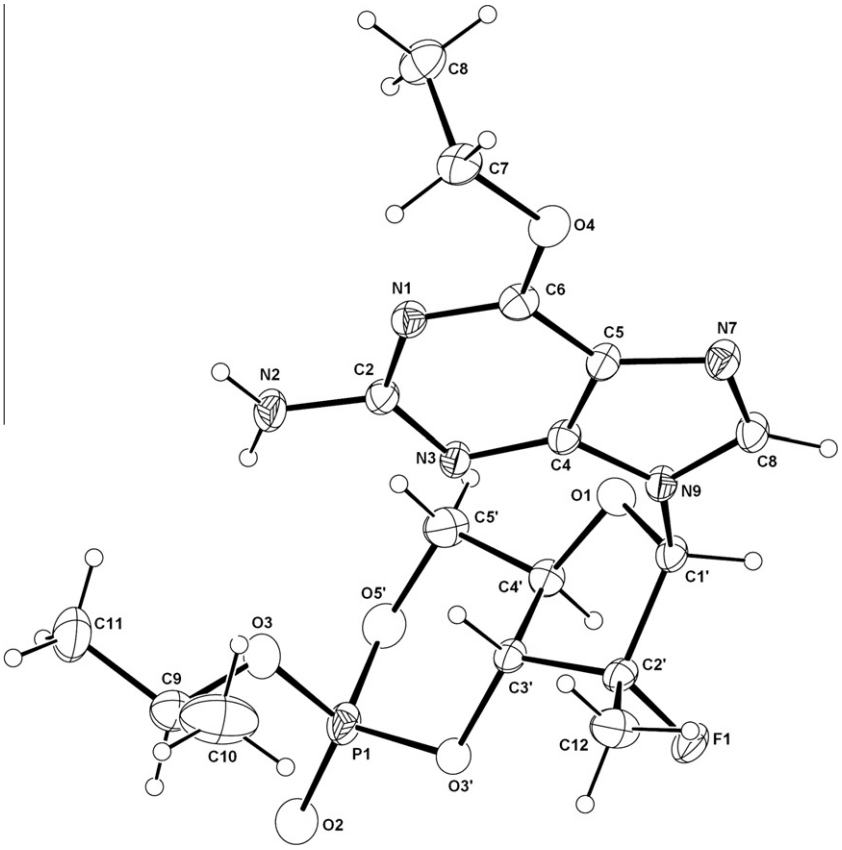


Figure 3. ORTEP drawing of **24a** showing Rp (*cis*) configuration at phosphorous.

Our initial SAR was focused on modifications around the cyclic phosphate ester and 6-position of 2′-deoxy-2′-α-F-2′-β-C-methyl guanosine analog. The desired cyclic phosphate prodrugs of this class were readily prepared by the method shown in Scheme 1.[12] Although several methods for preparing cyclic phosphates have been reported in the literature,[11,13,14] in our case a two step protocol via cyclic phosphite intermediate followed by oxidation gave improved results. The nucleoside starting material **10** was prepared by Mitsunobu coupling of lactol **7**[7] with 6-chloropurine **8** followed by nucleophilic displacement of chlorine with required alkoxide or amine. Cyclization of the nucleoside with tetraisopropyl phoshorodiamidite **11** using either tetrazole or 4,5-dicyanoimidazole (DCI)[15] as activators gave cyclic phosphite intermediate **12** as a mixture of *cis*/*trans* isomers. The *trans* isomer of the cyclic phosphite **12** can be converted to the thermodynamically more stable *cis* isomer by heating the reaction mixture at 50 °C for several hours.[13] However, since there was an interest in obtaining both isomers the reaction was performed at room temperature. Subsequent oxidation of the cyclic phosphite mixture with mCPBA or tBuOOH gave corresponding cyclic phosphates (**13a** and **13b**) in moderate yield as a mixture of diastereomers. Wherever possible, the two individual diastereomers were isolated by column chromatography and evaluated for their anti-HCV activity. Improved yields were observed when DCI was used as an activator in the first step. The tetraisopropyl phosphorodiamidite reagent **11** was prepared following a literature procedure.[16] Treatment of the bis(diisopropylamino)chlorophosphine with the corresponding alcohol in the presence of Et_3N gave the required tetraisopropyl phoshorodiamidite **11**. The guanosine methyl cyclic phosphate **15** was prepared by the debenzylation of the corresponding 6-benzyloxy methyl cyclic phosphate **14** as shown in Scheme 2.

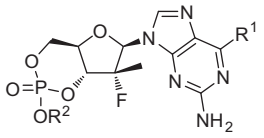
The structure and stereochemistry of one of the cyclic phosphates (**24a**) was determined by single crystal X-ray analysis. An ORTEP drawing of compound **24a** is shown in Figure 3 indicating the Rp (*cis*) configuration at phosphorous center. In the ^{31}P NMR spectra, the ^{31}P resonance for the *cis* isomer **24a** appears upfield from the *trans* isomer **24b**. This observation was applied by analogy to the other diastereomeric compounds to assign the stereochemistry.

The anti-HCV activities of these prodrugs were assessed using the clone A replicon and a quantitative real time PCR assay.[17] Cytotoxicity (CC_{50}) was simultaneously evaluated by assessing cellular RNA replication. Representative examples assessed to identify a subset of lead compounds are listed in Table 1. Our initial SAR had focused on the 6-position of the base keeping the methyl cyclic phosphate constant (Table 1, **15**–**22**). Unsubstituted guanosine methyl cyclic phosphate **15** did not provide the desired submicromolar potency presumably due to its poor cellular penetration. In order to address this, we chose to increase the lipophilicity by using simple 6-alkoxy or alkyamine substituents (double prodrug). As desired, simple alkoxy groups (**16** and **17**) provided submicromolar potency with no substantial cytotoxicity. Similarly, small alkylamine and azetidine substituents (**19** and **22**) were well tolerated. However, benzyloxy and cycloalkyl amines (**18**, **20**, and **21**) did not give the desired potency and were not used in subsequent SAR studies.

The initial SAR investigation around the 6-position of the base while maintaining the methyl cyclic phosphate moiety constant demonstrated that small alkoxy, alkyamine and azeditidine were preferred substituents. Subsequently, SAR at the phosphate ester was performed using these three substituents at the 6-position of the base. Linear alkyl, cycloalkyl and phenyl groups with all three preferred substituents at the 6-postion gave derivatives that were weak inhibitors of HCV. Both *cis* and *trans* isomers of 6-ethoxy isopropyl ester derivatives (**24a** and **24b**) afforded the desired potency with no significant cytotoxicity. The 6-azetidinyl and *n*-propylamino substituents of the isopropyl cyclic phosphate (**28** and **31**) offered no advantage over the 6-ethoxy derivates **24a** and **24b**.

In addition to the activity against HCV, the key compounds were further evaluated for stability and subjected to extensive cytotoxicity evaluation. In order to assess the ability of the cyclic phosphate prodrugs to survive exposure in the gastrointestinal tract and preferentially release the nucleotide monophosphate in the liver, compound stability was determined in simulated gastrointestinal fluid (SGF), simulated intestinal fluid (SIF), human plasma and human liver S9 fraction, a surrogate in vitro model for liver stability. Tables 2 and 3 list stability and expanded cytotoxicity data, respectively, for the key compounds (**16**, **19**, **22**, **24a**, and **24b**) that had passed the initial activity and cytotoxicity filter. Of these, compound **22** had poor stability in the liver S9 fraction. The stability half life ($t_{1/2}$) for this compound in human S9 was 2.53 h while the $t_{1/2}$ values for the other compounds were >24 h. In addition, compound **22** was shown to exhibit a cytostatic effect. Compound **19** had poor stability in the SIF ($t_{1/2}$ = 4 h) and showed cytotoxicity in the Huh 7 cell line. In addition, this compound was associated with mitochondrial toxicity (CC_{50} = 14 μM). Hence neither compound was considered for further development. On the other hand, compound **16** demonstrated acceptable stability profile and no significant cytotoxicity in all four cell lines. Compounds **24a** and **24b** were shown to be devoid of any cytotoxicity in the four cell lines tested and possessed prolonged stability in SGF, SIF, human plasma

Table 1
SAR data of cyclic phosphate prodrugs of 2′-deoxy-2′-α-fluoro-2′-β-C-methyl purine analogs



Compd	Isomer/ratio[a] (*cis*/*trans*)	R^1	R^2	HCV EC_{90} (μM)	Cytotoxicity[b] CC_{50} (μM)
3	—	—	—	3.9	>100
15	*cis*	–OH	Me	11.75	>65
16	1:1.5	–OEt	Me	0.41	>30
17	1:1.3	–OPr^n	Me	0.83	>30
18	1:1.6	–OBn	Me	5.05	—
19	1:1.3	–NHPr^n	Me	0.92	>30
20	1:1.4	–NH(*c*-butyl)	Me	1.56	—
21	1:1.3	–NH(*c*-pentyl)	Me	7.20	—
22	1:1.1	—N◇	Me	0.98	>30
23	*cis*	–OEt	*n*Pr	13.27	—
24a	*cis*	–OEt	*i*Pr	1.37	>100
24b	*trans*	–OEt	*i*Pr	0.69	>100
25	2:1	–OEt	Ph	1.72	—
26a	*cis*	–OPr^n	*n*Pr	23.83	—
26b	*trans*	–OPr^n	*n*Pr	5.27	>11
27	*cis*	–NHPr^n	Et	48.14	—
28	*cis*	–NHPr^n	*i*Pr	9.28	—
29	*cis*	–NHPr^n	Ph	4.76	—
30	*cis*	—N◇	Et	69.19	—
31	*cis*	—N◇	*i*Pr	18.6	—
32	*cis*	—N◇	*c*-Butyl	7.30	—
33	*cis*	—N◇	Ph	7.70	—

[a] Based on ^{31}P NMR chemical shifts.
[b] CC_{50} was determined for the compounds with desired potency.

Table 2
Stability data for selected purine cyclic phosphate analogs

Compd	Stability $t_{1/2}$ (h)			
	SIF	SGF	Plasma	S9
16	>20	>20	8.88	>24
19	4	>20	15.70	>24
22	>20	>20	8.33	2.53
24a	>20	>20	>24	>24
24b	20	18	>24	>24

Table 4
In vitro/in vivo triphosphate (**6**) levels of the key compounds in a rat PK study

Compd	Cellular triphosphate (**6**) levels (mM)		Rat liver triphosphate (**6**) levels	
	Clone A	Human hepatocytes	C_{max} (ng/g)	AUC (0–24 h, ng h/g)
16	0.08 (24)	0.12 (24)	613	4092
24a	0.41 (72)	0.59 (72)	4860	41893

and liver S9 fraction. Intriguingly, all of these purine analogs were equipotent against the NS5B S282T resistant replicon. It was shown previously that the S282T replicon cells were less susceptible to certain modified 2′-*C*-methyl nucleoside/tides.[18]

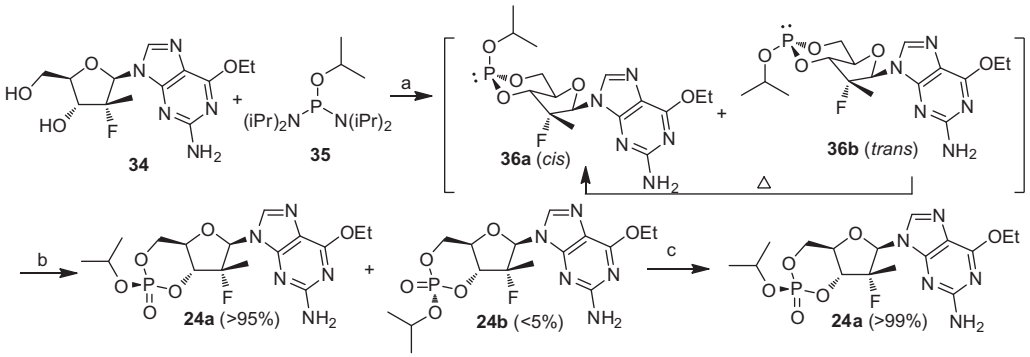
Based on the replicon potency, stability and cytotoxicity profile, compounds **16**, **24a**, and **24b** were selected for further evaluation. Of the two diastereomers, **24a** and **24b**, the *cis* isomer **24a** was shown to be the thermodynamically more stable isomer and could be made efficiently by equilibration of the initial phosphite intermediate mixture (**36a** and **36b**) to **36a** followed by oxidation and crystallization (Scheme 3). Consequently, compound **24a** was selectively prepared by first cyclization of the nucleoside **34** with isopropyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite **35** using DCI as an activator to give a mixture of **36a** and **36b** in about 85:15 ratio respectively. After heating the reaction mixture at 50 °C for 6 h, ^{31}P NMR indicated >95% of **36a**. Further oxidation of the mixture with tBuOOH followed by crystallization from ethyl acetate or acetone afforded **24a** in high purity (>99%).

Cyclic phosphates **16** and **24a** were further evaluated for their in vitro and in vivo liver levels of the active triphosphate **6** (Table 4). Since triphosphate levels are correlated to in vivo potency of nucleoside/tide analogs, in vitro triphosphate production in clone A replicon cells and primary human hepatocytes was assessed. In vitro analysis of triphosphate (**6**) production was accomplished by incubating clone A replicon cells and primary human hepatocytes with 100 μM of each compound at 37 °C for 72 h and then extracting triphosphate metabolites from the cells and analyzing by HPLC. Surprisingly, for compound **24a** triphosphate levels in clone A cells and primary human hepatocytes were shown to be much higher than that generated by **16**, the more potent compound in the replicon assay. Similar results were seen in vivo in the rat PK study when each compound was administered as a single 50 mg/kg oral dose and liver levels of the nucleoside triphosphate **6** were determined. The relative levels of triphosphate **6** found in the liver samples were considered an indication of anticipated in vivo potency and were the basis for selecting which compound to progress further. Table 4 shows the PK parameters for these two compounds. Compound **24a** produced the highest triphosphate C_{max} and AUC_{0-24} levels post dose indicating high drug exposure in the liver.[19] In addition, significant exposure to the parent prodrug **24a** was observed in rat liver (C_{max} = 2829 ng/g, AUC_{0-24} = 13234 ng h/g) compared to the compound **16** (C_{max} = 3052 ng/g, AUC_{0-24} = 8310 ng h/g). This suggests that compound **24a** survived through GI exposure, remained intact during the absorption phase and delivered the active triphosphate into the liver. Based on the relative liver exposure levels of both the prodrug and active triphosphate of the two compounds **16** and **24a**, it appears that the prodrug stability in plasma may correlate to liver triphosphate levels.

Based on its superior potency against both wild type and resistant mutants (S282T), ability to produce higher intracellular triphosphate levels both in vitro and in vivo and due to the synthetic accessibility of a single diastereomer, **24a** was selected for further development. Compound **24a** (PSI-352938) is currently in Phase I clinical trials for the treatment of HCV infection.

Table 3
Cytotoxicity data for selected purine cyclic phosphate analogs

Compd	HCV inhibition (clone A): EC_{90} (μM)		Cytotoxicity: CC_{50} (μM)				Mitochondrial toxicity: CC_{50} (μM)	
	WT	S282T	Huh 7	HepG2	BxBC3	CEM	CEM	HepG2
16	0.41	0.4	96	95	>100	>100	>100	>100
19	0.92	3.08	72	>100	>100	>100	>100	14
22	0.98	1.53	>100	>100	>100	>100	>100	>100
24a	1.37	1.61	>100	>100	>100	>100	>100	>100
24b	0.69	0.86	>100	>100	>100	>100	>100	>100



Scheme 3. Synthesis of compound **24a**. Reagents and conditions: (a) DCI, CH_3CN, rt to 50 °C, 6 h; (b) tBuOOH, CH_3CN, rt, 20 h; (c) Crystallization from EtOAc or acetone, 35% (three steps).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.035.

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19. Based on our preliminary metabolism studies on compound **24a**, the sequence of cyclic phosphate prodrug activation is as follows: Hydrolysis of isopropyl ester followed by hydrolysis of 3′-end of the cyclic phosphate to give 5′-monophospahte and then hydrolysis of 6-ethoxy group to give 5′-monophosphate of guanosine analog. Details of the metabolism study will be published in a subsequent publication.