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Discovery of a β-D-2′-Deoxy-2′-α-fluoro-2′-β-C-methyluridine Nucleotide Prodrug (PSI-7977) for the Treatment of Hepatitis C Virus

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Hepatitis C virus (HCV) is a global health problem requiring novel approaches for effective treatment of this disease. The HCV NS5B polymerase has been demonstrated to be a viable target for the development of HCV therapies. β-D-2′-Deoxy-2′-α-fluoro-2′-β-C-methyl nucleosides are selective inhibitors of the HCV NS5B polymerase and have demonstrated potent activity in the clinic. Phosphoramidate prodrugs of the 5′-phosphate derivative of the β-D-2′-deoxy-2′-α-fluoro-2′-β-C-methyluridine nucleoside were prepared and showed significant potency in the HCV subgenomic replicon assay (<1 μM) and produced high levels of triphosphate **6** in primary hepatocytes and in the livers of rats, dogs, and monkeys when administered in vivo. The single diastereomer **51** of diastereomeric mixture **14** was crystallized, and an X-ray structure was determined establishing the phosphoramidate stereochemistry as *S*p, thus correlating for the first time the stereochemistry of a phosphoramidate prodrug with biological activity. **51** (PSI-7977) was selected as a clinical development candidate.

Introduction

The hepatitis C virus (HCV[a]) presents a global health problem with approximately 180 million individuals infected worldwide with 80% of those progressing to chronic HCV infection.[1] Of those chronically infected individuals, approximately 30% will develop liver cirrhosis and 10% will go on to develop hepatocellular carcinoma.[2] The current standard of care (SOC) for HCV infected patients consists of regular injections of pegylated interferon (IFN) and oral ribavirin (RBV) administration. However, SOC has proven to be effective in producing a sustained virological response in only 40−60% of patients treated, dependent on viral genotype and other predictors of host immune responsiveness. In addition, drug discontinuations may be high because of adverse side effects associated with the SOC treatment regimen.[3,4] Consequently, the development of alternative treatment options is greatly needed. The search for novel therapies for the treatment of HCV infection has focused on the development of direct acting antiviral agents (DAAs).[5,6]

HCV is a plus strand RNA virus of the *Flaviviridea* family with a 9.6 kb genome encoding for 10 proteins: three structural proteins and seven nonstructural proteins. The nonstructural proteins, which include the NS5B RNA dependent RNA polymerase (RdRp), provide several attractive targets for the development of anti-HCV therapy.[7,8] The HCV RdRp is part of a membrane associated replication complex that is composed of other viral proteins, viral RNA, and altered cellular membranes.[3] The NS5B polymerase is responsible for replicating the viral RNA genome and thus is absolutely required for HCV replication. As in the case of other viral polymerases, two approaches have been pursued to identify small molecule HCV NS5B polymerase inhibitors. These approaches include the identification of nucleoside analogues that function as alternative substrate inhibitors that induce a chain termination event and non-nucleoside inhibitors that bind to allosteric sites on the polymerase leading to a nonfunctional enzyme.[5,6]

Several nucleoside classes have been or continue to be in development as inhibitors of HCV. These classes include the β-D-2′-deoxy-2′-α-F-2′-β-C-methylribose, the β-D-2′-β-methylribose, and the 4′-azidoribose classes.[9−13] These classes are represented by the clinical candidates **1** (RG7128), **2** (NM-283), and **3** (R1626), respectively (Figure 1).

Compound **1** is a 3′,5′-diisobutyrate ester prodrug of the cytidine nucleoside **4**. In clinical studies when administered at 1000 mg b.i.d., **1** demonstrated efficacy in genotype 1 infected patients (reduction of HCV RNA levels) in a 14-day monotherapy study (−2.7 $\log_{10}$ decrease in HCV RNA) and produced a 88% RVR in a 4-week combination study with SOC−pegylated interferon plus ribavirin.[14,15] In addition, **1** was shown to be efficacious in HCV genotype 2,3 patients who had not responded to prior therapy, the first direct-acting

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[a] Abbreviations: HCV, hepatitis C virus; SOC, standard of care; IFN, interferon; RBV, ribavirin; DAA, direct acting antiviral; RdRp, RNA dependent RNA polymerase; b.i.d., twice daily; q.d., once daily; YMPK, uridine−cytidine monophosphate kinase; NDPK, nucleoside diphosphate kinase; SAR, structure−activity relationship; NMI, *N*-methylimidazole; DCM, dichloromethane; EC_{90}, compound concentration that returns 90% of inhibition; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; NTP, nucleoside triphosphate; PK, pharmacokinetic; C_{max}, maximum concentration; AUC, area under the curve; t_{max}, time at maximum concentration; PAMPA, parallel artificial membrane permeability assay; IC_{50}, compound concentration that returns 50% of inhibition; NOAEL, no observed adverse effect level; TFA, trifluoroacetic acid, THF, tetrahydrofuran; IPE, isopropyl ether; DMSO, dimethylsulfoxide.

antiviral to show multiple genotype coverage in the clinic.[16] However, even with the positive clinical attributes of **1**, we were interested in investigating second generation agents with improved potency, enhanced pharmacokinetic properties (i.e., q.d. dosing), and the potential for generating high concentrations of the active triphosphate in the liver to enable low doses and potentially fixed-dose combinations of DAAs. To achieve this objective, we focused on β-D-2′-deoxy-2′-α-F-2′-β-*C*-methyluridine (**5**) (Figure 2). Earlier studies had shown that while **5** was inactive in the HCV replicon assay, its triphosphate was a potent inhibitor of HCV NS5B with a K_i of 0.42 μM.[17−19] In addition, metabolism studies with **4** showed that the monophosphate of **4** can be deaminated to the uridine monophosphate derivative and subsequently anabolized to the triphosphate **6** by uridine−cytidine monophosphate kinase (YMPK) and nucleoside diphosphate kinase (NDPK) (Figure 2). This uridine triphosphate was shown to have an intracellular half-life of 38 h.[17−19] Therefore, in order to leverage the desired attributes of the uridine derivative, we needed to deliver the monophosphate of uridine nucleoside **5**. To accomplish this, we required a monophosphate prodrug that would bypass the nonproductive phosphorylation step and that would potentially accomplish our other objective: the delivery of high liver concentrations of the desired triphosphate **6**.

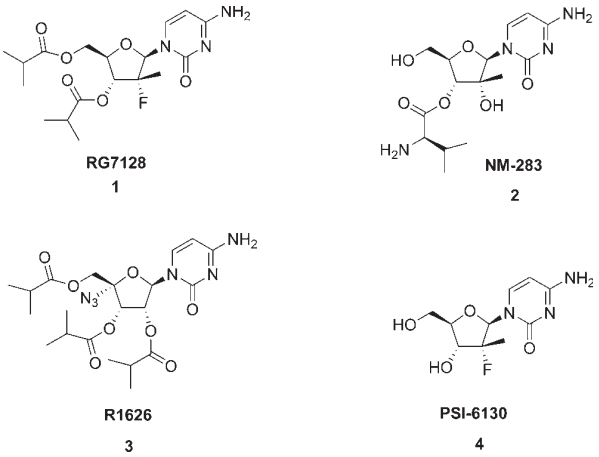


Figure 1. Structures of HCV nucleoside inhibitors **1**, **2**, **3**, and **4**.

Phosphoramidate prodrug strategies had been shown to enhance nucleoside potency in cell culture presumably by increasing intracellular concentrations of the active nucleotide.[20−22] However, at the time we began our work there was no example where phosphoramidate prodrug technology was applied to the inhibition of HCV. We speculated that application of the phosphoramidate prodrug method would be an ideal approach for delivering the desired uridine monophosphate to hepatocytes in an in vivo setting (Figure 3). We hoped to take advantage of first pass metabolism where the liver enzymes would hydrolyze the terminal carboxylic acid

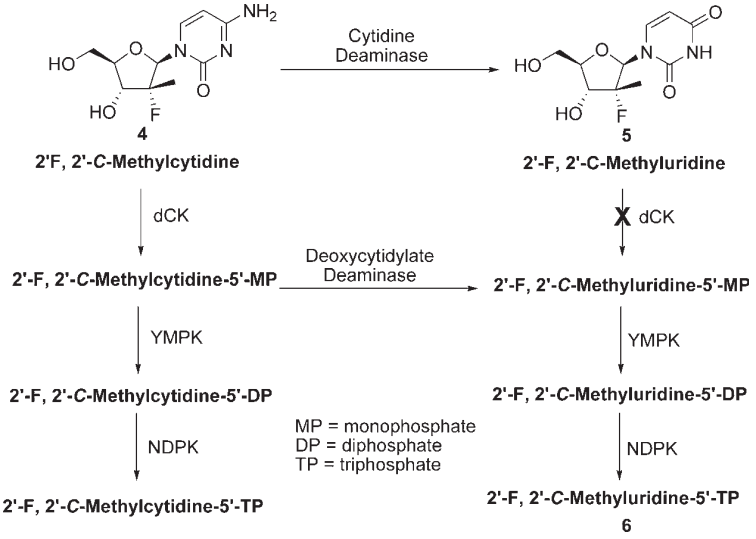


Figure 2. Metabolism of **4** leads to both the active triphosphate and the inactive nucleoside **5**. The monophosphate metabolite of **4** is also metabolized to the uridine monophosphate derivative which is then further phosphorylated to the active uridine triphosphate **6**.

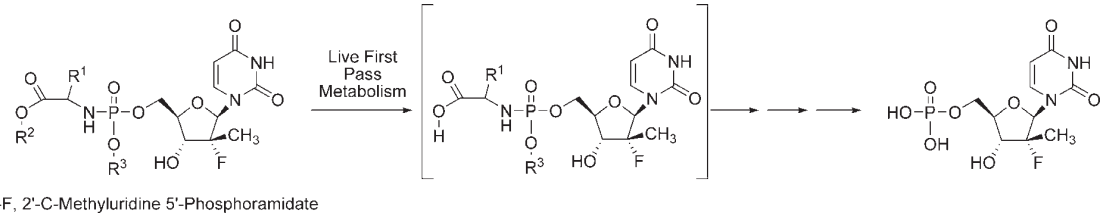
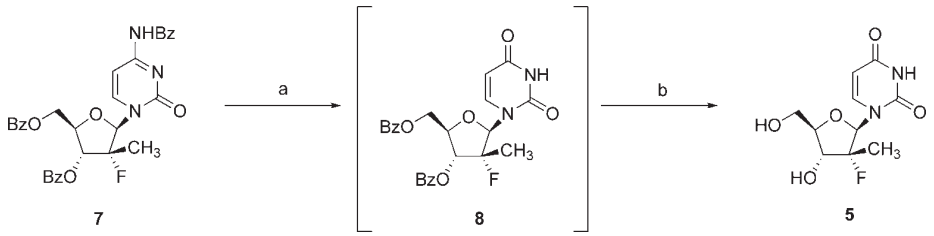


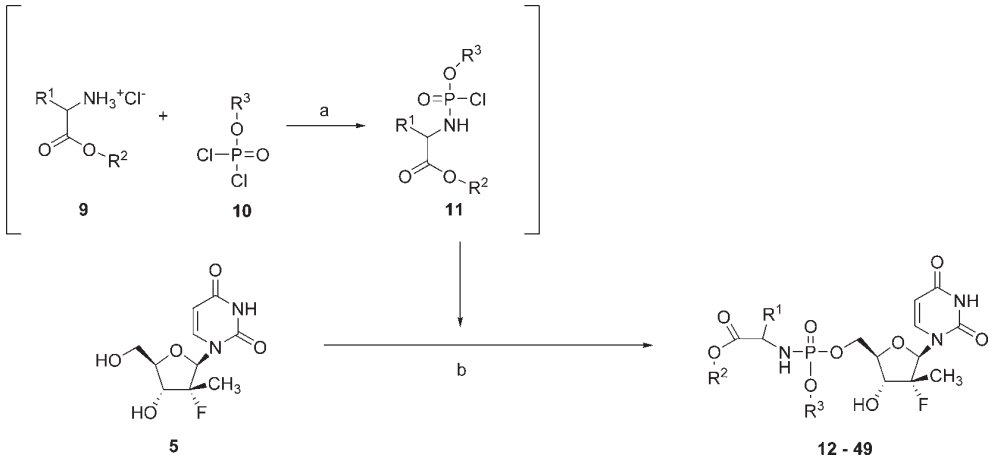
Figure 3. First pass metabolism of the phosphoramidate prodrug derivative of the monophosphate of **5** releases the monophosphate in the liver at the desired site of action.

Scheme 1[a]



[a] (a) 70% aqueous acetic acid, 100 °C; (b) 25% methanolic ammonia, 0−15 °C.

Scheme 2[a]



[a] NMI, DCM, −5 to 5 °C; (b) NMI, DCM, 5−25 °C.

ester of the phosphoramidate moiety triggering a cascade of chemical and enzymatic events that would produce the desired uridine monophosphate at the desired site of action, the liver. Subsequently, several reports demonstrated that phosphoramidates of several other anti-HCV nucleosides were able to improve potency, but these reports did not translate this improved in vitro potency into a clinical development candidate.[23−25] Here we describe the discovery of phosphoramidate prodrugs of 2′-deoxy-2′-α-F-2′-*β*-*C*-methyluridine 5′-monophosphate and the selection of **14** and ultimately of its single isomer **51** as clinical development candidates.

Results and Discussion

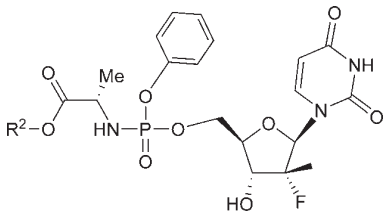
The development of phosphoramidate prodrugs of **5** began with the investigation of the anti-HCV SAR around the phosphoramidate portion of the molecule. The synthesis of the 2′-deoxy-2′-α-F-2′-*β*-*C*-methyluridine phosphoramidates began with the preparation of the uridine nucleoside **5**. To obtain the uridine nucleoside **5**, we started with the benzoyl protected 2′-deoxy-2′-α-F-2′-*β*-*C*-methylcytidine (**7**). We recently reported on the efficient synthesis of **7**.[26] From **7**, preparation of the uridine nucleoside was efficiently accomplished by a two-step process (Scheme 1). The benzoyl cytidine **7** was heated with 80% acetic acid overnight to afford the protected uridine **8**, which was then treated at room temperature with methanolic ammonia to provide **5** in 78% yield.

The phosphoramidate derivatives of the uridine nucleoside were prepared as shown in Scheme 2. The phosphoramidate moiety was appended by reacting **5** with a freshly prepared chlorophosphoramidate reagent **11** in the presence of NMI.[20] Each chlorophosphoramidate reagent was prepared by stirring an amino acid ester **9** with the appropriate phosphorodichloridate reagent **10** in the presence of an amine base (Et_3N or NMI) in either THF or dichloromethane. The reaction provided the desired 5′-phosphoramidates **12**−**49** as the major product with lesser amounts of the 3′- and 3′,5′-phosphoramidate derivatives. The desired 5′-phosphoramidates were purified by chromatography as a 1:1 mixture of diastereomers at the phosphorus center.

The anti-HCV activity of these prodrugs was assessed using the clone A replicon and a quantitative real time PCR assay.[12] Each compound was simultaneously evaluated for cytotoxicity by assessing for the levels of cellular rRNA.[12] The objective was to identify phosphoramidate prodrugs that exhibited submicromolar activity with the hope that this increased activity would translate into reduced drug load in the clinic relative to **1**. A survey of the terminal carboxylic acid ester of the phosphoramidate moiety in which the amino acid was alanine and the phosphate ester was simply phenyl showed that small simple alkyl and branched alkyl groups provided the desired submicromolar activity; however, in the case of the *n*-butyl (**15**), 2-butyl (**16**), and *n*-pentyl (**17**) esters, cytotoxicity was observed (Table 1). Small cycloalkyl (**18**) and benzyl (**22**, **23**) esters were also compatible; however, phenyl (**21**) and halogenated alkyl groups (**19**, **20**) did not provide the desired potency enhancement.

A survey of the phosphoramidate phosphate ester substituent (Table 2) demonstrated that the 1-naphthyl ester **29**

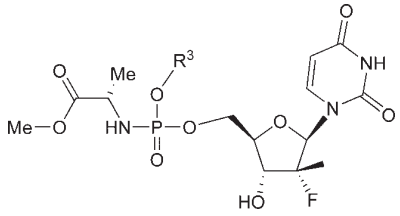
Table 1. HCV Replicon Activity of Phosphoramidate Prodrugs **12**−**23**: Modification of the Phosphoramidate Ester Moiety



compd	R^2	EC_{90} cloneA (μM)[a]	inhibition of cellular rRNA replication at 50 μM (%)[b]
1		3.9	0
12	Me	0.91	0
13	Et	0.98	36.9
14	*i*-Pr	0.52	25.9
15	*n*-Bu	0.09	79.6
16	2-Bu	0.06	93.8
17	*n*-Pen	>50	92
18	*c*-Hex	0.25	61
19	FCH_2CH_2	1.72	43.8
20	F_2CHCH_2	6.80	38.3
21	Ph	18.50	0
22	Bn	0.13	74.3
23	4-F-Bn	0.24	0

[a] Each value is a result of $n = 2$ determinations. [b] Clone A cells.

Table 2. HCV Replicon Activity of Phosphoramidate Prodrugs **12** and **24**−**30**: Modification of the Phosphoramidate Phenolic Ester Substituent



compd	R^3	EC_{90} cloneA (μM)[a]	inhibition of cellular rRNA replication at 50 μM (%)[b]
12	Ph	0.91	0.0
24	4-F-Ph	0.69	16.8
25	4-Cl-Ph	0.58	62.8
26	4-Br-Ph	2.11	30.8
27	3,4-Cl-Ph	0.45	63.7
28	2,4-Cl-Ph	0.69	10.9
29	1-Napth	0.09	95.4
30	Et	>50	16.8

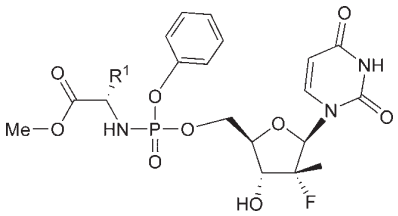
[a] Each value is a result of $n = 2$ determinations. [b] Clone A cells.

provided the greatest potency and that mono- and dihalogenated phenolic esters also gave inhibitors with submicromolar potency. The derivative with a simple alkyl phosphate ester (**30**) was not active against HCV. Although the 1-naphthol ester substitution produced the most potent HCV inhibitor, this substitution also led to substantial cytotoxicity and was therefore not considered a viable substituent.

Study of the amino acid side chain demonstrated that a small alkyl group (**12**, **32**) was accommodated, but α-substitution larger than ethyl showed substantial reduction in potency (Table 3). α-Disubstituted amino acids that included an α-cyclopropanylamino acid derivative **39** did not provide the target submicromolar potency (Table 4). Additionally, it was shown that the natural L-amino acid was required for activity, since the D-alanine derivative **40** was inactive (Table 4).

Results of the phosphoramidate moiety single substituent modifications showed that L-alanine was the preferred amino acid moiety, that methyl, ethyl, isopropyl, or cyclohexyl carboxylate esters provided the desired potency enhancement, and that the phosphate ester accommodated simple phenyl or halogenated phenyl substituents. Subsequently, select combinations of these preferred substitutions were prepared in which only the phenyl or para-halogenated phenyl phosphate ester analogues were examined. Polyhalogenated phosphate esters were excluded from further evaluation in order to preempt any potential toxicity issues that may arise from the release of polyhalogenated phenols upon conversion of the phosphoramidate to the desired nucleoside monophosphate (Table 5).[27−29] The most dramatic difference observed in the SAR for the phosphoramidate substituent combinations was associated with the terminal carboxylic acid ester substituent where the cyclohexyl ester derivatives (**18**, **47**−**49**) showed as much as a 10-fold improvement in potency relative to their methyl, ethyl, or isopropyl analogues. On the basis of replicon potency, initial cytotoxicity profile, and structural diversity,

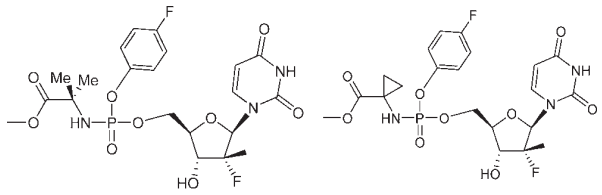
Table 3. HCV Replicon Activity of Phosphoramidate Prodrugs **12** and **31**−**37**: Modification of the Amino Acid Side Chain



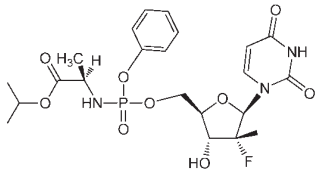
compd	R^1	EC_{90} clone A (μM)[a]	inhibition of cellular rRNA replication at 50 μM (%)[b]
31	H	22.11	0.0
12	Me	0.91	0.0
32	Et	1.61	0.0
33	Me_2CH	>50	0.0
34	Me_2CHCH_2	5.4	0.0
35	$MeSCH_2CH_2$	60.13	24.1
36	$PhCH_2$	57.65	20.6
37	indole-3-CH_2	15.6	68.4

[a] Each value is a result of $n = 2$ determinations. [b] Clone A cells.

Table 4. HCV Replicon Activity of Phosphoramidate Prodrugs **24** and **38**−**40**: α-Disubstituted Amino Acid Side Chains



38 **39**



40

compd	EC_{90} clone A (μM)[a]	inhibition of cellular rRNA replication at 50 μM (%)[b]
24	0.69	16.8
38	2.20	0.0
39	>50	0.0
40	>50	0.09

[a] Each value is a result of $n = 2$ determinations. [b] Clone A cells.

a select set of seven compounds (**12**, **14**, **18**, **41**, **44**, **45**, and **47**) was chosen for further evaluation.

To achieve the objective of identifying a phosphoramidate prodrug of the 2′-α-F-2′-*β*-*C*-methyluridine monophosphate suitable for clinical studies as a treatment for HCV, the prodrug moiety would need to survive exposure in the gastrointestinal tract and preferentially release the nucleotide monophosphate in the liver. Consequently, compounds **12**, **14**, **18**, **41**, **44**, **45**, and **47** were further evaluated for gastrointestinal stability using simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). In addition, stability in human plasma and stability on exposure to human liver S9 fraction were also evaluated (Table 6). The human liver S9 fraction was chosen as a surrogate in vitro model to test for liver stability. The ultimate objective was to select compounds that showed improved potency relative to **4** in the HCV replicon and stability in SGF, SIF, and plasma but showed a short half-life in liver S9, which could indicate rapid release in hepatocytes. Table 6 shows the in vitro stability data for the key compounds selected from combinations of the preferred phosphoramidate substituents. Each of these compounds exhibited prolonged stability ($t_{1/2} > 15$ h) in SGF, SIF, and human plasma but decomposed quickly to the monophosphate when incubated with human liver S9 fraction.

Having the desired target activity and stability profile, compounds **12**, **14**, **18**, **41**, **44**, **45**, and **47** were evaluated in vivo to determine liver levels of the active uridine triphosphate **6** after oral administration. Since HCV replicates in liver cells, measurable levels of nucleoside triphosphate (NTP) should be a strong indication of in vivo efficacy, the assumption being

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