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Letter

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Discovery of 2'-α-C-methyl-2'-β-C-fluorouridine phosphoramidate prodrugs as inhibitors of hepatitis C virus

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KEYWORDS: hepatitis C virus, phosphoramidate prodrugs, docking, inhibitor, Hint 1

ABSTRACT: 2'-α-C-Methyl-2'-β-C-fluorouridine and its phosphoramidate prodrugs were synthesized and evaluated for their inhibitory activity against HCV. The structure-activity relationship analysis of the phosphoramidate moiety found that **17m**, **17q** and **17r** exhibit potent activities against HCV, with EC_{50} values of 1.82 ± 0.19 μM, 0.88 ± 0.12 μM and 2.24 ± 0.22 μM, respectively. The docking study revealed that the recognition of the 2'-β-F by Arg158, 3'-OH by N291 and the Watson-Crick pairing with the template allowed **23** to form the in-line conformation necessary for its incorporation into the viral RNA chain.

Hepatitis C virus (HCV) is a small, single-stranded positive-sense RNA virus that was explicitly identified in 1989.[1] The 9.6 kb RNA genome shares similarities with the genomes of flaviviruses and pestiviruses.[2] HCV infection is a global health problem that impacts approximately 180 million individuals, of which approximately 150 million people may proceed to develop chronic liver disease. A significant percentage of patients may ultimately progress to liver cirrhosis and eventually develop hepatocellular carcinoma.[3] HCV infection is also the primary reason for liver transplants among adults. The therapy for HCV infection has long been regular injections of pegylated α-interferon with the daily oral administration of ribavirin (RBV).[4] This standard of care (SOC) functions by enhancing the host immune system rather than acting directly on the virus. However, HCV patients undergoing this therapy may also suffer significant adverse effects, including fatigue, hemolytic anemia, depression and flulike symptoms, which are poorly tolerated. In 2011, boceprevir and telaprevir, as HCV protease inhibitors, became available to treat HCV infection with genotype 1 in combination with ribavirin and pegylated interferon.[5] In 2013, two new direct-acting antivirals (DAAs), simeprevir and sofosbuvir, were approved by the FDA, which significantly enhanced the available armory.[6] However the high cost for a course of treatment with sofosbuvir creates a considerable economic burden for patients.[7] Hence, the search for novel DAAs that are safe and effective remains a necessary endeavor.

The HCV NS5B RNA-dependent RNA polymerase is a key enzyme in the replication of the virus. Therefore, it has become an attractive target for the development of small molecule inhibitors of viral replication. Nucleoside and nucleotide inhibitors of HCV (NIs) are a class of DAAs that show broad activity across HCV genotypes and a high barrier to the emergence of viral resistance.[8] Structural modifications have been made to both the base and ribose sugar portions of a ribonucleoside to develop potent and selective anti-HCV inhibitors.[9] Structural modifications of the ribose sugar portion focused primarily on the 1'-, 2'- and 4'-positions. Structural modifications of the 2'-position mainly consisted of introducing an alkyl or including a methyl at the β-position and the substitution of 2'-OH, for instance, NM107, EC_{50} = 1.23 μM, PSI-6310, EC_{50} = 4.5 μM, PSI-7977 (sofosbuvir), EC_{90} = 0.42 μM (Figure 1).[10-12] Additionally, some NIs containing 2'-spirocycloalkyl or 2'-spirocyclic ether groups showed anti-HCV activities. 2'-Deoxy-2'-spirocyclopropylcytidine (**1**, EC_{50} =7.3 μM, Figure 1) was discovered as a new member of the class of 2'-modified nucleoside derivatives and showed potent anti-HCV activity.[13] A 2'-Spiroetheruridine analogue (**2**, EC_{50} =14.9 μM, Figure 1) and its phosphoramidate prodrug (**3**, EC_{50} =20.6 μM, Figure 1) exhibited medium activity against HCV.[14] This may suggest that the 2'-α-C-alkyl in nucleosides/nucleotides might be tolerated and that the 2'-α-OH or 2'-α-F may not be essential for nucleosides/nucleotides to exhibit anti-HCV activity. Sofosbuvir, with the modification of 2'-β-C-CH_3-2'-α-C-F, shows potent activity against HCV, with few adverse effects. Inspired by 2'-spiro nucleosides/nucleotides and sofosbuvir, 2'-α-C-CH_3-2'-β-C-F uridine and its phosphoramidate prodrugs were synthesized and evaluated to determine their inhibitory activity against HCV and investigate the impact of stereochemistry at the 2'-position on the anti-HCV activity.

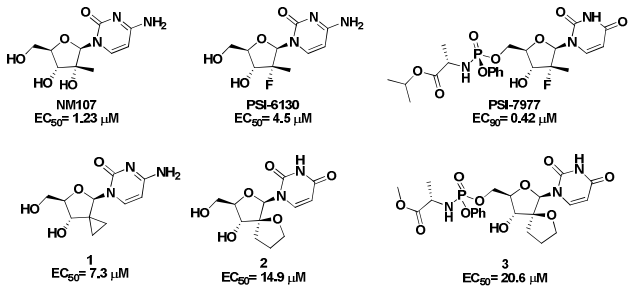
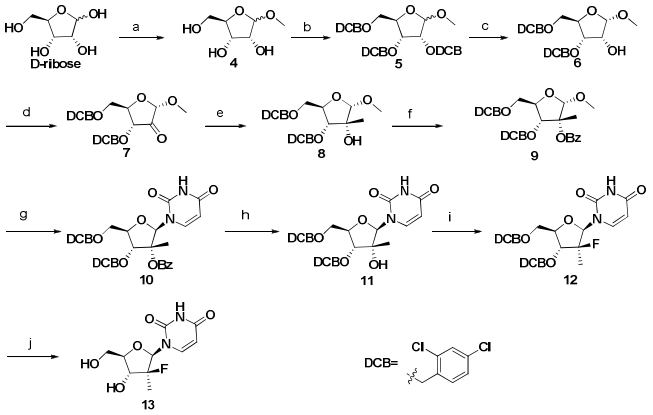


Figure 1. Nucleoside/nucleotide inhibitors of HCV.

The synthesis of intermediate 2'-α-C-CH_3-2'-β-C-F uridine **13** was illustrated in Scheme 1. D-ribose was treated with concentrated sulfuric acid in methanol to obtain 1-O-methyl-D-ribofuranose **4**. Then, the hydroxyl groups of **4** were protected by 2,4-dichlorobenzyl (DCB) groups to obtain **5**, followed by the selective removal of the 2-O-DCB group in the presence of tin (IV) tetrachloride to obtain the alcohol **6**. Ketone **7**, obtained by the oxidation of the 2-OH of alcohol **6**, was treated with methylmagnesium chloride to obtain **8**, followed by the protection of the 2-OH with benzoyl chloride to obtain **9**. The treatment of the resulting intermediate with a silylated base and tin (IV) tetrachloride gave the uridine derivative **10**. Then, the benzoyl group was removed to obtain alcohol **11**, followed by the fluorination of the 2'-OH to obtain the 2'-α-C-CH_3-2'-β-C-F uridine derivative **12**. Finally, the DCB groups were removed to obtain the desired 2'-α-C-CH_3-2'-β-C-F uridine **13**.

Scheme 1. Preparation of 13.



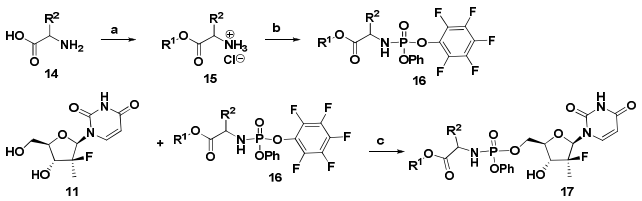
Reagents and conditions: a) H_2SO_4, MeOH, r.t., 24 h; b) 2,4-dichlorobenzyl chloride (DCBCl), NaH, DMF, 80 ° C, 3 h, 60% for two steps; c) $SnCl_4$, DCM, r.t., 3 h, 85%; d) 2,2,6,6-tetramethylpiperidinooxy, trichloroisocyanuric acid, DCM, r.t., 3 h, 90%; e) CH_3MgCl, THF, -20 °C, 4 h, 85%; f) BzCl, Et_3N, DMAP, DCM, r.t., 2 h, 88%; g) uracil, N,O-bis(trimethylsilyl)acetamide, $SnCl_4$, CH_3CN, 50 °C, 4 h, 88%; h) LiOH, CH_3OH, r.t., 4 h, 95%; i) diethylaminosulfur trifluoride, DCM, -40 °C, 2 h, 75%; j) BCl_3, DCM, -78 ° C to -30 °C, 2 h, 85%.

The anti-HCV activity of **13** was evaluated by an in vitro HCV replicon cell system,[15] and the results were expressed as EC_{50} values for antiviral activity and CC_{50} values for cytotoxicity. Uridine **13** presented medium anti-HCV activity, with an EC_{50} value of 14.65 ± 1.21 μM (Table 1). It was very interesting to find that the reverse stereochemistry of the 2'-carbon on the ribonucleoside compared to sofosbuvir was still active against HCV.

Generally, nucleoside inhibitors of antiviral were believed to work as a chain terminator and needed to be converted intracellularly to their 5'-triphosphate derivatives to be active inhibitors of viral polymerases. So a series of phosphoramidate prodrugs based on 2'-α-C-CH_3-2'-β-C-F uridine **13** were synthesized. As illustrated in Scheme 2, amino acid **14** was reacted with an alcohol and thionyl chloride to obtain the amino acid ester hydrochloride **15**, which reacted with phenyl dichlorophosphate and 2,3,4,5,6-pentafluoro phenol under basic conditions to obtain phosphoramidate **16**. The reaction of intermediates **13** and **16** under basic conditions gave the desired phosphoramidate prodrugs **17**.

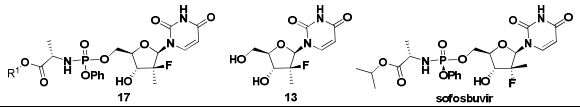
To explore the SAR of R^1, L-alanine was chosen as the amino acid, and various phosphoramidate prodrugs containing different ester groups were synthesized and evaluated (Table 1). With sofosbuvir (EC_{50} = 0.12 ± 0.02 μM) as the control, the results showed that phosphoramidate prodrugs presented decreased activities compared to **13**. The phosphoramidate prodrugs all exhibited weak activities (EC_{50} > 50 μM), except that **17d** showed medium activity, with an EC_{50} value of 34.21 ± 1.88 μM. Nevertheless, low toxicity (CC_{50} > 100 μM) was observed for all phosphoramidate prodrugs. The isopropyl group of **17d** as the fixed R^1 moiety for phosphoramidate prodrugs was investigated in the following study.

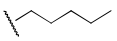
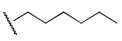
Scheme 2. Preparation of phosphoramidate prodrugs.



Reagents and conditions: a) $SOCl_2$, alcohol, reflux, 2 h - 24 h, 75% - 95%; b) phenyl dichlorophosphate, Et_3N, 2,3,4,5,6-pentafluoro phenol, DCM -78 ° C - rt, 4 h, 60% - 85%; c) *t*-BuMgCl, THF, 0 ° C, 12 h, 28% - 35%.

Table 1. Structures of phosphoramidate prodrugs 17a-17i and in vitro anti-HCV activities and cytotoxicities.



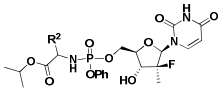
Cmpd No.	R^1	EC_{50}[a] (μM)	CC_{50}[a] (μM)
sofosbuvir		0.12 ± 0.02	>100
13	-	14.65 ± 1.21	>100
17a		> 50	>100
17b		> 50	>100
17c		> 50	>100
17d		34.21 ± 1.88	>100
17e		> 50	>100
17f		> 50	>100
17g		> 50	>100
17h		> 50	>100
17i		> 50	>100

[a]Measurements of in vitro activity and cytotoxicity were performed in triplicate and represent the mean ± SD of at least three experiment sets.

To explore the structure-activity relationship (SAR) of R^2, various phosphoramidate prodrugs containing different amino acids with different stereochemistries were synthesized and evaluated (Table 2). The results showed that most amino acid

side chains were accommodated. Phosphoramidate prodrugs with hydrogen, methyl or benzyl groups at R^2 (**17j**, **17d**, **17k, 17n** and **17o**) exhibited comparable anti-HCV activities, with EC_{50} values of from 22.88 ± 1.65 μM to 35.34 ± 1.85 μM, indicating that the chirality of the α-carbon of the amino acid moieties has little effect on the anti-HCV activities of these phosphoramidate prodrugs. Interestingly, it was found that phosphoramidate prodrugs (**17l** and **17p**) containing an L-valine or L-tryptophan moiety exhibited weak activities against HCV at the maximum testing concentration (50 μM). On the other hand, phosphoramidate prodrugs (**17n** and **17q**) with a D-valine or D-tryptophan moiety displayed a significant improvement in activity, with EC_{50} values of 2.24 ± 0.22 μM and 0.88 ± 0.12 μM, respectively. Surprisingly, phosphoramidate prodrugs containing a methionine moiety preferred an L-amino acid moiety (EC_{50} values: 2.24 ± 0.22 μM for **17r**, > 50 μM for **17s**). It was reported that sofosbuvir and its analogues containing a modification of 2'-β-C-CH_3-2'-α-C-F preferred L-amino acid moieties,[16] suggesting that the conformation inversion of the 2'- chiral carbon might influence the preference for the chirality of the α-carbon of the amino acid moieties. Favorable CC_{50} values (CC_{50} > 100 μM) were observed for all of the phosphoramidate prodrugs inhibitors in the in vitro cytotoxicity assay.

Table 2. Structures of phosphoramidate prodrugs 17j-17s and in vitro anti-HCV activities and cytotoxicities.



Cmpd No.	$R^{2\,a}$	$EC_{50}{}^{b}$ (μM)	$CC_{50}{}^{b}$ (μM)
17j	H	22.88 ± 1.65	>100
17d	(L) Me	34.21 ± 1.70	>100
17k	(D) Me	34.34 ± 1.85	>100
17l	(L) Me_2CH	> 50	>100
17m	(D) Me_2CH	1.82 ± 0.19	>100
17n	(L) $PhCH_2$	33.33 ± 1.54	>100
17o	(D) $PhCH_2$	35.34 ± 1.85	>100
17p	(L) indole-3-CH_2	> 50	>100
17q	(D) indole-3-CH_2	0.88 ± 0.12	>100
17r	(L) $MeSCH_2CH_2$	2.24 ± 0.22	>100
17s	(D) $MeSCH_2CH_2$	> 50	>100

[a]L means from L-amino acid, D means from D-amino acid. [b]Measurements of in vitro activity were performed in triplicate and represent the mean ± SD of at least three experiment sets.

Phosphoramidate prodrugs cannot directly exhibit anti-HCV activities. According to the mechanism of sofosbuvir against HCV,[12] phosphoramidate prodrugs **17** must be metabolized to the active triphosphate form by a metabolic pathway that may contain five steps (Figure 2A). The process from **17** to its corresponding 5'-monophosphate **21** involves two types of enzyme, human cathepsin A (CatA)/carboxylesterase 1 (CES1) and histidine triad nucleotide-binding protein 1 (Hint 1).[12] **17a-17c** and **17e-17i** displayed weak activities against HCV, probably because of the weak substrates for CatA/CES1. In 2007, Tsui-Fen Chou et al.[17] reported that indole-containing nucleoside phosphoramidates could be the substrates for Hint 1, and the k_{cat} value reached as high as approximately 2.5 s^{-1} for uridine phosphoramidates. Moreover, it was found that Hint 1 was sensitive to the configurations at the α-carbon of the amino acid, preferring D- over L-tryptophan. This may be the reason why **17q** was far more active against HCV than **17p**. To provide insight into the interactions between the phosphoramidate prodrugs and Hint 1, a docking study was performed using Autodock software.

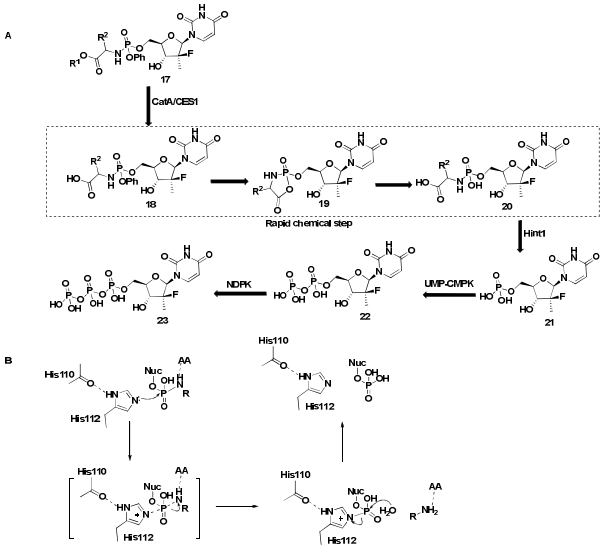


Figure 2. The formation of NTP. (A) Metabolic pathway for nucleoside phosphoramidates **17**. (B) Catalytic mechanism for Hint 1.

In Hint 1, His110 and His112 play important roles in the hydrolyzing the P-N bond of the phosphoramidate prodrugs according to the histidine triad (HIT) protein family[18] (Figure 2B). The carbonyl of His110 formed a hydrogen bond with the imidazole of His112 to increase the basicity of the nitrogen atom, which attacked the phosphorus atom of the nucleoside phosphoramidates in the next step. The formation of a hydrogen bond between the NH of the phosphoramidates and Hint 1 was useful for the cleavage of the P-N bond. In the docking study, the corresponding metabolites **20** of the phosphoramidate prodrugs were docked into Hint 1. According to the docking models, **20j**, **20d**, **20k**, **20n** and **20o** interacted with Hint 1 in almost the same pattern (models not shown). The distance between the nitrogen atoms of His112 and the phosphorus atom was between 3.8 Å and 4.1 Å. Ser107 formed a hydrogen bond with the NH of the phosphoramidates. The interaction pattern of **20m** and Hint 1 is illustrated in Figure 3. The distance between the nitrogen atom of His112 and phosphorus atom was 3.9 Å. The side chain of Asn99 interacted with the NH of phosphoramidate (3.3 Å), promoting the formation of monophosphate **21**. Moreover, the oxygen atom of the P-O bonds formed hydrogen bonds with the side chain of His114 and the backbone of Ser107 (2.8 Å and 3.0 Å, respectively). Additionally, the backbone of Ile44 donated a hydrogen bond to carbonyl of the base (3.0 Å). **20l** bonded to Hint 1 in almost

the same pattern as **20m**, except that no hydrogen bond interaction existed between the NH of **20l** and Hint 1 because of the chirality of the α carbon (Figure 3), which might be the reason why **20l** has a lower activity against HCV. The situations of **20p** and **20q** were similar to those of **20l** and **20m**. However, the chirality of the α carbon resulted in a great difference in the interactions between phosphoramidate prodrugs containing methionine and Hint 1 (Figure 4). The distance between the nitrogen atom of His112 and the phosphorus atom of **20r** was 3.4 Å, and the NH of the L-methionine moiety interacted with the side chain of Ser107 (3.3 Å). However, the distance between His112 and the phosphorus atom of **20s** containing the D-methionine moiety was as great as 6.8 Å, which resulted in a loss of activity against HCV.

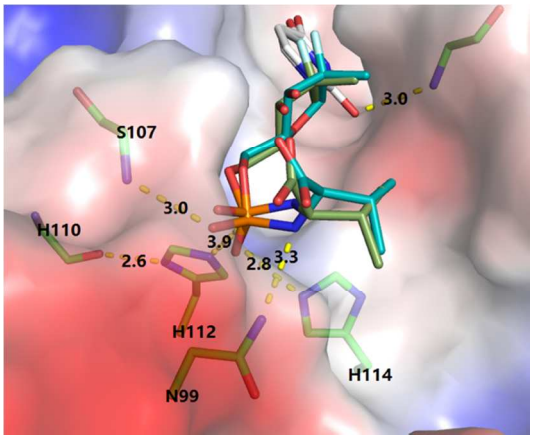


Figure 3. Docking models of **20l** (smudge) and **20m** (cyan) bound to Hint 1 (PDB code: 1KPF).

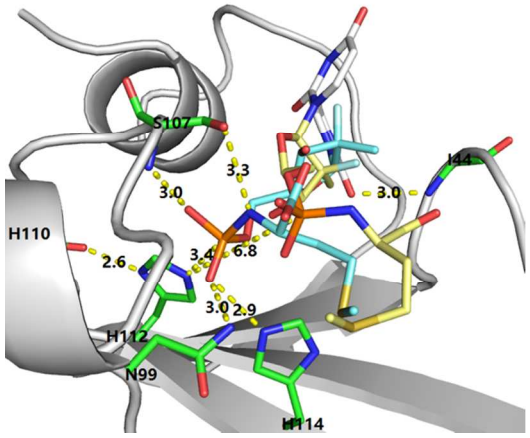


Figure 4. Docking models of **20r** (cyan) and **20s** (yellow) bound to Hint 1 (PDB code: 1KPF).

In 2015, Todd C. Appleby reported the crystal structure for HCV 2a NS5B triple-mutant Δ8 elongation state assemblies with sofosbuvir and proposed a model of HCV replication by NS5B,[19] which provided us with insight into the interactions between the nucleotide and NS5B polymerase. Herein, triphosphate 2'-α-C-CH_3-2'-β-C-F UTP **23**, the metabolite of phosphoramidate prodrugs **17**, was docked into NS5B polymerase using MOE software. **23** and 2'-β-C-Me-2'-α-C-F UDP (**24**) were overlaid to show the differences clearly (Figure 5A). It was reported that the recognition of the 2'-F by N291 and the Watson-Crick pairing with the template allow **24** to form the in-line conformation necessary for incorporation into the growing chain. The docking model of **23** with NS5B revealed that the 3'-OH of **23** moved close to the side chain of Asn291 and formed a hydrogen bond (2.5 Å), compared to **24**. Moreover, 2'-β-F was recognized by Arg158 (2.7 Å). Along with the Watson-Crick pairing with the template, **23** could be identified by NS5B polymerase. The conserved catalytic residues Asp220, Asp 318, and Asp 319 coordinated the two catalytic Mn^{2+} ions, which in turn coordinated the α and β phosphates of **23** (Figure 5B).

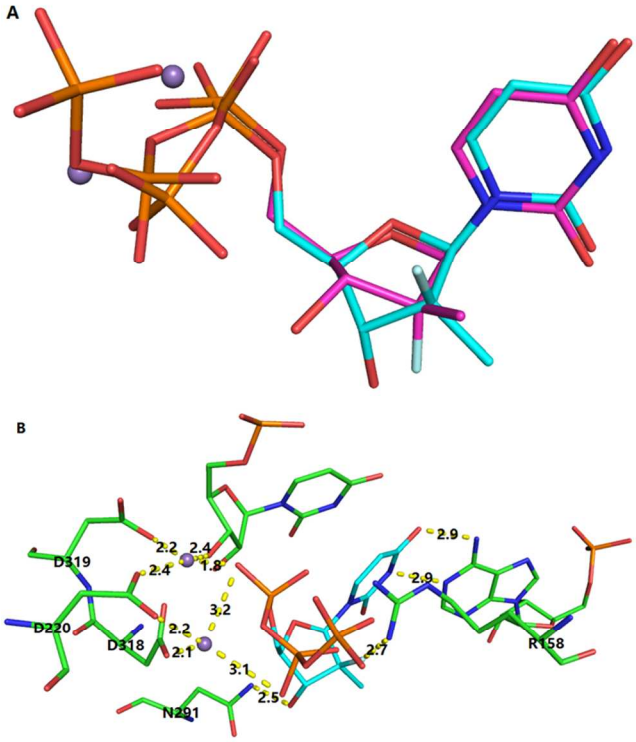


Figure 5. Docking models of **23** (cyan) bound to NS5B and co-crystal structure of **24** (magentas)/NS5B (PDB code: 4WTG). (A) **23** and **24** were overlaid to show the differences. (B) Recognition of **23** by NS5B.

In summary, 2'-α-C-CH_3-2'-β-C-F uridine **13** and a series of phosphoramidate prodrugs **17** were synthesized, and their anti-HCV activities were evaluated. Phosphoramidates **17m**, **17q** and **17r** exhibited potent activities against HCV, with EC_{50} values of 1.82 ± 0.19 μM, 0.88 ± 0.12 μM and 2.24 ± 0.22 μM, respectively. The results revealed that conformation inversion of the 2'- chiral carbon might influence the preference for the chirality of the α-carbon of the amino acid moieties compared to sofosbuvir. Favorable CC_{50} values (CC_{50} > 100 μM) were observed for all of the phosphoramidate inhibitors in the in vitro cytotoxicity assay. A docking study showed that the formation of a hydrogen bond between the NH of the phosphoramidates and Hint 1 had a significant impact on the cleavage of the P-N bond, thus influencing the activities of the phosphoramidate prodrugs against HCV. Finally, the triphosphate **23** of phosphoramidate prodrugs **17** docked into NS5B polymerase. This revealed that the recognition of the 2'-β-F by Arg158 and 3'-OH by N291 and the Watson-Crick pairing with the template allowed **23** to form the in-line conformation necessary for incorporation into the viral RNA chain.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Full experimental details, in vitro antiviral activity and cytotoxicity assay (PDF)

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Author Contributions

The manuscript was written through the contributions of all authors. All authors have given their approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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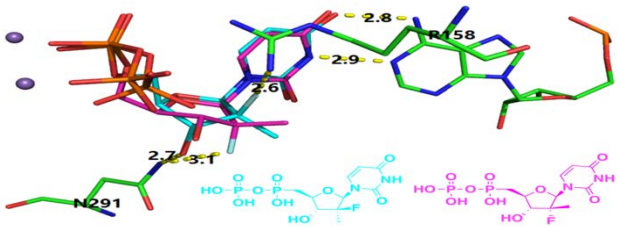

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