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Design, synthesis, and antiviral properties of 4′-substituted ribonucleosides as inhibitors of hepatitis C virus replication: The discovery of R1479

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Abstract—A series of 4′-substituted ribonucleoside derivatives has been prepared and evaluated for inhibition of hepatitis C virus (HCV) RNA replication in cell culture. The most potent and non-cytotoxic derivative was compound **28** (4′-azidocytidine, R1479) with an IC_{50} of 1.28 μM in the HCV replicon system. The triphosphate of compound **28** was prepared and shown to be an inhibitor of RNA synthesis mediated by NS5B (IC_{50} = 320 nM), the RNA polymerase encoded by HCV. Data on related analogues have been used to generate some preliminary requirements for activity within this series of nucleosides.

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease and it is estimated that more than 170 million individuals worldwide are infected with this pathogen.[1] After initial infection, the majority of HCV infections become chronic which can lead to cirrhosis of the liver, hepatocellular carcinoma, and eventually death. Furthermore, HCV infection is a leading cause of liver disease, necessitating organ transplantation. Currently, interferon-based therapies are the only FDA-approved regimens for the treatment of HCV infection. Response rates are in the 50–90% range depending on the genotype of the infecting virus. In addition, response rates are markedly reduced in individuals co-infected with HIV, with cirrhosis, and in older patients. Most individuals experience undesirable side effects with the currently available therapies and in many cases patients choose to discontinue their treatment. In addition, there are significant numbers of individuals who do not respond to interferon-based therapies, therefore, there is an urgent need to discover and develop new HCV therapeutic agents that are more effective either alone or in combination with interferon.

Historically, the majority and most successful antiviral agents in terms of efficacy and side-effect profile have been targeted against viral-encoded enzymes. The largest group of antiviral agents approved by regulatory authorities inhibit viral nucleic acid polymerases, and within that group nucleoside analogues represent the majority, exemplified by agents approved to treat HIV, hepatitis B (HBV), and the herpes group of viruses. All of these are inhibitors of DNA synthesis catalyzed by the virally encoded DNA polymerases. In fact, the active entity is the triphosphate of the nucleoside

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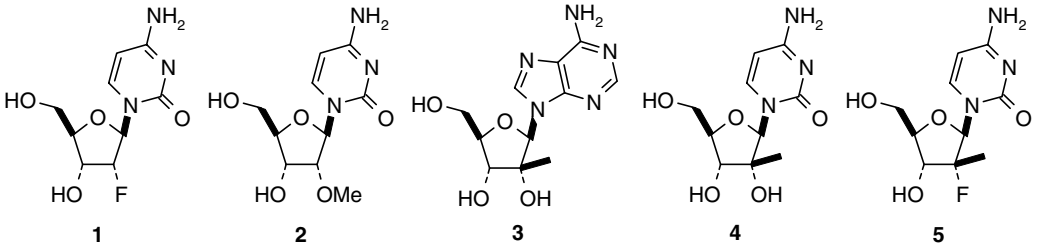


Figure 1. Recently reported nucleoside inhibitors of NS5B.

analogue, which is formed by metabolism within the infected cell. The polymerase encoded by HCV (NS5B) is an RNA dependent RNA polymerase, and since there are no marketed inhibitors of viral RNA polymerases, this provides both a challenge and an opportunity to discover potent and selective inhibitors of HCV replication that could form the basis of new treatment regimens.

There are recent reports of 2′-modified ribonucleoside analogues that inhibit HCV replication (Fig. 1).[2] One example is 2′-deoxy-2′-α-fluorocytidine **1**[2a–c], but this and other examples of 2′-deoxy-2′-α-fluoronucleosides have been shown to have low selectivity for HCV, which can probably be attributed to their ability to inhibit both viral and cellular polymerases. Because of their poor selectivity this class of nucleosides is unlikely to form the basis of a useful drug candidate. The 2′-*O*-methylcytidine analogue **2** has also been reported as an inhibitor of HCV, although the cellular activity reported for this compound is an order of magnitude lower than that of compounds currently in clinical development.[2d] Another group of nucleosides that possess a 2′-β-methyl substituent has provided several examples of inhibitors of HCV replication, and the corresponding triphosphates have been shown to be inhibitors of HCV polymerase. While **3**[2d] and its 7-deaza analogue[2e] have been reported to have interesting preclinical properties, of particular interest is 2′-β-methylcytidine **4**[2f] which is currently in Phase II clinical trials for the treatment of HCV infection. Another recent disclosure combines elements of both of the above structural classes, that is, 2′-deoxy-2′-β-methyl-2′-α-fluorocytidine **5**.[2c,g]

Here, we present our early studies directed toward the design and discovery of the totally different class of 4′-substituted ribonucleoside analogues that are potent and selective inhibitors of NS5B and of HCV replication.

All of the marketed HIV, HBV and herpes antiviral nucleoside analogues inhibit viral DNA synthesis mediated through virally encoded polymerases. The majorities of these are either 3′-deoxy- or 2′,3′-dideoxy-nucleoside analogues, and thus act as obligate chain terminators. Chain termination is effected by these molecules after the corresponding nucleoside analogue has been phosphorylated and becomes incorporated into the growing chain of DNA. Since there is no 3′-hydroxy group, further elongation of the growing nucleic acid chain is not possible. For a 4′-substituted ribonucleoside, which possesses the 3′-hydroxyl necessary for further chain elongation, an alternate avenue for chain termination after incorporation would have to be operative. In order to qualitatively explore the possible structure–activity relationships, we targeted a range of 4′-substituents which varied in size, shape, and polarity. Here we describe the SAR of cytidine and uridine analogues from this series (Fig. 2).

Synthetic routes used to prepare the target molecules were chosen according to the nature of the 4′-substituent and were based on procedures described in the literature. Cytidine derivatives were in general prepared from the corresponding uridine analogues.

Compound **10** (4′-azidouridine) was prepared as outlined in Scheme 1, which is a modification of the procedures described by Verheyden and Moffatt.[3] Treatment of **6**[4] with iodine azide, generated in situ from iodine chloride and sodium azide, gave compound **7** which was benzoylated with benzoyl chloride in pyridine to give **8**. Oxidative de-iodination of compound **8** with *m*-chloroperbenzoic acid gave **9**, which after deprotection with methanolic ammonia gave the desired 4′-azidouridine **10** in good overall yield.

4′-Propynyluridine **15** was readily prepared as illustrated in Scheme 2 from compound **11**[5] by treatment with propynyl magnesium bromide followed by ozonolysis of the isopropenyl group to give as a key intermediate the hemiacetal **12**. Removal of the isopropylidine protecting group from **12** with acetic acid, followed by peracetylation with acetic anhydride in pyridine, gave compound **13**. Condensation of **13** with persilylated uracil using the conditions of Vorbrüggen[6] gave the protected uridine **14**, which upon treatment with methanolic ammonia gave the target uridine **15** in good yield. Compound **16** was also prepared from **11** in a similar manner as described for **15**, but using ethyl Grignard in the first step. For the coupling step, persilylated *N*-benzoyl

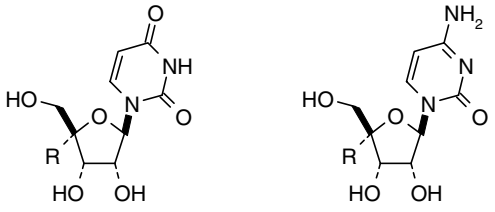
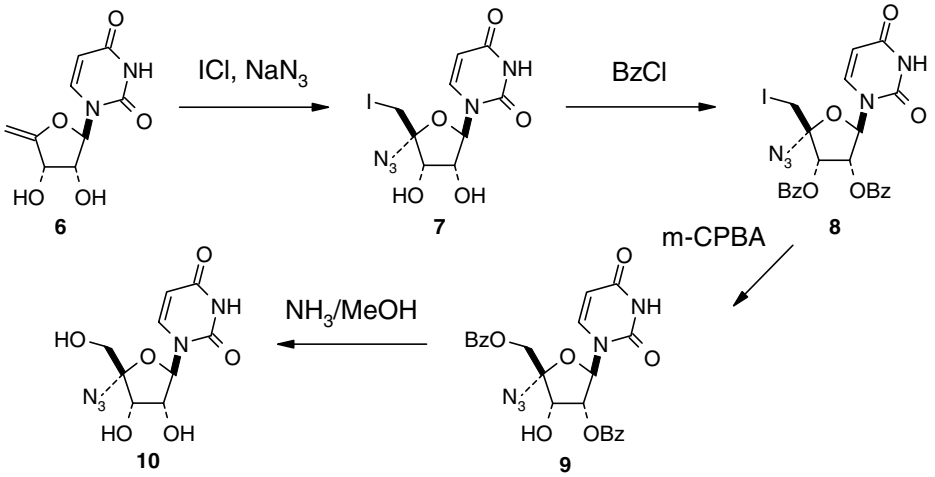
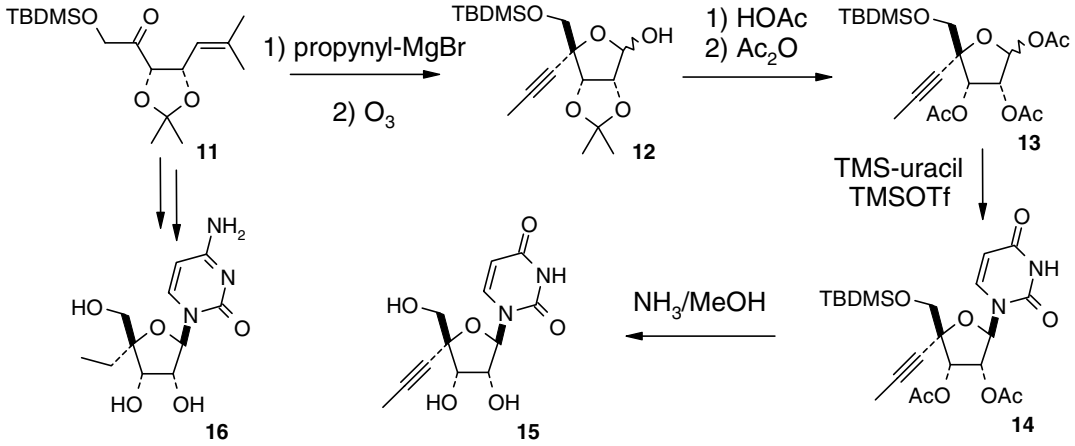


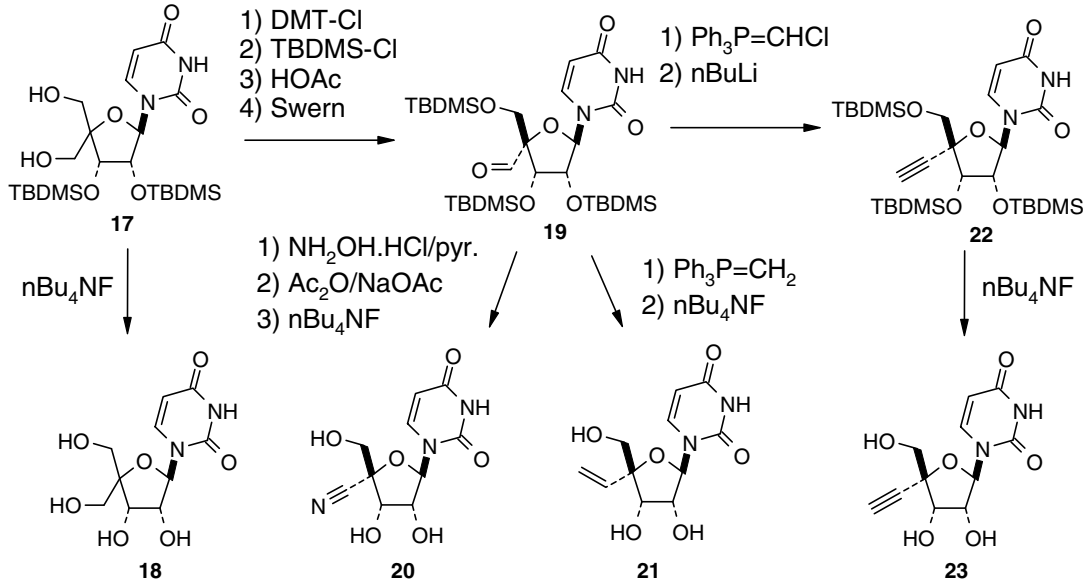
Figure 2. Ribonucleosides targeted in this work.



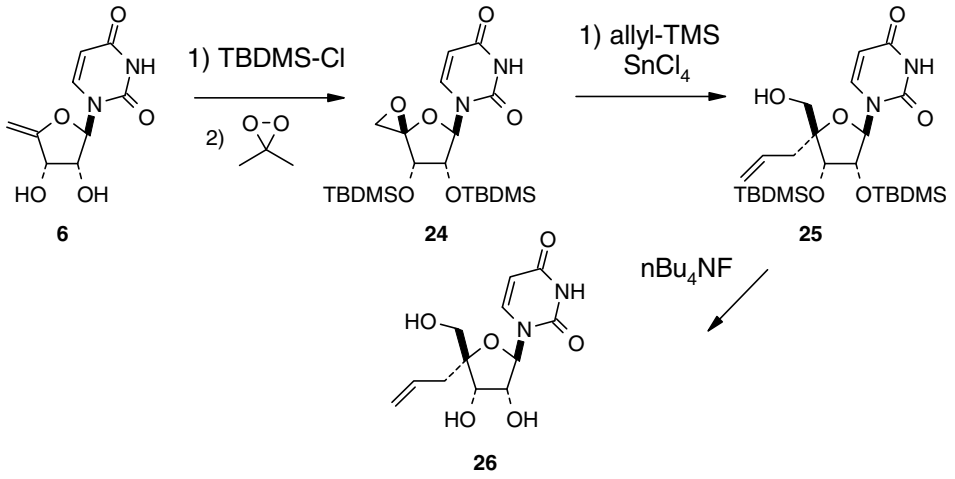
Scheme 1.



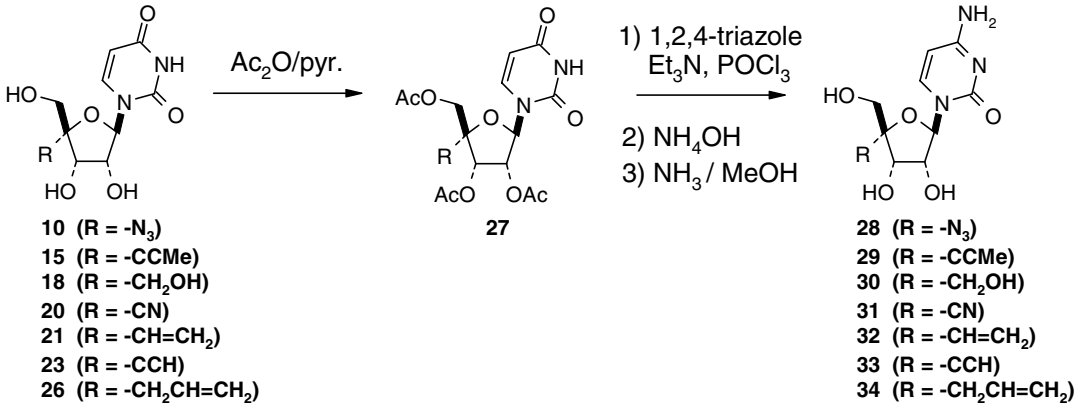
Scheme 2.



Scheme 3.



Scheme 4.



Scheme 5.

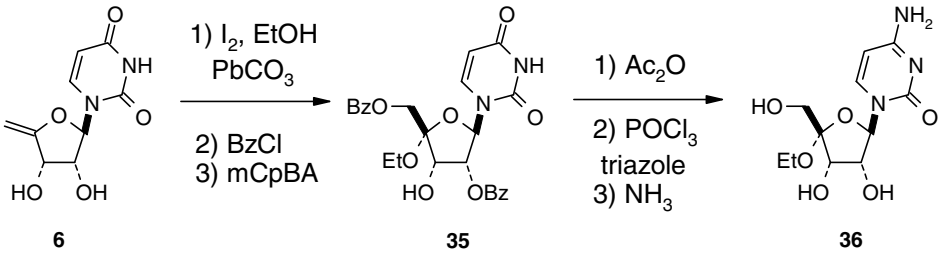
cytidine was used, and the corresponding uridine derivative was not prepared.

A range of other 4′-substituted uridines were prepared as shown in Scheme 3 from the key starting material **17**.[7] Deprotection of compound **17** with TBAF (tetrabutylammonium fluoride) gave 4′-hydroxymethyl uridine **18**[8] in excellent yield. Selective protection of the 4′-α-hydroxymethyl substituent of compound **17** was achieved by treatment with dimethoxytrityl chloride in pyridine, and was followed by protection of the 4′-β-hydroxymethyl group with TBDMS chloride. Removal of the dimethoxytrityl group with acetic acid, followed by Swern oxidation,[9] gave the aldehyde derivative **19**. Condensation of the aldehyde function in compound **19** with hydroxylamine gave the corresponding oxime which was dehydrated with acetic anhydride in the presence of sodium acetate to give the nitrile. Global deprotection with TBAF gave 4′-α-cyanouridine **20**. Alternatively, treatment of **19** with methylene triphenylphosphorane under Wittig conditions followed by deprotection with TBAF gave 4′-α-vinyl-uridine **21**. Next, intermediate **19** was condensed with a chloromethylene triphenylphosphorane Wittig reagent, which was followed by dehydrohalogenation with butyl lithium to provide compound **22**. Deprotection with TBAF was conducted to afford the target molecule, 4′-α-ethynyluridine **23**.

4′-Allyluridine was prepared according to Scheme 4. Protection of the previously described olefin **6** with TBDMS chloride followed by reaction with dimethyldioxirane gave the β-epoxide **24**. Treatment of the epoxide with allyl trimethylsilane in the presence of anhydrous stannic chloride gave the protected uridine **25**.[10] Deprotection of **25** with TBAF afforded the target uridine, compound **26**.

An efficient method for the conversion of uridines to the corresponding cytidines has been described by Divakar and Reese,[11] and was used in this work as shown in Scheme 5. Accordingly, each of the uridines **10**, **15**, **18**, **20**, **21**, **23**, and **26** was peracetylated with acetic anhydride in pyridine to give the corresponding protected derivatives represented by the generic structure **27**. Activation of the 4-keto group using phosphorus oxychloride and triazole was followed by treatment with aqueous ammonia to afford the corresponding triacetylated cytidine derivatives, which after treatment with methanolic ammonia gave the desired cytidines **28**–**34**, respectively, in good overall yield.

Finally, the synthesis of 4′-ethoxycytidine is presented in Scheme 6. For this compound, we did not discretely prepare the corresponding uridine analogue, but utilized a minor variation of the procedure reported by Verheyden



Scheme 6.

and Moffatt.[3b] Starting from **6**, addition of iodine in ethanol in the presence of lead carbonate installed the requisite 4′-ethoxy group. Following protection of the 2′- and 3′-hydroxyl groups with benzoyl chloride, displacement of the iodide as previously described for the conversion of **8**–**9** in Scheme 1 furnished **35**. Acetylation of the newly liberated 3′-hydroxy was followed by conversion of uridine to cytidine in the usual manner, with global deprotection using ammonia providing the target compound **36**.

HCV replicon assays.[12] The evaluation of compounds, inhibitory activity at 20 μM and/or the determination of the inhibitory concentrations at which a 50% reduction in replicon replication was observed (IC_{50}) were obtained using replicon cell line 2209-23. This cell line was established from the Huh-7 cells expressing a HCV genotype 1b subgenomic replicon including *Renilla* luciferase as a reporter gene. Quantification of *Renilla* luciferase activity was performed using the *Renilla* luciferase assay kit (Promega) according to manufacturer's instructions. The WST-1 or MTT assays (Roche Diagnostics) were used to measure cell viability.

Replicon proliferation assay.[12] The effect of compounds on the incorporation of tritiated thymidine into cellular DNA was measured using the SPA [^{3}H]-thymidine incorporation assay system from Amersham Biosciences.

HCV polymerase assay.[12] The enzymatic activity of NS5B570-BK proteins was measured as incorporation of radiolabeled nucleoside monophosphate into acid-insoluble RNA products using HCV cIRES RNA as a template.

Table 1 shows the results obtained with the compounds prepared and evaluated for inhibition of HCV replication. Only compounds **28** and **31** were active in the replicon system, with >50% inhibition at 20 μM. Importantly, compound **28** was not cytotoxic up to >2 mM, but compound **31** displayed a level of cytotoxicity similar to its inhibitory activity in the replicon. We concluded that the activity of compound **31** is due to inhibition of cell viability; therefore, further evaluation of this compound was not undertaken.

In Table 2, we present comparative data for compounds **28**, **26**, **1**, and **4**. Compounds **28** and **4** displayed good activity in the replicon assay with no measurable cytotoxic or cytostatic effect. Compound **26** showed a cytotoxic effect of the same order of its replicon potency, indicating the replicon activity observed is likely due to reduction in cell viability. Compound **1** showed cytostatic activity at levels similar to the IC_{50} value. Cytostatic activity is consistent with inhibition of DNA synthesis and of cell proliferation that has been shown to result from non-specific inhibition of HCV replicon RNA replication.[13]

Table 1. Initial evaluation of activity and cytotoxicity in the Huh-7 replicon assay

Compound (base C/U)	4′-α-Substituent (R)	Replicon inhibition at 20 μM	Cytotoxicity at 20 μM
10 (U)	N_3	13%	17%
28 (C)	N_3	97%	13%
16 (C)	Et	20%[a]	20%[a]
36 (C)	EtO	11%	0%
18 (U)	CH_2OH	1%	0%
30 (C)	CH_2OH	13%	2%
20 (U)	C≡N	0%	0%
31 (C)	C≡N	99%	100%
23 (U)	C≡CH	0%	0%
33 (C)	C≡CH	3%	0%
15 (U)	C≡CMe	76%[a]	19%[a]
29 (C)	C≡CMe	11%	0%
26 (U)	$CH_2CH{=}CH_2$	N.D.[b]	N.D.[b]
34 (C)	$CH_2CH{=}CH_2$	0%	0%
21 (U)	$CH{=}CH_2$	50%[a]	0%
32 (C)	$CH{=}CH_2$	53%[a]	0%

C, cytidine; U, uridine.
[a] Activity at 100 μM.
[b] IC_{50} and CC_{50} are reported in Table 2.

Table 2. IC_{50}, CC_{50}, and CT_{50} determination

Compound	Replicon IC_{50} (μM)	Cytotoxicity CC_{50} (μM)	Cytostaticity CT_{50} (μM)
28 4′-Azido-cytidine	1.28	>2000	>100
26 4′-Allyl-uridine	22	39	N.D.
1 2′-Deoxy-2′-α-*F*-cytidine	0.58	>100	0.8
4 2′-β-Me-cytidine	1.23	>33	>100

Table 3. Inhibition of the RNA polymerase activity of NS5B

Compound TP	NS5B inhibition (IC_{50} μM)
10 TP	0.30
28 TP	0.29
33 TP	2.7

In order to elucidate the mechanism of action of compound **28**, the corresponding triphosphate derivative was prepared. The triphosphate of **28** inhibited RNA synthesis by HCV polymerase in a template dependent manner, consistent with competitive inhibition of CMP incorporation by HCV polymerase (Table 3).[12] These data are consistent with compound **28** being efficiently metabolized to the corresponding triphosphate in cell culture and inhibition of HCV replication being mediated through competitive inhibition by the triphosphate. Once incorporated into the nascent RNA chain, a terminal **28** acts as a chain terminator with efficiency similar to that of 3′-deoxy-CTP.[12] Our favored mechanism to explain how a nucleoside bearing the 3′-hydroxyl can act as a chain-terminator is both conformational and steric in nature. Based on related work in 2′-deoxy systems, the 4′-α-azido substituent introduced into the carbohydrate moiety of a nucleoside will prefer to exist in a pseudo-axial orientation, which in turn will induce the nucleoside to exist predominantly in the northern conformation.[14] In this conformation, the 3′-hydroxyl group resides in a pseudo-equatorial orientation and is closely flanked by the adjacent 4′-azido group, which would serve to encumber the ability of the 3′-hydroxyl to act as a nucleophile.

The inactivity of the other derivatives in the replicon system might be explained through either lack of phosphorylation or through non-acceptance or poor fit of the 4′-substituted triphosphate in the active site of the HCV polymerase. We selected compounds **10** (4′-azidouridine) and **33** (4′-ethynylcytidine) to differentiate between these possibilities.

Compounds **10** and **33** did not inhibit HCV replication in the replicon system, but their triphosphates were inhibitors of NS5B mediated RNA synthesis (Table 3). From these data, we conclude that **10** and **33** were inefficiently phosphorylated in Huh-7 cells but that their triphosphates could productively bind in the active-site of NS5B.

The comparative results with **10** and **28** also suggest that uridine analogues may be less efficiently phosphorylated than cytidine analogues in Huh-7 cells. The synthesis and evaluation of additional triphosphate derivatives of nucleosides described in Table 1 would have helped to further delineate the SAR for binding of 4′-α-substituted nucleoside triphosphates to HCV polymerase. Unfortunately, the limited supply of key compounds precluded such studies from being undertaken.

In conclusion, data reported here show that compound **28**, designated as R1479, is a potent and highly specific inhibitor of HCV replication in cell culture, and that its triphosphate is a potent and highly selective inhibitor of NS5B mediated RNA synthesis, the HCV encoded RNA polymerase. These interesting preclinical results have led to the selection of R1479 as a clinical candidate. Further details of the advancement of R1479 into clinical studies have been reported elsewhere.[15]

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