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Design and evaluation of a potential mutagen for Hepatitis C virus

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Abstract—Pyrrolopyrimidine nucleoside **1** was designed and synthesized as a potential mutagen for HCV. An in vitro HCV NS5B enzymatic assay indicated that pyrrolopyrimidine triphosphate acts as a CTP analog rather than a UTP analog. The SATE-prodrug of pyrrolopyrimidine monophosphate showed a weak inhibitory activity in an HCV replicon system (EC_{50} = 60 μM) and did not exhibit cytotoxicity (CC_{50} > 100 μM). Investigation of phosphorylation events using nucleoside kinases and LC–MS analysis revealed that the second phosphorylation step, from monophosphate ester to diphosphate ester, is unfavorable.

Hepatitis C virus (HCV) infection is a leading cause of chronic liver diseases, such as cirrhosis and hepatocellular carcinoma. It is estimated that more than 170 million people are infected by HCV worldwide. HCV is a positive-stranded RNA virus in the family of *Flaviviridae*.[1] The only FDA-approved HCV therapies are interferon or pegylated interferon monotherapies or in combination with ribavirin.[2] However, their efficacy is less than ideal as only 52–54% of patients achieve a sustained virological response.[3] Clearly, there is urgent medical need to develop more antiviral agents to fight the HCV infection.

One idea to develop new antiviral agents is to exploit the lack of fidelity or proof-reading mechanism during RNA virus replication which is responsible for the extraordinary genetic variation among RNA viruses.[4] Such diverse RNA viral quasispecies are important to promote escape from immune selection and to adapt rapidly to new environments but also may put the RNA viruses on the threshold of error catastrophe at which a small increase of mutation rate may induce a genetic melt-down and eradicate a virus population.[5,6] Therefore, an RNA virus mutagen would be an effective antiviral drug. In fact, recent studies have indicated that ribavirin may act as such a mutagen. In the poliovirus model system, ribavirin increased the mutation rate of the viral genome in a dose-dependent manner, and a small increase of mutation rate (2- to 6-fold) reduced viral infectivity to as little as 0.00001% in cell culture. In a primer-extension assay of poliovirus polymerase, ribavirin triphosphate acted as a substrate and base-paired equally well with either uridine or cytidine. As a result, ribavirin monophosphate could be mis-incorporated into the nascent RNA product. Ribavirin in an RNA template also served as a base for incorporation of cytidine or uridine with equal efficiency, confirming that ribavirin mis-incorporation could be mutagenic.[7,8] The mutagenic effect of ribavirin on HCV infection has been subsequently investigated using in vitro studies.[9,10] In this paper, we report the design of a modified cytidine base as a potential mutagen during HCV replication and evaluation of its biological activities.

We designed pyrrolo[2,3-*d*]pyrimidin-2-one nucleoside **1** as a potential mutagen by fusing a five-membered ring on the pyrimidine of cytidine (Fig. 1). If this bicyclic ring system tautomerizes between **1** and **1a** in biological solutions, structure **1** could act as a cytidine and structure **1a**

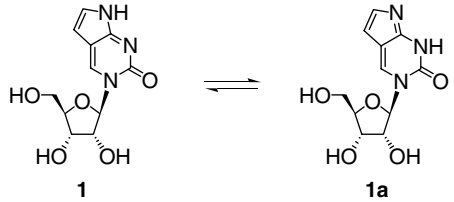

Figure 1. Proposed mutagen, pyrrolopyrimidine nucleoside **1**. The pyrrolopyrimidine nucleoside could potentially mimic a cytidine and it could also tautomerize to **1a** to mimic a uridine.

Keywords: Mutagen; Antiviral design; Nucleoside analog; HCV; RNA virus.
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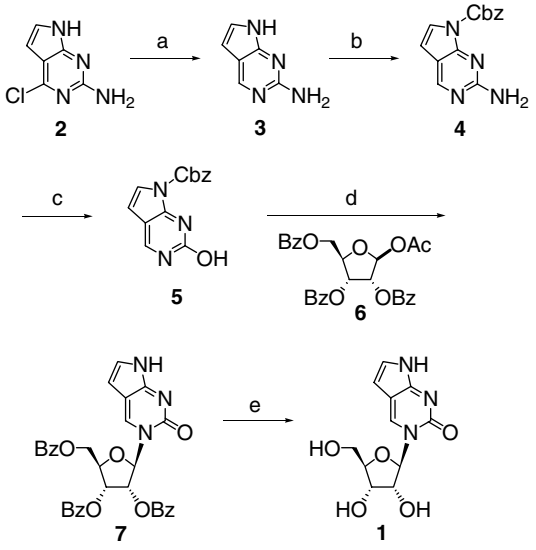
could act as a uridine. Synthesis of **1** was achieved following a route depicted in Scheme 1.[11] Hydrogenation of 2-amino-4-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine (**2**) in the presence of palladium on activated charcoal gave **3**. Protection of pyrrole nitrogen of **3** with benzyloxycarbonyl (Cbz) group was necessary for the next diazotization. The diazotization of **4** with sodium nitrite in acetic acid and water provided the pyrrolopyrimidine **5**. Glycosylation of **5** with ribofuranose **6** yielded nucleoside **7**, and deprotection of benzoyl groups of **7** provided the target nucleoside **1**.

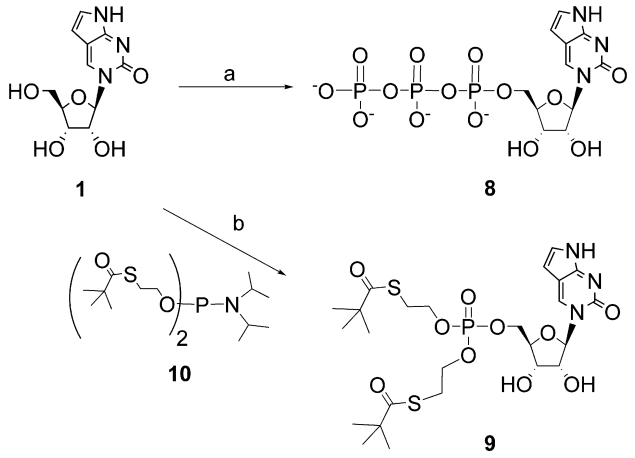
For biological evaluations, **1** was further converted to its triphosphate form **8** and an *S*-acyl-2-thioethyl (SATE) prodrug form **9** was synthesized following a well-established literature procedure.[12] The triphosphate **8** was synthesized from the reaction of **1** with phosphorus oxychloride in trimethylphosphate, followed by the reaction with tributylammonium pyrophosphate. Synthesis of prodrug **9** was accomplished by the treatment of **1** with phosphoramidite **10** in the presence of ^{1}H-tetrazole and the subsequent oxidation with *tert*-butyl hydroperoxide (Scheme 2).

To test if **1** could mimic both cytidine and uridine, we used triphosphate **8** in the HCV NS5B enzymatic nucleotide incorporation assay on different templates. Base-pairing of **8** with A into a product was examined by using an RNA template, 5′-AAAAAGGAGC-3′, and a ^{33}P-labeled primer, pGpC. The reaction will give a trinucleotide product or longer elongation products when additional CTP is present. Similarly, incorporation opposite to G was tested by using 5′-AAAAAAA GAU-3′ and ^{33}pApU.

The resulting products are shown in Figure 2 along with products from control experiments using UTP or CTP. It appears that **8** was not incorporated in significant amount into a product through base-pairing with A



Scheme 1. Reagents: (a) H_2, Pd–C, CH_3OH, 90%; (b) $C_6H_5CH_2OCOCl$, $N(CH_2CH_3)_3$, DMAP, DMF, 52%; (c) $NaNO_2$, H_2O, CH_3COOH, 51%; (d) *N*,*O*-bis(trimethylsilyl)acetamide, CH_3CN; **6**, $SnCl_4$, 45%; (e) NH_3, CH_3OH, 98%.



Scheme 2. Reagents: (a) $POCl_3$, $PO(OCH_3)_3$; TBAPP, tributylamine, 20%; (b) **10**, DMF, 1*H*-tetrazole; $(CH_3)_3COOH$, 60%.

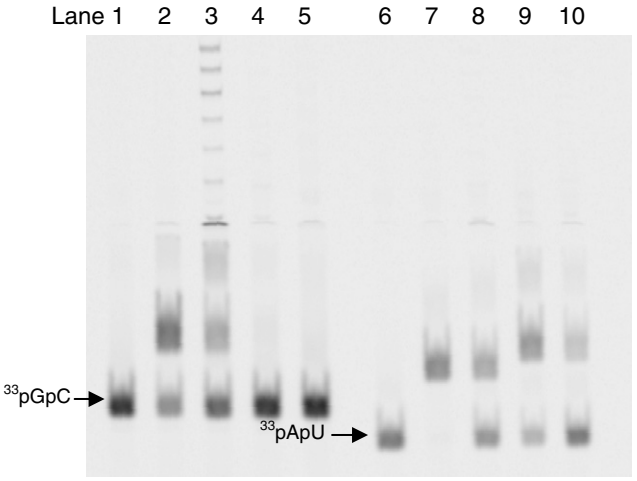


Figure 2. Incorporation of pyrrolopyrimidine triphosphate **8** by HCV NS5B using AAAAAGGAGC and ^{33}pGpC (lanes 1–5), and AAAAAAAGAU and ^{33}pApU (lanes 6–10). Each reaction mixture contained 5 μM of NS5B, 20 μM of the primer, and 20 μM of the RNA template, and 1 mM of a nucleotide as the following: lane 1, none; lane 2, UTP; lane 3, UTP and CTP; lane 4, compound **8**; lane 5, compound **8** and CTP; lane 6, none; lane 7, CTP; lane 8, CTP and UTP; lane 9, compound **8**; lane 10, compound **8** and UTP. The reactions were performed in 50 mM HEPES, pH 7.3, 10 mM $MgCl_2$, and 10 mM DTT at 30 °C for 1 h. The reaction products were resolved on a 25% polyacrylamide-7 M urea-TBE gel and were scanned on a PhosphorImager.

(lane 4), nor were there any significant elongation products (lane 5). However, the trinucleotide product was clearly seen when it was incorporated through base-pairing with G (lane 9). The intensity of the band is comparable to that from CTP incorporation (lane 7). Interestingly, elongation products from both CTP and **8** were not clearly visible (lanes 8 and 10). This result indicates that the pyrrolopyrimidine base of **8** favors the cytosine-like tautomer rather than the uracil-like tautomer under our experimental conditions.

The SATE-prodrug **9** was tested by using a cell-based HCV replication assay. It showed a weak inhibitory activity in an HCV replicon system (EC_{50} = 60 μM)

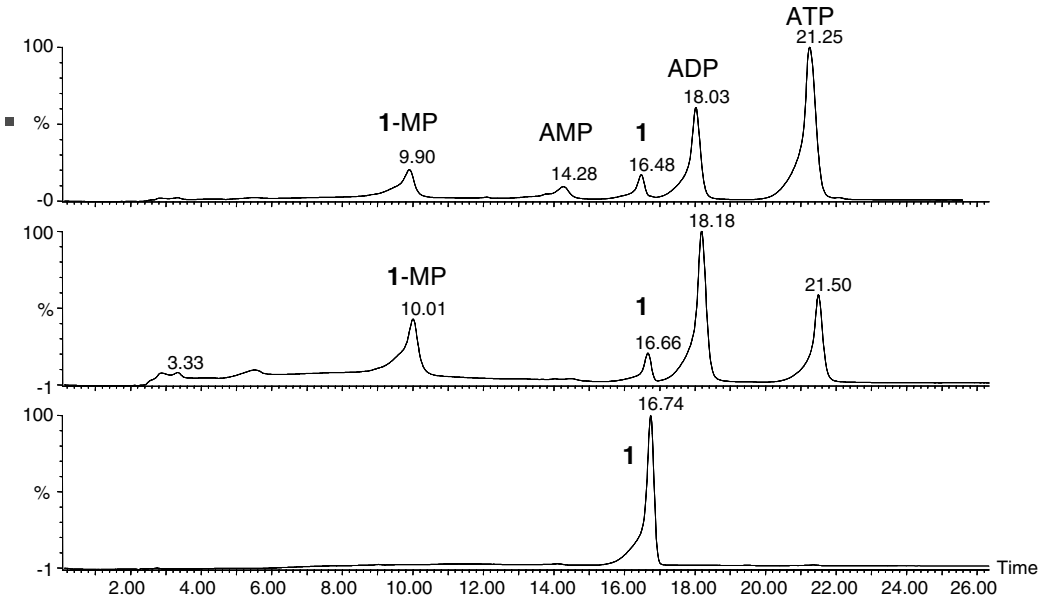


Figure 3. Chromatograms of UCK and NMPK reactions with pyrrolopyrimidine **1**; lower panel, no enzyme, pyrrolopyrimidine **1** alone; middle panel, UCK + **1**; top panel, UCK + NMPK + **1**. Reaction mixtures include 0.2 μM 1, 0.2 or 0.4 μM ATP, 2 μM UCK1 (human), and 0.014 U of NMPK (bovine liver) in 50 mM HEPES, pH 7.3, and 2 mM $MgCl_2$. Reactions were performed at 37 °C for 30 min and quenched with 10 μM EDTA.

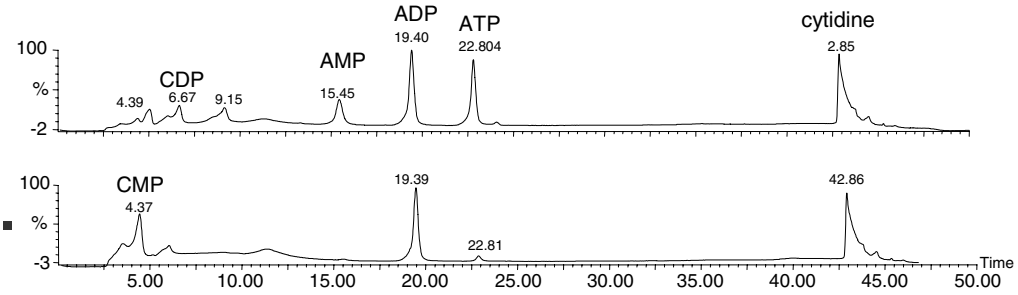


Figure 4. Chromatograms of UCK and NMPK reactions with cytidine; lower panel, UCK + cytidine; upper panel, UCK + NMPK + cytidine.

and did not exhibit any cytotoxicity (CC_{50} >100 μM). The lack of both inhibitory and cytotoxic activity may reflect poor phosphorylation events in the cells. Thus, we decided to examine phosphorylation efficiency of **1** in vitro. The pyrrolopyrimidine nucleoside **1** was incubated with uridine–cytidine kinase (UCK) and nucleoside monophosphate kinase (NMPK), and products were analyzed on an LC–MS (Fig. 3). In parallel, a control reaction was performed with cytidine to confirm the production of CMP and CDP (Fig. 4). The middle panel in Figure 3 clearly shows that a reaction with UCK alone produced the monophosphorylated product. However, a reaction with both UCK and NMPK did not produce any trace of a diphosphate product (top panel, Fig. 3). This is surprising because NMPK is known to accommodate a wide range of analogs of nucleoside monophosphates. Usually, the first phosphorylation of nucleoside, producing NMP, is known to be the rate-limiting step in cells, and to overcome this difficult step, prodrug forms of NMP are frequently utilized.

In conclusion, an in vitro enzymatic assay indicated that pyrrolopyrimidine triphosphate **8** preferentially acts as a CTP analog rather than as a UTP analog under our experimental conditions. Testing the pyrrolopyrimidine nucleoside **1** as a mutagen in vivo may not be feasible because, unlike other nucleoside analogs, the second step of phosphorylation in cells, from monophosphate ester to diphosphate ester, appears to be unfavorable.

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