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Synthesis and characterization of 2′-*C*-Me branched *C*-nucleosides as HCV polymerase inhibitors

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ABSTRACT

A series of 2′-*C*-methyl branched purine and pyrimidine *C*-nucleosides were prepared. Their anti-HCV activity and pharmacological properties were profiled, and compared with known 2′-*C*-Me *N*-nucleoside counterparts. In particular, 2′-*C*-Me 4-aza-7,9-dideazaadenosine *C*-nucleoside (**2**) was found to have potent and selective anti-HCV activity in vitro as well as a favorable pharmacokinetic profile and in vivo potential for enhanced potency over the corresponding *N*-nucleoside.

Search for effective direct-acting antiviral agents (DAAs) for treatment of HCV infection has been intense for the last decade. A number of compounds have been advanced to the development, and many have demonstrated antiviral activity clinically.[1] Among them, nucleoside polymerase inhibitors are promising in that they offer pan-genotype activity and a high barrier to the selection of viral resistance.[2]

Several nucleoside polymerase inhibitors have been in development. Compounds shown in Figure 1 are ones that are reported to be under active clinical developments at the present time.[2] Structurally, all of these compounds contain a 2′-*C*-Me branched sugar. 2′-*C*-Me *N*-nucleosides, the syntheses of which was initially described in the late 1960s[3], were among the first to show anti-HCV activity in vitro.[4] These inhibitors are metabolized to the active triphosphorylated nucleosides, which upon incorporation into RNA, inhibit further elongation catalyzed by the viral RNA-dependent RNA polymerase, nonstructural protein 5B (NS5B). The presence of the 2′-*C*-Me group prevents an incoming nucleoside triphosphate from binding to the active site of NS5B.[5]

Butora et al. reported HCV inhibitory activity of *C*-nucleosides containing a 2′-*C*-Me.[6] Although only moderately active, the results demonstrated that the *C*-nucleoside scaffold could be tolerated for HCV inhibitory activity. Very recently, we also reported a series of 1′-substituted 4-aza-7,9-dideazaadenosine *C*-nucleosides displaying antiviral activity against various RNA viruses including HCV.[7] As continuing efforts in this area, we decided to evaluate 2′-*C*-Me branched purine and pyrimidine *C*-nucleosides for their anti-HCV activity. Here, we report (1) preparation of a series of novel 2′-*C*-Me *C*-nucleoside analogs, (2) their in vitro anti-HCV activity compared with the *N*-nucleoside counterparts, and (3) relevant pharmacological properties.

Adenosine analog **1** was prepared in a highly convergent way (Scheme 1).[8] An *O*-benzyl protected 2′-*C*-Me ribonolactone **6**[9] and a fully elaborated bromo heterocycle **7**[10] were prepared first and then coupled together. Addition of ~3 equiv of BuLi to a cold suspension of **7** in THF in the presence of 'stabase' resulted in both protection of the free amino group and exchange of lithium–bromine, which was then reacted with the lactone **6**, affording the adduct **8**. Compound **8** appears to exist in equilibrium with the two anomers in solutions. Remarkably, subsequent reduction of **8** with triethylsilane and boron trifluoride etherate was highly stereoselective, leading to formation of **9** as an exclusive product. Final *O*-debenzylation either via hydrogenolysis or using BCl_3 provided the desired nucleoside **1**. Similarly, 2′-*C*-Me 4-aza-7,9-dideaza A (**2**) was prepared starting the bromo pyrrolotriazine **10**.[11] The stereochemistry at 1′-C was assigned based on NOE experiments.

Preparation of a guanosine *C*-nucleoside analog **3** started with coupling of the 2′-*C*-Me ribonolactone **6** and a bis(thiomethoxy) bromo imidazolotriazine **13** (Scheme 2).[12] The resulting 1′-OH intermediate **14** was reduced to a β-*C*-nucleoside derivative **15**. Similar to the previous observations for compounds **1** and **2**, the reduction was highly stereoselective. Next step was to properly replace the two thiomethoxy groups in **15** with the amino group at 2-C and the hydroxyl group at 6-C as in compound **3**. Treatment

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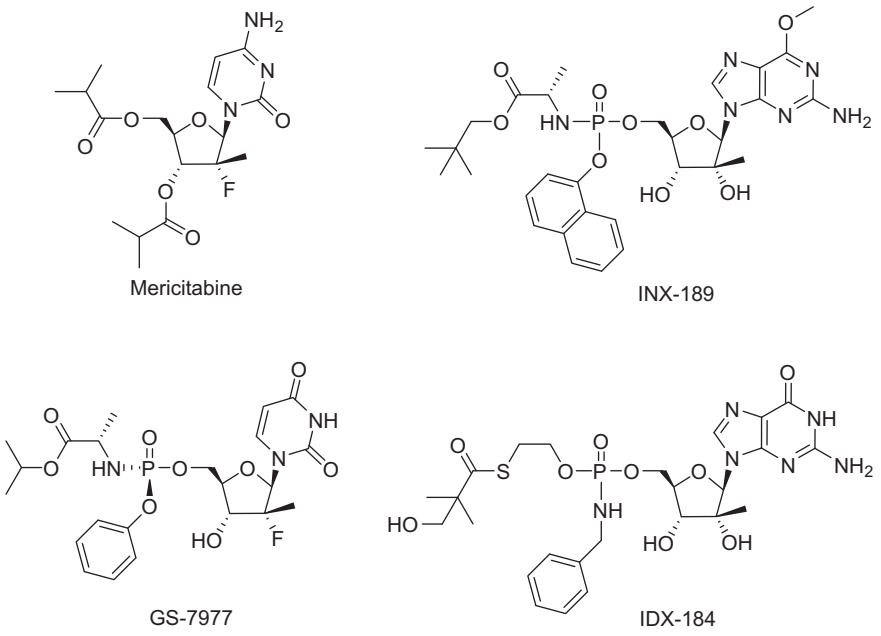
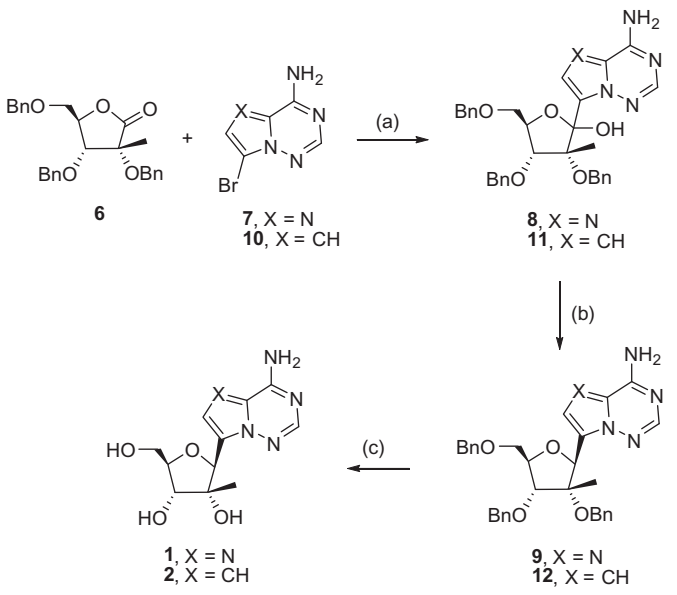


Figure 1. Nucleoside polymerase inhibitors under active clinical developments.



Scheme 1. Reagents and conditions: (a) **7** or **10,** 1,1,4,4-tetramethyl-1,4-dichlorodisilylethylene ('stabase', 1.2 equiv) and *n*-BuLi (3.3 equiv), THF, −78 °C followed by addition of **6**, 1 h, 56% for **8**/60% for **11**; (b) Et_3SiH (4 equiv), $BF_3{-}OEt_2$ (2 equiv), CH_2Cl_2, 0 °C to rt, 16 h, 35–85%; (c) H_2, 10% Pd/C, acetic acid, rt, alternatively BCl_3 (4–8 equiv), CH_2Cl_2, −78 °C, 1 h.

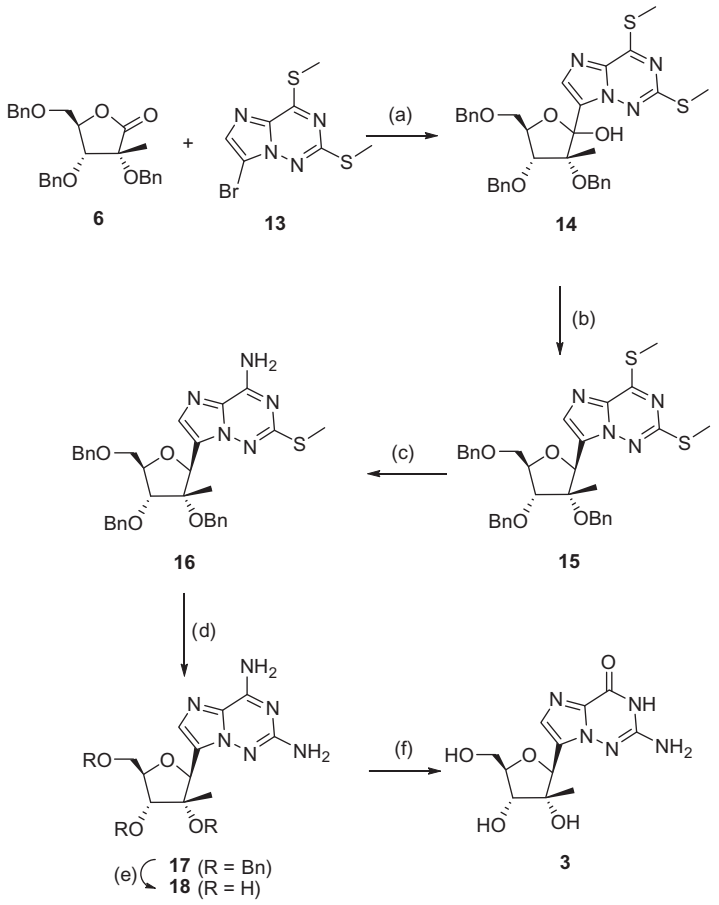
of **15** with 7 N ammonia in methanol at 45 °C gave the monosubstituted product **16** where the substitution occurred only at 6-C. Attempt to achieve further substitution to the diamino product **17** by elevating the reaction temperature (up to 110 °C) was unsuccessful. Ultimately, the resilient 2-thiomethoxy group was oxidized to the more reactive sulfone using oxone. Subsequent treatment with ammonia gas at 110 °C in a high-pressure steel reactor afforded **17**. After de-benzylation, a DAP analog **18** was treated with adenosine deaminase to give **3**.

Cytidine analog **4** was prepared in a manner similar to the method reported by Chu, et al (Scheme 3).[13] Accordingly, treatment of the lactone **18** with (ethoxycarbonylmethylene) triphenylphosphorane afforded compound **19**. The best way in our hands to construct the pyrimidine ring from **19** was to treat it sequentially with Bredereck's reagent and guanidine. The resulting **21** was found to be a ~1:1 mixture of the two anomers. After hydrogenolysis, the stereoisomeric mixture was separated by HPLC to obtain **4**.

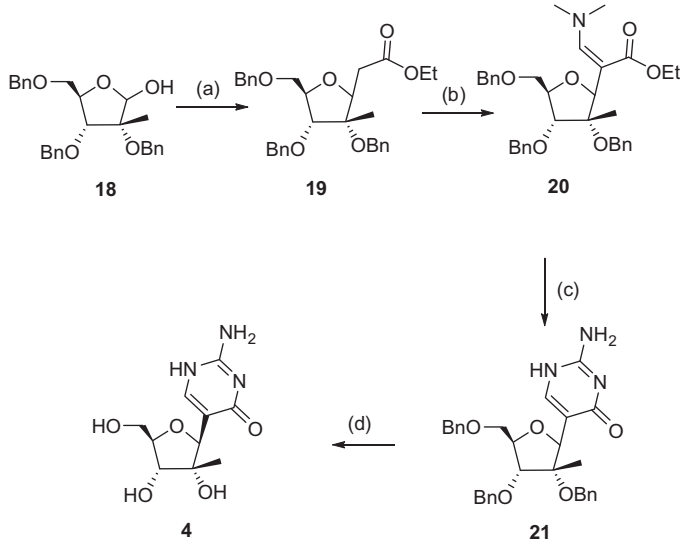
Uridine analog **5** was prepared by a modified method for preparation of the 2′-*C*-unsusbtituted peudouridine (Scheme 4).[14] The lactone **6** and the bromo pyrimidine **22**[15] were coupled, which was reduced to the diol **23**. Treatment of the diol with hydrochloric acid resulted in dehydrative ring closure and removal of the two *t*-butyl groups. The product **24** was a ~1:1 mixture of the two anomers. Final hydrogenolysis followed by HPLC separation of the two isomers afforded **5**.

The newly synthesized *C*-nucleosides, together with the corresponding known *N*-nucleosides, were evaluated for their anti-HCV activity (EC_{50}) using a subgenomic GT1b replicon and cytotoxicity (CC_{50}) from Huh-7 cells (Table 1).[7] In addition, enzymatic activity (IC_{50}) of the nucleoside triphosphates was determined using GT1b NS5B recombinant protein.[7] Consistent with the literature reports, all the *N*-nucleosides displayed selective anti-HCV activity in a range of EC_{50} of 0.08–15 μM in our replicon assay.[2] The activity varied depending on the nucleobase of the ribonucleosides, where the 7-deazaadenine base (i.e., MK-608) afforded the most potent activity. In the case of *C*-nucleoside analogs, the nucleobase was found to cause much greater impact on antiviral activity. Only adenosine analogs **1** and **2** showed inhibition of HCV. The EC_{50}s for **1** and **2** were poor relative to the corresponding *N*-nucleosides (up to EC_{50} >20-fold greater for *C*-nucleosides). Importantly, however, the intrinsic enzyme activity measured for the adenosine and guanosine *C*-nucleoside triphosphates were comparable to the corresponding *N*-nucleoside triphosphates. Consistent with their lack of cellular activity, the pyrimidine *C*-nucleosides **4** and **5** were found to inhibit NS5B only very weakly as their triphosphates and further studies showed no evidence of their functional incorporation into elongating RNA. Encouraged by the potent enzyme activity, further pharmacological properties for the purine *C*-nucleosides were profiled.

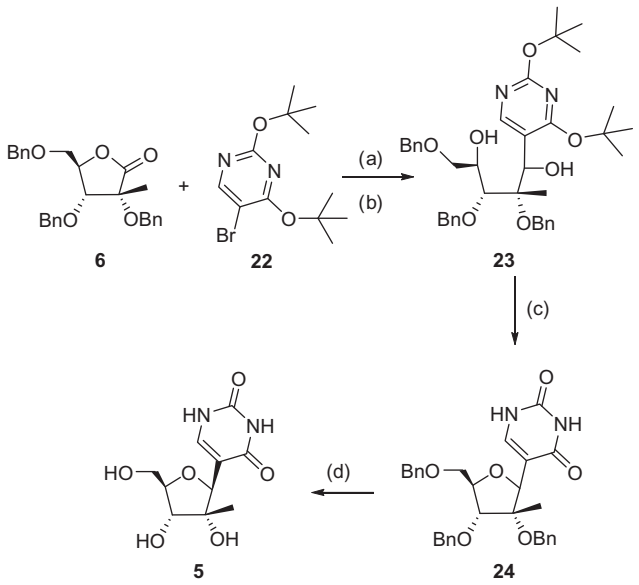
Strikingly, the guanosine analog **3** did not inhibit HCV replication despite the potent enzyme activity observed for its triphosphate metabolite. This lack of replicon activity could be



Scheme 2. Reagents and conditions: (a) **13**, *n*-BuLi, (1.0 equiv), THF, −78 °C followed by addition of **6**, 1 h, 85%; (b) Et_3SiH (4 equiv), BF_3–OEt_2 (2 equiv), CH_2Cl_2, 0 °C to rt, 16 h, 86%; (c) 7 N ammonia in methanol, 45 °C, 24 h; (d) oxone, acetone–water, 0 °C to rt; then liquid ammonia (5 mL/g), 110 °C, 40 h, 81% in three steps; (e) BCl_3 (4–8 equiv), CH_2Cl_2, −78 °C, 1 h, 69%; (e) adenosine deaminase from bovine spleen, water, 37 °C, 5 h, 100%.



Scheme 3. Reagents and conditions: (a) (ethoxycarbonylmethylene)tripheylphosphorane (1.5–2.5 equiv), MeCN, microwave 180 °C, 1 h, 55–65%; (b) *t*-butoxy bis(dimethylamino)methane (1.5 equiv), toluene, 120 °C, 6 h; (c) guanidine (10 equiv freshly prepared by treatment of sodium methoxide in methanol), methanol, rt, 24 h, 30% in two steps; (d) H_2, 10% Pd/C, 54%.



Scheme 4. Reagents and conditions: (a) **22**, *n*-BuLi, (1.0 equiv), THF, −78 °C followed by addition of **6**, 1 h; (b) $NaBH_4$ (4 equiv), methanol, 0 °C, 1 h; (c) c-HCl and ethanol (1/10, v/v), rt, 2 h, 43% in three steps; (d) H_2, 10% Pd/C, 30%.

due to the inability of **3** to be converted to its biologically active triphosphate derivative by cellular kinases. In fact, it is well established that the first phosphorylation of 2′-*C*-Me G *N*-nucleoside to its monophosphate derivative is inefficient in the phosphorylation cascade,[5] even though it still shows an appreciable level of activity (EC_{50} of 3.25 μM). Thus, monophosphate prodrug strate-

Table 1
Antiviral activity of the 2′-*C*-Me branched nucleosides

Base	Nucleoside	GT1b EC_{50} (μM)	Huh-7 CC_{50} (μM)	GT1b NS5B TP IC_{50} (μM)
A	2′-*C*-Me A	0.35	66	6.83
	2′-*C*-Me 4-aza-9-deaza A (**1**)	1.28	>89	2.10
7-deaza A	2′-*C*-Me 7-deaza A	0.08	>89	0.30
	2′-*C*-Me 4-aza-7,9-dideaza A (**2**)	1.98	85	0.31
G	2′-*C*-Me G	3.25	>89	0.25
	2′-*C*-Me 4-aza-9-deaza G (**3**)	>89	>89	0.19
C	2′-*C*-Me C	4.07	>89	0.73
	2′-*C*-Me iso-ψC (**4**)	>89	>89	13.4[a]
U	2′-*C*-Me U	15.2	>89	1.32
	2′-*C*-Me ψU (**5**)	>89	>89	13.4[a]

[a] No incorporation observed for triphosphates by NS5B.

gies have been devised to by-pass the first phosphorylation step. IDX-184 and INX-189 are such examples, where much improved antiviral activity was achieved (EC_{50} of <0.5 μM) as a result of efficient formation of the triphosphate metabolite.[16,17] In order to see if **3** can be rescued by the monophosphate prodrug strategy, an *S*-acetylthioethanol (SATE) monophosphate prodrug (**26**) was prepared and evaluated (Fig. 2). This *C*-nucleoside prodrug showed significant increase in activity (EC_{50} of 9.54 μM from >89 μM). However, the effect was less pronounced compared to the corresponding *N*-nucleoside prodrug (**25**, EC_{50} of 0.05 μM from 3.25 μM). To understand these results, intracellular levels of the phosphorylated metabolites from **25** and **26** were measured. Incubation of **26** in replicon cells resulted in accumulation of high levels of the monophosphate metabolite over time, with only minor

Table 2
Degradation of adenosine analogs in the presence of bovine adenosine deaminase

Nucleoside	(%) Remaining[a]
2′-*C*-Me A	94%[b]
2′-*C*-Me 4-aza-9-deaza A (**1**)	55%
2′-*C*-Me 7-deaza A	98%[c]
2′-*C*-Me 4-aza-7,9-dieaza A (**2**)	100%

[a] Results are the mean of 2 independent experiments done in duplicate. Percent nucleoside remaining measured after a 60 min incubation.[21]
[b] Formation of 2′-*C*-Me inosine correlating to the decrease in 2′-*C*-Me A was detected.
[c] No formation 2′-*C*-Me 7-deaza inosine detected corresponding to slight loss of 2′-*C*-Me 7-deaza A.

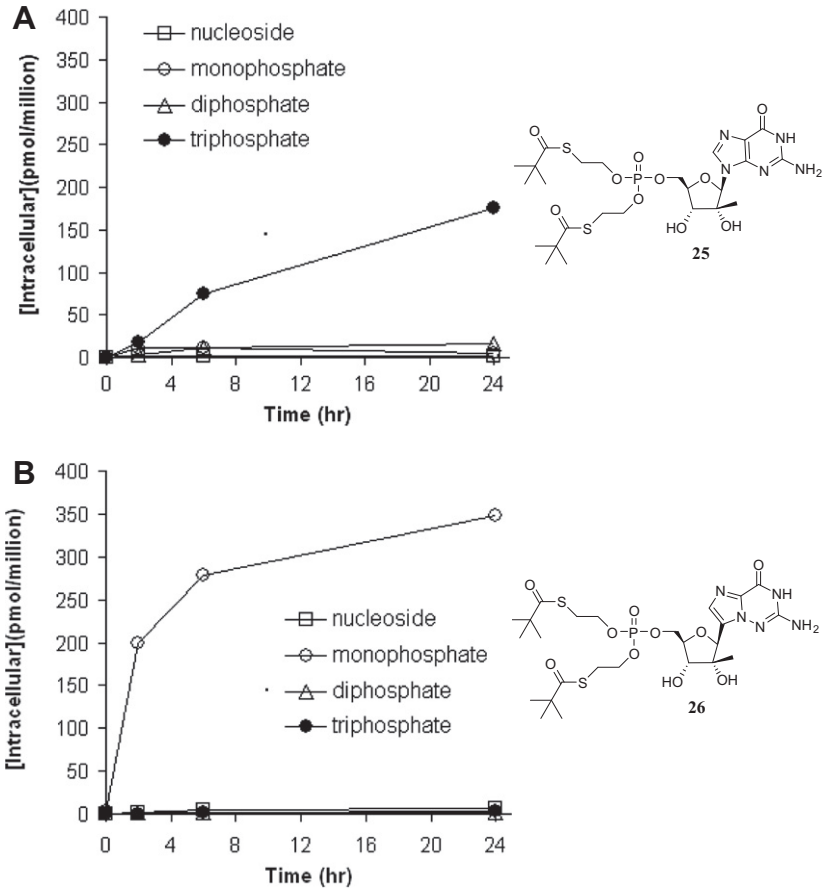


Figure 2. Intracellular metabolism of bis(SATE) prodrugs of 2′-*C*-Me G *N*-Nuc (**25**; A) and *C*-Nuc (**26**; B) during continuous 24 h incubations with Huh-7 replicon cells. Results are the average of duplicate wells from a single side-by-side experiment.

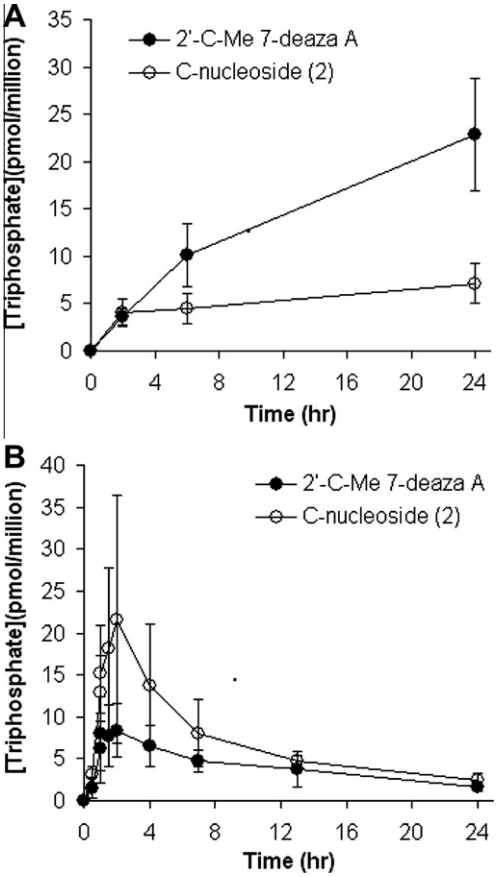


Figure 3. Comparison of intracellular tirphosphate levels formed following in vitro incubation of 2′-*C*-Me 7-deaza A and *C*-nucleoside **2** with Huh-7 replicon cells (A) and primary human hepatocytes (B). Results are the mean ± standard deviation from 2 independent experiments done in duplicate. Results in panel B are in primary human hepatocytes from two separate donors.

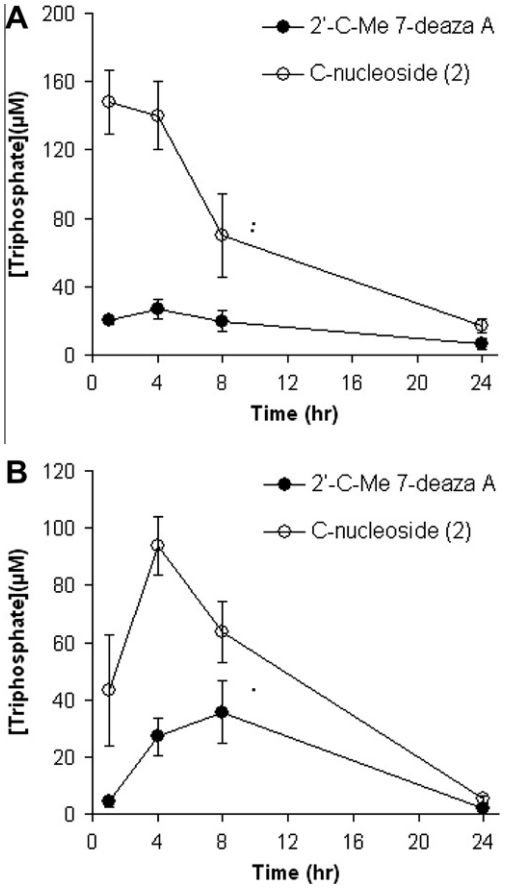


Figure 4. Comparison of hepatic triphosphate levels formed 2′-*C*-Me 7-deaza A and *C*-nucleoside **2** following oral administration to hamsters (A) and rats (B). Orally administered doses in rats were 2 mg/kg and 5 mg/kg and in hamsters were 20 mg/kg and 10 mg/kg for 2′-*C*-Me 7-deaza A and **2**, respectively. Results are the mean ± standard deviation from intracellular triphosphate measurements made from the livers from 3 animals at each time point.

levels of its di-and triphosphate metabolites. This suggests that **26** was efficiently metabolized to the monophosphate, but further phosphorylation leading to the triphosphate was not efficient. In contrast to this, high levels of the triphosphate were observed when cells were incubated with the *N*-nucleoside prodrug **25**.

An adenosine *N*-nucleoside, 2′-*C*-Me A, was reported to be susceptible to degradation by adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP), resulting in poor pharmacokinetic properties (e.g., plasma clearance >200 mL/min/kg, oral bioavailability 0% in rats).[18] Our *C*-nucleosides **1** and **2** should not be susceptible to PNP due to the incorporation of the carbon–carbon glycosidic bond. Accordingly, their instability to ADA was examined only relative to the corresponding *N*-nucleoside analogs. As summarized in Table 2, compound **1** was found to be degraded by ADA faster than 2′-*C*-Me A suggesting that it will have a poor pharmacokinetic profile and, for that reason, **1** was excluded from further characterization in vivo. In contrast to the analogs containing nitrogen at the 7 position of the respective heterocyclic base, both 2′-*C*-Me 7-deaza A and the *C*-nucleoside analog **2** were observed to be stable to ADA degradation. 2′-*C*-Me 7-deaza A has been reported to have a favorable pharmacokinetic profile and potent anti-HCV activity in chimpanzees.[19,20] Therefore, we decided to further characterize **2** for its potential as an orally administered therapeutic agent for chronic HCV infections.

As an initial step in characterizing the pharmacokinetic profile of **2**, the pharmacokinetic profile following intravenous and oral administration to dogs was assessed.[22] Intravenous administration of **2** at 0.5 mg/kg resulted in plasma clearance markedly below liver blood flow (0.4 L/hr/kg), a volume of distribution in large excess of extracellular water (1.66 L/kg) and a resulting long terminal elimination half life in plasma in excess of 7 h. Rapid absorption (time to maximum concentration of approximately 30 min) and high oral bioavailability (70%) were observed following oral administration at 1 mg/kg. The plasma pharmacokinetic profile for **2** was similar to that reported previously for 2′-*C*-Me 7-deaza A (MK-608).[19]

To cause antiviral activity, nucleosides must first permeate the target cells and be phosphorylated to their active triphosphate forms. The phosphorylation of **2** was compared to that of its *N*-nucleoside counterpart in Huh-7 replicon cells and primary human hepatocytes.[23,24] Compound **2** was not phosphorylated to its triphosphate form as efficiently in replicon cells as 2′-*C*-Me 7-deaza A (Fig. 3A), which is consistent with the relative potency observed in the replicon assays. In contrast to this, **2** was more efficiently phosphorylated in primary human hepatocytes (Fig. 3B). This improved phosphorylation in primary human hepatocytes suggest the potential for enhanced activation of *C*-nucleoside **2** over 2′-*C*-Me 7-deaza A in the liver where HCV replication takes place.

To gain a better understanding of the combined effect of oral absorption and intracellular activation described above on liver loading with the active triphosphate metabolite, the liver pharmacokinetic profiles of the triphosphate formed by **2** was compared to that of its *N*-nucleoside counterpart in rodents (hamsters and rats).[25] Following oral administration, dose-normalized triphosphate levels were 2- and 5-fold higher for **2** than for its

corresponding *N*-nucleoside (Fig. 4). Consistent with the high oral bioavailability observed in dogs for **2**, high nucleoside levels were observed in hamster plasma and, relative to a separate intravenous study, the oral bioavailability in rats was 50% (data not shown).

In summary, characterization of a series of 2′-*C*-Me *C*-nucleosides has identified nucleoside analogs with potent anti-HCV activity. While the pyrimidine *C*-nucleosides (**4** and **5**) lacked replicon activity due to poor enzymatic potency and, despite excellent activity as its triphosphate, the guanosine *C*-nucleoside (**3**) and its nucleotide prodrug (**26**) were inefficiently metabolized, adenosine *C*-nucleosides (**1** and **2**) were found to have selective replicon activity and potent inhibition of NS5B as their triphosphates. Furthermore, **2** was found to have a favorable pharmacokinetic profile and in vivo potential for enhanced potency over the corresponding *N*-nucleoside 2′-*C*-Me 7-deaza A. These results have prompted the subsequent further characterization of the toxicological profile of **2**, which will be published in due course. *C*-Nucleosides may hold promise as potential future therapies for HCV infection.

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21. Nucleosides (10 μM) were incubated at 37 °C with 0.0125 U/mL bovine adenosine deaminase (Sigma A5168) in 0.01 M potassium phosphate buffer pH 6.0 and samples collected over a 1 hour time course. The reactions were stopped by adding 2.5 volumes of 0.2% formic acid in ice cold methanol. Nucleoside levels were then measured using liquid chromatography (0.2% formic acid in water and an acetonitrile gradient) coupled to triple quadrapole mass spectrometry running in positive ion multiple reaction monitoring mode.
22. Groups of 3 non-naïve male beagle dogs were administered a single dose of 2 formulated as a solution in 5/5/5/85 ethanol/polypropylene glycol/polyethylene glycol/aqueous 50 mM citrate buffer (pH 3.3) either by intravenous infusion at 0.5 mg/kg or orally at 1 mg/kg. Blood samples were collected over a 24 h period postdose into VacutainerTM tubes containing EDTA-K3 (BD Biosciences) and plasma was isolated by the manufacturers suggested protocol. Plasma samples were processed via protein precipitation by adding acetonitrile to a final concentration of 60%. Following filtration to remove precipitated proteins, samples were dried and reconstituted in 20% acetonitrile in water. Samples were then analyzed by reversed phase liquid chromatography coupled to triple quadrapole mass spectrometry (LC/MS/MS) in positive ion and multiple reaction monitoring mode.
23. Replicon cells were maintained in Dulbecco′s modified eagle medium containing glutamax supplemented with 10% heat inactivated fetal bovine serum, penicillin–streptomycin, and G418 disulphate salt solution. Cells were transferred to 12-well tissue culture treated plates by trypsonization and grown to confluency (0.88 × 106 cells/well). Cells were treated for 24 h with a test compound (10 μM). Following 24 hours, cells were washed twice with 2.0 mL ice cold 0.9% sodium chloride saline. Cells were then scraped into 0.5 mL 70% methanol and frozen overnight to facilitate the extraction of nucleotide metabolites. Extracted cell material in 70% methanol was transferred into tubes and dried. After drying, samples were re-suspended in 1 mM ammonium phosphate pH 8.5. TP levels were quantified using liquid chromatography coupled to triple quadrapole mass spectrometry by methods similar to those previously reported in Durand-Gasselin, L.; Van Rompay, K.K.; Vela, J.E.; Henne, I.N.; Lee, W.A.; Rhodes, G.R. *Mol. Pharm.* **2009**, *6*, 1145.
24. Primary human hepatocytes were maintained in Williams E medium containing Cellz Direct′s proprietary supplement cocktail. Cells were purchased from Cellz Direct as a twelve well plate format grown to confluency (0.88 × 106 cells/well). Cells were treated for 1 h with a test compound (10 M) followed by a 23 h treatment with non-drug containing media. Cells were collected at 0, 0.5, and 1 h during compound incubation and at 0, 0.5, 1, 3, 6, 8 and 23 h following compound removal. Cells were collected and intracellular metabolites were analyzed as described for replicon cells.
25. Groups of **12** Golden Syrian hamsters or Sprague–Dawley rats were administered nucleosides formulated in water at respective doses. At 1, 4, 8 and 12 h, livers were harvested under isoflurane anesthesia. Collected livers were wrapped in aluminum foil and snap-frozen in liquid nitrogen immediately following removal to avoid sample dephosphorylation. Livers were processed by sectioning into smaller pieces with a razor blade and collecting into pre-weighed 15 mL conical tubes kept on dry ice. Four volumes of ice-cold extraction buffer (0.1% KOH and 67 mM EDTA in 70% MeOH, containing 0.25 μM Cl-ATP) were added and samples were promptly homogenized using an Omni-Tip THTM with disposable, hard tissue homogenizer probes (Omni International). Aliquots of homogenate were then centrifuged (20,000×*g* at 4 °C for 10 min). Aliquots of supernatant were transferred to clean tubes, evaporated to dryness in a heated centrifugal evaporator, and reconstituted with an equal volume of 1 mM ammonium phosphate (pH 7.0). Intracellular triphosphate levels were measured by LC/MS/MS as described for samples generated in vitro. Levels of endogenous adenosine triphosphate were also determined to assure the sample stability. Nucleoside levels in rat plasma were determined by the same methods described for dogs.