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# Discovery of a 2'-Fluoro-2'-*C*-Methyl *C*-Nucleotide HCV Polymerase Inhibitor and a Phosphoramidate Prodrug with Favorable Properties

Thorsten A. Kirschberg[a*], Sammy Metobo[a], Michael O. Clarke[a], Vangelis Aktoudianakis[a], Darius Babusis[a], Ona Barauskas[a], Gabriel Birkus[a], Thomas Butler[a], Daniel Byun[a], Gregory Chin[a], Edward Doerffler[a], Thomas E. Edwards[b], Martijn Fenaux[a], Rick Lee[a], Willard Lew[a], Michael R. Mish[a], Eisuke Murakami[a], Yeojin Park[a], Neil H. Squires[a], Neeraj Tirunagari[a], Ting Wang[a], Mark Whitcomb[a], Jie Xu[a], Huiling Yang[a], Hong Ye[a], Lijun Zhang[a], Todd C. Appleby[a], Joy Y. Feng[a], Adrian S. Ray[a], Aesop Cho[a], Choung U. Kim[a].

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## ABSTRACT

A series of 2'-fluorinated *C*-nucleosides were prepared and tested for anti-HCV activity. Among them, the triphosphate of 2'-fluoro-2'-*C*-methyl adenosine *C*-nucleoside (**15**) was a potent and selective inhibitor of the NS5B polymerase and maintained activity against the S282T resistance mutant. A number of phosphoramidate prodrugs were then prepared and evaluated leading to the identification of the 1-aminocyclobutane-1-carboxylic acid *iso*propyl ester variant (**53**) with favorable pharmacokinetic properties including efficient liver delivery in animals.

Keywords: Hepatitis C; Antiviral; C-nucleoside; NS5B polymerase

Hepatitis C Virus (HCV) infection is a major cause of chronic liver disease worldwide. When left untreated, it can lead to end stage liver diseases including cirrhosis and hepatocellular carcinoma. [1] Until 2011, the standard of care for patients with HCV infection was a regimen consisting of pegylated interferon-α and ribavarin, which has only limited efficacy. [2] More recently several direct-acting antivirals (DAAs) have been developed and licensed for use, resulting in combination regimens with markedly improved clinical outcomes. [3] A notable advance in this context was the discovery of the nucleotide prodrug sofosbuvir (**1**) (Figure 1). Combined with other DAAs, sofosbuvir affords high cure rates for infections across all genotypes of HCV. [4]

Nucleotide inhibitors are an important class of DAA as they have pan-genotype activity and a high barrier to emergence of resistant mutations. These inhibitors are phosphorylated within host cells to the bioactive nucleoside triphosphates (NTPs) that are then used as substrates by the HCV RNA-dependent RNA polymerase (non-structural protein 5B, NS5B) and incorporated into the growing RNA genome. Once integrated, the nucleotide analogs prevent incoming NTP substrates from further incorporation, thus stopping RNA elongation. [5]

We have recently reported a series of *C*-nucleosides such as compounds **2** and **3** as anti-HCV agents, and the discovery of a clinical candidate GS-6620 (**4**) through extensive exploration of phosphoramidate prodrugs of **3** (Figure 1). [6] GS-6620 displayed potent and selective inhibition of HCV replicons derived from genotypes 1 to 6 ($EC_{50}$ of 0.048 to 0.68 μM), but selected for the S282T mutant that was highly resistant in both enzymatic and cellular assays (>30 fold). In Phase 1 clinical studies, reductions in plasma viral load to below the limit of quantification (>4 logs) were observed in some individuals, but high doses were required and intersubject variability was unacceptable as a result of poor oral absorption. GS-6620 is a double prodrug with an *iso*butyryl ester masking the 3'-hydroxyl of phosphoramidate prodrug **5** (Figure 1). Blocking the polar hydroxyl group in this way was important for achieving the desired level of permeability in both in vitro assays and in vivo animal PK studies. However, follow-up studies suggested that intestinal metabolism of the *iso*butyryl pro-moiety was the likely cause for poor absorption and variability in humans. [7]

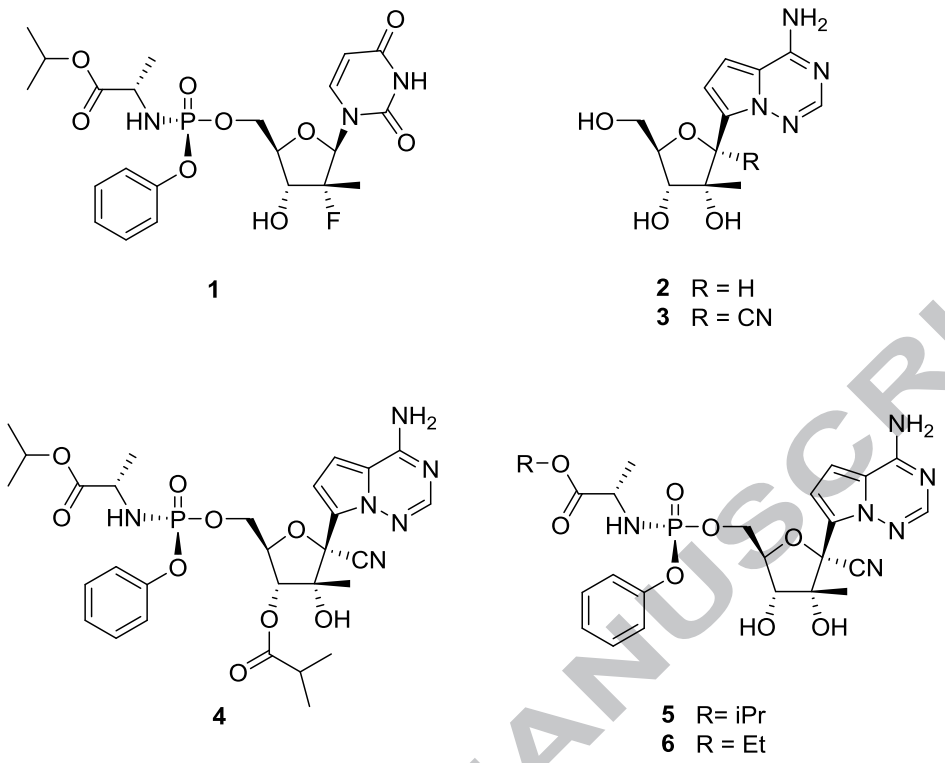



**Figure 1**: Structures of nucleoside derivatives targeting HCV

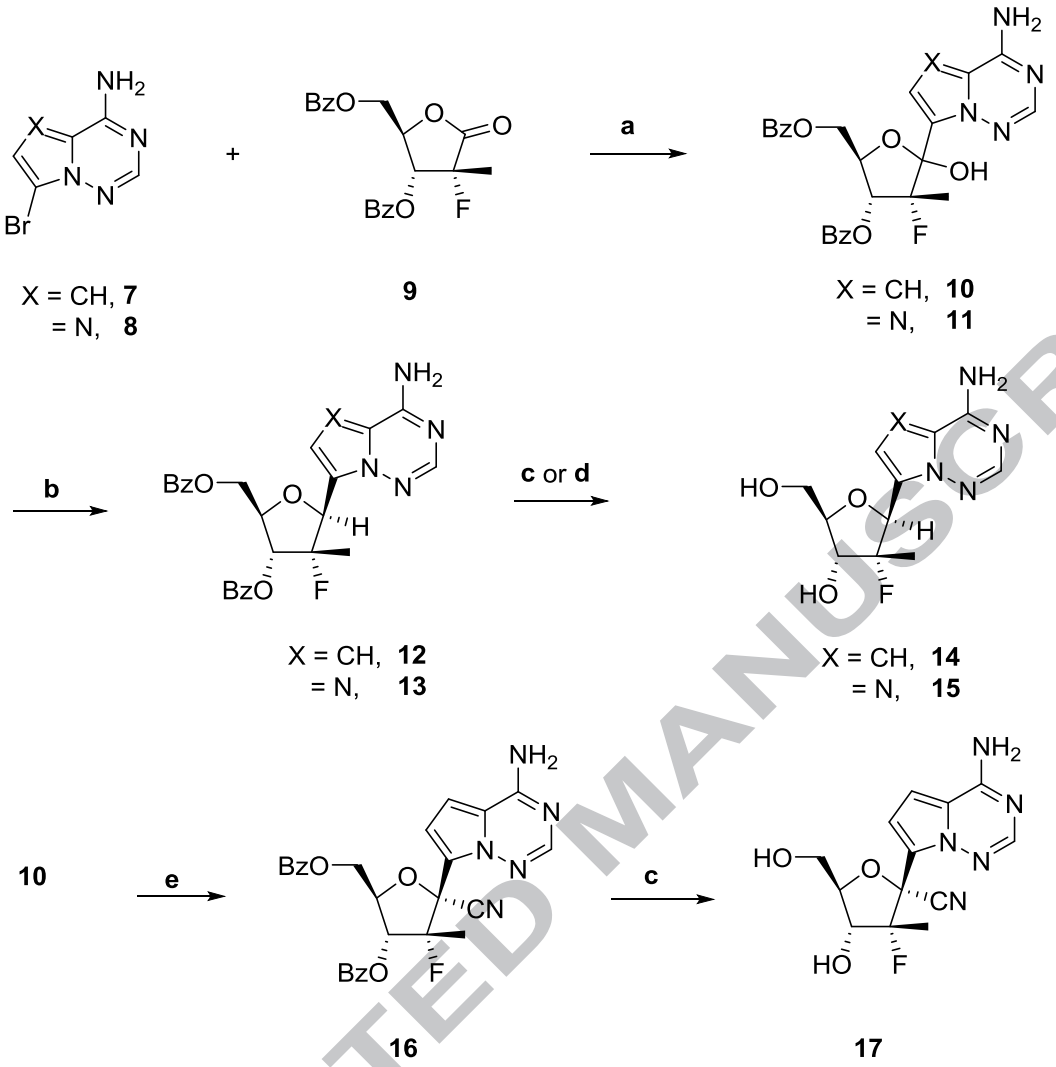
We sought to extend our initial clinical proof of concept in the *C*-nucleoside series by identifying a new agent with improved oral delivery and activity against the S282T resistance mutant. Toward this end, a series of nucleosides in which the 2'-hydroxyl group is replaced with a 2'-fluoro group was targeted; elimination of one hydrogen bond donor may affect intestinal permeability,[8] and purine analogs with this ribose modification had been reported to be relatively insensitive to the S282T resistance mutation. [9] Furthermore, we wanted to broaden our exploration of phosphoramidate promoieties in the hope that we might discover further motifs for cell-type specific prodrug activation. [10] Here, we present syntheses and characteristics of new 2'-fluoro-2'-*C*-methyl *C*-nucleosides, resulting in the discovery of orally bioavailable phosphoramidate prodrugs that are efficiently activated in the liver.

The new compounds were prepared by synthetic routes analogous to those of 2'-hydroxy-2'-*C*-methyl *C*-nucleosides recently reported (Scheme 1). [11] Transient silyl protection of the amino group and lithium-halogen exchange of the bromo heteroaromatics **7** and **8** in one pot were carried out at -78℃, and reaction with the lactone **9** furnished the hemiacetals **10** and **11** in moderate yields. Reduction to compounds **12** and **13** was achieved with high diastereoselectivity

(~95/5 ratio of β/α-isomers), as in the earlier 2'-hydroxy-2'-*C*-methyl series, using triethylsilane in the presence of boron trifluoride etherate.[11] The target nucleosides **14** and **15** were then obtained by hydrolysis of the benzoyl ester groups using aqueous ammonium hydroxide solution or ammonia in methanol.

To prepare the 1'-cyano substituted nucleoside **17**, the 1'-hydroxy intermediate **10** was treated with trimethylsilyl cyanide in the presence of indium trifluoromethanesulfonate, again providing ~95/5 ratio of β/α-isomers. The resulting product **16** was de-benzoylated using aqueous ammonium hydroxide solution to afford the desired product, while monitoring carefully to minimize cyanide hydrolysis.

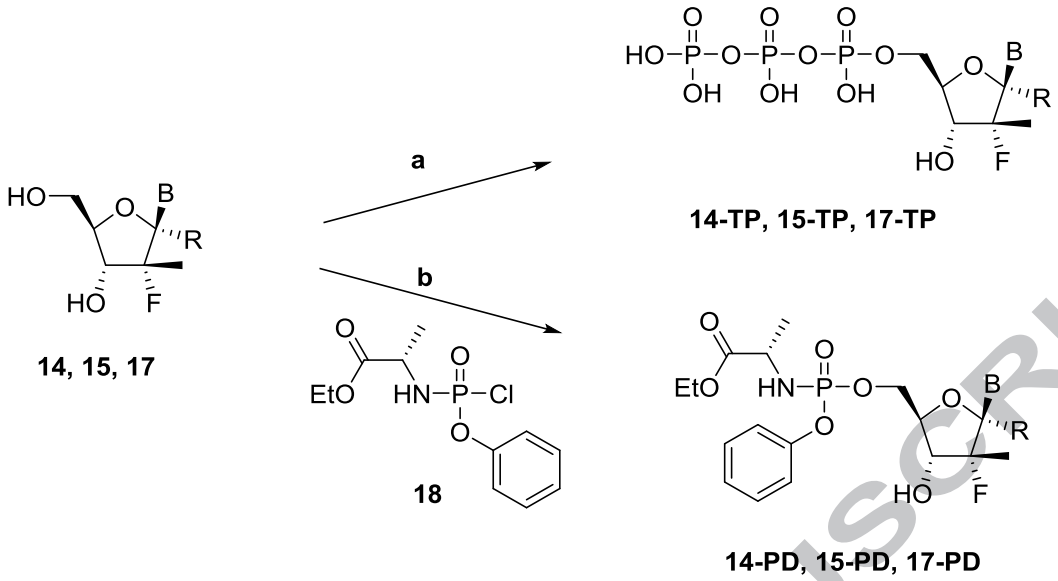
**Scheme 1**. Synthesis of *C*-Nucleosides




Reagents and conditions: (a) (i) **7** or **8**, $(ClSiMe_2CH_2)_2$, THF, -78 ˚C, BuLi (ii) then **9**; for **10**, 40%; for **11**, 59%. (b) $BF_3{\cdot}OEt_2$, triethylsilane, $CH_2Cl_2$, 91% (**12**); 64% (**13**) (~95/5 ratio of β/α-anomers) (c) conc. $NH_3$(aq), MeOH, 66% (**14**); 60% (**17**). (d) $NH_3$ MeOH, 54% (**15**). (e) TMSCN, $In(OTf)_3$, DCE, 58% (~95/5 ratio of β/α-anomers).

Nucleoside triphosphates and representative (*L*)-alanine ethyl ester phosphoramidate prodrugs were also prepared according to previously reported methods (Scheme 2). [12]

**Scheme 2**. Synthesis of nucleoside triphosphates and phosphoramidate prodrugs.




Reagents and conditions: (a) (i) $POCl_3$, $(MeO)_3PO$ (ii) $Bu_3N$·pyrophosphate (average yields ~10%) (b) *N*-methyl imidazole (NMI), **18**, THF, 0℃ to rt., (average yields ~ 30%).

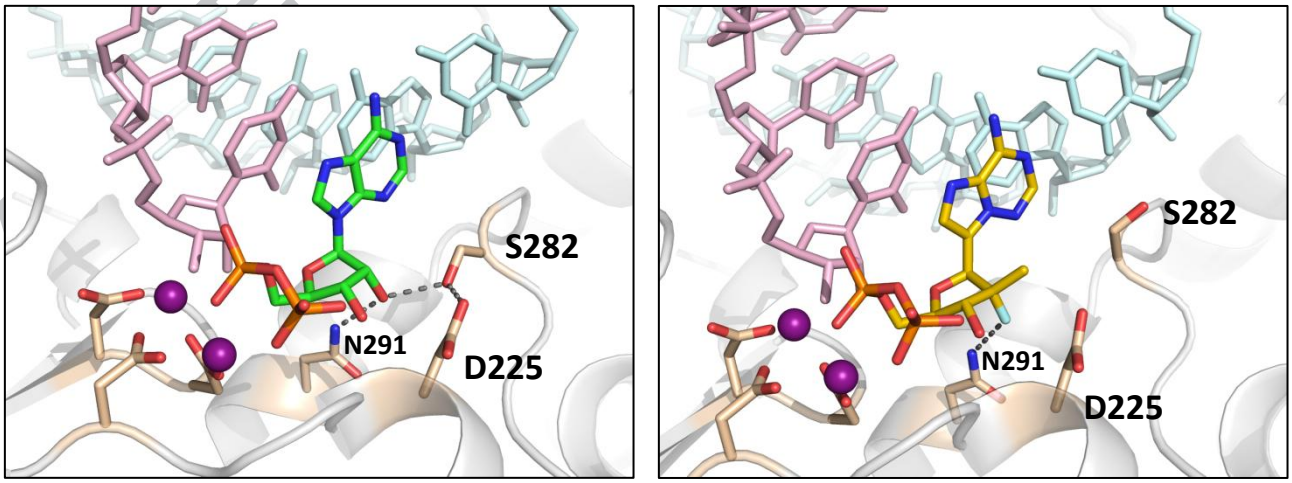
Data for these compounds elucidated the impact of key structural elements on intrinsic potency of the triphosphate against both the genotype 1b wild-type (WT) and the S282T mutant NS5B enzymes. Furthermore, the potency differential for the nucleoside / prodrug pair in the cellular replicon assay established the need for a prodrug strategy in order to bypass the first phosphorylation step (Table 1).

**Table 1**. Enzymatic activity ($IC_{50}$) against WT and S282T NS5B and antiviral activity ($EC_{50}$) in the genotype 1b replicon (GT 1b)

| Compound (triphosphate) | NS5B, WT $IC_{50}$ [μM] | NS5B, S282T Fold | Compound (Nuc/PD) | GT1b, $EC_{50}$ [μM][a] (Nuc / PD) |
|---|---|---|---|---|
| **14-TP** | 0.046 | 2.8 | **14** / **14-PD** | >89 / >89 |
| **15-TP** | 0.15 | 5.9 | **15** / **15-PD** | >89 / 0.59 |
| **17-TP** | 0.42 | 6.0 | **17** / **17-PD** | >89 / 3.24 |
| **3-TP** | 0.29 | >30 | **3** / **6** | >89 / 1.45 |

[a]TP = Triphosphate ; PD = Prodrug as in Scheme 2. [b] Effective concentration at which 50% inhibition occurs as determined by a luciferase-based genotype 1b replicon assay in huh-7 cells. All values reported are the averages of at least three repeats.

All three 2'-fluoro triphosphates displayed inhibitory potency in the wild-type NS5B biochemical assay that was similar to or better than the 2'-hydroxy analog (**3-TP**). A particular benefit was the much smaller differences in inhibitory potency between WT and S282T mutant. To understand this better, an X-ray structure of the elongation phase ternary complex of NS5B and **15** diphosphate (**15-DP**) was obtained (Figure 2B). [13] The bound conformations of **15-DP** and adenosine diphosphate (ADP) (Figure 2A) were almost identical in the structures of these two ternary complexes. The 2'-fluoro atom in **15-DP** forms a hydrogen bond with N291 that is analogous to that seen with the natural 2'-hydroxyl group in ADP, and the modified base in **15-DP** forms a Watson-Crick pairing with the template base, demonstrating that the non-natural carbon-carbon replacement of the glycosidic bond does not alter the ability of *C*-nucleotides to adopt the necessary conformation for incorporation into the elongating RNA strand. However, in contrast to ADP, in which the 2′-OH group interacts directly with the S282 Oγ atom, the 2′-fluoro-2′-*C*-methyl motif in **15-DP** engenders a conformational change in the side chain of S282, moving it away from the inhibitor. A similar S282 conformation was observed previously in the ternary complex of NS5B with 2′-fluoro-2′-*C*-methyl uridine diphosphate as the incoming nucleotide [14]. The altered S282 conformation provides a rationale for why the loss in activity against the S282T is reduced, as the steric clash between the 2'-*C*-Me group and this residue may be alleviated.




A B

**Figure 2**: Hydrogen bonding networks involved in the recognition at the 2′-position observed for (2A) the NS5B/ADP ternary elongation complex (PDB ID: 4WTD) and (2B) the NS5B/**15-DP** ternary elongation complex (PDB ID: 5UJ2).

While the *C*-nucleosides **14**-**17** were inactive in the replicon assay (Table 1), two of the monophosphate prodrugs (**15-PD** and **17-PD**) showed activity that was similar to the 2'-hydroxyl containing *C*-nucleoside comparator **6** with no apparent cellular toxicity (data not shown). Given the exceptional potency of **14-TP** in the enzyme assay, the lack of cellular activity of **14-PD** is notable.

Cellular antiviral activity is a function of intracellular levels of the triphosphate. To address the SAR apparent in Table 1, intracellular triphosphate levels after continuous prodrug incubation in huh-7 replicon cells were measured (Table 2). [15] The lack of formation of any quantifiable levels of **14-TP** from **14-PD** over the course of 24 hours explains the lack of replicon activity. The corresponding monophosphate of **14** was detected, yet the diphosphate of **14** was not seen. Incubation with **15-PD** and **17-PD** resulted in formation of high levels of **15-TP** and **17-TP**. The 6-fold higher antiviral activity of **15-PD** over **17-PD** can be qualitatively explained by both a higher intrinsic potency in biochemical assays and the higher triphosphate level. Notably, the 2'-hydroxy-containing nucleoside prodrug **6** (Figure 1) produced much higher levels of the corresponding triphosphate, suggesting some limitation of the 2′-F-2′-*C*-Me motif for intracellular activation.

**Table 2.** Nucleoside triphosphate levels after prodrug incubation in huh-7 replicon cells

| Time[a] [h] | TP Concentration [pmol / million cells] | | | |
|---|---|---|---|---|
| | **14-PD** | **15-PD** | **17-PD** | **6** |
| 2 | BLQ[b] | 2.4 | 1.6 | 26 |

| | | | | |
|---|---|---|---|---|
| 6 | BLQ | 13.0 | 5.8 | 117 |
| 24 | BLQ | 20.5 | 3.3 | 83 |

[a] Continuous 24 h incubation with 10 μM of prodrug. Results are the average of duplicate wells from a single side-by-side experiment. [b] Below lower limit of quantification (0.137 pmol / million cells).

The clinical development of ribonucleoside analogs has been hampered by toxicity. Insufficient selectivity for viral over host polymerases has been implicated for many analogs. In particular, the incorporation efficiency by human RNA polymerases needs to be monitored carefully. [16] The potential for toxicity with **15-TP** was studied, with no inhibition of the human DNA or RNA polymerases tested evident (Table 3). Furthermore, incorporation of **15-TP** by the mitochondrial RNA polymerase was minimal (0.04% relative to the rate of incorporation of natural adenosine triphosphate) – a result that stands in contrast to the 2'-hydroxyl series, in which a 1'-substituent was important for limiting incorporation. [6]. The control compound 4'-azido cytidine triphosphate used in this assay showed an incorporation efficiency equivalent to that of the natural substrate (cytidine triphosphate).

**Table 3**: Inhibition of human polymerases for **15-TP** and control compounds[a,b]

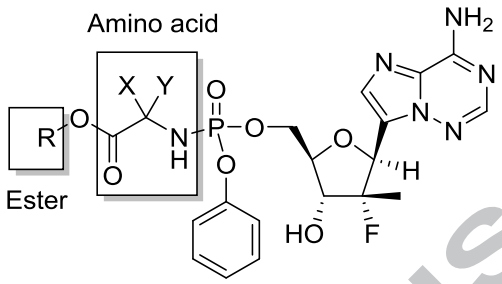
| | RNA Pol II | DNA Pol α | DNA Pol β | DNA Pol γ |
|---|---|---|---|---|
| **15-TP** | >200 | >200 | >200 | >200 |
| Control agent | 0.0024<br>α-Amanitin | 7.3<br>Aphidicolin | 1.4<br>3'-dTTP | 0.74<br>3'-dTTP |

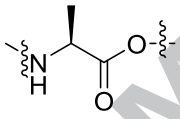
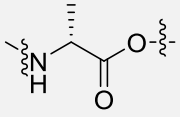
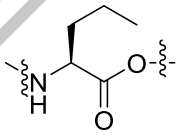
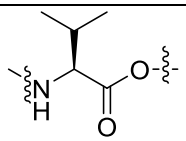
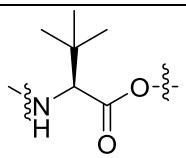
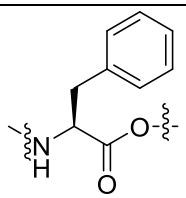
[a] $IC_{50}$ in [μM]. [b] Values represent the average of two independent experiments.

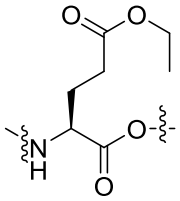
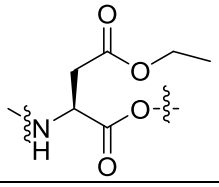
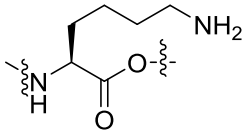
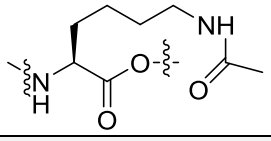
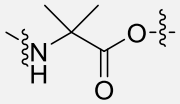
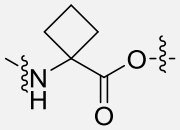
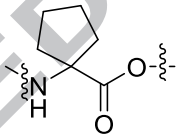
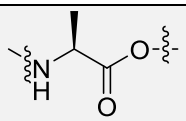
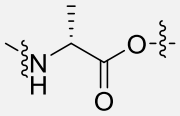
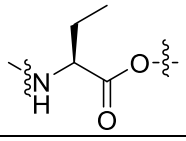
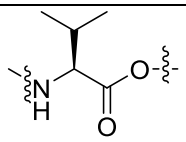
Encouraged by these results, we embarked on a prodrug campaign for nucleoside **15** in which the aminoacid and ester substituents were varied, while the phenol substituent was kept constant. In general, the compounds were tested as stereoisomeric mixtures at phosphorus [17] To monitor for activity beyond genotype 1, and to assess prodrug cleavage and phosphorylation

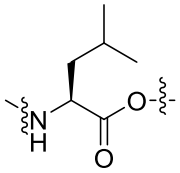
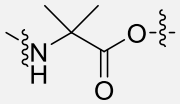
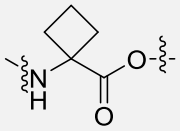
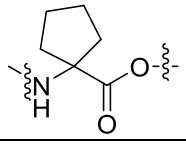
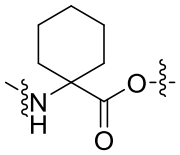
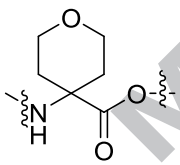
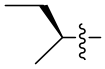
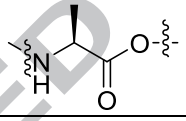
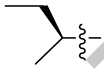
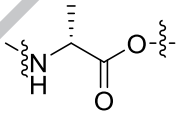
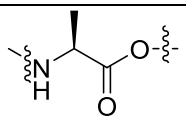
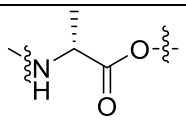
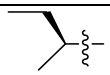
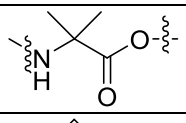
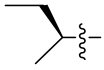
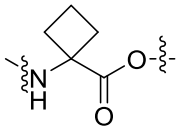
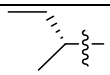
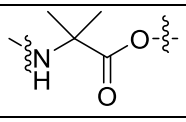
beyond huh-7 cells, a genotype 4a replicon assay constructed in 1C cells was also employed (Table 4).[18]

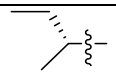
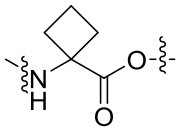
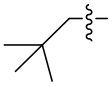
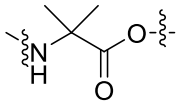
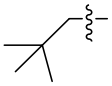
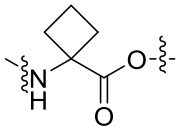
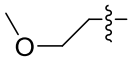
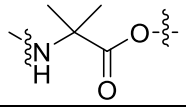
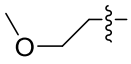
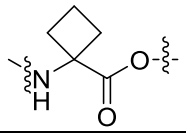
**Table 4**: Potency of phosphoramidate prodrugs in replicon systems for HCV genotypes 1a, 1b, and 4a.[a]




| Compound[b] | Ester (R) | Amino Acid | $EC_{50}$ GT1a [μM] | $EC_{50}$ GT1b [μM] | $EC_{50}$ GT4a [μM] |
|---|---|---|---|---|---|
| **15-PD** |  |  | 0.37 | 0.58 | 0.10 |
| **19** |  |  | 14.7 | 26.4 | 5.7 |
| **20** |  |  | 0.39 | 0.65 | 0.27 |
| **21** |  |  | 35.2 | 37.6 | 11.2 |
| **22** |  |  | >88 | >88 | >88 |
| **23** |  |  | 1.7 | 2.2 | - |

| | | | | | |
|---|---|---|---|---|---|
| **24** |  |  | 1.2 | 3.4 | 0.83 |
| **25** |  |  | 3.3 | 8.6 | 2.3 |
| **26** |  |  | >88 | >88 | 62.5 |
| **27** |  |  | 60.5 | >88 | 86.2 |
| **28** |  |  | 0.59 | 0.80 | 0.15 |
| **29** |  |  | 0.33 | 0.66 | 0.14 |
| **30** |  |  | 1.0 | 2.0 | 0.38 |
| **31**[c] |  |  | 0.51 | 0.67 | 0.11 |
| **32** |  |  | >88 | >88 | 58.1 |
| **33** |  |  | 2.6 | 5.6 | 2.0 |
| **34** |  |  | >88 | >88 | >88 |

| | | | | | |
|---|---|---|---|---|---|
| **35** |  |  | 0.74 | 0.69 | 0.59 |
| **36** |  |  | 5.0 | 41.8 | 1.1 |
| **37** |  |  | 2.3 | 18.8 | 0.38 |
| **38** |  |  | 11.3 | 51.6 | 3.9 |
| **39** |  |  | 68.3 | 83.7 | - |
| **40** |  |  | 14.4 | 29.1 | 7.0 |
| **41** |  |  | 0.81 | 0.67 | 0.46 |
| **42** |  |  | 7.9 | 7.3 | 2.1 |
| **43** |  |  | 0.53 | 0.56 | 0.37 |
| **44** |  |  | >88 | >88 | >88 |
| **45** |  |  | 36.0 | >88 | 3.6 |
| **46** |  |  | 18.8 | 16.1 | 2.1 |
| **47**[b] |  |  | 28.7 | >88 | 1.1 |

| | | | | | |
|---|---|---|---|---|---|
| **48** |  |  | 6.4 | >88 | 0.35 |
| **49** |  |  | 6.2 | 20.3 | 4.3 |
| **50** |  |  | 72.4 | >88 | 77.8 |
| **51** |  |  | 4.3 | 6.2 | 0.82 |
| **52** |  |  | 3.4 | 6.1 | 1.5 |

[a] Shaded rows for compounds further described in tables 5, 6, and 7. [b] Synthesized as described in footnote [23] using stereoisomeric mixtures of the phosphoramidate reagents. [c] Single isomer at phosphorus ($S_P$).

Across the ethyl esters (**15PD** – **30**), those of small, unbranched (*L*)-amino acids were preferred for activity, and the incorporation of a (*D*)-amino acid (**19**) was detrimental. Branching at the amino acid β position (**21**, **22**) or the introduction of side-chain polarity (e.g., **24**-**27**) reduced activity. Conversely, small α,α disubstituted derivatives (**28**, **29**) were particularly potent.

Branching at the ester in the form of the isopropyl derivatives led to a general decrease in cellular activity that was magnified in the sec-butyl analogs (compare, for example, **29**, **36**, **46** and **48**). In the latter, the chiral center at the alkyl ester exerted little influence on activity except in the interesting quartet of alaninyl derivatives **41** – **44**. Here the two (*L*)-alaninyl analogs **41** and **43** were of similar potency, whereas one of the (*D*)-alaninyl pair (**42**) was markedly more active than the other (**44**). The activity of **42** in the face of the lack of activity of **44** and its lower alkyl homologs is suggestive of the involvement of a different and specific esterase. Neopentyl and methoxyethyl esters were also less active.

In general, these analogs were slightly more active in the genotype 4A replicon assay than in the genotype 1 systems. However, for the prodrugs containing amino acids with small

α,α disubstitutions and secondary alkyl esters (**36**, **37**, **47**, **48**), activity in the 4a replicon was 5-20 fold greater. This increased selectivity is likely due to higher expression of carboxyl esterase 1 (CES1) and cathepsin A, hydrolases known to activate phosphoramidate prodrugs. [19] A heightened dependence on CES1, an enzyme known to be highly expressed in the liver [20] and implicated in cleavage of phosphoramidate prodrugs in vivo, was intriguing and stimulated further assessment; for a successful prodrug strategy we envisioned high plasma and intestinal stability coupled with high liver extraction leading to efficient and selective prodrug activation in the target tissue. Toward that end, several selected analogs were compared in hepatic subcellular fraction (S9), intestinal S9 and plasma stability assays in vitro (Table 5). [21]

**Table 5:** Stability assessment of selected prodrugs

| Compound | Plasma stab. human / hamster $t_{1/2}$ [min] | Intestinal S9 stab. human/ hamster $t_{1/2}$ [min] | Liver extraction human / hamster [%] |
|---|---|---|---|
| **15-PD** | 454 / >789 | 134 / 24 | 83 / 76 |
| **19** | 683 / 316 | >789 / 192 | 28 / 84 |
| **28** | 461/ 498 | 129 / 100 | 62 / 83 |
| **29** | 668 / 420 | 175 / 70 | 88 / 91 |
| **31**[a] | >789 / >789 | 590 / 11 | 65 / 97 |
| **32** | 380 / 673 | >789 / 415 | 17 / 85 |
| **36** | 155 / 124 | >789 / 106 | 65 / 86 |
| **37** | 534 / >789 | >789 / 48 | 79 / 93 |

[a] Single isomer at phosphorus ($S_P$).

Sufficient plasma stability was observed across the series. The (D)-alanine derived prodrugs **19** and **32** failed to activate efficiently in human liver S9 fraction and were not pursued further. Given the clinical experience with GS-6620, particular attention was paid to maximizing intestinal stability while retaining rapid liver activation. While the ethyl esters showed promise,

the isopropyl congeners exhibited the greatest stability differential, as desired, especially in combination with a disubstituted amino acid as in **36** and **37**.

Based on these promising results, liver triphosphate levels following oral dosing of **31**, **36**, and **37** at 5 mg-eq/kg in hamster were compared with those for the 2'-hydroxyl nucleoside prodrug **5** (Table 6). [22] (High instability in rat plasma limited the relevance of this species for pre-clinical assessment.)

**Table 6**: In-vivo triphosphate levels in hamster liver after oral dosing, solubility and permeability

| Compound | TP-levels hamster liver[a] [μM] | Solubility pH 7.4[b] [μg/mL] | Caco-2 permeability[c] [x$10^{-6}$cm/s] |
|---|---|---|---|
| **31** | 0.34 | 642 | 0.94 / 16.6 |
| **36** | 1.29 | 657 | 4.2 / 17.6 |
| **37** | 1.89 | 538 | 2.4 / 9.7 |
| **5** | 0.53 | 306 | 0.2 / 8.9 |

a) Determined at t = 1h post dose. b) Amorphous material . c) Measured in the apical (A) to basolateral (B) and B/A directions at 10 μM.

In these experiments, the disubstituted amino acid prodrugs **36**, and **37** out-performed the (*L*)-alanine example **31** as well as **5**. The Caco-2 data for this compound set suggests that higher permeability (and therefore reduced prodrug residence time in enterocytes upon absorption) may contribute to the overall outcome. In a follow-up experiment, **37** was dosed orally in male beagle dogs at 5 mg/kg and >70% fraction absorbed was determined via portal vein sampling. In stark contrast, only 10% of an oral dose of the 2'-hydroxyl nucleoside prodrug **5** was absorbed in comparative studies. [6]

In order to support further development, the two individual phosphorous stereoisomers of **37** were synthesized and tested separately. [23] Key data from these studies are reported in Table 7.

**Table 7**: Isomer comparison for compound **37**

| | $EC_{50}$: 1a [μM] | $EC_{50}$: 1b [μM] | $EC_{50}$: 4a [μM] | Plasma stab. human / hamster $t_{1/2}$ [min] | Intestinal S9 stab. human/ hamster $t_{1/2}$ [min] | Liver extraction[a] human / hamster [%] | Caco-2 permeability[b] [$x10^{-6}$ cm/s] |
|---|---|---|---|---|---|---|---|
| **53** | 0.80 | 3.0 | 0.44 | 546 / 218 | >789 / 99 | 76 / 90 | 1.7 / 9.3 |
| **54** | 7.3 | 31.2 | 0.74 | >789 / 430 | >789 / 18 | 78 / 96 | 1.3 / 9.2 |

[a] Human / hamster liver S9 fraction used. [b] Measured in the apical (A) to basolateral (B) and B/A directions at 10 μM compound concentration.

Although **53** exhibited greater potency in both genotype 1 replicons, the activity of the two isomers was within 2-fold in the more optimal enzymatic background of the 4a replicon assay. Overall, the profile of **53** was slightly better than that of **54** in terms of potency, oral absorption potential and hamster liver triphosphate levels (~30% increase).

The favorable characteristics of **53** led to its progression into toxicology studies in rats and monkeys. Following 28 days of dosing, **53** appeared well tolerated at 1,000 and 30 mg/kg/day in rats and monkeys, respectively. However, closer inspection during histopathology found vacuolization in the brains of rats treated with **53** in two independent studies in a somewhat dose dependent manner. This unexpected effect may have been associated with the unnatural amino acid but the exact mechanism remains to be investigated.

In summary, we discovered the 2'-fluoro-2'-*C*-methyl adenosine *C*-nucleoside **15** as a potent and selective inhibitor of HCV polymerase and a series of effective phosphoramidate prodrugs of it. Replacement of the 2'-hydroxyl to the 2'-fluoro afforded increased activity against WT enzyme, reduced S282T resistance, and resulted in high oral absorption potential. Importantly, efficient delivery of the triphosphate into liver tissue was achieved with prodrugs containing a matched combination of branched alkyl ester and disubstituted amino acid.

**References and Notes**

[1] Lavanchy, D., *Clin. Microbiol. Infect*. **2011**, *17*, 107-115

[2] Zeuzem, S.; Berg, T.; Moeller, B.; Hinrichsen, H.; Mauss, S.; Wedemeyer, H.; Sarrazin, C.; Hueppe, D.; Zehnter, E.; Manns, M. P., J. *Viral Hepatitis* **2009**, *16*, 75-90

[3] http://hepatitiscnewdrugresearch.com/approved-treatments-for-hepatitis-c.html

[4] Sofia, M. J., *Antiviral Chem. Chemother*., **2011**, *22*, 23-49.

[5] Migliaccio, G.; Tomassini, J. E.; Bosserman, M. R.; Ceccacci, A.; Colwell, L. F.; Cortese, R.; Francesco R. D.; Eldrup, A. B.; Getty, K. L.; Hou, Z. S.; LaFemina, R. L.; Ludmerer, S. W.; MacCoss, M.; MacMasters, D. R.; Stahlhut, M. W.; Olsen, D. B.; Hazuda, D. J.; Flores, O. A., *J. Biol. Chem*., **2003**, *278*, 49164-49170

[6] Cho, A.; Zhang, L.; Xu, J.; Lee, R.; Butler, T.; Matebo, S.; Aktoudianakis, V.; Lew, W.; Ye, H.; Clarke, M.; Doerffler, E.; Byun, D.; Wang, T.; Babusis, D,; Carey, A. C.; German, P.; Sauer, D.; Zhong, W.; Rossi, S.; Fenaux, M.; McHutchison, J. G.; Perry, J.; Feng, J.; Ray, A. S.; Kim, C.U. *J. Med. Chem*., **2014**, *57*, 1812-1825.

[7] Feng, J. Y.; Cheng, G.; Perry, J.; Barauskas, O.; Xu, Y.; Fenaux, M.; Eng, S.; Tirunagari, N.; Peng, B.; Yu, M.; Tian, Y.; Lee, Y.-J.; Stepan, G.; Lagpacan, L. L.; Jin, D.; Hung, M.; Ku, K. S.; Han, B.; Kitrinos, K.; Perron, M.; Birkus, G.; Wong, K. A.; Zhong, W.; Kim, C-U.; Carey, A.; Cho, A.; Ray, A. S. *Antimicrob. Agents Chemother*., **2014**, *58*, 1930-1942. Murakami, E.; Wang, T.; Babusis, D.; Lepist E.-I.; Sauer, D.; Park, Y.; Vela, J. E.; Shih, R.; Birkus, G.; Stefanidis, D.; Kim, C.U.; Cho, A. Ray, A. S., *Antimicrob. Agents Chemother*., **2014**, *58*, 943-1951.

[8] Lipinski, C. A.; Lombardo, F.; Dominy, B. W., Feeney P. J., *Adv. Drug Delivery Rev.*, **1997**, *23*, 3-25.

[9] Chang, W.; Bao, D.; Chun, B.-K.; Naduthambi, D.; Nagaranatham, D.; Rachakonda, S.; Reddy, P. G.; Ross, B. S.; Zhang, H.-R.; Bansal, S.; Espiritu, C. L.; Keilman, M.; Lam, A. M.; Niu, C.; Micolochick Steuer, H.; Furman, P. A.; Otto, M. J.; Sofia, M. J. *ACS Med. Chem. Letters* **2011**, *2*, 130-135.

[10] McGuigan, C.; Mehellou, Y.; Balzarini, J. *ChemMedChem.*, **2009**, *4*, 1779-1791. Sofia, M. J., *Antiviral Chem. Chemother.*, **2011**, *22*, 23-49. McGuigan, C.; Gilles, A.; Madela, K.; Aljarah, M.; Holl, S.; Jones, S.; Vernachio, J.; Hutchins, J.; Ames, B.; Bryant, K. D.; Gorovits, E.; Ganguly, B.; Hunley, D.; Hall, A.; Kolykhalov, A.; Liu, Y.; Muhammed, J.; Raja, N.; Walters, R.; Wang, J.; Chamberlain, S.; Henson, G. *J. Med. Chem.*, **2010**, *53*, 4949-4957.

[11] Cho, A.; Saunders, O. L.; Butler, T.; Zhang, L.; Xu, J.; Vela, J. E.; Feng, J. Y.; Ray, A. S.; Kim, C. U. Bioorg. Med. Chem. Letters, 2012, 22, 2705-2707. Cho, A. ; Zhang, L.; Xu, J.; Babusis, D,; Butler, T.; Lee, R.; Saunders, O. L.; Wang, T.; Parrish, J.; Perry, J.; Feng, J. Y.; Ray, A. S.; Kim, C. U. *Bioorg. Med. Chem. Letters*, **2012**, *22*, 4127-4132. For additional work enabling the efficient synthesis of 2'-*C*-Methyl *C*-Nucleosides please also see: Tokarenko, A.; Slavetinska, L. P.; Klepetarova, B.; Hocek, M. *Eur. J. Org. Chem.*, **2015**, 7962-7983. Draffan, A. G.; Frey, B.; Pool, B.; Gannon, C.; Tyndall, E. M.; Lilly, M.; Francon, P.; Hufton, R.; Halim, R.; Jahangiri, S.; Bond, S.; Nguyen, V. T. T.; Jeynes, T. P.; Wirth, V.; Luttick, A.; Tilmanis, D.; Thomas, J. D.; Pryor, M.; Porter, K.; Morton, C. J.; Lin, B.; Duan, J.; Kukolj, G.; Simoneau, B.; McKercher, G.; Lagace, L.; Amad, M.; Bethell, R. C.; Tucker, S. P. *ACS Med Chem Letters* **2014**, *5*, 679-684.

[12] McGuigan C.; Cahard, D.; Sheeka, H. M.; De Clercq, E.; Balzarini, J., *J. Med. Chem.*, **1996**, *39*, 1748-1753

[13] Brief description of crystallization conditions: Crystals were grown at 4 °C using the sitting drop vapor diffusion method with 0.4 μL of NS5B (2a JFH-1 C-terminal Δ21 with S15G, C223H, V321I mutations and a Δ8 β-hairpin loop deletion) at 5 mg/mL (~70 μM) in 5 mM Tris pH 7.5, 200 mM ammonium acetate, 1 mM EDTA, 1 mM DTT and an equal volume of precipitant against 80 μL of reservoir (25% PEG 550 MME, 50 mM $MgCl_2$, 0.1 M Hepes pH 7.5) over weeks to months. To obtain the ternary complex, crystals were soaked at 4 °C over three days in a modified reservoir (28% PEG 550 MME, 8% ethylene glycol, 0.2 M ammonium acetate, 50 mM BisTris propane pH 6.0, 50 mM Tris pH 7.2), **15-DP** (10 mM), $MnCl_2$ (6 mM), and the symmetrical RNA primer template pair, 5′-AUAAAUUU, (2 mM). For more details please see [14]. The diphosphate (**15-DP**) was synthesized in a way similar to that of the triphosphate, except that $Bu_4N$ phosphate was used instead of $Bu_4N$ pyrophosphate.

[14] Appleby, T. C.; Perry J. K.; Murakami, E.; Barauskas, O.; Feng, J.; Cho, A.; Fox III, D.; Wetmore, D. R.; McGrath, M. E.; Ray, A. S.; Sofia, M. J.; Swaminathan, S.; Edwards, T. E. *Science*, **2015**, *347*, 771-775.

[15] **Measurements of Triphosphate Levels in Replicon Cells.** Replicon cells (Huh-7 cells) were maintained in Dulbecco's modified eagle medium containing glutaMAX supplemented with 10% heat inactivated fetal bovine serum, penicillin-streptomycin, and G418 disulfate salt solution. The cells were transferred to 12-well tissue culture plates treated by trypsonization and grown to confluency ($0.88 \times 10^6$ cells/well). The cells were then treated for 24 hours with a test compound (10 μM), and then washed twice with 2.0 mL ice cold 0.9% sodium chloride saline, scraped into 0.5 mL 70% methanol, and frozen overnight to facilitate the extraction of nucleotide metabolites. The extract was transferred into tubes, dried, and re-suspended in 1 mM ammonium phosphate (pH 8.5). TP levels were quantified using liquid chromatography coupled to triple quadrapole mass spectrometry (LC-MS/MS) 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.

[16] Feng, J. Y.; Xu, Y.; Barauskas, O.; Perry, J.; Ahmadyar, S.; Stepan, G.; Yu, H.; Babusis, D.; Park, Y.; McCutcheon, K.; Perron, M.; Schultz, B. E.; Sakowicz, R.; Ray, A. S., *Antimicrob Agents Chemother*., **2016**, *60*, 806-817.

[17] Tyndall, E. M.; Draffan, A. G.; Frey, B.; Pool, B.; Halim, R.; Jahangiri, S.; Bond, S.; Wirth, V.; Luttick, A.; Tilmanis, D.; Thomas, J.; Porter, K.; Tucker, S. P., *Bioorg. Med. Chem. Letters*, **2015**, *25*, 869-873.

[18] Peng, B.; Yu, M.; Lee, Y.-J.; Tian, Y.; Yang, H.; Chan, K.; Mo, H.; McHutchison, J.; Delaney IV, W.; Cheng, G. *Gastroenterology*, **2013**, *144*, 59-61.

[19] Murakami, E.; Tolstykh, T.; Bao, H.; Niu, C.; Micolochick-Steuer, H. M.; Bao, D.; Chang, W.; Espiritu, C.; Bansai, S.; Lam, A. M.; Otto, M. J.; Sofia, M. J.; Furman, P. A., *J. Biol. Chem.*, **2010**, *285*, 34337-34347. Murakami, E.; Wang, T.; Park, Y.; Hao, J.; Lepist, E.-I.; Babusis, D,; Ray, A. S., *Antimicrob. Agents Chemother*., **2015**, *59*, 3563-3569.

[20] Imai, T.; Taketani, M.; Shii, M.; Hosokawa, M.; Chiba, K. *Drug Metab. Disp*., **2006**, *34*, 1734-1741. Imai, T. *Drug Metab. Pharmacokinet.*, **2006**, *21*, 173-185.

[21] **Stability in blood plasma:** Test compounds were incubated at 2 μM in hamster, rat, or human plasma samples for up to 4 hours at 37 °C. At the desired time points, aliquots from the incubation were quenched by adding 9 volumes of 100% acetonitrile supplemented with an internal standard. The samples were centrifuged at 3,000×*g* for 30 minutes and the supernatants were transferred to new plates containing an equal volume of water for analysis by LC-MS/MS. **Intestinal and Hepatic S9 Stability.** Test compounds were incubated at 2 μM in human intestinal and hepatic S9 fractions (obtained from In Vitro Technologies, Baltimore, MD) for 90 min at 37 °C in the presence of NADPH and UDPGA (Phase I and Phase II cofactor, Sigma-Aldrich). At specified time points following compound addition, the samples were quenched with 9 volumes of an aqueous solution containing an internal standard, 50% acetonitrile and 25% methanol. The sample plates were centrifuged at 3000g for 30 min, and 10 μL of the resulting solutions were analyzed by LC/MS/MS. The data (ratio of the sample to the internal standard peak area) were plotted on a semi log scale and fitted using an exponential fit. Assuming the first order kinetics, half-life and rate of metabolism were determined. Predicted hepatic extraction was calculated from the half-life by reported methods using the well-stirred model for hepatic clearance. LC/MS/MS Instrumentation.: Liquid chromatography was performed using an Agilent 1200-series quaternary pump system (Agilent Technologies, Santa Clara, CA) with a 2 μm 20 × 2.1 mm Mercury RP $C_{18}$ (Phenomenex, Torrance, CA). HPLC system was coupled to a Quattro Premier triple-quadrupole mass spectrometer (Waters, Milford, MA). Mass spectrometry was performed in positive-ion mode and multiple reaction monitoring modes using a Quattro Premier (Waters, Milford, MA). The test compound was eluted with a mobile phase consisting of 0.2% formic acid and a linear gradient from 0 to 95% acetonitrile over 2 minutes.

[22] **Hamster Liver Triphosphate Analysis.** Groups of 12 Golden Syrian hamsters were administered a test compound orally at 5 mg-eq/kg. The formulation consisted of EtOH, PEG400 and water, and buffered with 50 mM pH 4 citrate (2.5 mg-eq/mL) for oral dosing. At 1, 4, 8 and 12 hr, 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 TH$^{TM}$ with disposable, hard tissue homogenizer probes (Omni International). Aliquots of homogenate were then centrifuged (20,000xg at 4$^{o}$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. The levels of endogenous adenosine triphosphate were also determined to assure the sample stability.

[23] Synthesis: Nucleoside **15** (500 mg, 1.8 mmol) was dissolved in NMP (5 mL) and THF (5 mL), and the solution was cooled to 0 °C. A solution of *tert*-butylMgCl (1.0M in THF, 2.6 mL) was added dropwise. The cooling was removed and stirring was continued for 30 minutes. A solution of *iso*propyl 1-(((4-nitrophenoxy)(phenoxy)phosphoryl)amino)cyclobutane-1-carboxylate (1.3 g 1.6 eq.) in THF (13 mL) was added. The reaction mixture was stirred at room temperature for 18 hours, which was diluted with EtOAc and quenched with aqueous saturated ammonium chloride solution. The organic layer was washed sequentially with aqueous sodium hydroxide (0.1M), water, and brine, and dried over sodium sulfate. After filtration and evaporation of solvents, the residue was purified via flash silica gel chromatography (eluent: A: 20%MeOH in EtOAc and B: hexanes). The product was isolated in 65% yield. Compound **53**: $^{1}$H NMR (400 MHz, MeOH –d4) δ = 8.04 (s, 1H), 7.61 (s, 1H), 7.35-7.28 (m, 2H), 7.25 (m, 2H), 7.15 (t, *J* = 6.8 Hz, 1H), 5.64 (d, *J* = 25.0 Hz, 1H), 5.03-4.96 (m, 1H), 4.57 (dd, *J* = 10.7/4.8 Hz, 1H), 4.47-4.33 (m, 1H), 4.12 (m, 2H), 2.48 (m, 2H), 2.30 (m, 2H), 1.96-1.80 (m, 2H), 1.24 (m, 6H), 1.16 (d, J = 22 Hz, 3H) ppm. $^{19}$F NMR (376MHz, MeOH-d4) δ = -156ppm. $^{31}$P NMR (162MHz, MeOH –d4): δ = 2.58 ppm.

The phosphoramidate reagent (*iso*propyl 1-(((4-nitrophenoxy) (phenoxy)phosphoryl) amino) cyclobutane-1-carboxylate) was prepared as the racemic mixture according to [6]. The isomers were then separated via chiral chromatography [Chiralpak AY 20 μm; mobile phase EtOH; 40 °C]. The earlier eluding fraction was taken and crystalized from EtOAc and heptane, which was used for preparation of **53**.

Graphical abstract