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Discovery of GS-9131: Design, synthesis and optimization of amidate prodrugs of the novel nucleoside phosphonate HIV reverse transcriptase (RT) inhibitor GS-9148

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ABSTRACT

GS-9148 [(5-(6-amino-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-yloxymethyl)phosphonic acid] **4** is a novel nucleoside phosphonate HIV-1 reverse transcriptase (RT) inhibitor with a unique resistance profile toward N(t)RTI resistance mutations. To effectively deliver **4** and its active phosphorylated metabolite **15** into target cells, a series of amidate prodrugs were designed as substrates of cathepsin A, an intracellular lysosomal carboxypeptidase highly expressed in peripheral blood mononuclear cells (PBMCs). The ethylalaninyl phosphonamidate prodrug **5** (GS-9131) demonstrated favorable cathepsin A substrate properties, in addition to favorable in vitro intestinal and hepatic stabilities. Following oral dosing (3 mg/kg) in Beagle dogs, high levels (>9.0 μM) of active metabolite **15** were observed in PBMCs, validating the prodrug design process and leading to the nomination of **5** as a clinical candidate.

1. Introduction

The worldwide burden of HIV has continued to increase over the last decade and revised WHO estimates now place the infected population between 30.6 and 36.1 million.[1] No vaccine or cure is available for infected patients, but tremendous advances have been made in the development of chemotherapeutic treatments to suppress viral replication. These efforts have resulted in the clinical approval of more than two dozen HIV drugs, with diverse mechanisms of action, and their use in combination therapy, now known as, highly active anti-retroviral therapy (HAART).[2] The selection of two nucleos(t)ide HIV reverse transcriptase (RT) inhibitors (N(t)RTIs) from this portfolio of drugs has emerged as the backbone of choice in the most effective HAART regimens.[3] Two examples of commonly prescribed N(t)RTIs are TDF **1**, an ester based prodrug of the nucleoside phosphonate TFV **2**, and FTC **3**, which together with the non-nucleoside inhibitor, efavirenz, form Atripla™, a once daily (QD) fixed dose combination regimen recommended for treatment naïve HIV-infected patients (Fig. 1). The success of HAART therapy has now increased life expectancy among infected patients to >20 years, in effect, transforming HIV into a manageable chronic disease. In this context, the durability of therapy can be limited by drug resistance, side effects as a result of chronic use, and drug-drug interactions. Some of the adverse effects observed during chronic therapy include neuropathy, hepatotoxicity, peripheral lipoatrophy and lactic acidosis. The ability of the active triphosphate metabolites of currently approved N(t)RTIs to interfere with mitochondrial DNA replication are likely related to some of these side effects.[4] Therefore, we embarked on a program to identify a novel N(t)RTI with improved resistance profile, safety, and long term tolerability that resulted in the identification of GS-9148 (**4**) and its orally bioavailable amidate prodrug GS-9131 (**5**).[5,6]

In this report a second generation, more robust and scalable synthesis of **4** is described followed by the design, synthesis and SAR of an extensive array of amino acid based prodrugs, designed as substrates for lysosomal carboxypeptidase cathepsin A (Cat A). The high activity of Cat A in lymphoid cells provided a vehicle to preferentially 'target' the novel phosphonate to tissues where HIV replicates in vivo. Prodrugs were evaluated across a panel of stability assays to identify the optimal oral candidates from which prodrug **5** was selected as a clinical candidate.

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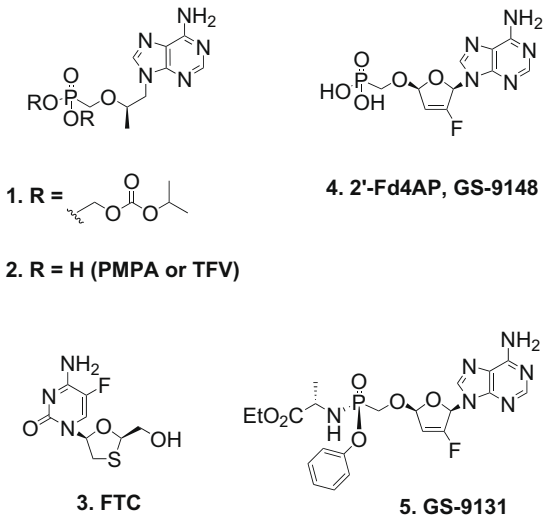
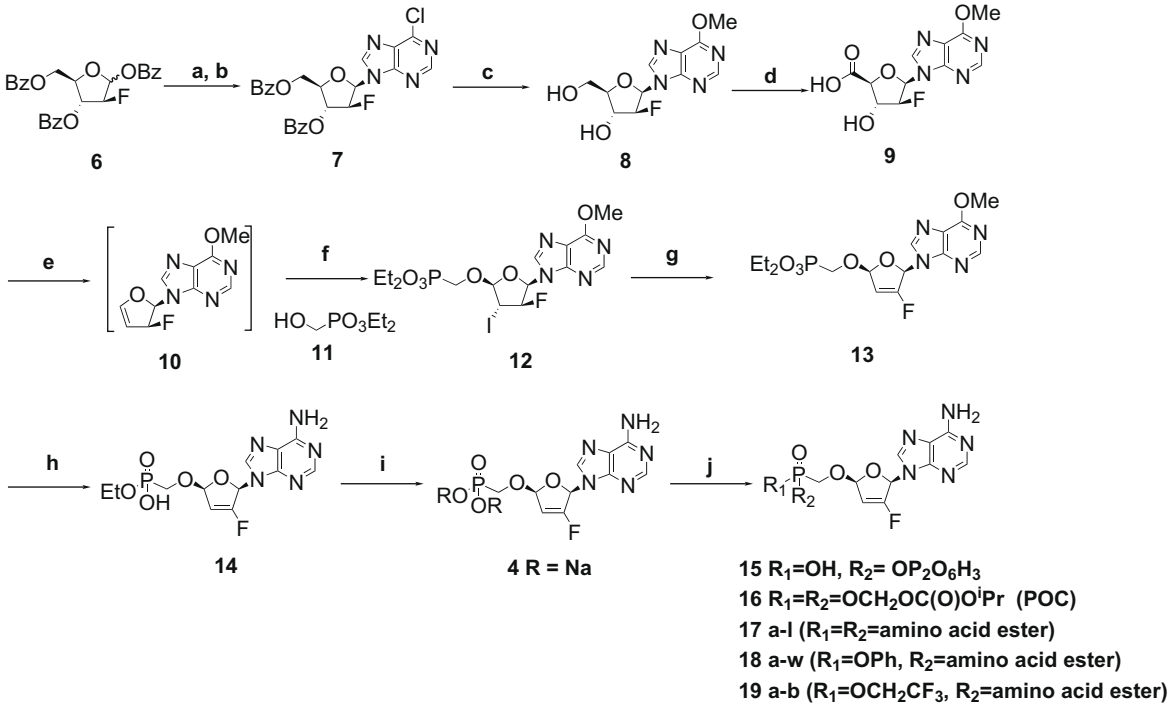


Figure 1. N(t)RTI and NRTI inhibitors of HIV reverse transcriptase.

2. Chemistry

The first generation synthesis of **4** was derived from an earlier route reported for the non-fluoro analog, but yielded only small amounts of material over 16 steps.[6] Clearly, prodrug development of **4**, especially in vivo optimization would require a more robust and scalable synthesis. The second generation synthesis is shown in Scheme 1 and includes some key distinctions from the first generation route. Commercially available fluoro sugar **6** was chosen as the ideal starting material since it was readily available in kilogram amounts. The route to **4** funnels through the glycal intermediate **10**, a key intermediate that allows for the stereocontrolled introduction of the hydroxymethyl phosphonomethoxy synthon, **11**, *cis* to the purine. Unlike the first generation route, glycal intermediate **10** has a 6-methoxy substituted purine, rather than 6-aminopurine. Introducing, the amino group early in the first generation route, ultimately required multiple protection and deprotections with trityl, formamidine and pivaloyl groups, thus contributing a significant number of additional steps. The 6-methoxy group avoided all these laborious steps and could be transformed to the 6-amino group late in the synthesis. In the forward route, fluoro sugar **6** was converted via the anomeric bromide and addition of 6-chloropurine sodium salt, into **7**.[7] Using precipitation, the pure N-9 substituted β-anomer **7** was readily isolated in 54% yield from the α-anomer and the regioisomeric N-7 isomers. Alternative 6-aminopurine precursors, for example, 6-NHBz, 6-Br, and 6-SMe purines were considered but none were found to be superior in yield and isolation ease to 6-chloropurine. The benzoyl esters were removed by treatment with potassium carbonate in methanol, which also introduced the 6-methoxy group to provide nucleoside **8**. Selective oxidation of the 5′-hydroxyl was efficiently carried out in one step using Jones reagent to generate acid **9** which was readily recrystallized to remove all chromium impurities. Glycal formation at elevated temperatures using dimethylformamidine dineopentyl acetal provided the desired intermediate **10** but only in low yield.[8] The high temperature required was well suited to the synthesis of the non-fluorinated analog but the presence of the 2-fluorine group in **10** led to elimination of HF. Milder methods for effecting the decarboxylative dehydration were therefore explored. Activation under Mitsunobu conditions proved to be the most effective at lower temperatures and allowed the glycal **10** to be formed in high yield.[9] In the first generation synthesis phenyl selenium chloride was used to activate the glycal for nucleophilic addition of the phosphonate synthon **11**, but in the interest of sca-



Scheme 1. Second generation synthesis of **4** and its prodrugs. Reagents and conditions: (a) 33% HBr/AcOH, CH_2Cl_2, quant.; (b) 60% NaH, CH_3CN, 6-chloropurine, 54%; (c) K_2CO_3, MeOH, 84%; (d) acetone, Celite545, Jones' reagent, 67%; (e) PPh_3, DIAD, CH_2Cl_2; (f) IBr, $HOCH_2PO_3Et_2$, CH_2Cl_2, 55% two steps; (g) NaClO, MeOH, AcOH, 88%; (h) NH_4OH (aq), 110 °C; (i) TMSBr, 2,6-lutidine, CH_3CN, 68%; (j) For **15** see Ref. 11a; for **16**, *N*-methyl pyrrolidinone, TEA, $ClCH_2OCO_2iPr$ (9 equiv), 7%; for **17a–l**, amino ester, Et_3N, 2,2′-dithiodipyridine, PPh_3, pyridine, 11–73%; for **18a–w**, amino ester, PhOH, Et_3N, 2,2′-dithiodipyridine, PPh_3, pyridine, 13–52%; for **19a–b**, amino ester, CF_3CH_2OH, Et_3N, 2,2′-dithiodipyridine, PPh_3, pyridine, 32–33%.

lability, less toxic alternatives were tried. Phenylsulfide activation led to multiple products of the desired mass by LC–MS which was interpreted to be the result of unselective facial activation of the glycal due to the smaller sulfur reagent compared to selenium. Iodide activation however, resulted in the observation of only one product with the desired mass. Phosphonate **12** was obtained in gram scale and a robust 55% yield when the intermediate glycal **10** was treated with IBr followed by the phosphonate synthon **11**. Unsaturated intermediate **13** was obtained in 88% yield using buffered sodium hypochlorite oxidation of the iodine, followed by elimination.[10] Triphenylphosphine oxide traces were removed by dissolution of the product in EtOAc followed by filtration. With all the essential groups of the target molecule introduced the remaining steps involved conversion of the 6-OMe group to adenine and removal of the phosphonate esters. Treatment of **13** with concentrated ammonium hydroxide generated 6-aminopurine **14**, and removed one of the phosphonate esters. Subsequent dealkylation using TMSBr in CH_3CN then afforded diacid **4** isolated as the sodium salt in an overall yield of ~10% over eight steps.

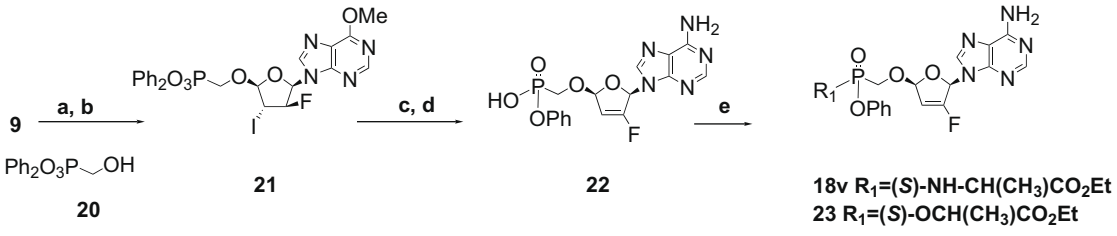
Diphosphate **15** and bisPOC prodrug **16** were prepared from the diacid **4** using reported procedures.[11a] Phosphonic diamide (often referred to as bisamidate) prodrugs **17a–l** were also prepared from **4** using 2,2′-dithiodipyridine and triphenylphosphine activation followed by treatment with the desired amino acid ester. Monoamidate prodrugs were prepared by utilizing a mixture of phenol and amino acid ester in the case of **18a–w**, or trifluoroethanol and amino acid ester in the case of **19a–b**. The monoamidate methods all afforded mixtures of isomers at phosphorus which, based on proton and phosphorus NMR analysis, were approximately 1:1. Separation of the diastereoisomers could be achieved using chiral HPLC chromatography as described in the experimental methods. The chiral separation of mixture **18v** resulted in the isolation of **18v**(**B**) (**5**) as the second eluting isomer.

An alternative method for generation of the phenol based monoamidate mixtures is shown in Scheme 2. In this alternative procedure, diphenyl hydroxymethyl phosphonate synthon **20** replaced the previous diethyl phosphonate **11**. Upon addition of **20** to the same glycal intermediate, diphenyl phosphonate **21** was obtained. Oxidation of iodine and elimination of HI, followed by conversion of the 6-methoxy to 6-aminopurine, provided the monoacid **22** suitable for the direct incorporation of amino acid esters to provide, for example, **18v** as an isomeric mixture that contains **5**. Lactate prodrug **23** was prepared from **22** by substituting the lactate ester for the amino acid ester.

2.1. Antiviral activity and cathepsin A cleavage of prodrugs

The 2′-fluoro analog **4** was discovered following broad SAR studies on a range of nucleoside phosphonates analogs.[6,11] Earlier reports had shown that the non-fluorinated analog of **4**, although an effective inhibitor of HIV RT with a promising resistance profile, interfered with host mitochondrial DNA replication in HepG2 cells (MTC_{50} = 3.6 μM).[5a,6,11] The 2′-fluoro analog was rationally designed as a more selective RT inhibitor based on structural differences between HIV RT and a molecular model of mitochondrial DNA polymerase γ, in addition to literature reports demonstrating reduced mitochondrial toxicity for 2′-F modified nucleoside analogs.[12] Upon synthesis and evaluation, the fluoro analog **4** reduced mitochondrial cytotoxicity in HepG2 cells (MTC_{50} >300 μM) and the inhibition of mitochondrial DNA polymerase γ by the active metabolite **15** exceeded 100 μM.[5,6] The inhibition of wild type RT by **15** was only marginally reduced by adding the fluorine leading to a selectivity of >130-fold over mitochondrial DNA polymerase γ inhibition. Finally, extensive resistance profiling toward many clinical isolates, demonstrated that analog **4** had a unique and promising resistance profile compared to the FDA approved N(t)RTIs.[5a] Thus, the favorable RT inhibition, selectivity, and resistance profile of **4** led to its selection for prodrug optimization to develop an orally bioavailable RT inhibitor.

A series of phosphonamidate and phosphonic diamide prodrugs were designed on the parent molecule to mask the diacid group, a strategy that had been developed for phosphate prodrugs and was recently applied to phosphonate **2** with considerable success.[13,14] Phosphonate monoamidate **24** (GS-7340), a second generation prodrug of **2**, was developed to improve systemic prodrug circulation and undergo selective cleavage inside target lymphatic cells (Fig. 2). In beagle dogs, oral dosing of **24**, improved the PBMC-to-plasma AUC_{0-24} ratio of **2** by 34-fold compared to that of bisPOC prodrug **1**.[14] Furthermore, in pivotal phase 1 studies, **24** achieved proof-of-concept for enhanced delivery of **2** to PBMCs in patients. Recently, **24** was reported to be an excellent substrate for lysosomal carboxypeptidase cathepsin A (Cat A) which initiates the intracellular breakdown of the prodrug inside PBMCs (Fig. 2).[15] Once the initial ester is cleaved to acid **25** the subsequent breakdown is proposed to go through an cyclized intermediate **26** by displacement of one phosphorous leaving group, in this case, phenol. The cyclic intermediate is then hydrolyzed and the remaining P–N bond cleaved to release the phosphonic diacid. Histidine triad nucleotide binding proteins (Hints) have been implicated in the cleavage of the P–N bond of phosphoramidate prodrugs.[16] These proteins or similar proteins may have a role in the phosphonate P–N bond cleavage but this late step in the phosphonate prodrug breakdown has not been elucidated yet. Prodrug design for lead compound **4** centered on targeting cleavage by the initial enzyme in the pathway, Cat A to preferentially deliver **4** to the PBMCs. The prodrugs were initially evaluated in vitro by measuring prodrug cleavage rates under exposure to recombinant Cat A, cleavage rates in human PBMC extracts, and also antiviral activity and toxicity in MT-2 cells (Table 1). Importantly, a positive correlation between Cat A activity and hPBMC extract activity (R^2 = 0.88 for all values represented in Table 1) was found for the prodrugs and provides support for Cat A being the dominant enzyme involved in the initial breakdown of the prodrugs inside PBMCs. Table 1 also shows that although many of the prodrugs demonstrated significantly



Scheme 2. Alternative synthesis of monoamidate prodrugs and lactate prodrug **23**. Reagents and conditions: (a) PPh_3, DIAD, CH_2Cl_2; (b) IBr, $HOCH_2PO_3Ph_2$, CH_2Cl_2; (c) NaClO, MeOH, AcOH; (d) NH_4OH (aq); (e) for **18v**, (L)-alanine amino acid ethyl ester·HCl, DMF, PyBOP, TEA, 30%; for **23**, 2-(*S*)-hydroxypropionic acid ethyl ester, DMF, PyBOP, TEA, 19%.

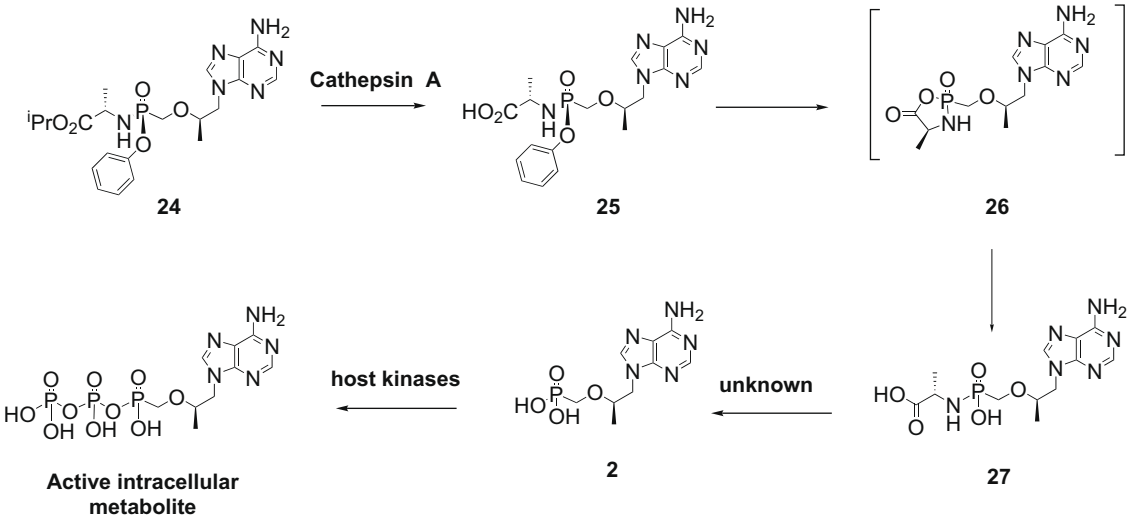


Figure 2. Proposed amidate prodrug breakdown pathway utilizing cathepsin A. Cathepsin A cleaves the isopropyl ester to acid **25**, which in turn cyclizes to **26** releasing phenol. Hydrolysis of the P–O bond to **27** is then followed by cleavage of the P–N bond to generate **2**.

improved antiviral activity compared to the parent **4**, cell based toxicities in MT-2 cells were mostly in the high micromolar range. One isomer of the monoamidate ethyl ester mixture, **18v**(**B**) (**5**), later chosen as the optimal prodrug, demonstrated good selectivity in MT-2 cells (CC_{50} >100 μM, Table 1).

The diamide prodrugs **17a–l** were explored extensively in the early stages of the optimization process since they avoided the generation of a phosphorous chiral centre. Lipophilic phenylalanine (Phe) ester prodrugs **17a–c** were the best substrates for Cat A, superior in some cases to **24**! Consistent with the high cleavage rate these prodrugs demonstrated potent antiviral activity below 10 nM, >10-fold more potent than the bisPOC prodrug **16**. The less hydrophobic alanine (Ala) esters **17g–j** were weaker Cat A substrates, but nevertheless, **17g**, for example, still demonstrated improved antiviral activity over **16**. Unnatural amino acid, 2-L-aminobutyric acid (ABA) analogs **17d–f**, and glycine (Gly) analogs, for example, **17k–l** were the poorest substrates.

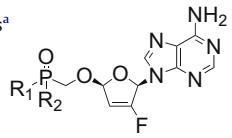
A large array of monoamidate prodrugs (diastereomeric mixtures) bearing phenol, **18a–w**, or trifluoroethoxy, **19a–b**, and one amino acid ester were also evaluated. Comparing four different isobutyl esters **18d**, **18k**, **18q** and **18w** established a similar trend to the diamides in that the Phe and Ala based prodrugs are superior Cat A substrates than ABA and Gly, leading to more potent antiviral activity. Similarly, the more lipophilic Phe esters **18a–i** had the highest Cat A activities and the most potent antiviral activity (EC_{50} range 3.8–199 nM) compared to the Ala esters **18l–v**. Proximal branching of the ester group in the Phe series, examples **18a–b** (*sec*-butyl(S) and *sec*-butyl(R), respectively) and **18f** (*i*Pr) led to significantly reduced Cat A cleavage rates and reduced antiviral activities relative to the linear *n*-alkyl esters. In the alanine series the proximally branched 3-pentyl ester **18l** and isopropyl ester **18s** also had very poor Cat A cleavage rates. The fact that the alanine isopropyl ester was a poor Cat A substrate whereas the same prodrug **24** (albeit a single isomer) is an excellent substrate, suggests that the parent nucleoside phosphonate is an important recognition aspect for Cat A. The purpose for exploring proximally branched esters was to try and modify the balance between Cat A activity and in vitro hepatic stability (see next section). Given the poor Cat A activity, constrained, proximally branched esters, for example, **18r** (cyclo-butyl) were attempted and found to be surprisingly good substrates. The lactate ester **20** was not a good substrate for Cat A and established that an amino acid is preferred for at least one of the two prodrug groups. In general, provided the ester group was linear, branched non-proximally, or proximally branched in a small ring, the natural L-configured amino acids Ala or Phe provided sufficient cleavage by Cat A to result in antiviral activities below 100 nM, more than two orders of magnitude improved over the diacid **4**. Examples **19a** and **19b** are interesting analogs in which the phenol is replaced with trifluoroethoxy group. Cat A breaks down the trifluoroethoxy prodrug **19a** with similar efficiency to the equivalent phenol analog **18d** yet has 20-fold reduced potency in the antiviral assay? This difference in antiviral activity, most likely, reflects differences in subsequent steps of the prodrug breakdown, perhaps displacement of the trifluoroethyl group by the amino acid in formation of the cyclic intermediate (Fig. 2).

The more promising monoamidate mixtures, examples **18r** (Ala-*c*Bu), and **18v** (Ala-Et), were separated using chiral chromatography and established that the second eluting isomer (B) was in all cases the better substrate for Cat A resulting in greater antiviral activity (Table 1). To establish absolute configuration, purified isomer **18v**(**A**) was crystallized and the X-ray structure determined (see Supplementary data). The prodrug **18v**(**A**) has P(*R*) stereochemistry so by inference the preferred Cat A substrate was the P(*S*) isomer **18v**(**B**) (**5**). The P(*S*) stereochemistry is the same stereochemistry at phosphorous reported for monoamidate **24** confirming the ability of Cat A to preferentially cleave a single phosphorus isomer.[17]

2.2. In vitro stability profiling of amidate prodrugs

Plasma stability, hepatocyte stability and intestinal S9 stability were evaluated in both dog and human (Table 1) fractions to determine the best prodrugs for in vivo oral evaluation. Rodents were avoided due to their high plasma esterase activities. Tenofovir monoamidate **24** served as a good comparitor prodrug for profiling both in vitro and in vivo stability since the clinical phase 1 studies had demonstrated good oral loading of PBMCs using this prodrug. Clearly, the goal for amidate prodrugs of **4** was to try and match, or exceed the stability properties exhibited by **24**. It was found that all amidate prodrugs of **4** demonstrated good plasma stability over 60 min in both dog and human species (Table 1). The best Cat A substrates in the diamide series of prodrugs, **17a–l** demonstrated low hepatic stability, and in general a correlation between high Cat A activity and low hepatic stability was observed throughout all the prodrugs tested. Therefore, designing the optimal prodrug

Table 1
In vitro stability profiles of amidate prodrugs[a]



Compd	R_1[b]	R_2[b]	WT HIV EC_{50} (nM)	MT-2 CC_{50} (nM)	*c* log *D*[c]	Human Cat A[d]	Human PBMC extract[d]	Dog/human Plasma% remain[e]	Dog/human hepatocytes $T_{1/2}$ (min)	Dog/human intestinal S9 $T_{1/2}$ (min)
4	H	H	10,600	>100,000	−5.7	—	—	—/—	—/—	—/—
2	POC	POC	13 (9)	>50,000	0.98	—	—	—/—	—/—	—/—
16	POC	POC	105 (33)	—	1.80	—	—	—/—	—/—	—/—
17a	Phe-*n*Bu	Phe-*n*Bu	8.4 (5.6)	4800	6.49	66,680	50	96/99	10/—	—/—
17b	Phe-*n*Pr	Phe-*n*Pr	5.2 (1.5)	—	5.47	27,642	20	99/100	—/—	—/—
17c	Phe-Et	Phe-Et	8.9 (4.9)	—	4.45	41,020	40	95/100	—/—	—/—
17d	ABA-*n*Bu	ABA-*n*Bu	90 (28)	>100,000	4.30	1872	1.3	100/99	73/—	—/—
17e	ABA-*i*Bu	ABA-*i*Bu	128 (38)	—	3.87	867	1.3	—/—	71/—	—/—
17f	ABA-*i*Pr	ABA-*i*Pr	>2000	—	3.33	0	0.05	100/100	690/—	—/—
17g	Ala-*n*Bu	Ala-*n*Bu	68 (32)	51,000	3.28	17,355	18	100/96	48/—	—/—
17h	Ala-*i*Bu	Ala-*i*Bu	119 (30)	>100,000	2.85	1770	2.1	100/100	193/—	—/—
17i	Ala-*n*Pr	Ala-*n*Pr	1065 (21)	—	2.26	—	—	—/—	—/—	—/—
17j	Ala-Et	Ala-Et	723 (258)	—	1.24	—	—	—/—	—/—	—/—
17k	Gly-*i*Bu	Gly-*i*Bu	1610	>100,000	1.78	0	0	—/—	—/—	—/—
17l	Gly-*i*Pr	Gly-*i*Pr	>2000	—	1.24	—	—	—/—	—/—	—/—
24	Ala-*i*Pr (B)	OPh	9.2 (7.9)	139,000	2.16	35,000	20	100/—	103/159	15/106
18a	Phe-*s*Bu(S)	OPh	199 (2)	>100,000	5.10	504	0.67	84/83	79/—	—/—
18b	Phe-*s*Bu(R)	OPh	141 (7)	>100,000	5.10	1510	2.1	84/74	56/—	—/—
18c	Phe-CH_2cBu	OPh	3.8 (2.2)	46,500	4.92	37,017	24	80/51	23/62	3.4/—
18d	Phe-*i*Bu	OPh	6.0 (3.8)	38,300	4.86	29,655	31	84/52	28/—	8.3/—
18e	Phe-cBu	OPh	20 (8)	>100,000	4.65	30,786	25	90/64	27/61	5.4/—
18f	Phe-*i*Pr	OPh	63 (32)	>100,000	4.59	1417	2.8	100/85	98/720	—/—
18g	Phe-*n*Pr	OPh	4.9 (0.5)	>100,000	4.56	33,250	21	98/65	14/37	—/—
18h	Phe-CH_2cPr	OPh	7.5 (3.7)	>100,000	4.42	39,270	41	85/63	17/40	—/—
18i	Phe-Et	OPh	33 (11)	25,000	4.05	39,246	35	78/74	37/78	—/—
19a	Phe-*i*Bu	OCH_2CF_3	119 (106)	>100,000	4.05	30,107	22	84/91	27/—	—/—
18j	ABA-*n*Bu	OPh	88 (17)	70,000	3.98	1377	1.4	95/100	91/—	—/—
18k	ABA-*i*Bu	OPh	103 (18)	>100,000	3.76	728	1.9	95/100	127/—	—/—
18l	Ala-3-Pentyl	OPh	662 (214)	8350	4.00	0	0	—/—	—/—	—/—
18m	Ala-*n*Pentyl	OPh	33 (3.3)	27,000	3.98	5088	6.7	79/91	57/—	—/—
18n	Ala-cPentyl	OPh	63 (22)	>100,000	3.55	5688	3.0	58/54	—/—	—/—
18o	Ala-*n*Bu	OPh	92 (21)	91,000	3.47	6183	7.3	100/89	63/—	—/—
18p	Ala-CH_2cBu	OPh	25 (12)	>100,000	3.32	5311	3.0	71/69	132/92	63/143
18q	Ala-*i*Bu	OPh	58 (45)	>100,000	3.25	7916	4.0	93/81	38/86	—/—
18r	Ala-cBu	OPh	36 (13)	57,000	3.05	18,150	7.7	87/84	100/98	25/122
18s	Ala-*i*Pr	OPh	728 (214)	—	2.98	216	0.3	78/97	88/127	—/—
18t	Ala-*n*Pr	OPh	52 (71)	31,000	2.96	19,103	26	100/96	88/—	—/—
18u	Ala-CH_2cPr	OPh	59 (34)	>100,000	2.82	28,235	28	64/69	85/69	50/98
18v	Ala-Et	OPh	96 (64)	74,000	2.45	7280	7.2	84/88	165/189	146/318
19b	Ala-Et	OCH_2CF_3	1050 (212)	>100,000	1.64	1132	0.69	99/95	86/185	—/—
23	Ala(O)-Et	OPh	>2000	—	2.77	—/—	—/—	—/—	—/—	—/—
18w	Gly-*i*Bu	OPh	1120	22,000	2.72	226	0.72	—/—	—/—	—/—
18r(A)	Ala-cBu(A)[f]	OPh	45 (16)	—	3.05	2527	3.2	80/84	140/—	32/—
18r(B)	Ala-cBu(B)[f]	OPh	21 (4.9)	—	3.05	23,997	19	60/82	52/—	3.9/—
18v(A)	Ala-Et(A)[f]	OPh	262 (94)	>100,000	2.45	645	0.87	57/94	165/—	108/—
18v(B)(5)	Ala-Et(B)[f]	OPh	157 (23)	>100,000	2.45	12,371	5.8	56/84	71/43	19/—

[a] Values for active compounds are results of at least two experiments in MT-2 cells, standard deviation is given in parentheses.
[b] Disoproxil (POC); phenylalanine (Phe); 2-(L)-aminobutyric acid (ABA); alanine (Ala); 2-(L)-hydroxypropionic acid (Ala(O)); glycine (Gly); cyclopentyl (cPentyl); *n*-butyl (*n*Bu); *n*-propyl (*n*Pr); ethyl (Et); *iso*-butyl (*i*Bu); *iso*-propyl (*i*Pr); (*S*)-*sec*-butyl (*s*Bu(S)); (*R*)-*sec*-butyl (*s*Bu(R)); cyclobutyl (cBu); cyclopropyl (cPr); phenol (OPh).
[c] *c* log *D* was calculated using Pallas 3.0 from CompuDrug International, Inc., Sedona, AZ.
[d] Activity is represented as pmol compound cleaved/min/μg of recombinant Cat A enzyme or PBMC extract.
[e] % prodrug remaining after 60 min incubation.
[f] A refers to first eluting isomer and B the second eluting isomer from the chiral chromatography method described in the experimental.

required balancing rapid Cat A mediated cleavage, with broader esterase stabilities in hepatocytes and intestinal fractions. For example, **17f** (ABA-*i*Pr) had very high hepatic stability but no cleavage by Cat A leading to poor antiviral activity. In contrast, **17g** (Ala-*n*Bu) showed promising Cat A mediated cleavage resulting in potent antiviral activity, and hepatic stability approximately half that of **24** leading to its later selection for in vivo evaluation.

Amongst the Phe monoamidates (**18a–i**) hepatocyte stability rarely reached that of **24** unless a proximally branched ester was used, for example, **18f** (Phe-*i*Pr). Despite the low Cat A activity, **18f** demonstrated good antiviral activity and was also selected for in vivo evaluation. In the Ala series of monoamidates, plasma stability and hepatocyte stability were generally improved, providing profiles that more closely resembled **24**. Some of the most promising prodrugs, for example, **18p** and **18v** (Ala-CH_2*c*Bu and Ala-Et respectively) demonstrated superior dog hepatocyte stability than **24**! The desire to obtain the best possible hepatic stabilities, yet also have optimal Cat A activity, drove the exploration of the constrained aliphatic esters such as cyclobutyl and cyclopentyl. The cyclobutyl analog **18r** was remarkable in that the constraint of the ring improved the Cat A cleavage rate substantially compared to the isopropyl ester **18s**, but hepatic stability did not decrease.

Table 2
In vivo pharmacokinetics of selected prodrugs **17** and **18** in Beagle dogs

Compd	Prodrug	IV clearance (l/h/kg)	PBMC C_{max} [a] **4** (μM)	Loading efficiency[b]	Oral plasma prodrug %F	Oral PBMC C_{max} **15** (μM)
24	*Ala-i*Pr	2.23 (0.25)	nd	0.58	21 (3)	—
17g	Bis Ala-*n*Bu	3.22 (1.17)	0.94 (0.26)	0.05	—	—
18d	Phe-*i*Bu	3.70 (0.30)	22 (7.3)	1.94	2.6 (0.9)	—
18f	Phe-*i*Pr	1.35 (0.31)	3.0 (0.5)	0.10	—	—
18p	Ala-CH_2*c*Bu	1.63 (0.4)	9.0 (2.5)	0.30	—	—
18q	Ala-*i*Bu	2.62 (0.54)	3.8 (0.9)	0.16	—	—
18r	Ala-cBu	2.76 (0.88)	21 (5.3)	0.62	—	—
18u	Ala-CH_2*c*Pr	1.56 (0.63)	18 (5.6)	0.48	6.3 (4.8)	—
18v	Ala-Et	1.71 (0.41)	17 (1.5)	0.47	12 (3.5)	7.8 (3.0)
18v(**A**)	Ala-Et(A)	—	—	—	9.8 (6.8)[c]	5.2 (1.0)
18v(**B**) (**5**)	Ala-Et(B)	—	—	—	18 (13)[c]	9.0 (2.3)
18r(**A**)	Ala-cBu(A)	—	—	—	7.7 (4.6)[c]	14 (5.4)
18r(**B**)	Ala-cBu(B)	—	—	—	9.1 (4.5)[c]	9.4 (4.3)

All values are the mean ± SD in parentheses from 3 dogs and dose normalized.
[a] Quantity of diacid **4** measured after dephosphorylation of samples with calf intestinal phosphatase.
[b] Loading efficiency ratio of $[AUC]_{0-24\ h}$ of **4** and its metabolites inside PBMCs divided by $[AUC]_{0-24\ h}$ plasma prodrug.
[c] Estimated based on IV parameters for isomeric mixture.

Several of the promising alanine ester prodrugs were selected for in vivo profiling.

2.3. In vivo pharmacological profiling of amidate prodrugs

Initial in vivo profiling was performed using a 30 min IV infusion dosing protocol in beagle dogs to establish plasma clearance of the prodrug and evaluate total PBMC exposure to **4** (and its metabolites) over 24 h (Table 2).[5] These measurements provided insight into the pharmacokinetics that would drive PBMC cell loading, that is, Cat A cleavage versus hepatic and other clearance routes of the prodrug. The ratio of PBMC cell loading of **4** over 24 h ($[AUC]_{0-24\ h}$ of **4** and its metabolites inside PBMCs) compared to the plasma prodrug exposure over 24 h ($[AUC]_{0-24\ h}$) was defined as the loading efficiency and provided a useful parameter to rate the best candidates for oral studies. It should be noted that IV dosing of diacid **4** resulted in low levels of **4** inside PBMCs, thereby establishing that circulating levels of **4** in the plasma following prodrug cleavage in sites other than PMBCs, would not contribute significantly to the intracellular PBMC levels of **4** and its metabolites (data not shown). Phosphonic diamide **17g** (Ala-*n*Bu) had rapid clearance approaching that of liver blood flow in dogs, a low PBMC C_{max} for **4** and more than a 10-fold reduced loading efficiency compared to **24**. This result suggested that hepatic extraction and loading of other tissues was more rapid than PBMC cell loading. In contrast, Phe monoamidate **18d** (*i*Bu) which also showed a high clearance rate had the highest C_{max} and the highest loading efficiency. The difference can be attributed to the faster cleavage by Cat A, essentially redressing the balance between rapid PBMC cell loading versus other clearance routes. Unfortunately, first pass hepatic extraction via oral administration was extensive for **18d**, resulting in an oral bioavailability of 2.6% and minimal intracellular delivery of **4** and its metabolites. The Phe-*i*Pr ester **18f**, showed reduced clearance, as expected based on its improved hepatocyte stability profile, but demonstrated a poor loading efficiency compared to **24** and was therefore not considered for oral evaluation. More success was achieved in the Ala series of monoamidates due to their improved hepatocyte stabilities. Prodrugs, **18u**, **18v**, and **18r** (CH_2*c*Pr, Et and *c*Bu respectively) all demonstrated lower IV clearance than the Phe monoamidates and comparable loading efficiencies to **24**. Consistent with the low clearance and excellent in vitro intestinal S9 stability of **18v** (Table 1) good oral bioavailability was observed for **18v** leading to intracellular PBMC levels of diphosphate **15** close to 8 micromolar. The separated diastereoisomers for both **18v** and **18r** were then evaluated orally to identify **5** (**18v**(**B**)) as the most promising isomer based on oral bioavailability and intracellular levels of active metabolite **15**.

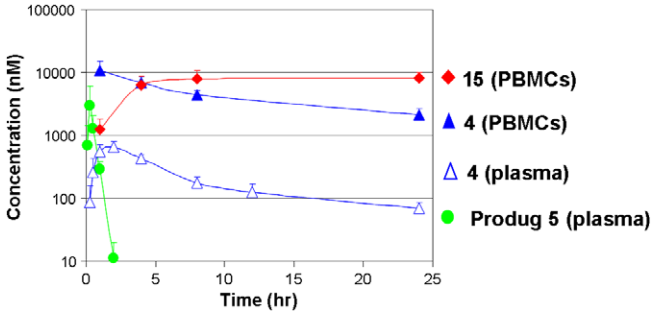


Figure 3. Oral plasma and PBMC pharmacokinetics of prodrug **5** in beagle dogs (n = 3).

The full pharmacokinetic profile of **5** upon oral administration to beagle dogs (3 mg/kg) is shown in Figure 3. The prodrug is cleared from circulation in the first 1–2 h with a half life of less than 20 min, but during this time the PBMCs are efficiently loaded with the parent diacid **4** to levels 10-fold higher than plasma levels. The early C_{max} for **4** inside PBMCs, is consistent with the rapid prodrug clearance from the plasma and supports that only the plasma prodrug is driving the intracellular levels of **4**. Critically, the intracellular accumulation of the active diphosphate metabolite **15**, peaks at ~5 h with a concentration of >9.0 μM, a level well above the RT IC_{50} of 1.9 μM. Furthermore, metabolite **15** persists inside the cell for periods in excess 24 h, supporting a QD dosing regimen for the prodrug. Based on this promising pharmacokinetic data, and the resistance and selectivity data, prodrug **5** was chosen as a candidate for preclinical toxicological evaluation in vivo.[5]

3. Conclusion

The design of amidate prodrugs as substrates for the lysosomal carboxypeptidase, cathepsin A, in combination with hepatocyte and plasma stability testing allowed for the rapid identification of an oral prodrug candidate of the novel nucleoside phosphonate RT inhibitor **4**. Cathepsin A recognized both phosphonic diamide and monoamidate prodrugs, and the best substrates demonstrated antiviral activity below 10 nM. The optimal prodrug, **5**, contained an ethyl ester of alanine and phenol, as the two masking moieties of the phosphonic acid. Monoamidate prodrug **5** effectively loaded

lymphoid cells following oral dosing in dogs, and was efficiently phosphorylated to its active metabolite **15**.

4. Experimental and methods

All reactions were performed with reagent-grade materials under an atmosphere of nitrogen. Solvents were reagent-grade or better. Silica gel chromatography was performed using EMD Chemicals Silica Gel 60. ^{1}H, ^{13}C, ^{31}P and ^{19}F NMR spectra were recorded on a Varian 300 MHz NMR and are reported in parts per million (ppm) and signals reported as singlet (s), doublet (d), triplet (t), quartet (q), broad (br), and multiplet (m). ^{1}H, and ^{13}C NMR shifts are reported relative to solvent residual. ^{19}F NMR are reported relative to trifluoroacetic acid external standard ($\delta = -77$ ppm). ^{31}P NMR are reported relative to phosphate ion in D_2O external standard $\delta = 0$ ppm. Low-resolution LC mass spectra (LC–MS) were recorded on a Thermo Finnigan LCQ Advantage, electrospray ionization mass spectrometer. The LC–MS was equipped with a Phenomenex Synergi 4 micron Polar-RP C18 column (30 × 4.6 mm), eluting with a gradient of 5–100% solvent B in solvent A over 2.5 min at a flow rate of 2 mL/min (solvent A = 0.1% AcOH in water, solvent B = 0.1% AcOH in CH_3CN). High resolution mass spectra were provided by Gilead Sciences Analytical Department using ESI. Melting points are uncorrected. Elemental analysis was carried out by Galbraith Laboratories Inc., Knoxville, TN. Analytical HPLC was carried out on a Agilent 1100 series analytical HPLC, equipped with a DIONEX DNAPac PA-100 column (250 × 4 mm) eluting with a gradient of 0–50% solvent B in solvent A over 12 min followed by 100% B for 5 min (solvent A = water, solvent B = 0.5 mM triethylammonium bicarbonate buffer pH 8.5. Compounds were >95% pure at 254 nM and 214 nM for bioassay. Preparative HPLC chromatography was carried out on a Gilson Liquid Handler 215 equipped with 322 pumps, a UV/Vis-156 detector and a Phenomemex Synergi Combi-HTS 4 micron hydro-RP C18 column (150 × 30 mm). The flow rate was 20 mL/min and a linear gradient of 5–100% ACN in water was typically used. Amino acids are abbreviated as follow glycine (Gly), alanine (Ala), phenylalanine (Phe), L-2-aminobutyric acid (ABA), and L-2-hydroxypropionic acid (Ala(O)). X-ray structure of **18v**(**A**) was determined by Rigaku Americas, TX, USA

4.1. 5-[(6-Amino-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-yloxymethyl] phosphonic acid disodium salt (4)

Compound **13** (62.4 g, 155 mmol) was treated with ammonium hydroxide (28–30%) (150–200 mL) and vigorously agitated for 20 min to dissolve the oily material. The resultant mixture was heated at 110–120 °C for 3 h in a steel bomb, allowed to cool and then concentrated under reduced pressure to approximately 50–100 mL volume. The residue was filtered through a plug of C18 to remove insoluble material and then concentrated to near dryness. MeOH (200 mL) was added and the mixture filtered once again to remove insoluble material. The eluant was concentrated to dryness (~60 g) and the residue containing monoacid **14** dried under high vacuum for 24 h. The resultant solid was suspended in CH_3CN (750 mL) and then treated with 2,6-lutidine (35 mL, 300 mmol) followed by TMSBr (115 g, 750 mmol, 5 equiv). The mixture was heated for 1 h at 40 °C and then cooled to 0 °C. The mixture was poured into cold aqueous $NaHCO_3$ and then evaporated under reduced pressure. The crude residue was purified on C-18 (40 μm) column, eluting with water. The aqueous fractions were freeze dried to give disodium salt **4** (40 g, 106 mmol, 68%). ^{1}H NMR (300 MHZ, D_2O) δ 8.22 (s, 1H), 8.02 (s 1H), 6.6 (d, J = 2.7 Hz, 1H), 5.92 (d, J = 4.8 Hz, 1H), 5.89 (s, 1H), 3.59 (dd, J = 12.6, 9 Hz, 1H), 3.40 (dd, J = 12.6, 9 Hz, 1H). ^{13}C NMR (77 MHz, D_2O) δ 158.1, 155.4, 154.3, 152.8, 148.6, 140.2, 118.3, 106.2, 79.6, 79.2, 66.9, 64.9. ^{19}F NMR (282.2 MHz, D_2O) δ −134.6 (m). ^{31}P NMR (D_2O) δ 12.4. LC–MS m/z 332 $[M+H]^+$. Anal. ($C_{10}H_9FN_5Na_2O_5P\cdot 2H_2O$) C, H, N.

4.2. 6-Chloro-9-(2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-β-D-arabinofuranosyl)-9*H*-purine (7)[7]

A solution of 2-fluoro-1,3,5-tri-*O*-benzoyl-D-arabinofuranose **6** (600 g, 1.3 mol) in CH_2Cl_2 (2 L) was treated with 33% HBr (1.5 equiv) in acetic acid (340 mL). The mixture was stirred at rt for 16 h, then cooled with ice water, and slowly neutralized over 1–2 h with $NaHCO_3$. The organic phase was separated and washed with $NaHCO_3$. The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure to yield the anomeric bromide (~540 g, 1.3 mol) as a viscous yellow oil. NMR data was consistent with data reported.[7] A suspension of NaH (56.4 g, 60%, 1.11 equiv) in CH_3CN (2 L), was treated with 6-chloropurine (208 g, 1.05 equiv) over 2–3 h. The mixture was stirred at rt for 1.5 h and then a solution of the anomeric bromide in CH_3CN (500 mL) was added over 1 h. The resultant mixture was stirred at rt for a further 16 h. The mixture was carefully quenched with acetic acid, and the solid collected by filtration. The solid was washed with water (3×), MeOH (3×), and then dried under high vacuum to provide the title product **7**. The mother liquor, following the acetic acid quench, was concentrated under reduced pressure and allowed to stand overnight to precipitate further solid material. The solid was washed with water (3×), MeOH (3×) and dried under vacuum to provide a second crop of **7** (total yield, 345.5 g, 0.70 mol, 54%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.75 (s, 1H), 8.41 (d, J = 2.7 Hz, 1H), 8.08 (d, J = 7.2 Hz, 4H), 7.4–7.7 (m, 6H), 6.7 (dd, J = 22, 2.7 Hz, 1H), 5.8 (dd, J = 16.5, 2.5 Hz, 1H), 5.4 (dd, J = 2.5, 50 Hz, 1H), 4.83 (d, J = 4.6 Hz, 2H), 4.64 (dd, J = 4.6, 4.6 Hz, 1H). ^{13}C NMR (77 MHz, $CDCl_3$) δ 166.1, 165.1, 152.2, 151.4, 151.1, 144.5, 144.4, 134.3, 133.5, 129.9, 129.7, 129.2, 128.7, 128.6, 128.0, 93.8, 91.2, 84.0, 83.7, 81.5, 77.5, 76.8, 76.4, 63.1. ^{19}F NMR (282.2 MHz, $CDCl_3$) δ −198.3 (m). LC–MS m/z 497 $[M+H]^+$.

4.3. 6-Methoxy-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-9*H*-purine (8)

A suspension of **7** (345.5 g, 0.70 mol) in MeOH (6 L) was treated with K_2CO_3 (384 g, 2.78 mol). The mixture was mechanically stirred at rt for 13 h, and then filtered. The eluant was neutralized with acetic acid to pH 7 and concentrated under reduced pressure (not to dryness). The resultant residue was partitioned between hexane and water. The aqueous layer was separated and concentrated under reduced pressure to precipitate a solid which was collected by filtration. The solid was washed with ice water to provide **8** (167 g, 0.59 mol, 84%) as a white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 8.5 (s, 2H), 6.55 (dd, J = 15.6, 4.0 Hz, 1H), 5.18 (dq, J = 52, 4.0, 3.0 Hz, 1H), 4.55 (dq, J = 18, 4.5, 3.0 Hz, 1H), 4.18 (s, 3H), 4.02 (m, 1H), 3.85 (m, 2H). ^{13}C NMR (77 MHz, CD_3OD) δ 160.7, 152.1, 151.2, 142.2, 120.1, 96.7, 94.2, 84.3, 83.2, 83.0, 73.4, 73.0, 60.6, 53.5. ^{19}F NMR (282.2 MHz, CD_3OD) δ −200.0 (m). LC–MS m/z 285 $[M+H]^+$. Recrystallization from EtOAc, mp = 146 °C. Anal. ($C_{11}H_{13}FN_4O_4$) C, H, N.

4.4. 6-Methoxy-9-(2-deoxy-2-fluoro-5-carboxy-β-D-arabinofuranosyl)-9*H*-purine (9)

Compound **8** (100 g, 0.35 mol) was dissolved in acetone (1.4 L) and treated with 200 g of vigorously agitated acetone-washed Celite 545. The mixture was cooled to 0 °C and Jones' reagent (2.63 M chromium(VI), 2 mol equiv or 1.5 redox equiv, 268 mL) was added dropwise with mechanical stirring over 2 h. The mixture was warmed to

rt and stirred for a further 12 h. The mixture was then cooled to 0 °C and the remaining chromium(VI) quenched by the addition of *i*PrOH (2 equiv, 54 mL) followed by stirring for 2 h. The mixture was filtered through a Celite pad and the pad washed generously with THF. The filtrate was reduced in volume and the residue re-dissolved in THF/EtOAc. The mixture was extracted with brine using color as indicator for the completion of extraction. The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to provide a white foam. The foam was crystallized from MeOH:H_2O to provide **9** (70 g, 0.235 mol, 67%) as a white solid. ^{1}H NMR (300 MHz, CD_3OD) δ 8.74 (d, J = 2.4 Hz, 1H), 8.5 (s, 1H), 6.76 (dd, J = 22.5, 2.4 Hz, 1H), 5.1 (d, J = 48 Hz, 1H), 4.8 (d, J = 9.6 Hz, 1H), 4.7 (s, 1H), 4.18 (s, 3H). ^{13}C NMR (77 MHz, CD_3OD) δ 171.1, 160.7, 152.1, 151.0, 143.2, 120.1, 94.1, 91.6, 86.1, 85.9, 83.6, 77.3, 76.9, 53.5. ^{19}F NMR (282.2 MHz, CD_3OD) δ −199.5 (m). LC–MS m/z 299 $[M+H]^+$. Recrystallized from H_2O, deposited as a fine flakes, mp = 158 °C (dec.). Anal. ($C_{11}H_{11}FN_4O_5$) C, H, N.

4.5. Diethyl 5-(*R*)-[(6-methoxy-purin-9-yl)-4-(*R*)-fluoro-3-(*R*)-iodo-tetrahydro-furan-2-(*R*)-yloxymethyl]phosphonic acid (12)

A solution of **9** (23 g, 71 mmol) in CH_2Cl_2 (1 L) was treated with PPh_3 (24.3 g, 92.6 mmol) and DIAD (18.8 g, 93 mmol). The resultant mixture was refluxed for 0.5 h to generate glycal **10** and then cooled to −40 °C. Diethyl (hydroxymethyl)phosphonate **11** (51.9 g, 309 mmol) was added followed by the dropwise addition of IBr (31.95 g, 154 mmol) in CH_2Cl_2 (100 mL). The reaction mixture was warmed to 0 °C and then stirred for 2 h. The reaction mixture was poured into aqueous $NaHCO_3$ (100 g) containing $Na_2S_2O_3$ (30 g). The organic phase was separated, dried over $MgSO_4$, and concentrated under reduced pressure to dryness. The reaction mixture was subjected to silica gel chromatography eluting with 0–100% EtOAc in CH_2Cl_2, followed by 0–10% MeOH in CH_2Cl_2, to provide **12** (22.5 g, 55%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.5 (s, 1H), 8.21 (d, J = 3.9 Hz, 1H), 7.05 (dd, J = 18.5, 3.9 Hz, 1H), 5.6 (s, 1H), 5.4 (dd, J = 50, 4.0 Hz, 1H), 4.55 (d, J = 16.5 Hz, 1H), 4.05–4.2 (m, 4H), 4.15 (s, 3H), 4.03 (dd, J = 13.7, 10.8 Hz, 1H), 3.82 (dd, J = 13.7, 9.3 Hz, 1H), 1.3 (m, 6H). ^{13}C NMR (77 MHz, $CDCl_3$) δ 166.0, 152.4, 151.7, 142.4, 142.3, 120.7, 111.7, 111.5, 96.1, 93.4, 84.0, 83.8, 62.7–63.3 (m), 61.0, 54.4, 21.3, 21.0, 16.5, 16.4. ^{19}F NMR (282.2 MHz, $CDCl_3$) δ −165.6 (m). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 19.3. LC–MS m/z 531 $[M+H]^+$. Anal. ($C_{15}H_{21}FN_4O_6P$) C, H, N.

4.6. Diethyl 5-[(6-methoxy-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-yloxymethyl] phosphonic acid (13)

A solution of compound **12** (6 g, 11.3 mmol) in acetic acid (2.5 mL) and MeOH (50 mL), was treated with the dropwise addition of NaClO (10–13%) (50 mL). The reaction mixture was stirred for 0.5 h and then concentrated under reduced pressure. The residue was treated with EtOAc and then filtered to remove solids. The filtrate was concentrated and the residue was purified by silica gel chromatography to give **13** (4.0 g, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.53 (s, 1H), 8.16 (s 1H), 6.82 (dd, J = 4.0, 1.2 Hz, 1H), 5.84 (dd, J = 4.0, 1.2 Hz, 1H), 5.78 (d, J = 1.2 Hz, 1H), 4.0–4.2 (m, 5H), 4.18 (s, 3H), 3.82 (m, 1H), 1.3 (m, 6H). ^{13}C NMR (77 MHz, $CDCl_3$) δ 161.1, 160.0, 156.2, 152.7, 151.9, 140.2, 121.4, 105.0–105.6 (m), 79.7, 79.3, 62.5–63.1 (m), 60.9, 54.3, 16.5, 16.4, 16.3. ^{19}F NMR (282.2 MHz, $CDCl_3$) δ −132.6 (m). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 19.9. LC–MS m/z 403 $[M+H]^+$.

4.7. 5-[(6-Amino-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-yloxymethyl] phosphonic acid diphosphate (15)

The diphosphate was prepared from the disodium salt **4** by Trilink Technologies, Inc, San Diego.

4.8. 9-(*R*)-[5-(*R*)-[(Bis-isopropyloxycarbonyloxymethyl) phosphonyl]methoxy-3-fluoro-2,5-dihydrofuran-2-yl]-9*H*-adenine (16)

Disodium salt **4** (20 mg, 0.060 mmol) was dissolved in anhydrous *N*-methyl pyrrolidinone (3 mL) and treated with triethylamine (0.03 mL, 0.21 mmol). The mixture was heated at 60 °C under nitrogen and then treated with isopropyloxycarbonyloxymethylchloride (0.082 g, 0.53 mmol). The mixture was stirred at 60 °C for 3 h and then cooled, diluted with EtOAc and washed with brine. The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resultant residue was then subjected to preparative HPLC (linear gradient, 5–95% CH_3CN in water) to afford compound **16** (2 mg, 7%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40 (s, 1H), 8.08 (s, 1H), 6.81 (d, J = 2.4 Hz, 1H), 5.90 (d, J = 4.5 Hz, 1H), 5.84 (s, 1H), 5.65 (m, 6H), 4.94 (m, 2H), 4.05 (m, 2H), 1.32 (t, J = 5.4 Hz, 12H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.5. LC–MS m/z 564 $[M+H]^+$

4.9. Phosphonic diamide example procedure: 9-(*R*)-[5-(*R*)-[[[(bis-(*S*)-1-(*n*-propyloxycarbonyl)-ethyl]amino]phosphonyl]methoxy-3-fluoro-2,5-dihydrofuran-2-yl]-9*H*-adenine (17i)

Disodium salt **4** (12 mg, 0.032 mmol) and (*S*)-alanine-*O*-*n*-Pr ester hydrochloride (32 mg, 6 equiv, 0.192 mmol) were mixed in anhydrous pyridine (1 mL). Et_3N (53 μL, 0.384 mmol) was added and the resultant mixture stirred at 60 °C under N_2. In a separate flask, 2,2′-dithiodipyridine (59 mg, 0.224 mmol) and PPh_3 (49 mg, 0.224 mmol) were dissolved in anhydrous pyridine (0.5 mL) and the resultant yellow solution was stirred for 15–20 min. The solution was then added to the 60 °C solution of **4** in one portion. The combined mixture was stirred at 60 °C under nitrogen for 16 h to give a clear yellow to light brown solution. The mixture was then concentrated under reduced pressure. The resultant oil was dissolved in CH_2Cl_2 and purified by silica gel chromatography (eluting with a linear gradient of 0–5% MeOH in CH_2Cl_2). The resultant oil was dissolved in CH_3CN and water and then purified by preparative reverse phase HPLC (linear gradient, 5–95% CH_3CN in water). Pure fractions were combined and freeze dried to give phosphonic diamide 17i as a white powder (7.0 mg, 39%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.29 (s, 1H), 8.25 (s, 1H), 6.84 (m, 1H), 6.00 (s, 1H), 5.96 (m, 1H), 4.04 (m, 8H), 1.66 (m, 4H), 1.38 (m, 6H), 0.98 (m, 6H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 22.8. LC–MS m/z 558 $[M+H]^+$.

The following phosphonic diamide prodrugs were prepared using the general procedure for preparation of **17i** except substituting the appropriate amino acid.

4.10. Prodrug 17a (2′-Fd4AP bis-Phe-*n*-butyl ester)

Yield = 42%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (s, 1H), 8.17 (s, 1H), 7.21 (m, 10H), 6.80 (m, 1H), 5.91 (s, 1H), 5.72 (m, 1H), 4.04 (m, 6H), 3.50 (m, 2H), 2.90 (m, 4H), 1.47 (m, 8H), 0.92 (m, 6H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 22.3. LC–MS m/z 738 $[M+H]^+$.

4.11. Prodrug 17b (2′-Fd4AP bis-Phe-*n*-propyl ester)

Yield = 60%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (s, 1H), 8.16 (s, 1H), 7.22 (m, 10H), 6.81 (m, 1H), 5.90 (s, 1H), 5.72 (m, 1H), 4.02 (m, 6H), 3.63 (m, 2H), 2.90 (m, 4H), 1.58(m, 4H), 0.87(m, 6H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 22.3. LC–MS m/z 710 $[M+H]^+$.

4.12. Prodrug 17c (2′-Fd4AP bis-Phe-ethyl ester)

Yield = 11%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (s, 1H), 8.16 (s, 1H), 7.24 (m, 10H), 6.84 (m, 1H), 5.91 (s, 1H), 5.75 (m, 1H), 4.08

(m, 6H), 3.60 (m, 2H), 2.90 (m, 4H), 1.21 (m, 6H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 22.4. LC–MS m/z 682 $[M+H]^+$.

4.13. Prodrug 17d (bis-ABA-*n*-butyl ester)

Yield = 73%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.38 (s, 1H), 8.12 (s, 1H), 6.80 (m 1H), 5.93 (m, 1H), 5.79 (s, 1H), 4.02 (m, 6H), 3.42 (m, 1H), 3.21 (m, 1H), 1.65 (m, 4H), 1.35 (m, 8H), 0.92 (m, 12H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.5. LC–MS m/z 614 $[M+H]^+$.

4.14. Prodrug 17e (bis-ABA-*iso*-butyl ester)

Yield = 35%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.39 (s, 1H), 8.12 (s, 1H), 6.82 (m, 1H), 5.81–5.96 (m, 4H), 3.79–4.03 (m, 10H), 3.49 (s, 1H), 3.2 (m, 2H), 1.69–1.96 (m, 10H), 1.26 (m, 4H), 0.91 (m, 12H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.4. LC–MS m/z 614 $[M+H]^+$.

4.15. Prodrug 17f (bis-ABA acid-*iso*-propyl)

Yield = 59%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.39 (s, 1H), 8.13 (s, 1H), 6.81 (m, 1H), 5.95 (m, 1H), 5.81(s, 1H), 4.98 (m, 2H), 3.90 (m, 2H), 3.37 (m, 1H), 3.19 (m, 1H), 1.71 (m, 4H), 1.25 (m, 12H), 0.90 (m, 6H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.4. LC–MS m/z 586 $[M+H]^+$.

4.16. Prodrug 17g (bis-Ala-*n*-butyl ester)

Yield = 55%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.37 (s, 1H), 8.17 (s, 1H), 6.80 (m, 1H), 6.18 (s, 1H), 5.93 (m, 1H), 5.79 (s, 1H), 4.02 (m, 6H), 3.46 (m, 1H), 3.37 (m, 1H), 1.61 (m, 4H), 1.32 (m, 10H), 0.92 (m, 6H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.0. LC–MS m/z 586 $[M+H]^+$.

4.17. Prodrug 17h (bis-Ala-*iso*-butyl ester)

Yield = 47%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.38 (s, 1H), 8.12 (s, 1H), 6.80 (m, 1H), 5.93 (m, 2H), 5.80 (s, 1H), 3.91 (m, 6H), 3.42 (m, 1H), 3.30 (m, 1H), 1.91 (m, 2H), 1.40 (m, 6H), 0.90 (m, 12H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 19.9. LC–MS m/z 586 $[M+H]^+$.

4.18. Prodrug 17j (bis-Ala-ethyl ester)

Yield = 22%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.30 (s, 1H), 8.25 (s, 1H), 6.84 (m, 1H), 6.00 (s, 1H), 5.95 (m, 1H), 4.06 (m, 8H), 1.31 (m, 12H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 22.8. LC–MS m/z 530 $[M+H]^+$.

4.19. Prodrug 17k (bis-Gly-*iso*-butyl ester)

Yield = 73%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.31 (s, 1H), 8.25 (s, 1H), 6.84 (m 1H), 6.01 (s, 1H), 5.98 (m, 1H), 4.03 (m, 2H), 3.86 (m, 4H), 3.68 (m, 4H), 1.92 (m, 2H), 0.93 (m, 12H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 25.5. LC–MS m/z 558 $[M+H]^+$.

4.20. Prodrug 17l (bis-Gly-*iso*-propyl ester)

Yield = 59%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.31 (s, 1H), 8.25 (s, 1H), 6.84 (m 1H), 6.02 (s, 1H), 5.98 (m, 1H), 4.98 (m, 2H), 4.01 (m, 2H), 3.66 (m, 4H), 1.23 (m, 12H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 25.5. LC–MS m/z 530 $[M+H]^+$.

4.21. Monoamidate example procedure 1, from disodium salt 4. 9-(*R*)-[5-(*R*)-[[(*S*)-1-[(ethoxycarbonyl)ethyl]amino]phenoxyphosphonyl]methoxy-3-fluoro-2,5-dihydrofuran-2-yl]-9*H*-adenine (18v)

Disodium salt **4** (1.5 g, 4.52 mmol) was mixed with alanine ethyl ester HCl salt (1.23 g, 8 mmol) and PhOH (1.88 g, 20 mmol). Anhydrous pyridine (35 mL) was added followed by Et_3N (6.7 mL, 48 mmol). The mixture was stirred at 60 °C under nitrogen for 15–20 min. The 2,2′-dithiodipyridine (7.3 g) was mixed in a separate flask with PPh_3 (6.2 g) in anhydrous pyridine (5 mL) and the resultant mixture was stirred for 10–15 min to give a clear light yellow solution. The solution was then added to the solution of **4** and the combined mixture stirred overnight at 60 °C. The mixture was concentrated under reduced pressure and the residue dissolved in EtOAc, washed twice with saturated aqueous sodium bicarbonate (2×), and then brine. The organic layer was separated, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The resultant oil was dissolved in CH_2Cl_2 and subjected to silica gel chromatography eluting with 0–5% MeOH in CH_2Cl_2. The product foam was dissolved in CH_3CN and further purified by preparative HPLC (linear gradient, 5–95% CH_3CN in water) to provide **18v** (0.95 g, 42%) as a mixture of P(*R*) and P(*S*) isomers (approx. 3:2). ^{1}H NMR (300 MHz, CD_3OD) δ 8.29 (s, 1H), 8.24 (m, 1H), 7.30 (m, 5H), 6.84 (m, 1H), 6.04 (m, 2H), 4.05 (m, 5H), 1.24 (m, 6H). ^{13}C NMR (77 MHz, DMSO-d_6) δ 173.3, 157.2, 156.1, 153.2, 150.1, 149.4, 138.4, 129.5, 124.5, 120.6, 118.6, 105.6, 104.9, 79.0, 63.2, 60.5, 49.1, 20.3, 13.9. ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.6, 22.3. LC–MS m/z 507.4 $[M+H]^+$.

4.22. Monoamidate example procedure 2: Preparation of 18v from monophenoxy ester 22

A solution of monoacid **22** (22 mg, 0.05 mmol) in DMF (2 mL) was treated with PyBoP (56 mg, 0.11 mmol) and stirred at rt for 30 min. The mixture was then treated with ethyl alanine ester·HCl salt (12 mg, 0.1 mmol) and Et_3N (10 μL, 0.072 mmol). The resultant mixture was stirred overnight and then diluted with EtOAc. The solution was washed with $NaHCO_3$, brine, dried over $MgSO_4$, filtered and concentrated to give a crude solid. The crude solid was subjected to HPLC chromatography (linear gradient, 5–95% CH_3CN in water) to give **18v** (8 mg, 30%) as a mixture of P(R) and P(S) isomers (approx. 3:2).

The following monoamidates were prepared from diacid **4** using the general procedure 1 shown above, except substituting the appropriate amino acid and phenol, or trifluoroethanol.

4.23. Prodrug 18a (Phe-*sec*-(*S*)-butyl, OPh ester)

Yield = 37%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (m, 2H), 7.20 (m, 9H), 6.96 (m, 1H), 6.81 (m, 1H), 5.97 (m, 2H), 5.73 (m, 1H), 4.71 (m, 1H), 4.05 (m, 2H), 3.60 (m, 1H), 3.02 (m, 1H), 2.81 (m, 1H), 1.49 (m, 2H) 1.07 (m, 3H), 0.82 (m, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.2, 22.5. LC–MS m/z 611 $[M+H]^+$, 609 $[M-H]^+$.

4.24. Prodrug 18b (Phe-*sec*-(*R*)-butyl, OPh ester)

Yield = 16%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (m, 2H), 7.20 (m, 9H), 6.96 (m, 1H), 6.81 (m, 1H), 5.97 (m, 2H), 5.73 (m, 1H), 4.71 (m, 1H)), 4.05 (m, 2H), 3.60 (m, 1H), 3.02 (m, 1H), 2.81 (m, 1H), 1.49 (m, 2H) 1.07 (m, 3H), 0.82 (m, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.3, 22.6. LC–MS m/z 611 $[M+H]^+$.

4.25. Prodrug 18c (Phe-CH_2-cyclobutyl, OPh ester)

Yield = 44%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (m, 2H), 7.12 (m, 10H), 6.83 (m, 1H), 5.99 (m, 2H), 5.72 (m, 1H), 4.10 (m, 4H), 3.65 (m, 1H), 3.02 (m, 1H), 2.79 (m, 1H), 2.50 (m, 1H), 1.89 (m, 6H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.3, 22.5. LC–MS m/z 623 $[M+H]^+$.

4.26. Prodrug 18d (Phe-*iso*-butyl, OPh ester)

Yield = 32%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40 (m, 1H), 8.09 (m, 1H), 7.27–6.74 (m, 8H), 5.30–5.93 (m, 4H), 4.39 (m, 1H), 4.14–3.77

(m, 4H), 3.58 (m, 2H), 2.95 (m, 2H), 1.90 (m, 3H), 1.26 (m, 1H), 0.85 (m, 6H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 21.0, 20.0. LC–MS m/z 611 $[M+H]^+$.

4.27. Prodrug 18e (Phe-cyclobutyl, OPh ester)

Yield = 31%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.23 (m, 2H), 7.18 (m, 10H), 6.96 (m, 1H), 6.81 (m, 1H), 5.94 (m, 2H), 5.72 (m, 1H), 4.81 (m, 1H), 4.05 (m, 2H), 3.60 (m, 1H), 3.02 (m, 1H), 2.81 (m, 1H), 2.25 (m, 2H) 1.81 (m, 4H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.3, 22.5. LC–MS m/z 609 $[M+H]^+$.

4.28. Prodrug 18f (Phe-*iso*-propyl, OPh ester)

Yield = 40%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (m, 2H), 7.20 (m, 9H), 6.96 (m, 1H), 6.81 (m, 1H), 5.97 (m, 2H), 5.73 (m, 1H), 4.05 (m, 2H), 3.60 (m, 1H), 3.02 (m, 1H), 2.81 (m, 1H), 1.13 (m, 6H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.3, 22.6. LC–MS m/z 598 $[M+H]^+$.

4.29. Prodrug 18g (Phe-*n*-propyl, OPh ester)

Yield = 13%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (m, 2H), 7.22 (m, 8H), 6.95 (m, 1H), 6.82 (m, 1H), 5.90 (m, 2H), 5.72 (m, 1H), 3.95 (m, 4H), 3.63 (m, 1H), 3.07 (m, 1H), 2.81 (m, 1H), 1.55 (m, 2H), 0.86 (m, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.3, 22.5. LC–MS m/z 597 $[M+H]^+$.

4.30. Prodrug 18h (Phe-CH_2-cyclopropyl, OPh ester)

Yield = 33%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (m, 2H), 7.15 (m, 10H), 6.82 (m, 1H), 5.99 (m, 2H), 5.73 (m, 1H), 3.99 (m, 4H), 3.65 (m, 1H), 3.05 (m, 1H), 2.85 (m, 1H), 1.02 (m, 1H), 0.51 (m, 2H), 0.20 (m, 2H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.4, 22.5. LC–MS m/z 609 $[M+H]^+$.

4.31. Prodrug 18i (Phe-ethyl, OPh ester)

Yield = 38%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.24 (s, 1H), 8.20 (m, 1H), 7.10 (m, 9H), 6.94 (m, 1H), 6.82 (m, 1H), 5.94 (m, 1H), 5.73 (m, 1H), 4.05 (m, 4H), 3.63 (m, 1H), 3.03 (m, 1H), 2.92 (m, 1H), 1.14 (m, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.3, 22.5. LC–MS m/z 583 $[M+H]^+$.

4.32. Prodrug 18j (ABA-*n*-butyl ester, OPh ester)

Yield = 39%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.39 (m, 1H), 8.11 (m, 1H), 7.10–7.28 (m, 5H), 6.82 (s, 1H), 5.76–5.98 (m, 3H), 3.56–4.18 (m, 4H), 3.59 (m, 1H), 0.70–1.74 (m, 12H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 21.0, 20.1. LC–MS m/z 549 $[M+H]^+$.

4.33. Prodrug 18k (ABA-*iso*-butyl ester, OPh ester)

Yield = 40%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.22–8.26 (m, 2H), 7.22–7.34 (m, 2H), 7.10–7.16 (m, 3H), 6.83–6.84 (m, 1H), 5.9–6.01 (m, 2H), 4.05–4.22 (m, 2H), 3.76–3.86 (m, 3H), 1.79–1.88 (m, 1H), 1.68–1.74 (m, 1H), 1.51–1.68 (m, 1), 0.72–0.99 (m, 9H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.8, 22.6. LC–MS m/z 549 $[M+H]^+$.

4.34. Prodrug 18l (Ala-3-pentyl, OPh ester)

Yield = 14%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (m, 2H), 7.17 (m, 5H), 6.85 (m, 1H), 5.99 (m, 2H), 4.66 (m, 1H), 4.12 (m, 3H), 1.56 (m, 4H), 1.28 (m, 3H), 0.88 (m, 6H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.5, 22.4. LC–MS m/z 549 $[M+H]^+$.

4.35. Prodrug 18m (Ala-*n*-pentyl, OPh ester)

Yield = 40%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.39 (s, 1H), 8.13 (s, 1H), 7.27–7.14 (m, 5H), 6.85 (s, 1H), 5.97–5.77 (m, 4H), 4.19–4.05 (m, 7H), 1.60 (m, 3H), 1.29 (m, 7H), 0.90 (m, 3H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.7, 19.8. LC–MS m/z 549 $[M+H]^+$.

4.36. Prodrug 18n (Ala-cyclopentyl, OPh ester)

Yield = 34%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.28 (m, 1H), 8.24 (m, 1H), 7.17 (m, 5H), 6.85 (m, 1H), 6.04 (m, 2H), 5.15 (m, 1H), 4.14 (m, 3H), 1.64 (m, 8H), 1.25 (m, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.6, 22.3. LC–MS m/z 547 $[M+H]^+$.

4.37. Prodrug 18o (Ala-*n*-butyl, OPh ester)

Yield = 25%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25–8.27 (m, 2H), 7.25–7.34 (m, 2H), 7.10–7.19 (m, 3H), 6.83–6.84 (m, 1H), 5.93–6.02 (m, 2H), 3.95–4.26 (m, 5H), 1.48–1.58 (m, 3H), 1.24–1.38 (m, 6H), 0.85–0.95 (m, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.6, 22.2. LC–MS m/z 535 $[M+H]^+$.

4.38. Prodrug 18p (Ala-CH_2-cyclobutyl, OPh ester)

Yield = 37%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.25 (m, 2H), 7.15 (m, 5H), 6.83 (m, 1H), 5.98 (m, 2H), 4.10 (m, 5H), 2.57 (m, 1H), 1.80 (m, 6H), 1.25 (m, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.6, 22.2. LC–MS m/z 548 $[M+H]^+$.

4.39. Prodrug 18q (Ala-*iso*-butyl, OPh ester)

Yield = 17%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.39 (s, 1H) δ 8.13 (s, 1H), 7.27–7.11 (m, 5H), 6.82 (s, 1H), 5.97–5.77 (m, 4H), 4.14–3.79 (m, 6H), 3.64 (m, 1H), 2.00–1.88 (bm, 4H), 1.31 (m, 3H), 0.91 (m, 6H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.8, 19.8. LC–MS m/z 535 $[M+H]^+$.

4.40. Prodrug 18r (Ala-cyclobutyl, OPh ester)

Yield = 37%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.29 (m, 1H), 8.25 (m, 1H), 7.20 (m, 5H), 6.85 (m, 1H), 5.97 (m, 2H), 4.85 (m, 1H), 4.15 (m, 2H), 3.95 (m, 1H), 2.28 (m, 2H), 1.99 (m, 2H), 1.77 (m, 2H) 1.26 (m, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.6, 22.2. LC–MS m/z 533 $[M+H]^+$.

4.41. Prodrug 18s (Ala-*iso*-propyl, OPh ester)

Yield = 43%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.22–8.27 (m, 2H), 7.25–7.34 (m, 2H), 7.10–7.18 (m, 3H), 6.83–6.84 (m, 1H), 5.94–6.02 (m, 2H), 4.90–5.05 (m, 1H), 3.90–4.26 (m, 3H), 1.15–1.31 (m, 9H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.5, 22.3. LC–MS m/z 521 $[M+H]^+$.

4.42. Prodrug 18t (Ala-*n*-propyl, OPh ester)

Yield = 52%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.38 (m, 1H), 8.11 (s, 1H), 7.11–7.31 (m, 5H), 6.82 (s, 1H), 5.76–5.96 (m, 4H), 3.63–4.22 (m, 6H), 2.17 (bm, 2H), 1.65 (m, 2H), 1.30 (m, 4H), 0.88 (m, 3H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.8, 19.8. LC–MS m/z 521 $[M+H]^+$.

4.43. Prodrug 18u (Ala-CH_2-cyclopropyl, OPh ester)

Yield = 28%. ^{1}H NMR (300 MHz, CD_3OD) δ 8.29 (s, 1H), 8.24 (m, 1H), 7.28 (m, 5H), 6.85 (m, 1H), 6.00 (m, 2H), 4.16 (m, 5H), 1.05 (m, 1H), 0.48 (m, 3H), 0.25 (m, 4H). ^{31}P NMR (121.4 MHz, CD_3OD) δ

23.6, 22.2. LC–MS *m*/*z* 533 $[M+H]^+$. HRMS (ESI) *m*/*z* Calc. for $C_{23}H_{26}FN_6O_6P$, 532.1635. Found, 532.1662.

4.44. Prodrug 18w (Gly-*iso*-butyl, OPh ester)

Yield = 43%. ^1H NMR (300 MHz, $CDCl_3$) δ 8.39 (s, 1H), 8.17 (m, 1H), 6.82–7.32 (m, 5H), 6.82 (s, 1H), 5.81–5.98 (m, 3H), 3.64–4.27 (m, 6H), 1.94 (m, 1H), 0.90 (m, 6H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 21.5, 21.4. LC–MS *m*/*z* 521 $[M+H]^+$.

4.45. Prodrug 19a (Phe-*iso*-butyl, OCH_2CF_3 ester)

Yield = 33%. ^1H NMR (300 MHz, CD_3OD) δ 8.20 (m, 2H), 7.25 (m, 6H), 6.82 (m 1H), 5.95 (m, 2H), 5.68 (m, 1H), 3.93 (m, 6H), 3.50 (m, 1H), 3.20 (m, 1H), 2.81 (m, 1H), 1.90 (m, 1H), 0.95 (m, 6H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 27.4, 26.8. LC–MS *m*/*z* 617 $[M+H]^+$.

4.46. Prodrug 19b (Ala-ethyl, OCH_2CF_3 ester)

Yield = 32%. ^1H NMR (300 MHz, CD_3OD) δ 8.24 (m, 2H), 6.85 (m, 1H), 6.01 (m, 2H), 4.43 (m, 2H), 4.09 (m, 5H), 1.38 (m, 3H) 1.23 (m, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 28.0, 26.9. LC–MS *m*/*z* 513 $[M+H]^+$.

4.47. Separation of monoamidate phosphorus diastereoisomers, example prodrug 18v

The isomer mixture of monoamidate **18v** (950 mg, 1.87 mmol) was dissolved in a small amount of CH_3CN and allowed to stand at rt overnight. The solid that formed (isomer A) was collected by filtration and washed with a small amount of cold CH_3CN. The filtrate was reduced under vacuum and then loaded onto Chiralpak AS-H column equilibrated in Buffer A, 2% ethanol in CH_3CN. Isomer A, was eluted out with Buffer A at 10 mL/min for 17 min. The eluting buffer was then changed to Buffer B, 50% MeOH in CH_3CN, and isomer B eluted from the column in 8 min. All solvent was removed under reduced pressure, and then the samples re-dissolved in CH_3CN and water. Samples were freeze dried to provide isomer A (475 mg, 50%) and isomer B (348 mg, 37%).

4.48. Prodrug 18v(A) (Ala-ethyl, OPh ester, diastereoisomer A, 50% recovery)

^1H NMR (300 MHz, CD_3OD) δ 8.29 (s, 1H), 8.24 (s, 1H), 7.30 (m, 5H), 6.84 (d, *J* = 2.7 Hz, 1H), 6.04 (s, 1H), 5.99 (d, *J* = 5.1 Hz, 1H), 4.05 (m, 5H), 1.26 (d, *J* = 7.2 Hz, 3H) 1.19 (t, *J* = 7.2 Hz, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 23.6. HRMS (ESI) *m*/*z* Calc. for $C_{21}H_{24}FN_6O_6P$, 506.1479. Found, 506.1480

4.49. Prodrug 5 or 18v(B) (Ala-ethyl, OPh ester, diastereoisomer B, 37% recovery)

^1H NMR (300 MHz, CD_3OD) δ 8.29 (s, 1H), 8.25 (s, 1H), 7.30 (m, 5H), 6.85 (m, 1H), 5.98 (s, 1H), 5.95 (d, *J* = 4.5 Hz, 1H), 4.11 (m, 5H), 1.28 (d, *J* = 6.9 Hz, 3H) 1.21 (t, *J* = 7.5 Hz, 3H). ^{31}P NMR (121.4 MHz, CD_3OD) δ 22.3. HRMS (ESI) *m*/*z* calcd for $C_{21}H_{24}FN_6O_6P$, 506.1479. Found, 506.1492.

Prodrug **18r** was isolated as pure isomers at phosphorous using the same chiral separation procedure reported above for **18v**.

4.50. Prodrug 18r(A) (Ala-cyclo-butyl, OPh ester, diastereoisomer A, 48% recovery)

^1H NMR (300 MHz, $CDCl_3$) δ 8.39 (s, 1H), 8.10 (s, 1H), 7.21 (m, 5H), 6.82 (m, 1H), 5.97 (m, 1H), 5.79 (d, *J* = 1.2 Hz, 1H), 5.68 (br s, 1H), 4.96 (m, 1H), 4.01–4.20 (m, 4H), 3.60 (t, *J* = 9.9 Hz, 1H), 2.29 (m, 2H), 2.02 (m, 2H), 1.62–1.80 (m, 4H) 1.28 (d, *J* = 6.9 Hz, 3H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.7. HRMS (ESI) *m*/*z* Calc. for $C_{23}H_{26}FN_6O_6P$, 532.1635. Found, 532.1659.

4.51. Prodrug 18r(B) (Ala-cyclo-butyl, OPh ester, diastereoisomer B, 36% recovery)

^1H NMR (300 MHz, $CDCl_3$) δ 8.39 (s, 1H), 8.10 (s, 1H), 7.14–7.30 (m, 5H), 6.80 (m, 1H), 5.86 (m, 1H), 5.77 (m, 2H), 4.98 (m, 1H), 3.92–4.22 (m, 4H), 3.72 (m, 1H), 2.32 (m, 2H), 2.04 (m, 2H), 1.60–1.90 (m, 4H) 1.33 (d, *J* = 7.8 Hz, 3H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 19.7.

4.52. Diphenyl-hydroxymethylphosphonate (20)

A suspension of paraformaldehyde (1.6 g, 53 mmol) in CH_2Cl_2 (25 mL) at 0 °C was treated with TMSI (8 mL, 56 mmol). The resultant mixture was stirred at 0 °C for 30 min. The ice bath was removed, and a solution of diphenylphosphite (12.5 g, 50 mmol) in CH_2Cl_2 (25 mL) was added dropwise. The resultant solution was stirred in the dark for 16 h. The reaction mixture was then partitioned between CH_2Cl_2 and cold saturated aqueous $NaHCO_3$ solution. The organic phase was separated and washed with brine. The organic phase was concentrated under reduced pressure and the crude residue subjected to silica gel chromatography eluting with 0–40% EtOAc in CH_2Cl_2 to afford **20** (7.3 g, 55%). ^1H NMR (300 MHz, $CDCl_3$) δ 7.34–7.38 (m, 4H), 7.20–7.33 (m, 6H), 4.21 (m, 2H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 17.1.

4.53. Diphenyl 5-(*R*)-[(6-methoxy-purin-9-yl)-4-(*R*)-fluoro-3-(*R*)-iodo-tetrahydro-furan-2-(*R*)-yloxymethyl]phosphonic acid (21)

A suspension of carboxylic acid **9** (4.0 g, 13.4 mmol) in CH_2Cl_2 (100 mL) was heated to 40 °C. The mixture was treated with PPh_3 (4.2 g, 16.1 mmol) and DIAD (3.3 g, 16.1 mmol). The resultant mixture was heated at 40 °C for 2 h and then cooled to −50 °C. Diphenyl-(hydroxymethyl)phosphonate **20** (7.3 g, 27 mmol) was added followed by the dropwise addition of a solution of IBr (6.8 g, 33 mmol) in CH_2Cl_2 (30 mL). The resultant mixture was stirred at −20 °C for 30 min and then at rt for 2 h. The reaction mixture was poured into aqueous $NaHCO_3$ containing $Na_2S_2O_3$. The organic phase was separated, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography eluting with EtOAc in hexanes to afford **21** (3.5 g, 40%). ^1H NMR (300 MHz, $CDCl_3$) δ 8.56 (s, 1H), 8.17 (d, *J* = 3.9 Hz, 1H), 7.20–7.38 (m, 10H), 7.10 (dd, *J* = 19.3, 4.0 Hz, 1H), 5.67 (s, 1H), 5.4 (dd, *J* = 49.2, 3.9 Hz, 1H), 4.55 (d, *J* = 16.5 Hz, 1H), 4.35–4.42 (m, 4H), 4.19 (s, 3H), 4.13–4.19 (m, 1H). ^{13}C NMR (77 MHz, $CDCl_3$) δ 166.1, 152.5, 151.7, 150.0, 149.6, 142.1, 142.0, 130.0, 129.9, 125.8, 125.7, 120.7, 120.6, 111.8, 111.6, 96.1, 93.4, 84.1, 84.8, 62.9, 60.6, 57.6, 54.4. ^{19}F NMR (282.2 MHz, $CDCl_3$) δ −166.14(m). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 12.12.

4.54. Phenyl 5-(*R*)-[(6-amino-purin-9-yl)-4-fluoro-2,5-dihydro-furan-2-(*R*)-yloxymethyl]phosphonic acid (22)

Diphenyl ester **21** (2 g, ~3 mmol) was dissolved in acetic acid (2 mL) and MeOH (20 mL). The mixture was cooled to 0 °C and then treated by dropwise addition with NaClO (10–13%) (13 mL). The reaction mixture was stirred for 1 h and then concentrated under reduced pressure. The residue was partitioned between CH_2Cl_2 and saturated aqueous $NaHCO_3$. The organic phase was separated, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was redissolved in 1,4-dioxane (8 mL) and. NH_4OH (20 mL). The mixture was heated at 100 °C for 5 h

and then cooled to rt. The resultant mixture was concentrated under reduced pressure and the residue subjected to silica gel chromatography eluting with 50% EtOAc in CH_2Cl_2 followed by 15% water in CH_3CN. The collected product was then further purified by HPLC chromatography (linear gradient, 5–95% CH_3CN in water) to give the title product **22** (240 mg, 18%). ^1H NMR (300 MHz, CD_3OD) δ 8.38(s, 1H), 8.27 (s, 1H), 6.98–7.20 (m, 5 H), 6.82 (d, J = 3.0 Hz, 1H), 5.96–5.98 (m, 2H), 3.96–4.01 (m, 1H), 3.80–3.86 (m, 1H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 12.1. LC–MS m/z 408 $[M+H]^+$.

4.55. Prodrug 23 (ethyl-(*S*)-2-hydroxypropionic acid ester, OPh ester)

A solution of monoacid **22** (22 mg, 0.05 mmol) and PyBoP (52 mg, 0.1 mmol) in DMF (1.5 mL) was stirred at rt for 30 min. The mixture was then treated with ethyl, 2-(*S*)-hydroxypropionic acid ester (18 mg, 0.15 mmol) and Et_3N (7.6 mg, 0.075 mmol). The resultant mixture was stirred overnight and then diluted with EtOAc. The solution was washed with saturated $NaHCO_3$, brine, dried over $MgSO_4$, filtered and concentrated to give a crude solid. The crude solid was subjected to HPLC chromatography (linear gradient, 5–95% CH_3CN in water) to provide **23** (5 mg, 19%). ^1H NMR (300 MHz, $CDCl_3$) δ 8.40 (s, 1H), 8.11 (s, 1H), 7.22 (m, 5H), 6.82 (d, J = 2.4 Hz, 1H), 5.97 (d, J = 3.3 Hz, 1H), 5.81 (s, 1H), 5.65 (s, 2H), 5.03 (m, 1H), 4.19 (m, 5H), 1.44 (d, J = 6.9 Hz, 3H), 1.23 (t, J = 6.9 Hz, 3H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 18.2. LC–MS m/z 508 $[M+H]^+$.

4.56. Antiviral activity

The methods used for the evaluation of the antiviral activity have been described previously.[5]

4.56.1. In vitro Cat A, PBMC, hepatic and intestinal S9 stabilities and in vivo methods

The method describing the profiling of compounds toward human recombinant cathepsin A has been reported.[15] Extracts from human PBMC were prepared as described previously.[18] The prodrugs (30 μM) were incubated with PBMC extracts (134 mg/mL) for 5, 10, 30, and 120 min at 37 °C in a reaction buffer containing 25 mM Mes-Na, pH 6.5, 100 mM NaCl, 0.1% Nonidet P-40 and 1 mM DTT. After the incubation, the reactions were quenched by adding ice-cold methanol (final concentration, 70%) and centrifuged at 13,000*g* for 30 min. The supernatants were evaporated, re-solubilized in 100 μL of buffer A (25 mM potassium phosphate, pH 6.0, 5 mM tetrabutylammonium bromide), and injected onto a C_{18} reverse-phase column (Prodigy 5 micron ODS(2); Phenomenex, Torrance, CA) equilibrated with buffer A. Bound prodrugs and metabolites were resolved using a linear gradient of acetonitrile (0–70%, 11 min, 0.25 mL/min) with buffer B (25 mM KPO_4, pH 6.0, 5 mM tetrabutylammonium bromide, 70% acetonitrile). The peak areas of parent nucleotide, parent nucleotide–amino acid conjugate and prodrug was used for the calculation of the reaction rates. The activity was expressed as picomoles of metabolites produced per minute per minute per μg protein. The intestinal and hepatic stability of prodrugs were assessed in pooled intestinal and hepatic cytosolic supernatant fractions collected after centrifugation at 9000*g* (S9) obtained from In Vitro Technologies (Baltimore MD). Briefly, 2 μM of the prodrugs were incubated with S9 extract at 37 °C and samples taken over 90 min. Hepatic S9 studies were done in the presence of mixed cofactors (NADPH regenerating system, UDPGA and alamethicin). Amounts of remaining prodrug at each collected time point were quantified by LC/MS/MS and the half life determined by fitting the data to an exponential decay curve. The methods for the in vivo evaluation of prodrugs have been reported.[4]

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Supplementary data

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