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Synthesis, anti-HIV activity, and resistance profile of thymidine phosphonomethoxy nucleosides and their bis-isopropyloxymethylcarbonyl (bisPOC) prodrugs

Richard L. Mackman,* Lijun Zhang, Vidya Prasad, Constantine G. Boojamra, Janet Douglas, Deborah Grant, Hon Hui, Choung U. Kim, Genevieve Laflamme, Jay Parrish, Antitsa D. Stoycheva, Swami Swaminathan, KeYu Wang and Tomas Cihlar

Gilead Sciences, Inc., 333 Lakeside Drive, Foster City, CA 94404, USA

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Abstract—Phosphonomethoxy nucleoside analogs of the thymine containing nucleoside reverse transcriptase inhibitors (NRTIs), 3′-azido-2′,3′-dideoxythymidine (AZT), 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T), and 2′,3′-dideoxythymidine (ddT), were synthesized. The anti-HIV activity against wild-type and several major nucleoside-resistant strains of HIV-1 was evaluated together with the inhibition of wild-type HIV reverse transcriptase (RT). Phosphonomethoxy analog of d4T, **8** (d4TP), demonstrated antiviral activity with an EC_{50} value of 26 μM, whereas, phosphonomethoxy analogs of ddT, **7** (ddTP), and AZT, **6** (AZTP), were both inactive at concentrations up to 200 μM. Bis-isopropyloxymethylcarbonyl (bisPOC) prodrugs improved the anti-HIV activity of **7** and **8** by >150-fold and 29-fold, respectively, allowing for antiviral resistance to be determined. The K65R RT mutant virus was more resistant to the bisPOC prodrugs of **7** and **8** than bisPOC PMPA (tenofovir DF) **1**. However, bisPOC prodrug of **7** demonstrated superior resistance toward the RT virus containing multiple thymidine analog mutations (6TAMs) indicating that new phosphonate nucleoside analogs may be suitable for targeting clinically relevant nucleoside resistant HIV-1 strains.

1. Introduction

Since the introduction of AZT-based antiretroviral therapy, a suite of nucleoside and nucleotide HIV reverse transcriptase inhibitors (N(t)RTIs) have been approved for clinical use in the treatment of HIV.[1] Tenofovir disoproxil fumarate (tenofovir DF, Viread™) **1** is a prodrug of PMPA **2**, a unique member of this class of drugs since it is the only nucleoside phosphonate and the only acyclic ribose analog (Fig. 1).[2] The favorable resistance and toxicity profile of tenofovir DF, in addition to the benefits of a once-daily administration, has led to its widespread use in antiretroviral combinations.[3] However, a subset of treatment-experienced patients with either K65R mutation or multiple ($\geqslant$3) thymidine analog mutations (TAMs), including M41L and/or L210W in RT, respond sub-optimally to tenofovir DF treatment.[4] The identification of HIV strains with reduced susceptibility to tenofovir prompted our search to identify new N(t)RTIs that possess a superior resistance profile to tenofovir DF.

2. Strategy

The phosphonomethoxy moiety of **2** is a bioisostere of a nucleoside monophosphate. However, compared to the phosphate group, the phosphonate is chemically stable and insensitive to enzymatic hydrolysis. Once delivered into cells, via a prodrug (e.g., **1**), the released phosphonate diacid **2** is effectively metabolized to the active diphospho-phosphonate (triphosphate equivalent).[5] Moreover, the high polarity and limited permeability of the phosphonate diacid can be a significant advantage when combined with a long intracellular half-life of the active diphospho-phosphonate species. Together, these properties favor the intracellular accumulation of the active metabolite resulting in optimal pharmacodynamics.[5] For these reasons, we focused our design efforts toward new nucleoside phosphonate analogs and their prodrugs.

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* Corresponding author. Tel.: +1 650 522 5258; fax: +1 650 522 5899; e-mail: rmackman@gilead.com

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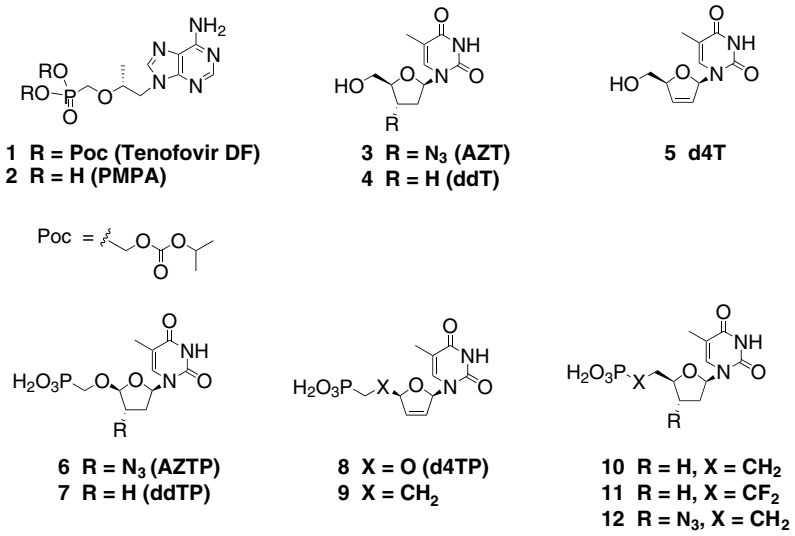
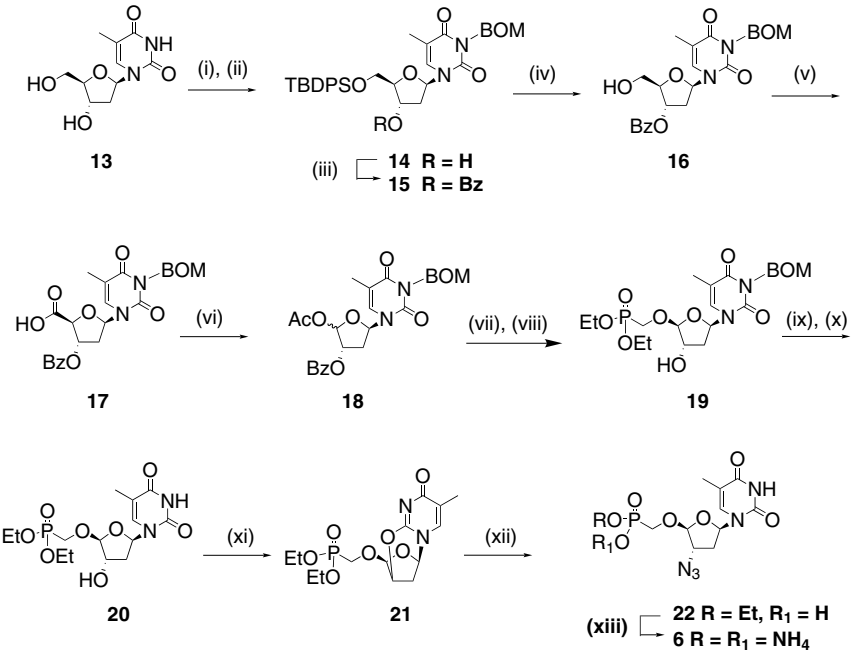


Figure 1. Structures of phosphonate and nucleoside analogs.

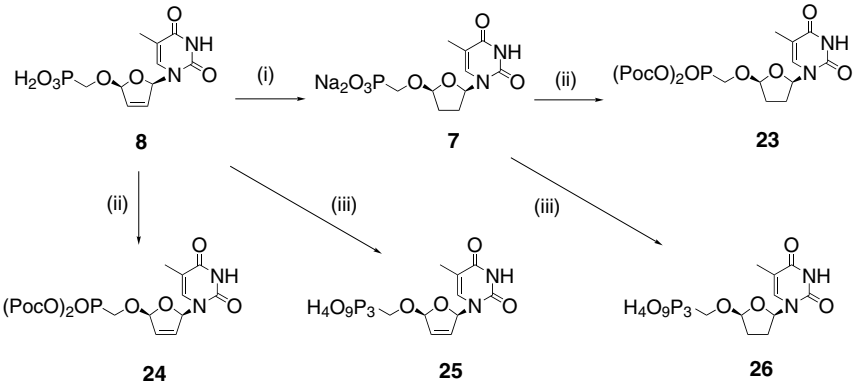
In comparison with other clinically approved and investigational N(t)RTIs, the anti-HIV activity of thymidine nucleosides AZT, ddT, and d4T (**3**–**5**) is only modestly affected by the K65R mutation in cell culture assays.[6,7] Evaluation of 2′,3′-dideoxy (dd) analogs of inosine, guanosine, cytidine, and thymidine toward a recombinant K65R virus indicated that the thymidine analog, ddT **4**, was the least susceptible to K65R mutation.[7] It was therefore reasoned that nucleoside phosphonates of thymidine based HIV RT inhibitors AZT, ddT, and d4T, for example, **6**–**8**, may be highly effective against HIV strains containing the K65R mutation. Phospho*nomethoxy* nucleoside **8** and several phospho*nonalkyl* thymidine nucleosides (**9**–**12**) have been reported in the literature but all, apart from **8**, are inactive toward HIV.[8–11] However, the presence of anti-HIV activity for phospho*nomethoxy* analog, **8**, and lack of anti-HIV activity for the corresponding phospho*nonalkyl* analog **9**, suggests that the phospho*nomethoxy* group may be critical for anti-HIV potency. Therefore, our strategy was to synthesize the thymidine based phospho*nomethoxy* analogs **6**–**8**, compare and contrast their respective anti-HIV potency and resistance profiles, and evaluate prodrugs accordingly.

3. Synthesis

The phosphonomethoxy analog **6** was prepared from thymidine in 13 steps (Scheme 1). The two key transformations in the sequence were first, the stereoselective introduction of the phosphonate group in the β-configuration, and second, replacement of the 3′-OH with an azide. A Lewis acid-mediated glycosidation of a phosphonate alcohol onto an anomeric acetate such as **18** could be used to introduce the desired phosphonate as reported previously.[12] Stereoselectivity would be controlled by anchiomeric assistance from the 3′-OBz group. To begin, 5′-*tert*-butyl-diphenylsilyl-N^3-benzyloxymethyl 2′-deoxythymidine, **14**, was prepared from thymidine, **13**, following literature procedures.[13] Benzoyl protection of 3′-OH was followed by TBAF mediated 5′-*O*-silyl deprotection to afford **16** in high yield.[12] The 5′-OH was then efficiently oxidized to carboxylic acid **17** by treatment with catalytic TEMPO and stoichiometric bisacetoxyiodobenzene.[14] Acid **17** was then transformed into α and β anomeric acetates **18** by treatment with $Pb(OAc)_4$. The β anomer predominated but the mixture of anomers was carried forward to the next step. Treatment of the acetate anomers with diethyl hydroxymethylphosphonate in the presence of Lewis acid TMSOTf, followed by removal of the 3′-benzoyl group using ammonium hydroxide, resulted in the formation of the desired phosphonate **19**. Unfortunately, the overall yield for this reaction was relatively poor (28% from **18**) compared to the literature.[12] An equivalent amount of the *C*-1′-α-isomer was obtained, presumably through TMSOTf-mediated isomerization over the prolonged reaction time. The structures of **19** and its *C*-1′-α-isomer were established using 2D NMR spectroscopy. Similar *C*-1′-epimerizations in the presence of TMSOTf have been reported.[15] The *N*-benzyloxymethyl group was then removed by hydrogenolysis followed by treatment with ammonium hydroxide to provide **20**. Dehydration of **20** to give the anhydro intermediate **21** proceeded smoothly using DAST.[16] Mitsunobu dehydration conditions for the formation of the anhydro intermediate **21** were not successful. Finally, acid promoted opening of **21** by treatment with sodium azide proved quite difficult, requiring high temperatures and prolonged reaction times. Under the forcing conditions required one of the phosphonate alkyl groups was cleaved to give the mono acid **22** as the major product. To complete the synthesis of **6**, the remaining phosphonate ester group was removed by treatment with TMSBr followed by isolation as the ammonium salt. Saturated analog **7** was prepared from **8** by hydrogenation using catalytic Pd/C (Scheme 2).[17] The bisPOC prodrugs, **23** and **24**, were prepared from the phosphonate diacids **7** and **8**, respectively, by treatment with chloromethyl isopropyl carbonate in the presence of triethylamine.[18] Diphospho-phosphonates, **25** and **26** were prepared



Scheme 1. Reagents and conditions: (i) TBSCl, DMAP, pyridine, rt, 16 h; (ii) BOMCl, DBU, DMF, 2 h; (iii) BzCl, pyridine, DMAP; (iv) TBAF, THF, 0 °C, 1.5 h; (v) TEMPO, $PhI(OAc)_2$, CH_3CN, H_2O, rt, 16 h; (vi) $Pb(OAc)_4$, pyridine/DMF, 16 h; (vii) TMSOTf, hydroxymethyl phosphonic acid diethyl ester, CH_2Cl_2, −25 °C to rt, 16 h; (viii) NH_4OH, MeOH, rt, 6 h; (ix) $Pd(OH)_2$, H_2, EtOH, rt, 16 h; (x) NH_4OH, MeOH, 40 °C, 16 h; (xi) diethylaminosulfur trifluoride, CH_2Cl_2, −78 °C, 30 min; (xii) NaN_3, DMF, benzoic acid, 120 °C, 36 h; (xiii) TMSBr, DMF, 2,6-lutidine, 0 °C to rt, 16 h, then NH_4OH.



Scheme 2. Reagents and conditions: (i) Pd/C, H_2, EtOH, rt, 2 h; (ii) chloromethylisopropyl carbonate, Et_3N, DMF, 70 °C, 3 h, then rt, 16 h; (iii) bis(tributylammonium)pyrophosphate, CDI, DMF, rt, 16 h.

from diacids **8** and **7**, respectively, following literature procedures (Scheme 2).[10]

4. Results and discussion

The anti-HIV activity of the thymidine phosphonates, **6**–**8**, is reported in Table 1. Neither **6** nor **7** displayed anti-HIV activity at concentrations up to 200 μM. However, the unsaturated 2′,3′-didehydro-2′,3′-dideoxy (d4) analog **8**, consistent with earlier reports,[8] demonstrated low micromolar activity, only 9-fold less active than PMPA **2**. To probe the basis for the different antiviral activities of **7** and **8**, their respective active metabolites, diphospho-phosphonates, **26** and **25** were prepared. 2′,3′-Dideoxy (dd) analog **26** was approximately 14-fold less active toward HIV RT than the d4 analog **25**. Thus, the weak antiviral activity of dd analog **7** can be attributed, at least in part, to the weaker inhibition of HIV RT by the active metabolite produced inside the cell. It is worth noting that both phosphonates are poorer inhibitors of RT than the corresponding nucleosides ddT and d4T (Table 1). The phosphonate bioisostere replacement for the nucleoside phosphate group is clearly inferior with respect to RT inhibition. The trends in RT activity between analogs **7** and **8**, (the d4 analog being more potent than the dd analog) are mirrored by the respective nucleoside triphosphates of d4T and ddT (Table 1). Favorable π–π interactions between tyrosine 115 in the active site of HIV RT and the double bond of the d4 analogs are proposed to be responsible for the superior activity.[19] The parallel activity trends

Table 1. HIV antiviral activity, cytotoxicty, RT inhibition, and resistance profile

Compound	Diphospho-phosphonate	Code	WT HIV[a] EC_{50}	HIV RT[b] IC_{50}	MT-2[c] CC_{50}	6TAMs[d] fold	K65R fold	M184V fold
2		PMPA	3.6 (1.5)	0.38 (0.20)	>200	8.8 (3.7)	4.3 (1.5)	0.7 (0.2)
3		AZT	0.16 (0.12)	0.05 (0.03)	>200	>50	1.2 (0.7)	0.7 (0.2)
4		ddT	>200	0.12 (0.09)	—	—	—	—
5		d4T	4.8 (2.3)	0.06 (0.01)	286 (122)	5.5 (2.5)	2.3 (1.1)	1.2 (0.2)
6		AZTP	>200	—	—	—	—	—
7	**26**	ddTP	>200	5.4 (2.1)	>500	—	—	—
8	**25**	d4TP	26 (8.8)	0.39 (0.08)	>500	13 (3.9)	5.2 (0.3)	1.1
1		BP[e]-PMPA	0.015 (0.007)	—	>50	7.7 (2.5)	3.1 (0.4)	—
23		BP[e]-ddTP	1.3 (0.8)	—	>50	2.6 (0.5)	5.9 (0.6)	—
24		BP[e]-d4TP	0.91 (0.42)	—	10	6.8 (0.7)	6.7 (2.6)	—

Results are expressed in μM with standard deviations in parentheses.
[a] Antiviral activity in MT-2 cells using HIV-IIIa.
[b] Diphospho-phosphonates or nucleoside triphosphates were used in this assay.
[c] Cytotoxicity measured in MT-2 cells.
[d] 6TAMs virus contains M41L/D67N/K70R/L210W/T215Y/K219Q mutations in reverse transcriptase.
[e] BP, bis-isopropyloxymethylcarbonyl prodrug.

also suggest that the weaker IC_{50} of the phosphonates is not the result of gross differences in binding mode in the active site of RT but, more likely, differences in the polymerization step. Indeed AZT phosphonate **12**, when compared to AZT using pre-steady state kinetics, was found to have a significantly reduced rate of polymerization but a similar binding constant.[10,20]

Despite the parallel RT inhibition trends, the dd analog **7** is a surprisingly weak RT inhibitor, being 45-fold weaker than the corresponding nucleoside ddT, **4**, triphosphate, whereas the unsaturated d4 analog **8** is only 7-fold weaker than d4T (**5**) triphosphate. This incremental increase in the IC_{50} for the dd analog **7** implies there may be an additional factor influencing the RT activity of this particular type of phosphonate. One key structural difference between the phosphono*methoxy* analogs and the corresponding nucleosides is the generation of a second anomeric center. To probe the impact of the additional anomeric center, the solution conformation of **7** was determined from the ^{1}H NMR *J* couplings and NOEs in D_2O at 25 °C (see experimental Section 6.3).[21] 2′,3′-Dideoxy nucleosides typically have a predominantly Northern (P = 0–20°; 3′-endo, 2′-exo) conformation.[21–23] In contrast **7** has a dramatically different (P = 246°) conformation (Fig. 2). The anomeric effect drives the phosphono*methoxy* group closer to a *pseudoaxial* position unlike the *psuedoequatorial* orientation found in a typical Northern nucleoside conformation. As a result, it is likely that there is an unfavorable energy associated with dd analog **7** adopting a Northern conformation similar to ddT. This is important, because the Northern conformation has been proposed to be the required conformation for substrate binding to HIV RT and is supported by the X-ray structure and reported data on constrained carbocyclic inhibitors.[19,24] In the case of the d4 phosphonate analog **8**, the sp^2 carbonsrender the furanose ring planar and more rigid, thus minimizing the impact of the additional anomeric center on the conformation.[25] It is anticipated that the conformation of the d4 phosphonate analog **8** is therefore similar to nucleoside d4T, **5**. Further studies have been performed on other dd phosphonate analogs

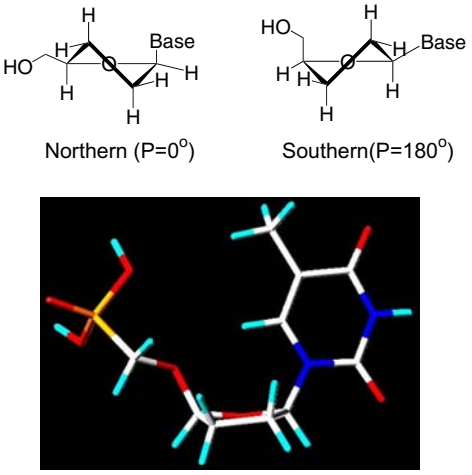


Figure 2. NMR derived conformation of **7** (P = 246°, ψ = 20°) and schematic showing ribose conformations defined as Northern (P = 0°) and Southern (P = 180°).

leading to similar IC_{50} trends and conformational differences (data not shown).

In addition to enzymatic potency, poor cellular uptake and/or intracellular phosphorylation can also contribute to poor antiviral activity. Therefore, to evaluate the effects of poor cellular uptake on the antiviral activity, bisPOC prodrugs, **23** and **24**, were prepared from **7** and **8**, respectively (Scheme 2). The bisPOC prodrugs improved antiviral potency of **8** by 29-fold and **7** by >150-fold, whereas the bisPOC prodrug of PMPA **2** improved activity by 250-fold (Table 1). Interestingly, bis(POC) prodrug **23** now displays comparable antiviral activity to bis(POC) prodrug **24** despite the lower inhibition of HIV RT. Thus, the prodrugs can compensate for reduced RT activity by facilitating permeability of the phosphonate across the cell membrane leading to the generation of higher intracellular levels of active diphosphate (triphosphate equivalent) species. Further studies on AZT analog **6** to probe the basis for its poor antiviral potency have been hampered by a lack of sufficient

material. However, given that dd analog **7** is a poor inhibitor of HIV RT, it is likely that **6**, being a saturated ribose analog, also suffers from poor RT potency. Furthermore, intracellular phosphorylation of **6** may be limited, similar to that observed for AZT monophosphate.[26]

The resistance profile toward viruses containing six thymidine analog mutations (6TAMs), K65R or M184V was evaluated for analog **8** and its bis(POC) prodrug **23**, and also bis(POC) prodrug **24** (Table 1). The susceptibility of many clinically approved and investigational N(t)RTI toward K65R has been reported in the literature.[6,7] Thymidine analogs, for example, AZT and d4T, have, in general, been found to be less susceptible in cell culture to the K65R recombinant viruses than other inhibitors, including PMPA **2**.[6] In this study, nucleoside d4T **5** was found to have a 2.3-fold drop in potency toward K65R, whereas, d4 analog **8** and its bis-POC prodrug **24** both exhibited 5- to 7-fold reduced activity against K65R recombinant virus. A comparison cannot be made between ddT **4** and the dd analog **7** due to its poor antiviral potency, but the bis(POC) prodrug **23** showed a 5.9-fold reduction in the potency against K65R compared to 2.2-fold reported for ddT.[7] The phosphonate therefore introduces a small unfavorable contribution toward K65R resistance compared to the nucleosides. The K65 residue lies in the fingers domain of HIV RT and H-bonds to the γ-phosphate of the incoming dNTP substrate, thereby aligning it correctly for incorporation.[19] Mutation to arginine is proposed to reduce the conformational mobility of RT active site, and, in turn, reduce binding and incorporation of the dNTP.[27,28] In the case of phosphonate analogs **7** and **8**, the increased K65R resistance is likely due to subtle changes in the binding mode that lead to further drops in incorporation efficiency. The O–C–P atom arrangement compared to the natural C–O–P is often regarded as isosteric but a more strict analysis indicates that the bond lengths and angles are subtly different.[29] Moreover, the ether oxygen creates an anomeric center at C4′ which was shown above to impact the ribose conformation. Thus, the increased ability of the K65R mutant to resist **7** and **8** could easily be caused by subtle differences in the positioning of the α-phosphonate for attack by the primer 3′-hydroxyl.

The 6TAMs mutant promotes ATP- or pyrophosphate-mediated unblocking of the N(t)RTI terminated primer in the Nucleotide-site (N-site) of RT.[30] It has been proposed that some NRTIs, for example, AZT, preferentially reside in the N-site leading to more efficient excision by RT containing TAMs mutations.[31] The 6TAMs mutant was marginally more resistant toward analog **8** than the corresponding nucleoside d4T **5**. Bis(POC) prodrug **24** was not substantially different given the experimental error in the antiviral assay. The 6TAMs mutant, however, is only 2.6-fold resistant toward dd prodrug **23**. Indeed the 6TAMs profile for **23** is superior to all the other phosphonate and nucleoside thymidine analogs shown in Table 1. The underlying reason for the improved profile may lie in the more puckered dd ring that in turn positions the alpha phosphonate group unfavorably for the excision mechanism to take place. Further kinetic studies will be required to understand the basis for the improved TAMs profile.

The M184V mutation is located in the active site of RT underneath the primer and in proximity to the ribose oxygen. It is primarily selected by L-nucleosides, that is, 3TC and FTC, and represents the most prevalent NRTI mutation occurring in >40% of all NRTI-experienced patients.[32] Similar to PMPA, and many natural d-configuration nucleosides with a 1′-O atom, the activity of analog **8** was not significantly affected by M184V (1.1-fold, Table 1). The level of M184V resistance was essentially identical to that of d4T **5**.

5. Conclusions

The synthesis of several thymidine phosphono*methoxy* nucleosides has established that phosphonates **7** (ddT analog) and **8** (d4T analog) are both inhibitors of HIV RT, albeit weaker than the corresponding nucleosides. The second anomeric center in the saturated dd analogs promotes an unfavorable ribose ring conformation which may account for the greater loss in RT inhibition for the dd analog. BisPOC prodrugs effectively improved cellular uptake leading to low micromolar antiviral activity and allowed the resistance profile toward K65R, 6TAMs, and M184V to be determined. Although bis(POC) prodrug **23** shows a superior TAMs profile than bis(POC) PMPA **1**, the poor K65R resistance profile has reduced the potential for further development. Efforts to identify alternative phosphonates with excellent antiviral activity and resistance profiles continue.

6. Experimental

6.1. General

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 and ^{31}P NMR spectra were recorded on a Varian 300- or 500-MHz NMR. NMR shifts are reported relative to an internal standard as noted. Low-resolution LC mass spectra (LR LCMS) were recorded on two different Thermo Finnigan LCQ Advantage, electrospray ionization (ESI) mass spectrometers. LR LCMS(A) 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 acetonitrile). LR LCMS(B) was equipped with a Phenomenex Luna C18 (30 × 2.0 mm) column eluting with a gradient of 0–100% solvent B in solvent A over 8 min at a flow rate of 1.75 mL/min (solvent A = 1% AcOH in water, solvent B = 1% AcOH in MeOH). High resolution mass spectroscopy (HRMS) was determined by UC Berkeley mass spectrometry facility using FAB ionization. Reversed phase analytical HPLC for phosphonate diacids and bisPOC prodrugs was performed on a Agilent 1100

series, equipped with a Phenomenex Luna C18 5 μm 100 A column (250 × 4.6 mm), eluting with a gradient of 2–70% solvent B in solvent A over 30 min (solvent A = 0.1% TFA in water; solvent B = 0.1% TFA in acetonitrile). Ion exchange HPLC analysis for diphosphophosphonates was performed 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).

6.2. Chemistry

6.2.1. (2*R*,5*R*)-[5-(5-Methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-2,5-dihydro-furan-2-yloxymethyl]-phosphonic acid disodium salt (8). Compound **8** was prepared according to the method of Kim et al.[17] Compound **8**: ^{1}H NMR (300 MHz, D_2O) δ 7.30 (s, 1H), 6.68 (s, 1H), 6.34 (d, J = 8.7 Hz, 1H), 6.05 (d, J = 8.7 Hz, 1H), 5.85 (s, 1H), 3.40–3.62 (m, 2H), 1.75 (s, 3H). ^{31}P NMR (121.4 MHz, D_2O) δ 12.72. LR LCMS(A) (M+Na) 327.2 (0.25 min). FABHRMS (M+Na) calc. for $C_{10}H_{13}N_2O_7NaP$ 327.0358, found 327.0354. HPLC 8.62 min, 99.5%.

6.2.2. (2*R*,3*S*,5*R*)-Benzoic acid 5-(3-benzyloxymethyl-5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-2-(*tert*-butyl-diphenyl-silanyloxymethyl)-tetrahydro-furan-3-yl ester (15). The protected thymidine **14**[11] (5.0 g, 8.3 mmol) was dissolved in pyridine (10 mL) and the mixture cooled to 0 °C. The mixture was then treated with benzoyl chloride (1.45 mL, 12.5 mmol) followed by DMAP (0.31 g, 2.5 mmol). The resultant mixture was stirred for 3 h and then concentrated under reduced pressure. The crude solid was redissolved in ethyl acetate and the insoluble material was collected by filtration. The solution was then concentrated under reduced pressure and the resultant solid was subjected to silica gel chromatography eluting with a gradient of 15–30% ethyl acetate in hexanes to give the title product **15** (5.56 g, 94%). Compound **15**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (dd, J = 1.2, 8.6 Hz, 2H), 7.24–7.73 (m, 9H), 6.56 (dd, J = 5.1, 9.3 Hz, 1H), 5.71 (d, J = 6.1 Hz, 1H), 5.53 (s, 2H), 4.73 (s, 2H), 4.26 (d, J = 1.9 Hz, 1H), 4.08 (d, J = 1.9 Hz, 2H), 2.64 (dd, J = 5.4, 13.9 Hz, 1H), 2.32–2.42 (m, 1H), 1.60 (d, J = 1.0 Hz, 3H), 1.41 (s, 9H). LR LCMS(B) (M+H) 705.3.

6.2.3. (2*R*,3*S*,5*R*)-Benzoic acid 5-(3-benzyloxymethyl-5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-2-hydroxymethyl-tetrahydro-furan-3-yl ester (16). The benzoyl protected thymidine **15** (1.55 g, 2.2 mmol) was dissolved in THF (20 mL) and the mixture cooled to 0 °C. The cooled solution was then treated with a solution of TBAF in THF (1 M, 2.8 mL, 2.8 mmol). The reaction mixture was stirred for 1.5 h and then quenched by the addition of saturated aqueous ammonium chloride. The mixture was extracted with ethyl acetate (3× 20 mL) and the combined organic extracts dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was subjected to silica gel chromatography, eluting with a gradient of 15–70% ethyl acetate in hexanes, to give the title product **16** (0.83 g, 81%). Compound **16**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.05 (dd, J = 1.2, 8.6 Hz, 2H), 7.22–7.63 (m, 9H), 6.39 (dd, J = 6.2, 8.0 Hz, 1H), 5.60 (m, 1H), 5.51 (s, 2H), 4.71 (s, 2H), 4.25 (d, J = 2.1 Hz, 1H), 4.00 (d, J = 2.4 Hz, 2H), 2.46–2.60 (m, 2H), 1.94 (d, J = 1.2 Hz, 3H). LR LCMS(B) (M+Na) 489.3.

6.2.4. (2*S*,3*S*,5*R*)-Benzoyloxy-5-(3-benzyloxymethyl-5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-2-carboxylic acid (17). The alcohol **16** (0.83 g, 1.78 mmol) was dissolved in acetonitrile (8 mL). BAIB (1.26 g, 3.92 mmol) was added to the solution followed by water (8 mL). The mixture was then treated with TEMPO (0.056 g, 0.36 mmol) and stirred for 16 h. The mixture was then concentrated under reduced pressure and the crude product subjected to silica gel chromatography, eluting with a gradient of 30–50% ethyl acetate in hexanes, to give the title product **17** (0.65 g, 76%). Compound **17**: ^{1}H NMR ($CDCl_3$) δ 8.05–8.10 (m, 3H), 7.25–7.64 (m, 9H), 6.57 (dd, J = 5.2, 9.2 Hz, 1H), 5.77 (d, J = 5.2 Hz, 1H), 5.53 (s, 2H), 4.80 (s, 1H), 4.72 (s, 2H), 2.66 (dd, J = 5.5, 14.1 Hz, 1H), 2.20–2.29 (m, 1H), 1.97 (s, 3H). LR LCMS(B) (M−H) 479.7.

6.2.5. (2*RS*,3*S*,5*R*)-Benzoic acid 2-acetoxy-5-(3-benzyloxymethyl-5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl ester (18). The acid **17** (1.89 g, 3.94 mmol) was dissolved in DMF (10 mL) and treated with pyridine (1.15 mL, 15.8 mmol). The reaction mixture was flushed with nitrogen and then treated with lead tetraacetate (8.5 g, 19.7 mmol). The reaction mixture was protected from light and stirred for 16 h. The mixture was then concentrated under reduced pressure and the crude residue subjected to silica gel chromatography, eluting with a gradient of 30–60% ethyl acetate in hexanes, to give the title product **18** (1.67 g, 86%) as a mixture of isomers (predominantly β-isomer). A sample of the β-isomer was purified for analysis. Compound β-**18**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.06 (d, J = 7.7 Hz, 1H), 7.23–7.66 (m, 9H), 6.74 (dd, J = 6.4, 8.2 Hz, 1H), 6.43 (s, 1H), 5.54 (d, J = 5.1 Hz, 1H), 5.51 (s, 2H), 4.71 (s, 2H), 2.77 (dd, J = 6.3, 15.2 Hz, 1H), 2.29–2.39 (m, 1H), 2.20 (s, 3H), 1.97 (s, 3H). LR LCMS(B) (M+Na) 517.2.

6.2.6. (2*R*,3*S*,5*R*)-[5-(3-Benzyloxymethyl-5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-3-hydroxy-tetrahydro-furan-2-yloxymethyl]-phosphonic acid diethyl ester (19). The acetate **18** (1.67 g, 3.38 mmol) was dissolved in DCM (50 mL) and then cooled to −25 °C. The cooled solution was then treated with hydroxyethyl phosphonic acid diethyl ester (1.5 mL, 10.1 mmol) followed by TMSOTf (2.45 mL, 13.5 mmol). The mixture was stirred for 16 h and then quenched with triethylamine (2.1 mL, 15 mmol). The resultant solution was concentrated under reduced pressure and the crude residue used in the next reaction. The crude residue was dissolved in methanol (4 mL) and ammonium hydroxide (4 mL) and the reaction mixture stirred for 6 h. The reaction mixture was concentrated under reduced pressure and the crude residue subjected to HPLC chromatography to isolate the title product 5*R*-**19** (232 mg, 14%). Eluant was a gradient of 20–90% solvent B in solvent A over 26 min (sovent A = water, solvent B = acetonitrile). An equivalent amount (14%) of the 5*S*-**19** was also obtained.

Compound 5*R*-**19**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.21–7.45 (m, 6H, Ph and 6-H), 6.78 (dd, J = 6.5, 8.0 Hz, 1H, 2′-H), 5.49 (s, 2H, NCH_2O), 5.05 (s, 1H, 5′-H), 4.69 (s, 2H, OCH_2Ph), 4.41 (d, J = 4.6 Hz, 1H, 4′-H), 4.10–4.24 (m, 4H, Ethyl-CH_2), 3.96 (dd, J = 9.5, 13.7 Hz, 1H, CH_2P), 3.76 (dd, J = 9.5, 13.7 Hz, 1H, CH_2P), 2.38 (dd, J = 6.4, 14.0 Hz, 1H, 3′-H_α), 2.08 (ddd, J = 5.3, 8.6, 14.3 Hz, 1H, 3′-H_β), 1.98 (s, 3H, 5-CH_3), 1.35 (t, J = 7.0 Hz, 6H, Ethyl-CH_3). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.72.

Compound 5*S*-**19**: ^{1}H NMR (300 MHz, CD_3CN) δ 7.74 (d, J = 1.2 Hz, 1H, 6-H), 7.30-7.39 (m, 5H, Ph), 6.36 (dd, J = 2.8, 8.6 Hz, 1H, 2′-H), 5.44 (s, 2H, NCH_2O), 5.21 (s, 1H, 5′-H), 4.66 (s, 2H, OCH_2Ph), 4.27 (dd, J = 3.4, 5.5 Hz, 1H, 4′-H), 4.05-4.16 (m, 4H, Ethyl-CH_2), 3.98 (dd, J = 9.7, 14.0 Hz, 1H, CH_2P), 3.82 (dd, J = 12.2, 14.1 Hz, 1H, CH_2P), 2.63-2.73 (m, 1H, 3′-H_β), 1.85-1.91 (m, 1H, 3′-H_α), 1.88 (d, J = 1.2 Hz, 3H, 5-CH_3), 1.33 (m, 6H, Ethyl-CH_3). ^{31}P NMR (121.4 MHz, CD_3CN) δ 20.61.

6.2.7. (2*R*,3*S*,5*R*)-[3-Hydroxy-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-2-yloxymethyl]-phosphonic acid diethyl ester (20). The phosphonate **19** (232 mg, 0.47 mmol) was dissolved in ethanol (4 mL) and then treated with $Pd(OH)_2$ (160 mg). The mixture was stirred under an atmosphere of hydrogen for 16 h and then the catalyst was removed by filtration. The eluant was concentrated under reduced pressure and the crude residue dissolved in methanol (1.6 mL) and ammonium hydroxide (1.6 mL). The resultant solution was heated at 40 °C for 16 h. The mixture was concentrated under reduced pressure and the crude residue subjected to silica gel chromatography, eluting with a gradient of 0–5% methanol in dichloromethane, to give the title product **20** (110 mg, 63%). Compound **20**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.15 (br s, 1H), 7.33 (s, 1H), 6.77 (dd, J = 6.8, 7.9 Hz, 1H), 5.10 (s, 1H), 4.45 (d, J = 4.6 Hz, 1H), 4.19 (m, 4H), 3.99 (dd, J = 9.3, 13.6 Hz, 1H), 3.81 (dd, J = 9.1, 13.7 Hz, 1H), 2.42 (dd, J = 6.3, 14.2 Hz, 1H), 2.08–2.19 (m, 1H), 1.98 (s, 3H), 1.36 (t, J = 7.2 Hz, 6H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.63.

6.2.8. (4-Methyl-5-oxo-8,11-dioxa-2,6-diaza-tricyclo-[7.2.1.02,7]dodeca-3,6-dien-10-yloxymethyl)-phosphonic acid diethyl ester (21). The phosphonate **20** (21 mg, 55 μmol) was dissolved in DCM (1 mL) and cooled to −78 °C. The mixture was then treated with DAST (9 μL, 69 μmol) and stirred for 30 min before being warmed to RT over 30 min. The mixture was treated with saturated sodium hydrogen carbonate (0.35 mL) and then concentrated under reduced pressure. The crude residue was subjected to silica gel chromatography, eluting with a gradient of 0–15% methanol in dichloromethane, to give the title product **21** (15 mg, 75%). Compound **21**: ^{1}H NMR (300 MHz, CD_3CN) δ 7.26 (d, J = 0.9 Hz, 1H), 5.62 (d, J = 3.6 Hz, 1H), 5.47 (m, 1H), 5.12 (m, 1H), 3.91–4.10 (m, 6H), 2.60 (d, J = 13.1 Hz, 1H), 2.49 (dd d, J = 3.1, 3.6, 13.1 Hz, 1H), 1.87 (d, J = 1.2 Hz, 3H), 1.21–1.28 (m, 6H).

6.2.9. (2*R*,3*S*,5*R*)-[3-Azido-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-2-yloxymethyl]-phosphonic acid monoethyl ester (22). The anhydro compound **21** (33 mg, 90 μmol) was dissolved in DMF (8 mL) and then treated with sodium azide (18 mg, 275 μmol) followed by benzoic acid (11 mg, 90 μmol). The resultant mixture was heated at 120 °C for 36 h and then concentrated under reduced pressure. The crude residue was subjected to silica gel chromatography eluting with a gradient of 0–15% methanol in dichloromethane to give the title product **22** (24 mg, 71%). Compound **22**: ^{1}H NMR (300 MHz, D_2O) δ 7.43 (d, J = 1.2 Hz, 1H), 6.41 (dd, J = 6.7, 11.3 Hz, 1H), 5.07 (s, 1H), 4.34 (d, J = 4.9 Hz, 1H), 3.85 (m, 1H), 3.73 (dd, J = 8.9, 13.2 Hz, 1H), 3.56 (dd, J = 9.6, 13.3 Hz, 1H), 2.30–2.47 (m, 2H), 1.81 (s, 3H), 1.10 (t, J = 4.0 Hz, 3H). ^{31}P NMR (121.4 MHz, D_2O) δ 16.26. LR LCMS(B) (M+H) 376.1.

6.2.10. (2*R*,3*S*,5*R*)-[3-Azido-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-2-yloxymethyl]-phosphonic acid ammonium salt (6). The monoacid **22** (8.9 mg, 24 μmol) was dissolved in DMF (2 mL) and evaporated (2×). The solid was then dissolved in dry DMF (1.5 mL) and cooled to 0 °C. The solution was then treated with 2,6-lutidine (30 μL, 240 μmol) followed by the dropwise addition of TMSBr (15 μL, 120 μmol). The solution was allowed to warm to RT and stirred for 16 h. The reaction was then cooled to −78 °C and treated with methanol (30 μL). The mixture was allowed to warm to room temperature, treated with ammonium hydroxide, and then concentrated under reduced pressure. The residue was triturated with acetone and the solid that formed was then collected by filtration. The crude solid was subjected to C18 chromatography eluting with water to give the title product **6** as the bis ammonium salt (2 mg, 22%). Compound **6**: ^{1}H NMR (300 MHz, D_2O) δ 7.46 (d, J = 1.2 Hz, 1H), 6.42 (t, J = 7.3 Hz, 1H), 5.08 (s, 1H), 4.35 (d, J = 4.2 Hz, 1H), 3.67 (dd, J = 8.8, 12.8 Hz, 1H), 3.47 (dd, J = 10.1, 12.8 Hz, 1H), 2.30–2.45 (m, 2H), 1.80 (s, 3H). ^{31}P NMR (121.4 MHz, D_2O) δ 14.21. LR LCMS(B) (M−H) 346.3 (1.43 min). FABHRMS (M+H) calc. for $C_{10}H_{15}N_5O_7P$ 348.0709, found 348.0718.

6.2.11. (2*R*,5*R*)-[5-(5-Methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-2-yloxymethyl]-phosphonic acid disodium salt (7). Disodium salt of **8** (50 mg, 0.14 mmol) was dissolved in ethanol (5 mL) and then treated with 10% Palladium on carbon (5 mg). The mixture was stirred under an atmosphere of hydrogen for 2 h. The mixture was filtered over Celite and the eluant concentrated under reduced pressure to give the title product **7** (40 mg, 80%). Compound **7**: ^{1}H NMR (300 MHz, D_2O) δ 7.56 (d, J = 1.2 Hz, 1H), 6.20 (m, 1H), 5.23 (m, 1H), 3.57 (dd, J = 8.7, 12.9 Hz, 1H), 3.35 (dd, J = 9.6, 12.9 Hz, 1H), 2.25 (m, 1H), 2.04 (m, 1H), 1.81 (d, J = 1.2 Hz, 3H). ^{31}P NMR (121.4 MHz, D_2O) δ 13.6. LR LCMS(B) (M−H) 304.9 (0.28 min). FABHRMS (M+Na) Calc. for $C_{10}H_{15}N_2O_7NaP$ 329.0515, found 329.0510. HPLC 8.92 min, 99.4%.

6.2.12. (2*R*,5*R*)-[5-(5-Methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-2-yloxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester (23). The diso-

dium salt **7** (20 mg, 0.057 mmol) was dissolved in acetonitrile (2 mL) and water (0.4 mL) and cooled to 0 °C. The stirred solution was treated with Dowex-H+ resin for 10 min and then the resin was removed by filtration. The resin was washed with methanol and the combined eluants were treated with triethylamine (0.1 mL). The solution was concentrated under reduced pressure and then redissolved in DMF (1 mL) and triethylamine (0.1 mL). The solution was concentrated under reduced pressure once again and the residue then treated with DMF (1 mL) and triethylamine (0.07 mL). The mixture was stirred and chloromethylisopropylcarbonate (0.06 mL) was added. The mixture was heated at 70 °C for 3 h and then stirred at room temperature overnight. The mixture was concentrated under reduced pressure and the residue added to ethyl acetate (5 mL) and water (0.2 mL). The mixture was filtered and the eluant was then dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was subjected to silica gel chromatography eluting with a gradient of 50–100% ethyl acetate in hexanes to give the title product **23** (7.9 mg, 26%). Compound **23**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.12 (s, 1H), 7.38 (d, J = 1.2 Hz, 1H), 6.48 (m, 1H), 5.75 (m, 4H), 5.19 (d, J = 3.9 Hz, 1H), 4.93 (m, 2H), 4.07 (dd, J = 9.0, 13.8 Hz, 1H), 3.88 (dd, J = 10.2, 13.8 Hz, 1H), 2.07–2.38 (m, 4H), 1.33 (m, 15H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 21.2. LR LCMS(A) (M+Na) 561.2 (2.11 min). FABHRMS (M+Li) calc. for $C_{20}H_{31}LiN_2O_{13}P$ 545.1724, found 545.1715. HPLC 25.32 min, 100%.

6.2.13. (2*R*,5*R*)-[5-(5-Methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-2,5-dihydro-furan-2-yloxymethyl]-phosphonic acid diisopropoxycarbonyloxymethyl ester (24). Prepared from **8** according to the procedure described for **23**. Compound **24**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.45 (s, 1H), 7.15 (s, 1H), 6.98 (s, 1H), 6.32 (d, J = 8.7 Hz, 1H), 6.1 (d, J = 8.7 Hz, 1H), 5.6–5.8 (m, 5H), 4.95 (m, 2H), 4.0–4.12 (m, 2H), 1.92 (s, 3H), 1.35 (d, J = 7.6 Hz, 12H). ^{31}P NMR (121.4 MHz, $CDCl_3$) δ 20.77. LR LCMS(A) (M+Na) 559.6. FABHRMS (M+Li) calc. for $C_{20}H_{29}LiN_2O_{13}P$ 543.1567, found 543.1563. HPLC 24.84 min, 99.3%.

6.2.14. (2*R*,5*R*)-[5-(5-Methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-2,5-dihydro-furan-2-yloxymethyl]-phosphonic acid diphosphate (25). Prepared from **8** according to the procedure described for **26**. The product was purified using TEA bicarbonate buffer, instead of ammonium carbonate, to isolate the bis-triethylamine salt of compound **25**: ^{1}H NMR (300 MHz, D_2O) δ 7.21 (s, 1H), 6.66 (s, 1H), 6.27 (d, J = 6.0 Hz, 1H), 6.02 (d, J = 6.3 Hz, 1H), 5.76 (s, 1H), 3.77 (m, 2H), 2.98 (m, 12H), 1.98 (s, 3H), 1.12 (m, 18H). ^{31}P NMR (121.4 MHz, D_2O) δ 6.8 (d, J = 22 Hz, P_α), −12.6 (d, J = 22 Hz, P_γ) −25.3 (m, P_β). LR LCMS(A) (M+H) 464. HPLC 9.05 min, 100%.

6.2.15. (2*R*,5*R*)-[5-(5-Methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-2-yloxymethyl]-phosphonic acid diphosphate (26). The ddTP disodium salt **7** was treated as described in the preparation of **23** to give the free acid of **7**. The acid (24 mg, 79 μmol) was dissolved in DMF (2 mL) and then CDI (65 mg, 0.40 mmol), dissolved in DMF (1 mL), was added. The mixture was stirred for 16 h and then quenched with methanol (24 μL). The mixture was stirred for 2 h and then treated with bis(tributylammonium)pyrophosphate (190 mg, 0.4 mmol). The resultant mixture was stirred for 16 h and then filtered. The eluant was concentrated under reduced pressure and the residue chromatographed on a DEAE column. The column was eluted with 0–0.4 M ammonium carbonate in water. The fractions containing product were collected and concentrated under reduced pressure. The residue was dissolved in water and concentrated under reduced pressure (repeat 4 times). The residue was then dissolved in ethanol and concentrated under reduced pressure four times. The crude residue was then freeze-dried to give **26** as a white solid (3 mg, 9%). Compound **26**: ^{1}H NMR (300 MHz, D_2O) δ 7.54 (s, 1H), 6.25 (m, 1H), 5.27 (s, 1H), 3.79 (m, 2H), 2.28 (m, 1H), 2.05 (m, 3H), 1.82 (s, 3H). ^{31}P NMR (121.4 MHz, D_2O) δ 7.9 (d, J = 25 Hz, P_α), −5.5 (d, J = 20 Hz, P_γ), −21.3 (m, P_β). HPLC 8.84 min, 97.4%.

6.3. NMR conformation determination

The J_{HH} coupling constants were measured from one-dimensional proton spectrum, collected with a spectral width of 5000 Hz and 32K complex points. Two hundred milliseconds of ROESY spectra were collected with 2048 complex points in t2, and 150 fids. The spectral width was 5000 Hz. Gaussian function was used in both dimensions. Zero filling was used to end up with 2048 × 2048 points. The NOE volumes were from the 200 ms ROESY data. All data processing, including NOE volume integration, was done by the Varian NMR software, VNMR. The ensemble solution conformations of nucleosides and phosphonates were derived from J_{HH} and NOE data. Coupling constant data were used to extract vicinal hydrogen dihedral angles according to the Altona–Karplus equation.[21] H–H distance constraints falling off as 1/r6 were derived from ROESY NOE data. The molecular species of interest were generated and manipulated using the SYBYL computational package [SYBYL 6.92, Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA]. Each molecule was minimized to convergence using the MMFF94s force field, as implemented in Sybyl 6.92, applying the experimentally derived dihedral and distance constraints. All calculations were carried out in vacuum with a constant dielectric of ε = 4.0. The resulting molecular conformation was used to calculate ensemble-average values for the pseudorotation angle P and the amplitude of sugar pucker ψ.[21]

6.4. Biological assays

HIV RT inhibition assay wild-type p66/p51 heterodimer HIV-1 reverse transcriptase was cloned, expressed, and isolated as described previously.[6] Enzymatic RT reactions contained the following components: 50 mM Tris–HCl, pH 7.8, 60 mM KCl, 5 mM $MgCl_2$, 3 mM DTT, 150 μg/mL activated calf thymus DNA, 500 μg/mL bovine serum albumin, 5% glycerol, cognate [^{33}P]dNTP at the K_m concentration (0.15–0.25 μM depending on dNTP), 25 μM each of the remaining three dNTPs, and serial dilutions of the tested nucleotide triphosphate analog. Each inhibitor concentration was set up in duplicate and pre-incubated at 37 °C. Reactions were

started by adding RT enzyme to a final concentration of 5 nM. Following the incubation for 6 and 12 min, reaction aliquotes were transferred into EDTA and spotted onto Unifilter 96-well CE81 DEAE plate. Filter plate was washed with 125 mM Na_2HPO_4 and the radioactivity on filter was counted after adding liquid scintillant. Percent inhibition and IC_{50} values were determined using GraphPad Prizm program.

6.5. Antiviral activity and cytotoxicity

Appropriate compound dilutions were prepared in triplicate in 96-well plates. MT-2 cells were infected in bulk with HIV-1 IIIb for 3 h at 37 °C and added to plates at a density of 20,000/well in a final volume of 200 μL. After a 5-day incubation at 37 °C, the virus-induced cytopathic effect was determined using a cell viability assay. 100 μL of media was removed from each well and replaced with 100 μL of phosphate-buffered saline containing 1.7 mg/mL XTT [2,3,-bis(methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] and 5 μg/mL phenazine methosulfate. Following a 1-h incubation at 37 °C, 20 μL/well of 2% Triton X-100 was added and absorbance was read at 450 nm. The data were plotted as cell death versus drug concentration. Cell death was expressed as a percentage of the signal from samples with fully suppressed virus replication following the subtraction of signal from untreated infected control. The concentration of each drug that inhibited the virus-induced cytopathic effect by 50% (EC_{50}) was calculated using GraphPad Prizm program. Cytotoxicity in MT-2 cells was determined under identical conditions except that cells were uninfected and higher drug concentrations were tested.

6.6. Resistance profiling

All HIV-1 variants containing drug resistance mutations in reverse transcriptase were recombinant strains and were kindly provided by Nicolas Margot of Gilead Sciences Inc. Molecular construction and characterization of viruses with K65R or M184V has been previously described.[33] HIV-1 strain designated 6TAMs contains the reverse transcriptase gene isolated from an NRTI-experienced patient with M41L/D67N/K70R/L210W/T215Y/K219Q mutations. The virus has been produced by homologous recombination as described for the other two mutants.[33] All viruses were propagated in MT-2 cells, titered, and used in parallel with wild-type HIV-1 HXB2 strain to determine the fold resistance of tested inhibitors using the above described XTT-based cytopathicity assay.

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