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Bioorganic & Medicinal Chemistry Letters 17 (2007) 6785–6789
Bioorganic & Medicinal Chemistry Letters

Synthesis, anti-HIV activity, and resistance profiles of ribose modified nucleoside phosphonates

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Received 27 August 2007; revised 11 October 2007; accepted 11 October 2007
Available online 17 October 2007

Abstract—A series of nucleoside phosphonate reverse transcriptase (RT) inhibitors have been synthesized and their anti-HIV activity and resistance profiles evaluated. The most potent analog [5-(6-amino-purin-9-yl)-2,5-dihydro-furan-2-yloxymethyl]-phosphonic acid (d4AP) demonstrated a HIV EC_{50} = 2.1 μM, and the most favorable resistance profile against HIV-1 variants with K65R, M184V or multiple thymidine analog mutations in RT.

The recommended first line therapy for treatment-naïve HIV-infected patients includes a combination of two nucleos(t)ide reverse transcriptase inhibitors (N(t)RTIs) and one non-nucleoside RT or protease inhibitor. There are 8 licensed N(t)RTIs for the treatment of HIV, with most of the drugs exhibiting some limitations due to clinical resistance, toxicities or drug–drug interactions.[1] Consequently, there is continued interest in developing new N(t)RTIs with improved cross-resistance and toxicological profiles.

Nucleotide, 9-*R*-(2-phosphonomethoxypropyl)adenine (tenofovir) **1**,[2] the phosphonate component of tenofovir disoproxil fumarate (tenofovir DF, Viread®), stands out amongst the N(t)RTI for two reasons (Fig. 1). First, it is a nucleoside phosphonate and second, it does not have a formal 5-membered ring ribose core. The phosphonomethoxy group is a bioisostere of the α-phosphate group of nucleotide monophosphates but differs in that it is more enzymatically stable than the α-phosphate group.[3] A limiting property of the phosphonate diacids is that they are charged at physiological pH and therefore exhibit poor membrane permeability. However, once the diacid is delivered into cells, via a prodrug, the high polarity and reduced permeability now favor intracellular accumulation of the diacid and its metabolites. The bis-isopropyloxymethylcarbonyl (bisPOC) prodrug is used to deliver tenofovir orally and more recently an amidate prodrug (GS-7340) has been demonstrated to be an effective alternative enhancing the nucleotide delivery into lymphocytes.[4,5] Only a handful of reports have described antiviral activity of phosphonomethoxy nucleoside analogs on cyclic 5-membered ring ribose scaffolds, for example, **2**–**7** (Fig. 1).[6–9] Therefore, we have synthesized a variety of novel NtRTI inhibitors and compared their anti-HIV activity and resistance profiles toward several key clinical HIV RT mutants.

Phosphonomethoxy analogs of 2′,3′-didehydro-2′,3′-dideoxy (d4) adenosine **2** (d4AP), and 2′,3′-dideoxy (dd) adenosine, **4** (ddAP), were prepared following the procedures of Kim et al.[6] Thymidine analogs **3** (d4TP) and **5** (ddTP), were prepared according to reported procedures.[6,7] Uridine analogs, **12** (d4UP) and **13** (5-F-d4UP) were prepared from commercially available 2′-deoxyuridine **8a** and 5-fluoro-2′-deoxyuridine **8b**, respectively (Scheme 1). Initial oxidation using platinum oxide[10] was followed by decarboxylative dehydration to the intermediate glycal.[11] Subsequent activation of the glycal with PhSeCl on the least hindered α-face of the ribose ring directed introduction of the dimethyl hydroxymethylphosphonate ester to the β-face.[6] Olefin

Keywords: Anti-HIV; Phosphonates; Resistance; Reverse transcriptase.
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doi:10.1016/j.bmcl.2007.10.038

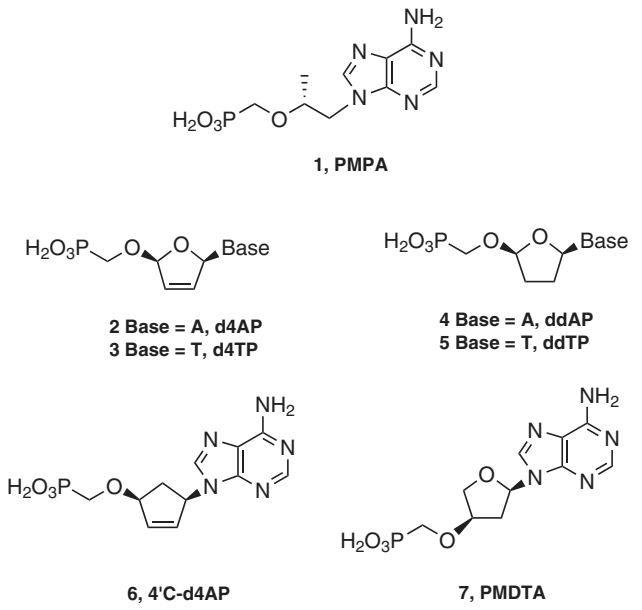
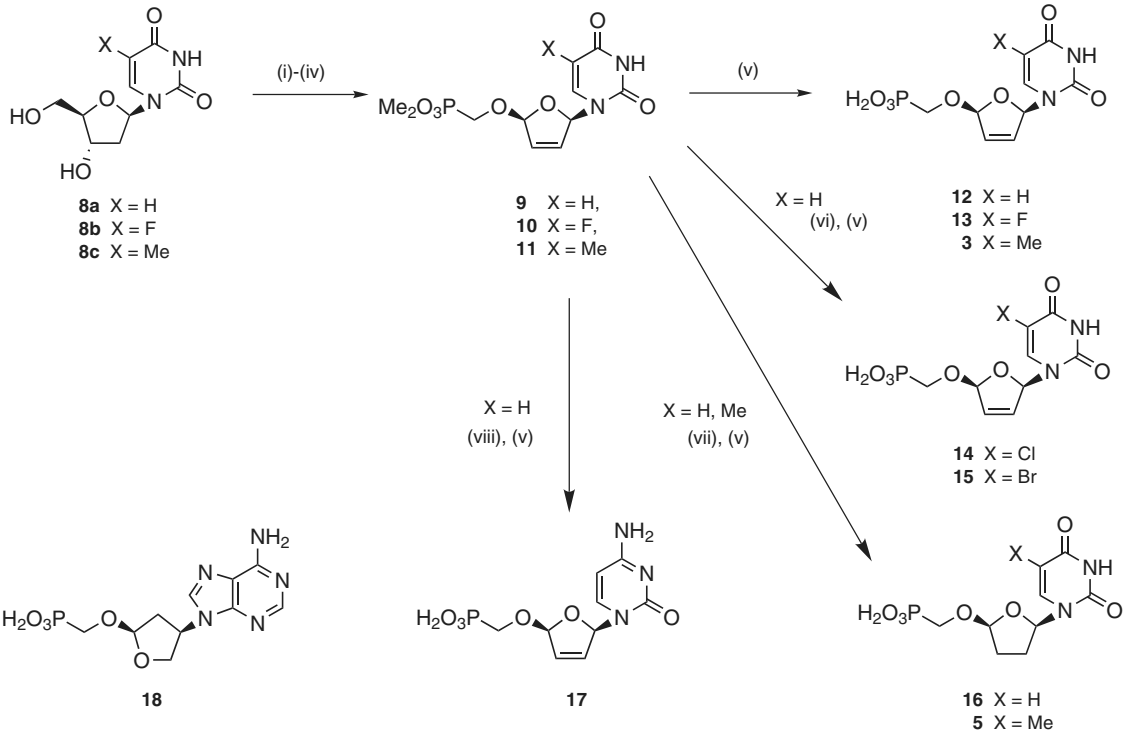


Figure 1. Structures of tenofovir and ribose modified phosphonates. The nomenclature describing the phosphonate analogs is derived by adding the 'P' to the typically accepted nucleoside notation, for example, the equivalent phosphonate analog of nucleoside d4T is referred to as d4TP.

formation was effected via hydrogen peroxide-mediated oxidation of selenium and subsequent elimination. TMSBr hydrolysis of the phosphonate ester groups provided compounds **12** and **13**.[6] Further halogenated uridine analogs, **14** and **15**, were prepared from **9** by treatment with NCS or NBS, respectively, followed by TMSBr mediated ester hydrolysis.[12] Carbocyclic analog **6** was prepared according to modified literature procedures.[13] The dd analog, **16** (ddUP), was prepared from **9**, by hydrogen reduction over 10% Pd/C prior to removal of the phosphonate ester groups. Cytidine analog **17** (d4CP) was prepared from uridine analog **9**.[14] The procedure for the preparation of **18** (*iso*-ddAP) has been briefly described and will be reported in full elsewhere.[15] The active diphospho-phosphonate (triphosphate equivalents) metabolites of the phosphonate diacids **2**–**5**, **12**, **16**, and **18** were prepared by treatment of the phosphonates with CDI followed by pyrophosphate.[7] Antiviral testing methods and resistance profiling have been reported previously.[7]

The wild-type (WT) antiviral activity of the unsaturated d4 phosphonate analogs is dependent on the nucleobase (Table 1, entries **2**–**3**, **12**–**15**, and **17**). Adenine analog, **2** (d4AP), has the most potent antiviral (EC_{50}) activity, 12-fold more potent than thymidine **3**, and significantly more potent than the uridine analogs **12** and **13**. 5-Halogenated uridines, **14** (5-Cl) and **15** (5-Br), have comparable antiviral activity to thymidine analog **3**, but increased cellular toxicity. The saturated dd analogs, **4**, **5**, and **16**, have weaker antiviral activity than their corresponding d4 analogs (**2**, **3**, and **12**, respectively). Antiviral activity is dependent on the ability of the phosphonate diacid to penetrate cells, become metabolized to the active diphospho-phosphonate (triphosphate equivalent) species, and inhibit HIV RT. Diphospho-phosphonate concentrations, measured in the CEM-CCRF



Scheme 1. Reagents and conditions: (i) Pt/C, O_2, 70 °C, H_2O, $NaHCO_3$, 3 days, 45%; (ii) DMF–dineopentylacetal, DMF, 80 °C, 75%; (iii) PhSeCl, −70 °C, CH_2Cl_2 then $HOCH_2PO(OMe)_2$, $AgClO_4$ 71%; (iv) H_2O_2, H_2O, dioxane, 85%; (v) TMSBr, DMF, 0 °C then NH_4OH; (vi) NBS (or NCS), pyr, DMF, 90 °C, 50–60%; (vii) Pd/C, H_2, EtOH, 75%; (viii) *p*-ClPhOP(=O)Cl_2, triazole, pyridine, then NH_4OH, 55%.

Table 1. Anti-HIV activity, RT potency, and antiviral resistance profile

Compound	Code	WT HIV[a] $EC_{50}/\mu M$	MT-2 $CC_{50}/\mu M$	WT RT $IC_{50}/\mu M$	Nucleoside[c] WT RT $IC_{50}/\mu M$	M184V[b] Fold res.	6TAMs[b] Fold res.	K65R[b] Fold res.
2	d4AP	2.1 (1.0)	>1000	0.60 (0.16)	0.14 (0.07)	0.9 (0.6)	2.9 (1.0)	2.9 (1.1)
3	d4TP	26 (8.8)	>1000	0.39 (0.08)	0.06 (0.03)	1.1	12.7 (3.9)	5.2 (0.3)
17	d4CP	>200	—	—	—	—	—	—
12	d4UP	>200	>1000	5.7	0.55 (0.15)	—	—	—
13	5-F-d4UP	>200	—	—	—	—	—	—
14	5-Cl-d4UP	28.5 (16.2)	413 (174)	—	—	0.8	>10	6.7
15	5-Br-d4UP	15	935	—	—	1.3	>10	7.5
6	4′C-d4AP	51.7 (13.4)	—	—	—	—	—	—
4	ddAP	12 (2.5)	>1000	4.1 (1.8)	0.26 (0.14)	1.1	3.4 (0.3)	4.9 (1.4)
5	ddTP	>200	—	5.4 (2.1)	0.12 (0.02)	—	—	—
16	ddUP	>200	—	31	0.83 (0.28)	—	—	—
18	*iso*-ddAP	9.5 (5.6)	863 (238)	0.32 (0.14)	—	11.0 (3.0)	3.2 (0.6)	2.2 (1.1)
1	Tenofovir	3.6 (1.5)	>1000	0.38 (0.20)	—	0.7 (0.2)	8.8 (3.7)	4.3 (1.5)
—	Abacavir	0.32 (0.15)	190 (45)	0.13 (0.07)	—	7.1 (4.4)	5.7 (3.1)	3.8 (1.8)

[a] Values are results of at least 2 experiments, standard deviation is given in parentheses.
[b] Resistance determined in MT-2 cell lines.
[c] Data for the equivalent nucleoside triphosphate analog.

T-cell line, were shown to be higher for d4 analogs, **2** and **3**, compared to dd analogs, **5** and **16** (data not shown), consistent with the more potent antiviral activity of the d4 analogs.[16]

Examination of the RT inhibition trends between unsaturated d4 and saturated dd phosphonates shows that the d4 analogs are consistently more potent inhibitors of RT than the saturated dd analogs. This trend is also found in the d4 and dd nucleosides suggesting that the phosphonate bioisostere of the nucleoside α-phosphate does not lead to grossly different binding modes to HIV RT. However, despite this similar trend, all the phosphonate analogs are weaker RT inhibitors than their nucleoside counterparts, especially the dd phosphonate analogs. For example, the dd phosphonate analogs are 16-fold (A analog **4**), 45-fold (T analog **5**), and 37-fold (U analog **16**) less potent than the equivalent dd nucleosides (Table 1, column 6). In contrast, the d4 phosphonates are all within 10-fold of the d4 nucleosides with respect to RT inhibition. This more substantial drop in activity for the dd phosphonates has been noted previously and hypothesized to be the result of introducing a second anomeric center that significantly perturbs the conformation of the dd phosphonate analogs compared to their nucleoside dd counterparts.[7] Conformational analysis using NMR has shown that the second anomeric center is more powerful than the nucleobase anomeric center and drives the conformation to the 3′-exo, 2′-endo (Southern) conformation. This perturbation is minimized in unsaturated d4 analogs due to the more rigid and planar geometry of the ribose ring. We therefore sought to evaluate the effects of reducing the number of anomeric centers by transposing the 4′-O around the ring, for example, *iso*-analog **18**. Inhibition of RT improved by >10-fold resulting in the most potent phosphonate RT inhibitor of the series. However, antiviral activity was weaker than d4 analog **2** due to decreased phosphorylation relative to **2** (Table 1).[16] Replacement of the 4′-O with carbon yielded analog **6** (4′C-d4AP).[9] However, this conversion to the carbocyclic ring led to a 25-fold drop in the antiviral activity. Nevertheless, the corresponding diphosphate of **6** is reported[9] to be a potent inhibitor of HIV RT, suggesting that, once again the replacement of the 4′-O with carbon reduces phosphorylation.

Antiviral resistance profiles against HIV variants with the major N(t)RTI mutations M184V, K65R, and 6TAMs were determined for the most active analogs and compared to tenofovir **1**. As anticipated, the d4 and dd phosphonomethoxy analogs, **2**–**4** and **14**–**15**, all retain their full activity against HIV strain containing M184V. In contrast, *iso*-ddAP **18** suffered a 11-fold drop in antiviral activity similar to that observed for abacavir (Table 1). The common feature between these 2 inhibitors is the replacement of the 4′-O with carbon. Recent studies have determined that the M184V mutation reduces the incorporation efficiency of abacavir more than the corresponding 4′-O ribose analog, d4G triphosphate.[17] Thus, the 4′-O of the ribose ring is critical for optimal binding and incorporation kinetics toward the M184V mutation. Modeling of *iso*-ddAP **18** in HIV RT indicates that the ring pucker changes to alleviate interactions between the adenine C-8 hydrogen and 4′-CH_2, thus placing the 4′-CH_2 in closer proximity to residue M184 (Fig. 2). This explains the susceptibility to the M184V mutation that increases the steric interactions in this part of the active site.

Pyrimidine analogs, **3**, **14**, and **15** showed the greatest loss in susceptibility (>10-fold) due to multiple thymidine analog mutations (6TAMs) whereas purines **2**, **4**, and **18** were all superior to tenofovir, **1**. The TAM mutations promote primer unblocking by excision of the nucleotide chain terminator prior to translocation from the Nucleotide-site (N-site) to the Primer-site (P-site).[18] The increased effect of 6TAMs on the activity of thymidine analog **3** compared to adenine **2** clearly

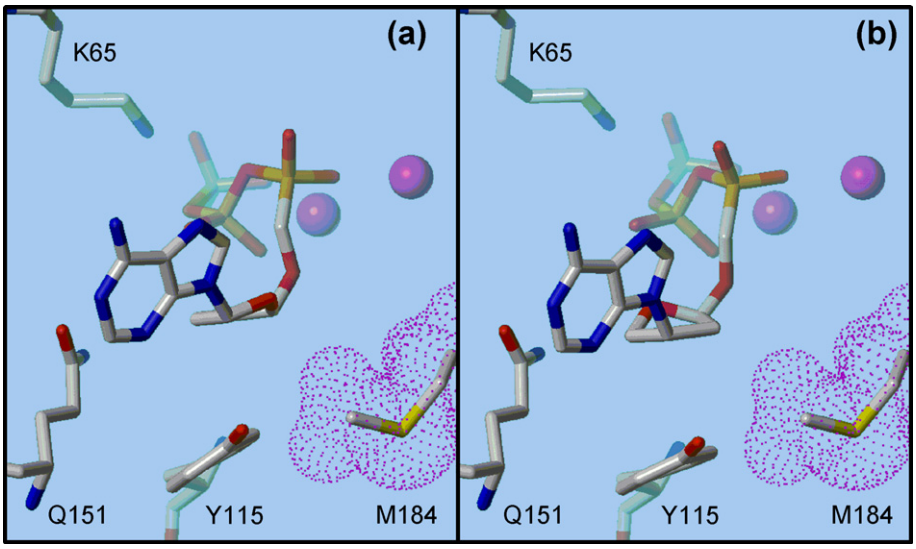


Figure 2. (a) Compound **2** and (b) compound **18** bound in WT HIV RT. M184 interacts more closely to the 4′-CH_2 in (b) due to the different puckering of the isomeric ribose ring compared to compound **2**.

indicates that the nucleobase has an important role in reducing the propensity for excision. Comparing the TAMs profiles for four adenine analogs, acyclic phosphonate tenofovir **1** (8.8-fold resistance), d4 analog **2** (2.9-fold resistance), dd analog **4** (3.4-fold resistance), and *iso*-analog **18** (3.2-fold resistance), indicates how differences in the ribose portion of the molecule affect the antiviral resistance due to TAMs. Further studies that examined the in vitro removal of the four adenine analogs from the 3′-terminated DNA primer by RT indicated that the cyclic analogs displayed reduced efficiency of excision compared to tenofovir (data not shown). These data corroborate the differences in the TAMs susceptibility observed in the antiviral assays. Taken together, cyclic ribose phosphonates containing adenine base exhibit the most favorable TAMs resistance profile among all tested nucleotide analogs.

Finally, the lysine 65 residue is located in the finger subdomain of RT and donates a hydrogen bond to the γ-phosphate oxygen of the dNTP upon its binding in the enzyme active site.[19] Mutation to arginine at position 65 introduces additional hydrogen bonds that reduce the conformational mobility of the RT fingers domain resulting in diminished incorporation and excision of nucleotide analogs. Compared to tenofovir **1**, the purine analogs, d4AP **2** and *iso*-ddAP **18**, have marginally reduced susceptibility due to K65R whereas the pyrimidines **3**, **14**–**15** tended to show more significant loss of activity. However, the small differences are not substantial enough to draw any firm structural or mechanistic conclusions.

A series of nucleoside phosphonates that combine different ribose modifications and base changes led to the identification of 2 potent RT inhibitors, d4AP **2** and *iso*-ddAP **18**. Resistance profiling against HIV strains with clinically relevant RT mutations determined that d4AP **2** has a superior resistance profile to both *iso*-ddAP **18** and tenofovir **1**, the only FDA-approved NtRTI.

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