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Amidate Prodrugs of *O*-2-Alkylated Pyrimidine Acyclic Nucleosides Display Potent Anti-Herpesvirus Activity

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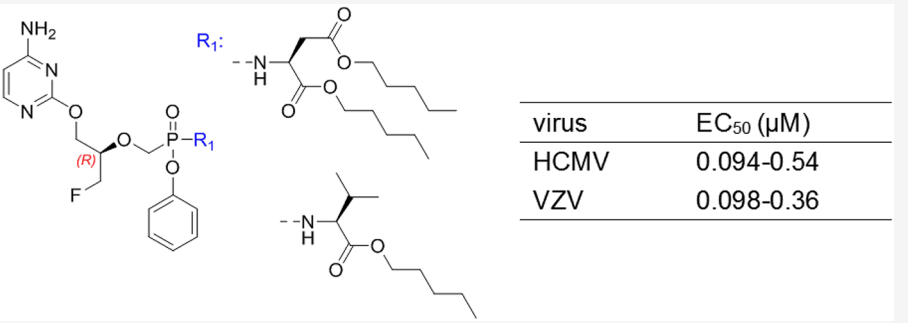

ABSTRACT: Three series of amidate prodrugs of *O*-2-alkylated acyclic nucleosides of the 3-fluoro-2-(phosphonomethoxy)propyl (FPMP), cyclic 3-hydroxy-2-(phosphonomethoxypropyl) (cHPMP), and 2-(phosphonomethoxypropyl) (PMP)-type featuring cytosine and 5-fluorocytosine as nucleobases were readily synthesized. Both the aspartic acid ester and valine ester prodrugs of (*R*)-*O*-2-alkylated FPMPC exhibited potent anti-HCMV and VZV activity in the micromolar range. In addition, the valine ester prodrugs of 5-fluorocytosine (*R*)-*O*-2-alkylated FPMP and (*R*)-*O*-2-alkylated cHPMPC showed inhibitory activity at molar concentrations against these viruses.

KEYWORDS: *Acyclic nucleoside phosphonates, prodrugs, human cytomegalovirus, varicella zoster virus, antiviral activity*

Acyclic nucleoside phosphonates (ANPs) have gained increasing attention in nucleos(t)ide research due to a number of significant advantages, including potent antiviral activity, easy and economical chemical synthesis, and potential as genetic materials.[1−4] Extensive investigation of their synthesis and therapeutic properties has led to the marketing approval of three ANPs as antiviral agents (Figure 1).[5,6]

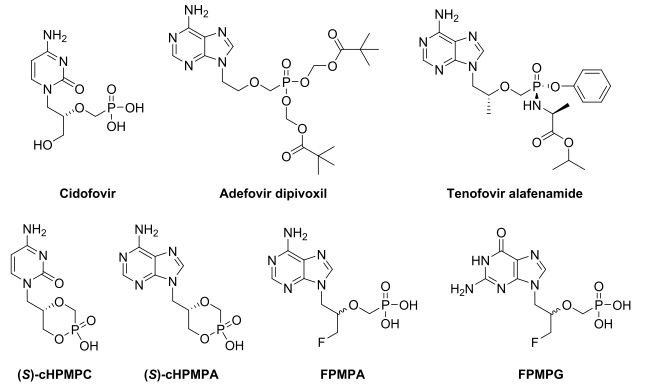

Figure 1. Chemical structures of cidofovir (HPMPC), adefovir dipivoxil, tenofovir alafenamide, (*S*)-cHPMPA, (*S*)-cHPMPC, FPMPA, and FPMPC.

Cidofovir (HPMPC, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine) is currently being used for the treatment of human cytomegalovirus (HCMV) retinitis in acquired immune deficiency syndrome (AIDS) patients, while adefovir (PMEA, 9-(2-phosphonylmethoxyethyl)-adenine) was approved as antihepatitis B virus (HBV) agent. Upon derivatization of the active drug tenofovir (PMPA, (*R*)-9-(2-phosphonylmethoxypropyl)adenine), two nucleoside phosphonate prodrugs, i.e., tenofovir disoproxil and alafenamide, have been developed and licensed for the treatment of HBV as well as human immunodeficiency virus (HIV) infections.

The generation of structurally modified ANPs remains an attractive platform for the development of new bioactive compounds (Figure 1).[7] Among these, the cyclic forms of HPMP-type nucleosides such as (*S*)-cHPMPA and (*S*)-cHPMPC, which were prepared aiming to reduce the toxic side effects of the parent ANPs, retained a remarkable antiviral

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potency against DNA viruses.[8,9] On the other hand, the replacement of the hydroxyl group of HPMC derivatives with a fluorine atom led to 3-fluoro-2-(phosphonomethoxy)propyl (FPMP) nucleoside analogues that lacked anti-DNA virus activity, while being moderately active inhibitors of HIV and HBV.[10−12] Similarly, the PMP series characterized by the presence of a methyl group at the acyclic side chain, as exemplified by tenofovir, showed potent activity against retroviruses but were completely devoid of anti-DNA virus activity.[12]

It should be noted that the majority of reported nucleoside phosphonate analogues belong to the *N*-nucleoside series, i.e., where a heterocyclic base (purine or pyrimidine) is linked to an acyclic chain through a nitrogen atom (Figure 1). On the other hand, *O*-alkylated nucleosides, especially *O*-alkylated cytosine derivatives, were not widely investigated and never commercialized as either anticancer or antiviral agents. The best studied class of *O*-alkylated acyclic phosphonate nucleosides comprises substituted 4-amino-6-hydroxypyrimidine derivatives as nucleobase moiety (Figure 2).[13,14] For instance,

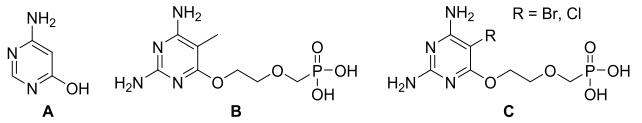


Figure 2. 4-Amino-6-hydroxypyrimidine (**A**) and selected examples of 6-*O*-alkylated acyclic nucleosides (**B** and **C**).

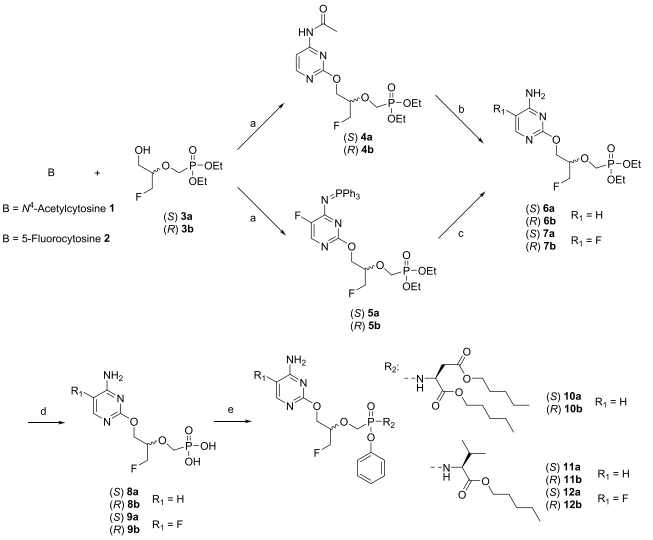
5-methyl derivative **B** (Figure 2) showed excellent antiviral activity against HIV and Moloney murine sarcoma virus in vitro (EC_{50} = 0.00016−0.00043 μmol/mL). In addition, 5-halogen-substituted derivatives **C** (Figure 2) were endowed with pronounced antiretroviral activity with EC_{50} values in the 0.0023−0.0110 μmol/mL range.

Among the various available strategies for nucleotide prodrug design,[15] amidate prodrugs have been shown on many occasions to lead to superior antiviral activities by masking the negatively charged phosphate or phosphonate group, which commonly limits the ability of the parent compounds to penetrate the lipid-rich cell membrane, consequently contributing to higher levels of active cellular metabolites.[16−19] In particular, we previously demonstrated that the use of L-aspartic acid diamyl ester[18] and L-valine amyl ester[20] moieties afforded analogues with an enhanced antiviral efficacy.

Herein, we report the discovery of a new family of *O*-alkylated acyclic nucleoside phosphonates and their amidate prodrugs. For this specific class of molecules, various aliphatic pseudosugar side chains (i.e., FPMP, cHPMP, and PMP) were linked to the 2-*O* position of a pyrimidine base rather than the 4-*O*-position.

As shown in Scheme 1, N^4-acetylcytosine **1** was successfully condensed under Mitsunobu conditions (Ph_3P, DIAD) with **3a** and **3b** to provide compounds **4a** and **4b**. The initial enantiomeric fluorinated acyclic phosphonate ester synthons **3a** and **3b** were synthesized according to a previously reported method.[18,21] Subsequent removal of the N^4-acetyl protecting group using methanolic ammonia furnished compounds **6a** and **6b** in 48 and 50% yield, respectively. On the other hand, the alkylation of 5-fluorocytosine **2** with **3a** and **3b** generated the corresponding triphenylphosphine adducts **5a** and **5b** in agreement with a previous report,[22] which could be

Scheme 1. Synthesis of (*S*)/(*R*)-Phosphonamidates 10a, 10b, 11a, 11b, 12a, and 12b[a]



[a]Reagents and conditions: (a) Ph_3P, DIAD, THF, rt, 12 h; (b) NH_3/MeOH, 45 °C, 15 h, 48−50% over 2 steps; (c) 1 M HCl, CH_3CN/H_2O 12 h, 30−31% over 2 steps; (d) TMSBr, 2,6-lutidine, CH_3CN, rt, 12 h, 65−70%; (e) L-Aspartic acid amyl diester HCl salt or L-valine amyl ester HCl salt, PhOH, 2,2′-dithiodipyridine, PPh_3, Et_3N, Pyr, 60 °C, 12 h, 28−54%.

successfully deprotected by treatment with 1 M HCl at room temperature to afford the desired products **7a** and **7b**. All phosphonate esters underwent hydrolysis in the presence of TMSBr to give compounds **8a**, **8b**, **9a**, and **9b** in good yields. Phosphonic acids **8a**, **8b**, **9a**, and **9b** were then converted to their aryloxyphosphonamidates **10a**, **10b**, **11a**, **11b**, **12a**, and **12b** upon reacting with either L-aspartic acid amyl diester HCl salt or L-valine amyl ester HCl salt and phenol using 2,2′-dithiodipyridine and triphenylphosphine as activating agents. All compounds were isolated as diastereoisomeric mixtures (ratios in the 1/1−1/1.5 range).

Subsequent efforts were directed toward the synthesis of phosphonamidates **22a**, **22b**, **23a**, and **23b**, as illustrated in Scheme 2. In this case, compounds **13a** and **13b** were prepared according to the same method used for the synthesis of diethyl alcohols (Scheme S-1, Supporting Information).[23] Under Mitsunobu conditions (Ph_3P, DIAD), the condensation reaction between **13a** and **13b** and either nucleobase **1** or **2** occurred smoothly to afford nucleoside phosphonates **14a**, **14b** and **15a**, **15b**, respectively. After deprotection of either the acetyl group or triphenylphosphine adduct, standard TMSBr-promoted hydrolysis of the phosphonate diesters afforded compounds **18a**, **18b**, **19a**, and **19b** in 70−75% yields. The key aryloxyphosphonamidate intermediates **20a**, **20b**, **21a**, and **21b** were then obtained as described above. Lewis-acid assisted removal of the benzyl moiety using boron trichloride and sequential treatment with triethylamine in DCM led to a concomitant cyclization, affording the desired cyclic L-valine amyl ester containing (*S*)/(*R*)-phosphonamidates **22a**, **22b**, **23a**, and **23b** as diastereoisomeric mixtures (ratios in the 1/1.1−1/1.2 range) in 25−30% yields.

Lastly, phosphonamidates **31a**, **31b**, **32a**, and **32b** were easily accessible from nucleobases **1** and **2** and compounds **24a**[24] and **24b** (Scheme S-2, Supporting Information), as illustrated in Scheme 3. Compounds **27a**, **27b**, **28a**, and **28b**

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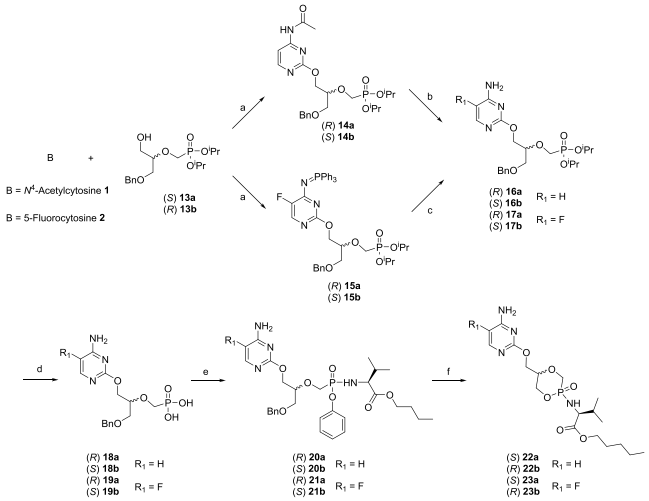
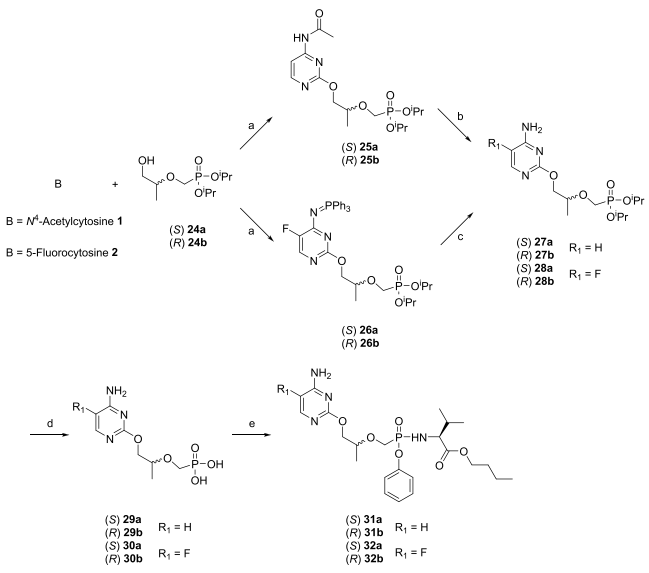
Scheme 2. Synthesis of (*S*)/(*R*)-Phosphonamidates 22a, 22b, 23a, and 23b[a]



[a]Reagents and conditions: (a) Ph_3P, DIAD, THF, rt, 12 h; (b) NH_3/MeOH, 45 °C, 15 h, 42−53% over 2 steps; (c) 1 M HCl, CH_3CN/H_2O, 12 h, 31−46% over 2 steps; (d) TMSBr, 2,6-lutidine, CH_3CN, rt, 12 h, 70−75%; (e) L-Valine amyl ester HCl salt, PhOH, 2,2′-dithiodipyridine, PPh_3, Et_3N, Pyr, 60 °C, 12 h; (f) (i) BCl_3, DCM, −78 to 0 °C, 2 h; (ii) Et_3N, DCM, rt, 1 h, 25−30% over 2 steps.

Scheme 3. Synthesis of (*S*)/(*R*)-Phosphonamidates 31a, 31b, 32a, and 32b[a]



[a]Reagents and conditions: (a) Ph_3P, DIAD, THF, rt, 12 h; (b) NH_3/MeOH, 45 °C, 15 h, 35% over 2 steps; (c) 1 M HCl, CH_3CN/H_2O, 12 h, 30−35% over 2 steps; (d) TMSBr, 2,6-lutidine, CH_3CN, rt, 12 h, 65−70%; (e) L-Valine amyl ester HCl salt, PhOH, 2,2′-dithiodipyridine, PPh_3, Et_3N, Pyr, 60 °C, 12 h, 40−45%.

were obtained under the established Mitsunobu conditions as diastereoisomeric mixtures (ratios in the 1/1−1/1.3 range), followed by hydrolysis of either the acetyl moiety or triphenylphosphine adduct. Cleavage of the phosphonate ester groups furnished the corresponding phosphonic acids, which were converted to the desired prodrugs.

Next, we proceeded with the antiviral activity evaluation of all synthesized phosphonamidate prodrugs and their parent phosphonates against HCMV (strains AD-169 and Davis) and VZV [strains (TK^+) Oka and thymidine kinase deficient (TK^-) 07-1] in human embryonic lung (HEL) cells, along with the assessment of their toxic effects on the same cell line (Table 1). Ganciclovir, cidofovir, acyclovir, and brivudin were also included in this study as reference drugs.

As discussed earlier, *N*-alkylated FPMP nucleoside derivatives bearing canonical and modified nucleobases showed no or very weak antiviral activity against HCMV and VZV at subtoxic concentrations.[18,25] However, we previously established that the use of a diamyl aspartate phenoxyamidate group as phosphonate prodrug moiety was beneficial for enhancing the antiviral activity of FPMP nucleosides and particularly enlarging their spectrum of activity against herpesviruses.[18] In particular, amyl aspartate phosphonamidate prodrugs of purine containing analogues exhibited submicromolar anti-VZV potency, while (*S*)-aspartate-FPMPC displayed an EC_{50} value of 0.76 μM against HCMV.

Interestingly, for the *O*-2-alkylated counterparts, (*R*)-*O*-2-alkylated FPMPC **8b** showed moderate antiviral activity against both HCMV and VZV with EC_{50} values in the 0.80−2.36 and 3.79−25.3 μM range, respectively, while its (*S*)-enantiomer **8a** was found to be inactive.

Further derivatization of (*R*)-*O*-2-alkylated FPMPC **8b** afforded amidate prodrugs **10b** and **11b**, which exhibited potent antiviral activity against different strains of HCMV and VZV (including a thymidine kinase mutant virus) with EC_{50} values ranging between 0.094 and 0.54 μM and concomitantly low cytotoxicity and cytostatic effects. These compounds displayed interesting selectivity indices (ratio CC_{50}/EC_{50}), i.e., 96−551 (**10b**) and 220−818 (**11b**). In contrast, the corresponding (*S*)-enantiomers **10a** and **11a** showed minimal antiviral activity against HCMV and VZV with EC_{50} values in the range of 7.61 to >100 μM. The L-valine amyl ester prodrug of (*R*)-*O*-2-alkylated FPMP carrying 5-fluorocytosine (**12b**) was found to be moderately active against both HCMV strains with EC_{50} values in the 2.94−11.2 μM range, while no activity was observed for its (*S*)-counterpart **12a**. It is worth mentioning that the lack of anti-herpesvirus activity was associated with the opposite configuration at the pseudosugar chiral center for the *N*- and *O*-alkylated series of prodrug analogues [(*R*) and (*S*), respectively]. Moreover, when compared to (*R*)-O-2-alkylated FPMPC **8b**, the corresponding prodrugs **10b** and **11b** displayed a dramatically improved cellular permeability due to increased lipophilicity, as evidenced by their clogP values (Table 1).

In previous studies, *N*-alkylated (*S*)-cHPMPC and (*S*)-cHPMPA (Figure 1) were synthesized and displayed good antiviral potency against DNA viruses.[26,27] It was also demonstrated that their corresponding amidate prodrugs showed excellent antiviral potency against DNA viruses.[20] However, the alkylation of cytosine or 5-fluorocytosine at the 2-*O*-position as in the case of compounds **22a**, **22b**, **23a**, and **23b** generally resulted in loss of antiviral activity, except for the prodrug of (*R*)-*O*-2-cHPMPC (**22b**) that proved to be moderately active against both VZV strain mutants expressing either a functional or deficient thymidine kinase with EC_{50} values of 14.0 and 4.76 μM, respectively.

With regard to the *O*-2-alkylated nucleoside phosphonates and prodrug analogues of the PMP-type, i.e., compounds **29a, 29b**, **30a**, **30b**, **31a**, **31b**, **32a**, and **32b**, no inhibition of HCMV and VZV replication was observed.

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Table 1. Antiviral Activity and Cytotoxicity of Acyclic Nucleoside Phosphonates and Phosphonamidates against HCMV and VZV in HEL Cells

	Antiviral activity EC_{50}[a] (μM)				Cytotoxicity (μM)		
	HCMV		VZV		Cell morphology (MCC)[b]	Cell growth (CC_{50})[c]	
Compound	AD-169 strain	Davis strain	TK^+ VZV strain (OKA)	TK^- VZV strain (07-1)	HEL	HEL	clogP[d]
8a	>100	>100	>100	>100	>100	>100	−0.70
8b	2.36 ± 0.24	0.80	3.79 ± 0.30	25.3 ± 9.75	>100	>100	−0.70
10a	62.8 ± 4.20	48.2 ± 7.60	38.2 ± 7.04	24.7 ± 12.9	>100	>100	6.28
10b	0.54 ± 0.37	0.094	0.36 ± 0.16	0.098 ± 0.003	>100	51.84	6.28
11a	>100	7.61	38.6	35.9	>100	ND[e]	5.25
11b	0.26 ± 0.14	0.097 ± 0.004	0.36 ± 0.035	0.18 ± 0.014	>100	79.3	5.25
12a	>100	>100	>100	>100	>100	ND[e]	5.34
12b	11.2 ± 3.64	4.70 ± 2.76	6.73 ± 3.64	2.94 ± 1.76	>100	>100	5.34
22a	>100	>100	>100	>100	>100	ND[e]	4.00
22b	14.0 ± 1.88	4.76 ± 1.07	53.6 ± 25.1	35.7 ± 13.5	>100	>100	4.00
23a	>100	>100	>100	>100	>100	ND[e]	4.09
23b	>100	>100	>100	>100	>100	ND[e]	4.09
31a	>100	>100	>100	>100	>100	ND[e]	5.38
31b	>100	>100	>100	>100	>100	ND[e]	5.38
32a	>100	>100	>100	>100	>100	ND[e]	5.47
32b	>100	>100	>100	>100	>100	ND[e]	5.47
Ganciclovir	13.0 ± 3.40	4.44 ± 2.23	ND[e]	ND[e]	>394	>394	
Cidofovir	1.74 ± 0.77	0.94 ± 0.86	ND[e]	ND[e]	>317	>317	−2.39
Acyclovir	ND[e]	ND[e]	7.06 ± 3.56	42.7 ± 13.7	>444	>444	
Brivudin	ND[e]	ND[e]	0.089 ± 0.058	0.18	>300	>300	

[a]Effective concentration required to reduce virus-induced cytopathicity (HCMV) or plaque formation (VZV) by 50%. [b]Minimum concentration required to cause a microscopically detectable alteration of cell morphology. [c]Cytotoxic concentration required to reduce cell viability by 50%. [d]cLogP values were calculated using ChemBioDraw Ultra version 14.0 from CambridgeSoft. [e]Not determined.

In addition, the ability of all synthesized amidate prodrugs to inhibit the replication of HBV was also assessed in the human hepatoblastoma cell line HepG2.2.15, which is a widely used cell line containing two copies of the HBV wild-type strain ayw1 genome and constitutively produces high levels of HBV.[28] Real-time qPCR (TaqMan) was used to measure the extracellular HBV DNA copy number associated with virions released from HepG2 2.2.15 cells. A tetrazolium dye uptake assay was employed to measure cell viability and calculate the CC_{50} values. Only compounds **10b**, **11b**, and **12b** proved moderately active against HBV with EC_{50} values in the range of 2.10−8.71 μM (Table S1).

The amidate prodrugs of (*R*)-*O*-2-alkylated FPMPC, i.e., compounds **10b** and **11b**, showed promising antiviral activity against HCMV and VZV in vitro. To achieve this efficacy, prodrugs **10b** and **11b** must efficiently penetrate the target cells and undergo further intracellular metabolism. Previously, we suggested that deoxythreosyl and 3-fluoro-2-(phosphonomethoxy)propyl aryloxyphosphonamidate prodrugs containing a L-aspartate acid diester could be hydrolyzed to afford the nucleoside phosphonates in the cell, which then formed the pharmacologically active diphosphates of phosphonates.[18,19] Therefore, these two prodrugs can be expected to generate intracellularly phosphonate **8b**, which then undergoes activation to its diphosphate species.

In summary, a number of amidate prodrugs of *O*-2-alkylated acyclic nucleosides bearing cytosine or 5-fluorocytosine as nucleobase in combination with three structurally diverse aliphatic side chains were synthesized. Such modification proved to be especially successful within the FPMP series, as demonstrated by the potent activity exhibited by the prodrugs of (*R*)-*O*-2-alkylated FPMPC against both HCMV and VZV. Furthermore, prodrugs of (*R*)-*O*-2-alkylated FPMP containing 5-fluorocytosine and (*R*)-*O*-2-cHPMPC showed a moderate ability to inhibit HCMV replication or VZV plaque formation. These results can help guide the development of novel potential drug candidates against DNA viruses.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00090.

Experimental details and characterization data for the reported compounds, NMR spectra, and biological assays (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ANPs, acyclic nucleoside phosphonates; HPMPC, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine); PMEA, 9-(2-phosphonylmethoxyethyl)adenine; PMPA, (*R*)-9-(2-phosphonylmethoxypropyl)adenine; FPMP, 3-fluoro-2-(phosphonomethoxy)propyl; HIV, human immunodeficiency virus; HCMV, human cytomegalovirus; VZV, varicella zoster virus; cHPMPC, cyclic cidofovir; TMSBr, bromotrimethylsilane; HEL, human embryonic lung; TK, thymidine kinase; Val, valine

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