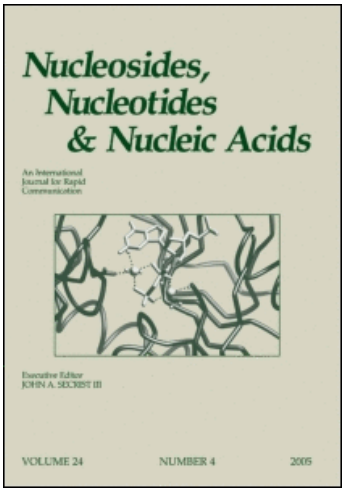

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Antiviral Potential of a New Generation of Acyclic Nucleoside Phosphonates, the 6-[2-(Phosphonomethoxy)Alkoxy]-2,4-Diaminopyrimidines

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ANTIVIRAL POTENTIAL OF A NEW GENERATION OF ACYCLIC NUCLEOSIDE PHOSPHONATES, THE 6-[2-(PHOSPHONOMETHOXY)ALKOXY]-2,4-DIAMINOPYRIMIDINES

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▫ *Three acyclic nucleoside phosphonates (ANPs) have been formally approved for clinical use in the treatment of 1) cytomegalovirus retinitis in AIDS patients (cidofovir, by the intravenous route), 2) chronic hepatitis B virus (HBV) infections (adefovir dipivoxil, by the oral route), and 3) human immunodeficiency virus (HIV) infections (tenofovir disoproxil fumarate, by the oral route). The activity spectrum of cidofovir {(S)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine [(S)-HPMPC)]}, like that of (S)-HPMPA {(S)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine} and (S)-HPMPDAP {(S)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine}, encompasses a broad spectrum of DNA viruses, including polyoma-, papilloma-, adeno-, herpes-, and poxviruses. Adefovir {9-[2-(phosphonomethoxy)ethyl]adenine (PMEA)} and tenofovir {(R)-9-[2-(phosphonomethoxy) propyl]adenine [(R)-PMPA)]} are particularly active against retroviruses (i.e., HIV) and hepadnaviruses (i.e., HBV); additionally, PMEA also shows activity against herpes- and poxviruses. We have recently identified a new class of ANPs, namely 6-[2-(phosphonomethoxy)alkoxy]-2,4-diaminopyrimidines, named, in analogy with their alkylpurine counterparts, HPMPO-DAPy, PMEO-DAPy, and (R)-PMPO-DAPy. These compounds exhibit an antiviral activity spectrum and potency that is similar to that of (S)-HPMPDAP, PMEA, and (R)-PMPA, respectively. Thus, PMEO-DAPy and (R)-PMPO-DAPy, akin to PMEA and (R)-PMPA, proved particularly active against HIV-1, HIV-2, and the murine retrovirus Moloney sarcoma virus (MSV). PMEO-DAPy and (R)-PMPO-DAPy also showed potent activity against both wild-type and lamivudine-resistant strains of HBV. HPMPO-DAPy was found to inhibit different poxviruses (i.e., vaccinia, cowpox, and orf) at a similar potency as cidofovir. HPMPO-DAPy also proved active against adenoviruses. In vivo, HPMPO-DAPy proved equipotent to cidofovir in suppressing vaccinia virus infection (tail lesion formation) in immunocompetent mice and promoting healing of disseminated vaccinia lesions in athymic-nude mice. The 6-[2-(phosphonomethoxy)alkoxy]-2,4-diaminopyrimidines offer substantial potential for the treatment of a broad range of retro-, hepadna-, herpes-, adeno-, and poxvirus infections.*

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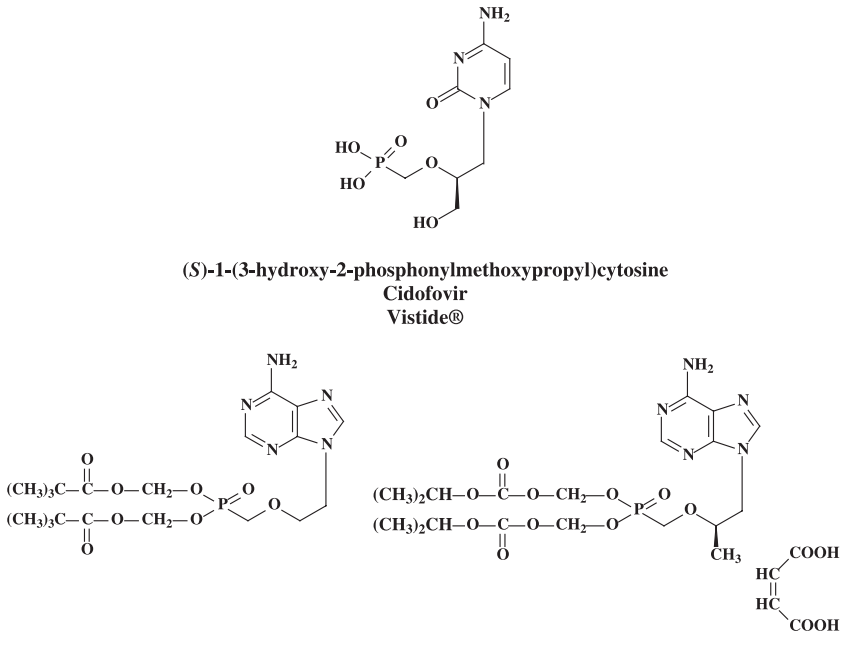
INTRODUCTION

Of the almost forty licensed antiviral drugs that are in current clinical use for the treatment of viral infections,[1] three compounds can be considered as nucleotide analogues, namely the acyclic nucleoside phosphonates (ANPs) cidofovir, adefovir, and tenofovir. Cidofovir (by the intravenous route: Vistide™) (Figure 1) has been formally approved for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients, but it has also demonstrated efficacy in the (experimental and/or clinical) treatment of various other DNA virus infections, i.e., polyomavirus, human papilloma virus (HPV), adenovirus, herpesvirus (herpes simplex virus type 1 [HSV-1] and type 2 [HSV-2], varicella-zoster virus [VZV], Epstein-Barr virus [EBV], human herpesvirus type 6 [HHV-6], type 7 [HHV-7], and type 8 [HHV-8]), and poxvirus (variola [smallpox], vaccinia, cowpox, molluscum contagiosum, and orf [sheeppox]) infections.[2,3] Adefovir has been approved, in its oral prodrug form adefovir dipivoxil (Hepsera™) (Figure 1), for the treatment of chronic hepatitis B virus (HBV) infections. Tenofovir has been approved, in its oral prodrug form tenofovir disoproxil fumarate (TDF) (Viread™) (Figure 1), for the treatment of human immunodeficiency virus (HIV) infections (AIDS), and this in combination with other anti-HIV drugs, in particular, the combination of TDF with lamivudine



(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine
Cidofovir
Vistide®

9-(2-phosphonylmethoxyethyl)adenine
bis(pivaloyloxymethyl) ester
Adefovir dipivoxil
Hepsera®

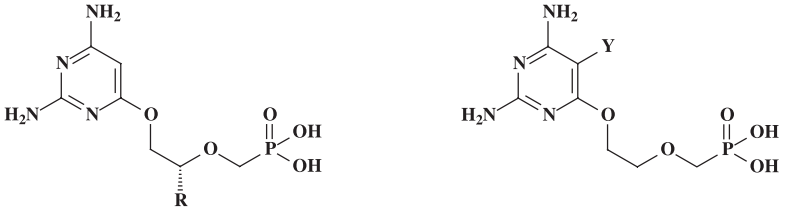
(*R*)-9-(2-phosphonylmethoxypropyl)adenine
bis(isopropyloxycarbonyloxymethyl) ester
Tenofovir disoproxil fumarate
Viread®

FIGURE 1 Formulae of the formally approved antiviral drugs Vistide®, Hepsera®, and Viread®.

and efavirenz has proven highly effective (and virtually free of side effects) over a 3-year study period in patients with AIDS.[4] In addition, TDF has also proven efficacious, even more so than adefovir dipivoxil (at the doses used [daily 300 mg and 10 mg, respectively]) in the treatment of chronic HBV infections.[5]

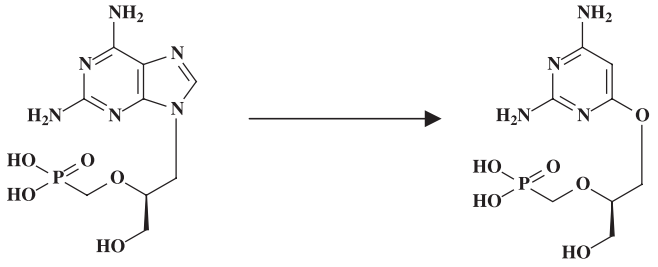
As the acyclic nucleoside phosphonates, apparently due to the presence of their negatively charged phosphonate moiety, are not readily taken up by the oral route, the prodrug forms, i.e., bis(pivaloyloxymethyl) ester for adefovir and bis(isopropyloxycarbonyloxymethyl) ester for tenofovir, had to be designed to ensure their oral bioavailability (Figure 1). Similarly, various ether lipid, i.e., hexadecyloxypropyl, octadecyloxyethyl, oleyloxypropyl, and oleyloxyethyl, esters of cidofovir were constructed to increase its oral bioavailability, and these ether lipid esters of cidofovir proved indeed effective, when given orally, in mouse models for cowpox,[6] vaccinia,[6] and mousepox.[6]

In 2002, Holý et al.[8] reported the synthesis and antiviral activity of a new class of acyclic nucleoside phosphonates, *viz*. 6-[2-(phosphonomethoxy)alkoxy]pyrimidines. The prototypes of this class of compounds, 2,4-diamino-6-[2-(phosphonomethoxy)ethoxy]pyrimidine (PMEO-DAPy) and 2,4-diamino-6-(*R*)-[2-(phosphonomethoxy)propoxy]-pyrimidine [(*R*)-PMPO-DAPy] were found to inhibit the replication



R = CH_3 : (*R*)-PMPO-DAPy
R = H : PMEO-DAPy

Y = CHO : 5-Formyl-PMEO-DAPy
Y = CH_3 : 5-Methyl-PMEO-DAPy
Y = CN : 5-Cyano-PMEO-DAPy
Y = Cl : 5-Chloro-PMEO-DAPy
Y = Br : 5-Bromo-PMEO-DAPy



(*S*)-HPMPDAP **HPMPO-DAPy**

FIGURE 2 Formulae of (*R*)-PMPO-DAPy, PMEO-DAPy, 5-substituted derivatives of PMEO-DAPy, (*S*)-HPMPDAP, and HPMPO-DAPy.

TABLE 1 Antiretroviral Activity of PMEO-DAPy, (*R*)-PMPO-DAPy, and 5-Substituted PMEO-DAPy Derivatives[10]

	EC_{50} (μg/mL)[a]		
Compounds	HIV-1(III_B)(CEM)	HIV-2(ROD)(CEM)	MSV(C3H)
PMEA	2.9	5.7	0.4
(*R*)-PMPA	1.1	1.4	4.2
(*R*)-PMPO-DAPy	1.9	1.3	0.05
PMEO-DAPy	0.90	0.66	0.15
5-Formyl-PMEO-DAPy	1.3	0.83	6.0
5-Methyl-PMEO-DAPy	0.09	0.09	0.05
5-Cyano-PMEO-DAPy	3.3	1.3	2.7
5-Chloro-PMEO-DAPy	0.93	1.3	0.85
5-Bromo-PMEO-DAPy	2.5	3.2	2.4

[a]Fifty percent effective concentration, or compound concentration required to inhibit HIV-induced giant cell formation in CEM cells or MSV-induced transformation of C3H cells by 50%.

of herpesviruses (HSV-1, HSV-2, VZV) as well as retroviruses (HIV-1, HIV-2).[8] In particular, the antiretroviral activity of PMEO-DAPy and (*R*)-PMPO-DAPy appeared interesting, as this was comparable to that of adefovir (PMEA) and tenofovir [(*R*)-PMPA] and extended to the in vivo situation, i.e., newborn mice infected with Moloney murine sarcoma virus (MSV).[9] Furthermore, the antiretroviral potency of these compounds, i.e., PMEO-DAPy, could be further enhanced by introduction of the appropriate substituent (i.e., methyl group) at the C-5 position of the pyrimidine ring.[10,11]

The new acyclic nucleoside phosphonates that are the subject of the present review are PMEO-DAPy (and 5-substituted derivatives thereof), (*R*)-PMPO-DAPy, and 2,4-diamino-6-(*R*)-[3-hydroxy-2-(phosphonomethoxy)propoxy]pyrimidine [HPMPO-DAPy].* The latter can be viewed as analogous to (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-2,6-diaminopurine [(*S*)-HPMPDAP]. The structures of these compounds are shown in Figure 2.

ANTI-RETROVIRUS ACTIVITY

Akin to PMEA (adefovir) and (*R*)-PMPA (tenofovir), PMEO-DAPy, (*R*)-PMPO-DAPy, and 5-substituted PMEO-DAPy derivatives exhibited marked activity against HIV-1, HIV-2, and MSV. PMEO-DAPy was about 3- to 9-fold more potent than PMEA, and this potency could be further enhanced (by 3- to 10-fold) upon introduction of a methyl group at the C-5 position (as in 5-methyl-PMEO-DAPy). The latter compound inhibited HIV and MSV replication at an EC_{50} of 0.05–0.1 μg/mL (Table 1).[12]

*Apparent discrepancy in the designation of HPMP and HPMPO compounds with the same absolute configuration is due to formal change in the priority of substituents at the assymetric carbon atom according to Ingold-Kahn-Prelog notation. This does not apply to PMP derivatives.

TABLE 2 Anti-HBV Activity of PMEO-DAPy, (*R*)-PMPO-DAPy, and 5-Substituted PMEO-DAPy Derivatives[11,12]

	EC_{50} (μg/mL)[a]	
Compounds	Hep AD38	Hep AD79
PMEO-DAPy	0.08 ± 0.05	0.065 ± 0.05
(*R*)-PMPO-DAPy	1.2 ± 0.3	3 ± 0.5
(*S*)-PMPO-DAPy	17 ± 4	~ 100
5-Methyl-PMEO-DAPy	0.9 ± 0.5	0.6 ± 0.2
5-Bromo-PMEO-DAPy	3 ± 1	6 ± 0.5
5-Formyl-PMEO-DAPy	6 ± 3	2.6 ± 0.4

[a]Fifty percent effective concentration, or compound concentration required to inhibit viral DNA synthesis in HepAD38 cells (producing wild-type HBV) and HepAD79 cells (producing 3TC-resistant HBV) by 50%.

ANTI-HBV ACTIVITY

PMEO-DAPy, (*R*)-PMPO-DAPy, and 5-substituted PMEO-DAPy derivatives also exhibited marked activity against HBV, whether wild-type (WT) or lamivudine (3TC)-resistant ($3TC^r$) type. Here, PMEO-DAPy emerged as the most potent congener. It inhibited the replication of wild-type and 3TC-resistant HBV at an EC_{50} of 0.08 and 0.06 μg/mL, respectively (Table 2).[13,14]

ANTI-ADENOVIRUS ACTIVITY

The (*R*)-enantiomer of HPMPO-DAPy and the racemic (*R*,*S*)-HPMPO-DAPy were found to inhibit adenovirus (type 2) replication at an EC_{50} of *circa* 1.6 to 4 μg/mL, as monitored by three different assays (cytopathogenicity [CPE], cell viability [MTS], viral DNA [Q-PCR]) in human embryonic lung (HEL) cells. However, cidofovir, and the prototype of all acyclic nucleoside phosphonates, (*S*)-HPMPA {(*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine},[15] proved about 3- to 10-fold more potent as anti-adenovirus agents than HPMPO-DAPy (Table 3).[16]

TABLE 3 Anti-Adenovirus Activity in HEL Cells[14]

	EC_{50} (μg/mL)[a] for adenovirus type 2			CC_{50} (μg/mL)[b]
Compound	CPE assay	MTS assay	Q-PCR assay	MTS assay
Cidofovir	0.64	0.65	0.99	>70
(*S*)-HPMPA	0.20	0.42	0.19	>70
HPMPO-DAPy	1.60	2.22	2.94	>70
(*R*,*S*)-HPMPO-DAPy	3.00	3.97	3.80	>70

[a]Fifty percent effective concentration, or compound concentration required to inhibit viral replication by 50%, as measured by evaluation of the adenovirus-induced cytopathic effect (CPE assay), colorimetric cell viability testing (MTS assay), or quantitation of viral DNA (Q-PCR assay).

[b]Fifty percent cytotoxic concentration, or compound concentration causing 50% reduction in cell viability, measured by the colorimetric MTS assay.

TABLE 4 Activity of HPMPC, HPMPA, HPMPDAP, and HPMPO-DAPy Against Vaccinia Virus (Lederle Strain), Cowpox Virus (Brighton Strain), and Orf Virus (NZ2 Strain) in HEL (Human Embryonic Lung) Cells[15]

	EC_{50} (μg/mL)[a]		
Compounds	Vaccinia virus	Cowpox virus	Orf virus
(*S*)-HPMPC	3.57	4.6	0.47
(*S*)-HPMPA	0.17	0.23	0.13
(*S*)-HPMPDAP	0.51	0.75	0.47
HPMPO-DAPy	0.76	1.65	0.58

Minimum cytotoxic concentration was >50 μg/mL.

[a]Fifty percent effective concentration, or compound concentration required to inhibit poxvirus-induced cytopathogenicity by 50%.

ANTI-POXVIRUS ACTIVITY (IN VITRO)

When evaluated for its activity (in HEL cells) against different poxviruses (vaccinia, cowpox, and orf), HPMPO-DAPy showed an antiviral potency that was comparable to that of cidofovir, (*S*)-HPMPA and (*S*)-HPMPDAP. Its EC_{50} for vaccinia, cowpox, and orf was in the range of 0.6–1.6 μg/mL. For (*S*)-HPMPA, the most active congener, the EC_{50} for these three poxviruses varied from 0.13 to 0.23 μg/mL (Table 4).[17]

ANTI-POXVIRUS ACTIVITY (IN VIVO)

We have recently elaborated a mouse model for disseminated vaccinia, reminiscent of disseminated vaccinia that may develop in immunocompromised individuals following vaccination against smallpox.[18] Athymic-nude mice inoculated intracutaneously with vaccinia virus develop disseminated/progressive cutaneous vaccinia lesions that invariably lead to death within 4 to 5 weeks after the infection. When cidofovir was administered subcutaneously (at 100 mg/kg/day) starting on day 15 post-infection, and continued for 21 days, it caused a complete healing of the vaccinia lesions, as recorded on day 36 post-infection.[19] Similarly, HPMPO-DAPy administered subcutaneously (at 100 mg/kg), on days 15, 17, and 19, conferred a healing of established vaccinia lesions within one week after the first dose (Figure 3).[20] HPMPO-DAPy proved equally effective as cidofovir in suppressing vaccinia virus infection, as monitored by the formation of tail lesions, in immunocompetent mice.[19]

ANTI-POXVIRUS ACTIVITY (RAFT CULTURE)

The anti-poxvirus activity of the "old" and "new" acyclic nucleoside phosphonates was further demonstrated in organotypic epithelial raft cultures of primary human keratinocytes (PHKs) infected with vaccinia virus[17] and primary

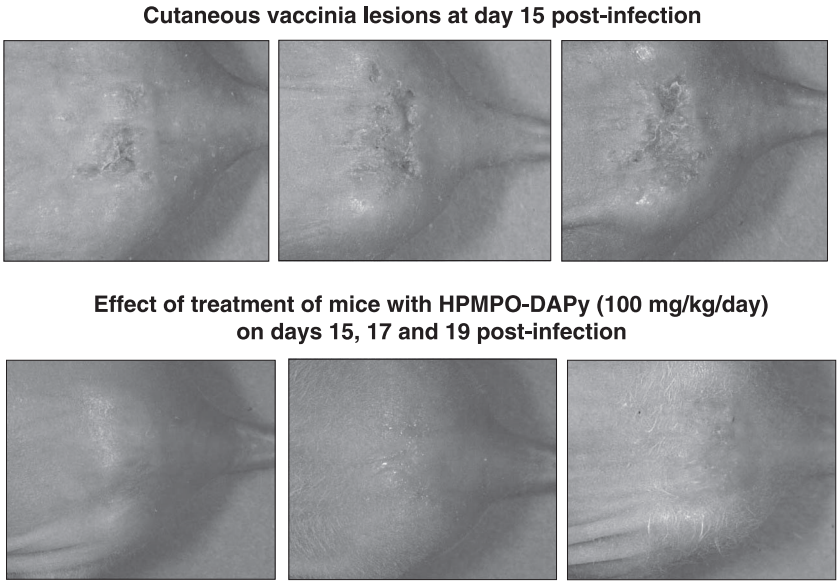


FIGURE 3 Healing of established vaccinia lesions following systemic (subcutaneous) administration of HPMPO-DAPy in athymic-nude mice.[20] Pictures taken at day 22 post-infection.

lamb keratinocytes (PLKs) infected with orf virus.[21] In PHKs infected with vaccinia virus, both (*S*)-HPMPDAP and HPMPO-DAPy (Figure 4), as well as the reference compound cidofovir, were found to completely inhibit virus replication at a concentration of 20, 40, or 50 μg/mL, and to partially inhibit virus replication at a concentration of 4 or 5 μg/mL. This could be readily monitored microscopically

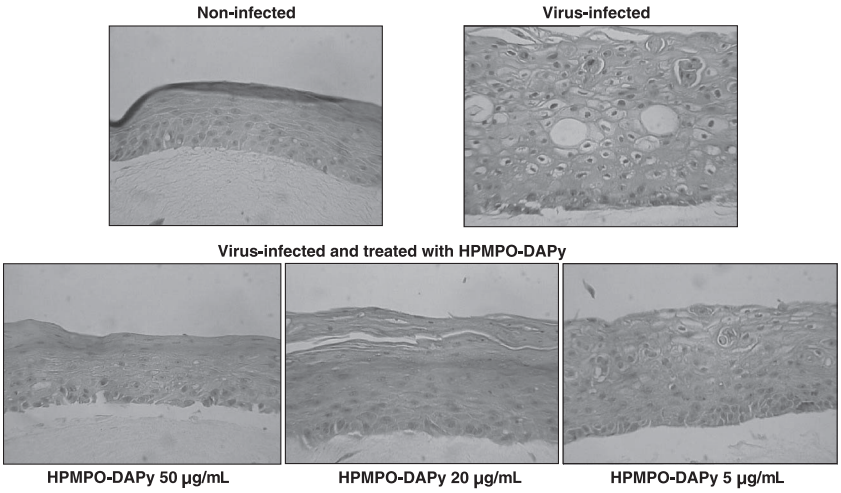


FIGURE 4 Epithelial raft cultures of primary human keratinocytes (PHKs) infected with vaccinia virus after 9 days of differentiation and treatment with HPMPO-DAPy.[17]

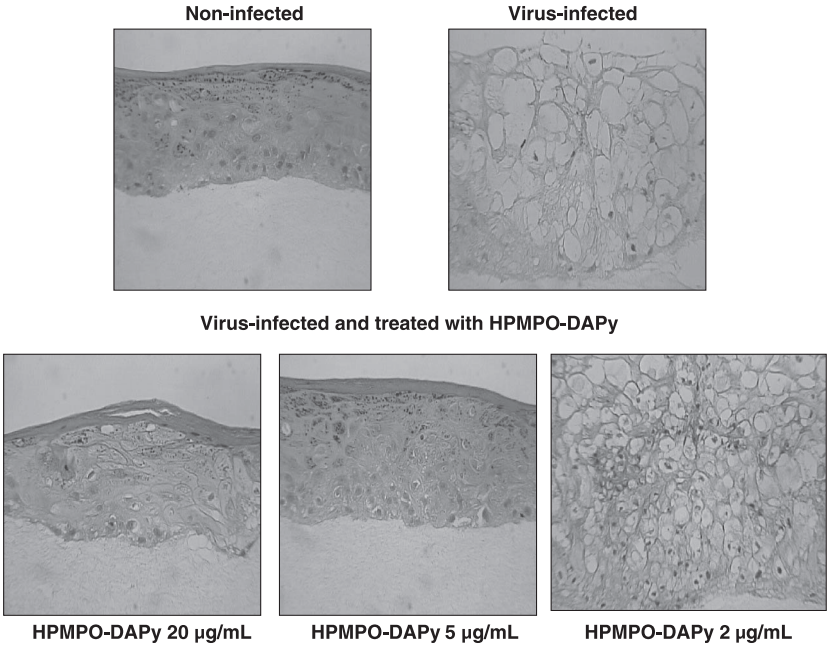


FIGURE 5 Epithelial raft cultures of primary lamb keratinocytes (PLKs) infected with orf virus after 6 days of differentiation and treatment with HPMPO-DAPy.[21]

based on the morphological appearance of the raft cultures at 12 days after infection (Figure 4).[17]

In PLKs infected with orf virus, again cidofovir, (*S*)-HPMPDAP, and HPMPO-DAPy were found to completely block virus replication at a concentration of 20 μg/mL, only partially at a concentration of 5 μg/mL, and virtually not at all at a concentration of 2 μg/mL. This is illustrated in Figure 5[21] for HPMPO-DAPy, which at a concentration of 5 and 20 μg/mL gave a microscopic appearance of the treated virus-infected cells that was similar to that of the non-infected cells, whereas in the presence of HPMPO-DAPy at 2 μg/mL, the microscopic appearance of the treated virus-infected cells was very much alike the untreated virus-infected cells.

ANTI-HPV ACTIVITY

As for the poxviruses, an organotypic epithelial raft culture system has been elaborated to monitor the inhibitory effect of the acyclic nucleoside phosphonates on the proliferation of HPV-transformed keratinocytes. Here, normal human keratinocytes were co-cultured with HPV (type 16)-transformed SiHa cells or HPV (type 33)-transformed CK-1 cells.[22] In these co-cultures (ratio 1:1) of primary human keratinocytes (PHKs) with either HPV-16-transformed (SiHa) epithelial cells or HPV-33-transformed (CK-1) epithelial cells, acyclic nucleoside phosphonates, such as cidofovir, were found to selectively suppress the proliferation of the tumor

(HPV-16- or HPV-33-transformed) cells. Also, PMEG [9-(2-phosphonylmethoxyethyl)guanine] and its prodrug cPr-PMEDAP [9-(2-phosphonyl-methoxyethyl)-N^6-cyclopropyl-2,6-diaminopurine] have been found to selectively suppress the proliferation of HPV-positive keratinocytes in co-cultures with normal keratinocytes.[22] Studies with the organic epithelial raft co-cultures of normal keratinocytes and HPV-transformed tumor cells are currently being extended to a variety of acyclic nucleoside phosphonates, including HPMPO-DAPy.

CONCLUSION

The novel acyclic nucleoside phosphonates described here, namely the 6-[2-(phosphonomethoxy)alkoxy]-2,4-diaminopyrimidines, fall into two categories (Figure 6). Starting off from (*S*)-HPMPA, (*S*)-HPMPC (cidofovir), and (*S*)-HPMPDAP as the first lead, HPMPO-DAPy can be viewed as, structurally, most closely related to (*S*)-HPMPDAP, and may thus be expected to exhibit a similar antiviral activity spectrum encompassing, in particular, adeno-, pox-, and other DNA viruses. A second lead, starting from PMEA (adefovir) and (*R*)-PMPA (tenofovir) yielded PMEO-DAPy (and 5-substituted derivatives thereof) and (*R*)-PMPO-DAPy with prominent activity, especially against retro- and hepadnaviruses.

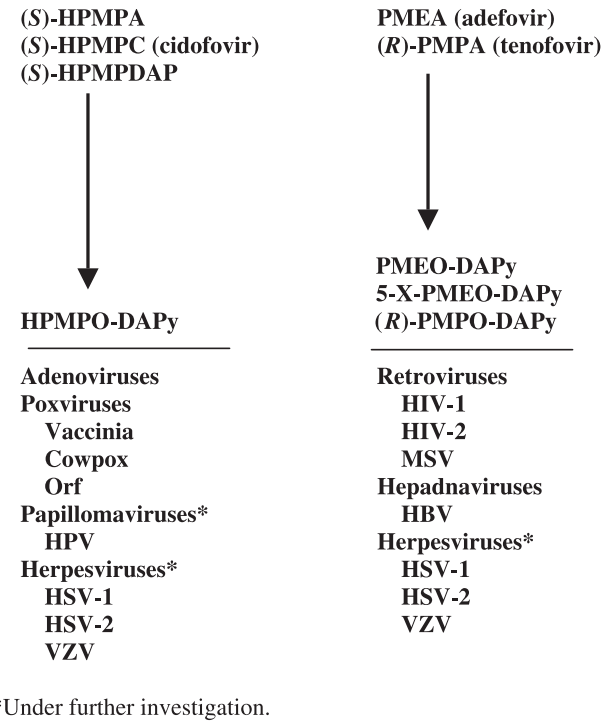


FIGURE 6 The two lines of 6-[2-(phosphonomethoxy)alkoxy]-2,4-diaminopyrimidines.

From a mechanistic viewpoint (i.e., cell uptake, intracellular metabolism, mode of action), much remains to be learned for HPMPO-DAPy, (*R*)-PMPO-DAPy, and PMEO-DAPy. An intriguing question is whether these compounds, being pyrimidine (Py) derivatives but isosteric to the purine derivatives, are recognized by the purine or pyrimidine nucleotide metabolizing enzymes, and, if eventually incorporated into the (viral) DNA, whether they occupy a purine or pyrimidine site. Also much further work remains to be done with regard to the in vivo efficacy, pharmacokinetics, bioavailability, safety, and virus-drug resistance, before the true potential of the 6-[2-(phosphonomethoxy)alkoxy]-2,4-diaminopyrimidines in the clinical context could be fully assessed.

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