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Acyclic nucleotide analogues: synthesis, antiviral activity and inhibitory effects on some cellular and virus-encoded enzymes in vitro*

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Summary

Several *N*-(*S*)-(3-hydroxy-2-phosphonylmethoxypropyl) (HPMP) and *N*-(2-phosphonylmethoxyethyl) (PME) derivatives of purine bases (adenine, guanine, 2-aminoadenine, 3-deazaadenine) and cytosine inhibit the growth of various DNA viruses. PME-derivatives (PMEA, PMEG and PMEDAP) are also active against retroviruses. Both types of nucleotide analogues undergo phosphorylation by cellular nucleotide kinases to their mono- and diphosphates. The phosphorylation with crude extracts of L-1210 cells is potentiated by an ATP-regenerating system. HPMPA is phosphorylated faster than PMEA with or without the ATP-regenerating system.

The HPMP and PME analogues inhibit several virus-encoded target enzymes and their cellular counterparts: (1) HSV-1 DNA polymerase is inhibited by the diphosphates of the PME series; the virus-encoded enzyme is more sensitive than HeLa DNA pol α and β. PMEApp terminates the growing DNA chain; it specifically replaces dATP. HPMPApp also acts as an alternative substrate of dATP, but, in contrast with PMEApp, it permits limited chain growth. (2) Diphosphates of both series inhibit HSV-1 ribonucleotide reductase; the greatest inhibition of

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CDP reduction to dCDP is exhibited by HPMPApp and PMEApp. The enzyme isolated from a PMEA-resistant HSV-1 mutant proved less sensitive to PMEApp, hydroxyurea and HPMPApp. (3) Diphosphates of PME derivatives efficiently inhibit AMV(MAV) reverse transcriptase. (4) The purine HPMP and PME analogues and, even more so, their monophosphate derivatives inhibit purine nucleoside phosphorylase from L-1210 cells.

N-(3-Hydroxy-2-phosphonylmethoxypropyl) derivatives; *N*-(2-phosphonylmethoxyethyl) derivative; Acyclic nucleotide analogue; HPMPA; HPMPC; PMEA

Introduction

Since the discovery of the selective antiviral activity of 9-(*S*)-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) (Chart 5) (De Clercq et al., 1986) our efforts have concentrated on the investigation of this novel structural type of antiviral agents. HPMPA can be regarded as an acyclic nucleotide analogue of dAMP, in which the sugar moiety is replaced by a 2,3-dihydroxypropyl residue and the phosphoric acid ester group by an isopolar phosphonylmethyl ether group linked to the acyclic chain. The structural elements of the HPMPA molecule guarantee its conformational adaptability to the stereochemistry of its natural counterpart (and/or of its metabolites) as well as its resistance against catabolic degradations including dephosphorylation (Holý, 1986). In fact, HPMPA is the first nucleotide analogue of which the antiviral activity can be assigned to the integral molecule including the phosphate unit and, also, the first biologically active nucleotide molecule which is transported through the cell membrane without undergoing dephosphorylation reaction. Its structure resembles that of 5′-nucleotides and clearly eliminates the need for primary phosphorylation which is otherwise required for activation of most nucleoside antivirals known so far. Such a process is particularly important for anti-herpesvirus agents (i.e. acyclovir, BVDU and others) of which the action depends on an activation by viral thymidine kinase (TK). Therefore, HPMPA is also active against TK-deficient mutants of herpes simplex viruses which are resistant to acyclovir, BVDU or other TK-dependent antiherpetic drugs.

Structure–activity relationship

We have synthesized a series of HPMPA analogues containing phosphonic acid residue bound by different types of C–P linkages to varying side-chains. Chart 1 shows several representatives of isomers and isosters of the parent molecule as well as additional related compounds substituted at the side-chain. This series comprises about forty 9-substituted adenine compounds (Rosenberg et al., 1988; Holý, 1989; Holý et al., 1989b, 1990). It has been established that of the four geometrical isomers of HPMPA, only the 2′-*O*-phosphonylmethyl ether of (*S*)-9-(2,3-di-

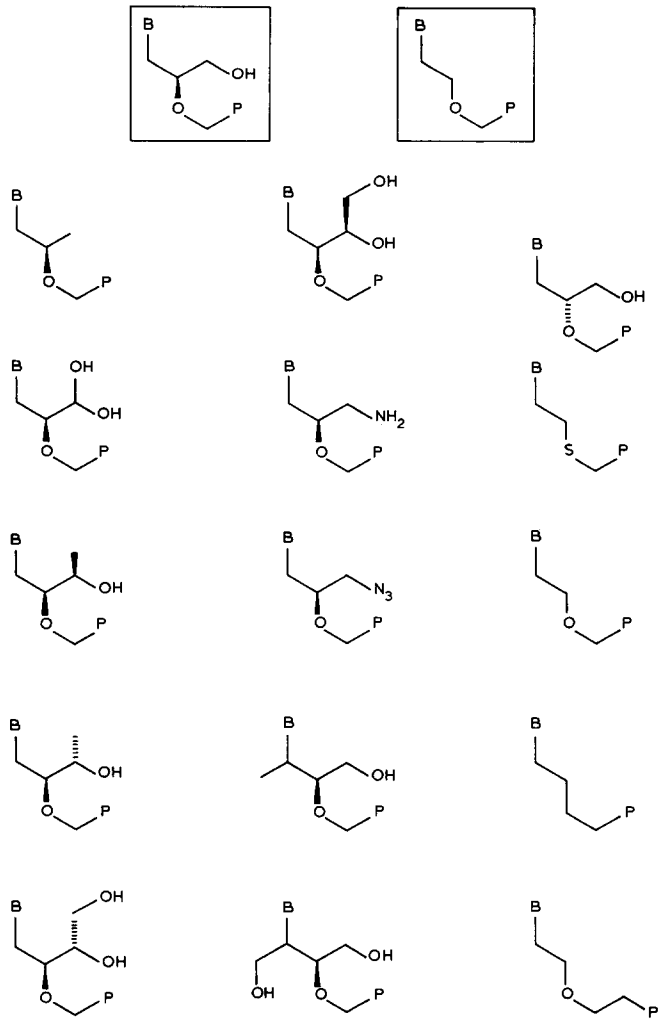


Chart 1.

hydroxypropyl)adenine [(*S*)-DHPA] exhibits antiviral activity (De Clercq et al., 1986). Although the latter compound also acts as an antiviral agent (De Clercq et al., 1978), the antiviral activity spectrum of the two drugs is clearly different.

The structural features of HPMPA are unique; none of its side-chain modified derivatives demonstrated any significant antiviral activity. In addition to the absolute configuration at C(2′), also the presence of the hydroxymethyl group at this position is crucial for the activity. Its hydroxyl group may not be etherified, esterified, or replaced by an hydrogen, or an amino or azido group; also additional substitutions which branch the side chain at C(1′), C(2′) or C(3′)-positions are inadequate. All these HPMPA derivatives show little, if any, antiviral activity.

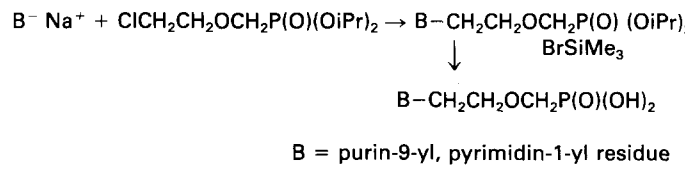


Chart 2.

9-(2-Phosphonylmethoxyethyl)adenine (PMEA) is the only congener in the series of HPMPA derivatives which is an exception to the rule (De Clercq et al., 1986). However, in view of the stringent requirement for the hydroxymethyl group with (2S)-configuration in the HPMPA molecule, it is questionable whether the two compounds are indeed related. As in the HPMPA series, the isomers, isosters and chain-substituted derivatives of PMEA (Rosenberg et al., 1988; Holý et al., 1990) are devoid of antiviral activity. If the antiviral action of HPMPA could be interpreted by its resemblance to dAMP, then PMEA should be considered an analogue of 2′,3′-dideoxynucleotide ddAMP. Consequently, it is not surprising that

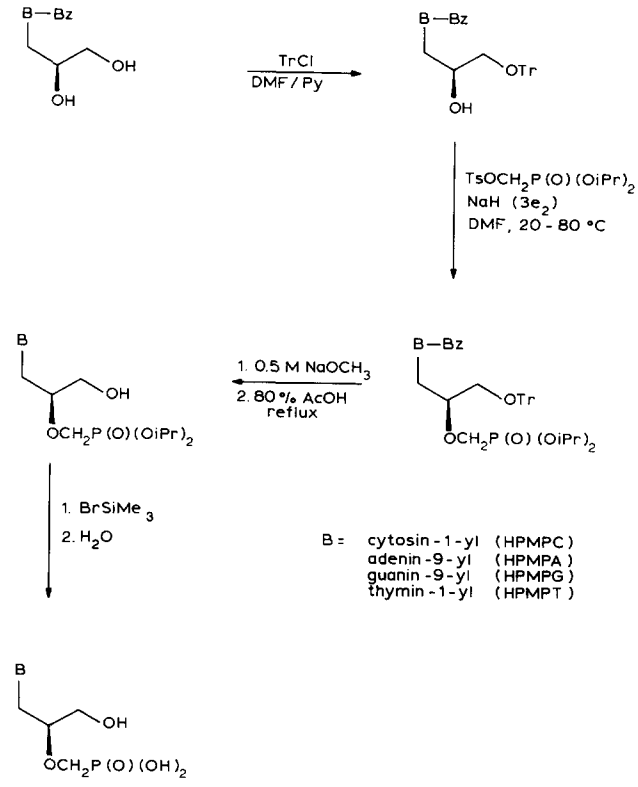


Chart 3. General synthesis of HPMP-derivatives, method (A).

the antiviral activity spectrum of these two compounds is not identical.

The decisive role of the heterocyclic base in the antiviral activity of nucleoside analogues is well known and apparently extends to *N*-(3-hydroxy-2-phosphonylmethoxypropyl)- and *N*-(2-phosphonylmethoxyethyl) derivatives of purine and pyrimidine bases. The relevant synthetic principles have already been described (Holý and Rosenberg, 1987). We now present optimized variants for the preparation of PME and HPMP derivatives which are currently used in our laboratory (Holý et al., 1989a,b). The process employed for the preparation of PMEA and its base-modified analogues is depicted in Chart 2. This process differs from the described route by the use of a di(2-propyl) instead of diethyl ester group in the synthon. This replacement excludes *N*-ethylation of the heterocyclic base and substantially simplifies the work-up procedure. The intermediary di(2-propyl) esters of the PME compounds are easily transformed to the phosphonates by $BrSiMe_3$ treatment, and the final products can be purified (after deionisation) by anion-exchange chromatography. The PME derivatives derived from cytosine, adenine, guanine and their base-modified congeners can be isolated as zwitter-ionic forms, although some of them are hardly soluble in water.

The use of a common synthon for the HPMP series of compounds (Webb and Martin, 1988; Bronson et al., 1989) is unsatisfactory. The routes (Charts 3 and 4) which have been developed in our laboratory for routine purposes start from the corresponding *N*-(2,3-dihydroxypropyl) derivatives (Holý, 1975). The simpler method (Chart 4) suits best for small-scale preparation of novel racemic or enantiomerically pure base-modified compounds (Holý and Rosenberg, 1987). Treatment with chloromethylphosphonic dichloride converts the starting materials

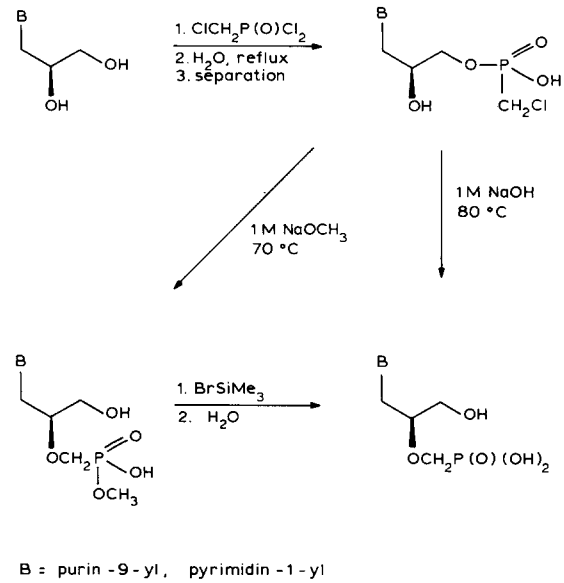
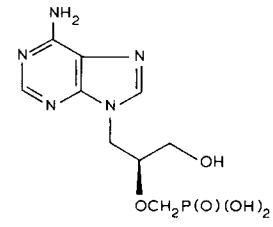


Chart 4. General synthesis of HPMP-derivatives, method (B).

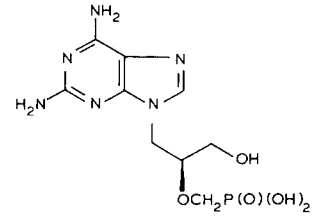
into mixtures of isomeric esters which can be enriched to 70–75% of the required 3′-isomer by boiling in acidic aqueous solution. The individual isomers can be separated by anion-exchange chromatography or by preparative HPLC on hydrophobized silica gel. The 3′-isomers afford, upon treatment with methanolic sodium methoxide under anhydrous conditions, methyl esters of HPMP-derivatives, which are converted to the final products by $BrSiMe_3$ treatment (Holý et al., 1989b).

The second route (Chart 3) which should be regarded as an improvement over the published procedure (Webb and Martin, 1987) is aimed at large scale preparation; it starts from the 3′-*O*-trityl derivatives (amino groups at the base must be eventually protected by *N*-benzoylation) which are treated with di(2-propyl) *p*-toluenesulfonyloxymethylphosphonate in the presence of three equivalents sodium hydride. Methanolysis of the reaction mixture followed by detritylation affords di(2-propyl) esters; the final deprotection by $BrSiMe_3$ results in the required HPMP-derivatives. This method has been successfully applied to the preparation of those compounds that demonstrate the most promising antiviral activity; they are listed in Chart 5 (Holý et al., 1989b).

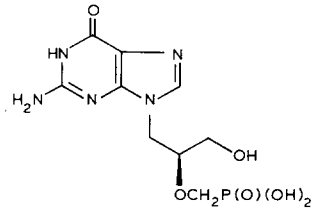
Previous studies have demonstrated that HPMPA, HPMPG and PMEA undergo intracellular phosphorylation to their mono- and diphosphates (Votruba et al., 1987). These metabolites were synthesized chemically for in vitro investigations of their interaction with cellular and viral target enzymes. The synthesis is readily ap-



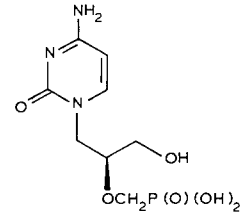
HPMPA



HPMPDAP



HPMPG



HPMPC

Chart 5.

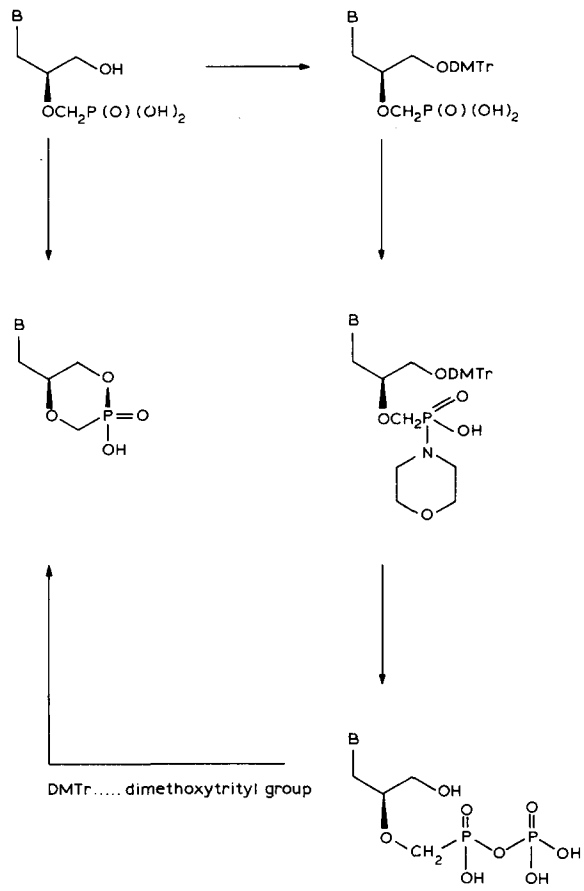


Chart 6.

plicable to the PME derivatives, and affords the required mono- and diphosphates (analogues of nucleoside di- and triphosphates) by the morpholidate procedure (Rosenberg and Holý, 1987). However, this method is not applicable to the HPMP compounds in which the participation of the primary hydroxyl group results in an intramolecular cyclization providing the cyclic phosphonate derivatives. This difficulty can be circumvented by protection of the 3′-hydroxyl group with the highly acid-sensitive substituted trityl group (Chart 6). Nonetheless, the products are very labile and must be handled with the utmost care.

Antiviral activity

N-(*S*)-(3-Hydroxy-2-phosphonylmethoxypropyl) (HPMP) derivatives of adenine, 2-aminoadenine, guanine and cytosine selectively inhibit multiplication of DNA

TABLE 1

Activity of acyclic nucleotide analogues against cytomegalovirus (CMV) in HEL cells

Compound	Antiviral activity ID_{50} (μg/ml)[a]		Cytotoxicity ID_{50} (μg/ml)	SI[b]
	AD-169 strain	Davis strain		
HPMPC	0.25	0.15	200	1000
cHPMPC	0.4	1	>200	>285
HPMPA	0.1	0.2	20	133
HPMPDAP	2.5	6	30	7
3-DeazaHPMPA	0.02	0.2	10	90
HPMPG	1	0.7	10	67
PMEG	0.025	0.04	1.5	46
PMEG ethyl ester	0.15	0.2	5	30
7-PMEG	2	1.5	7	4
DHPG	4	1	100	33

[a]Required to inhibit virus plaque formation by 50 % at a virus input of 100 PFU.
[b]Selectivity index (average value).

viruses, e.g. herpesviruses (HSV, CMV, VZV, EBV), adenoviruses, poxviruses (vaccinia virus) and iridoviruses (African swine fever virus) both in vitro (De Clercq et al., 1986, 1987, 1989; Baba et al., 1987a,b; Gil-Fernandez and De Clercq, 1987; Lin et al., 1987; Gil-Fernandez et al., 1987) and in vivo (De Clercq et al., 1986, 1989; Maudgal et al., 1987). The antiherpetic activity extends to both TK^- and TK^+ mutants of HSV-1. In the series of the *N*-(2-phosphonylmethoxyethyl) (PME) derivatives, those of adenine, 2-aminoadenine and guanine exhibit the most marked antiviral activity (De Clercq et al., 1987). This activity is mainly directed against the herpes viruses. The PME derivatives exhibit no activity against vaccinia virus or adenoviruses. On the other hand, the PME derivatives of adenine and 2-ami-

TABLE 2

Activity of acyclic nucleotide analogues against varicella-zoster virus (VZV) in HEL cells

Compound	Antiviral activity ID_{50} (μg/ml)[a]				Cytotoxicity ID_{50} (μg/ml)	SI[b]
	TK^+		TK^-			
	OKA strain	YS strain	YSR strain	07-1 strain		
HPMPC			0.25		50	200
HPMPA			0.02		20	1000
HPMPDAP			1		> 200	> 200
3-DeazaHPMPA	0.06	0.09	0.06	–	9	129
HPMPG			0.07		10	143
PMEG	0.001	0.001	0.001	0.001	1.5	1500
PMEG ethyl ester	0.008	0.01	0.004	0.007	5	715
7-PMEG	0.2	0.03	0.05	0.15	7	64

[a]Required to inhibit virus plaque formation by 50% at a virus input of 20 PFU.
[b]Selectivity index (average value).

TABLE 3

Apparent kinetic constants of DNA polymerase inhibition by acyclic nucleotide analogues

Inhibitor	Competing dNTP	HSV-1 DNA pol		DNA pol α		DNA pol β	
		K_i	K_m/K_i	K_i	K_m/K_i	K_i	K_m/K_i
dATP (K_m)		0.73		2.05		1.47	
dTTP (K_m)		1.25		1.65		2.97	
dGTP (K_m)		0.70		0.88		1.69	
dCTP (K_m)		0.90		1.48		1.69	
PMEApp	dATP	0.11	6.95	0.87	2.36	370	0.004
PMETpp	dTTP	1.01	1.24	8.36	0.20	820	0.004
PMEUpp	dTTP	5.90	0.21	47.3	0.035	>500	<0.006
PMEGpp	dGTP	0.09	7.78	1.15	0.77	130	0.013
PMECpp	dCTP	1.27	1.41	3.04	0.49	140	0.012
PMEDAPpp	dATP	0.03	25.1	0.18	11.5	>250	<0.006
HPMPApp	dATP	1.42	0.51	6.4	0.32	>250	<0.006
ddTTP	dTTP	21.0	0.06	38.5	0.043	1.7	1.75
AZT-TP	dTTP	330	0.004	116	0.014	1.1	2.70
araCTP	dCTP	0.30	3.02	2.56	0.58	0.71	2.38
ACV-TP	dGTP	0.12	5.83	2.53	0.35	350	0.005

noadenine display marked activity against some retroviruses, i.e. human immunodeficiency virus (HIV) (Pauwels et al., 1988; Balzarini et al., 1989).

The two structural groups (HPMP and PME) show differences in the dependence of the biological activity on the character of the heterocyclic base. In both series, purine derivatives exhibit the highest in vitro antiviral potency against herpesviruses (HSV-1, HSV-2, CMV, VZV), which decreases in the order guanine > 2-aminoadenine > adenine. The same order is observed for the cytotoxicity of these compounds. In addition to these compounds, several other purine bases (6-hydrazino-, 6-hydroxylamino- or 2-aminopurine derivatives) are also compatible with appreciable antiviral activity, but only in the HPMP series. However, N6-substitution or additional C2-substitution at the adenine base or replacement of adenine by hypoxanthine or xanthine does not lead to any active analogues. Further investigations have revealed an additional purine derivative with interesting antiviral properties, namely the 3-deaza analogue of HPMPA (Holý et al., 1989b). Its in vitro activity against CMV and VZV is demonstrated in Tables 1 and 2.

Most pyrimidine derivatives are devoid of an inhibitory effect on virus multiplication. However, in the HPMP series, the cytosine derivative HPMPC displays an activity comparable to that of HPMPA (Snoeck et al., 1988), which makes this compound worth further pursuing (Bronson et al., 1989). In contrast, the 5-methylcytosine derivative is inactive. The differences in activity observed with the purine and pyrimidine derivatives suggest that the two series of compounds act through different mechanisms of action.

Antiviral effects have also been observed with additional derivatives which can be considered as prodrugs of the active PME or HPMP compounds, e.g. cyclic

TABLE 4

Inhibitory effects of acyclic nucleotide analogues on reduction of CDP[a] to dCDP by HSV-1 induced ribonucleotide reductase

Compound[b]	IC_{50} (μM)	Ribonucleotide reductase (residual activity, %)
PMEA	> 1000	98
PMEAp	96	64.5
PMEApp	8	19
2-NH_2PMEAp	> 1000	80
2-NH_2PMEApp	19	15
PMEGp	> 1000	96
PMEGpp	> 1000	64
PMEUp	> 1000	108
PMEUpp	> 1000	74
PMETp	> 1000	83
PMETpp	> 1000	26
PMECp	> 1000	107
PMECpp	> 1000	68
HPMPAp	480	12
HPMPApp	0.90	...[c]
araC	> 1000	90
araCTP	9.50	...[c]
ACV-TP	> 1000	61

[a] [CDP] = 5.6 μM.
[b] At a concentration of 1 mM.
[c] Below the limit of detection.

phosphonates (De Clercq et al., 1987) or alkyl esters (cf. Tables 1 and 2). It remains to be established whether the compounds are active per se or need to be converted first to the active phosphonates. The prodrugs are particularly attractive as therapeutic modalities to circumvent the limited oral bioavailability of the phosphonates in vivo.

Biochemical investigations

The acyclic nucleotide analogues HPMPA and PMEA are phosphorylated in the presence of ribonucleoside 5'-triphosphates by crude extracts from murine L-1210 leukemic cells affording the corresponding mono- and diphosphoryl derivatives. The presence of an ATP-regenerating system markedly stimulates the metabolism of both compounds. The rate of phosphorylation of PMEA is 5 times slower than that of HPMPA, with or without an ATP-regenerating system. Surprisingly, CTP and UTP are very efficient phosphate donors in the absence of the ATP-regenerating system. The donor efficiency decreases in the order CTP > UTP >> ATP > GTP (Merta et al., 1989a).

In contrast with acyclovir (Miller and Miller, 1982), neither PMEA nor HPMPA are markedly phosphorylated by NMP kinase, AMP kinase, GMP kinase or NDP

TABLE 5

Inhibition of AMV(MAV) reverse transcriptase by acyclic analogues of nucleoside 5′-triphosphates

Inhibitor	Competitive substrate (20 μM)	IC_{50} (μM)	
		3 min	5 min
PMEDAPpp	dATP	0.23	0.18
PMEApp	dATP	1.35	1.00
PMEGpp	dGTP	2.50	2.10
PMECpp	dCTP	3.10	2.30
PMETpp	dTTP	3.60	3.25
PMEUpp	dTTP	3.90	3.10
AZT-TP	dTTP	1.05	1.13
ddTTP	dTTP	1.50	1.00

TABLE 6

Inhibitory effects of acyclic nucleotide analogues on inosine phosphorolysis by L-1210 purine nucleoside phosphorylase

Compound	v_i/v_o		K_i (μM)[a]	K_i/K_m[a,b]
	([P_i] = 41 mM)	([P_i] = 1 mM)		
PMEA	0.89	0.09	0.5	0.012
PMEAp	0.86	0.07	0.27	0.006
PMEApp	0.84	ND	ND	ND
PMEG	ND	0.89	ND	ND
PMEGp	0.39	0.04	0.055	0.001
PMEGpp	0.89	ND	ND	ND
PMEDAP	0.89	0.97	ND	ND
PMEDAPp	0.56	0.05	0.08	0.002
PMEDAPpp	0.90	ND	ND	ND
PMEHx	0.90	0.16	ND	ND
PMEHxp	0.73	0.10	0.75	0.017
PMEHxpp	0.93	ND	ND	ND
HPMPA	0.92	0.21	2.00	0.046
HPMPAp	0.94	0.19	1.80	0.042
HPMPApp	1.00	ND	ND	ND
HPMPG	0.93	0.77	ND	ND
HPMPGp	ND	0.03	0.02	0.0005
HPMPHx	0.89	0.92	ND	ND
ACV	0.65	ND	ND	ND
ACV-MP	1.00	0.56	ND	ND
ACV-DP	0.14	0.02	0.005	0.0001
ACV-TP	0.64	ND	ND	ND

[a][P_i] = 1 mM; P_i, inorganic phosphate.
[b]K_m (μM) : Ino 43, Guo 27.
ND, not determined.

kinase in vitro, even at enzyme concentrations exceeding by two or more orders of magnitude those necessary for efficient phosphorylation of the natural substrates. Also 3-phosphoglycerate kinase, glycerol kinase and fructose-6-phosphate kinase fail to catalyze the phosphorylation of HPMPA. These data are at variance with the data published on the phosphorylation of (RS)-HPMPG in the presence of GMP kinase (Terry et al., 1988).

Inhibition of HSV-1 DNA polymerase and HeLa cell DNA polymerases α and β by the diphosphates of the HPMP and PME derivatives was studied and compared with the inhibitory effects of ACV-TP, araCTP, ddTTP and AZT-TP (Merta et al., 1989b). The inhibitory effects on HSV-1 DNA pol of the PME diphosphates (Table 3) decreased in the order: PMEDAPpp >> PMEGpp > PMEApp > PMETpp > PMECpp > PMEUpp. HPMPApp proved to be a relatively weak inhibitor of HSV-1 DNA pol. The inhibitors tested can be divided in three groups: (a) The diphosphates of the PME and HPMP derivatives and ACV-TP specifically inhibit HSV-1 DNA pol and cell DNA pol α; they do not significantly inhibit cell DNA pol β; (b) AZT-TP and ddTTP inhibit only DNA pol β; (c) araCTP inhibits all three DNA polymerases.

When dATP was omitted from the reaction mixtures, the addition of HPMPApp stimulated DNA synthesis by HSV-1 DNA pol indicating that HPMPApp is an alternative substrate for in vitro DNA synthesis catalyzed by this enzyme. These results were confirmed by sequencing analysis of the DNA transcript formed in the presence of HPMPApp and PMEApp. The transcripts were obtained from the template prepared by short incubation of denaturated superhelical plasmid with annealing primer in the presence of Klenow polymerase. The data suggest that HPMPApp is incorporated inside the growing chain of the transcript and eventually causes its termination at distant A-rich sites. PMEApp seems to be efficiently incorporated at the A-sites, but, because of the absence of an hydroxyl group in the side-chain, such incorporation immediately causes chain termination.

Ribonucleotide reductase catalyzes reduction of all four ribonucleoside 5′-diphosphates. The T-even bacteriophages, some herpesviruses and poxviruses encode ribonucleotide reductases which differ from the eukaryotic enzymes by their insensitivity to allosteric regulation by ATP, dATP or dTTP (Averett et al., 1983). This enzyme probably plays a specific role in the regulation of viral DNA synthesis (Leary et al., 1983; Preston et al., 1983) and might constitute an important target for antiviral chemotherapy (Turk et al., 1986; Cameron et al., 1988). The enzyme was purified by partial ammonium sulfate precipitation of the 100000 × *g* supernatant from the lysates of VERO or REF cells infected with HSV-1 (KOS). Its maximum level appeared between 14 and 16 h post-infection. The data in Table 4 demonstrate the effects of various nucleotide analogues on CDP reduction catalyzed by the viral enzyme. The reaction was most efficiently inhibited by HPMPApp followed by PMEApp and araCTP. Our data demonstrate that (a) in both series the diphosphoryl derivatives are more efficient inhibitors than the monophosphoryl derivatives or the parent compounds; (b) of the compounds tested, adenine derivatives are the most inhibitory; (c) dose-dependent response of the CDP reduction has a saturation character; (d) ADP and GDP reduction is less efficiently

inhibited by acyclic nucleotide analogues than the reduction of CDP (Černý et al., 1989).

A PMEA resistant strain (HSV-1 $KOS^{PMEA\text{-}res}$), which was selected by 30 passages of the parental strain in increasing concentrations of PMEA (up to 100 μg/ml), completely preserved its sensitivity toward HPMPA. Ribonucleotide reductase obtained from the cells infected with this mutant was inhibited at PMEApp and HPMPApp concentrations which were markedly higher than those required for inhibition of the enzyme encoded by the parental KOS strain. CDP reduction to dCDP was not inhibited by PMEApp even at 200 μM. Inactivation of HSV-1 $KOS^{PMEA\text{-}res}$ ribonucleotide reductase by hydroxyurea occurred at 4-fold higher concentration than inactivation of the enzyme from the parental strain. Thus, the anti-HSV activity of the parent compound (PMEA) could, at least in part, be attributed to an inhibition of the viral ribonucleotide reductase. The fact that the wild-type HSV-1 strain and the PMEA-resistant HSV-1 mutant showed the same sensitivity to HPMPA suggests that HPMPA must therefore also act by a second mechanism that is different from that of PMEA.

The activity of 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and some of its base-modified congeners against HIV-1 replication and Moloney murine sarcoma virus (MSV)-induced transformation of fibroblasts in vitro (Pauwels et al., 1988; Balzarini et al., 1989; Ghazzouli et al., 1989; Mathes et al., 1989) led us to investigate the affinity of the diphosphates of the PME derivatives toward retroviral reverse transcriptase (Votruba et al., 1989).

Detergent disrupted AMV(MAV) retrovirions were selected as the source of both the reverse transcriptase and the template. This system thus utilizes natural template [genomic AMV(MAV)-RNA-primer ($tRNA^{trp}$)]; furthermore, it can be stimulated by addition of $oligo(dT)_{12-18}$ primer. In contrast to the reactions directed by a synthetic template-primer (Chen and Oshana, 1987; Wu et al., 1988), the AMV(MAV)-RNA system permits to establish inhibition of the reverse transcriptase in the presence of all four natural 2′-deoxynucleoside 5′-triphosphates (Table 5).

The diphosphates of the purine and pyrimidine PME derivatives were found to inhibit AMV(MAV) reverse transcriptase, the order of decreasing activity being: PMEDAppp > PMEApp > PMEGpp > PMECpp > PMEUpp > PMETpp. The 2-aminoadenine derivative proved to be more potent, and PMEApp about equally potent, as AZT-TP and ddTTP. PMEApp inhibited both the RNA-dependent and DNA-dependent reactions, as demonstrated by comparison of the inhibition of the enzyme reactions directed by activated DNA and carried out in the presence or absence of actinomycin D.

These results are consistent with the observed anti-retrovirus potency of the parent nucleotide analogues in vitro (Pauwels et al., 1988) as well as the activity of PMEA against murine retrovirus infection in vivo (Balzarini et al., 1989). The finding that PMEA is superior to AZT as an antiretroviral agent (equivalent increase in life-span of MSV-inoculated mice achieved by PMEA at a 25-fold lower dose than with AZT) (Balzarini et al., 1989) must be related to pharmacokinetic and/or pharmacological factors other than inhibition of the reverse transcriptase by the diphosphoryl derivatives of these compounds.

Purine nucleoside phosphorylase is one of the key enzymes in purine nucleoside metabolism. It catalyzes the phosphorolysis of inosine and guanosine to the corresponding bases which can then be re-utilized by the salvage pathway reactions. Some of the purine nucleoside analogues and their metabolites, e.g. ACV-DP (Stein et al., 1987) and 9-(ω-phosphonylalkyl)hypoxanthines (Nakamura et al., 1986) are potent inhibitors of this enzyme.

Data on the inhibitory action of the acyclic nucleotide analogues on the purine nucleoside phosphorylase purified from murine L-1210 leukemic cells are presented in Table 6. It is evident that the acyclic guanine nucleotide analogues HPMPGp and PMEGp are potent inhibitors of purine nucleoside phosphorylase. Also, PMEDAPp strongly inhibits purine nucleoside phosphorylase, whereas HPMPAp and PMEAp are much less effective. The monophosphates (analogues of NDP) are much stronger inhibitors than the parent compounds or their diphosphates. Since normal cells are able to convert the acyclic nucleotide analogues to their monophosphates, the higher cytotoxicity of both the guanine and 2-aminoadenine acyclic nucleotides might be explained by an inhibitory effect on purine nucleoside phosphorylase and the concomitant disturbance of purine nucleoside metabolism in the host cells.

Conclusion

Several *N*-(*S*)-(3-hydroxy-2-phosphonylmethoxypropyl) (HPMP) and *N*-(2-phosphonylmethoxyethyl) (PME) derivatives of purine bases (adenine, guanine, 2-aminoadenine, 3-deazaadenine) and cytosine inhibit the growth of various DNA viruses. PME derivatives (PMEA, PMEG and PMEDAP) are also active against retroviruses. The optimal preparation of HPMP compounds is based on the reaction of *N*-(2,3-dihydroxypropyl) derivatives of heterocyclic bases (protected by 3′-*O*-trityl and *N*-benzoyl) with di(2-propyl) *p*-toluenesulfonyloxymethylphosphonate in the presence of excess sodium hydride followed by methanolysis and acidic treatment. PME derivatives are easily obtained by alkylation of the corresponding base with di(2-propyl) 2-chloroethoxymethylphosphonate; the intermediary di(2-propyl) esters obtained in both cases are finally hydrolyzed by successive treatment with bromotrimethylsilane and water.

Both types of nucleotide analogues undergo phosphorylation by cellular nucleotide kinases to their mono- and diphosphates. The phosphorylation with the crude extract of L-1210 cells is potentiated by an ATP-regenerating system. HPMPA is phosphorylated 5-fold faster than PMEA in both the presence and the absence of the ATP-regenerating system.

The analogues interfere with several virus-encoded target enzymes and their cellular counterparts: (1) HSV-1 DNA polymerase is inhibited by the diphosphates of the analogues in the order PMEDAPpp >> PMEGpp > PMEApp > PMETpp > PMECpp >> HPMPApp. The virus-encoded enzyme is significantly more sensitive than HeLa cell DNA pol α, the effects on HeLa cell DNA pol β being only marginal. PMEApp terminates the growing DNA chain. It specifically replaces

dATP. HPMPApp also acts as an alternative substrate to dATP, but, in contrast with PMEApp, HPMPApp can be incorporated into the interior of the DNA chain, thereby allowing short oligonucleotide chain growth. (2) Diphosphates of both series inhibit HSV-1-encoded ribonucleotide reductase, the greatest inhibition of CDP reduction to dCDP is exhibited by HPMPApp and PMEApp. The enzyme isolated from a PMEA-resistant HSV-1 mutant proved much less sensitive to PMEApp, hydroxyurea and HPMPApp. (3) Diphosphates of PMET derivatives inhibit AMV (MAV) reverse transcriptase with the activity decreasing in the order of PMEDAPpp >> PMEApp > PMEGpp > PMECpp > PMEUpp > PMEUpp. PMEDAPpp is more potent than either AZT-TP or ddTTP, while PMEApp has approximately the same potency as these two reference compounds. (4) The purine HPMP and PME derivatives and their monophosphates inhibit purine nucleoside phosphorylase from L-1210 cells, the monophosphates being more efficient than the parent compounds.

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