Antiviral Research, 13 (1990) 287–294
Elsevier

AVR 00417

Inhibition of avian myeloblastosis virus reverse transcriptase by diphosphates of acyclic phosphonylmethyl nucleotide analogues

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(Received 27 July 1989; accepted 7 February 1990)

Summary

Diphosphates of *N*-(2-phosphonylmethoxyethyl) derivatives of heterocyclic bases were studied in the endogenous oligo(dT)$_{12-18}$ primed reaction of reverse transcriptase from detergent-disrupted AMV(MAV) retrovirions. These diphosphates (analogues of nucleotide 5′-triphosphates) exhibited an inhibitory activity towards reverse transcriptase. This inhibitory activity was dependent on the character of the heterocyclic base and decreased in the order: 2-aminoadenine > adenine > guanine >> cytosine >> thymine > uracil. The 2-aminoadenine derivative was more potent than either AZT-TP or ddTTP, while PMEApp had approximately the same potency as the two reference compounds ($IC_{50} \approx 1$ μM at 20 μM competing substrate). This finding is consistent with the antiviral activity of the parent nucleotide analogues against retroviruses (including HIV).

Reverse transcriptase; Retrovirus; PMEA; Avian myeloblastosis virus

Introduction

Acyclic nucleotide analogues containing a phosphonylmethyl ether linkage were shown to efficiently inhibit the growth of DNA viruses and retroviruses (De Clercq et al., 1986, 1987) (Fig. 1). Recently, Pauwels et al. (1988) and Balzarini et al. (1989) demonstrated that 9-(2-phosphonylmethoxyethyl)adenine (PMEA) is a po-

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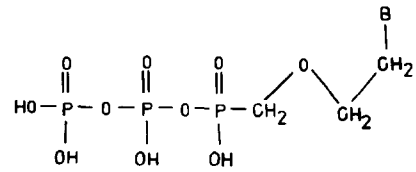

Fig. 1. Structure of PME-derivatives (acyclic nucleoside 5′-triphosphates). B = purine or pyrimidine residue.

tent and selective inhibitor of human immunodeficiency virus (HIV) replication and of Moloney murine sarcoma virus (Mo-MSV)-induced cell transformation. Moreover, Balzarini et al. (1989) demonstrated that PMEA is more effective than 3′-azido-2′,3′-dideoxythymidine (AZT) against MSV-induced tumor formation in vivo. The phosphorylation of PMEA by cellular kinases (Merta et al., 1990a) to the corresponding diphosphate (analogue of nucleoside 5′-triphosphate) and the high antiretroviral efficacy of PMEA prompted us to investigate the affinity of the diphosphates of the *N*-(2-phosphonylmethoxyethyl) (PME-) purine and pyrimidine derivatives toward retroviral reverse transcriptase as a potential target of the antiretroviral action of these acyclic nucleotide analogues.

The detergent disrupted retrovirions of replication-defective avian myeloblastosis associated virus and its replication-competent helper virus myeloblastosis associated virus (AMV(MAV)) were used as the source of both the reverse transcriptase and the template. This system utilizes the natural template (genomic AMV(MAV)-RNA)-primer ($tRNA^{trp}$); furthermore, it can be stimulated by addition of $oligo(dT)_{12-18}$ primer. Unlike the reaction directed by a synthetic template (Chen and Oshana, 1987; Wu et al., 1988), our system permits the establishment of the relative inhibitory efficacy of the acyclic nucleotide analogues toward reverse transcriptase in the presence of all four natural 2′-deoxynucleoside 5′-triphosphates.

Materials and Methods

Chemicals

The 2′-deoxynucleoside 5′-triphosphates were obtained from Pharmacia (Uppsala, Sweden) and ^3H-labelled 2′-deoxynucleoside 5′-triphosphates were products of Amersham (U.K.). Nonidet NP 40 was purchased from LKB Bromma (Sweden). *N*-(2-Diphosphorylphosphonylmethoxyethyl) derivatives of heterocyclic bases (2-aminoadenine, adenine, guanine, cytosine, uracil, thymine) were synthesized from the corresponding *N*-(2-phosphonylmethoxyethyl) compounds by the morfolidate procedure in an analogy to the reaction described for PMEA diphosphate (Holý and Rosenberg, 1987). 3′-Azido-2′,3′-dideoxythymidine 5′-phosphomorpholidate and 2′,3′-dideoxythymidine 5′-phosphomorpholidate were prepared from the corresponding nucleoside analogues and 'hydrolyzed' morpholinophosphodi-

chloridate by the modified method described by Efimov (Efimov et al., 1982). Their transformation to 3′-azido-2′,3′-dideoxythymidine or 2′,3′-dideoxythymidine 5′-triphosphates (AZT-TP, ddTTP, respectively) was performed by the Moffatt's procedure (Moffatt, 1964). Compounds were isolated as sodium salts homogeneous on HPLC, paper electrophoresis (1 M acetic acid) and TLC on PEI cellulose.

Source and purification of the virus

The BAI strain A avian myeloblastosis virus [AMV(MAV)] was used in all experiments. The virus was isolated and purified from blood plasma of leukemic chicks (Leghorn White) in the terminal stage of myeloblastic leukemia as previously described (Trávníček and Říman, 1973).

Enzyme assay

The reaction mixture consisted of 50 mM TRIS-HCl (pH 8.3), 8 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, appropriate 20–50 μM [^{3}H]2′-deoxynucleoside 5′-triphosphate (competitive substrate), 100 μM unlabelled 2′-deoxynucleoside 5′-triphosphates, oligo(dT)$_{12-18}$ (10 $\mu g \cdot ml^{-1}$) or activated DNA (125 $\mu g \cdot ml^{-1}$), AMV(MAV)-lysate (2.3 $mg \cdot ml^{-1}$, lysed in 0.2% Nonidet NP 40), the appropriate amount of acyclic nucleoside 5′-triphosphate analogue (diphosphate of PME-derivative), or, alternatively, AZT-TP or ddTTP. The reaction was stopped after 5 min incubation at 37°C by addition of excess cold 5% TCA; the radioactive precipitates were collected by membrane filtration and counted in a liquid scintillator.

In the experiments with actinomycin D, this compound was added to the reaction mixture to reach the final concentration of 100 $\mu g \cdot ml^{-1}$.

Results and Discussion

The influence of *N*-(2-diphosphorylphosphonylmethoxyethyl) derivatives of 2-aminoadenine, adenine, guanine, cytosine, uracil and thymine upon the reverse transcriptase activity is shown in Fig. 2a,b.

The inhibitory effects of these compounds were compared with those of AZT-TP and ddTTP. The most marked inhibitory effect was exerted by the 2-aminoadenine derivative (2-aminoPMEApp). A less pronounced inhibition, equivalent to that of AZT-TP and ddTTP, was exhibited by PMEApp, whereas the inhibitory effect of PMEGpp was slightly inferior to that of PMEApp. Pyrimidine (cytosine, uracil, thymine) derivatives are not as efficient inhibitors of AMV(MAV)-reverse transcriptase as the purine compounds (Fig. 2b). The same order of inhibitory potency against AMV(MAV)-reverse transcriptase was found following quantitative analysis of the kinetic data (Table 1). The most efficient inhibitor of all the PME-derivatives studied – 2-amino-PMEA – exhibits a K_m/K_i value which is 4–10 times higher than that of either PMEApp, AZT-TP or ddTTP. All diphosphates of PME-derivatives are competitive inhibitors of the enzyme (data not shown).

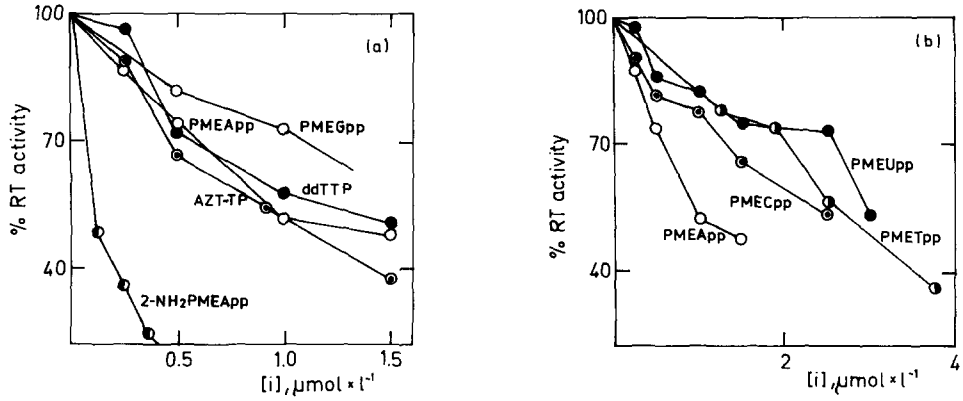

Fig. 2. Effects of *N*-(2-diphosphorylphosphonylmethoxyethyl) derivatives of purine (a) and pyrimidine (b) bases on AMV(MAV)-reverse transcriptase (RT activity). Reactions were performed with oligo$(dT)_{12-18}$ stimulated AMV(MAV)-lysate. The incorporation of (^{3}H)-labeled dNTPs (competing substrates) was measured by TCA precipitation and the residual activity of AMV(MAV)-reverse transcriptase was calculated after scintillation counting (for details, see Materials and Methods).

In another experiment, we studied the time course of the effect of PMEApp upon the reverse transcriptase catalyzed reaction with the aim to establish whether there is any difference in its potency to inhibit reactions (a) directed by endogeneous template-primer only, (b) stimulated by oligo $(dT)_{12-18}$, (c) stimulated by activated DNA, (d) stimulated by activated DNA and simultaneously inhibited by actinomycine D. The results shown in Fig. 3 clearly demonstrate that there is no difference in the inhibitory potency of PMEApp in any of the above reactions. The inhibitory activity of PMEApp is independent upon the character of the template (RNA or DNA). The reverse transcriptase of retroviruses is one of the obvious

TABLE I

Inhibition of reverse transcriptase and DNA polymerase α by acyclic nucleoside 5′-triphosphate analogues ('PME'-derivatives)

Inhibitor	Competing substrate	Reverse transcriptase		DNA polymerase α	
		K_i (μM)	K_m/K_i	K_i (μM)	K_m/K_i
2-AminoPMEApp	dATP	0.056	357	0.18	11.5
PMEApp	dATP	0.22	91	0.87	2.36
PMEGpp	dGTP	0.44	55	1.15	0.77
PMECpp	dCTP	2.00	14	3.04	0.49
PMETpp	dTTP	10.00	3	8.36	0.20
PMEUpp	dTTP	17.00	2	47.3	0.035
AZT-TP	dTTP	0.48	69	116.00	0.014
ddTTP	dTTP	0.58	59	38.5	0.043

[1]The inhibition of HeLa DNA polymerase α was measured as described by Merta et al. (Antiviral Res., 1990b).

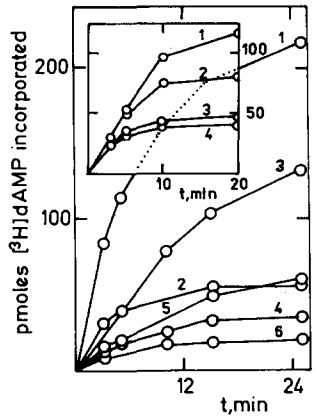

Fig. 3. Time course of the inhibition by PMEApp of RNA- and DNA-dependent reactions catalysed by AMV(MAV)-reverse transcriptase. [^{3}H]dAMP incorporation was measured by TCA precipitation and scintillation counting (a) in AMV(MAV)-lysate stimulated by oligo(dT)$_{12-18}$: (1) control experiment; (2) 1.5 μM PMEApp added; (b) in AMV(MAV)-lysate directed by activated DNA: (3) control experiment; (4) 1.5μM PMEApp added; (c) without exogenous stimulation (endogenous primer only): (5) control experiment; (6) 1.5 μM PMEApp added. Insert: [^{3}H]dAMP incorporation in AMV(MAV)-lysate directed by activated DNA: (1) control experiment; (2) actinomycin D (100 μg·ml^{-1}) added; (3) 1 μM PMEApp added; (4) 1 μM PMEApp and actinomycine D (100 μg·ml^{-1}) added.

targets for antivirals (De Clercq, 1989; Prusoff et al., 1986). To elucidate the antiretrovirus activity of the acyclic nucleotide analogues (PME-derivatives), of which PMEA in particular has marked activity in vitro (Pauwels et al., 1988) and in vivo (Balzarini et al., 1989), we have chosen the non-ionic detergent disrupted AMV(MAV)-retrovirions as a model system. It serves simultaneously as the source of both the enzyme and the template for the oligo(dT)$_{12-18}$ primed reverse transcriptase reaction. The above data show that the diphosphates of the PME-derivatives of purine and pyrimidine bases (analogues of nucleoside 5′-triphosphates) exhibit an inhibitory activity toward AMV(MAV)-reverse transcriptase that is dependent upon the character of the heterocyclic base and decreases in the order of 2-aminoadenine > adenine > guanine > cytosine > thymine > uracil. The 2-aminoadenine derivative is more potent than both AZT-TP and ddTTP, while the potency of the adenine derivative (PMEApp) is comparable with that of the two reference compounds. PMEApp inhibits both the RNA-dependent and DNA-dependent reactions catalyzed by AMV(MAV)-reverse transcriptase as demonstrated by comparison of the inhibition of the enzyme reactions stimulated by activated DNA and performed in the presence or absence of actinomycin D.

The results presented here are consistent with the observed antiretroviral potency of the parent nucleotide analogues in vitro (Pauwels et al., 1988) and their activity in various retrovirus models in vivo (Balzarini et al., 1989; Mathes et al., 1982; Naesens et al., 1989; Balzarini et al., submitted). PMEA is superior to AZT as an antiretroviral agent: the same survival of Mo-MSV-inoculated NMRI mice is effected by PMEA at a 25-fold lower dose than for AZT (Balzarini et al., 1989).

This differential effect cannot merely be explained by the inhibition of reverse transcriptase by the nucleoside 5′-triphosphate analogues, since the latter are equally inhibitory to the reverse transcriptase.

PMEA and its base-modified congeners also display significant anti-herpes activity (De Clercq et al., 1987). We have investigated the affinity of the diphosphates of the purine and pyrimidine PME-derivatives for HSV-1 DNA polymerase and cellular DNA polymerases α and β. The inhibitory effects of 2-amino-PMEApp, PMEGpp and PMEApp on HSV-1 DNA-polymerase follow approximately the same pattern as the antiviral potency of the parent phosphonates (for more details, see Merta et al., 1990b). However, the diphosphates of the PMEA-derivatives are also efficient inhibitors of DNA-polymerase α.

In comparison with their activity against HSV-1 DNA-polymerase and cellular DNA-polymerase α, the effect of these diphosphates on cellular DNA-polymerase β is only marginal. In all cases, DNA polymerase inhibition is strictly competitive with respect to the natural substrate. The structure of PME-derivatives (Fig. 1) excludes the possibility of an internal (5′-3′)-phosphodiester bond formation inside the growing DNA chain.

Additional investigations are needed to elucidate whether these analogues act as DNA chain-growth terminators for DNA viruses and/or retroviruses.

Acknowledgement

The authors are indebted to Mrs H. Miklová for excellent technical assistance.

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