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INHIBITION OF REVERSE TRANSCRIPTASE ACTIVITY OF AVIAN MYELOBLASTOSIS VIRUS BY PYROPHOSPHATE ANALOGUES

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Several pyrophosphate analogues have been studied for their effects on avian myeloblastosis virus reverse transcriptase and on cellular DNA polymerase α. Examination of structure–activity relationships for these compounds revealed that two acidic groups connected by a short bridge were necessary, but not sufficient, for inhibition of the enzyme activities. Foscarnet sodium (trisodium phosphonoformate) was the most potent inhibitor of reverse transcriptase, giving non-competitive inhibition of reactions primed by $(rA)_n \cdot (dT)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$, $(dC)_n \cdot (dG)_{12-18}$, and activated DNA. Carbonyldiphosphonate and 2-hydroxyphosphonoacetate also caused non-competitive inhibition patterns, whereas hypophosphate and imidodiphosphonate inhibited AMV reverse transcriptase in a competitive, non-linear manner. The reverse transcriptase reactions directed by $(rA)_n \cdot (dT)_{12-18}$ and activated DNA were most affected by the non-competitive inhibitors. Hypophosphate and imidodiphosphonate inhibited preferentially reactions primed by $(dC)_n \cdot (dG)_{12-18}$ and activated DNA. In all cases the $(rC)_n \cdot (dG)_{12-18}$ directed reaction was the least affected.

AMV reverse transcriptase phosphonoformate pyrophosphate analogues inhibitors

INTRODUCTION

Avian myeloblastosis virus (AMV) induces an acute myeloblastic leukemia in birds and transforms myeloid hematopoietic cells in vitro [17]. Following infection, a double-stranded DNA intermediate is synthesized by retrovirus reverse transcriptase and integrated into host cell DNA. The expression of integrated viral genes seems to be responsible for initiation and maintenance of the transformed state of cells [2]. A selective inhibition of retrovirus reverse transcriptase activity could prevent the virus-mediated spread of a tumor. Compounds inhibiting enzyme functions also constitute important tools in the study of enzyme mechanisms. Several inhibitors of reverse transcriptase have been described (for reviews see refs. 7, 8, 23).

Foscarnet sodium (trisodium phosphonoformate, PFA) has been found to inhibit reverse transcriptase activity of several retroviruses [29] and to prevent the growth of visna virus in cell culture [28] at concentrations not inhibitory to cellular proliferation [25, 26]. Surprisingly, the structurally related compound phosphonoacetic acid is

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not an inhibitor of the activity of reverse transcriptase [1, 29]. Recently, Reno et al. [19] reported that foscarnet is a competitive inhibitor of the pyrophosphate exchange reaction catalyzed by AMV reverse transcriptase. The inhibition of herpesvirus DNA polymerase activities by foscarnet and phosphonoacetic acid also seems to depend on a similar interaction with a pyrophosphate binding site on the enzyme [9, 13].

In this communication we report the effects of several pyrophosphate analogues on AMV reverse transcriptase activity. Kinetic experiments revealed the mechanism of inhibition and apparent kinetic constants of the active analogues.

METHODS AND MATERIALS

Enzymes

Avian myeloblastosis virus (AMV) reverse transcriptase was purchased from Boehringer Mannheim GmbH, Mannheim, F.R.G., and calf thymus DNA polymerase α from Worthington Biochemical Corporation, Freehold, NJ.

Enzyme assays

The following assay conditions were used if not otherwise indicated.

Reverse transcriptase. 100 μl reaction mixture contained 100 mM Tris-HCl, pH 8.0; 6 mM $MgCl_2$; 5 mM dithiothreitol; 40 μg bovine serum albumin (BSA), fraction V; 1 μg $(rA)_n \cdot (dT)_{12-18}$; the indicated concentrations of [^{3}H]dTTP (0.6–72 μM; specific activity 165–17,100 c.p.m./pmol), and 1.4 units of enzyme protein. (One unit catalyzes the incorporation of 1 pmol dTMP/min at 37°C into an acid-precipitable product with $(rA)_n \cdot (dT)_{12-18}$ as template-primer.) In $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ directed synthesis, 1 μg of synthetic template–primer was added to 100 μl reaction mixture and [^{3}H]dGTP (0.01–4.0 μM; specific activity 1200–8970 c.p.m./pmol) was used as substrate. In DNA synthesis directed by activated calf thymus DNA, 20 μg of template was used together with 100 μM each of dATP, dCTP and dGTP and [^{3}H]dTTP as indicated (0.2–8.0 μM; specific activity 1300–17,100 c.p.m./pmol). After an incubation at 37°C for 60 min 40 μl samples were withdrawn and processed as described previously [10].

DNA polymerase α: 100 μl reaction mixture, incubated at 37°C for 30 min contained 100 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$; 1.5 mM dithiothreitol; 40 μg BSA; 100 μM each of dATP, dCTP, dGTP and 5 μM [^{3}H]dTTP (200–1000 c.p.m./pmol); 12.5 μg activated calf thymus DNA and one unit of enzyme. (One unit catalyzes the incorporation of 1 nmol dAMP in 1 h at 37°C with $(dT)_n \cdot (rA)_{12-18}$ as template–primer.) Cellular DNA polymerase α activity directed by $(dT)_n \cdot (rA)_{12-18}$ was analyzed as described above with 100 μM each of dCTP, dGTP and dTTP and 5 μM [^{3}H]dATP (500 c.p.m./

pmol) as substrates and 1.25 μl of $(dT)_n \cdot (rA)_{12-18}$, (17.7 A_{260}/ml) as template–primer. The incubation was carried out at 37°C for 30 min. The acid-soluble precipitates collected on filter papers were processed as described previously [10].

Pyrophosphate analogues and related compounds

Pyrophosphate, oxalic acid and malonic acid were purchased from E. Merck, Darmstadt, F.R.G. Phosphoglycolic acid, and dilithium carbamyl phosphate were purchased from Sigma Chemical Co., St. Louis, MO. Disodium sulfonoacetate was from Eastman Kodak Co., Rochester, NY. 2-Amino-4-phosphonobutyric acid was obtained from Calbiochem AG, Lucerne, Switzerland. Tetrasodium imidodiphosphonate was purchased from Boehringer Mannheim GmbH, Mannheim, F.R.G. Disodium ethane-1-hydroxy-1,1-diphosphonate was a gift from The Proctor Gamble Company, Cincinnati, OH. *N*-(phosphonoacetyl)-L-aspartic acid was a gift from Dr. L.H. Khedda, Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, MD.

Methanediphosphonic acid, tetrasodium carbonyldiphosphonate, disodium methanehydroxydiphosphonate, trisodium phosphonoformate, phosphonoacetic acid, 2-phosphonopropionic acid, 3-phosphonopropionic acid, 2-phenylphosphonoacetic acid, phosphonoacetamide, and disodium hypophosphate were synthesized as previously described [10].

2-Hydroxy-2-phosphonopropionic acid was synthesized according to Blum and Worms [3]. 2-Hydroxyphosphonoacetic acid, 4-phosphonobutyric acid, and phosphonomethanesulfonic acid were synthesized by B. Lindborg and S. Kovacs at this laboratory and will be published elsewhere.

Other chemicals and isotopes

The deoxyribonucleoside triphosphates were from Sigma Chemical Co., St. Louis, MO. [^{3}H]dTTP, [^{3}H]dGTP, and [^{3}H]dATP were purchased from New England Nuclear, Boston, MA. Calf thymus DNA was purchased from Sigma Chemical Co., St. Louis, MO and activated as described by Schlabach et al. [22]. The synthetic template–primers $(rA)_n \cdot (dT)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ were obtained from Collaborative Research, Waltham, MA.

Kinetic studies

The nomenclature of Cleland [5, 6] was used. Except for the competitive inhibitors, the kinetic experiments were performed in substrate intervals with little or no observed deviation from linearity. The analysis of each reaction mixture was done in duplicate. The amount of product formed was proportional to reaction time and amount of enzyme. Data for the different plots were evaluated using a computer program based on linear regression analysis. The kinetic constants, K_m and K_i, were determined graphically from Lineweaver–Burk and Dixon plots, respectively.

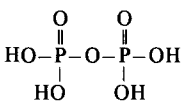
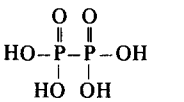
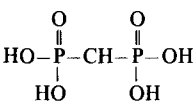
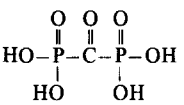
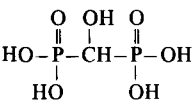
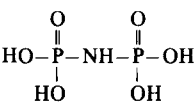
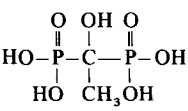
RESULTS

Structural requirements of pyrophosphate analogues for inhibitory effect on AMV reverse transcriptase

Table 1 lists the concentrations of various diphosphonates which caused a 50% inhibition of the activities of AMV reverse transcriptase directed by $(rA)_n \cdot (dT)_{12-18}$ and

TABLE 1

Effects of diphosphonates on AMV reverse transcriptase and cellular DNA polymerase-α activities

Compound		Concentration (μM) giving 50% inhibition		
		AMV reverse transcriptase	DNA polymerase-α	
		$(rA)_n \cdot (dT)_{12-18}$	$(dT)_n \cdot (rA)_{12-18}$	Activated DNA
I		500	⩾1000	⩾1000
II		25	250	250
III		⩾500	⩾500	⩾500
IV		200	500	100
V		⩾500	⩾250	⩾500
VI		100	nd	nd
VII		⩾500	⩾500	⩾500

Assay conditions were as described in Methods. nd = not determined.

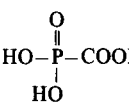
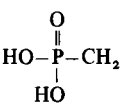
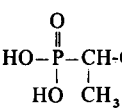
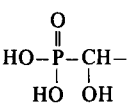
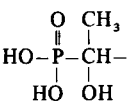
of cellular DNA polymerase α directed either by $(dT)_n \cdot (rA)_{12-18}$ or activated DNA.

Pyrophosphate (I) reduced the activity of reverse transcriptase to 50% at 500 μM. No significant inhibition of cellular α-polymerase could be observed even at 1 mM concentration. Under the reaction condition used, hypophosphate (II) strongly affected reverse transcriptase activity. About ten times higher concentration had to be used in order to affect the α-polymerase to the same extent. Imidodiphosphonate (VI) and carbonyldiphosphonate (IV) were also found to inhibit reverse transcriptase activity at 100 μM and 200 μM, respectively. Diphosphonates without inhibitory potential at 500 μM were methanediphosphonate (III), methanehydroxydiphosphonate (V), and ethane-1-hydroxy-1,1-diphosphonate (VII).

The effects of compounds containing one phosphono group connected to a carboxyl group either directly or via a methylene bridge are summarized in Table 2. Compound VIII, foscarnet (phosphonoformate or PFA), reduced the activity of reverse transcrip-

TABLE 2

Effects of compounds containing phosphono and carboxylic groups on AMV reverse transcriptase and cellular DNA polymerase-α activities

Compound	Concentration (μM) giving 50% inhibition		
	AMV reverse transcriptase	DNA polymerase-α	
	$(rA)_n \cdot (dT)_{12-18}$	$(dT)_n \cdot (rA)_{12-18}$	Activated DNA
VIII 	8	150	40
IX 	⩾2000	100	40
X 	⩾2000	⩾1000	⩾1000
XI 	700	nd	100
XII 	⩾1000	⩾1000	⩾1000

nd = not determined.

tase to 50% at 8 μM. DNA polymerase α was affected to the same extent by 40 μM or 150 μM of foscarnet, depending on the template–primer used. An increased distance between the two acidic groups led to inactive compounds. This was evident for phosphonoacetate (IX) as well as for 3-phosphonopropionate and 4-phosphonobutyrate (data for the latter two compounds not shown). 2-Hydroxyphosphonoacetate (XI) was the only exception found among α-substituted phosphonoacetates which slightly inhibited AMV reverse transcriptase activity. However, this compound like phosphonoacetate (IX) seemed to preferentially affect the cellular α-polymerase. 2-Phosphonopropionate (X) and 2-hydroxy-2-phosphonopropionate (XII) were analogues without effect.

Compounds containing two carboxylic groups, e.g. oxalate or malonate, did not affect AMV reverse transcriptase activity at a concentration of 1 mM. At 500 μM the following structurally related compounds inhibited neither AMV reverse transcriptase nor α-polymerase: 2-phenylphosphonoacetate; 2-amino-4-phosphonobutyrate; phosphoglycolate; sulfonoacetate; phosphonomethanesulfonate; carbamyl phosphate; phosphonoacetamide, and *N*-(phosphonoacetyl)-L-aspartate.

Mechanism of inhibition of AMV reverse transcriptase by pyrophosphate analogues

The inhibitors in the present study were further examined kinetically in order to elucidate their mode of action on AMV reverse transcriptase. In double reciprocal plots, the inhibition by foscarnet gave a linear non-competitive pattern with $(rA)_n \cdot (dT)_{12-18}$ as template–primer and dTTP as the varied substrate (Fig. 1). The same mechanism of inhibition was observed when either $(rC)_n \cdot (dG)_{12-18}$ (data not shown) or $(dC)_n \cdot (dG)_{12-18}$ (Fig. 2) were used as template–primers and the corresponding substrate, dGTP, was varied.

Also carbonyldiphosphonate and 2-hydroxyphosphonoacetate gave linear non-competitive inhibition patterns of the AMV reverse transcriptase reaction with $(rA)_n \cdot (dT)_{12-18}$ as template–primer and dTTP as varied substrate (data not shown). This indicates that foscarnet as well as carbonyldiphosphonate and 2-hydroxyphosphonoacetate affect the polymerization enzyme at sites other than those involved in the binding of deoxyribonucleoside triphosphates.

However, when the reverse transcriptase activity was studied under the influence of hypophosphate or imidodiphosphonate a different type of inhibition pattern was observed. The results from the effect of imidodiphosphonate (Fig. 3) clearly indicated a substrate dependency. An increase in substrate concentration will overcome the inhibition caused by imidodiphosphonate. Hypophosphate gave an almost identical inhibition pattern (data not shown). This mechanism of inhibition was not readily determined, as the reciprocal plots were not linear within the substrate interval. Although atypical, the inhibition could be referred to as a competitive-like inhibition. The inset panels in Figs. 1 and 3, which are alternative ways of presenting identical data, strengthened the findings that different kinetic behaviour existed between foscarnet and imidodiphosphonate.

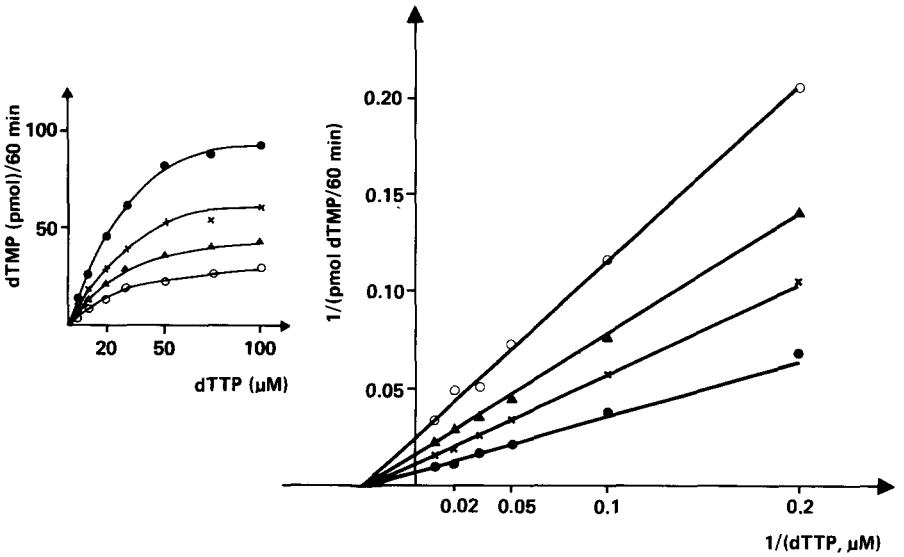


Fig. 1. Inhibition of AMV reverse transcriptase by foscarnet (PFA), using $(rA)_n \cdot (dT)_{12-18}$ as template–primer and dTTP as variable substrate. The concentration of dTTP ranged from 5 μM to 100 μM. The concentrations of PFA were: 0 μM (●); 5 μM (x); 10 μM (▲); and 20 μM (○). Conditions and assay procedure were as described in Methods. Data are expressed as the average of two determinations. The inset shows the effect of substrate concentration on the rate of the AMV reverse transcriptase reaction in the presence of 0 μM (●), 5 μM (x); 10 μM (▲); and 20 μM PFA (○).

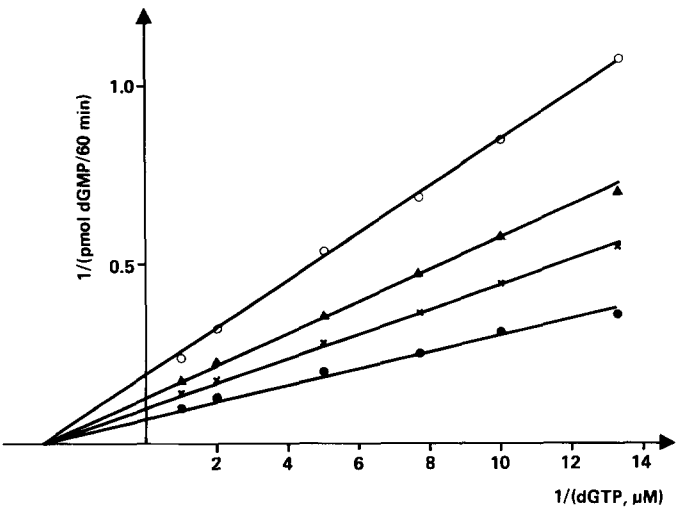


Fig. 2. Inhibition of AMV reverse transcriptase by foscarnet (PFA) using $(dC)_n \cdot (dG)_{12-18}$ as template–primer and dGTP as the variable substrate. The concentration of dGTP ranged from 0.075 μM to 1 μM. The concentrations of PFA were: 0 μM (●); 10 μM (x); 20 μM (▲); and 50 μM (○). Conditions and assay procedure were as described in Methods. Data are expressed as the average of two determinations.

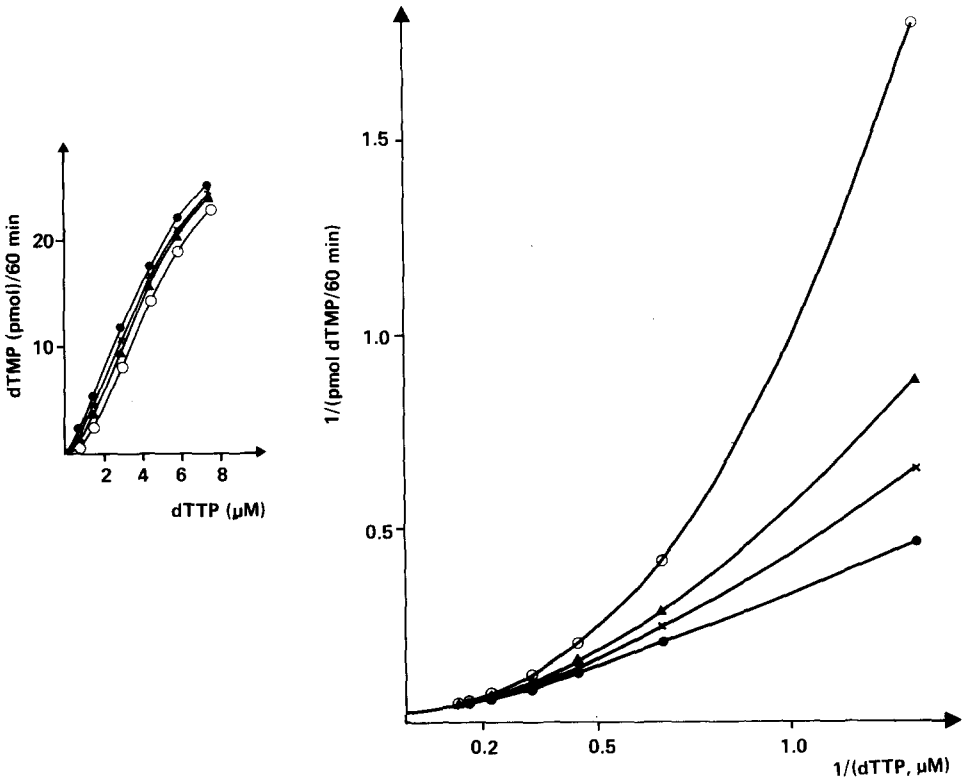


Fig. 3. Inhibition of AMV reverse transcriptase by imidodiphosphonate using $(rA)_n \cdot (dT)_{12-18}$ as template–primer and dTTP as variable substrate. The concentration of dTTP ranged from 0.75 μM to 7.5 μM. The concentrations of imidodiphosphonate were: 0 μM (●); 50 μM (x); 100 μM (▲); and 200 μM (○). Data are expressed as the average of two determinations. The inset shows the effect of substrate concentration on the rate of AMV reverse transcriptase reaction in the presence of 0 μM (●); 50 μM (x); 100 μM (▲); and 200 μM (○) imidodiphosphonate.

The effect of foscarnet on the DNA synthesis catalyzed by the AMV reverse transcriptase directed by various template–primers is summarized in Table 3. The Michaelis-Menten (K_m) and inhibition (K_i) constants were calculated from double reciprocal and Dixon plots, respectively. The constants were found to be highly dependent on the choice of template–primer. Under the reaction conditions used, the highest K_m value (36 μM) was observed with $(rA)_n \cdot (dT)_{12-18}$ as template–primer and dTTP as substrate. On the other hand, the reaction directed by $(dC)_n \cdot (dG)_{12-18}$ gave the lowest K_m value, 0.25 μM of dGTP. Activated DNA and $(rC)_n \cdot (dG)_{12-18}$ as template–primers gave intermediate K_m values, 4 μM and 2 μM, respectively. Concerning the inhibition constants (K_i), it was apparent that the synthesis directed by activated DNA or $(rA)_n \cdot (dT)_{12-18}$ were most susceptible to inhibition by foscarnet. The inhibition of DNA synthesis directed by the other synthetic template–primers required much higher concentrations of foscarnet.

TABLE 3

Apparent kinetic constants for inhibition of AMV reverse transcriptase activity by foscarnet in the presence of different template–primers

Template–primer	Substrate(s)	K_m (μM)	K_i (μM)	Mode of inhibition
$(rA)_n \cdot (dT)_{12-18}$	dTTP	36	8	NC
$(rC)_n \cdot (dG)_{12-18}$	dGTP	2.0	100	NC
$(dC)_n \cdot (dG)_{12-18}$	dGTP	0.25	30	NC
Activated DNA	dATP, dCTP dGTP, dTTP	4.0	5	NC

The assay conditions were as described in Methods.
NC = non-competitive inhibition.

Effect of different template–primers and substrate concentration on the inhibition by pyrophosphate analogues

The rate of DNA synthesis was characteristically dependent on the template primer used. Also the K_i values, obtained from inhibition by foscarnet, differed widely (Table 3). In order to compare the effect of an inhibitor on the AMV reverse transcriptase reactions primed by activated DNA, $(dC)_n \cdot (dG)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$ or $(rA)_n \cdot (dT)_{12-18}$ the following experiment was performed. With a chosen concentration of inhibitor held constant, the rates of the four differently directed sets of reactions were determined at specific substrate levels, related to the K_m value of the studied reaction. Fig. 4 shows the results and indicates that two kinds of inhibition mechanisms were found among the pyrophosphate analogues. The inhibition caused by one set of compounds, exemplified by foscarnet, carbonyldiphosphonate and 2-hydroxyphosphonoacetate (panels a, b and c), was not altered by changes in substrate concentrations. The effects of the remaining three inhibitors, pyrophosphate, hypophosphate and imidodiphosphonate (panels d, e and f), were substantially decreased with increasing substrate concentrations. Synthesis directed by $(rA)_n \cdot (dT)_{12-18}$ and activated DNA were most susceptible to inhibition by foscarnet, carbonyldiphosphonate and 2-hydroxyphosphonoacetate. Hypophosphate and imidodiphosphonate preferentially inhibited the synthesis directed by $(dC)_n \cdot (dG)_{12-18}$ and activated DNA. In all experiments the $(rC)_n \cdot (dG)_{12-18}$ directed reaction was the least inhibited.

Effect of preincubation of AMV reverse transcriptase

Since foscarnet and phosphonoacetate were reported to have the same mode of action on isolated herpesvirus DNA polymerase [9, 19], it was of interest to examine whether a preincubation of AMV reverse transcriptase with phosphonoacetate or pyrophosphate could protect the enzyme from inhibition by foscarnet (Table 4). Apparently, a preincu-

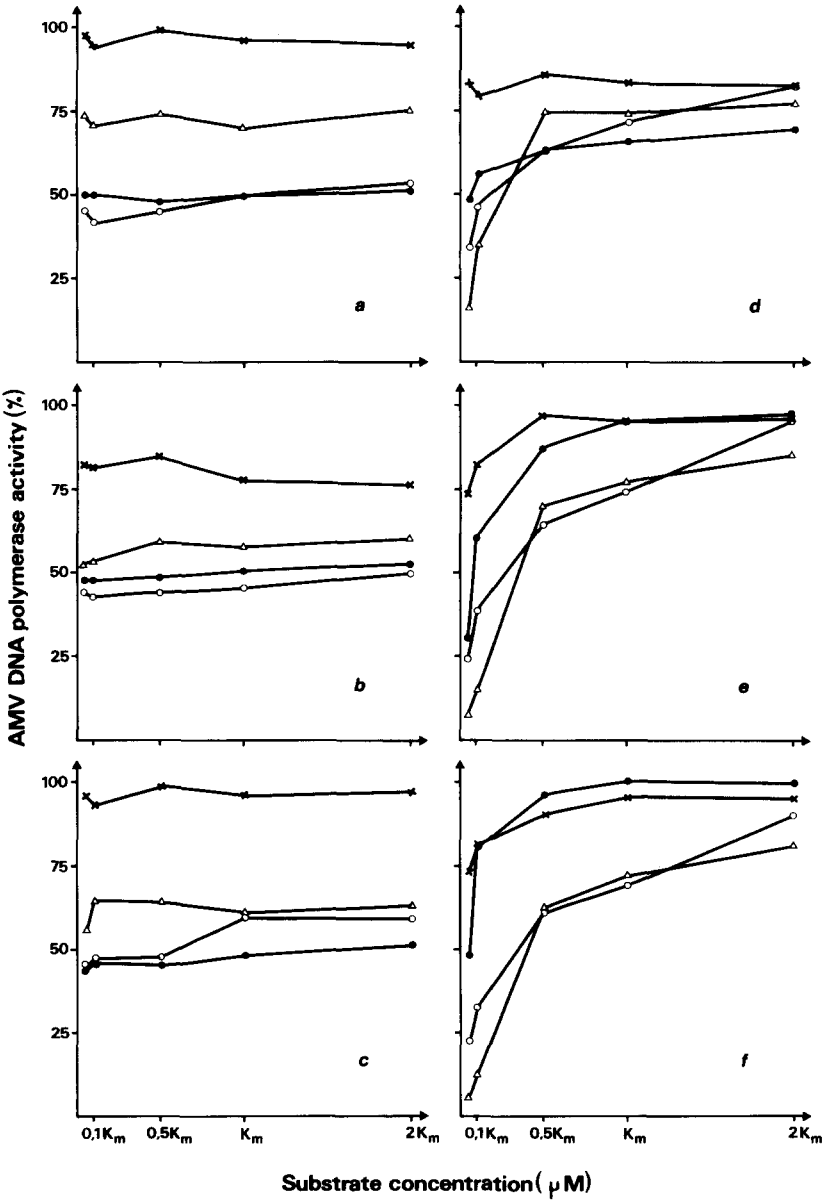


Fig. 4. Influence of substrate concentration on the effect of pyrophosphate analogues on AMV reverse transcriptase reactions. The inhibitors studied were for a) 8 μM foscarnet; b) 700 μM 2-hydroxyphosphonoacetate; c) 200 μM carbonyldiphosphonate; d) 1 mM pyrophosphate; e) 100 μM hypophosphate; and f) 300 μM imidodiphosphonate. The various reactions were represented by the following symbols: x, $(rC)_n \cdot (dG)_{12-18}$; △, $(dC)_n \cdot (dG)_{12-18}$; ●, $(rA)_n \cdot (dT)_{12-18}$; and ○, activated DNA directed synthesis. The individual substrate concentrations were dependent on the K_m value of the reaction and varied between 0.1 to 4 μM dGTP for $(rC)_n \cdot (dG)_{12-18}$ directed synthesis; 0.0125–0.5 μM dGTP for $(dC)_n \cdot (dG)_{12-18}$ directed synthesis; 1.8–72 μM dTTP for $(rA)_n \cdot (dT)_{12-18}$ directed

TABLE 4

Effect of preincubation of AMV reverse transcriptase (E) with foscarnet (PFA), pyrophosphate (PP), and phosphonoacetate (PAA)

Additions to		Inhibition (%)	(%)
preincubation mix	reaction mix		
E	10 μM PFA	(58)[a]	56
E	50 μM PFA	(85)	84
E	1 mM PP	(48)	52
E	1 mM PAA	(20)	20
E	10 μM PFA + 1 mM PP	(66)	66
E	50 μM PFA + 1 mM PP	(85)	86
E	10 μM PFA + 1 mM PAA	(61)	62
E	50 μM PFA + 1 mM PAA	(84)	84
E	1 mM PP + 1 mM PAA	(42)	47
E, 10 μM PFA	10 μM PFA		56
E, 50 μM PFA	50 μM PFA		82
E, 1 mM PP	1 mM PP		33
E, 1 mM PAA	1 mM PAA		11
E, 1 mM PP	1 mM PP + 10 μM PFA		61
E, 1 mM PP	1 mM PP + 50 μM PFA		85
E, 1 mM PAA	1 mM PAA + 10 μM PFA		58
E, 1 mM PAA	1 mM PAA + 50 μM PFA		84

Preincubation was carried out in a volume of 20 μl for 15 min at 37°C. The reaction was initiated by addition of 80 μl reaction ingredients and incubated for 60 min. The concentration of inhibitor in the 20 μl preincubation mix is given in the first column. The final amounts of inhibitors in 100 μl reaction mixture are given in the second column. Assay conditions were as indicated in Methods with 1 μg $(rA)_n \cdot (dT)_{12-18}$ and 36 μM (K_m) of [^{3}H]dTPP (spec. act. 303 c.p.m./pmol) present. The uninhibited reaction incorporated 28 pmol dTMP/60 min and 40 μl.

[a] Inhibition values in parentheses were obtained from parallel experiments without preincubation of enzyme. The uninhibited control reaction incorporated 39 pmol dTMP/60 min and 40 μl.

bation of enzyme with either 1 mM of pyrophosphate or 1 mM of phosphonoacetate did neither decrease nor increase the inhibitory effect of foscarnet on the $(rA)_n \cdot (dT)_{12-18}$ directed reaction. The preincubation of reverse transcriptase alone resulted in a loss of about 25% of its catalytic activity compared with the standard reaction. However, this

synthesis; and 0.2–8 μM of all four dNTPs for activated DNA. The assay conditions were as described in Methods. The incubations of $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ directed synthesis were for 15 min. No significant change of the inhibition values were observed at incubations for 60 min (data not shown). Controls incorporated at two K_m substrate levels 2.9 and 2.4 pmol dGMP/40 μl and 15 min for $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ directed synthesis. Corresponding values for $(rA)_n \cdot (dT)_{12-18}$ and activated DNA directed synthesis were 51 and 1.0 pmol dTMP/40 μl and 60 min, respectively.

did not influence the effect of the inhibitors. Preincubation of enzyme with either foscarnet, pyrophosphate or phosphonoacetate did not result in higher inhibition values than was obtained in the standard assays.

DISCUSSION

As has been shown previously for herpes virus DNA polymerase [4, 9, 10, 13], hepatitis B DNA polymerase [18] and influenza virus RNA polymerase [23], a rather narrowly defined structural requirement exists for pyrophosphate analogues possessing inhibitory effect. This was found also for AMV reverse transcriptase in the present investigation. The two most active inhibitors have an unsubstituted phosphono group linked to either an unsubstituted carboxyl group, as for foscarnet, or to another phosphono group, as for hypophosphate. Phosphonoacetate and analogues having a longer carbon chain length did not inhibit the enzyme reaction. However, some variations around the methylene position of phosphonoacetate or the corresponding diphosphonate are possible as carbonyldiphosphonate, imidodiphosphonate, and 2-hydroxyphosphonoacetate inhibited AMV reverse transcriptase activity, although at higher concentrations.

The preferential inhibition of reverse transcriptase by foscarnet is in agreement with previous reports in which reverse transcriptases of various origin were inhibited at concentrations not inhibitory to cellular DNA polymerases [9, 10, 12, 29]. Modak et al. [16] recently reported foscarnet to equally affect AMV reverse transcriptase and cellular DNA polymerase α reactions, directed by activated DNA. A 50% inhibition was found at a concentration of 55 μM. Our investigation on the effect of foscarnet on the α-polymerase gave similar results. We observed, however, a difference in susceptibility to foscarnet (Table 2) with AMV reverse transcriptase being the most susceptible enzyme. Furthermore, the reaction directed by the other deoxyribonucleotide polymer, $(dC)_n \cdot (dG)_{12-18}$, was also inhibited at lower concentrations than reported by Modak et al. Our results on the inhibition of AMV reverse transcriptase activity by foscarnet using $(rA)_n \cdot (dT)_{12-18}$ or $(rC)_n \cdot (dG)_{12-18}$ as template–primers are consistent with the results of Modak et al. [16]. The reasons for the observed discrepancies are not clear but might be due to different reaction conditions.

The existence of a strong preferential inhibition of some reverse transcriptases by foscarnet is evident from our results and strengthened by the following observations: i) replication of visna virus is inhibited by more than 90% by 100 μM foscarnet, and ii) at this concentration no effect is observed on cell growth [25, 26, 28].

The mechanism of inhibition by foscarnet was previously reported to be linear noncompetitive with $(rA)_n \cdot (dT)_{12-18}$ as template–primer and the corresponding deoxyribonucleotide triphosphate as the varied substrate [24]. This was confirmed in the present investigation and further extended to reactions directed by $(rC)_n \cdot (dG)_{12-18}$, $(dC)_n \cdot (dG)_{12-18}$, and activated DNA. Previous reports on the action of foscarnet show the same inhibition patterns on herpes simplex virus DNA polymerase [9], cytomegalovirus DNA polymerase [10], and hepatitis B virus DNA polymerase [15].

The mechanism of inhibition by carbonyldiphosphonate and 2-hydroxyphosphonoacetate was also found to be linear non-competitive, with dTTP as varied substrate. On the other hand, both hypophosphate and imidodiphosphonate gave a non-linear competitive type of inhibition. In this investigation the latter two inhibitors were examined under reaction conditions containing such low levels of substrates that deviations from linearity were clearly visible in the double reciprocal plots. The fact that structurally related compounds gave rise to different inhibition patterns was strengthened by the comparison of the effects of inhibitors in the four different reverse transcriptase reactions (Fig. 4). The inhibitory effects by one set of compounds (pyrophosphate, hypophosphate, and imidodiphosphonate) were highly dependent on the concentrations of deoxyribonucleoside triphosphates used whereas foscarnet, carbonyldiphosphonate, and 2-hydroxyphosphonoacetate were not. Our results also indicated that $(rA)_n \cdot (dT)_{12-18}$ and activated DNA directed synthesis were preferentially and about equally affected by the non-competitive inhibitors whereas the substrate dependent inhibitors (especially imidodiphosphonate) preferentially affected the $(dC)_n \cdot (dG)_{12-18}$ and activated DNA directed reactions. The reason for the difference in susceptibility between the synthetic template–primers is unclear.

The various pyrophosphate analogues were supposed to mimic parts of the triphosphate moiety of the deoxyribonucleoside triphosphate substrates. Despite the competitive mode of inhibition of hypophosphate and imidodiphosphonate it seems unlikely to expect these compounds to compete directly with the deoxyribonucleoside triphosphates for the substrate binding site of the enzyme. If, however, a binding of inhibitor to a site other than the substrate binding site should produce an enzyme complex with reduced affinity for its substrate(s) without affecting the rate of breakdown of enzyme–substrate complex, then this mode of inhibition will be competitive [21]. Such an inhibition will reach a maximum when all enzyme molecules are complexed to the inhibitor. A further increase in inhibitor concentration will not result in higher inhibition values. This is representative for a partially (or hyperbolic) competitive inhibition. Studies are in progress to determine whether this actually is the case for hypophosphate, imidodiphosphonate and pyrophosphate.

Large differences in K_m values of AMV reverse transcriptase depending on the template–primer and substrate(s) were found (Table 4). This suggests that the reverse transcriptase could utilize some deoxyribonucleoside triphosphate(s) more efficiently depending on the template–primer and these effects would be especially pronounced in reactions containing limited amounts of substrate(s).

A preincubation of enzyme with either pyrophosphate or phosphonoacetate could not protect the enzyme from inhibition by foscarnet. The inhibition of reverse transcriptase by foscarnet could not be increased by preincubating enzyme and inhibitor.

Neither foscarnet nor pyrophosphate inhibits the RNase H activity of AMV DNA polymerase [13, 23] and evidence supports the hypothesis that the active site for RNase H and template binding may be the same [14, 15]. As there obviously exist differences in the mechanisms of inhibition by pyrophosphate analogues, it seems reasonable to

expect not only the pyrophosphate binding site but also other site(s) to be involved. Although foscarnet seems to have an optimal structural requirement for inhibition, the other inhibitors found may be of importance in the further characterization of other catalytic reactions mediated by AMV reverse transcriptase.

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