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OBSERVATIONS ON THE PHOSPHONOFORMIC ACID INHIBITION OF RNA DEPENDENT DNA POLYMERASES

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ABSTRACT: A critical analysis of phosphonoformic acid inhibition of enzymatic DNA synthesis revealed it to be both enzyme and template specific but that retroviral DNA polymerases were not selectively inhibited. AMV reverse transcriptase and cellular DNA polymerase α showed identical sensitivity while RLV reverse transcriptase, DNA polymerase β, E. coli DNA polymerase I and terminal deoxynucleotidyl transferase exhibited significant resistance. The sensitivity of individual enzyme activities was strongly dependent upon the type of template primer used to measure the synthesis. The inhibitory effect of phosphonoformic acid was reversible and was characterized to be noncompetitive with substrate triphosphate although increasing concentrations of substrate triphosphates protected against phosphonoformic acid inhibition. The target of phosphonoformicacid action appears to be at the substrate level and probably at the PPi binding site which results in interference with substrate binding and/or phosphodiester bond formation.

INTRODUCTION: A study of site-specific reagents provides a convenient means of analyzing certain stages of complicated process of enzymatic DNA synthesis. We have recently identified two site-specific reagents, pyridoxal 5'phosphate and phenylglyoxal, that react with substrate and template binding sites of reverse transcriptase* (1-3). As a continuation of these analyses (1-5), we have examined the effects of phosphonoformic acid (PFA), for its possible site specific effects. In this communication we report, for the first time, a detailed survey of the effects of PFA on the purified retroviral and cellular DNA polymerases as well as its probable mode of inhibition. Contrary to previous reports (6-8), we did not find this reagent to be specific for inhibition of oncornaviral enzymes and furthermore the sensitivity of individual enzyme depended upon the template primer used to assay DNA synthesis.

MATERIALS AND METHODS: Sodium salt of phosphonoformic acid was a product of Astra Lakemedal, Sweden and was kindly donated by Drs. Modak and Fox of College of Phys. and Surgeons of Columbia University, New York. Radioactive deoxyribonucleotides were purchased from New England Nuclear while template

* Abbreviations used: RT, reverse transcriptase; PFA, phosphonoformic acid; AMV, Avian Myeloblastosis virus; RLV, Rauscher murine leukemia virus; DTT, dithiothreitol

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primers and non radioactive substrates were obtained from P.L. Biochemicals Inc. Activated calf thymus DNA and globin mRNA.oligo $(dT)_{10}$ were generously donated by Dr. Stuart Marcus. Rauscher leukemia virus and purified AMV reverse transciptase were provided by the Division of Cancer Cause and Prevention, National Cancer Institute. Poly rC-agarose chromatography was employed for the purification of RLV DNA polymerase (9) while purification of DNA polymerase β and terminal deoxynucleotidyl transferase is essentially as described before (10). E. coli DNA polymerase I was a generous gift of Dr. L. Loeb of University of Washington, Seattle, Wash. while purified DNA polymerase α from mouse thymus was kindly donated by Dr.S. Marcus of this institute.

DNA polymerase, RNAse H and Terminal Transferase assays: Assay conditions for various enzymatic activities have been described before (1-5,10,11).

Agarose gel electrophoresis; DNA product isolated from mRNA directed reaction was analysed on 1.6% agarose gels under alkaline condition as described by Modak and Beard (12).

Results:

Effect of PFA on various DNA polymerases: PFA has been reported to be a specific inhibitor of viral DNA polymerases (7-8) and therefore, we examined the sensitivity of various retroviral and non-viral DNA polymerases to this agent. Activated DNA was chosen as a template primer in this study due to the fact that synthetic template primers conventionally used for RT assays are not used by all the enzymes tested. Furthermore, comparative study of this nature was considered to be more meaningful when DNA synthesis, catalysed under the direction of a natural template primer, such as activated DNA, was employed to measure inhibitory effects of the drug. Of the various DNA polymerases tested, only AMV RT and mammalian DNA polymerase α were sensitive to PFA at low concentrations, while RLV RT, E. coli DNA polymerase I, DNA polymerase β and

Table 1: Differential susceptibility of DNA directed DNA synthesis catalysed by various DNA polymerizing enzymes

Enzyme	PFA concentration required for 50% inhibition
AMV reverse Transcriptase	55 μM
RLV reverse Transcriptase	> 500 μM
E. coli DNA polymerase I	> 300 μM
DNA polymerase α	55 μM
DNA polymerase β	> 500 μM
Terminal Deoxynucleotidyl transferase	> 500 μM

A dose response study for each enzyme was carried out using activated DNA as a template primer and concentration of PFA required for 50% inhibition was determined.

terminal deoxynucleotidyl transferase required nearly 10 fold higher inhibitor concentration (Table 1). Further studies to analyze PFA mediated inhibition of DNA synthesis was carried out using AMV and RLV reverse transcriptases.

Effect of PFA on the DNA synthesis directed by various template primers: It is apparent from the result summarized in Table 2 that synthesis directed by Poly(rA).$(dT)_{12-18}$ is the most sensitive to PFA inhibition whereas the inhibition of synthesis directed by Poly (rC). $(dG)_{12-18}$, Poly (dC).$(dG)_{12-18}$, as well as activated DNA and globin mRNA required several fold higher concentration of PFA. This difference is most pronounced with RLV enzyme catalysed synthesis.

Effect of PFA on the polymerization and RNAse H activity and mechanism of inhibition: A typical dose response of PFA on the polymerization and RNase H activity associated with AMV and RLV reverse transcriptase is shown in Fig. 1. It is clear that RNase H activity is totally insensitive to PFA addition while polymerization reaction monitored by Poly(rA).(dT) directed synthesis appeared quite sensitive to PFA inhibition. Since RNase H expression has been thought to involve template binding site of reverse transcriptase (4,15,16), we conclude that PFA mediated inhibition is not effected through its action on the template binding site.

Since the polymerization reaction is also known to require the participation of a substrate binding site, intrinsic zinc and sulfhydryl groups present on the enzyme, interaction of any one of these components with PFA may be responsible for the observed inhibition. Pretreatment of enzyme with PFA

Table 2: Inhibition of AMV and RLV DNA polymerase by PFA in the presence of various template primer

Template primer	μ M concentration of PFA required for 50% inhibition of	
	AMV polymerase	RLV polymerase
$Poly(rA.dT)_{10}$	10	2
$Poly(rC.dG)_{12-18}$	100	> 300
$Poly(dC.dG)_{12-18}$	100	> 300
Activated DNA	55	> 500
Globin mRNA.dT_{10}	60	> 300

A dose response of PFA on AMV and RLV DNA polymerase activity was determined with each one of the listed template primer and concentration required for 50% inhibition was computed.

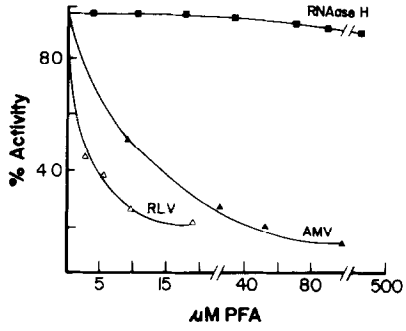

Figure 1: Response of AMV and RLV polymerase to increasing PFA concentration: Both AMV and RLV DNA polymerase were assayed using poly (rA).$(dT)_{12-18}$ as a template primer and Ø x 174 DNA: RNA hybrid as RNase H substrate. One hundred percent activity corresponds to 50 pmol of TTP polymerized and 10 pmol of nucleotide released from hybrid for nuclease assay.

in the presence or absence of reducing agent such as DTT or an equimolar concentration of zinc ions, did not alter the degree of inhibition (Table 3). However, increasing the substrate (but not the template) concentration did protect RLV RT activity from PFA inhibition to some degree (Table 4). Furthermore the protective effect was restricted only to substrate triphosphate

Table 3: Effect of preincubation of AMV and RLV DNA polymerases with PFA.

Additions		% inhibition	
In preincubation mix	In reaction mix	AMV Pol.	RLV Pol
-	50 μMPFA	70	95
-	100 μM PFA	90	98
50 μM PFA	-	20	40
100 μM PFA	-	45	85
500 μM PFA	-	73	96
-	10 μM Zn^{2+}	-	15
-	10 μM Zn^{2+} plus 10 μM PFA	-	83
1-5 mM DTT + 10 μM PFA	-	42	81
10 μM PFA	5 mM DTT	40	79

Additions in the preincubations were carried out in the final volume of 20 μl and preincubation was for 15 min at 37°C. Reaction was started by addition of 80 μl of reaction components.

Table 4: Effect of increasing substrate and template concentration on the PFA inhibition of RLV DNA polymerase.

Addition	3H-dTMP incorporated		% Inhibition
	Control	+PFA (10 μM)	
20 μM TTP	16700	3370	79
100 μM TTP	7265	2425	65
300 μM TTP	4875	2260	53
500 μM TTP	3348	1862	44
500 μM dGTP	16200	4050	75
500 μM dCTP	15300	3824	75
1 μg rA.dT	16850	4885	71
5 μg.rA. dT	17800	4805	

Standard assay mixture contained desired amount of substrate or template.

(Table 4). The kinetic analysis of PFA inhibition revealed that it was non-competitive withrespect to substrate triphosphate concentration (data not shown).

Effect of other substrate site specific reagents on PFA inhibition: Since PFA appears to interfere in the process of substrate binding, effects of other substrate site specific reagents on the inhibition by PFA was examined. The reagents used were pyrophosphate(ppi) and pyridoxal 5'phosphate both of which have been shown to inhibit polymerization but not RNAse H activity catalysed by reverse transcriptases (5,13). In the presence of either of these reagents, inhibitory action of PFA is significantly retarded (Fig. 2). In a typical dose response curve both AMV and RLV polymerase required significantly greater concentrations of PFA for 50% inhibition(shown by dotted line in Fig. 2) in the presence of PPi or pyridoxal phosphate. These results further support the contention that PFA inhibition is at the level of substrate binding, and is probably similar to that effected by pyrophosphate.

Effect of PFA on the size of DNA product: Presence of pyrophosphate in an RNA directed reaction has been reported to increase the size of the cDNA product presumably due to an inhibition of nuclease activity (5,14) since PFA inhibition appears to be similar in nature to that of PPi, the size of the DNA product synthesized in the presence of PFA was determined. Using globin mRNA (9S) as a template with oligo dT_{10} as a primer, a heteropolymeric product was synthesized in the presence and absence of PFA, electrophoresis of the product on alkaline gels revealed that the size of synthesized cDNA

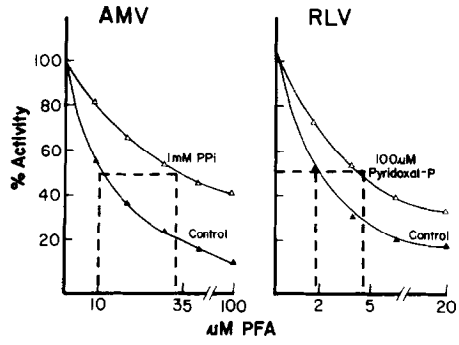

Figure 2: Effect of pyrophosphate and pyridoxal 5'phosphate addition on the PFA inhibition of AMV and RLV DNA polymerases. Dose response studies with increasing PFA concentration was carried out in the presence of 1 mM PPi for AMV enzyme or 100 μM Pyridoxal 5'phosphate for RLV polymerase. One hundred percent activity for control was 48 pmol for AMV and 32 pmol for RLV enzyme.

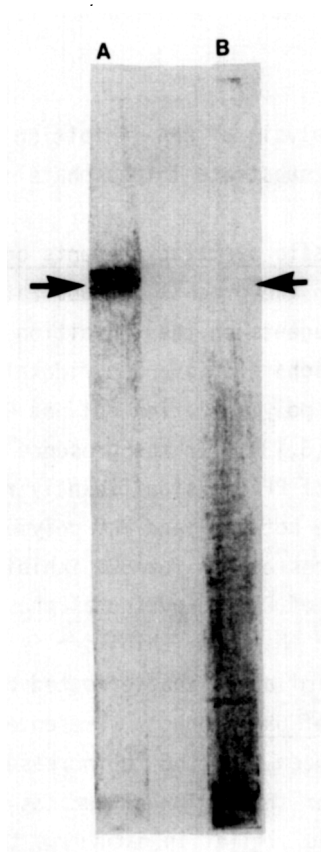

Figure 3: Agarose gel electrophoresis of the DNA product made using globin mRNA.oligo dT as a template and (^{3}H) dGTP as a substrate in an AMV DNA polymerase catalyzed reaction that contained PFA (Lane B). Lane A represents product synthesized in the absence of inhibitor. Approximately, 6,000 cpm were loaded on each gel. Arrow indicates the position of 450 nucleotide marker.

in the presence of PFA was quite heterogeneous, compared with a single band of full length product synthesized in the absence of PFA (Figure 3).

Discussion:

Effectiveness of PFA as an antiviral agent has been indicated in a diverse experimental viral infections and nucleic acid replicating enzymes of the viruses have been suggested as a target for PFA action (6-8). Our investigation of mechanism of PFA action was primarily motivated by its possible site-specific effects on reverse transcriptase enzyme. PFA inhibition was found to be quite reversible and no preference for inhibition between the 2 viral or other non viral enzymes could be detected. However, the inhibition appeared to be both enzyme and template specific with respect to the concentrations of PFA required for 50% inhibition of synthesis (Table 1 and 2). Studies on the mechanism of PFA action using AMV and RLV DNA polymerases revealed that PFA had no effect on the expression of RNase H activity of tumor viral enzymes (Fig 1). Since RNase H activity associated with RTs appears to be expressed through the template binding site (4,15,16),we conclude that PFA does not affect binding of the enzyme to a template. Inhibition of polymerization reaction, therefore, appeared to be in the domain of substrate binding and/or elongation process. Several structural feactures of AMV RT have been identified as essential for this process. For example, in addition to a substrate binding site, presence of active sulfhydryl groups and intrinsic zinc has been known to be required for substrate binding and elongation (3). Preincubation of PFA and enzyme in the presence or absence of a reducing agent (DTT) had no protective effect nor could the supplementation of zinc ions reverse or protect the enzyme from PFA inhibition (Table 3). The product size analysis of globin cDNA synthesized with AMV RT in the presence and absence of PFA indicated that the size of the DNA product in the presence of drug was significantly reduced in contrast to a rather homogenous size class produced in the absence of PFA (Fig 3). It therefore, appears that synthesis of DNA in the presence of PFA may occur via distributive mode, a switch from normal processive mode, as a result of premature dissociation of polymerase during the synthesis, a situation analogous to that reported for adriamycin action (17). The action of PFA on the substrate binding process by AMV RT was suggested by the fact that substrate triphosphate could at least partially protect and reverse the inhibitory effect of PFA (Table 4). Kinetic analyses of the inhibitory effects revealed a non competitive mode with respect to substrate triphosphate which is very similar to that observed with PPi (unpublished observations). Indeed, in the presence of PPi as well as pyridoxal phosphate, a substrate binding site directed reagent (1), sensitivity of DNA sy-

nthesis to PFA is significnatly reduced suggesting a common site for their action. Recently, Reno et al (18) have reported that PFA is a competitive inhibitor of PPi exchange activity of AMV RT. The basis for the differential inhibition of DNA synthesis directed by various template primer to PFA inhibition is not clearly understood. But it is noteworthy that similar differences in the susceptibility of various enzymes have also been noted with pyridoxal 5'phosphate (1) and pyrophosphate (unpublished observations).

Finally, the relative insensitivity of RLV RT and some cellular polymerases and the sensitivity of both AMV and cellular DNA polymerase α to PFA, together with the fact that PFA is a reversible inhibitor, strongly suggests that PFA may not be a replicase directed inhibitor and that an alternate target for its antiviral activity may exist.

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References:

1) Modak, M.J.(1976) Biochemistry 15, 3620 - 3626.
2) Srivastava, A. and Modak, M.J. (1980) J. Biol. Chem. 255, 2000 - 2004.
3) Srivastava, A. and Modak, M.J. (1980) J. Biol. Chem. 255, 917 - 921.
4) Modak, M.J. and Srivastava, A. (1979) J. Biol. Chem. 254, 4756 - 4759.
5) Srivastava, A. and Modak, M.J. (1979) Biochem. Biophys. Res. Comm. 91,892-899.
6) Sundquist, B. and Larner, E. (1977) J. Virol. 30, 847 - 851.
7) Helgstrand, E. et al. (1978) Science 201, 819 - 821.
8) Sundquist, B. and Oberg, B. (1980) J. Gen. Virology 45, 273 - 281.
9) Modak, M.J. and Marcus, S.L. (1977) J. Biol. Chem. 252, 11 - 19.
10) Modak, M.J. (1979) Biochemistry 18, 2679 - 2684.
11) Modak, M.J., Bhatt, H., Seidner, S., Hahn, E.C., Gupta, S. and Good, R.A. (1978) Biochem. Biophys. Res. Commun. 83, 266 - 273.
12) Modak, S.P. and Beard, P. (1980) Nucleic Acids Res. 8: 2665 - 2678.
13) Modak, M.J. (1976) Biochem. Biophys. Res. Commun. 71, 180 - 187.
14) Myers, J.C. and Speigelman, S. (1978) Proc. Natl. Acad. Sci. 75, 5329 - 5333.
15) Modak, M.J. and Marcus, S.L. (1977) J. Virol. 22, 243 - 246.
16) Marcus, S.L., Smith, S.W. and Modak, M.J. (1978) J. Virol. 27, 576 - 581.
17) Matson, S.W., Fay, P.J. and Bambara, R.A. (1980). Biochemistry 19, 2089-2096.
18) Reno, J.M., Kung, H, and Boezi, J.A. (1980) Fed. Proc. 39, 1956.