Phosphonoformate Inhibits Reverse Transcriptase

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SUMMARY

The new antiviral substance phosphonoformate (PFA) has been tested in a cell-free system for its effect on reverse transcriptases from an avian retrovirus (avian myeloblastosis virus, AMV) and from mammalian retroviruses (Rauscher leukaemia virus, RMuLV; bovine leukaemia virus; baboon endogenous virus; simian sarcoma virus; visna virus). The observed inhibitory effect of PFA has been compared with that of a structurally related substance, phosphonoacetate (PAA). Phosphonoformate, at a concentration of 100 μM, reduced the activities of all the above mentioned polymerases by 90% when (rA)n.(dT)10 was used as a template/primer. The dose-response curves for AMV and RMuLV polymerases primed with (rA)n.(dT)10 showed PFA to be a 1000-fold more active than PAA; the RMuLV polymerase activity was reduced to 50% after incubation with 0.7 μM-PFA and 0.7 mM-PAA, respectively. There was no difference in PFA inhibition of virus-associated and purified reverse transcriptase activity. Results with various synthetic templates showed that both the RNA- and the DNA-dependent polymerase activities of reverse transcriptase were inhibited by PFA. The endogenous polymerase activity of AMV was inhibited to 50% at 100 μM-PFA, while PAA had no effect. The PFA inhibition was dependent on whether Mg2+ or Mn2+ was used as divalent cation in the assay. Phosphonoformate arrested DNA synthesis immediately after being added to the assay system. The mechanism of inhibition of the AMV polymerase was non-competitive with respect to substrate and template and the apparent inhibition constants were 16 μM and 9 μM, respectively.

INTRODUCTION

Ever since the discovery of reverse transcriptase (Baltimore, 1970; Temin & Mizutani, 1970), efforts have been made to find compounds that specifically interfere with the activity of this enzyme (for review see Chandra et al. 1977). So far, the compounds that have been described have not been very active nor selective either as inhibitors of reverse transcriptase or for therapy of retrovirus-induced tumours in animals (Chirigos & Papas, 1975; Shannon, 1976; Chandra et al. 1977; Verma, 1977; Wehrli, 1977). One of these substances, phosphonoacetate (PAA), inhibits both Gross murine leukaemia virus replication in mouse cells (Shannon, 1976) and reverse transcriptase released from the lymphoid leukaemia cell line, L 1210 (Allaudeen & Bertino, 1978). However, using the PAA concentration range (350 μM to 1500 μM) necessary to inhibit reverse transcriptase, it is apparent that PAA will also affect cellular polymerases and host cell proliferation (Shannon, 1976; Allaudeen & Bertino, 1978; Helgstrand et al. 1978; Stenberg & Larsson, 1978).

A related compound, phosphonoformate (Fig. 1) was recently found to inhibit herpesvirus DNA polymerase and influenza virus RNA polymerase activities at concentrations not inhibitory for cell proliferation (Helgstrand et al. 1978; Reno et al. 1978; Stenberg & Larsson, 1978). As part of an investigation to determine the effect of PFA on different
polymerases, a number of reverse transcriptases acting on different template/primers have been tested with PFA and the results are described in this report. Also a comparison of the effects of PFA and PAA is presented.

**METHODS**

*Viruses and enzymes.* Rauscher murine leukaemia virus (RMuLV), simian sarcoma virus (SSV) and baboon endogenous virus (BaEV) were generously provided by Dr K. Nilsson (Wallenberg Laboratory, University of Uppsala). Bovine leukaemia virus (BLV) was produced in permanently BLV-infected foetal lamb kidney cells (van der Maaten et al. 1974). Generous supplies of avian myeloblastosis virus (AMV) and purified reverse transcriptase from AMV were obtained through the Office of Resources and Logistics, National Cancer Institute, from Life Sciences Inc., Florida. Visna virus (VV) strain L154 was kindly provided by Dr G. Pétursson (University of Iceland). Visna virus was produced in monolayer cell cultures derived from sheep choroid plexus which were grown in medium 199 supplemented with antibiotics, glutamine and 10 to 15% lamb serum.

Tissue culture medium from virus-producing cells was clarified by low-speed centrifugation. Virus was sedimented from the supernatant at 85,000 g for 90 min and the pellets were suspended in TEN buffer (50 mm-tris-HCl, pH 7.5, 0.15 M-NaCl, 0.001 M-EDTA) and banded twice in 10 to 50% linear sucrose gradients. Fractions at a density of 1.15 to 1.16 g/ml were pooled and diluted to less than 10% sucrose. Virus was again pelleted, dissolved in TEN buffer and stored at -70 °C until use. Virus was diluted in 0.05 M-tris-HCl, pH 8.3, and incubated for 10 min at 0 °C in 0.03% Triton X-100, 10 mm-dithiothreitol to activate the reverse transcriptase and then used in the enzyme assay.

*Chemicals and template/primers.* Trisodium phosphonoformate and phosphonoacetic acid were prepared according to Nylén (1924), and were used as the sodium salts in solutions adjusted to pH 7.2. Methyl-3H-dTTP (60 Ci/mmol) and 3H-dGTP (9 Ci/mmol) were purchased from New England Nuclear (Dreieichenhain, Germany) and unlabelled triphosphates were from Sigma Chemical Company (St Louis, Mo., U.S.A.). Template/primers, (rA)n.(dT)10, (rC)n.(dG)12-18 and (dC)n.(dG)12-18 were obtained from PL-Biochemical Inc. (Milwaukee, Wisc., U.S.A.).

*Assay conditions.* DNA polymerase activity was assayed in 100 µl reaction mixtures. Assays with synthetic template/primers were performed in 50 mm-tris-HCl, pH 8.3; 80
Phosphonoformate inhibits reverse transcriptase

Table 1. Inhibition of various reverse transcriptase activities by PFA and PAA

<table>
<thead>
<tr>
<th>Enzyme source*</th>
<th>Template/primer</th>
<th>³H-dTMP incorporated without inhibitor (ct/min)</th>
<th>% inhibition</th>
<th>μM PFA</th>
<th>μM PAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMuLV</td>
<td>(rA)₀(rT)₁₀</td>
<td>162 660</td>
<td>10 100 500</td>
<td>10 100 500</td>
<td></td>
</tr>
<tr>
<td>VV</td>
<td>(rA)₀(rT)₁₀</td>
<td>68 85</td>
<td>ND 95 99</td>
<td>ND 3 25</td>
<td></td>
</tr>
<tr>
<td>BLV</td>
<td>(rA)₀(rT)₁₀</td>
<td>10 70</td>
<td>ND 83 93</td>
<td>ND 10 0</td>
<td></td>
</tr>
<tr>
<td>BaEV</td>
<td>(rA)₀(rT)₁₀</td>
<td>46 600</td>
<td>ND 96 &gt;99 &gt;99</td>
<td>5 58 71</td>
<td></td>
</tr>
<tr>
<td>SSV</td>
<td>(rA)₀(rT)₁₀</td>
<td>60 860</td>
<td>ND 92 &gt;99 &gt;99</td>
<td>0 44 58</td>
<td></td>
</tr>
<tr>
<td>AMV</td>
<td>(rA)₀(rT)₁₀</td>
<td>72 700</td>
<td>ND 43 94 99</td>
<td>0 34 86</td>
<td></td>
</tr>
<tr>
<td>AMV, purified enzyme</td>
<td>(rA)₀(rT)₁₀</td>
<td>43 340</td>
<td>ND 15 49 70</td>
<td>0 10 0</td>
<td></td>
</tr>
<tr>
<td>AMV, purified enzyme</td>
<td>(rA)₀(rT)₁₀</td>
<td>10 6950</td>
<td>ND 45 96 &gt;99</td>
<td>0 0 19</td>
<td></td>
</tr>
</tbody>
</table>

* The RMuLV, BaEV, SSV, BLV and AMV virion polymerases were assayed at a final concentration of about 150 μg/ml and purified AMV polymerase was assayed at 0.1 μg/ml.
† Per cent inhibition is the inhibition by PFA and PAA in comparison with a control without drug. Negative values indicate stimulation over the control value.
‡ Not determined.

mm-KCl; 10 mm-dithiothreitol; 0.05% Triton X-100; 250 μg/ml bovine serum albumin (Sigma). Unless otherwise indicated, 6 mm-MgCl₂ was used in the assay with AMV, BLV, VV and 0.2 mm-MnCl₂ in the assay with RMuLV, BaEV and SSV. With (rA)₀(rT)₁₀ as template/primer, 1 μM-dTTP, 0.4 μM-methyl-³H-dTTP (50 Ci/mmol) was included in the reaction mixture. With (rC)₀(rG)₁₂₋₁₅ and (dC)₀(rG)₁₂₋₁₅ as template/primer 1 μM-dGTP, 2 μM-³H-dGTP (9 Ci/mmol) was used. Fifty μg/ml of synthetic template/primer was added. Enzyme assays containing only endogenous AMV virus nucleic acid as template/primer were performed in 50 mm-tris-HCl, pH 8.3; 25 mm-KCl; 2 mm-MgCl₂; 0.01% Triton X-100; 2 mm-dithiothreitol; 200 μmol each of dGTP, dCTP and dATP; 5 μM dTTP, 2 μM-methyl-³H-dTTP (60 Ci/mmol).

The reaction was initiated by the addition of 10 μl of purified enzyme or detergent treated virus to the reaction mixture and the reaction was stopped after 30 min incubation at 30 °C by the addition of 2.5 ml cold 10% trichloroacetic acid containing 0.05 M-sodium pyrophosphate. After 30 min on ice the precipitates were collected on Millipore nitrocellulose filters, washed, dried and counted in a liquid scintillation spectrometer. The reaction rates were linear for at least 45 min. For evaluation of the apparent inhibition constants, a computer programme to obtain double reciprocal plots was used.

RESULTS

Effect of PFA and PAA on different reverse transcriptases

The inhibitory effect of PFA and PAA on reverse transcriptase activities of detergent-treated retrovirus particles was tested using the synthetic template/primer (rA)₀(rT)₁₀ (Table 1). Addition of 10 μM-PFA decreased the activity of the BaEV, SSV and RMuLV enzymes by 99% while it had a moderate effect (43%) on the AMV enzyme and only a weak effect (15%) on the endogenous AMV activity. Phosphonoformate, at concentrations of 100 and 500 μM, caused a further reduction in polymerase activity. Again, endogenous AMV activity was less sensitive to PFA inhibition while the activity of detergent treated virions, primed with synthetic templates, was inhibited to a greater extent at the same concentration. The purified enzyme from AMV was inhibited to the same extent as the enzyme from detergent-disrupted virions. At 10 μM, PAA did not inhibit any of the polymerases (Table 1) whilst concentrations of 100 and 500 μM significantly inhibited the enzymes from RMuLV,
Fig. 2. Inhibition by PFA and PAA of reverse transcriptase from AMV and RMuLV. Purified AMV reverse transcriptase (0.1 μg/ml) and detergent treated RMuLV (150 μg/ml) were assayed for DNA synthesis as described in Methods with (rA)ₙ, (dT)₀ as the template/primer. The inhibition is expressed as percentage reverse transcriptase activity remaining at different concentrations of PFA or PAA. The activity was determined as the ΔH-dTMP incorporation during 30 min incubation at 30 °C. In the uninhibited reaction 106300 ct/min were incorporated with the AMV enzyme and 169600 ct/min with the RMuLV enzyme. • • •, AMV with PFA; ○ ○ ○, AMV with PAA; ▲ ▲ ▲, RMuLV with PFA; △ △ △, RMuLV with PAA.

BaEV and SSV. The other enzymes were less inhibited. Table 1 shows that the enzymes most sensitive to PAA inhibition were also most sensitive to PFA.

The DNA synthetic capacity of the AMV enzyme showed an 80% increase in the presence of 500 μM-PAA. The stimulatory effect of PAA was not dependent on the source of AMV enzyme or on the synthetic template used (data not shown). However, it was dependent on the enzyme concentration. This was shown in an experiment where the effect of PAA on AMV was assayed, in a standard reaction mixture with (rA)ₙ, (dT)₁₀ as template/primer at two enzyme concentrations (0.005 μg/ml and 0.1 μg/ml). At the lower enzyme concentration, the enzymic activity was unaffected by PAA at 10, 100, or 1000 μM. However, when the enzyme concentration was increased 20 times to 0.1 μg/ml, PAA showed a stimulatory effect at 100 μM (80%) but not at 10 μM. The inhibition by PFA was not affected by this variation in enzyme concentration.

Fig. 2 shows the dose-response curves for the purified AMV enzyme and the RMuLV virion-associated enzyme when assayed at increasing concentrations of PFA and PAA using (rA)ₙ, (dT)₁₀ as template/primer. Phosphonoformate was an effective inhibitor of both enzyme activities. However, the enzymes were not equally sensitive to PFA; the RMuLV enzyme activity was reduced to 50% at a concentration of 0.7 μM whereas 10 μM was needed to reduce the AMV enzyme activity to the same level. The concentration of PAA which reduced the enzyme activities by 50% was a 1000-fold higher than the concentrations of PFA giving the same inhibition. The dose-response curve for the virion-associated AMV enzyme activity was identical to that of the purified AMV enzyme.

Template/primer dependence for inhibition by PFA

The template dependence for PFA inhibition of reverse transcriptase with two different RNA–DNA hybrids, (rA)ₙ, (dT)₁₀ and (rC)ₙ, (dG)₁₂–₁₈ and one DNA–DNA hybrid, (dC)ₙ, (dG)₁₂–₁₈ is shown in Table 2. The polymerase activities of AMV, RMuLV and BLV were inhibited 90% by 100 μM-PFA using (rA)ₙ, (dT)₁₀ as a template/primer. When (rC)ₙ, (dG)₁₂–₁₈ was used as a template/primer, 100 μM-PFA inhibited the RMuLV and BLV polymerase activities by about 90%, but the AMV polymerase was not inhibited. Using the DNA–DNA template/primer (dC)ₙ, (dG)₁₂–₁₈, the RMuLV polymerase was
Table 2. Inhibition of reverse transcriptase activity stimulated by various template/primers

<table>
<thead>
<tr>
<th>Template/primer</th>
<th>3H-dTMP or 3H-dGMP incorporated without inhibitor (ct/min $\times 10^{-3}$)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMV</td>
<td>RMuLV</td>
</tr>
<tr>
<td>(rA)$<em>n$-(dT)$</em>{10}$</td>
<td>60</td>
<td>21</td>
</tr>
<tr>
<td>(rC)$<em>n$-(dG)$</em>{12-18}$</td>
<td>426</td>
<td>32</td>
</tr>
<tr>
<td>(dC)$<em>n$-(dG)$</em>{12-18}$</td>
<td>211</td>
<td>9</td>
</tr>
</tbody>
</table>

* Assay conditions as in Methods.

Table 3. Effect of Mg$^{2+}$ and Mn$^{2+}$ ions on inhibition of reverse transcriptase activity by PFA and PAA

<table>
<thead>
<tr>
<th>Reaction buffer*</th>
<th>Enzyme</th>
<th>3H-dTMP incorporated without inhibitor (ct/min)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMV</td>
<td>67265</td>
<td>88</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>AMV</td>
<td>67457</td>
<td>43</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>RMuLV</td>
<td>82027</td>
<td>9</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>RMuLV</td>
<td>3110</td>
<td>56</td>
</tr>
</tbody>
</table>

* Virion-associated enzyme activity was tested using (rA)$_n$-(dT)$_{10}$ as template/primer. Standard buffer contained Mg$^{2+}$ or Mn$^{2+}$ as described in Methods.

effectively inhibited by 100 $\mu$M-PFA while the AMV and BLV polymerases were less affected.

In no combination of template/primers and virus polymerase was PAA as inhibitory as PFA. Table 2 demonstrates that 100 $\mu$M-PAA did not decrease the activity of the AMV and BLV polymerases with any of the synthetic template/primers but reduced the RMuLV enzyme activity between 40 and 80%.

**Influence of Mg$^{2+}$ and Mn$^{2+}$ on PFA and PAA inhibition**

The AMV and RMuLV polymerases, with 6 mM-Mg$^{2+}$ or 0.2 mM-Mn$^{2+}$ in the assay mixture, were assayed for inhibition by PFA and PAA as shown in Table 3. The AMV virion-associated enzyme activity was inhibited to a greater extent by 10 $\mu$M-PFA in the presence of Mn$^{2+}$ (88%) than in the presence of Mg$^{2+}$ (43%). The reverse effect was seen for the RMuLV virion enzyme activity. The AMV enzyme was inhibited (73%) by 100 $\mu$M-PAA in the presence of Mn$^{2+}$, but not in the presence of Mg$^{2+}$. The opposite effect was noticed with the RMuLV enzyme: 9% inhibition with Mn$^{2+}$ and 56% inhibition with Mg$^{2+}$ at 100 $\mu$M-PAA.

**Kinetics of inhibition by PFA**

The rate of inhibition of AMV polymerase by PFA was determined by addition of PFA at zero time or 15 min after initiation of polynucleotide synthesis. As seen from Fig. 3, the radioactive substrate was incorporated into the polynucleotide at a linear rate for 60 min. Addition of PFA at zero time resulted in an immediate inhibition of the reaction, without any lag period, but low levels of enzyme activity remained for at least 60 min. The addition of PFA at 15 min after initiation caused an immediate and complete arrest of the enzyme activity.
Mechanism of inhibition

The inhibition by PFA of AMV polymerase activity using (rA)_n.(dT)_10 as template/primer was non-competitive with respect to dTTP concentration as is evident from the Lineweaver-Burk plot in Fig. 4(a). Variation of the (rA)_n.(dT)_10 concentration showed (Fig. 4b) that PFA is also a non-competitive inhibitor with respect to template concentration. The inhibition constants were 16 μM with varying substrate concentration and 9 μM with varying template concentration. Variation of the triphosphate concentrations from 1 to 100 μM did not change the PFA inhibition of the AMV polymerase activity.

DISCUSSION

Our results indicate that PFA is a general inhibitor of reverse transcriptase. As shown in Table 1, it acts on reverse transcriptase not only from an avian virus but also on reverse transcriptase from several mammalian species. In addition, PFA is a very potent inhibitor giving a 50% inhibition at 0.7 μM for the R MuLV reverse transcriptase. As observed by Stenberg & Larsson (1978), a concentration of 1000 μM-PFA was required to reduce cell proliferation and cellular DNA synthesis to 50% in HeLa cells and human lung cells. These results imply that PFA could be used as an inhibitor of reverse transcriptase at concentrations not inhibitory to macromolecular synthesis and cell proliferation.

PAA at 100 μM showed a slight effect with some of the tested enzymes but significant inhibition was not noted until a concentration of 500 μM was reached. Our results are in agreement with those of Allaudeen & Bertino (1978) who reported that reverse transcriptase purified from C particles released by L1210 cells were inhibited 62% by 100 μM-PAA. We have no explanation so far for the stimulatory effect of PAA.
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Fig. 4. (a) Double reciprocal plot of the reaction rate in the presence of different concentrations of dTTP and with PFA as inhibitor. The reaction mixture contained 50 μg/ml of (rA)₅₋₁₀(dT)₁₀, purified AMV polymerase and indicated concentrations of dTTP. The initial velocities were expressed as ct/min of methyl-³H-dTMP incorporated into DNA. PFA concentrations were 0 (○○○○) and 50 μM (●●●●). (b) Double reciprocal plot of the reaction rate in the presence of different concentrations of template/primer and with PFA as inhibitor. The reaction mixture contained 10 μM-dTTP as substrate, purified AMV polymerase and indicated concentrations of (rA)₅₋₁₀(dT)₁₀. The initial velocities were expressed as ct/min of methyl-³H-dTMP incorporated into DNA. PFA concentrations were 0 (○○○○); 10 μM (●●●●) and 50 μM (▲▲▲▲).

Although all the enzymes that were tested were sensitive to PFA at rather low concentrations, it is obvious from Table 1 that some enzymes were more sensitive than others. The RMuLV virion-associated enzyme was ten times more sensitive to PFA than both the AMV purified and virion-associated enzymes (Fig. 2). Despite this difference, both enzymes were still a 1000-fold more sensitive to PFA inhibition than to PAA inhibition. Similar results have been reported with two other polymerases, influenza virus RNA polymerase and hepatitis B DNA polymerase, which were both inhibited by PFA but not by PAA (Helgstrand et al. 1978; Nordenfelt et al. 1978), while, for example, the herpesvirus DNA polymerase is equally well inhibited by PFA and PAA (Helgstrand et al. 1978; Reno et al. 1978; Sabourin et al. 1978).

When we assayed the AMV enzyme activity in the purified form or from detergent disrupted virions, with endogenous and synthetic template/primers, we found that PFA inhibited both the RNA- and DNA-dependent steps of reverse transcriptase. No significant preference for either of the two steps was observed, i.e. templates with ribo- and deoxyribonucleotide compositions were equally sensitive to PFA inhibition (Table 2). This would be expected if the two enzymic activities shared a common active site.

Divalent ions, usually Mg²⁺ or Mn²⁺, are necessary for in vitro DNA synthesis with reverse transcriptase (Verma, 1977). It has been shown that Mg²⁺ or Mn²⁺ can significantly alter the base-stacking properties of the template/primer, thus probably affecting the enzyme activity by altering the conformation of template/primer (Vamvakopoulos et al.
Table 3 shows that these suggested conformational changes could possibly influence the inhibitory effect of PFA or PAA. The AMV enzyme activity was inhibited to a greater extent in the presence of Mn²⁺ than Mg²⁺ while the opposite effect was observed for the RMuLV enzyme activity.

The effectiveness of the PFA inhibition was dependent on the enzymes tested (Table 2); with (rC)ₙ-(dG)₁₂₋₁₈ as template/primer 100 μM-PFA was not inhibitory for the AMV polymerase but the RMuLV and BLV polymerases were inhibited, making it unlikely that PFA acts by binding to the template. This was confirmed by the experiment shown in Fig. 4(b), where it is obvious from the Lineweaver–Burk plot that PFA is a non-competitive inhibitor with respect to the template. This is in agreement with the non-competitive mechanism of inhibition observed for herpesvirus DNA polymerase inhibited by PFA at varied template concentrations (Reno et al. 1978). The constant for PFA inhibition (Kᵢ) of the AMV polymerase was determined at 9 μM. Thus PFA is quite different in its action when compared with non-selective inhibitors which bind to nucleic acids, such as actinomycin, proflavin and daunomycin (Chandra et al. 1977).

Our results (Fig. 3) show that PFA added 15 min after the start of DNA synthesis immediately stopped the reaction. The same result has been reported by Mao & Robishaw (1975) for the PAA inhibition of herpesvirus DNA polymerase. The structural similarity between PFA and PAA makes it likely that both compounds exert their activities in an analogous fashion by interacting at the pyrophosphate-binding site (Leinbach et al. 1976; Reno et al. 1978). The similarity to the inhibition of herpesvirus DNA polymerase by PFA (Reno et al. 1978) was further stressed by the observation that the inhibition of AMV polymerases by PFA was non-competitive with respect to dTTP as variable substrate (Fig. 4a) with a Kᵢ value at 16 μM. The same inhibition by PFA was observed at triphosphate concentrations from 1 to 100 μM.

The inhibition of reverse transcriptase by PFA at low concentrations and a low cellular and animal toxicity (Alenius et al. 1978; Helgstrand et al. 1978; Stenberg & Larsson, 1978; H. Flodh, personal communication) make PFA a potentially valuable compound. It could be used as a tool to analyse the molecular biology of retroviruses and to determine what effect, if any, an inhibitor of reverse transcriptase will have on transformed cells and established tumours. A study of the effect of PFA on retrovirus-infected cell cultures and retrovirus induced animal tumours has been initiated. It has already been established that 20 to 80 μM-PFA reduces visna virus multiplication to 50% in cell cultures while more than 500 μM-PFA was needed to affect cell growth (Sundquist & Larner, 1979).

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