Selective Excision of Chain-terminating Nucleotides by HIV-1 Reverse Transcriptase with Phosphonoformate as Substrate*

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A major mechanism for human immunodeficiency virus 1 (HIV-1) reverse transcriptase (RT) resistance to nucleoside analogs involves the phosphorolyltical removal of the chain-terminating nucleotide from the 3’-end of the primer. In this work, we analyzed the effect of phosphonoformate (PFA) and other pyrophosphate (PPi) analogs on PPi- and ATP-dependent phosphorolysis catalyzed by HIV-1 RT. Our experimental data demonstrated that PFA did not behave as a linear inhibitor but as an alternative substrate, allowing RT to remove AZT from a terminated primer through a PFA-dependent mechanism. Interestingly, in non-terminated primers, PFA was not a substrate for this reaction and competitively inhibited PPi- and ATP-dependent phosphorolysis. In fact, binding of PFA to the RT-template/primer complex was hindered by the presence of a chain terminator at the 3’-end of the primer. Other pyrophosphate analogs, such as phosphonoacetate, were substrates for the excision reaction with both terminated and non-terminated primers, whereas pamidronate, a bisphosphonate that prevents bone resorption, was not a substrate for these reactions and competitively inhibited the phosphorolytic activity of RT. As expected from their mechanisms of action, pamidronate (but not PFA) synergistically inhibits HIV-1 RT in combination with AZT-triphosphate in the presence of PPi or ATP. These results provide new clues about the mechanism of action of PFA and demonstrate that only certain pyrophosphate analogs can enhance the effect of nucleosidic inhibitors by blocking the excision of chain-terminating nucleotides catalyzed by HIV-1 RT. The relevance of these findings in combined chemotherapy is discussed.

Most of the drugs currently used for the clinical treatment of human immunodeficiency virus type 1 (HIV-1) are targeted against the viral reverse transcriptase (RT). Two major classes of RT inhibitors have been identified, nucleoside analogs and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (1, 2). Nucleoside analogs, such as ddC or 3'-azido-3'-deoxythymidine (AZT), are phosphorylated within the cell and compete with natural deoxynucleotides for their incorporation into the growing DNA chain. NNRTIs, such as nevirapine or efavirenz, are a diverse set of compounds that bind to a hydrophobic pocket in close proximity to the active site, inhibiting DNA polymerization.

Although there is no cure available at present, the successful management of patients with AIDS requires the combination of three or more drugs to abrogate viral replication. Most combination therapies for HIV include AZT. Long term therapy with AZT leads to the emergence of resistant viral strains harboring RT with classical zidovudine-associated amino acid substitutions (generally referred to as thymidine analog mutations or TAMs). Mutant reverse transcriptase (RTAZT) is able to remove the incorporated AZT in the presence of ATP (3, 4). Moreover, ATP-dependent phosphorolysis (and probably PPi-dependent phosphorolysis) is also the most frequent mechanism of resistance to other nucleoside analogs such as d4T (5–7). In fact, using combinations of various mutations, it is likely that some mutants of RT will be able to excise most nucleoside analogs with varying efficiencies (8). For this reason, there is an urgent need for new drugs that can inhibit the excision reaction.

Previously, we and others have described that NNRTIs inhibit the excision of the chain-terminating AZTMP catalyzed by HIV-1 RT, resensitizing the AZT-resistant enzyme to this compound (9, 10). Thereafter, it was shown that NNRTIs inhibit the excision of other nucleoside analogs (11) and that this inhibition is responsible for the synergy found in antiviral combinations between AZT and NNRTIs (12). Although the clinical relevance of these findings needs to be established, the inhibition of RT-catalyzed nucleotide excision from a terminated primer seems to be related to the superior long term efficacy of combinations containing a NNRTI over combinations containing only nucleoside analogs. As an example, a phase III trial (13) has shown that a combination of three nucleoside analogs is clearly inferior to other efavirenz-containing treatment regimens.

There are other possible strategies to inhibit the excision reaction. It has been proposed that compounds that interfere with the binding of ATP, or analogs of the dinucleoside tetraphosphate product of the reaction, might be suitable for this purpose (8). Other straightforward candidates are pyrophosphate analogs (1, 12). Phosphonoformate (foscarnet or PFA) is a classic PPi analog that has been shown to inhibit a broad spectrum of DNA polymerases, including herpes viruses and HIV-1
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RT (14). Despite its activity toward HIV-1, PFA is used exclusively to treat opportunistic viral infections such as human cytomegalovirus, acyclovir-resistant herpes simplex, and varicella-zoster virus infections in patients with immunodeficiency. Based on its putative mechanism of action, it was expected that foscarnet would block phosphorylase and therefore would enhance the effect of AZT. Moreover, it has been reported that zidovudine resistance is suppressed by mutations conferring resistance to foscarnet (15, 16), making this an attractive combination for chemotherapy. The combination of AZT with foscarnet in antiviral assays, however, shows low synergy or is merely additive (17–20), and clinical data indicate that even this additive effect is transient (21).

The aim of this work was to analyze whether PFA and some other pyrophosphate derivatives were able to inhibit the RT-catalyzed excision of chain-terminated primers. Our study shows for the first time that PFA is a substrate for phosphorylase and that this compound, unlike other natural or artificial substrates described to date, is not a suitable substrate for the RT-catalyzed nucleotide excision from non-terminated primers. These results not only provide new clues about the mechanism of action of PFA but also support the notion that the inhibition of RT-catalyzed nucleotide excision from terminated primers is a relevant parameter for the interaction between pyrophosphate analogs and chain-terminating nucleotides.

EXPERIMENTAL PROCEDURES

Enzymes and Nucleic Acids—Recombinant p66/p51 wild type (RT) and AZT-resistant HIV-1 RT (RTAZT) were obtained as described previously (10, 12). In the following oligonucleotides, “d” refers to deoxynucleotides and “r” to ribonucleotides: r39 template, 5’-AAAAAUAAGAAGGGUCGACUCUAGAGGUAUCCC-3‘; r39G template, 5’-AAAAAUAAGAAGGGUCGACUCUAGAGGUAUCCC-3‘; d39 template, 5’-AAAAAAAAAAAGAAGGTCGACTCTAGGAGTCCCC-3‘; r36 template, 5’-AAAAAAAAAAAGAAGGTCGACTCTAGGAGTCCCC-3‘; d21 primer, 5’-GGGGATCTTCTGAGTCGACC-3‘; d22T primer, 5’-GGGGATCTTCTGAGTCGACC-3‘; d22 primer, 5’-GGGGATCTTCTGAGTCGACC-3‘; and d22C primer, 5’-GGGGATCTTCTGAGTCGACC-3‘. These and dT20 were purchased from Proligo and purified by PAGE. Poly(rA) and dNTPs were purchased from Amersham Biosciences. AZTMP-, ddCMP-, and ddTMP-terminated d21 primers were obtained from d21 as previously described (10).

Phosphorylase Analogs—Phosphonoacetic acid and potassium pyrosulphate were purchased from Aldrich. Sodium disulphite was purchased from Merck. Methyleneediphosphonic acid, dichloromethyleneediphosphonic acid (disodium salt), sodium phosphonofomate, sodium orthovanadate, and sodium paminodinate (3-amino–1-hydroxy–1-phosphono propyl phosphonic acid, trisodium salt) were purchased from Sigma.

Phosphorylase Assays—To analyze the kinetics of nucleotide removal using PFA or phosphonoacetate as a substrate, d21-AZTMP, d21-ddTMP, d21-ddCMP, and d21 were end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. The labeled primer was annealed to a 3-fold excess of RNA template (r39 or r39G). The annealed template/primer (1 nM) was incubated with 25 nM RT in Buffer A (50 mM Tris-HCl, 1.25 mM EGTA, 0.5 mM EDTA, 0.05% Nonidet P-40, and 10 mM MgCl2 at pH 8) containing 20 mM NaCl in the presence of the indicated amounts of PFA or phosphonoacetate. Reactions were stopped at different times by adding the same volume of loading buffer (90% formamide, 10 mM EDTA, 0.025% bromphenol blue, and 0.025% xylene cyanol), and samples were analyzed by denaturing PAGE as described previously (10).

To measure the inhibition of RT-catalyzed phosphorylase by NNRTIs or pyrophosphate analogs, the annealed template/primer (1 nM) was incubated in a final volume of 50 μl with 25 nM RT in Buffer A containing the indicated amounts of NaCl, inhibitor, and PPi, or ATP. Reactions were stopped by the addition of the same volume of loading buffer, and samples were analyzed by denaturing PAGE (12).

Detection of RT-Template/Primer Complexes with Foscarnet by Mobility Shift Assays—The ability of HIV-1 RT to form a stable complex with the template/primer was assessed as described previously (22). For this purpose, the 5’-32P-labeled d21-AZTMP or d21 primer was annealed to d39 template. The annealed template/primer (4 nM) was incubated for 5 min at 37 °C with PFA in Buffer A containing 20 mM NaCl. The reaction mixture was placed on ice for 5 min, and the putative ternary complexes formed were challenged by the addition of 1.5 μM poly(rA)-(dT)20. After 5 min of incubation, 5 μl of loading buffer (30% glycerol, 0.025% bromphenol blue) were added, and the ternary complexes resistant to this trap were resolved in a non-denaturing 5% polyacrylamide gel.

Inhibition by PFA of DNA Polymerase Activity—RT was incubated in Buffer A containing 100 mM NaCl with the 5’-32P-labeled d21 primer at a concentration of 1 nM annealed to the template (r39 or r36) and 10 μM dNTPs in a final volume of 50 μl. Reactions were quenched at the indicated times by the addition of an equal volume of loading buffer (90% formamide, 10 mM EDTA, 0.025% bromphenol blue, and 0.025% xylene cyanol). The samples were analyzed by denaturing PAGE using 12% polyacrylamide gels containing 7 M urea.

Synergy Analysis—To measure the combined effect of two inhibitors on RT activity, 10 nM RT was added to a mixture prepared in Buffer A containing 100 mM NaCl, 3 nM poly(rA)-(dT)20, 20 μM [α-32P] dTTP and the indicated amounts of the inhibitors with or without 250 μM PPi. After 20 min of incubation at 30 °C, they were quenched by adding 5 μl of 0.5 mM EDTA. Aliquots of 15 μl were spotted onto a DE81 paper (Whatman), washed three times with 0.5 M phosphate buffer (pH 7.5), and then dried and counted. Synergy analysis was done by two approaches, as previously explained (12). Briefly, in a series of experiments, inhibitors were mixed in a constant proportion and interaction indexes calculated for each inhibition level. In the second approach, inhibitor concentrations were mixed in a checkerboard design and data plotted in a Yonetani-Theorell graph (12). In this plot, synergy is detected by lines converging at the left of the y-axis, whereas parallel lines indicate zero interaction (mutually exclusive effects). Diverging lines, i.e., lines converging at the right of the y-axis, indicate antagonism. The absissa value of the intercept point in this plot depends both on the potency of the inhibitor and on the magnitude of the interaction; therefore, the extent of the interaction can be quantified by calculating the −intercept:IC50 ratio (12, 23). For synergistic com-
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RESULTS

Kinetics of PFA-dependent Phosphorolysis—First, we tested the ability of PFA to inhibit the ATP- and PPi-dependent removal of AZTMP from a terminated primer catalyzed by HIV-1 RT. The results obtained led us to the conclusion that PFA did not behave as a linear inhibitor but as an alternative substrate (see below). For this reason, we measured the kinetics of nucleotide excision using PFA as a substrate. Two template/primers were used for this purpose, an AZTMP-terminated template/primer (r39/d21-AZTMP) and a non-terminated template/primer (r39/d21) (Fig. 1). Excision of AZTMP followed apparent first order kinetics with a maximum rate of PFA-lysis ($k_{\text{rem}}$) of 0.0084 $\pm$ 0.0004 min$^{-1}$ and an apparent dissociation constant ($K_d$) of 750 $\pm$ 90 $\mu$M. It is important to note that, with PFA as a substrate, RT selectively catalyzed the removal of AZTMP but did not proceed further (Fig. 1A). Consequently, RT was unable to remove the last nucleotide from d21, even when incubated for 8 h with 2.5 mM PFA (Fig. 1B). The same result was obtained with r39/d22T and with a similar template/primer having dCMP at its 3'-end (r39G/d22C) (data not shown).

To determine whether the lack of phosphorolysis with the non-terminated template/primer was because of a reduced affinity of RT for PFA, we used a gel binding assay. In these experiments, the amount of template/primer detected in complex in the mobility shift assay was measured as a function of the concentration of PFA. As shown in Fig. 2, PFA binds far better to the RT-d39/d21 complex ($K_d = 49 \pm 5 \mu$M) than to the RT-d39/d21-AZTMP complex ($K_d = 730 \pm 80 \mu$M). These results indicated that the inability of RT to excise AZTMP using PFA as a substrate was not a result of PFA binding poorly to RT but to the fact that PFA binds in a different manner to RT, depending on the nature of the 3'-end of the primer.

Inhibition by PFA of RT-catalyzed Phosphorolysis—PFA is a well known inhibitor of cellular and viral DNA polymerases. However, its effect on ATP- or PPi-dependent phosphorolysis catalyzed by HIV-1 RT has not yet been studied. In the absence of dNTPs, RT catalyzed the successive excision of nucleotide residues from the 3'-end of blocked and unblocked primers using PPi as a substrate (Fig. 3). PFA inhibited the PPi-dependent nucleotide excision by HIV-1 RT from r39/d21 (Fig. 3A) or r39G/d22C in the micromolar range. However, this compound does not completely block the PPi-dependent AZTMP (Fig. 3B), ddTMP (Fig. 3C), or ddCMP excision from terminated primers, even in the millimolar range. The apparent inhibition constants ($K_i$) determined from Dixon plots with r39/d21-
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AZTMP, r39/d21-ddTMP, or r39G/d21-ddCMP were > 20 mM (Fig. 4), whereas the apparent $K_{ic}$ values obtained with d21 or d22C primers were 170 ± 10 and 110 ± 29 μM, respectively. Moreover, Dixon plots obtained when the template/primer was blocked with AZTMP or ddTMP showed obvious deviations from linearity, particularly at low PPi concentrations. Non-linearity arose because PFA was a substrate for the phosphorolysis reaction. In fact, the pattern of inhibition found in Fig. 4 corresponds to the expected plot obtained with two alternative substrates when varying the poorer substrate (PFA) at the constancy of the better one (PPi) (24).

Although PFA was unable to completely block the excision of AZTMP, ddTMP, or ddCMP from a terminated primer, the inhibition of the PPi-mediated excision of the second and following nucleotides of the primer is quite efficient. Fig. 4 also shows the inhibition patterns obtained with PFA when the removal of the second nucleotide from AZTMP- or ddTMP-terminated primers was measured. Dixon plots gave inhibition constants of 86 ± 15 and 79 ± 5 μM, respectively. These values are consistent with the $K_{d}$ obtained for the binding of PFA to the RT-d39/d21 complex (49 ± 5 μM) (Fig. 2) and confirm that of the terminated primer because of the PFA-dependent phosphorolysis.

Inhibition of HIV-1 RT-dependent DNA Polymerization by PFA—To go more deeply into the mechanism of action of PFA, we analyzed the inhibition of HIV-1 RT-dependent DNA polymerization by this compound (Fig. 6). In these experiments, a heteropolymeric template was used to restrict the number of nucleotides sequentially incorporated by the enzyme. In the presence of only dTTP, RT incorporated a single nucleotide into the primer. Interestingly, the incorporation of dTMP was not inhibited by millimolar concentrations of PFA (Fig. 6A). In the presence of both dTTP and dCTP, up to 4 nucleotides were incorporated, and the amount of PFA needed to inhibit 50% of the production of full-length DNA decreased to 0.1 mM. If three rather than two deoxynucleotides were present, the enzyme incorporated up to 9 residues.

AZTMP was only slightly inhibited by 1 mM PFA. However, as the concentration of PFA was raised because of the PFA-dependent excision activity of HIV-1 RT, the incorporation of AZTMP decreased, and the IC$_{50}$ value decreased to the low micromolar range. At high concentrations, this compound enhances the rescue both the affinity and the mode of binding of PFA to RT depend on the nature of the last nucleotide of the primer.

It has been described that resistance to AZT displayed by mutant RT (RT<sub>AZT</sub>) is due to the increased ability of this enzyme to unblock the AZTMP-terminated primer in the presence of ATP (3, 4). For this reason, we also analyzed the effect of PFA on ATP-dependent phosphorolysis catalyzed by RT<sub>AZT</sub>. PFA inhibited the ATP-dependent removal of the 3'-terminal nucleotide from d21 primer with an estimated $K_{ic}$ of 37 ± 9 μM (Fig. 5C). Under the same conditions, PFA was unable to completely block the rescue of AZTMP catalyzed by RT<sub>AZT</sub> with ATP as substrate (Fig. 5B). Even more, at 0.5 mM ATP, the amount of d21-AZTMP substrate decreased rather than increased as the concentration of PFA was raised because of the PFA-dependent excision activity of HIV-1 RT. As a consequence, Dixon plots clearly deviate from linearity, particularly at low ATP concentrations (Fig. 5D); therefore, PFA shows a dual effect. At low concentrations, it inhibits the ATP- or PPi-mediated removal of a terminated primer, because PFA is a poorer substrate for RT than PPi or ATP. However, at high concentrations, this compound enhances the rescue process.
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**FIGURE 4. Inhibition of PP₈-dependent phosphorolysis by PFA.** A, Dixon plots obtained for the inhibition of the PP₈-mediated excision of the first (left) and second (right) nucleotides from the 3'-end of d21-AZTMP primer by PFA. Apparent \( K_{ic} \) values estimated by these graphs were >20 mM and 86 \( \mu M \pm 15 \), respectively. Concentrations of PP₈ were 75 (○), 150 (●), 600 (□), 1200 (▲), and 4800 \( \mu M \) (△). B, Dixon plots obtained for the inhibition by PFA of the PP₈-mediated excision of the first (left) and second (right) nucleotides from the 3'-end of d21-ddTMP primer. Apparent \( K_{ic} \) values estimated from these graphs were >20 mM and 79 ± 5 \( \mu M \), respectively. C, scheme showing the mechanism of inhibition of phosphorolysis by PFA. With AZTMP- or ddTMP-terminated primers, PFA did not act as a linear inhibitor but as an alternative (poorer) substrate for HIV-1 RT. With non-terminated or unblocked primers, PFA was not a substrate for phosphorolysis but a competitive inhibitor of this reaction.

From 1 to 15. In addition, polymerized DNA chains shortened as PFA concentrations increased, as happens with chain-terminating nucleotides.

As a control, we also included a NNRTI (4-arylmethylpyridinone) that inhibited both DNA polymerase and phosphorolysis reactions by binding to a site on the RT adjacent to, but distinct from, the catalytic site. This inhibitor clearly showed a different inhibition pattern. Fig. 6B shows that the total amount of polymerized chains (but not their size) was reduced upon the addition of the NNRTI. Moreover, the IC₅₀ value for 4-arylmethylpyridinone was the same irrespective of the number of nucleotides incorporated by the enzyme (Fig. 6C).

**Inhibition of RT-catalyzed Phosphorolysis by Other PP₈ Analogs—**To find a more effective inhibitor of the RT-catalyzed phosphorolysis, we selected a variety of structural PP₈ analogs. Compounds analyzed included phosphonoacetate, orthovanadate, disulfite, pyrosulfate, methylenediphosphonate, dichloromethylene diphosphonate, and pamidronate. From these, only pamidronate and orthovanadate inhibited RT-catalyzed phosphorolysis (Fig. 7A). On the other hand, high concentrations of phosphonoacetate enhanced rather than inhibited phosphorolysis, suggesting that phosphonoacetate was a substrate for this reaction. As shown in Fig. 7B, phosphonoacetate, unlike PFA, is a suitable substrate for RT-catalyzed nucleotide excision from both terminated and non-terminated primers. In fact, the rate of excision (\( k_{obs} \)) for RT in the presence of 2.5 mM phosphonoacetate was about three times greater with a non-terminated template/primer than with a template/primer terminated with AZTMP. Fig. 7C shows the inhibition of PP₈-dependent phosphorolysis by pamidronate. At low (<150 \( \mu M \)) PP₈ concentrations, pamidronate effectively inhibited this reaction at submillimolar concentrations. As PP₈ concentration was raised, inhibition by pamidronate decreased, as expected for a competitive inhibitor (\( K_{ic} = 400 ± 47 \mu M \)). On the other hand, pamidronate was not a suitable substrate for this reaction, even at the higher concentration tested (5 mM).

**Effect of the Combination of AZTTP with PP₈ Analogs on RT Activity—Removal of AZTMP from the terminated primer mediated by the human HIV-1 reverse transcriptase has been found to be a relevant mechanism for the resistance of HIV to AZT. For this reason, we analyzed the effect of the combination of PFA and pamidronate with AZTTP on HIV-1 reverse transcriptase under conditions in which phosphorolysis takes place.** As a control, we included two NNRTIs, efavirenz, and 4-arylmethylpyridinone, which exhibit a strong synergy when combined with AZTTP due to the inhibition of the RT-catalyzed phosphorolysis by the NNRTI (10, 12).

In the absence of ATP or PP₈, the combination of PFA and AZTTP resulted in parallel lines in a Yonetani-Theorell plot, indicating that this combination showed mutually exclusive effects (data not shown). In fact, in the absence of PP₈ or ATP, none of the combinations tested resulted in synergistic inhibition of the RT activity. In the presence of 250 \( \mu M \) PP₈, the combination of PFA with AZT resulted in a moderate amount of synergy. Interaction indexes for this combination were ~0.8, irrespective of the degree of inhibition (Fig. 8). It
is important to note that, for a synergistic combination, it is expected that this index decreases as the total inhibition increases (25). As an example, the combination of AZTTP with either efavirenz or 4-arylmethylpyridinone resulted in interaction indexes that decreased from 0.51 or 0.85 at 50% of inhibition to 0.17 or 0.35 at 95% inhibition, respectively (Fig. 8). The lack of synergy between AZTTP and PFA can be explained by the low potency of PFA as an inhibitor of RT-catalyzed phosphorolysis. As expected, pamidronate synergistically inhibited RT activity when combined with AZTTP in the presence of pyrophosphate. Combination indexes obtained in this case decreased from 0.93 at 50% of inhibition to 0.39 at 95% of inhibition.

A more quantitative parameter for measuring the interaction present in the combination of two inhibitors can be obtained from Yonetani-Theorell plots by determining the intercept:IC_{50} ratio (12, 23). For synergistic combinations, this parameter takes positive values that decrease as synergy increases. Consistent results were obtained by this approach, and values of 2.6, 0.23, 0.09, and 0.08 were found in the presence of 250 μM PPi for the combination of AZTTP with PFA, pamidronate, 4-arylmethylpyridinone, and efavirenz, respectively.

**DISCUSSION**

Although the inhibition of viral DNA polymerases by PFA has been described many years ago (14, 26, 27), the detailed mechanism of action of this compound remains elusive (1, 28). Previous biochemical studies about the mechanism of action of PFA focused on the inhibition of DNA polymerization. These studies lead to the conclusion that PFA does not compete with the incoming nucleotide. On the other hand, it has been consistently found that PFA competitively inhibited the ATP-PPi exchange reaction (26, 29), and PFA has been used as a pyrophosphate analog for product inhibition studies in a variety of DNA polymerases (29, 30). The interaction between foscarnet and pyrophosphate is not completely straightforward, however, because high levels of resistance to foscarnet are usually not associated with high levels of resistance to PPi (14, 26, 28).

In this work, we have analyzed the effect of phosphonofomate and other pyrophosphate analogs on RT catalyzed phosphorolysis. The fact that PFA is a substrate for the RT-catalyzed excision of AZTMP, ddTMP, or ddCTMP from a template/primer or that it binds in a different way. This would imply that either PFA does not bind to the N-form of the complex formed by RT and a non-terminated template/primer or that it binds in a different way. Taking into account that PFA inhibited PPi- and ATP-dependent nucleotide excision in a competitive manner, it can be concluded that PFA probably binds also to the pretranslocational form of the complex, although in a catalytically incompetent form.

As shown in Fig. 2, binding of PFA to the RT-template/primer complex is hindered by the presence of AZTMP at the
primer complex is important for the binding of PFA to the enzyme. It is interesting to note that several of the amino acids that mutated in PFA-resistant enzymes, such as Glu<sup>89</sup>, Asp<sup>113</sup>, Ala<sup>114</sup>, Gln<sup>151</sup>, or Tyr<sup>183</sup>, are the same or are in close proximity to those implicated in the binding of reverse transcriptase to the primer during the excision process (Asp<sup>113</sup>, Tyr<sup>115</sup>, Gln<sup>151</sup>, and Asp<sup>185</sup> in the p66 subunit of RT) (31). Interestingly, the structure of a ternary complex of HIV-1 RT, double-stranded DNA, and a bound dTTP revealed that the 3'-OH of the incoming nucleotide projects toward a small pocket containing a series of RT residues including Asp<sup>113</sup>, Tyr<sup>115</sup>, Phe<sup>116</sup>, and Gln<sup>151</sup> (32), and functional studies (33, 34) show that amino acid Gln<sup>151</sup> directly interacts with the 3'-OH of the incoming deoxynucleotide. It is possible that one or several of these residues may also interact with the terminal 3'-OH of the primer in the N-form of the complex.

On the other hand, the maximum rate for PFA-dependent excision of AZTMP for RT is ~700-fold lower than those obtained with PP<sub>i</sub> (5). Taking into account that the affinity of the RT-template/primer-AZTMP complex for PFA is ~2.4-fold greater than for PP<sub>i</sub>, it may be estimated that PP<sub>i</sub> is ~300-fold more efficient as a substrate than PFA. When compared with ATP, the efficiency of PFA as a substrate for HIV-1 RT is reduced only by 9-fold. However, physiological concentrations of ATP are in the millimolar range, and it seems unlikely to us that pharmacological concentrations of PFA may contribute to AZT excision <i>in vivo</i>, because very high concentrations would be needed to compete with physiological substrates. In any case, the results obtained in this work clearly demonstrated that, even if higher concentrations were reached within the cell, PFA would not be able to completely block the excision of AZTMP from a terminated primer.

The non-competitive mode of inhibition of DNA synthesis by PFA (27) has led some authors to the notion that foscarnet binds to the RT at a site that is distinct from the nucleoside triphosphate binding site. However, the analysis of the inhibi-
although binding studies demonstrate that PFA binds to the catalytic site of RT but to different mechanistic forms of the enzyme in such a way that they do not compete with each other. The notion that PFA binds to the catalytic site is also supported by the displacement of radiolabeled L693-593 (a NNRTI) from its binding site on RT by PFA (35). This displacement would confirm the connection between NNRTI and polymerase binding sites, as demonstrated by presteady-state kinetic studies (36) and by the inhibition of nucleotide excision by NNRTIs (10, 11). Taken together, it can be concluded that the efficiency of PFA as a DNA polymerase inhibitor is not due to the high affinity binding of this compound to the active site of the enzyme but to the fact that this inhibition may occur at each catalytic cycle. This effect will increase the inhibition measured during processive polymerization with long templates, as previously described for chain-terminating nucleotides (25, 37).

It is interesting to note that other pyrophosphate analogs behaved differently. For example, phosphonoacetate, unlike PFA, was a substrate in the phosphorolysis reaction, both with terminated and with non-terminated template/primers (Fig. 7B). As a consequence, RT catalyzed the successive excision of nucleotide residues from the 3’-end of both blocked and unblocked primers using phosphonoacetate as substrate. An extreme case would be hypophosphoric acid, a compound that has been recently reported to be a better substrate for RT-catalyzed excision than pyrophosphate (38). It has been shown that this analog is able to excise dTMP, AZTMP, and dTMP(3’-NH2) with similar efficiency. On the other hand, pamidronate was a weak inhibitor of phosphorylase (Kic = 400 μM), but it is not a substrate for RT and effectively blocked AZTMP excision at submillimolar concentrations. As expected for their mechanisms of action, the combination of AZTTP with pamidronate, but not with PFA, was highly synergistic when tested in the presence of PP, or ATP on purified HIV-1 reverse transcriptase. The low potency of pamidronate as the inhibitor prevented us from testing this combination in viral systems. However, a short report has been published indicating that another bisphosphate, at the low micromolar range and that this compound enhances the effect of AZT both on wild-type and AZT-resistant virus (39). This finding would support the notion that inhibition of phosphorylase is responsible for the synergy found between RT inhibitors and that only certain pyrophosphate analogs would be useful for combined chemotherapy with chain terminating nucleotides.
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FIGURE 8. Effect of the combination of AZTTP with PFA or pamidronate on HIV-1 RT activity. AZTTP and either pamidronate (circles), PFA (squares), or 4-arylmethylpyridinone (triangles) were combined at fixed molar ratios and assayed on HIV-1 RT in the absence (open symbols) or in the presence (closed symbols) of 250 μM PP, interaction indexes calculated for each inhibition level were plotted against the effect of the combination. Interaction indexes in <1 or >1 indicate synergism, no interaction, or antagonism, respectively.

It would be conceivable to generate new AZT analogs consisting of AZTMP attached to the pyrophosphate mimic. Once incorporated by the enzyme, the pyrophosphate analog would be released, inhibiting the phosphorolytic rescue of the terminated primer. However, these derivatives would probably be incorporated less efficiently by RT, and the concentration of the pyrophosphate analog reached within the cell by this mechanism might be insufficient to inhibit the excision reaction, because nucleoside analogs are effective at submicromolar concentrations. In any case, it is clear that the inhibition of phosphorolysis by some pyrophosphate analogs offers new opportunities for the rational design of more effective combination treatments.

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