Inhibition of Phosphorolysis Catalyzed by HIV-1 Reverse Transcriptase Is Responsible for the Synergy Found in Combinations of 3′-Azido-3′-deoxythymidine with Nonnucleoside Inhibitors†

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ABSTRACT: In spite of the growing attention to the combined chemotherapy in the treatment of AIDS, the molecular mechanisms underlying the antiviral synergy of combinations of reverse transcriptase (RT) inhibitors are in most cases unknown. Most combinations of nonnucleoside inhibitors (NNRTI) with nucleoside analogues synergistically inhibit HIV-1 replication in cell culture, though they fail to show synergy in enzymatic assays. In this work we have examined the mechanisms mediating the synergy in combinations of AZTTP with NNRTIs on HIV-1 RT and their possible relevance in antiretroviral therapy. We found that if two inhibitors bind either to different sites on the RT or to the same site but to different mechanistic forms, it is always possible to find conditions in which their combination results in synergistic inhibition of DNA polymerase activity. Though these analyses are interesting from a biochemical point of view, this kind of synergy is unlikely to play any role in vivo, since this positive interaction is lost under the conditions present in viral replication. Here we describe that the synergy found for combinations of NNRTI with AZT is due not to the inhibition of the DNA polymerase activity but to the inhibition of the RT-catalyzed phosphorolysis by the NNRTI. While phosphorolytical removal of the AZT-terminated primer has been related to the mechanism of resistance toward AZT, our data suggest that a basal phosphorolysis occurs even with the wild-type enzyme, and that the inhibition of this activity could explain the synergy found in antiviral assays.

The introduction of highly active antiretroviral treatments against the human immunodeficiency virus type 1 (HIV-1)[1] has dramatically decreased the mortality linked to AIDS in developed countries (*1*). Multiple drug combinations have been shown to suppress viral load for longer periods of time when compared to monotherapy, though the long-term success of such treatments is yet to be determined. Most of the drugs currently used for the clinical treatment of HIV-1 are targeted against reverse transcriptase (RT), an essential enzyme for the viral cycle, which is responsible for the conversion of viral RNA to proviral DNA. The inhibitors of this enzyme can be divided into two broad classes: dideoxynucleoside analogues, which inhibit viral replication by acting in their triphosphate forms as chain terminators of DNA synthesis (*2*), and nonnucleoside RT inhibitors (NNRTIs), including a large number of structurally different molecules which bind to a common site on the RT adjacent to, but distinct from, the polymerase active site (*3*, *4*). The antiretroviral agents currently approved by FDA include 8 nucleoside analogues (AZT, ddI, ddC, d4T, 3TC, abacavir, FTC, and tenofovir disoproxil fumarate), 3 NNRTIs (nevirapine, delavirdine, and efavirenz), 7 inhibitors of viral protease (saquinavir, indinavir, ritonavir, nelfinavir, amprenavir, lopinavir, and atazanavir), and 1 inhibitor of viral fusion (T-20). The most commonly used combinations include two or three nucleoside analogues with or without a protease inhibitor or a NNRTI, though multiple combinations are possible. Given the high number of potential combinations of RT inhibitors, understanding the molecular mechanisms that mediates synergy between antiretroviral agents would be invaluable for the rational design of more effective therapies.

This work was intended to analyze the biochemical mechanism mediating the synergy between NNRTIs and AZT on HIV-1 RT. It has been previously shown that most combinations of NNRTIs with nucleosidic inhibitors, such as nevirapine or delarvidine with AZT or ddC, synergistically inhibit HIV-1 replication in cell culture (*5*−*16*), though the same combinations showed no synergy in inhibiting RT

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[1] Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZTMP, AZT-5′-monophosphate; AZTTP, AZT-5′-triphosphate; RT, reverse transcriptase; wt RT, wild-type reverse transcriptase; HIV-1, human immunodeficiency virus type 1; 4-arylmethylpyridinone, 3-dimethylamino-4-(3,5-dimethylbenzyl)-5-ethyl-6-methylpyridin-2(1*H*)-one; NNRTI, nonnucleoside reverse transcriptase inhibitor; PP_i, inorganic pyrophosphate; T/P, template/primer.

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activity in conventional enzymatic assays (*6*, *10*, *17*−*21*). From a biochemical point of view this was unexpected, since both types of inhibitors can bind simultaneously to the enzyme (*22*, *23*) and therefore a positive interaction was predicted. In a previous report we analyzed this discrepancy and found that synergistic inhibition of HIV-1 RT can only be detected under distributive DNA synthesis, i.e., when the potential number of sites of termination in the template is very low (*19*). Under processive DNA synthesis, the effect of the chain terminator is amplified and the combination behaves as if the effects of both inhibitors were mutually exclusive, as usually found in most combination assays. To make things more confusing, other authors have reported that certain NNRTIs, such as efavirenz (*24*), PPO464 (*15*), and some quinazolinone derivatives (*25*, *26*) synergistically inhibit HIV-1 RT when combined with AZTTP under processive DNA synthesis in conventional reverse transcriptase assays. Taken together, these results indicate that there is not a direct correlation between enzymatic and antiviral data, showing that the molecular mechanism mediating synergy in vivo is in fact unknown.

In the present study we analyzed the factors affecting the synergy between NNRTIs and AZT on HIV-1 RT. We tested the interaction between AZTTP and four structurally dissimilar NNRTIs: efavirenz, nevirapine, TIBO 82913, and a compound related both to HEPT and pyridinone, the 3-dimethylamino-4-(3,5-dimethylbenzyl)-5-ethyl-6-methylpyridin-2(1*H*)-one, called 4-arylmethylpyridinone for short (*27*, *28*). Previous work dealing with this subject have only focused on the inhibition of the DNA polymerase activity of HIV-1 RT. The role of phosphorolysis on the interaction between inhibitors was not considered. The first observation about the inhibition of phosphorolysis by NNRTIs was reported by Borkow et al. (*16*). They showed that the nonnucleoside inhibitor UC781 was able to block the PP_i-dependent phosphorolysis catalyzed by AZT-resistant HIV-1 RT. It was also suggested that this inhibition might be a relevant mechanism for the delayed development of resistance to combinations of AZT plus UC781 in antiviral assays. In a previous work we analyzed and extended this observation (*27*). We showed that the inhibition of the phosphorolytical activity catalyzed by wt and AZT-resistant RTs was a general property to most NNRTIs, rather than specific of only UC781. We also demonstrated that this inhibition was responsible for the strong synergy found when combinations of NNRTIs with AZT affected the AZT-resistant enzyme. Recently, Basavapathruni et al. (*29*) reported that NNRTIs inhibited the phosphorolytical removal of several nucleoside analogues and altered the catalytic rate of ATP hydrolysis and its binding constant to RT. In addition these authors postulated that the origin of the synergy between NNRTIs and nucleoside analogues was related to the interplay between their respective binding pockets.

In the present work we make a critical analysis on the factors affecting the interaction between NNRTIs and nucleoside analogues and their possible relevance in combined chemotherapy. We show that synergistic inhibition between NNRTI and AZTTP is consistently found when an excess of enzyme over the template-primer is added, and phosphorolysis is allowed to take place. Our data suggest that the inhibition of a basal phosphorolytical activity of wt RT by NNRTIs may account for the synergy found in antiviral assays. This result makes sense, since phosphorolytic removal from the terminated primer can severely decrease the potency of a chain terminating nucleotide. This work also provides a rationale to understand the results obtained in combinations of RT inhibitors in enzymatic assays, that may contribute to design more effective combination treatments.

EXPERIMENTAL PROCEDURES

Preparation of HIV-1 RT. Recombinant p66/p51 wt and AZT-resistant HIV-1 RTs were obtained as previously described (*27*). Enzyme preparations were homogeneous as judged by gel filtration and SDS−PAGE. The amount of active enzyme, as determined by pre-steady-state burst kinetics of single nucleotide incorporation on an RNA/DNA primer-template, yielded an active site concentration of about 50%, using an absorption molar coefficient of 260450 $M^{-1} \times cm^{-1}$ at 280 nm for the heterodimer (*30*).

Oligonucleotides and Nucleotides. In the following nucleotides, "d" refers to oligodeoxynucleotides and "r" to oligoribonucleotides: r39 template (5′-AAAAAAAAUAAAAGAACAGGUCGACUCUAGAGGAUCCCC-3′), d39 template (5′-AAAAAAAATAAAAGAACAGGTCGACTCTAGAGGATCCCC-3′), d24 template (5′-TGAGGTCGACTCTAGAGGATCCCC-3′), d21 primer (5′-GGGGATCCTCTAGAGTCGACC-3′), and dT_{20} were purchased from Proligo and purified by PAGE. Poly(rA) and dNTPs were purchased from Amersham Pharmacia Biotech. AZT-terminated d21 primer was obtained from d21 as previously described (*27*).

DNA Polymerase Activity. When RT activity was tested under distributive DNA synthesis, reactions were prepared in a final volume of 50 μL in buffer A (50 mM Tris-HCl, 1.25 mM EGTA, 0.5 mM EDTA, 0.05% NP40 at pH 8), containing 100 mM NaCl, 8 nM RT, 600 nM d21-d24, 1 μM [α-^{33}P] dTTP, and the indicated concentrations of inhibitors. The reactions were started with 10 mM $MgCl_2$, and after 20 min of incubation at 30 °C, they were quenched by adding 5 μL of 0.5 M EDTA. Aliquots of 15 μL were spotted onto a DE81 paper (Whatman), washed three times with 0.5 M Na_2HPO_4 (pH 7.5), dried, and counted. When RT was tested under processive DNA synthesis, 1 nM RT was added to a mixture prepared in buffer A containing 100 mM NaCl, 500 nM poly(rA)-dT_{20} (with respect to dT_{20}), 20 μM [α-^{33}P] dTTP, and the indicated amounts of NNRTI and AZTTP. Reactions were started, incubated, and processed as before. In some combination experiments RT was tested under processive DNA synthesis, but using an excess of RT over template-primer. In these cases enzyme activity was measured in buffer A containing 100 mM NaCl, 3 nM poly(rA)-dT_{20}, 10 nM RT, and 20 μM [α-^{33}P] dTTP with or without ATP or PP_i in the medium.

Phosphorolysis Assay. To measure the inhibition of RT catalyzed phosphorolysis by NNRTIs, d21-AZTMP was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. After purification, the labeled primer was annealed to a 3-fold excess of RNA template r39. The annealed primer-template (1 nM) was incubated with 25 nM RT in buffer A containing 100 mM NaCl, the indicated amount of NNRTI, and 150 μM PP_i as substrate in a final volume of 50 μL. Reactions

were stopped by addition of the same volume of loading buffer (90% formamide, 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol), and samples were analyzed by denaturing PAGE as previously described (*27*).

To analyze the rate of AZT removal, 5′-^{32}P-labeled d21 AZTMP-terminated primer was annealed to a 3-fold excess of template (r39 or d39). The annealed primer-template (1 nM) was incubated with 25 nM enzyme in buffer A containing 20, 100, or 150 mM NaCl in the presence of 150 μM PP_i or 3 mM ATP. Reactions were stopped at different times by adding the same volume of loading buffer, and samples were processed as before.

Stable Ternary Complex Formation Assays. The ability of HIV-1 RT to form a stable complex with template/primer in the presence of NNRTIs was assessed as described (*31*, *32*). For this purpose the 5′-^{32}P-labeled d21 AZTMP-terminated primer (4 nM) annealed to d39 template was incubated for 5 min at 37 °C in the presence of NNRTIs with RT in buffer A containing 50 mM NaCl and 10 mM $MgCl_2$. 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% bromophenol blue) was added and the ternary complexes resistant to this trap were resolved in a nondenaturing 5% polyacrylamide gel. Apparent K_d for efavirenz was estimated from the fraction of template/primer detected in complex in the mobility shift assay as a function of NNRTI concentration. Data were fitted by nonlinear regression to the quadratic equation describing the binding of a ligand to a single site in the enzyme (*33*). Quantification of the radioactivity and curve fitting were performed with ImageMaster software (Amersham-Pharmacia) and Grafit (Erithacus software), respectively.

Synergy Analysis. To evaluate the interaction between inhibitors two approaches were used. The first relied on isobolographic analysis and was done as previously explained (*19*). Briefly, dose−response curves for each inhibitor alone were obtained within a wide range of effects by fitting experimental data to eq 1 by unweighted nonlinear regression using the commercially available fitting program Grafit (Erithacus software)

$$f = \frac{1}{1 + \left(\frac{IC_{50}}{D}\right)^m} \quad (1)$$

where f is the fractional inhibition, D represents the concentration (dose) of the inhibitor, IC_{50} the concentration of the inhibitor giving 50% of inhibition, and m a parameter giving the sigmoidicity of the dose−response curve. Inhibitors were then combined at a fixed molar ratio, and the interaction between inhibitors was then evaluated by means of the interaction index (I) as defined by Berenbaum (*34*),

$$I = \frac{d_1}{D_1} + \frac{d_2}{D_2} \quad (2)$$

D_1 and D_2 being the concentrations of inhibitors 1 and 2 individually producing the same effect as the combination ($d_1 + d_2$). When $I = 1$, agents in the combination do not interact; if $I > 1$, the combination is antagonistic, and if $I < 1$, the combination is synergistic.

This method, though widely used to evaluate the interaction between RT inhibitors, has some drawbacks. For example, inhibitors must be mixed in an arbitrarily fixed ratio and the interaction index must be calculated for each effect level, so this analysis does not provide a clear quantitative parameter that measures the intensity of the interaction between inhibitors. A better approach to quantify the synergy is the Yonetani and Theorell plot (*35*). In this plot synergistic inhibition is detected by lines converging at the left of the *y*-axis, while parallel lines indicate mutually exclusive effects. Diverging lines (lines crossing at the right of the *y*-axis) indicate antagonism. For synergistic combinations, the abscissa value of the intercept point in the Yonetani−Theorell plot depends both on the potency of the inhibitor and on the magnitude of the interaction, so the extent of the interaction can be quantified by calculating the −intercept/IC_{50} ratio (*36*). This value decreases as synergy increases. Negative values, lines crossing at the right of the *y*-axis, indicate antagonism between inhibitors. Though this method was originally based on kinetic grounds, subsequent analyses demonstrated that it is more generally applicable than previously thought (*36*). In fact, the validity of this method does not depend on any assumption about the mechanism of action of the inhibitors, but only on the shape of the dose response curve, since it is based on isobolographic analysis (*34*). Consequently, both methods of evaluating the synergy for combinations of inhibitors giving hyperbolic dose−response curves must be consistent.

RESULTS

Effect of the Combination of AZTTP and NNRTIs in the Presence of a Saturating Amount of Primer-Template over wt HIV-1 RT. We and others (*6*, *10*, *17*−*21*) have previously described that NNRTIs in combination with chain terminating nucleotides do not show synergy in the inhibition of HIV RT in conventional kinetic assays, i.e., with catalytic amounts of enzyme in processive DNA synthesis. However, we demonstrated that a positive interaction is found when inhibition of polymerization is tested under distributive DNA synthesis (*19*). Figure 1A shows the Yonetani−Theorell plots obtained under these conditions for the combination of AZTTP with nevirapine or efavirenz. In both cases the lines intersect at the left of *y*-axis, which shows that these combinations synergistically inhibit HIV-1 RT. The intensity of the interaction measured by the −intercept/IC_{50} ratio gave values of 1.1 and 1.9 for nevirapine and efavirenz, respectively (Table 1). Two other structurally unrelated NNRTIs, 9-Cl-TIBO and 4-arylmethylpyridinone (*27*), were also assayed with AZTTP. The −intercept/IC_{50} ratios were also close to 1 (1.1 and 1.2, respectively), as expected for the combination of two inhibitors that bind independently to the enzyme. Under the same conditions the combination of AZTTP with ddTTP, two mutually exclusive inhibitors, resulted in parallel lines (data not shown). Consistent results were found when the interaction between inhibitors was assessed by means of the interaction index. As seen in Table 1 synergy was diagnosed for the combination of NNRTIs with AZT when polymerization was tested under distributive DNA synthesis, while mutually exclusive effects were found

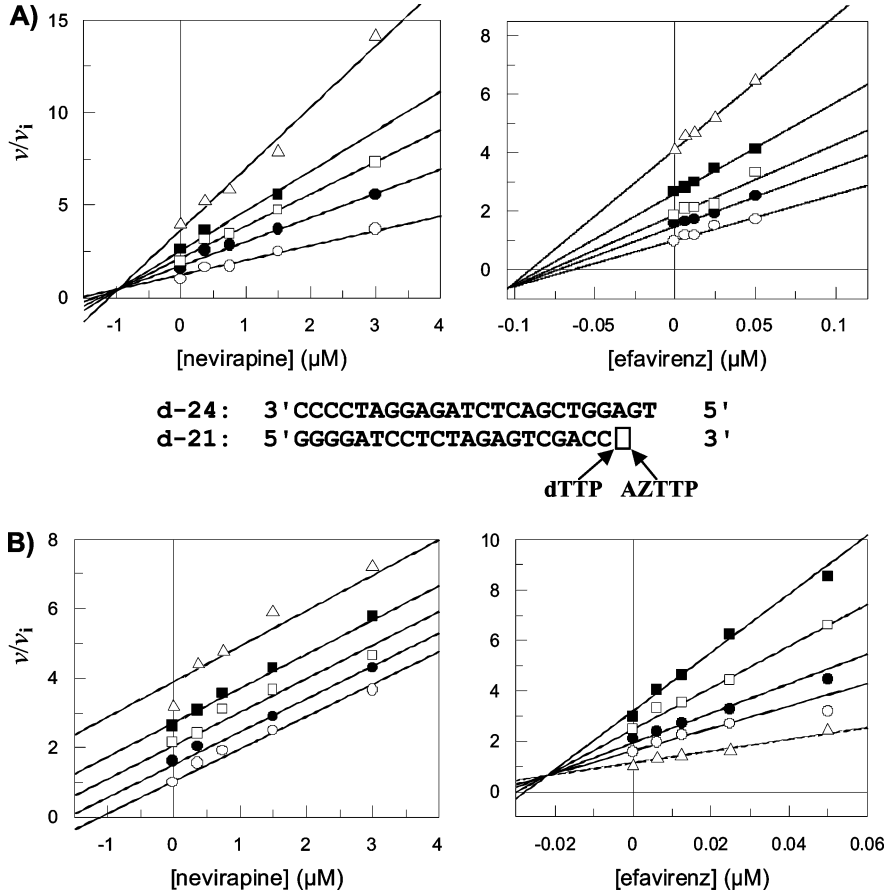

FIGURE 1: Effect of the combination of a NNRTI with AZTTP under distributive or processive DNA synthesis with an excess of primer-template over HIV-1 RT. Synergistic inhibition of RT activity is shown by converging lines at the left of the *y*-axis, while parallel lines indicate mutually exclusive effects. (A) Effect of the combination under distributive DNA synthesis. RT (8 nM) was incubated for 20 min at 30 °C in a mixture containing 600 nM d24-d21, the indicated amount of the NNRTI, and 0 (○), 0.6 (●), 1.25 (□), 2.5 (■), or 5 (△) μM AZTTP. Under these conditions only a single molecule of dTMP can be incorporated into the primer (see scheme). (B) Effect of the combination under processive DNA synthesis. RT (1 nM) was added to a mixture containing 500 nM poly(rA)-dT_{20} (with respect to dT_{20}), 20 μM [α-^{33}P] dTTP, the indicated amount of the NNRTI, and 0 (○), 0.075 (●), 0.15 (□), 0.3 (■), or 0.6 (△) μM AZTTP, and incubated as before.

Table 1: Interaction between AZTTP and NNRTIs under Processive or Distributive DNA Synthesis Using an Excess of Template-Primer over wt HIV-1 RT

DNA polymerization mode[a]	combination: AZTTP +	Yonetani−Theorell plot			interaction index at % inhibition[d]				
		IC_{50} (μM)	intercept[b] (μM)	−intercept/IC_{50}[c]	30	50	70	90	95
distributive	ddTTP	4.2 ± 0.6	−90 ± 22	21 ± 5.3	1.00	1.00	1.01	1.01	1.02
	TIBO	22 ± 1.58	−24 ± 3.5	1.1 ± 0.16	0.69	0.64	0.59	0.53	0.49
	nevirapine	0.85 ± 0.14	−0.97 ± 0.17	1.1 ± 0.19	0.60	0.59	0.58	0.55	0.54
	efavirenz	0.053 ± 0.008	−0.10 ± 0.02	1.9 ± 0.19	1.16	0.98	0.84	0.67	0.60
	4-arylmethylpyridinone	0.037 ± 0.0016	−0.044 ± 0.008	1.2 ± 0.02	0.69	0.66	0.63	0.59	0.58
processive	nevirapine	0.85 ± 0.013	−32 ± 4.2	38 ± 5	0.99	0.98	0.98	0.97	0.98
	efavirenz	0.035 ± 0.031	−0.022 ± 0.003	0.64 ± 0.06	0.80	0.61	0.49	0.42	0.42
	4-arylmethylpyridinone	0.036 ± 0.007	−0.015 ± 0.002	0.42 ± 0.07	0.84	0.74	0.62	0.47	0.40

[a] Assays under distributive and processive DNA synthesis were performed as indicated in the captions of Figure 1A and Figure 1B, respectively. Interactions between inhibitors were assessed both by the Yonetani−Theorell plot and by means of the interaction index (see Experimental Procedures). [b] Abscissa value of the point of intersection in the Yonetani−Theorell plot. [c] This value measures the intensity of the interaction between inhibitors and decreases as synergy increases (see Experimental Procedures). [d] Interaction index (*I*) as defined by Berenbaum (*31*). $I < 1$ indicates synergy, $I = 1$ mutually exclusive effects, and $I > 1$ antagonism. For synergistic combinations this value usually decreases as % of inhibition increases (*19*).

for the combination of AZTTP and ddTTP under the same conditions.

We have previously described that under processive DNA synthesis this favorable interaction is lost, at least for the combination of AZTTP with delarvidine or L-697,661 (*19*). Our results showed that this was also the case with nevirapine (Figure 1B). The combination of AZTTP with nevirapine gave parallel lines in the Yonetani−Theorell plot, indicating that under processive DNA synthesis the effects of nevirapine and AZTTP were mutually exclusive. However, a substantial amount of synergy was measured for the combination of AZTTP with efavirenz or 4-arylmethylpyridinone (Figure 1B and Table 1), resulting in −intercept/IC_{50} values of 0.64 and 0.42, respectively. It is important to note that these results

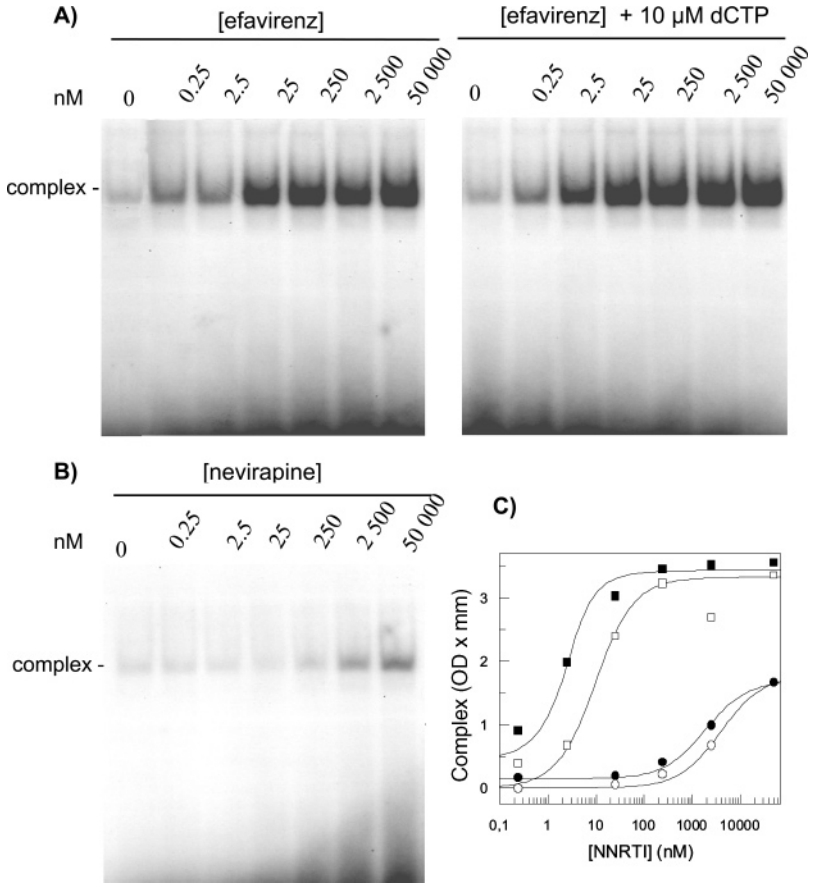

FIGURE 2: Efavirenz preferentially binds to the RT·template-primer complex. Electrophoretic mobility shift assays were carried out to measure the formation of a stable complex between NNRTI, HIV-1 RT and AZT-terminated primer-template. A duplex formed by the 5′-^{32}P-labeled d21-AZTMP-terminated primer (4 nM) annealed to d39 template was incubated with 220 nM wt RT and the indicated concentrations of NNRTIs and then challenged by the addition of 1.5 μM polyrA-dT$_{20}$. (A) Efavirenz in the absence or presence of 10 μM dCTP. Positions of the stable complexes formed are shown on the left. (B) Nevirapine in the absence of dCTP. (C) The amount of template/primer detected in complex in the mobility shift assay is shown as a function of NNRTI concentration. The solid lines represent the best fit of the data to the quadratic equation describing the binding of a ligand to a site in the enzyme (*33*). Efavirenz (□), efavirenz + 10 μM dCTP (■), nevirapine (○), and nevirapine + 10 μM dCTP (●).

were found under nonphysiological conditions, with a catalytic amount of enzyme with respect to primer-template. Under these conditions, the inhibition of the DNA synthesis critically depends on the rate of dissociation of the RT from the AZT-terminated primer-template (*33*, *37*). So we tested whether this synergy could be due to the stabilization of the RT·AZT-terminated primer-template upon binding of these NNRTIs.

Formation of a Stable Complex between NNRTI and HIV-1 RT Bound to AZT-Terminated Template-Primer. We used a gel retardation assay to examine the ability of NNRTIs to induce the formation of a stable complex with HIV-1 RT and the AZT-terminated primer-template. A large excess of RT (220 nM) over terminated primer-template (4 nM) was used in these experiments. A ternary NNRTI·RT·T/P$_{AZTMP}$ complex was detected at low nanomolar concentrations of efavirenz (Figure 2A), even in the presence of a large excess of free enzyme, which shows that efavirenz selectively binds to and stabilizes the RT·template-primer complex. To determine the apparent K_d for efavirenz, the amount of template/primer detected in complex in the mobility shift assay as a function of the concentration of efavirenz was measured by densitometry. The apparent K_d ($K_{d,app}$) cannot be estimated graphically because enzyme concentrations are in the same range as $K_{d,app}$. So data were fitted by nonlinear regression to the quadratic equation describing the binding of a ligand to a single site in the enzyme (*33*). Assuming that, as indicated by gel shift binding assay, efavirenz preferentially binds to the forms of the enzyme attached to the template/primer, the apparent K_d for efavirenz in this assay was 7.3 ± 1.0 nM. This assumption is further supported by the fact that the value of $K_{d,app}$ measured in the same assay with 20 nM RT, i.e., reducing the concentration of free enzyme by 10-fold, was 5.2 ± 0.5 nM (data not shown). These values are consistent with the reported K_i for efavirenz obtained in kinetic studies (*21*, *24*). Affinity of the RT−T/P complex for efavirenz was further increased by the addition of the next complementary nucleotide (Figure 2A). Addition of 10 μM of the next complementary nucleotide (dCTP) increased the affinity of the inhibitor for the complex by 6-fold, reducing the $K_{d,app}$ to 1.1 ± 0.08 nM, due to the formation of a dead-end efavirenz·RT·T/P$_{AZTMP}$·dNTP quaternary complex.

Similar complexes were also detected with nanomolar concentrations of 4-arylmethylpyridinone. Apparent dissociation constants measured in gel shift assays for 4-arylmethylpyridinone in the absence or presence of 10 μM dCTP were 36 ± 5 nM and 1.1 ± 0.04 nM, respectively (data not shown). On the other hand, micromolar concentrations of nevirapine were needed to detect the formation of some

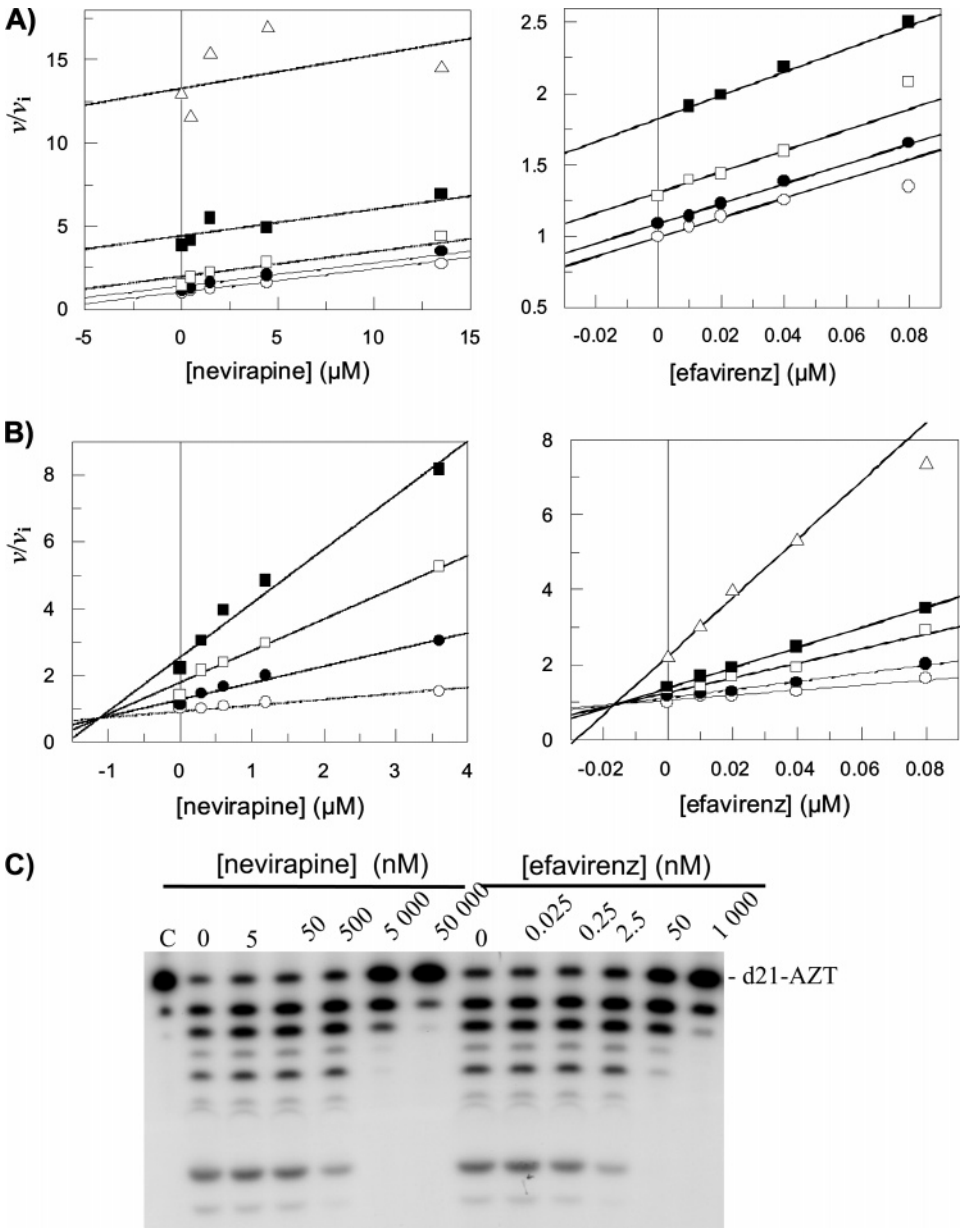

FIGURE 3: Effect of the combination of a NNRTI with AZTTP under processive DNA synthesis with an excess of HIV-1 RT over primer-template. (A) Effect of the combination in the absence of PP_i. RT (10 nM) was incubated for 20 min at 30 °C in a mixture containing 3 nM poly(rA)-dT_{20}, 20 μM [α-^{33}P] dTTP, and the indicated amounts of NNRTI and AZTTP. Concentrations of AZTTP used in the combination with nevirapine were 0 (○), 0.006 (●), 0.02 (□), 0.06 (■), and 0.18 (△) μM, while in the combination with efavirenz they were 0 (○), 0.0125 (●), 0.025 (□), and 0.05 (■) μM. Synergistic inhibition of RT activity is shown by converging lines at the left of the *y*-axis, while parallel lines indicate mutually exclusive effects. (B) Effect of the combination in the presence of 250 μM of PP_i. Concentrations of AZTTP used in the combination with nevirapine were 0 (○), 0.06 (●), 0.125 (□), and 0.25 (■) μM, while in the combination with efavirenz they were 0 (○), 0.05 (●), 0.1 (□), 0.2 (■), and 0.4 (△) μM. (C) Inhibition of the phosphorolytic activity of wt RT by NNRTIs. RT (25 nM) was added to a mixture containing 150 μM PP_i, 1 nM 5′-^{32}P-labeled d21-AZTMP-terminated primer annealed to r39 template and incubated at 37 °C for 60 min as described in Experimental Procedures. Lane C represents a control reaction carried out in the absence of RT. Reactions were then quenched and products were analyzed by denaturing PAGE.

complex (Figure 2B), and addition of 10 μM dCTP was without effect (Figure 2C).

Effect of the Combination of AZTTP and NNRTIs in the Presence of an Excess of HIV-1 RT over Primer-Template. It has been pointed out that an excess of enzyme over genomic RNA is present during viral replication (*38*). Under these conditions the rate of dissociation of the enzyme from the primer-template does not contribute to the inhibition measured, since all primer ends are occupied by RT molecules. When we tested the AZTTP/NNRTI combinations in vitro using an excess of RT over primer-template, none of the combinations showed synergy in the inhibition of the polymerase activity (Figure 3A and Table 2). These results confirmed that the synergy found in Figure 2 with efavirenz and AZTTP was due to the stabilization of the enzyme•template-primer complex by the NNRTI, leaving the question on the actual molecular mechanism mediating the synergy found in antiviral combinations unanswered.

Previous works dealing with this subject have centered their attention on the effect on DNA polymerase activity. However, we have recently shown that NNRTIs inhibit the phosphorolytic activity catalyzed by HIV-1 RT (*27*). In addition, we detected a strong antagonism between AZTTP and PP_i (Table 2), due to the PP_i-dependent removal of

Table 2: Effect of PP_i on the Interaction between AZTTP and NNRTIs under Distributive DNA Synthesis Using an Excess of wt HIV-1 RT over Template-Primer[a]

addition	combination: AZTTP +	Yonetani−Theorell plot: IC_{50} (μM)	Yonetani−Theorell plot: intercept[b] (μM)	Yonetani−Theorell plot: −intercept/IC_{50}[c]	interaction index at % inhibition[d]: 30	50	70	90	95
no addition	PP_i	2270 ± 178	*e*	*e*	4.04	6.65	7.96	14.2	19.9
	ddTTP	0.39 ± 0.02	−4.17 ± 0.89	10.69 ± 1.20	0.85	0.86	1.02	1.18	1.28
	TIBO	10 ± 2.4	−50.2 ± 12.00	4.9 ± 1.2	0.82	0.69	0.74	0.88	0.98
	nevirapine	7.55 ± 1.00	−148 ± 5.00	20 ± 0.7	0.73	0.73	0.76	0.87	0.95
	4-arylmethylpyridinone	0.32 ± 0.066	−37.4 ± 12	117 ± 37	0.81	0.84	0.87	0.82	0.95
	efavirenz	0.14 ± 0.02	−0.64 ± 0.13	4.6 ± 0.91	1.14	1.17	1.15	1.16	1.17
+ 250 μM PP_i	ddTTP	0.71 ± 0.035	−1.38 ± 0.9	1.94 ± 1.2	0.87	0.87	0.87	0.87	0.88
	TIBO	32 ± 9.8	−2.45 ± 0.7	0.1 ± 0.01	0.47	0.45	0.44	0.43	0.43
	nevirapine	7.35 ± 0.59	−1.14 ± 0.17	0.15 ± 0.02	0.63	0.55	0.48	0.38	0.34
	4-arylmethylpyridinone	0.33 ± 0.15	−0.028 ± 0.004	0.085 ± 0.01	1.09	0.85	0.66	0.45	0.36
	efavirenz	0.14 ± 0.021	−0.017 ± 0.001	0.12 ± 0.009	0.88	0.65	0.49	0.31	0.24

[a] Assays with or without PP_i were performed as indicated in the captions of Figure 3A and Figure 3B, respectively. Interactions between inhibitors were assessed both by the Yonetani−Theorell plot and by means of the interaction index (see Experimental Procedures). [b−d] See footnotes below Table 1. [e] The intercept point for this combination cannot be calculated, because total inhibition decreased as [PP_i] increased in the range tested (100 to 800 μM), as a consequence of the strong antagonism present, resulting in a nonlinear Yonetani−Theorell plot.

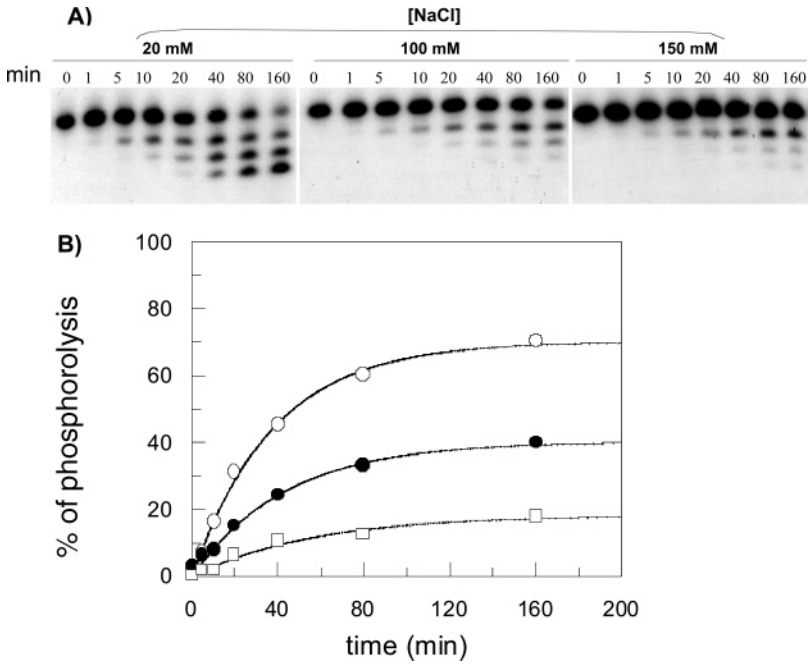

FIGURE 4: Effect of salt concentration on the rate of ATP-dependent phosphorolysis catalyzed by AZT-resistant HIV-1 RT. (A) ATP-dependent removal of AZT-terminated primer by AZT-resistant RT. The 5′-^{32}P-labeled d21 AZTMP-terminated primer (1 nM) annealed to r39 template was incubated with 25 nM RT in buffer A containing 20, 100, or 150 mM NaCl, in the presence of 3 mM ATP. Aliquots were taken at different reaction times, and the products were analyzed by electrophoresis. (B) Time courses of ATP-dependent removal catalyzed by AZT-resistant RT in buffer A containing 20 (○), 100 (●), and 150 (□) mM NaCl. The solid lines represent the best fit obtained by nonlinear regression of the data to equation $A(1 - e^{-kt}) + C$, where A is the amplitude of the burst, k is the apparent first-order rate constant of AZT excision, t is the reaction time, and C is a constant.

AZTTP from the AZTMP-terminated primer. So we decided to test the effect of PP_i on the interaction between AZTTP and NNRTIs. In the presence of PP_i the combinations of AZTTP with TIBO, nevirapine, 4-arylmethylpyridinone, or efavirenz were highly synergistic (Figure 3B and Table 2), resulting in −intercept/IC_{50} ratios of about 0.1. It is interesting to note that in the presence of PP_i the IC_{50} for AZT increased about 10-fold, from 31 nM to 330 nM, suggesting that the origin of the interaction was the inhibition by the NNRTI of the RT-catalyzed pyrophosphorolysis. In fact, a closer inspection of the Yonetani−Theorell plots shows that this strong synergy is detected at NNRTI concentrations that barely affected the DNA polymerase activity of HIV-1 RT (see Figure 3B), but effectively inhibited the excision of AZT from the terminated primer (Figure 3C).

Kinetics of ATP- and PP_i-Dependent Phosphorolysis. While ATP-dependent phosphorolysis has been implicated in the mechanism of resistance to AZT, a functional role of AZT excision catalyzed by wt HIV-1 RT has not been considered. According to our results the inhibition of this activity seems to be implicated in the synergy found in antiviral assays for combinations of AZT and NNRTIs. For this reason we analyzed the factors affecting excision of AZT from the terminated primer catalyzed by wt or AZT-resistant HIV-1 RTs. Figure 4 shows the effect of salt concentration on the kinetics of ATP-dependent phosphorolysis catalyzed

Table 3: Effect of Salt Concentration on AZT Excision Catalyzed by wt and AZT-Resistant HIV-1 RTs [a]

		AZT-resistant RT				wt RT			
		150 μM PP_i		3 mM ATP		150 μM PP_i		3 mM ATP	
primer-template	[NaCl] (mM)	k[b] (min^{-1})	amplitude[b] (%)	k (min^{-1})	amplitude (%)	k (min^{-1})	amplitude (%)	k (min^{-1})	amplitude (%)
d21/r39	20	0.070 ± 0.005	77	0.027 ± 0.001	70	0.12 ± 0.01	78	0.009 ± 0.002	50
	100	0.029 ± 0.002	54	0.023 ± 0.002	40	0.049 ± 0.005	62	*c*	10
	150	0.028 ± 0.006	36	0.018 ± 0.006	18	0.049 ± 0.007	37	*c*	4
d21/d39	20	0.18 ± 0.025	75	0.055 ± 0.004	71	0.37 ± 0.061	82	0.01 ± 0.001	44
	100	0.072 ± 0.009	66	0.024 ± 0.002	47	0.11 ± 0.005	73	*c*	7
	150	0.020 ± 0.003	31	*c*	12	0.028 ± 0.002	41	*c*	2

[a] AZT excision was measured as explained in Figure 4 using either wt RT or AZT-resistant RT in the presence of ATP and PP_i. [b] Values obtained by nonlinear regression of the data to equation $A(1 - e^{-kt}) + C$, where A is the amplitude of the burst, k is the apparent first-order rate constant of AZT excision, t is the reaction time, and C is a constant. [c] Not determined. The kinetic parameter could not be accurately determined because of insufficient amplitude.

by AZT-resistant HIV-1 RT on a DNA-RNA primer-template. In our hands, the rate of excision and the maximum amount of primer repaired critically depended on the salt concentration used. The maximum amount of primer repaired in the presence of 3 mM ATP with 20, 100, and 150 mM NaCl was 70%, 40%, and 18%, respectively, and the rate of removal of AZT was also reduced. Table 3 shows the results obtained with wt and AZT-resistant RTs. Pyrophosphorolysis of the AZTMP-terminated primer was a more efficient process for wt RT than for AZT-resistant RT, though both enzymes efficiently removed AZT from the terminated primer at physiological salt concentrations on DNA and RNA templates. The rate of excision was slightly higher for DNA than for RNA-dependent phosphorolysis. On the other hand ATP-dependent excision of AZT from terminated primers catalyzed by wt RT was a rather inefficient process, particularly at physiological salt concentrations (Table 3).

Effect of the Nature of the Pyrophosphate Donor on the Interaction between AZTTP and NNRTI. We tested whether it was possible to obtain synergy between NNRTIs and AZTTP using ATP as pyrophosphate donor. The effect obtained by changing the nature of the pyrophosphate donor cannot be derived only from AZT excision rates, because ATP-lysis is sensitive to the presence of the next incoming nucleotide (*39*, *40*). Figure 5A shows the interaction indexes obtained at each inhibition level for the combination of nevirapine and AZTTP on wt RT in the absence and presence of 3 mM ATP or 250 μM PP_i. Interaction indexes obtained at 90% of inhibition without or with ATP or PP_i in the medium were 1.19, 0.42, and 0.14, respectively. Similar data were found for efavirenz (0.93, 0.63, and 0.39) and for 4-arylmethylpyridinone (0.89, 0.51, and 0.17). In each case the synergy obtained with PP_i exceeded those obtained with ATP, and was proportional to the loss of sensitivity to AZTTP due to the presence of these pyrophosphate donors. It is interesting to note that the intensity of the interaction increased by extending the time of incubation. For example, interaction indexes obtained at 90% of inhibition on wt RT for the combination of AZTTP and nevirapine with 3 mM ATP after 20, 60, and 120 min of incubation decreased from 0.61, to 0.54 and 0.24, respectively. Under the same conditions the IC_{50} for AZTTP increased from 0.083, to 0.11 and 0.28 μM, respectively. These data support the notion that the origin of this interaction is due to the inhibition of the phosphorolytical activity catalyzed by wt RT.

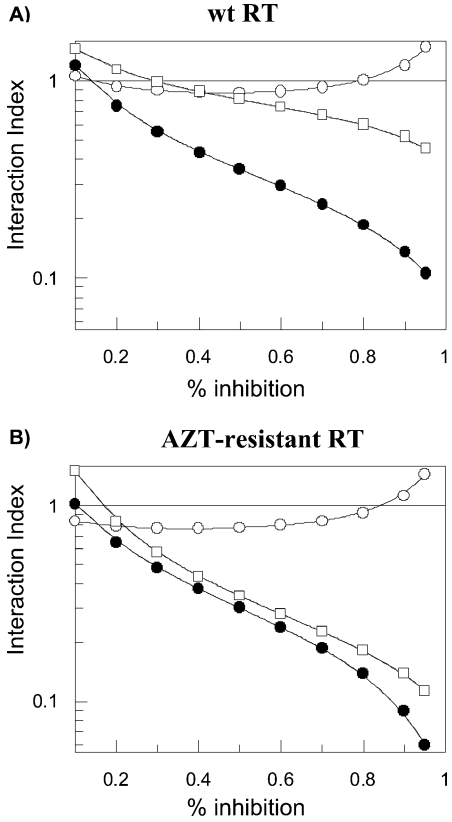

FIGURE 5: Effect of the nature of pyrophosphate donor on the interaction between nevirapine and AZTTP on wt and AZT-resistant HIV-1 RTs. AZTTP and nevirapine were combined at fixed molar ratios and assayed on wt and AZT-resistant HIV-1 RT. Interaction indexes calculated for each inhibition level were plotted against the effect on the combination. Enzyme activity was measured in buffer A containing 100 mM NaCl, 3 nM poly(rA)-dT_{20}, 20 μM [α-^{33}P] dTTP, and 10 nM wt RT without (○) or with 250 μM PP_i (●) or 3 mM ATP (□). Reactions were started with 10 mM $MgCl_2$, and incubated for 60 min at 30 °C. (A) Effect of the combinations on wt HIV-1 RT. IC_{50} values for AZTTP on wt RT measured without or with ATP or PP_i were 0.056, 0.13, and 1.0 μM, respectively. (B) Effect of the combinations on AZT-resistant HIV-1 RT. IC_{50} values for AZTTP measured without or with ATP or PP_i were 0.06, 0.81, and 1.19 μM, respectively. Interaction indexes <1, $=1$, or >1 indicate synergism, no interaction, or antagonism, respectively.

We then compared these results to those obtained with the AZT-resistant enzyme (Figure 5B). Interaction indexes obtained at 90% of inhibition for the combination of AZTTP and nevirapine in the absence and presence of 3 mM ATP or 250 μM PP_i in the reaction medium were 1.1, 0.14, and

0.09, respectively. Similar values were found for the combination of AZTTP with efavirenz (1.05, 0.41, and 0.44) or 4-arylmethylpyridinone (1.04, 0.20, and 0.22). It is important to mention that in all cases the amount of synergy found in combinations containing ATP was greater for the AZT-resistant than for the wt RT, in agreement with the ATP-dependent rates of excision of AZT presented in Table 3.

DISCUSSION

While many combinations of RT inhibitors synergistically inhibit HIV replication (*5*−*16*), most of them usually fail to show synergy when tested in vitro on purified HIV-1 RT (*6*, *10*, *17*−*21*). Several groups have evidenced synergy for combinations of RT inhibitors, but only under selected conditions (*15*, *24*−*26*, *41*, *42*). In our opinion these results must be critically reviewed in order to distinguish whether the synergy found is related to parameters that, affecting the inhibition measured in enzymatic assays, are irrelevant to viral replication. In this work we demonstrate that if two inhibitors of HIV-1 RT bind either to different sites on the enzyme or to the same site but to different mechanistic forms, it is always possible to find conditions in which their combination results in synergistic inhibition of the DNA polymerase activity. While this information is interesting from a biochemical point of view, this interaction is doubtful to be relevant in vivo. For example, the combination of a NNRTI with a chain terminator gives rise to synergy if RT is forced to polymerize under distributive conditions (Figure 1A). However, this kind of synergy is unlikely to play any role in vivo, since in most cases this positive interaction is lost under normal processive conditions (Figure 1B).

In addition there are some potent NNRTIs which synergistically inhibit polymerase activity under normal processive conditions when combined with a chain terminator (*24*, *42*−*44*). This is the case for the combination of AZTTP with efavirenz (Figure 1B) or 4-arylmethylpyridinone (Table 1), and it seems to be the case for other combinations described in the bibliography with PPO-464 (*15*), and some quinazolinone derivatives (*25*). According to our results, the synergy observed in combinations of these NNRTIs with AZT with catalytic amounts of enzyme is due to the accumulation of the NNRTI·RT·T/P_{AZTMP} ternary complex, which in turn can be converted to a dead-end NNRTI·RT·T/P_{AZTMP}·dNTP quaternary complex with a low dissociation rate. This work shows for the first time that some NNRTIs can induce the formation of a stable complex with the RT bound to template-primer, as judged by gel retardation assays (Figure 2). These data are consistent with kinetic studies showing that efavirenz, PPO-464, or 4-arylmethylpyridinone selectively bind to these forms of the enzyme (*15*, *24*, *27*). While dissociation rate is a critical factor under conventional steady-state conditions ([RT] ≪ [T/P]) (*33*, *37*), it seems to be irrelevant in vivo, where an excess of enzyme over template-primer is present (*38*). Consequently this kind of synergy disappears when combinations are tested under these conditions (Figure 3A).

As far as we know, nobody has found synergy in the combination of a nucleoside analogue with a NNRTI under processive DNA polymerization with an excess of RT over template-primer. It is important to note that this is just the expected result for these combinations if the mechanisms of action of these inhibitors are taken into account. According to the present work, the synergy found in antiviral assays for combinations of AZT and NNRTIs seems to be more related to the inhibition of the HIV-1 RT-dependent phosphorolytical activity caused by the NNRTI than to their combined effect on the polymerase activity. We consistently found synergy between NNRTIs and AZTTP as long as phosphorolysis is allowed to take place, i.e., when an excess of enzyme over the template-primer is added, and ATP or PP_i is present as substrate for this reaction. Both conditions are fulfilled in vivo where an excess of RT over genomic RNA exists (*38*), and intracellular levels of ATP and PP_i are in the range used in this work.

Our experimental evidence demonstrates that the inhibition of RT catalyzed phosphorolysis is indeed the actual mechanism that mediates the synergy found. First, the degree of synergy obtained is proportional to the loss of AZTTP sensitivity due to the presence of PP_i or ATP (Figure 5), in agreement with the hypothesis. In fact, it is possible to increase the synergy obtained by increasing reaction times or by increasing the concentration of ATP or PP_i, allowing a more efficient rescue of the AZT-terminated primer. Second, the synergy is easily detected at concentrations of the NNRTI that barely inhibit the polymerase activity (Figure 3B), but effectively block the phosphorolysis (Figure 3C). This is consistent with the observation that, under the conditions used in combination assays, phosphorolysis is more sensitive to inhibition by NNRTIs than DNA polymerase activity (*27*). It is important to note that NNRTIs block phosphorolysis and polymerization by binding to different starting complexes. Third, the results obtained in combination studies on wt and AZT-resistant RT clearly showed that the inhibition of RT catalyzed phophorolysis was implicated in the synergy found. Combinations on wt RT are far more effective in the presence of 150 μM PP_i than in the presence of 3 mM ATP (Figure 5), as expected by the rate of phosphorolysis with both substrates (Table 3). In addition, the synergy detected in combinations containing ATP is higher for AZT-resistant enzyme than for wt RT, as one would predict by analyzing the rate of phosphorolysis of both enzymes with these substrates.

While ATP-dependent phosphorolysis has been implicated in the mechanism of resistance to AZTTP, the role of PP_i in this removal is controversial. Both wt and AZT-resistant RTs efficiently excise AZT from a terminated primer/template using PP_i as substrate donor. However, low rates of excision are found with wt RT using physiological concentrations of ATP, whereas the enzyme carrying the four mutations shows an increase in this activity by at least 10-fold (*39*, *40*, *45*−*47*). The difference between wt and resistant RTs points at ATP as the possible substrate responsible for the rescue of the terminated primer by the AZT-resistant enzyme. Anyway, this does not rule out the possibility that PP_i may also act as a substrate for this reaction. To date only indirect evidence about the in vivo role of ATP and PP_i is available. Detailed kinetic analyses with ATP and PP_i on the resistant enzyme were performed by Ray et al. (*48*). They concluded that "it is reasonable to believe that both removal substrates are responsible for resistance and perhaps other biochemical properties mediate PP_i's role in AZT resistance". Many details of the role of pyrophosphate in the AZT-resistance

mechanism remain to be clarified, but in our hands physiological concentrations of pyrophosphate can severely decrease the sensitivity of the wt RT to AZTTP. These results might indicate that the inhibition of the PP_i-dependent RT-catalyzed phosphorolysis, though apparently not related to the resistance to AZT, may also play a role in the synergy found between nucleosidic and nonnucleosidic inhibitors with the wt enzyme. The inhibition of phosphorolysis catalyzed by HIV-1 RT by NNRTIs may also help to explain why combinations containing NNRTIs, such as efavirenz, were clearly superior to those including only nucleoside analogues (*27*). Several clinical trials have demonstrated that virologic failure occurred sooner and more often in patients receiving 3 nucleoside analogues than in regimens containing NNRTIs. We think that these studies can be better understood in the light of the results presented in this work.

Recently Basavapathruni et al. (*29*) addressed the inhibition of phosphorolytic removal of chain-terminating nucleotides by NNRTIs with wt HIV-1 RT. Thought they did not make combination studies, their data were consistent with the results presented here and in our previous studies (*27*). However, there is an important point in which we disagree with these authors, namely, on the implications of these results in combination chemotherapy. Basavapathruni et al. postulated that the origin of the synergy between NNRTIs and nucleoside analogues is related to the interplay between their respective binding pockets. Consequently, they concluded that a bifunctional inhibitor combining the functionalities of a nucleoside analogue and NNRTI could bind very tightly and specifically to RT and could be effective in the treatment of AIDS. We found this proposal unlikely. The mechanism of action of nucleoside analogues has been studied in detail (*2*, *49*). These inhibitors exert their function not by direct binding to the enzyme but by acting as chain terminators of DNA polymerization. In fact, competition with natural deoxynucleotides in the absence of chain termination has been found to be irrelevant in the mechanism of action of these compounds. If they are potent inhibitors of viral replication, it is because of the high number of potential sites of chain termination in the viral genome, not because they bind very tightly to the RT (*37*). Given that the structural requirements for a nucleoside analogue and for a NNRTI are very different, joining a chain terminator and a NNRTI in a single molecule would result in a poor substrate for RT. In addition, such a molecule would be poorly phosphorylated within the cell. And most important, several of these chimeras have been synthesized and when tested they have not shown the predicted properties (*50*−*52*).

However, a new strategy for the development of more effective combinations can be conceived. From the result of the present work it is expected that any inhibitor of the phosphorolytic activity, not only NNRTIs, would enhance the effect of several nucleosidic inhibitors, resulting in a more efficient therapy. This is an important point because often a single mutation may cause resistance to a variety of NNRTIs, precluding the inhibition of both the polymerase and the phosphorolytic activities by these compounds. While NNRTIs are effective inhibitors of phosphorolysis, there is a chance that other molecules, such as certain pyrophosphate analogues, were effective in inhibiting this activity. Our results predict that these inhibitors would be synergistic when combined with some chain-terminatingnucleotides, and they might also be useful to overcome the resistance to some drugs, such as AZT or d4T.

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