Phosphorolysis and Resensitize the 3′-deoxythymidine 5′-triphosphate (AZT)-resistant Polymerase to AZT-5′-triphosphate

Received for publication, December 12, 2002, and in revised form, June 6, 2003
Published, JBC Papers in Press, August 13, 2003, DOI 10.1074/jbc.M212673200

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Human immunodeficiency virus, type 1 (HIV-1)1 reverse transcriptase (RT) is responsible for the conversion of single-stranded viral RNA into double-stranded DNA prior to integration into the genome of the host. Numerous compounds that inhibit the DNA polymerase activity of RT have been described. They can be divided into two broad classes. The first group, that of nucleoside analogs, includes dideoxynucleoside compounds, such as 2′,3′-dideoxyxycytidine and AZT, that inhibit viral replication by acting as chain terminators of DNA synthesis (1). The second group, the non-nucleoside reverse transcriptase inhibitors (NNRTI), includes a large number of structurally dissimilar hydrophobic compounds that bind to a site on the RT palm subdomain adjacent to but distinct from the polymerase active site (2).

The FDA-approved HIV-1 therapies involve drugs that inhibit two viral enzymes, reverse transcriptase and protease. AZT was the first drug approved against HIV-1 and is still widely used in combination with other antiretroviral drugs. The prolonged clinical use of this nucleoside analog in monotherapy gives rise to highly resistant viruses containing mutations in the RT enzyme, D67N, K70R, T215F/Y, K219E/Q (3), and, in some cases, M41L and L210W. Viruses carrying at least four mutations are more than 100-fold less sensitive to AZT than wild type strains in cell culture. Although the genotype for AZT resistance is well characterized, it has been impossible to detect resistance to AZT 5′-triphosphate (AZTTP) in polymerization assays using recombinant RTs carrying these mutations. Recently, it has been demonstrated that HIV-1 RT can remove some 3′-terminal chain-terminating residues from blocked DNA using either pyrophosphate or ATP as substrate (4, 5). The prevailing hypothesis nowadays is that mutations conferring resistance to AZT do not interfere with the incorporation of the inhibitor but increase the excision rate of AZTTP from the 3′-end of the nascent DNA (4, 6, 7).

It has been reported that the thiocarboxanilide non-nucleoside inhibitor UC781, in combination with AZTTP, synergistically inhibits the replication of AZT-resistant HIV-1 virus (8). Because UC781 was also able to block the pyrophosphorylolytic reaction, it has been suggested that this inhibition could enhance the effect of AZT and might be a relevant mechanism for the delayed development of resistance to combinations of AZT plus UC781 in antiviral assays. Our work was aimed to find the biochemical mechanism mediating the synergy between AZT and NNRTIs obtained with the AZT-resistant RT. At present it is not known whether the inhibition of the pyrophosphorylolytic activity is specific for UC781, because no other NNRTI has been reported to block this activity. Moreover, the effect of NNRTIs on ATP-dependent phosphorolysis has not been tested. We therefore analyzed the effect of two structurally dissimilar compounds, nevirapine and TIBO 82913 (9, 10), along with a NNRTI related to both HEPT and pyridinone, the

Non-nucleoside Inhibitors of HIV-1 Reverse Transcriptase Inhibit Phosphorolysis and Resensitize the 3′-Azido-3′-deoxythymidine (AZT)-resistant Polymerase to AZT-5′-triphosphate

Removal of 3′-azido-3′-deoxythymidine (AZT) 3′-azido-3′-deoxythymidine 5′-monophosphate (AZTMP) from the terminated primer mediated by the human HIV-1 reverse transcriptase (RT) has been proposed as a relevant mechanism for the resistance of HIV to AZT. Here we compared wild type and AZT-resistant (D67N/K70R/T215Y/K219Q) RTs for their ability to unblock the AZTMP-terminated primer by phosphorolysis in the presence of physiological concentrations of pyrophosphate or ATP. The AZT-resistant enzyme, as it has been previously described, showed an increased ability to unblock the AZTMP-terminated primer by an ATP-dependent mechanism. We found that only mutations in the p66 subunit were responsible for this ability. Moreover, we found that three structurally divergent non-nucleoside reverse transcriptase inhibitor (NNRTI), nevirapine, TIBO, and a 4-arylmethylpyridinone derivative, were able to inhibit the phosphorolytic activity of the enzyme, rendering the AZT-resistant RT sensitive to AZTTP. The 4-arylmethylpyridinone derivative proved to be about 1000-fold more potent in inhibiting phosphorolysis than nevirapine or TIBO. Moreover, combinations of AZTTP with NNRTIs exhibited an exceptionally high degree of synergy in the inhibition of AZTTP in polymerization assays using recombinant RTs carrying these mutations. Recently, it has been demonstrated that HIV-1 RT can remove some 3′-terminal chain-terminating residues from blocked DNA using either pyrophosphate or ATP as substrate (4, 5). The prevailing hypothesis nowadays is that mutations conferring resistance to AZT do not interfere with the incorporation of the inhibitor but increase the excision rate of AZTTP from the 3′-end of the nascent DNA (4, 6, 7).

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* This work was supported by a joint grant from the Gobierno de Navarra and the Conseil Regional d’Aquitaine (Fonds Commun de Coopération Navarra-Aquitaine), the Agence Nationale Française pour la Lutte contre le SIDA, and the Plan de Investigación de la Universidad de Navarra.

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§The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; AZT, 3′-azido-3′-deoxythymidine; AZTMP, 5′-monophosphate; AZTTP, AZT-5′-triphosphate; RT, reverse transcriptase; NNRTI, non-nucleoside reverse transcriptase inhibitor(s); wt, wild type; TIBO, tetrahydroimidazo benzodiazepinone; HEPT, 1-[2-hydroxyethoxy]methyl]-6-phenylthio)thymine.

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chain-terminated primer-template was precipitated with ethanol, resuspended in 90% formamide and 10 mM EDTA, and finally purified by denaturing PAGE using a 12% polyacrylamide gel containing 7 M urea. The bands were visualized on a TLC silica gel plate under UV light, and the band corresponding to the terminated primer was cut out. The oligonucleotide was eluted from the gel in 0.3 M sodium acetate and 2 mM EDTA (pH 7.5), precipitated with ethanol, and dissolved in 25 μl of 10 mM Tris-HCl, 1 mM EDTA (pH 8).

**Phosphorolysis Assay**—The d21-AZTMP oligonucleotide was labeled with [γ-32P]ATP at the 5′-end and then annealed to a 39-nucleotide RNA template called r39 (5′-AAAAAAAAUAAAAGAACAGGUCGACUCUAGGAGAUCCUC-3′). This primer-template was incubated with the enzyme in the presence of the indicated concentrations of PPi or ATP in Buffer B (50 mM NaCl, 50 mM Tris-HCl, 0.05% Nonidet P-40, pH 8), and the reaction was started by adding 10 mM MgCl2 (pH 8) in a final volume of 25 μl. After incubation at 37 °C, the reactions were stopped by the addition of the same 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. The bands were visualized by autoradiography and quantified by densitometry as before. Because RT eliminates several nucleotides from the terminated primer, the amount of total product obtained was calculated by densitometric quantification of all oligonucleotides smaller than 22 nucleotides.

**Combination Assays**—The enzyme was incubated in Buffer A with 3 mM poly(rA)-dT20, 10 μM [γ-32P]dTTP in the presence or absence of 3 mM ATP or 250 μM PPi, in a final volume of 50 μl. The reactions were started with 10 mM MgCl2, and after 1 h of incubation at 37 °C, the reactions were quenched by adding 5 μl of EDTA 0.5 M. Fifteen μl of the mixture were spotted onto a DES1 (Whatman), washed three times with 0.5 M Na2HPO4 (pH 7.5), dried, and counted. The interaction index was calculated as explained previously (20, 24). Briefly, dose-response curves for each inhibitor alone were obtained within a wide range of effects by fitting experimental data to Equation 1 by unweighted nonlinear regression.

**RESULTS**

**Rate of Phosphorolysis by wt RT and Mutant AZT-RT**—AZT resistance has been related to an increased phosphorolytic activity of the mutant RT, leading to the removal of AZTMP from a chain-terminated primer (4, 5, 7). We measured the rate of phosphorolysis for wt and resistant RTs (Fig. 1A). At saturating RT concentrations, excision of AZT followed apparent first order kinetics. The addition of 150 μM PPi resulted in a similar rate of pyrophosphorolysis for both wt and resistant enzymes, with rate constants of 0.081 ± 0.009 and 0.074 ± 0.007 min⁻¹, respectively (Fig. 1B). Interestingly, when 3 mM ATP was used as substrate instead of PPi, wt RT showed a very slow removal of the chain terminator, with a rate constant of 0.001 ± 0.0002 min⁻¹. Under these conditions, this reaction was more efficient for resistant RT, which showed a rate constant of 0.011 ± 0.0001 min⁻¹.

The experimental procedure employed to purify the heterodimeric RT (see “Experimental Procedures”) allowed us to obtain a nonphysiological RT chimera, containing the four mu-
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ATP upon binding of the NNRTI. In previous work, we found that 4-arylmethylpyridinone was a linear noncompetitive inhibitor against dGTP polymerization reactions, with a $K_i$ of 35 nM (11). Steady-state kinetics analysis of inhibition by 4-arylmethylpyridinone against template-primer was examined by varying the concentration of poly(rA)-dT$_{20}$ while maintaining [${\alpha}^{32}$P]dTTP at a fixed concentration of 10 nM. 4-Arylmethylpyridinone displayed a complex inhibition pattern with respect to template-primer. At low concentrations of template-primer (<10 nM), the pattern was essentially uncompetitive, indicating that this compound preferentially binds to the RT-template-primer complex. Above this value the pattern changed to mixed noncompetitive (data not shown).

To confirm that this compound preferentially binds to the DNA-RT complex, 2 nM poly(rA)-dT$_{20}$ was incubated for 2 min with 10 nM [${\alpha}^{32}$P]dTTP, variable concentrations of 4-arylmethylpyridinone and 2, 5, 10, or 25 nM of AZT-RT. Under these conditions, $IC_{50}$ values for 4-arylmethylpyridinone were 72, 59, 47, and 24 nM, respectively. In the same experiment, $IC_{50}$ values for nevirapine were 0.87, 0.85, 2.2, and 2.8 nM. As a control, inhibition by 4-arylmethylpyridinone was measured under the same conditions except that a fixed concentration of AZT-RT was used (50 nM), and increasing concentrations of poly(rA)-dT$_{20}$ were added (2–25 nM). In this case, the $IC_{50}$ values for 4-arylmethylpyridinone increased from 87 to 250 nM. Taken together, these data support the hypothesis that 4-arylmethylpyridinone preferentially binds to the RT-template-primer complex rather than to the free enzyme. In fact, the concentration of this compound needed to half-inhibit the ATP-dependent phosphorolysis reaction using 2 nM r39-d21-AZTMP, 3 mM ATP, and 5–50 nM of RT is about 1–2 nM, irrespective of the total enzyme concentration added.
The effect of NNRTIs on ATP binding is difficult to determine. Direct binding assays using labeled ATP are not possible, because millimolar concentrations of the nucleotide are needed, whereas the RT concentration is in the nanomolar to low micromolar range. We tested by an indirect approach whether the concentration of ATP or PPi affects the inhibition caused by 4-arylmethylpyridinone. Useful ATP concentrations were 1–9 mM, because higher concentrations result in substrate inhibition of the phosphorolytic reaction. In the range 0–9 mM ATP, the IC_{50} for 4-arylmethylpyridinone in the RT-catalyzed phosphorolysis reaction only increased about 1.5-fold. The inhibition of DNA polymerization by NNRTI in the absence of AZTTP was also slightly sensitive to the presence of ATP, with the IC_{50} values increasing about 1.5–2-fold. These results suggest that NNRTIs do not exert their activity by impacting on ATP binding, although some interference with ATP may be present.

**Effect of ATP and PPi on the Rescue of AZTMP-terminated Primer**—Because ATP lysis is sensitive to the presence of the next incoming nucleotide (6, 26), we measured the sensitivity of RT to AZTTP in the polymerization reaction. These assays were performed with an adenine-rich template to allow the incorporation of AZTTP and the subsequent excision of the incorporated AZT during reaction. When AZTTP was not added to the reaction medium, RT was able to add bases in the absence of template once the primer was completely polymerized (Fig. 3A). In agreement with earlier observations (3), in the absence of pyrophosphorolysis, AZTTP was equally potent in the inhibition of DNA synthesis catalyzed by both wt and AZT-RT enzymes, with IC_{50} values of 0.20 and 0.26 M, respectively. Inhibition by AZTTP was lower in the absence of PPi. This effect was similar for both the wt and the mutant RTs (IC_{50} values were 0.93 and 1.1 M, respectively). Inhibition by AZTTP was also reduced in the presence of ATP. The reduction was less pronounced for the wt RT than for the AZT-RT (Fig. 3B). These results were consistent with the observed rates of pyrophosphorolysis for the enzymes with ATP and PPi.

**Effect of the NNRTIs on the Sensitivity of Resistant RT to AZTTP in the Presence of ATP or PPi**—We have demonstrated that the presence of physiological concentrations of PPi or ATP reduces the sensitivity of RT to AZTTP. This decrease was due to the phosphorolysis-mediated rescue of the AZTMP-terminated chain. On the other hand, we also found that NNRTIs can inhibit the phosphorolysis catalyzed by AZT resistant RT. Because resistance toward AZTTP is due to the increased unblocking capacity of AZT-terminated primer by mutant RT, it should be expected that inhibition of phosphorolysis by a NNRTI would resensitize the AZT-RT to AZTTP.

To test this hypothesis we measured the effect of several combinations of AZTTP and different NNRTIs in the presence and absence of PPi, or ATP. Fig. 4 (A and B) shows the combination of AZTTP with nevirapine in the presence of PPi. When different concentrations of nevirapine were added, the IC_{50} values decreased (Fig. 4C). Under these conditions, the inhibition of AZTTP was similar to that obtained in the absence of PPi and nevirapine (0.20 ± 0.01 M). It should be noted that this reduction was effectively due to a resensitization of the AZT-RT, because the inhibition values of the polymerase activity obtained with 0.1 and 1 M nevirapine in the presence of PPi were 1 and 15%, respectively. Furthermore, the addition of 0.1 M nevirapine in the absence of PPi, had no effect on the sensitivity of AZT-RT to AZTTP (0.24 ± 0.01 M), whereas 1 M nevirapine only decreased the IC_{50} value to 0.16 ± 0.01 M. These results confirm that the enhancement of the effect of AZTTP by nevirapine was not due to the additive inhibition of

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**TABLE I**

|            | Nevirapine | 9-Cl-TIBO | 4-Arylmethylpyridinone |
|------------|------------|-----------|------------------------|
| IC_{50} μM |            |           |                        |
| wt RT      |            |           |                        |
| Polymerization^{a} | 2.5 ± 0.36 | 23 ± 9.5 | 0.47 ± 0.03 |
| Pyrophosphorolysis^{b} | 2.4 ± 0.91 | 4.5 ± 0.55 | 0.002 ± 0.001 |
| AZT-RT     |            |           |                        |
| Polymerization^{a} | 3.8 ± 0.60 | 17 ± 3.5 | 0.12 ± 0.01 |
| Pyrophosphorolysis^{b} | 3.4 ± 0.46 | 4.6 ± 0.72 | 0.003 ± 0.001 |
| ATP-dependent | 0.78 ± 0.08 | 5.7 ± 1.3 | 0.001 ± 0.0001 |

^{a} 25 nM of RT was incubated with 2 nM [32P]d21-r36 and the NNRTI in the presence of 10 μM dTTP in buffer A and started by adding 10 mM MgCl2. After 1 h of incubation, products were analyzed by denaturing PAGE, and terminated chains were quantified by densitometry.

^{b} The experiments were carried out as described in the legend to Fig. 2.
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DNA synthesis but to the fact that nevirapine resensitizes the mutant enzyme by inhibiting the phosphorolytic reaction.

This effect was not exclusive of nevirapine, because similar results were obtained with other NNRTIs, although the concentration needed to obtain this effect varied considerably. For example, in the presence of PPi, 50 nm 4-arylmethylpyridinone decreased the IC50 of AZTTP for the mutant RT from 2.3 to 0.2 to 0.20 ± 0.02 μM. In the absence of pyrophosphate, the addition of 50 nm 4-arylmethylpyridinone had no effect on the IC50 of AZTTP (0.2 ± 0.02 μM). On the other hand, 9-Cl-TIBO was a weak inhibitor because 20 μM was needed to increase 5-fold the effect of AZT. This concentration of 9-Cl-TIBO inhibited the polymerization reaction by 15% in the presence of PPi.

The Synergy of AZTTP and NNRTIs in the Inhibition of the AZT-resistant Enzyme—Because NNRTIs were able to resensitize the AZT-resistant RT to AZTTP, it was expected that their combination with AZTTP would be synergistic in the presence of ATP or PPi. To analyze the interaction of these inhibitors, we measured the inhibition caused by each NNRTI alone or in combination with AZTTP in the presence and absence of either ATP or pyrophosphate. With ATP the combination of AZTTP with a NNRTI is far more effective than without ATP (Table II). This synergy is more obvious in the case of 4-arylmethylpyridinone, because in the presence of ATP, the combination was even more efficient than when each inhibitor was tested alone. The interaction index at 50% inhibition for the combination of AZTTP and nevirapine with ATP was 0.27, showing an exceptionally high degree of synergy (Fig. 5). Consequently, the interaction index for 4-arylmethylpyridinone in the presence of ATP was also very low, 0.39 at 50% of inhibition and 0.21 at 90% of inhibition. The same combinations were also synergistic when tested in the presence of pyrophosphate, resulting in interaction indexes of 0.30 at 50% of inhibition for the combination of nevirapine with AZTTP and 0.36 for the combination of 4-arylmethylpyridinone with AZTTP. Only additive effects (no interaction) were found when these combinations were tested in the absence of pyrophosphate or ATP (Fig. 5). These results show that the presence of a substrate of the phosphorolytic reaction is essential to mediate the synergy found in the combinations we tested.

We also analyzed the combination of AZTTP with 4-arylmethylpyridinone using a checkerboard design, i.e. varying AZTTP concentrations over a range of NNRTI concentrations (Fig. 6). In this plot, parallel lines are found if inhibitors do no interact, whereas intersecting lines are found if both inhibitors act synergistically (24). From this plot it can be concluded that in the presence of ATP the interaction is at least 10-fold greater than in its absence, as judged by the abscissa intersection points of both graphs. It should be noted that the IC50 for AZTTP in the absence and presence of 3 mM ATP in this experiment increased 13-fold, so the high synergy found can be attributed to the inhibition of the phosphorolytical activity of the resistant enzyme, in agreement with the hypothesis. It is noteworthy that the concentrations of 4-arylmethylpyridinone needed to detect this interaction are in fact very low. For example, the concentration of 4-arylmethylpyridinone needed to half-inhibit the polymerase activity of AZT-RT in the presence of ATP 3 mM was 0.33 μM, whereas synergy is easily detected at 0.025 μM of 4-arylmethylpyridinone (Fig. 6B). As expected, both methods of evaluating the synergy between RT inhibitors are fully consistent (24). In fact, the combination of 0.2 μM of 4-arylmethylpyridinone with 1 μM on the AZT-RT showed a 90% of inhibition (Fig. 6B), and the interaction index calculated for this combination is 0.26. This value is very close to the interaction index of 0.21 reported for the combination of AZTTP and 4-arylmethylpyridinone for the same inhibition level when both inhibitors were mixed at a 1:0.78 ratio (Table II and Fig. 5).

**DISCUSSION**

In this work we show that NNRTIs, besides inhibiting the polymerase activity of HIV-1 RT, can also block the phosphorolytic reaction. The fact that three unrelated NNRTIs, nevirapine, TIBO, and 4-arylmethylpyridinone, were able to affect the excision of the incorporated AZT suggests that most NNRTIs may have the potential to inhibit the phosphorolytic activity to some extent. Our results suggest that NNRTIs do not exert their activity by impacting template-primer or ATP binding, although some interference with ATP may be present. Taking into account that pyrophosphorylation is the reverse of the polymerization reaction, it seems to us more reasonable that the mode of inhibition of phosphorylation was similar to that of inhibition of DNA synthesis. The inhibition of PPi-dependent phosphorylation by NNRTIs also gives new clues in understanding the influence of NNRTI on the active site. Pre-steady-state kinetic analysis of inhibition of the polymerization reaction shows that NNRTIs block the chemical reaction but do not interfere with dNTP binding or with nucleotide-induced conformational changes (12). In contrast to dNTPs, PPi lacks the sugar ring and the nitrogenated base. Thus, inhibition of the pyrophosphorylative activity by NNRTIs support the hypothesis that these compounds modify the conformation of the catalytic site, where the crucial Mg2+ ions are not anymore in the proper alignment with the carboxyl groups for efficient catalysis, as previously suggested (12). These changes at the active
The combinations were only additive. Open symbols denote the presence of ATP (interaction indexes as the effect of combination increased. In the absence of ATP, both combinations were synergistic. 4-arylmethylpyridinone (squares), as shown by the decrease of filled symbols in the presence of ATP. Both combinations were synergistic.

AZTr-RT as enzyme. The mutant enzyme can reach about 0.012 min⁻¹ the first order constant for ATP-dependent phosphorolysis for the NNRTI on AZT r-RT in the presence and absence of ATP. Carrying the four mutations shows an increase in this activity (5).

Site of RT would prevent the catalysis in both the forward and the reverse reactions. Moreover, a chimeric enzyme, carrying the AZT-resistant mutations only in the p66 subunit, behaved as the AZT-resistant RT. This fact confirms that only mutations on the p66 are relevant for the resistance of the enzyme, as suggested by previous structural studies (27, 28).

We consistently found that the phosphorolysis catalyzed by RT is more sensitive to NNRTI than the polymerization reaction. In addition, synergy is easily detected at concentrations of the NNRTI that barely inhibit the polymerase activity but effectively block the phosphorolysis catalyzed by the AZT⁻RT (Figs. 4 and 6). The fact that phosphorolysis catalyzed by RT is more sensitive to NNRTI than DNA polymerization, is probably related to the absolute rate of both reactions. Depending on the conditions used, DNA polymerase activity catalyzed by RT may be about 5,000–20,000-fold faster than ATP-dependent phosphorolysis. For example, typical kcat values for RT catalyzed DNA polymerization are about 3 s⁻¹, whereas apparent first order constant for ATP-dependent phosphorolysis for the mutant enzyme can reach about 0.012 min⁻¹ = 0.0002 s⁻¹.

Pyrophosphorolysis of the AZTMP-terminated primer is a more efficient process than ATP lysis, because higher concentrations are needed to catalyze the reaction in the presence of the nucleotide. However, wild type RT is almost unable to catalyze ATP-dependent phosphorolysis, whereas the enzyme carrying the four mutations shows an increase in this activity by at least 10-fold. The difference found between wt and resistant RTs points at ATP as the possible substrate responsible for the rescue of the terminated primer in vivo (5). Many details of

### Table II

**Inhibition of DNA polymerase activity of AZT⁻RT by combinations of NNRTIs and AZTTP in the presence and absence of ATP**

Combination experiments were carried out as described under “Experimental Procedures” in the absence or presence of 3 mM ATP, using AZT⁻RT as enzyme.

|        | AZTTP | Nevirapine | 4-Arylmethyl-pyridinone |
|--------|-------|------------|-------------------------|
| No ATP | IC₅₀ (μM) | Molar ratio¹ | IC₅₀ (μM) | Molar ratio¹ | IC₅₀ (μM) | Molar ratio¹ |
| Molar ratio² | IC₅₀ (μM) | Molar ratio¹ | IC₅₀ (μM) | Molar ratio¹ | IC₅₀ (μM) | Molar ratio¹ |
| 3 mM ATP | 0.060 ± 0.86 | 5.8 ± 0.17 | 0.21 ± 0.01 | 3.13 ± 0.29 | 1:250 | 0.15 ± 0.09 | 1:12.5 |

¹ AZTTP/NNRTI ratio used in the combination. This ratio reflects the different potency of the compounds, ensuring in this way that both inhibitors significantly contributed to the total inhibition measured.

#### Experimental Procedures

Inhibition of DNA polymerase activity of AZT⁻RT by combinations of NNRTIs and AZTTP in the presence and absence of ATP. Interactions for combinations shown in Table II were calculated as described under “Experimental Procedures.” Interaction indexes of <1, 1, or >1 indicate synergism, no interaction, or antagonism, respectively. The combinations tested were AZT + nevirapine (circles) and AZT + 4-arylmethylpyridinone (squares). Both combinations were synergistic in the presence of ATP (filled symbols), as shown by the decrease of interaction indexes as the effect of combination increased. In the absence of ATP (open symbols), the combinations were only additive.

Fig. 5. Interaction indexes for the combination of AZT + NNRTI on AZT⁻RT in the presence and absence of ATP. Interaction indexes for combinations shown in Table II were calculated as described under “Experimental Procedures.” Interaction indexes of <1, 1, or >1 indicate synergism, no interaction, or antagonism, respectively. The combinations tested were AZT + nevirapine (circles) and AZT + 4-arylmethylpyridinone (squares). Both combinations were synergistic in the presence of ATP (filled symbols), as shown by the decrease of interaction indexes as the effect of combination increased. In the absence of ATP (open symbols), the combinations were only additive.

Fig. 6. Effect of the combination of AZTTP and 4-arylmethylpyridinone on AZT⁻RT in the presence and absence of ATP. Four concentrations of 4-arylmethylpyridinone (0.025, 0.05, 0.1, and 0.2 μM) were combined over a range of AZTTP concentrations, providing a matrix of data. The reverse of the relative activity of each combination was then plotted against the concentration of 4-arylmethylpyridinone. The concentrations of AZTTP were selected to obtain total inhibition values in the range 10–90%. A, no ATP. The reactions were incubated in buffer A containing 3 mM poly(rA)-dT20, 20 mM dTTP, 10 mM AZTr-RT at 37 °C and started by adding 10 mM MgCl2. After 1 h of incubation at 37 °C, the reactions were quenched by the addition of EDTA, and 15 μl of the mixture were spotted onto a DE81 paper, washed, and counted. The concentrations of AZTTP were 0 (○), 0.0125 (●), 0.025 (□), 0.05 (■), and 0.1 μM (△). B, 3 mM ATP. The experiments were performed as before, but 3 mM ATP was present during the incubation. The concentrations of AZTTP were 0 (○), 0.125 (●), 0.25 (□), 0.5 (■), and 1 μM (△).
the role of pyrophosphate in the AZT resistance mechanism remains to be clarified, but in our hands physiological concentrations of pyrophosphate can severely decrease the sensitivity of the wild type RT to AZTTP. Arion et al. (4) observed that the presence of 150 µM pyrophosphate decreased by 3-fold the sensitivity of the AZT-resistant RT for the chain terminator. However, no significant changes were found with the wild type RT. In our study, the presence of PPi at physiological concentrations not only decreased the inhibition of the RT-catalyzed DNA synthesis by AZTTP, but this reduction was similar with both enzymes. These results are consistent with the phosphorolytical activity of wild type and AZT RTs.

This work was intended to analyze the biochemical mechanism mediating the synergy between NNRTIs and AZT on resistant strains. Despite many studies devoted to this topic, the molecular mechanism underlying the antiviral synergy of combinations of reverse transcriptase inhibitors is in most cases unknown. The synergistic inhibition of HIV replication in cell culture has been reported for many combinations of nucleosidic and NNRTI inhibitors including, among others, bisphosphateyl-piperazine derivatives (29), pyridinone derivatives (30), nevirapine (13), HEP derivative (14), TIBO derivatives (15, 31), or canololide A (16). However, other studies have shown that the same combinations showed no synergy in inhibiting RT activity or canalolide A (16). However, other studies have shown that the molecular mechanism underlying the antiviral synergy of NNRTIs mediating the synergy between NNRTIs and AZT on resistant viruses to AZT. Borkow et al. (8) have suggested that this inhibition might also be a relevant mechanism for the delayed development of resistance to combinations of AZT plus UC781 in antiviral assays. This proposal deserves further attention and can be tested by analyzing the long term antiviral effect of combinations of AZT and a NNRTI able to effectively inhibit the phosphorylolytic activity of HIV-RT. In this context, it would be interesting to know whether the reported observations that combinations of two nucleoside inhibitors (including AZT) with efavirenz are clearly superior to combinations including only nucleoside analogs, could be related to the inhibition of the phosphorylolytic activity by this NNRTI.

Our results indicate that 4-aryl methyl pyridinone is a very good candidate to be used with AZT in the multietherapy strategy. Although nevirapine is slightly more synergistic than 4-aryl methyl pyridinone when combined with AZTTP, 4-aryl methylpyridinone is far more effective in inhibiting DNA polymerase (Ki = 20 nM) (11), and it is about 1,000-fold more effective in inhibiting ATP and PPi-dependent phosphorolysis (Table 1).

In addition, 4-aryl methylpyridinone can also block the DNA polymerase activity of RT carrying K103E or Y181C mutations, in agreement with the demonstrated ability of this compound to inhibit a nevirapine-resistant virus with an IC50 of 40 nM (11). Taken together, our data support the importance of screening the ability of the currently used NNRTIs as inhibitors of RT-catalyzed phosphorylolytic, leading to the design of new compounds based on their capacity to inhibit the phosphorolytical-mediated RT excision.

Acknowledgments—We thank the Drug Synthesis and Chemistry Branch, Development Therapeutics Program, Division of Cancer Treatment, NCI, National Institutes of Health, Bethesda, Maryland, for supplying us with nevirapine.

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J. Biol. Chem. 2003, 278:42710-42716.
doi: 10.1074/jbc.M212673200 originally published online August 13, 2003

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