JBC Papers in Press. Published on August 13, 2003 as Manuscript M212673200

Nonnucleoside inhibitors of HIV-1 reverse transcriptase inhibit phosphorolysis and resensitize the 3'-azido-3'-deoxythymidine (AZT)-resistant polymerase to AZTTP

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Running title: NNRTIs resensitize AZT-resistant HIV-1 RT

SUMMARY

Removal of AZT 3'-azido-3'-deoxythymidine 5'-monophosphate (AZTMP) from the terminated primer mediated by the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) has been proposed as a relevant mechanism for the resistance of HIV to 3'-azido-3'-deoxythymidine (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. We also found that three structurally divergent non nucleoside inhibitors (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 AZT-resistant enzyme only when ATP or PP_i were present, indicating that inhibition of phosphorolysis was responsible for the synergy found in the combination. Our results not only demonstrate the importance of phosphorolysis concerning HIV-1 RT resistance to AZT, but also point out to the implication of this activity in the strong synergy found in some combinations of NNRTIs with AZT.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-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 human 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 ddC 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 which 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 combinations 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, in positions 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 virus 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 AZTMP from the 3'-end of the nascent DNA (4,6,7).

It has been reported that the thiocarboxanilide nonnucleoside inhibitor UC781, in combination with AZTTP, synergistically inhibits the replication of AZT-resistant HIV-1 virus (8). Since UC781 was also able to block the pyrophosphorolytic 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 pyrophosphorolytic activity is specific for UC781, since 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 both to HEPT and pyridinone, the 3-dimethylamino-4-(3,5-dimethylbenzyl)-5-ethyl-6-methylpyridin-2(1*H*)-one (compound **1**) called 4-arylmethylpyridinone for short (see scheme I) (11), on ATP and PP_i-dependent phosphorolysis, using wild type and AZT-resistant enzymes. We found that all NNRTI were able to inhibit the phosphorolytic activity of the enzyme, though with different potencies.

Furthermore we analyzed the interaction between AZTTP and NNRTIs with the AZT-resistant enzyme. Since both groups of inhibitors bind to different sites on RT in a nonexclusive manner (12), combinations of nucleoside analogs and nonnucleoside inhibitors might have a potential synergistic inhibitory effect on HIV-1 RT. Synergistic inhibition of HIV replication in cell culture has been reported for many combinations of nucleoside and nonnucleoside RT inhibitors (13-16). Nevertheless, several other studies have shown that the same combinations showed no synergy in inhibiting RT activity *in vitro* (17-21). In this report we show that combinations of AZTTP with NNRTIs show an exceptionally high

degree of synergy in inhibiting AZT-resistant enzyme only when ATP or PP_i are present, showing that inhibition of phosphorolysis may be implicated in the mechanism of this interaction *in vivo*. The relevance of these findings in combined anti-HIV chemotherapy is discussed.

EXPERIMENTAL PROCEDURES

Preparation of HIV-1 RT. Recombinant p66/p51 wild type RT was obtained as follows. The p66 plasmid cloned in *E. coli* JM109 (22) was used to generate p51 by introducing two stop codons in amino acid positions 441 and 442 using a mutagenesis kit from Stratagene. Both clones were grown and induced by IPTG separately, and cell pellets were mixed maintaining an excess of the p51 subunit. Cells were disrupted in a French press and purified by immobilized metal affinity chromatography as described (20). Excess of monomeric p51 was removed by gel filtration through a Superdex 200 column (Pharmacia) (23). AZT-resistant RT (AZT^r-RT) contained 4 amino acid substitutions at positions D67N, K70R, T215Y and K219Q, introduced in the genes of both subunits using the same mutagenesis kit. The presence of expected mutations was verified by the complete sequencing of all RT clones. All enzyme preparations were homogeneous as judged by gel filtration and SDS-PAGE.

PAGE analysis of polymerization assay. The DNA oligonucleotide termed d21 (5'-GGGGATCCTCTAGAGTCGACC-3') was labeled with [γ-^{32}P]ATP at the 5'-end and then annealed to a 36-nt RNA template called r36 (5'-AAAAAAAAAAAAAAAGGUCGACUCUAGAGGAUCCCC-3'). This primer-template was incubated with the enzyme in the presence of 10 μM dTTP in buffer A (100 mM NaCl,

50 mM Tris-HCl, 1.25 mM EGTA, 0.5 mM EDTA, 0.05% NP40 at pH 8) and started by adding 10 mM $MgCl_2$ (pH 8), in a final volume of 25 µl. After 1 hour of incubation at 37º C, reactions were quenched by the addition of an equal volume of 25 µl of loading buffer (90% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol). Samples were analyzed by denaturing PAGE using 12% polyacrylamide gels containing 7 M urea. The electrophoretically resolved products were visualized by autoradiograpy and the amount of total product obtained was calculated by densitometric quantification of all oligonucleotides longer than 35-nt using Imagemaster software (Pharmacia).

Preparation of AZTMP-terminated primer. Oligonucleotide d21 (1 nmol) annealed with r36 (2 nmol) was incubated with 10 nM RT and 100 µM AZTTP in 100 mM NaCl, 50 mM Tris-HCl, 0.05% NP40 and 4 mM $MgCl_2$ (pH 8), for 4 hours at 37ºC, in a final volume of 200 µl. The 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. 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-^{32}P]ATP at the 5'-end and then annealed to a 39-nt RNA template called r39 (5'-AAAAAAAAUAAAAGAACAGGUCGACUCUAGAGGAUCCCC-3'). This primer-template was incubated with the enzyme in the presence of the indicated concentrations of PP_i or ATP in Buffer B (50 mM NaCl, 50 mM Tris-HCl, 0.05% NP40 at pH 8) and the reaction was started by adding 10 mM $MgCl_2$ (pH 8), in a final volume of 25 µl. After incubation at 37 ºC, reactions were stopped by the addition of the same volume of loading

buffer (90% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol). Samples were analyzed by denaturing PAGE using 12% polyacrylamide gels containing 7 M urea. Bands were visualized by autoradiography and quantified by densitometry as before. As 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-nt.

Combination assays. The enzyme was incubated in Buffer A with 3 nM poly(rA)-dT_{20}, 10 µM [α-^{33}P]dTTP, in the presence or absence of 3 mM ATP or 250 µM PP_i in a final volume of 50 µl. Reactions were started with 10 mM $MgCl_2$, and after 1 hour incubation at 37 ºC, reactions were quenched by adding 5 µl of EDTA 0.5 M. Fifteen µl of the mixture were spotted onto a DE81 (Whatman), washed three times with Na_2HPO_4 0.5 M 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 non-linear regression

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

where *f* is the fractional inhibition, *D* represents the concentration (dose) of the inhibitor when tested alone, IC_{50} the concentration of the inhibitor giving 50% of inhibition, and *m* a parameter giving the sigmoidicity of the dose-response curve, using the commercial available fitting program Grafit (Erithacus software). Interaction between inhibitors was evaluated by means of the interaction index (*I*).

$$I = \frac{d_1}{D_1} + \frac{d_2}{D_2} \qquad [2]$$

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) (24,25), calculated from [1]. 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.

RESULTS

Rate of phosphorolysis by wt RT and mutant AZT^r-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. Addition of 150 μM PP_i resulted in a similar rate of pyrophosphorolysis for both wt and resistant enzymes, with rate constants of 0.081 ± 0.009 min^{-1} and 0.074 ± 0.007 min^{-1}, respectively (Fig. 1B). Interestingly, when 3 mM ATP was used as substrate instead of PP_i, 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, that showed a rate constant of 0.011 ± 0.001 min^{-1}.

The experimental procedure employed to purify the heterodimeric RT (see Methods) allowed us to obtain a non physiological RT chimera, containing the four mutations (D67N, K70R, T215Y and K219Q) only in the p66 subunit while the p51 subunit contained the wild type sequence. This hybrid RT showed a rate of pyrophosphorolysis similar to the wild type and AZT resistant enzymes (k_{app}= 0.065 ± 0.004 min^{-1}). Unlike wild type enzyme, the chimeric RT efficiently catalyzed ATP-dependent phosphorolysis (k_{app}= 0.020 ± 0.005 min^{-1}). These data clearly show that only mutations in the p66 subunit were responsible for the

increased ability of the mutant enzyme to unblock the AZTMP-terminated primer by an ATP-dependent mechanism.

Inhibition of phosphorolysis by NNRTIs. Non-nucleoside compounds are well-known inhibitors of HIV-1 RT DNA-polymerase. We tested whether these compounds could also inhibit the phosphorolysis catalyzed by wt and AZT-resistant RTs, using a primer blocked at its 3'-end by AZTMP at saturating RT concentrations. As shown in figure 2 and Table 1, nevirapine, 9-Cl-TIBO, and 4-arylmethylpyridinone inhibit the phosphorolytic activity, though with a different efficiency. 9-Cl-TIBO was the weakest inhibitor, while the 4-arylmethylpyridinone was the most efficient. The latter compound was about 1000-fold more potent to block pyrophosphorolysis than nevirapine, and inhibited both ATP and PP_i dependent phosphorolysis in the nanomolar range.

We analyzed whether the inhibition by 4-arylmethylpyridinone was due to the impaired binding of the template-primer or ATP upon binding of the NNRTI. In previous work, we found that 4-arylmethylpyridinone was a linear noncompetitive inhibitor against dGTP in 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 [á-^{33}P]dTTP at a fixed concentration of 10 µM. 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).

In order 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 µM [á-^{33}P]dTTP, variable concentrations of 4-arylmethylpyridinone and 2, 5, 10 or 25 nM of AZT^r-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 µM. As a control, inhibition by 4-arylmethylpyridinone was measured under the same conditions except than a fixed concentration of AZT^r-RT was used (50 nM), and increasing concentrations of poly(rA)-dT_{20} were added (2 to 25 nM). In this case, the IC_{50} values for 4-arylmethylpyridinone increased from 87 nM 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 to 50 nM of RT is about 1-2 nM, irrespective to 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, since millimolar concentrations of the nucleotide are needed while the RT concentration is in the nano to low micromolar range. We tested by an indirect approach whether the concentration of ATP or PP_i affects the inhibition caused by 4-arylmethylpyridinone. Useful ATP concentrations were 1-9 mM, since higher concentrations results in substrate inhibition of the phosphorolytic reaction. In the range 0 to 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, 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, though some interference with ATP may be present.

Effect of ATP and PP_i on the rescue of AZTMP-terminated primer. Since 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 A-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^r-RT enzymes, with IC_{50} values of 0.20 and 0.26 μM, respectively. Inhibition by AZTTP was lower in the presence of PP_i. This effect was similar for both the wt and the mutant RTs (IC_{50} values: 0.93 and 1.1 μM, 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^r-RT (Fig. 3B). These results were consistent with the observed rates of phosphorolysis for the enzymes with ATP and PP_i.

Effect of the NNRTIs on the sensitivity of resistant RT to AZTTP in the presence of ATP or PP_i. We have demonstrated that the presence of physiological concentrations of PP_i 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. Since resistance towards 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^r-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 PP_i or ATP. Fig. 4 (A and B) shows the combination of AZTTP with nevirapine in the presence of PP_i. 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 PP_i and nevirapine (0.20 ± 0.01 μM). It should be noted that this reduction was effectively due to a resensitization of the AZT^r-RT, since the inhibition values of the polymerase activity obtained with 0.1 and 1 μM nevirapine in the presence of PP_i were 1% and 15%, respectively. Furthermore, addition of 0.1 μM nevirapine in the absence of PP_i had no effect on the sensitivity of AZT^r-RT to AZTTP (0.24 ± 0.01 μM), while 1 μM nevirapine only decreased the IC_{50} value to 0.16 ± 0.01 μM. These results confirm that the enhancement of the effect of AZTTP by nevirapine was not due to the additive inhibition of DNA synthesis, but to the fact that nevirapine resensitizes the mutant enzyme by inhibiting the phosphorolytic reaction.

This effect was not exclusive of nevirapine, since similar results were obtained with other NNRTIs, though the concentration needed to obtain this effect varied considerably. For example, in the presence of PP_i, 50 nM 4-arylmethylpyridinone decreased the IC_{50} of AZTTP for the mutant RT from 2.3 ± 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 IC_{50} of AZTTP (0.2 ± 0.02 μM). On the other hand, 9-Cl-TIBO was a weak inhibitor since 20 μM was needed to increase five fold the effect of AZT. This concentration of 9-Cl-TIBO inhibited the polymerization reaction by 15% in the presence of PP_i.

The synergy of AZTTP and NNRTIs in the inhibition of the AZT-resistant enzyme. Since 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 PP_i. 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 2). This synergy is more obvious in the case of

4-arylmethylpyridinone, since 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) was 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, while 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 IC_{50} for AZTTP in the absence and in the 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^r-RT in the presence of ATP 3 mM was

0.33 µM, while synergy are 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 AZTr-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 1:0.78 ratio (Table 2 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, though some interference with ATP may be present. Taking into account that pyrophosphorolysis is the reverse of the polymerization reaction, it seems to us more reasonable that the mode of inhibition of phosphorolysis was similar to that of inhibition of DNA synthesis. The inhibition of PP_i-dependent phosphorolysis 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, PP_i lacks the sugar ring and the nitrogenated base. Thus, inhibition of the pyrophosphorolytic activity by NNRTIs support the hypothesis that these compounds modify the conformation of

the catalytic site, where the crucial Mg^{++} ions are not anymore in the proper alignment with the carboxyl groups for efficient catalysis, as previously suggested (12). These changes at the active 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^r-RT (Fig. 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 to 20 000-fold faster than ATP-dependent phosphorolysis. For example, typical k_{cat} values for RT catalyzed DNA polymerization are about 3 s^{-1}, while 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, since 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 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 three-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 PP_i 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^r RTs.

This work was intended to analyze the biochemical mechanism mediating the synergy between NNRTIs and AZT on resistant strains. In spite of 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, BHAP derivates (29), pyridinone derivates (30), nevirapine (13), HEPT derivates (14), TIBO derivates (15,31), or canalolide A (16). However, other studies have shown that the same combinations showed no synergy in inhibiting RT activity *in vitro* (17-21). In previous studies, the relevance of the phosphorolysis on the interaction between inhibitors has not been considered. We found that these combinations were highly synergistic only when tested in the presence of PP_i or ATP. Fig. 4 and 6 clearly shows that this high level of synergy is due to the fact that complete inhibition of the phosphorolytic activity of AZT^r-RT is attained at concentrations of NNRTI that barely affect its polymerase activity. According to our results, the synergy found is more related to the inhibition of the phosphorolytical activity by the NNRTI than to the combined effect on the polymerase activity. Consequently it is clear that, along with its ability to block

the polymerase reaction, the inhibition of the phosphorolytic mediated AZT-excision activity by NNTIs should also be taken into account.

Since AZT has become the most accessible anti-HIV agent, a better understanding of the role played by other inhibitors in the resistance to AZT should help to design more efficient therapies against HIV. Nowadays, anti-HIV chemotherapy is based in the use of HAART, where three or more drugs are combined to inhibit virus replication. Since resistance to AZTTP is due to an increased rate in the phosphorolytic reaction by AZT^r-RT, the use of a NNRTI blocking efficiently this activity would resensitize resistant viruses to AZT. Borkow *et al.* (8) have suggested that this inhibition might be also 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 phosphorolytic 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 NRTI, could be related to the inhibition of the phosphorolytical activity by this NNRTI.

Our results indicate that 4-arylmethylpyridinone is a very good candidate to be used with AZT in the multitherapy strategy. Although nevirapine is slightly more synergistic than 4-arylmethylpyridinone when combined with AZTTP, 4-arylmethylpyridinone is far more effective in inhibiting DNA polymerase (K_i = 20 nM) (11) and it is about 1000-fold more effective in inhibiting ATP and PP_i-dependent phosphorolysis (Table 1). In addition, 4-arylmethylpyridinone 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 IC_{50} 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 phosphorolysis, leading to the design of new compounds based on their capacity to inhibit the phosphorolytic-mediated AZT excision.

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FOOTNOTES

This work was supported by a joint grant from the Gobierno de Navarra and the Conseil Regional d'Aquitaine (Fonds Commun de Cooperation Navarra-Aquitaine), the Agence Nationale Française pour la lutte contre le SIDA (ANRS) and the PIUNA.

ACKNOWLEDGEMENTS

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

The **ABBREVIATIONS** used are: AZT, 3'-azido-3'deoxythymidine; AZTMP, 5'-monophosphate; AZTTP, AZT-5'-triphosphate; RT, reverse transcriptase; AZT^r-RT, reverse transcriptase containing the D67N/ K70R/T215Y/K219Q mutations; HAART, highly active antiretroviral treatments; HIV-1, human immunodeficiency virus type 1; NNRTI, non-nucleoside reverse transcriptase inhibitor; wt, wild type; PP_i, inorganic pyrophosphate.

FIGURE LEGENDS

Scheme I. Structure of the 4-arylmethylpyridinone derivative.

Figure 1. Time course of phosphorolysis catalyzed by wt RT and AZT^r-RT. 2 nM (^{32}P-labeled) d21-AZTMP primer annealed to the r39 template was incubated in buffer B with 10 mM $MgCl_2$ and 25 nM RT in the presence of 150 μM PP_i or 3 mM ATP. Aliquots were taken at different time points, and the reactions were analyzed by electrophoresis. (A) PAGE analyses of phosphorolytic products. Gels show the unblocking of d21-AZTMP primer in the presence of 150 μM PP_i or 3 mM ATP by wt and AZT^r RTs. Phosphorolysis is revealed by the disappearance of the AZTMP-terminated primer and the appearance of shorter products. (B) Rates of phosphorolytic removal of AZT by wt RT (○) and AZT^r-RT (●). The gels presented in (A) were quantified by densitometry and experimental data fitted to a burst equation. The solid lines represents the best fit obtained by non-linear regression of the data to equation $A \times e^{-kt} + C$, where *A* is the amplitude of the burst, *k* is the apparent first order rate constant of AZT excision and *C* is a constant.

Figure 2. Inhibition of phosphorolytic activity of wt RT and AZT^r-RT by NNRTIs. 25 nM RT were added to a mixture containing 2 nM (^{32}P-labeled) d21-AZT annealed to the r39 template, the indicated amount of the NNRTI, and 150 μM PP_i or 3 mM ATP in buffer B and 10 mM $MgCl_2$ at 37º C. Reactions containing PP_i were incubated for 60 min and those containing ATP were incubated for 120 min. Under these conditions, no ATP-dependent phosphorolysis was detectable for the wt RT. Reactions were quenched at the indicated time and products were analyzed by denaturing PAGE. As RT eliminated several nucleotides from the terminated primer, phosphorolysis was calculated by quantifying all the oligonucleotides of 21-nt length or less by densitometry.

Figure 3. Effect of ATP and PP_i on the rescue of AZT-terminated primer. (A) Inhibition of AZT^r-RT by AZTTP in the presence or absence of PP_i or ATP was measured by denaturing PAGE and densitometric quantification. Experiments were carried out by incubating 2 nM (^{32}P-labeled) d21-r36 with 25 nM RT and AZTTP in buffer A containing 10 mM $MgCl_2$ and 10 µM dTTP at 37 ºC, with or without 150 µM PP_i or 3 mM ATP. Reactions were stopped after 1 h and products were analyzed by denaturing PAGE. (B) Inhibition of reverse transcriptase activity in the absence (○) or in the presence of 150 µM PP_i (●) or 3 mM ATP (□) was obtained by densitometric quantification in each reaction of all oligonucleotides longer than 35-nt.

Figure 4. NNRTIs resensitize AZT-resistant HIV-1 RT to AZTTP by inhibiting phosphorolysis. (A) Inhibition by AZTTP and nevirapine of AZT^r-RT in the presence or absence of PP_i or ATP was analyzed by denaturing PAGE. Experiments were carried out by incubating in buffer A 10 nM (^{32}P-labeled) d21 annealed to r36 template with 25 nM AZT^r-RT, 10 mM $MgCl_2$, 250 µM PP_i and 10 µM dTTP (pH 8) and the indicated amounts of inhibitors for 1 hour at 37º C. (B) Dose-response curves were obtained by densitometry as described in Fig. 3. The IC_{50} for AZTTP increased from 0.20 ± 0.01 µM, without PP_i, to 1.0 ± 0.12 µM with 250 µM PP_i (○). In the presence of 0.1 µM nevirapine and 250 µM PP_i, the IC_{50} for AZTTP decreased to 0.30 ± 0.05 µM (□) or to 0.19 ± 0.04 µM if 1 µM nevirapine was present (■). (C) Inhibition of AZT^r-RT by AZTTP with PP_i and nevirapine.

Figure 5. Interaction indexes for the combination of AZT + NNRTI on AZT^r-RT in the presence and absence of ATP. Interaction indexes for combinations shown in Table 2 were calculated as described in Experimental Procedures. Interaction indexes <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 (full symbols), as shown by the decrease of interaction indexes as the effect of combination increased. In the absence of ATP (empty symbols), the combinations were only additive.

Figure 6. Effect of the combination of AZTTP and 4-arylmethylpyridinone on AZT^r-RT in the absence and presence of 3 mM 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 (24). Concentrations of AZTTP were selected to obtain total inhibition values in the range 10-90%. (A) No ATP. Reactions were incubated in buffer A containing 3 nM poly(rA)-dT_{20}, 20 μM dTTP, 10 nM AZT^r-RT at 37 ºC and started by adding 10 mM $MgCl_2$. After 1 h of incubation at 37 ºC reactions were quenched by the addition of EDTA and 15 μl of the mixture were spotted onto a DE81 paper, washed and counted. Concentrations of AZTTP were 0 (○), 0.0125 (●), 0.025 (□), 0.05 (■), and 0.1 μM (△). (B) 3 mM ATP. Experiments were performed as before but 3 mM ATP was present during the incubation. Concentrations of AZTTP were 0 (○), 0.125 (●), 0.25 (□), 0.5 (■), and 1 μM (△).

Table 1. Inhibition of polymerization and phosphorolysis catalyzed by wt RT and AZT^r-RT

	IC_{50} (μM)		
	Nevirapine	9-Cl-TIBO	4-arylmethyl-pyridinone
wt RT			
Polymerization [(a)]	2.5 ± 0.86	23 ± 9.5	0.47 ± 0.03
Pyrophosphorolysis [(b)]	2.4 ± 0.91	4.5 ± 0.55	0.002 ± 0.001
AZT^r -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 phosphorolysis [(b)]	0.76 ± 0.08	5.7 ± 1.3	0.001 ± 0.0001

(a) 25 nM of RT was incubated with 2 nM [^{32}P]d21-r36 and the NNRTI in the presence of 10 μM dTTP in buffer A and started by adding 10 mM $MgCl_2$. After 1 h of incubation products were analyzed by denaturing PAGE, and terminated chains were quantified by densitometry.

(b) Experiments were carried out as described in Fig. 2.

Table 2. Inhibition of DNA polymerase actvity of AZT^r-RT by combinations of NNRTIs and AZTTP in the presence and absence of ATP [(a)]

				AZTTP +	
	AZTTP	**nevirapine**	**4-arylmethyl-pyridinone**	**nevirapine**	**4-arylmethyl-pyridinone**
No ATP					
IC_{50} (μM)	0.060 ± 0.86	5.8 ± 0.17	0.21 ± 0.01	3.13 ± 0.29	0.15 ± 0.09
Molar ratio[(b)]				1:250	1:12.5
3 mM ATP					
IC_{50} (μM)	0.82 ± 0.04	10.7 ± 0.38	0.43 ± 0.01	1.69 ± 0.10	0.23 ± 0.04
Molar ratio[(b)]				1:15.6	1:0.78

(a) Combination experiments were carried out as described under Experimental Procedures in the absence or in the presence of 3 mM ATP, using AZT^r-RT as enzyme.

(b) 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.

Scheme 1, Odriozola et al.

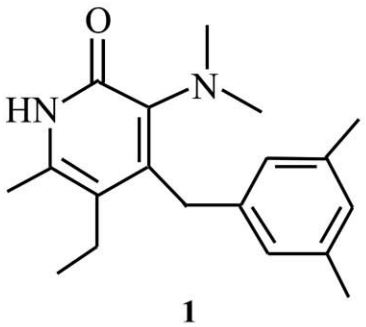

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Figure 1, Odriozola et al.

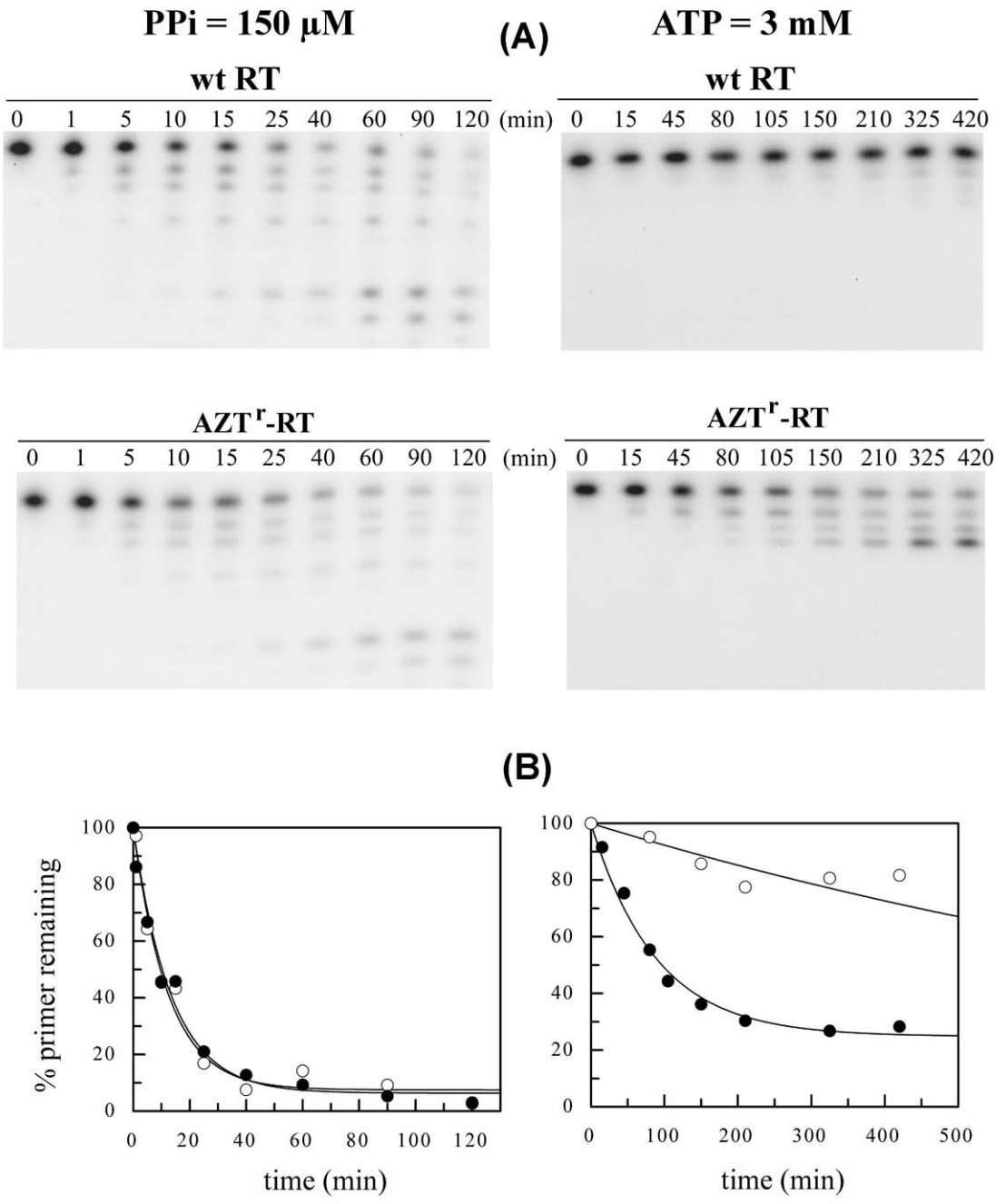


Figure 2, Odriozola et al.

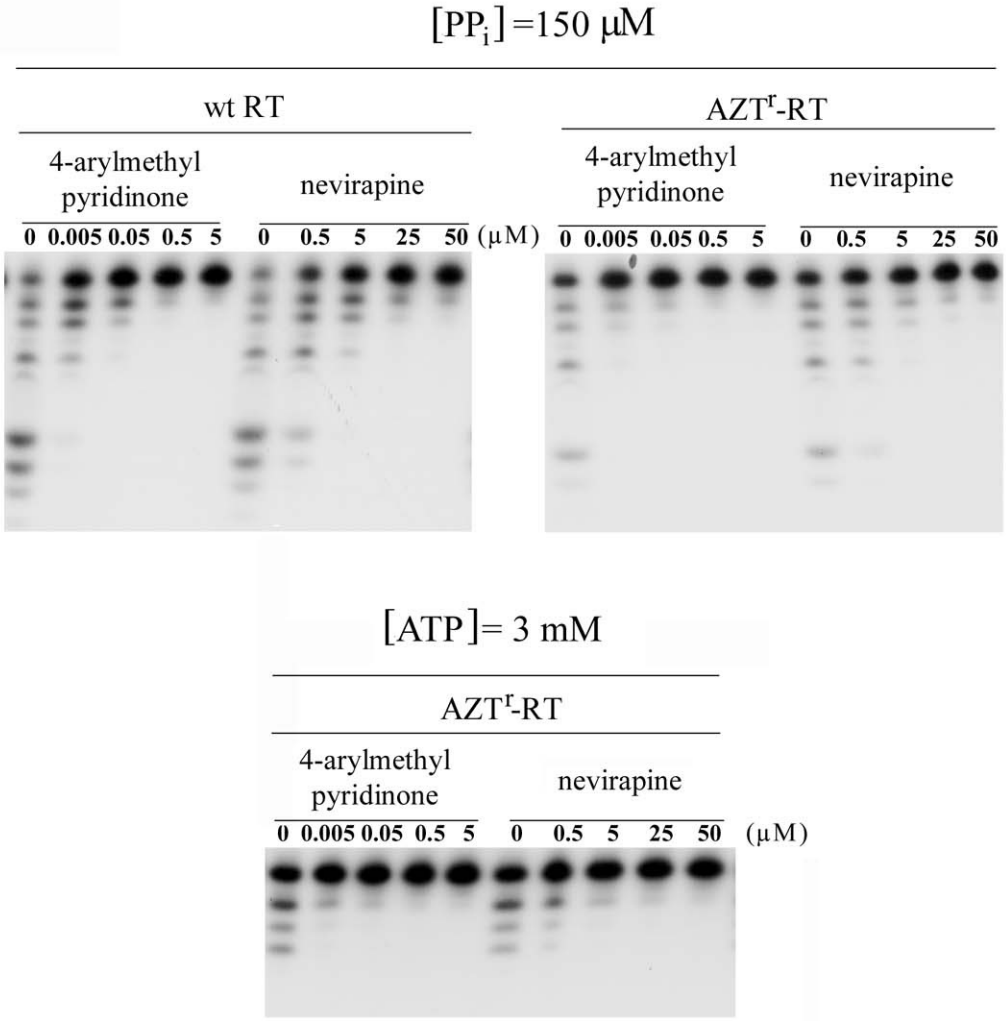


Figure 3, Odriozola et al.

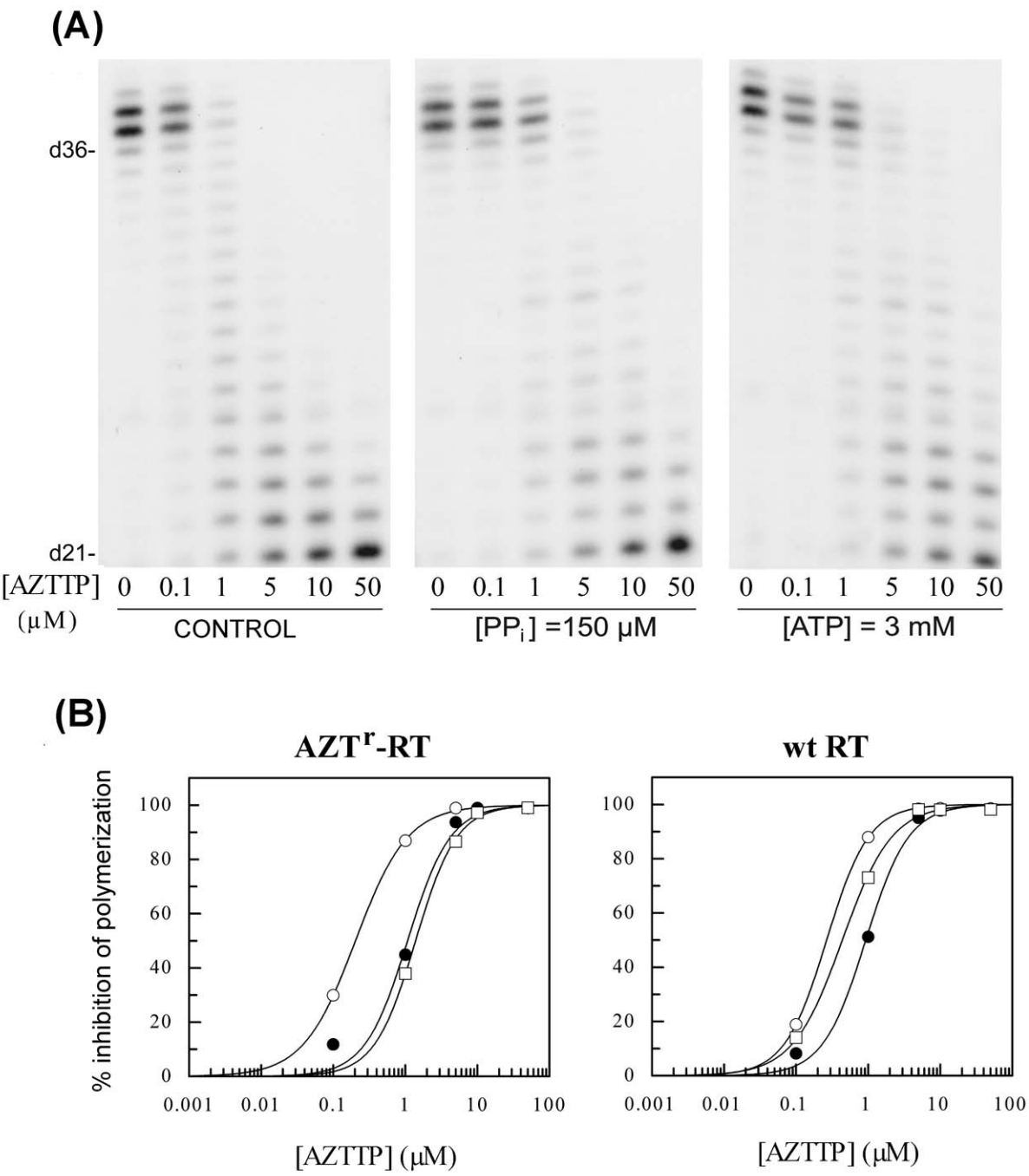
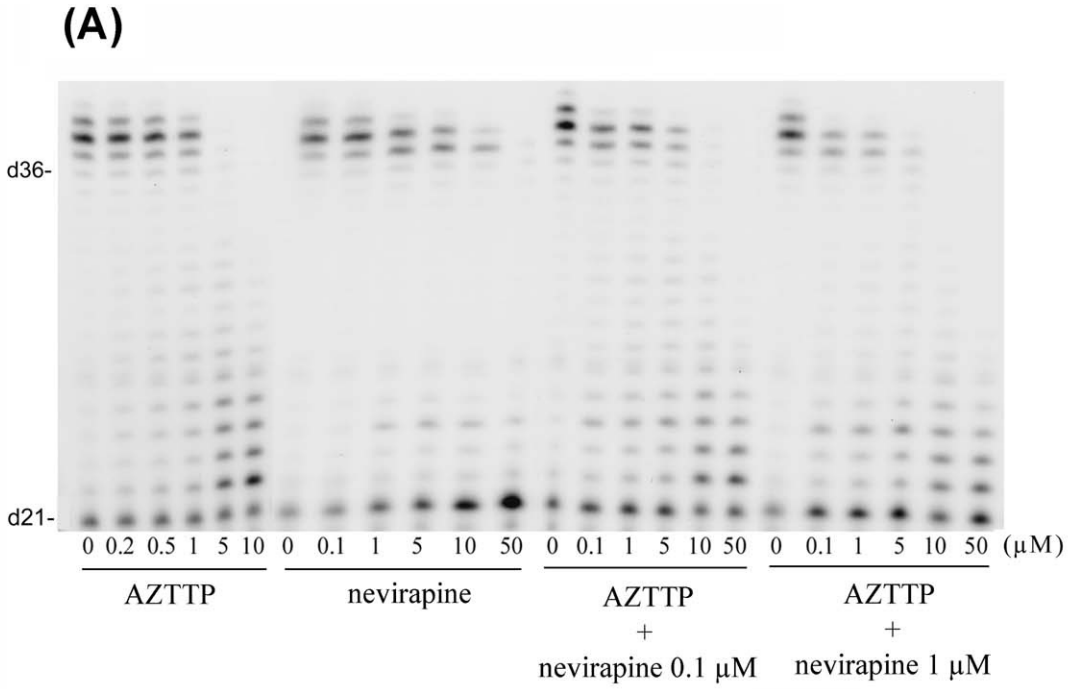
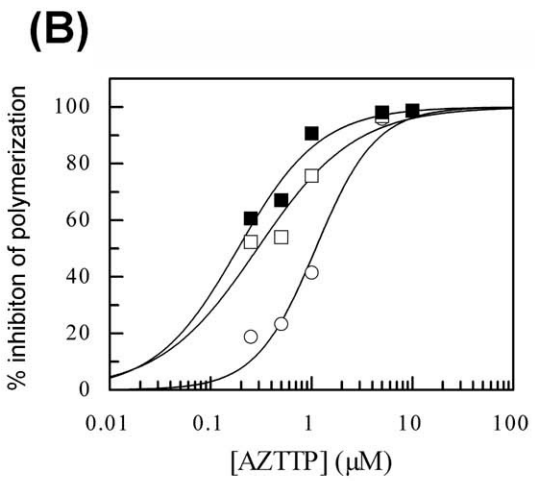


Figure 4, Odriozola et al.





(C)

Addition	IC_{50} (μM)
None	0.20 ± 0.01
PPi	1.00 ± 0.12
PPi + 0.1 μM nevirapine	0.30 ± 0.05
PPi + 1 μM nevirapine	0.19 ± 0.04

Figure 5, Odriozola et al.

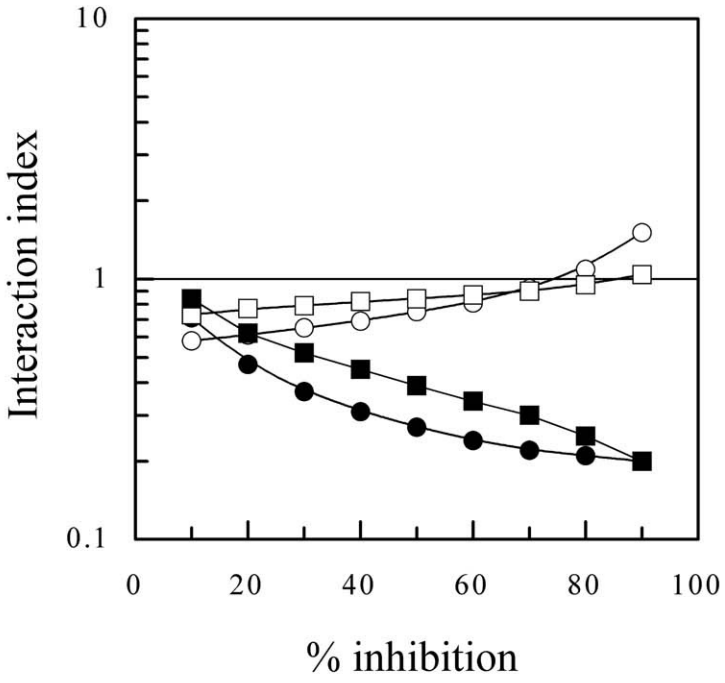


Figure 6, Odriozola et al.