Mechanism by Which Phosphonoformic Acid Resistance Mutations Restore 3’-Azido-3’-deoxythymidine (AZT) Sensitivity to AZT-resistant HIV-1 Reverse Transcriptase*

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The development of phosphonoformic acid (PFA) resistance against the background of 3’-azido-3’-deoxythymidine (AZT) resistance in human immunodeficiency virus type 1 (HIV-1) restores viral sensitivity to AZT. High level AZT resistance requires multiple mutations (D67N/K70R/T215F/K219Q). In order to characterize the mechanism of PFA resistance-mediated re-sensitization to AZT, the A114S mutation associated with PFA resistance was introduced into the reverse transcriptase (RT) of both wild type and drug-resistant virus. We previously showed that phosphorolytic removal of chain-terminating AZT is the primary mechanism of the AZT resistance phenotype (Arion, D., Kaushik, N., McCormick, S., Borkow, G., and Parniak, M. A. (1998) Biochemistry 37, 15908–15915). Introduction of A114S into the AZT resistance background significantly diminishes both the enhanced pyrophosphorolytic activity and the DNA synthesis processivity associated with the AZT-resistant RT. The A114S mutation also alters the nucleotide-dependent phosphorolysis activity associated with AZT resistance. The presence of the A114S mutation therefore severely impairs the mutant enzyme’s ability to excise chain-terminating AZT. The decrease in phosphorolytic activity of RT conferred by the PFA resistance A114S mutation resensitizes AZT-resistant HIV-1 to AZT by allowing the latter to again function as a chain terminator of viral DNA synthesis. These data further underscore the importance of phosphorolytic removal of chain-terminating AZT as the primary mechanism of HIV-1 AZT resistance.

The most widely used clinical therapeutic against the human immunodeficiency syndrome virus type 1 (HIV-1) is the nucleoside analogue 3’-azido-3’-deoxythymidine (AZT). Unfortunately, the long term utility of AZT therapy in HIV-1-infected individuals is limited by the emergence of drug-resistant strains, which exhibit up to 200-fold decreases in sensitivity to AZT compared with wild type (WT) virus. High level viral resistance to AZT correlates with multiple mutations in HIV-1 reverse transcriptase (RT), namely D67N, K70R, T215Y/F, and K219Q, (1), as well as M41L and L210W in some cases (2–4).

While the genotype for AZT resistance has been well characterized for more than a decade, the phenotypic mechanism of AZT resistance remained unclear. AZT inhibits primarily by acting as a chain terminator of viral DNA synthesis. In the past year, we and others have shown that phosphorolytic excision of chain-terminating AZT is an important feature of the AZT resistance mechanism. We first demonstrated that the mutant RT shows an enhanced rate of pyrophosphorolysis compared with wild-type drug-sensitive enzyme at physiologically relevant concentrations of PPi (5, 6). It was subsequently shown that nucleotide-dependent phosphorolysis is also enhanced in AZT resistance (7, 8). While these two mechanisms use different substrates for the phosphorolytic removal of chain-terminating AZT, the chemistry involved in both mechanisms is identical (6). The net result is the decreased ability of AZT to act as a chain terminator of viral DNA synthesis, resulting in HIV-1 resistance to the drug.

It has been known for some time that mutations in RT associated with HIV-1 resistance to foscartern (phosphonoformic acid; PFA) resensitize AZT-resistant virus to AZT (9–14). Since PFA is a pyrophosphate analog, we suspected that mutations conferring PFA resistance might affect the enhanced pyrophosphorolytic activity of AZT-resistant RT. We therefore introduced the PFA resistance A114S mutation into both WT and AZT-resistant mutant RT. In this report, we show that the A114S mutation, introduced into a background of AZT resistance, eliminates the increased phosphorolytic removal of chain-terminating AZT. Both pyrophosphorolysis and nucleotide-dependent phosphorolysis are inhibited by the A114S mutation. The A114S-mediated diminution of RT-catalyzed phosphorolysis allows AZT to again act as a chain terminator of viral DNA synthesis, thereby restoring antiviral activity of the drug against AZT-resistant HIV-1.

EXPERIMENTAL PROCEDURES

Materials—AZT triphosphate (AZTTP) was purchased from Moravek Biochemicals (Brea, CA). Phosphonoformic acid and inorganic pyrophosphate were from Sigma. [γ-32P]ATP, and the homopolymeric template-primer (TP) poly(rA)-oligo(dT)12–18 were obtained from Amersham Pharmacia Biotech. Heteropolymeric TP for the measurement of RT RNA-dependent DNA polymerase (RDDP) activity was prepared as described previously (15). All other reagents

AZTTP, AZT triphosphate; PAGE, polyacrylamide gel electrophoresis; nt, nucleotide(s).
of the highest quality available and were used without further purification.

Expression and Purification of WT and Mutant RT—The cloning of AZT-resistant RT has been described previously (5). The A114S mutant was introduced into WT and AZT-resistant RT using the Sculptron in vitro mutagenesis system (Amersham Pharmacia Biotech). The presence of the expected mutations was verified by sequencing of positive clones using the T7 sequencing kit from Amersham Pharmacia Biotech. Recombinant heterodimeric p66/p51 WT and mutant RT were expressed and purified as described previously (16). All enzyme preparations were more than 95% pure as assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Assays of RT DNA Polymerase Activity—HIV-1 RT RDDP activity was determined in a fixed time assay. Reaction mixtures (50 μl) containing 50 mM Tris-HCl (pH 7.8, 37 °C), 60 mM KCl, 10 mM MgCl2, 50 mM poly(rA)-oligo(dT)12–18, and varying concentrations of [3H]TTP. Reactions were initiated by the addition of 10–20 ng of RT. Reaction mixtures were incubated at 37 °C for 20 min and then quenched with 250 μl of ice-cold 10% trichloroacetic acid containing 20 mM NaPPi. Quenched samples were left on ice for 20 min and then filtered on Whatman 934-AH glass fiber filters and washed sequentially with 10% trichloroacetic acid containing 20 mM NaPPi and ethanol. The extent of radioactive incorporation was determined by liquid scintillation spectrometry.

PAGE Analysis of RT Continuous and Single Processive Cycle DNA Synthesis—Assay conditions were identical to those previously described (5). Briefly, WT and mutant RT were preincubated with 32P-labeled heteropolymeric RDDP T/P at 37 °C for 10 min. DNA synthesis was started by the addition of the appropriate combination of dNTP substrates (for continuous polymerization experiments), or dNTPs plus the polymerization trap heparin (0.2 mg/ml) for analysis of the processivity of RT-catalyzed DNA synthesis. After appropriate incubations at 37 °C, reactions were quenched by the addition of an equal volume of sequencing gel loading buffer (98% deionized formamide, 10 mM EDTA, and a 1 mg/ml concentration each of bromphenol blue and xylene cyanol). The samples were heated for 5 min at 100 °C and then analyzed by denaturing PAGE using 16% polyacrylamide gels containing 7 M urea. The electrophoretically resolved products were visualized by autoradiography and quantified by densitometry.

As assay of RT-catalyzed Pyrophosphorolysis—Pyrophosphorolysis was assessed as described previously (5). Briefly, RT (25 ng p66/p51 heterodimer) was preincubated with heteropolymeric RDDP T/P possessing 32P-labeled primer in 50 mM Tris-HCl (pH 7.8, 37 °C) containing 60 mM KCl and 10 mM MgCl2 for 10 min to allow the formation of the RT/T/P binary complex (5). Pyrophosphorolysis was initiated by the addition of 150 μM NaPPi. Aliquots were removed at various time intervals and added to equivalent volumes of sequencing gel loading buffer (see above) in order to stop the reaction. The samples were denatured at 100 °C for 5 min prior to denaturing PAGE resolution of products using 16% polyacrylamide, 7 M urea. The resolved products were visualized by autoradiography and quantified by densitometry.

RT-catalyzed DNA "Rescue" Synthesis Using an AZT-terminated Primer—A 24-nt DNA oligonucleotide termed RQ-1 (5'-CTG TTC GGG CGC CAC TGC TAG AGA-3') was labeled with [32P]ATP at the 5'-end and then annealed to a 72-nt DNA template oligonucleotide corresponding to the HIV-1 genomic sequence comprising the 18-nt primer binding site plus the next 54 downstream nucleotides. RQ-1 was then added to the 3'-end of the RQ-1 primer by incubation at 37 °C for 16 h with WT RT and AZTTP (10:1 ratio, to ensure completion of the reaction, as determined in control reactions). 32P-Labeled, AZT-terminated RQ-1 primer was purified by extraction of the appropriate band after denaturing gel electrophoretic separation. The labeled AZT-terminated RQ-1 primer was then annealed to the 72-nt template for use in rescue experiments.

RT-catalyzed phosphorolytic rescue DNA synthesis using AZT-terminated T/P was assessed by incubating 20 nM of RT with 40 nM chain-terminated T/P in 50 mM Tris-HCl, pH 7.8, 60 mM KCl, and 10 mM MgCl2. The reaction was initiated by the addition of either 150 μM pyrophosphate or 3 mM ATP (pretreated with inorganic pyrophosphatase to remove any contaminating PPi), 100 μM TTP, and 100 μM ddCTP. Under these conditions, the extended primer rescue DNA polymerization product was 4 nt longer than the starting AZT-terminated primer (excision of AZTTP; the addition of four TMP residues followed by termination with ddCMP). Aliquots were removed after various time intervals, quenched by the addition of an equal volume of sequencing gel loading buffer, and then analyzed by denaturing PAGE on 16% polyacrylamide, 7 M urea gels. Products were visualized by autoradiography and quantified by densitometry.

Results

Inhibition of WT and Mutant RT by AZT and PFA—All of the recombinant RT preparations used in these experiments (WT, A114S, D67N/K70R/T215F/K219Q, and A114S plus D67N/K70R/T215F/K219Q) had similar enzyme RDDP specific activities (data not shown). In addition, none of these showed any significant differences in affinity for dNTP substrates under normal RT RDDP assay conditions in the absence of PPi (Table I). As previously noted (5, 6, 17), the D67N/K70R/T215F/K219Q mutant RT was equally sensitive to inhibition by AZTTP as WT RT in vitro in the absence of PPi.

The A114S mutation provides a discernible resistance to PFA both in cell culture (9, 10) and in RT in vitro assays, when introduced into either WT or AZT-resistant mutant RT (Table I). Interestingly, the presence of the A114S mutation resulted in a 20-fold reduction in RT sensitivity to AZTTP inhibition in vitro whether in WT RT or in a background of the D67N/K70R/T215F/K219Q AZT resistance mutations (Table I).

DNA Polymerization of WT and Mutant RT under Continuous and Single Processive Cycle Conditions—The AZT resistance mutations T215F/K219Q provide increased DNA synthesis processivity to RT and are directly related to a decrease in T/P dissociation from the enzyme (5). As previously found, the D67N/K70R/T215F/K219Q mutant RT (Fig. 1, lane 2) shows significantly greater DNA synthesis processivity compared with WT RT (Fig. 1, lane 1). Introduction of the A114S mutation into the WT background had no effect on the RT-catalyzed DNA synthesis processivity (Fig. 1, compare lanes 1 and 2). In contrast, introduction of the A114S mutation into the D67N/K70R/T215F/K219Q mutant RT completely abolished the increased DNA polymerization processivity associated with the AZT-resistant RT (Fig. 1, lane 4).

Pyrophosphorolysis Catalyzed by WT and Mutant RT—The D67N/K70R/T215F/K219Q mutant RT shows a significant increase in the rate of pyrophosphorolysis compared with WT RT (5, 6). This phenotype is critical to the mutant enzyme's ability to remove chain-terminating AZT from the primer 3' terminus, thereby allowing reinitiation of viral DNA synthesis (5, 6). As seen in Fig. 2, A and B, introduction of the A114S mutation into the D67N/K70R/T215F/K219Q mutant RT significantly diminishes the pyrophosphorolytic activity of the AZT-resistant mutant enzyme.

TABLE I

| Data are the means ± S.D. from three or more independent experiments. |
| --- | --- | --- |
| **Km (μM)** | **Vmax (μM)** | **IC50 (μM)** |
| WT | A114S | D67N/K70R/T215F/K219Q | A114S | D67N/K70R/T215F/K219Q |
| dTTP | 6.6 ± 2.1 | 8.2 ± 1.4 | 6.9 ± 2.4 | 7.0 ± 0.3 |
| dGTP | 1.8 ± 0.3 | 1.9 ± 0.4 | 1.7 ± 0.02 | 2.1 ± 0.2 |
| PFA | 0.26 ± 0.04 | 0.85 ± 0.04 | 0.14 ± 0.02 | 0.87 ± 0.04 |
| AZT | 0.068 ± 0.002 | 0.068 ± 0.077 | 0.004 ± 0.001 | 0.006 ± 0.010 |
| ddCTP | 0.068 ± 0.007 | 0.105 ± 0.009 | 0.041 ± 0.003 | 0.138 ± 0.004 |
Ability of WT and Mutant RT to Carry Out Rescue DNA Synthesis from an AZT-terminated Primer—Removal of AZT-MP from a chain-terminated primer has been identified as a critical event in resistance to AZT (5–8). Therefore, primer extension reactions were carried out under conditions where the obligatory removal of AZT-MP preceded DNA polymerization. In these experiments, 100 μM of ddCTP was included in the reaction to terminate DNA polymerization after the incorporation of the first four nucleotides, in order to facilitate quantification of rescue DNA polymerization product. As shown in Fig. 3, neither WT nor mutant RT catalyzed the formation of rescue DNA polymerization product in the absence of PPi. However, when physiological concentrations of PPi were present, significant amounts of rescue DNA polymerization product were noted in reactions catalyzed by the D67N/K70R/T215F/K219Q mutant enzyme (Fig. 3, B and C). Similar results were noted when PPi was replaced with 3 mM ATP (Fig. 3, A and C). Introduction of the A114S mutation into the D67N/K70R/T215F/K219Q background completely eliminated the ability of RT to carry out rescue DNA synthesis from the AZT-terminated primer in the presence of PPi, or of ATP (Fig. 3, A–C).

Fig. 1. Continuous and single processive cycle DNA synthesis by WT and mutant RT. RT-catalyzed DNA synthesis was carried out as described under “Experimental Procedures.” The full-length DNA product was 191 nt in size (18-nt primer plus 173 nt added during DNA polymerization), corresponding to HIV-1 (-) strong stop DNA. Lanes 1–4 show DNA synthesis under single processive cycle conditions (with heparin polymerization trap); lanes 5–8 show DNA synthesis under continuous conditions (without heparin polymerization trap). Lanes 1 and 5, reactions catalyzed by WT RT; lanes 2 and 6, reactions catalyzed by A114S RT; lanes 3 and 7, reactions catalyzed by D67N/K70R/T215F/K219Q RT; lanes 4 and 8, reactions catalyzed by D67N/K70R/T215F/K219Q plus A114S RT.

Fig. 2. Pyrophosphorolysis catalyzed by WT and mutant RT. Pyrophosphorolysis kinetic experiments were carried out as described under “Experimental Procedures.” Pyrophosphorolysis was initiated by adding 150 μM PPi, to a solution containing preformed RT/P complexes. Aliquots were removed after 0-, 10-, 30-, 60-, and 90-min incubation at 37 °C. A, PAGE analyses of pyrophosphorolysis products. Pyrophosphorolysis by each of the indicated enzymes is shown by the disappearance of the 18-nt 3'-32P-labeled primer band and the appearance of smaller molecular size products. Each of the RT species used in this experiment had the same specific activity for forward direction DNA synthesis, as illustrated in lanes marked 1–4. These lanes correspond to WT, A114S, AZT-quad, and AZT-quad/A114S mutant RT, respectively. B, rates of pyrophosphorolysis. Shown is a semilog plot of the residual amount of the 18-nt 3'-32P-labeled primer bands (obtained by densitometric analysis of the bands shown in Fig. 2A) as a function of time of reaction. ○, WT RT; ■, A114S RT; ▲, AZT-quad RT; ▼, AZT-quad plus A114S RT. The first order rate constants for pyrophosphorolysis were calculated to be as follows: WT RT, 0.0078 min⁻¹; A114S RT, 0.0057 min⁻¹; AZT-quad RT, 0.0163 min⁻¹; AZT-quad plus A114S RT, 0.0078 min⁻¹.
The A114S mutation associated with PFA resistance results in a 20-fold decreased sensitivity to AZTTP in vitro, whether present against a WT background or on a background of the D67N/K70R/T215F/K219Q mutations in RT associated with high level resistance to AZT (Table I). It might thus be expected that the A114S mutation should actually increase HIV-1 resistance to AZT. In fact, viral clones that contain this mutation are hypersensitive to AZT (9, 10). Moreover, introduction of PFA resistance mutations such as A114S into AZT-resistant virus reseensitizes the virus to AZT (9–14).

It is now apparent that the primary mechanism of HIV-1 resistance to AZT involves an increased ability of the mutant viral RT to excise chain-terminating AZT from the primer 3'-terminus. The “unblocking” of the AZT-terminated primer is accomplished by phosphorylcleavage of the terminal AZT, mediated either by PPi (pyrophosphorylation) (5, 6) or ATP (ribo-nucleotide-dependent phosphorylation) (7, 8).

Under the conditions used in our experiments, it appeared that 150 μM PPi was more effective than 3 mM ATP in allowing the D67N/K70R/T215F/K219Q mutant RT to carry out rescue DNA synthesis from an AZT-terminated primer (Fig. 3), suggesting a preference for pyrophosphorylation. Nonetheless, both pyrophosphorylation and ATP-dependent phosphorylation are able to restore DNA synthesis, and we cannot predict which mechanism would be preferentially utilized in vivo. What is unequivocal however, is that the presence of the A114S mutation completely eliminates the ability of the AZT-resistant enzyme to carry out rescue DNA synthesis from an AZT-terminated primer. The A114S mutation is equally effective at preventing both pyrophosphorylation and ATP-mediated phosphorylation. This is not surprising given that the chemistry of these phosphorylation reactions is identical. Nucleophilic attack of a polyphosphate oxygen on the phosphodiester bond between the last two nucleotides of the primer results in the excision of the 3'-terminal nucleotide, either as nucleotide triphosphate (due to pyrophosphorylation) or as a dinucleotide tetr phosphosphate (from ATP-mediated phosphorylation).

The A114S mutation also eliminates the increased DNA synthesis processivity of the D67N/K70R/T215F/K219Q enzyme, in addition to diminishing the rate of phosphorylation carried out by the AZT-resistant mutant enzyme. We have previously shown that increased RT-catalyzed DNA synthesis processivity directly correlates with decreased RT/TPP dissociation (5, 18). Furthermore, T/P possessing 3'-terminal AZT dissociate more slowly from the D67N/K70R/T215F/K219Q mutant enzyme than do T/P lacking the chain-terminating AZT (19). We suggest that the A114S mutation counteracts the effect of the AZT resistance, thereby restoring WT T/P dissociation rates. The resulting decreased RT resident times of the 3'-AZT chain-terminated T/P would then provide less opportunity for phosphorylytic removal of the 3'-chain-terminating AZT.

The net result of the diminished rate of phosphorylation and the increased rate of AZT-terminated T/P dissociation from RT containing the A114S mutation is a restoration of the chain-terminating activity of AZT, despite the continued presence of the D67N/K70R/T215F/K219Q mutations in the enzyme.

The precise structural basis by which A114S acts to decrease both the phosphorylytic activity and DNA synthesis processivity of the D67N/K70R/T215F/K219Q mutant RT is unclear. Ala$^{114}$ is one of four highly conserved amino acids (Asp$^{113}$, Ala$^{114}$, Tyr$^{115}$, and Gln$^{151}$) that form a “3-pocket,” which accommodates the 3'-OH of the incoming dNTP (20). The A114S mutation may alter the structural dynamics of this pocket such that the binding of AZTTP and incorporation of AZTMP into nascent viral DNA is selectively impaired. This is consistent with the 20-fold decreased sensitivity to AZTTP shown by RT with the A114S mutation (Table I). In contrast, ddTTP inhibition of the A114S RT is only slightly reduced (2-fold), and affinities for dNTP substrates are unaffected. However, it is difficult to understand how a selective decrease in AZTTP binding could contribute to resensitization of AZT-resistant HIV-1 to AZT. Residue Ala$^{114}$ also interacts with the dNTP β-phosphate in the RT/TαdNTP ternary complex (20). Some studies have suggested that mutagenesis of RT residues interacting with the dNTP phosphates may directly affect RT pyrophosphorylytic activity (21, 22).

In addition to the A114S mutation, a number of other resistance mutations appear to resensitize AZT-resistant HIV-1 to AZT. For example, the L74V mutation that appears under 3'-3'-dideoxynosine (ddI) drug pressure has been reported to restore phenotypic sensitivity to AZT in AZT-resistant virus strains (20). PFA appears to select for a variety of resistance

Figure 3. Rescue DNA polymerization by WT and mutant RT initiated from an AZT-terminated primer. Experiments were carried out as described under “Experimental Procedures.” AZT-terminated T/P was incubated with RT for 5 min at 37 °C, and reactions were carried out as described under “Experimental Procedures.” AZT-terminated primer. The “unblocking” of the AZT-terminated primer is accomplished by phosphorylcleavage of the terminal AZT, mediated either by PPi (pyrophosphorylation) (5, 6) or ATP (ribonucleotide-dependent phosphorylation) (7, 8).
mutations in RT in addition to A114S, including substitutions at amino acids 88, 89, 90, 92, and 113. All of these suppress HIV-1 resistance to AZT when introduced into the background of the D67N/K70R/T215F/K219Q mutations (13). It is tempting to speculate that all of these mutations diminish the increased phosphorolytic activity of the AZT-resistant RT and that such diminution may be a general mechanism for the resensitization of AZT-resistant HIV-1 to AZT. In this respect, our recent findings with the M184V mutation, which confers HIV-1 resistance to 2',3'-dideoxy-3'-thiacytidine (3TC), are of interest. The appearance of the M184V mutation in AZT-resistant HIV restores viral sensitivity to AZT (24). We have recently found that the M184V mutation dramatically reduces the ability of RT to carry out PP1- or ATP-mediated phosphorolytic removal of chain-terminating AZT.3 This further underscores the importance of phosphorolysis in the AZT resistance mechanism and may account for the prolonged therapeutic benefit associated with AZT plus 3TC combination therapy.

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