Probing the Molecular Mechanisms of AZT Drug Resistance Mediated by HIV-1 Reverse Transcriptase Using a Transient Kinetic Analysis†

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ABSTRACT: Several hypotheses have been proposed to explain the development of resistance to the anti-HIV drug AZT. Clinical findings show that AZT resistance mutations in HIV-1 reverse transcriptase (RT) not only reduce susceptibility to thymidine analogues but may also confer multi-dideoxynucleoside resistance. In this report, we describe transient kinetic studies establishing the biochemical effects of AZT resistance mutations in HIV-1 RT on the incorporation and removal of natural and unnatural deoxynucleotides. While the physiological role remains to be elucidated, the largest biochemical difference between wild-type and AZT resistant HIV-1 RT manifested itself during ATP-mediated deoxynucleotide removal. Enhanced removal resulted from an increase in the maximum rate of chain terminator excision, suggesting that mutated residues play a role in the optimal alignment of substrates for ATP-mediated removal. The efficiency of pyrophosphorolysis was not increased by the presence of AZT resistance mutations. However, a 2-fold decrease in the extent of inhibition caused by the next correct nucleotide during pyrophosphorolytic cleavage of a D4TMP chain-terminated primer may illustrate how this mutant can utilize pyrophosphate to enhance resistance. The inability of RT to catalyze removal of a chain terminator from an RNA−RNA primer−template may show how slight changes in selectivity against AZTMP incorporation during the initiation of DNA synthesis can contribute to high-level resistance. Taken together, these results suggest that multiple modes of resistance may be conferred by these mutations. Structure−activity studies of chain terminator removal suggest that analogues that form tight interactions with residues in the RT active site may be more prone to resistance mechanisms mediated by removal.

Human immunodeficiency virus (HIV), the etiological agent of acquired immunodeficiency syndrome (AIDS), requires reverse transcriptase (RT)[1] to copy its single-stranded RNA genome into a double-stranded DNA copy for integration into the host cell genome during the early stages of viral infection. Although almost all aspects of the HIV-1 life cycle have been targeted (*2*−*4*), a majority of the drugs that have been effective are nucleoside reverse transcriptase inhibitors (NRTIs) which lack a 3′-hydroxyl group and serve to chain-terminate viral transcripts. However, treatment with NRTIs is limited by their toxicity to the host [often through their interaction with mitochondrial DNA polymerase γ (*5*−*8*)] and the ability of the virus to mutate and acquire resistance (*9*). Other factors that govern the efficacy of these inhibitors include uptake, transport, metabolism, and incorporation of the drugs (*10*).

Several hypotheses have been put forth to explain the decreased susceptibility of HIV to AZT following prolonged treatment. Some of these theories stem from cellular factors, including kinases (*11*−*14*), transporters (*15*, *16*), and exonucleases (*17*, *18*). While these factors potentially play important roles in drug sensitivity, high-level AZT resistance most likely resides within the virally encoded RT. The high

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[1] Abbreviations: RT, reverse transcriptase; NRTI, nucleoside reverse transcriptase inhibitor; dNMP, 2′-deoxynucleoside 5′-monophosphate; dNTP, 2′-deoxynucleoside 5′-triphosphate; abacavir, (1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol succinate; AZT, β-D-(+)-3′-azido-3′-deoxythymidine; CBV, carbovir; D4T, β-D-(+)-2′,3′-didehydro-3′-deoxythymidine; 3TC, β-L-(−)-2′,3′-dideoxy-3′-thiacytidine; ddC, β-D-(+)-2′,3′-dideoxycytidine; D4C, β-D-(+)-2′,3′-didehydro-2′,3′-dideoxycytidine; PMPA, (*R*)-9-(2-phosphonylmethoxypropyl)adenine; PP_i, pyrophosphate; PP_ilysis, pyrophosphorolysis; phys, values that represent kinetic constants at physiological concentrations of PP_i (125 μM) or ATP (3 mM, concentrations from ref *1*); WT, wild type; TAMs, thymidine-associated mutations; AZTR, AZT resistant quadruple mutant containing D67N, K70R, T215Y, and K219Q mutations. The suffixes -MP and -TP are added to the drug abbreviations to indicate their monophosphate and triphosphate forms, respectively.

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rate of HIV replication and the lack of proofreading by RT during reverse transcription lead to frequent mutations (*19*−*22*). One class of mutations includes those originally found to confer resistance to AZT (*23*, *24*). Later findings showed that these mutations also decreased sensitivity to D4T in patients (*13*) despite little or no cross resistance in cell culture (*25*). Their association with thymidine analogues led them to be termed thymidine-associated mutations (TAMs). Common TAMs include M41L, D67N, K70R, L210W, T215Y/F, and K219Q (*23*, *26*). Reports have suggested these mutations cause resistance through slight decreases in the level of AZTMP incorporation during RNA-directed processes (*27*, *28*) and an increase in the extent of chain terminator removal by ATP (*29*) or pyrophosphate (PP_i) (*30*). Removal reactions release the chain terminator as either a nucleoside triphosphate or a nucleoside tetraphosphate linked to adenosine for PP_i- or ATP-mediated removal reactions, respectively (*29*). Although each of these hypotheses superficially explains AZT resistance, there has been much discussion about their individual contributions to resistance observed during clinical treatment.

Adding more complexity to the understanding of TAMs is the finding that the presence of various combinations of these mutations correlates with the reduced activity of non-thymidine analogues, including abacavir, a prodrug of the guanosine analogue carbovir (CBV) (*31*, *32*), and PMPA, an acyclic adenosine monophosphate analogue (*33*). In fact, characteristic TAMs have been found in the resistance mutation spectra for all NRTIs currently available in the clinic. Therefore, TAMs appear to be involved in multidrug resistance, and the prevalence of these mutations poses a serious threat to the efficacy of nucleosides already approved by the Food and Drug Administration as well as those being developed as future treatments.

This report is a quantitative exploration of the underlying mechanisms that govern the resistance of TAM-containing RT to AZT and other compounds. Using a pre-steady-state kinetic approach, we studied PP_i- and ATP-mediated deoxynucleotide removal as well as AZTMP incorporation into a model initiation complex containing synthetic $tRNA_3^{Lys}$. Observations made during these studies suggest that ATP- and PP_i-mediated deoxynucleotide removal, as well as increased selectivity against AZTMP incorporation during RNA-directed processes, may all contribute to high-level resistance to AZT. Studies aimed at understanding how structural features of the deoxynucleotide analogue affect removal suggest that analogues with the potential to form strong interactions in the RT active site may be more prone to excision.

MATERIALS AND METHODS

Preparation of HIV-1 RT. RT^{WT} and RT^{AZTR} clones were generously provided by S. Hughes, P. Boyer, and A. Ferris (Frederick Cancer Research and Development Center, Frederick, MD). The N-terminal histidine-tagged heterodimeric p66 and p51 enzymes were purified as previously described (*34*, *35*). Protein active site concentrations were determined using pre-steady-state burst experiments where the primer−template (300 nM) is in slight excess over RT (100 nM total) and the amplitude of the initial exponential phase of product formation is correlated with the amount of active protein in the experiment (*36*). This methodology allowed for the accurate normalization of protein concentrations of different mutants and protein preparations. It is imperative that an accurate determination of protein active site concentration be carried out to allow for meaningful comparisons between wild-type and mutant RT.

Table 1: Sequences of Primers and Templates Used To Study Incorporation and Removal

model system	Name	Sequence
Initiation	syn $tRNA_3^{Lys}$ (76-mer)	5′-..... G UCC CUG UUC GGG CGC CA
	RNA 19-AZTMP[a]	5′- G UCC CUG UUC GGG CGC CAC Z[b]
	RNA 36-mer[a]	3′-CGA AAG UCC AGG GAC AAG CCC GCG GUG ACG AUC UCU
DNA/DNA and DNA/RNA	DNA 22-dNMP	5′-GCC TCG CAG CCG TCC AAC CAA CX[c]
	DNA 23-dNMP	5′-GCC TCG CAG CCG TCC AAC CAA CTY[d]
	DNA 45-mer	3′-CGG AGC GTC GGC AGG TTG GTT GN[e]G TTG GAG CTA GGT TAC GGC AGG
	RNA 45-mer	3′-CGG AGC GUC GGC AGG UUG GUU GAG UUG GAG CUA GGU UAC GGC AGG
Aberrant Replication	DNA 30C	5′-GCC TCG CAG CCG TCC AAC CAA CTC AAC CTC C
	DNA 45R	3′-CGG AGC GTC GGC AGG TTG GTT GAG TTG CAG CGA GGT TAC GGC AGC
	DNA 21-mer	5′- TTG GTT GGA CGG CTG CGA GGC
	DNA 22-mer	3′-C AAC CAA CCT GCC GAC GCT CCG

[a] DNA 36-mer has the same sequence with 2′-deoxyribonucleotides and U's changed to T's. [b] Z is AZTMP. [c] X is AZTMP, D4TMP, dCMP (D23-mer), ddCMP, 3TCMP, or D4CMP. [d] Y is dCMP (D24-mer) or dCMPαS. [e] N is A (D45). N is G (D45C).

Nucleoside Triphosphates. Natural 2′-deoxynucleotides were purchased from Amersham. AZTTP and D4TTP were obtained from Moravek Biochemicals.

Oligonucleotides. Primers and templates used for incorporation and removal studies are shown in Table 1, and all of the DNA oligos were synthesized on an Applied Biosystems DNA synthesizer (Keck DNA synthesis facility, Yale University, New Haven, CT) and purified using 20% polyacrylamide denaturing gel electrophoresis. The R18-, R36-, and R45-mers were synthesized and purified by New England Biolabs. Chain-terminated oligos were made by incorporation with RT by previously reported methods (*7*). DNA and RNA primers were 5′-^{32}P-labeled with T4 polynucleotide kinase (New England Biolabs) as previously described (*36*). [γ-^{32}P]ATP was purchased from Amersham. Biospin columns for the removal of excess [γ-^{32}P]ATP were purchased from Bio-Rad. Synthetic $tRNA_3^{Lys}$ was T7 transcribed from cut plasmid kindly provided by J. Pata and T. A. Steitz (Yale University). Transcription was followed by 5′- and 3′-hammerhead ribozyme cleavage to create homogeneous ends by methods previously described (*37*, *38*). Synthetic $tRNA_3^{Lys}$ was labeled with ^{32}P by internal labeling during transcription by the addition of 250 μCi of each α-labeled NTP (1 mCi total). [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]CTP, and [α-^{32}P]UTP were purchased from Amersham.

Annealing of the DNA and RNA primer−templates was carried out by adding a 1:1.4 molar ratio of purified primer

to template at 90 °C for 5 min, 50 °C for 10 min, and 0 °C for 10 min. The annealed primer and template were then analyzed using 15% nondenaturing polyacrylamide gel electrophoresis to ensure complete annealing. Concentrations of the oligonucleotides were estimated by UV absorbance at 260 nm using calculated extinction coefficients.

Pre-Steady-State Removal Assays. ATP- and PP_i-mediated removal were studied as previously described (*39*) by measuring the decrease in the length of the primer either at a physiological concentration of PP_i (125 μM) or ATP (3 mM) or by determining the dependence of concentration on rate by titrating the removing agent. Pyrophosphate exchange experiments for studying the removal of AZTMP were performed as described previously (*40*, *41*) by assessing the exchange of ^{32}P-labeled PP_i into an unlabeled pool of AZTTP during the process of incorporation and removal by HIV-1 RT. The reaction mixture contained 250 nM D22−D45, 20 μM AZTTP, 10 mM $MgCl_2$, 500 nM RT, and varying concentrations of ^{32}P-labeled PP_i. Formation of the labeled AZTTP was assessed by thin-layer chromatography using PEI-cellulose plates and 0.3 M potassium phosphate buffer (pH 8.0) as a mobile phase. The products were visualized and quantitated by phosphorimaging on a Bio-Rad GS-525 Molecular Imager System.

The inhibition constant (K_i) for the next correct nucleotide on removal was determined by examining PP_i binding in the presence of different concentrations of the next correct nucleotide (dCTP). Two approaches were used to determine the concentration necessary to inhibit ATP-mediated removal by 50% (IC_{50}): (i) the decrease in primer length measured directly (as described above) or (ii) a "limited rescue" methodology (described below). Comparison of the rates obtained from the two methodologies revealed similar results (data not shown). For limited rescue, 10 μM TTP was added as well as varying concentrations of the next correct nucleotide. A physiological concentration of ATP (3 mM) was used to stimulate removal. The ATP was pretreated with pyrophosphatase to ensure that there was no contaminating PP_i that would complicate interpretation of results. These experiments allowed for the quantitation of dNMP removal by assessing the elongation of the primer. A distinct advantage of the limited rescue methodology over direct measurement of removal by decreased primer length was that the reactions were found to go to completion, allowing for more precise quantitation. This method also eliminated possible interference from the presence of all four dNTPs and/or the addition of a separate polymerase for elongation. The IC_{50} was obtained by plotting the percent inhibition versus the concentration of next correct nucleotide and fitting the data to hyperbolic curves.

Pre-Steady-State Single-Turnover Incorporation Experiments. Rapid chemical quench experiments were performed as previously described with a KinTek Instruments model RQF-3 rapid-quench-flow apparatus (*34*, *36*). A pre-steady-state kinetic analysis was used to examine incorporation. The reactions were carried out by rapidly mixing a solution containing the preincubated complex of 250 nM HIV-1 RT (active site concentration) and 50 nM 5′-labeled duplex with a solution of 10 mM $MgCl_2$ and varying concentrations of the next correct dNTP in the presence of 50 mM Tris-HCl and 50 mM NaCl at pH 7.8 and 37 °C (all concentrations represent the final concentration after mixing). Single-turnover experiments were also used to study incorporation into the synthetic $tRNA_3^{Lys}$ as previously described (*27*). This required the addition of both dCTP (2 mM) and varying concentrations of TTP or AZTTP. Control experiments showed dCMP incorporation not to be rate-limiting (data not shown). Products and reactants were then separated on a 10% polyacrylamide gel containing 7 M urea and 40% formamide and visualized and quantitated using phosphorimaging.

Data Analysis. Data were fit by nonlinear regression using the program KaleidaGraph (Synergy Software, Reading, PA). Single-turnover incorporation and removal experiments were fit to single-exponential equations: $[\text{product}] = A[1 - \exp(-k_{obsd})]$ and $[\text{product}] = A[\exp(-k_{obsd}t)]$, respectively, where A is the amplitude of product formation and k_{obsd} is the observed rate at a specific substrate concentration. The dissociation constant (K_d) of the substrate (dNTP, PP_i, or ATP) binding to the complex of RT and the primer−template during dNMP incorporation or removal was calculated by fitting observed rate constants at different concentrations of dNTP to the hyperbolic equation $k_{obsd} = k_{pol}[\text{dNTP}]/(K_d + [\text{dNTP}])$, where k_{pol} is the maximum first-order rate constant for dNMP incorporation and K_d is the equilibrium dissociation constant for the productive interaction of dNTP with the E·DNA complex. PP_i exchange rates were calculated by the previously derived equation (*42*) $k_{exchange} = \{-[[PP_i]-[\text{AZTTP}]/([PP_i] + [\text{AZTTP}])](1/T)\ln(1 - F)\}/[\text{complex}]$, where F is the fraction of isotopic equilibrium at time T and [complex] is the concentration of enzyme-bound DNA (250 nM). A slope of the linear region from the plot of $\ln(1 - F)$ versus T is a function of the rate of isotope exchange.

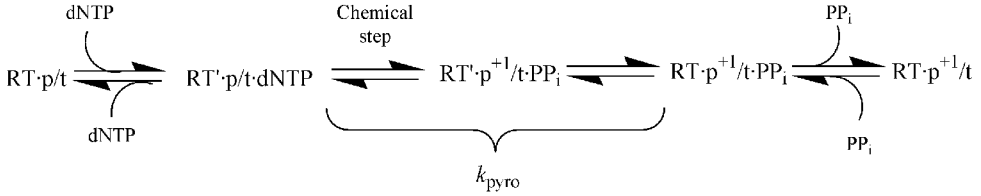
To determine the mechanism of inhibition of PP_i-mediated removal by the next correct nucleotide, hyperbolic curves were fit to the rate of PP_i lysis removal versus concentration for the data generated in the absence or presence of increasing concentrations of the next correct nucleotide. A significant increase in the K_d or decrease in the k_{pol} indicated competitive or noncompetitive mechanisms of inhibition, respectively. Consistency was observed for the mechanism of inhibition at differing concentrations of the next correct nucleotide for a specific RT enzyme and chain-terminated primer−template substrate. K_i values were determined by fitting the data generated in the presence of inhibitor to the hyperbolic binding equation for competitive $\{k_{obsd} = (k_{pol}[\text{dNTP}])/[[\text{dNTP}] + K_d(1 + [I]/K_i^{com})]\}$, noncompetitive $\{k_{obsd} = [(k_{pol}[\text{dNTP}])/(1 + [I]/K_i^{non})]/(K_d + [\text{dNTP}])\}$, or mixed inhibition $\{k_{obsd} = [(k_{pol}[\text{dNTP}])/(1 + [I]/K_i^{non})]/[[\text{dNTP}] + K_d(1 + [I]/K_i^{com})]\}$. Brackets denote concentration, and [I] equals the concentration of inhibitor at which the binding curve was determined. Reported errors represent the deviation of points from the curve fit generated by KaleidaGraph or were calculated by standard statistical analysis (*43*).

RESULTS

Mechanism of Nucleotide Removal by HIV-1 RT. The first step in understanding AZT resistance is to fully define the mechanism of nucleotide incorporation and/or removal by RT. Previous reports provide information about the forward and reverse reactions catalyzed by HIV-1 RT (*36*, *44*−*46*). The small changes with respect to incorporation for AZT resistant RT (RT^{AZTR}) (*27*, *28*, *47*, *48*) further focus attention

Scheme 1: Models for the Kinetic Mechanism of PP_i Release after dNMP Incorporation by HIV-1 RT

Model 1: $k_{PPilysis} = k_{PPi\ exchange}$. PP_ilysis rates are valid



Model 2: $k_{PPilysis} < k_{PPi\ exchange}$. PP_i can bind to an activated form of the enzyme just after incorporation and pyrophosphorolysis rates may represent an irrelevant conformational change.

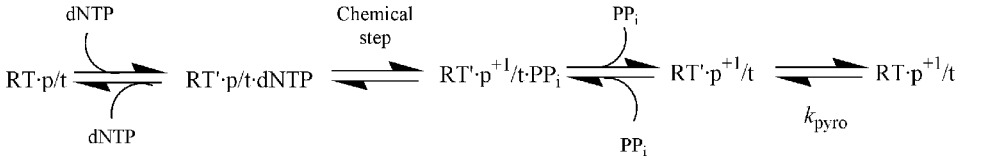


Table 2: Phosphorothioate Effect for the Forward versus Reverse Reaction and PP_ilysis versus PP_i Exchange by RT^{WT}

reaction	forward k_{obsd} (s^{-1})
D23 + dCMP	1.7 ± 0.1
D23 + dCMPαS	1.3 ± 0.1
reaction	**reverse initial velocity (nM/s^{-1})[a]**
D24 − dCMP	0.28
D24 − dCMPαS	0.043
reaction	**k_{obsd} (s^{-1})**
PP_ilysis[b]	0.010 ± 0.002
PP_i exchange[b]	0.014 ± 0.001

[a] The velocity before the rapid attainment of equilibrium. [b] PP_ilysis and PP_i exchange reactions were carried out in the presence of 125 μM PP_i.

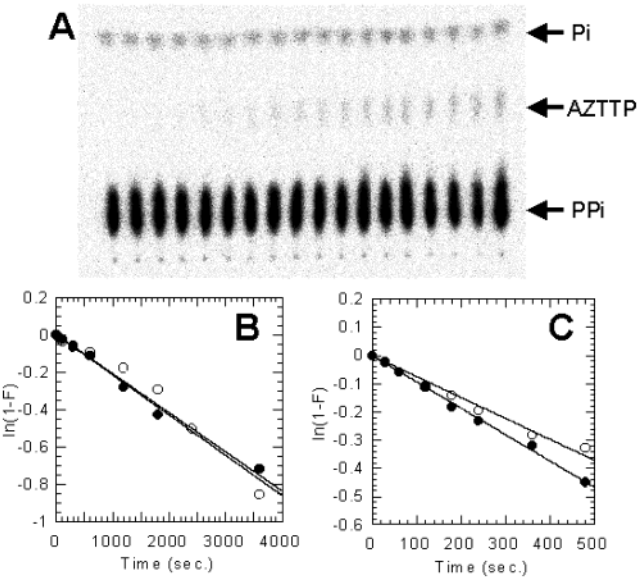


FIGURE 1: Determination of the PP_i exchange rate. (A) Products from a PP_i exchange reaction in which ^{32}P-labeled PP_i was exchanged into an unlabeled pool of AZTTP during rounds of incorporation and removal were separated using thin-layer chromatography. The positions of PP_i, AZTTP, and contaminating P_i are labeled. (B) Rate of PP_i exchange for RT^{WT} (●) and RT^{AZTR} (○) in the presence of 125 μM PP_i. The rates of exchange (k_{ex}) were 0.014 ± 0.001 and 0.015 ± 0.001 s^{-1} for RT^{WT} and RT^{AZTR}, respectively. (C) PP_i exchange in the presence of 1 mM PP_i. k_{ex} equaled 0.073 ± 0.002 and 0.063 ± 0.001 s^{-1} for RT^{WT} and RT^{AZTR}, respectively.

on the reverse reaction as a possible means of resistance. One aspect of the mechanism of removal that has not been thoroughly addressed is whether a rate-limiting step precedes or occurs after pyrophosphate (PP_i) release after the incorporation of deoxynucleotide (Scheme 1). Model 2 is potentially interesting because PP_i binding to the RT·primer−template complex in an activated form may allow for more rapid removal. This faster step could be masked by earlier slower steps in experiments that were done with pre-chain-terminated primer−templates, yielding data irrelevant to the physiologically pertinent removal reaction.

A comparison of the rates of pyrophosphorolysis (PP_ilysis) and PP_i exchange has proven to be useful in addressing the mechanism of the reverse reaction for other DNA polymerases (*40*, *41*). To distinguish between the two models, the PP_ilysis rate for AZTMP removal was compared to the rate of exchange of $^{32}PP_i$ with unlabeled AZTTP during rounds of both incorporation and removal with D22−D45 (primers and templates shown in Table 1). The similarity in rates of PP_ilysis and PP_i exchange (Table 2) suggests that, as for T7 DNA polymerase (*40*) and DNA polymerase I (*41*), the rate-limiting step occurs before PP_i release, suggesting that model 1 correctly reflects the mechanism followed by RT^{WT}. To determine if mutations in RT^{AZTR} caused any changes in the rate of PP_ilysis or PP_i exchange, similar experiments were carried out with the mutant enzyme. Rates of PP_i exchange by RT^{WT} and RT^{AZTR} were found to be similar (Figure 1A−C). These results further suggest that studies looking directly at the removal reaction from chain-terminated primers used throughout this paper were valid.

Large phosphorothioate elemental effects have previously been thought to indicate a rate-limiting chemical step (*40*, *49*), although results call into question the expected magnitude of the rate change (*50*). Experiments were carried out in an effort to examine the effects of a nonbridging sulfur atom on single-turnover incorporation and PP_ilysis in the presence of dCTPαS and dCMPαS-terminated primer, respectively. An excess of enzyme and high concentrations of both enzyme and primer−template were used to limit the

Table 3: Kinetic Parameters for AZTMP Removal during Various Stages of Replication

primer−template	substrate	protein	k_{rem} (s^{-1})[a]	K_d (mM)	efficiency[b]	k_{phys} (s^{-1})[c]
DNA−DNA	PP_i	WT	0.15 ± 0.02	0.97 ± 0.36	0.15	0.017
		AZTR	0.22 ± 0.03	4.2 ± 1.2	0.052	0.0064
	ATP	WT	0.00056 ± 0.00004	0.87 ± 0.23	0.00064	0.00043
		AZTR	0.0028 ± 0.0002	0.32 ± 0.10	0.0088	0.0025
DNA−RNA	PP_i	WT	0.098 ± 0.006	1.8 ± 0.3	0.054	0.0064
		AZTR	0.014 ± 0.001	0.21 ± 0.04	0.067	0.0052
	ATP	WT	0.00017 ± 0.00001	—[d]	—[d]	0.00015
		AZTR	0.0022 ± 0.0003	1.3 ± 0.5	0.0017	0.0015
RNA−RNA	PP_i/ATP	WT/AZTR	no removal observed after 3 h			

[a] k_{rem} is the maximum rate of nucleotide removal. [b] Efficiency equals k_{rem}/K_d and is expressed in units of s^{-1} mM^{-1}. [c] k_{phys} is the calculated rate of removal at a physiological concentration of the appropriate substrate (125 μM PP_i or 3 mM ATP). [d] The kinetic parameter could not be accurately determined because of insufficient amplitude.

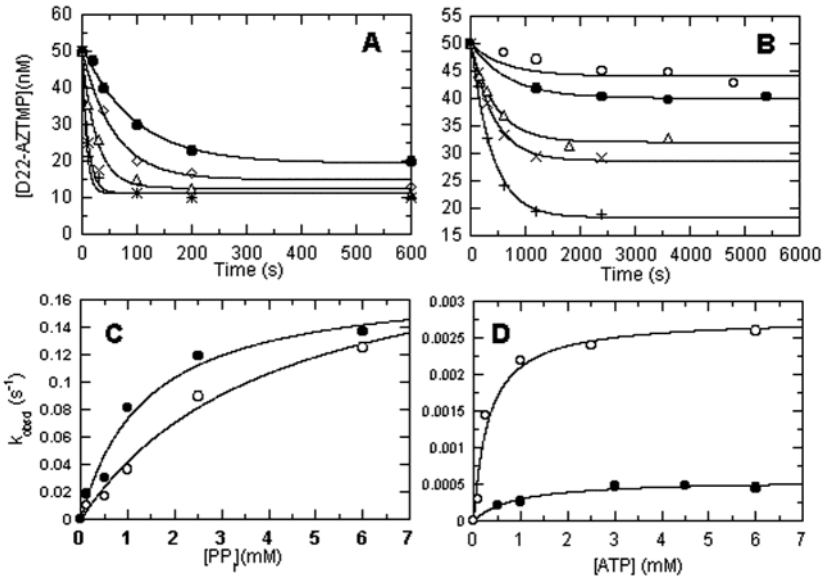


FIGURE 2: Removal of AZTMP from the DNA−DNA primer−template by RT^{WT} and RT^{AZTR}. (A) Family of curves for the removal of AZTMP by RT^{AZTR} and 0.125 (●), 0.5 (◇), 1 (△), 2.5 (×), and 6 mM PP_i (+). (B) Family of curves for the removal of AZTMP by RT^{AZTR} by 0.1 (○), 0.25 (●), 1 (△), 2.5 (×), and 6 mM ATP (+). (C) Dependence of the first-order rate constant (k_{obsd}) for AZTMP removal on PP_i concentration for RT^{WT} (●) and RT^{AZTR} (○). Data fit to hyperbolic curves giving maximum rates of removal (k_{rem}) of 0.15 ± 0.02 and 0.22 ± 0.03 s^{-1} and dissociation constants (K_d) of 0.97 ± 0.36 and 4.2 ± 1.2 mM for RT^{WT} and RT^{AZTR}, respectively. (D) Dependence of k_{obsd} for AZTMP removal on ATP concentration for RT^{WT} (●) and RT^{AZTR} (○). k_{rem} values were 0.00056 ± 0.00004 and 0.0028 ± 0.0002 s^{-1} and K_d values 0.87 ± 0.23 and 0.32 ± 0.10 mM for RT^{WT} and RT^{AZTR}, respectively.

possibility of large phosphorothioate binding effects observed with other polymerases, further confounding mechanistic conclusions (*41*). Phosphorothioate effects of 1.3- and 6.5-fold were observed during incorporation and removal, respectively (Table 2). These data tentatively suggest differences in the rate-limiting steps for catalysis in the forward and reverse direction.

Equilibrium Binding Constant (K_d) and Maximum Rate of Removal (k_{rem}) for PP_i- and ATP-Mediated Removal by RT^{WT} and RT^{AZTR} during Different Stages of HIV-1 Replication. The PP_i and ATP concentration dependence on the rate of removal of a terminal AZTMP from different RNA and DNA primer−template substrates was determined for RT^{WT} and RT^{AZTR} in an effort to further understand the mechanistic consequences of D67N, K70R, T215Y, and K219Q mutations within the AZT resistant quadruple mutant on removal (Table 3 and Figure 2). The kinetic data show that RT^{AZTR} is slightly impaired during DNA- and RNA-directed PP_i-mediated removal compared to RT^{WT} (Figure 2A,C). Conversely, during ATP-mediated removal from both DNA−DNA and DNA−RNA primer−templates, an as much as 10-fold increase in rate led RT^{AZTR} to be more efficient than RT^{WT} (Figure 2B,D). During both PP_i- and ATP-mediated removal, no AZTMP excision was observed from an RNA−RNA primer−template.

AZT resistant RT mutations can be selected by, and are cross resistant to, D4T (*51*). To understand the kinetics of D4TMP removal by RT^{AZTR}, similar removal studies were carried out with PP_i or ATP and a DNA−DNA primer−template (Table 4). Only small differences in the kinetic data were noted between D4TMP and AZTMP removal. Both had similar efficiencies of removal and predicted physiological removal rates (k_{phys}), suggesting that, in the absence of other factors, they are similarly removed by resistant virus. RT^{AZTR} once again had kinetic values similar to those of RT^{WT} during PP_ilysis but was 1 order of magnitude more efficient during ATP-mediated removal. For both D4TMP and AZTMP removal, the k_{phys} (rate constant for removal determined at physiological concentrations of PP_i or ATP) values of PP_i-lysis were greater than the rate of ATP-mediated removal for either RT^{WT} or RT^{AZTR}.

Physiological Role for ATP-Mediated Removal. (i) The observation that ATP-mediated removal is catalyzed by RT^{WT} (*29*) and (ii) inhibition of blunt-ended addition by PP_i or ATP

Table 4: Parameters for D4TMP Removal by ATP- and PP_i-Mediated Removal

primer−template	substrate	RT	k_{rem} (s^{-1})[a]	K_d (mM)	efficiency[b]	k_{phys} (s^{-1})[c]
DNA−DNA	PP_i	WT	0.17 ± 0.02	1.4 ± 0.4	0.12	0.014
		AZTR	0.23 ± 0.03	1.3 ± 0.4	0.18	0.020
	ATP	WT	0.00025 ± 0.00001	0.16 ± 0.07	0.0016	0.00024
		AZTR	0.0030 ± 0.0003	0.43 ± 0.15	0.0070	0.0026

[a] k_{rem} is the maximum rate of nucleotide removal. [b] Efficiency equals k_{rem}/K_d and is expressed in units of s^{-1} mM^{-1}. [c] k_{phys} is the rate of removal at a physiological concentration of the appropriate substrate (125 μM PP_i or 3 mM ATP).

Table 5: Inhibition by the Next Correct Nucleotide (dCTP) on PP_i- or ATP-Mediated Removal

		PP_i			ATP
D22-dNMP−D45	RT	K_i^{non} (μM)[a]	K_i^{com} (μM)[b]	IC_{50}^{phys} (μM)[c,d]	IC_{50}^{phys} (μM)[c]
D4TMP	WT	−	8.2 ± 0.72	8.8	7.2 ± 0.8
	AZTR	39 ± 16	36 ± 12	17	9.9 ± 1.8
AZTMP	WT	1130 ± 165	−	1200	>500 (18 ± 1[e])
	AZTR	522 ± 244	−	520	>500 (39 ± 1[e])

[a] K_i for noncompetitive inhibition. [b] K_i for competitive inhibition. [c] Concentration necessary to inhibit removal 50% at 125 μM PP_i or 3 mM ATP. [d] Calculated. [e] Determined with D19-AZTMP−D36 primer−template and dGTP as the next correct nucleotide.

have led to the hypothesis that ATP-mediated removal may have a physiological role (*52*). As a first step in elucidating this role, experiments were designed to determine the effects of PP_i and ATP on several different model systems of aberrant replication, to provide a quantitative assessment of the catalytic activities. The effect on either blunt-ended addition or removal from the 3′-blunt end or 3′-overhang sides of the D21−D22 primer−template was studied in the presence and absence of PP_i or ATP. No effect with either PP_i or ATP was observed on the slow process of blunt-ended addition (approximately 10% product formation after 3 h), and neither PP_i nor ATP was able to remove the product of blunt-ended addition (data not shown). Studies with the D30C−D45R primer−template showed physiological concentrations of PP_i or ATP to be unable to remove a mismatch. Direct examination of misincorporation in the presence of PP_i or ATP was complicated by the comparable rates of PP_i-lysis and misincorporation. To avoid the shortening of the primer during the process of misincorporation, a primer−template with a mismatch at the 3′-end was used (D30C/D45R). RT's misincorporation after a mismatch was found to be unaffected by physiological concentrations of ATP or PP_i. PP_i was also found to have no effect on correct single-nucleotide incorporation of a natural dNTP at physiological concentrations (data not shown).

Inhibition by the Next Correct Nucleotide on Removal. Previous work has shown that the next correct nucleotide can inhibit ATP-mediated chain terminator removal (*52*, *53*). Furthermore, it has been suggested that a decreased level of next nucleotide inhibition of AZTMP removal relative to D4TMP removal may account for AZT having a disproportionately greater decrease in activity than D4T when tested in cell culture with AZT resistant virus (*52*−*55*). To determine the effect of AZT resistance mutations on the magnitude and nature of inhibition by the next correct nucleotide during the pyrophosphorolytic removal of AZTMP and D4TMP, binding curves for PP_i were determined in the presence of varying concentrations of the next correct nucleotide (Figure 3). Differences in competitive and noncompetitive inhibition by the next correct nucleotide were noted between D4TMP and AZTMP as well as RT^{WT} and RT^{AZTR} (Table 5). During D4TMP removal, RT^{WT} was found

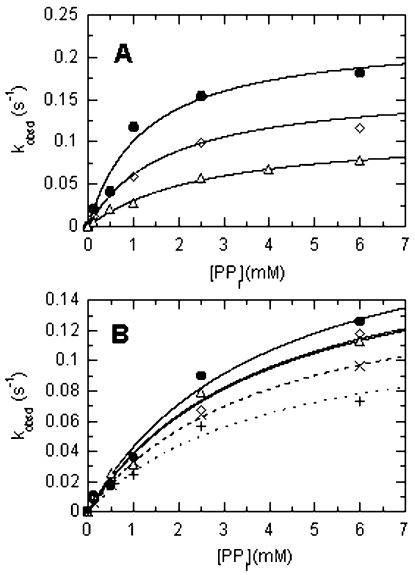


FIGURE 3: Inhibition by the next correct nucleotide on AZTMP and D4TMP PP_i-mediated removal by RT^{AZTR}. (A) Determination of the inhibition constant (K_i) for the next correct nucleotide on D4TMP removal. Hyperbolic curves were generated for the rate dependence of D4TMP removal on PP_i concentration in the presence of 0 (●), 10 (◇), and 50 μM dCTP (△). The curves are consistent with mixed inhibition with a noncompetitive inhibition constant (K_i^{non}) of 39 ± 16 μM and a competitive inhibition constant (K_i^{com}) of 36 ± 12 μM. (B) Determination of K_i for the next correct nucleotide on AZTMP removal. Hyperbolic curves were generated for the rate dependence of AZTMP removal on PP_i concentration in the presence of 0 (●), 10 (◇), 50 (△), 100 (×), and 500 μM dCTP (+). Only noncompetitive inhibition was detected, and K_i^{non} was equal to 522 ± 244 μM.

to be competitively inhibited by the next correct nucleotide while RT^{AZTR} showed data consistent with mixed inhibition (Figure 3A). Unlike during D4TMP removal, both RT^{WT} and RT^{AZTR} showed noncompetitive inhibition by the next correct nucleotide during AZTMP removal, and inhibitory concentrations were found to be in great excess over expected cellular dNTP concentrations (Figure 3B). In studies comparing ATP-mediated dNMP removal by RT^{AZTR} with D22-D4TMP−D45 and D22-AZTMP−D45 primer−template, it was observed that at low concentrations of the next correct

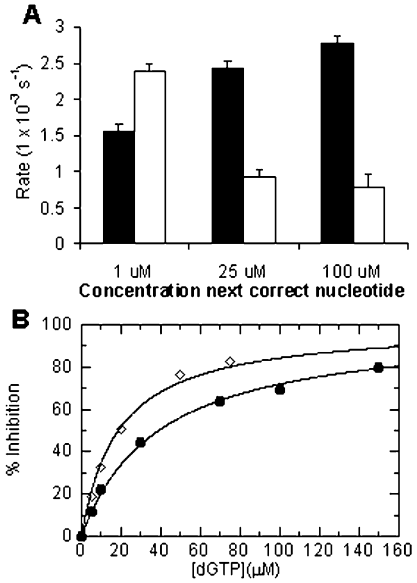


FIGURE 4: Inhibition by the next correct nucleotide on AZTMP and D4TMP ATP-mediated (3 mM) removal by RT^{WT} and RT^{AZTR}. (A) Comparison of the rates of dNMP removal from the D22-AZTMP−D45 (filled bars) or D22-D4TMP−D45 substrate (empty bars) by RT^{AZTR} at different concentrations of the next correct nucleotide (dCTP). (B) Percent inhibition of the next correct nucleotide on ATP-mediated AZTMP removal from the D19-AZTMP−D36 substrate by RT^{WT} (◇) and RT^{AZTR} (●). The hyperbolic fits gave IC_{50} values of 18 ± 1 and 39 ± 1 μM for RT^{WT} and RT^{AZTR}, respectively.

nucleotide D4TMP was removed more rapidly than AZTMP by RT^{AZTR} (1.5-fold faster at 1 μM dCTP). Conversely, at higher next nucleotide concentrations, the rate of AZTMP removal by RT^{AZTR} surpassed that of D4TMP (2.6- and 3.6-fold greater at 25 and 100 μM dCTP, respectively; Figure 4A). These data illustrate the decreased sensitivity of an AZTMP-terminated primer relative to the D4TMP-terminated primer with respect to inhibition by the next correct nucleotide during PP_i- and ATP-mediated removal by RT^{WT} and RT^{AZTR}.

A comparison between RT^{WT} and RT^{AZTR} showed that RT^{WT} was slightly more sensitive to the next correct nucleotide than RT^{AZTR} during both PP_i- and ATP-mediated D4TMP removal (Table 5). During AZTMP removal, neither RT^{WT} nor RT^{AZTR} was inhibited by the next correct nucleotide at concentrations of ≤500 μM when using the D22-AZTMP−D45 primer−template. Interestingly, as dCTP concentrations were increased, an enhancement in the rate of AZTMP ATP-mediated removal was observed, perhaps demonstrating dCTP's ability at high concentrations to catalyze the removal reaction (Figure 4A) (*29*). Thus, to assess if there is a difference in the sensitivity to inhibition by the next correct nucleotide between RT^{WT} and RT^{AZTR} for ATP-mediated removal of a chain-terminating AZTMP, a different sequence context was used. During removal from an HIV genomically derived D19-AZTMP−D36 primer−template, RT^{AZTR} was found to be 2-fold less sensitive to the next correct nucleotide (dGTP) than RT^{WT} (Figure 4B).

Structure−Activity Relationships Based on Removal of Cytidine Analogues. The association of TAMs with multidrug resistance makes it evident that an understanding of how nucleotide structural features interact with RT^{AZTR} is critical for the creation of new drugs that are less susceptible to

Table 6: Initial Velocity for the Removal of Cytidine Analogues from a D23−D45 Primer−Template by RT^{AZTR} and PP_i or ATP

	initial velocity (nM/s)[a]	
chain terminator	PP_i[b]	ATP[b]
AZTMP	0.22	0.036
D4TMP	0.52	0.039
ddCMP	0.054	0.002
D4CMP	0.20	0.015
3TCMP	0.032	0.0004

[a] The velocity before the rapid attainment of equilibrium. [b] PP_ilysis reactions were carried out in the presence of 125 μM PP_i and ATP removal reactions in the presence of 3 mM ATP, except for ATP removal of AZTMP and D4TMP which were carried out at 2.5 and 2 mM ATP, respectively.

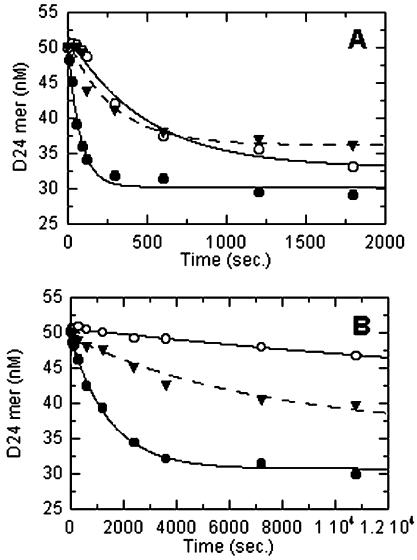


FIGURE 5: Structure−activity relationship for the removal of cytidine analogues. Removal studies were carried out with a D23−D45 primer−template where the 3′-terminal nucleotide was either ddCMP (▼), D4CMP (●), or 3TCMP (○). (A) Removal by RT^{AZTR} in the presence of 125 μM PP_i. The initial velocities of nucleotide removal were 0.054, 0.20, and 0.032 nM/s^{-1}, respectively. (B) Removal by RT^{AZTR} in the presence of 3 mM ATP. The initial velocities of nucleotide removal were 0.002, 0.015, and 0.0004 nM/s^{-1}, respectively.

resistance. A considerable effort has been made to obtain a detailed structure−activity relationship for the incorporation of cytidine analogues by both RT^{WT} (*35*, *56*, *57*) and RT^{M184V} (*57*, *58*). Here we studied the rate of removal of three structurally diverse cytidine analogues by RT^{AZTR} and physiological concentrations of PP_i or ATP. To avoid error in comparisons due to the differential establishment of equilibrium, removal is represented by the initial velocity derived from a line tangent to the initial phase of the exponential decay curve. D4CMP was found to have initial velocities of removal similar to those found with AZTMP and D4TMP (Table 6). All compounds that were studied for removal showed 7−27-fold faster rates of PP_i-mediated than ATP-mediated removal with RT^{AZTR}, except 3TC where PP_i-mediated removal was 80-fold faster than ATP-mediated removal. The order of effectiveness for removal of cytidine analogues was found to be as follows: D4CMP > ddCMP > 3TCMP (Figure 5).

Efficiency of TMP and AZTMP Incorporation by RT^{WT} and RT^{AZTR} during the Initiation of HIV Reverse Transcription.

Table 7: Kinetic Constants for Binding and Incorporation of TTP or AZTTP with a Synthetic $tRNA_3^{Lys}$−R36 Primer−Template and RT^{WT} or RT^{AZTR}

RT	nucleotide	k_{pol} (s^{-1})	K_d (μM)	efficiency[a]	selectivity[b]
WT	TTP	0.46 ± 0.02	84 ± 13	0.0055 ± 0.0009	3.4 ± 0.7
	AZTTP	0.27 ± 0.01	170 ± 20	0.0015 ± 0.0002	
AZTR	TTP	0.64 ± 0.06	130 ± 30	0.0049 ± 0.0013	4.9 ± 1.4
	AZTTP	0.24 ± 0.01	240 ± 20	0.0010 ± 0.0001	

[a] Efficiency equals k_{pol}/K_d and is expressed in s^{-1} μM^{-1}. [b] Selectivity = $efficiency^{TTP}/efficiency^{AZTTP}$.

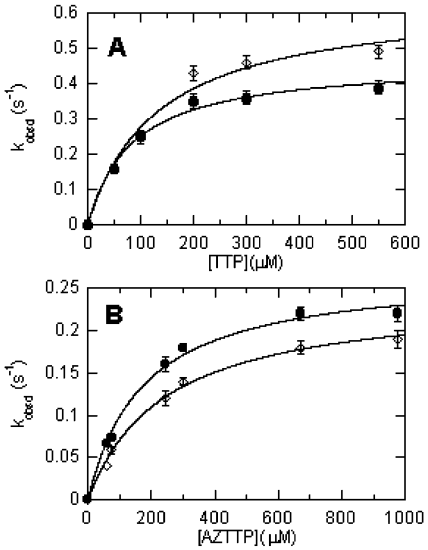


FIGURE 6: Dependence of the first-order rate constant (k_{obsd}) for incorporation into a synthetic $tRNA_3^{Lys}$−R36 primer−template on TTP (A) or AZTTP (B) concentration for RT^{WT} (●) and RT^{AZTR} (○). The observed rates of dNMP incorporation into the model initiation complex were plotted against the concentration of dNTP and fit to hyperbolic equations. The fit showed that TMP was incorporated with maximum rates (k_{pol}) of 0.46 ± 0.02 and 0.64 ± 0.06 s^{-1} and binding constants (K_d) of 84 ± 13 and 130 ± 30 μM for RT^{WT} and RT^{AZTR}, respectively. AZTMP had k_{pol} values of 0.27 ± 0.01 and 0.24 ± 0.01 s^{-1} and K_d values of 170 ± 20 and 240 ± 20 μM for RT^{WT} and RT^{AZTR}, respectively.

Slight changes in the incorporation efficiency for RT^{WT} and RT^{AZTR} during RNA-directed processes have been observed in previous work by our laboratory (*27*, *28*). Incorporation studies with a model 18-mer RNA primer representing the priming sequence from $tRNA_3^{Lys}$ and a 36-mer RNA template containing the primer binding site found that low-level selectivity against AZTMP incorporation was conferred by AZT resistance mutations. This finding led to the hypothesis that increased selectivity on the part of HIV-1 RT^{AZTR} during initiation may play a part in AZT resistance (*27*). To further test this hypothesis, pre-steady-state single-turnover experiments were used to compare the incorporation of TMP with AZTMP by RT^{WT} and RT^{AZTR} into a model initiation system that included a synthetic $tRNA_3^{Lys}$ primer and an R36 template. The observed rates from single-turnover experiments were plotted against dNTP concentration to determine the maximum rate of incorporation (k_{pol}) and binding constant for productive complex formation (K_d), and these values were used to calculate the efficiency of incorporation in the presence of either TTP or AZTTP (Table 7 and Figure 6). Only slight differences were recognized between RT^{WT} and RT^{AZTR}, but consistent with previous results, AZTMP was less efficiently incorporated by RT^{AZTR} than by RT^{WT}. These experiments revealed data that were very similar to those found with the R18-mer primer used previously (*27*), suggesting that the rest of the tRNA sequence does not enhance selectivity between TTP and AZTTP in the presence of synthetic $tRNA_3^{Lys}$.

DISCUSSION

Mechanism of Removal of Nucleotides by RT^{WT} and RT^{AZTR}. The comparison of PP_ilysis and PP_i exchange for RT^{WT} and RT^{AZTR} suggests that HIV-1 RT, like other DNA polymerases, has a rate-limiting step for the reverse reaction that occurs after PP_i binding. The lack of a difference between RT^{WT} and RT^{AZTR} in PP_ilysis and PP_i exchange suggests that TAMs do not have any marked effect on the rate or the mechanism of the reverse reaction when PP_i is responsible for the removal of the terminal dNMP. Studies were also carried out to further identify the rate-limiting step of the reaction through experiments using a nucleotide in which one of the α-phosphate oxygens is substituted with a sulfur. A much larger phosphorothioate effect was noted during removal in comparison to incorporation [where it is believed that a rate-limiting step precedes chemistry (*36*)], suggesting that chemistry is rate-limiting during removal. However, the elemental effect has been illustrated as an unreliable indicator of rate-limiting chemistry by the measurement of a smaller elemental effect for a reaction closely related to polymerization (*50*). Issues surrounding the elemental effect have been discussed at length in other papers (*59*, *60*). In light of these arguments, it can only be suggested that the rate-limiting step in the reverse reaction is the chemical step, and in Scheme 1 (model 1), the rate-limiting step (k_{pyro}) is loosely defined as either the chemical step or a preceding conformation change which includes the RT·primer−template·PP_i complex. From the slow relative rate of removal in comparison to the rate of primer−template release, it can also be inferred that the rate of primer−template release and reassociation may also play a critical role in dictating the maximum rate of removal. These parameters have been found to be affected by TAMs and a chain-terminating AZTMP (*61*), and changes in the dissociation rate of chain-terminated primer−templates from RT have been suggested as a mechanism for enhancing removal by mutants resistant to abacavir (*39*).

Comparison of Removal Kinetics for AZTMP and D4TMP by RT^{WT} and RT^{AZTR} Using either PP_i- or ATP-Mediated Removal during Different Stages of Viral Replication. Kinetic constants for the removal of AZTMP and D4TMP were found to be very similar. For both analogues, the maximum rate of PP_ilysis (k_{rem}) and the physiologically expected rate of removal (k_{phys}) were always faster than the corresponding rates of ATP-mediated removal, independent of the protein that was used. However, the rate of PP_ilysis was not increased by TAMs, while the rate of ATP-dependent removal was enhanced as much as 10-fold. On the basis of the observed biochemical differences of the removal reaction between RT^{WT} and RT^{AZTR}, it may be suggested that ATP is the relevant mediator of resistance. It was notable, however, that with RT^{WT} under estimated physiological concentrations of PP_i (125 μM) or ATP (3 mM), PP_ilysis occurs at a rate 40- to 60-fold faster than the rate of ATP-mediated removal for AZTMP and D4TMP removal (calculated from values presented in Tables 3 and 4). This difference was only

reduced for RT^{AZTR} where PP_ilysis was on average 5-fold faster than ATP-mediated removal of AZTMP and D4TMP (taking into account the values for cytidine analogues presented in Table 6, we determine the average is 20-fold). Results showing that the AZT resistant virus is more sensitive to foscarnet (*23*, *62*), a PP_i analogue, would also be seemingly unexplained without the involvement of PP_i in the resistance mechanism. In light of the faster rate of PP_i-lysis and the enhanced sensitivity of the resistant virus to foscarnet, it is reasonable to believe that both removal substrates are responsible for resistance and perhaps other biochemical properties mediate PP_i's role in AZT resistance.

Most of the research that has been done to date has focused on the effects of TAMs on removal of nucleotides from a DNA−DNA primer−template. However, removal during RNA-directed incorporation would be critical in resistance caused by TAMs. Comparison of the efficiencies of removal of AZTMP from a DNA−DNA, DNA−RNA, and RNA−RNA primer−template shows that removal is less efficient during RNA-directed processes, and no analogue removal was observed from our model RNA19-AZTMP−R36 initiation system. These results are consistent with the lack of removal observed with a $tRNA_3^{Lys}$ chain-terminated primer by others (*63*) and illustrates that AZT resistant virus may be more sensitive to chain termination during the RNA-directed process, especially initiation.

A Physiological Role for ATP-Dependent Removal during Normal Viral Replication? It is unclear whether ATP may influence RT fidelity either by decreasing the extent of aberrant incorporation or removing its products. It has been suggested that ATP-dependent removal may play a role in limiting blunt-ended addition (*52*). The slow rate of blunt-ended addition, however, raises doubt about its physiological relevance. Results presented in this paper also show ATP to be unable to directly affect blunt-ended addition or remove a 3′-overhang. An alternative explanation for the results observed previously is that a decreased level of blunt-ended addition was due to an inhibition of processive synthesis caused by PP_i or high concentrations of ATP. This would also be consistent with an overall inhibition of multiple incorporations observed by others in the presence of PP_i or ATP (*64*).

TAMs Play a Critical Role in the Optimal Alignment of a Chain-Terminated Primer−Template and ATP Rather than Increasing the Extent of ATP Binding. The kinetic data summarized in Table 3 illustrate that TAMs cause the greatest effect on the rate of removal, suggesting that ATP is more effectively positioned for catalytic attack of the terminating dNMP. An early report suggested that the mutations at positions 67 and 70 are important in dictating the enhanced rate of ATP-mediated removal (*53*), and perhaps these residues are responsible for positioning ATP and the chain-terminated primer−template for effective removal. Our data are not consistent with the hypothesis that ATP is bound more tightly by RT^{AZTR} (*52*). During DNA−DNA removal of AZTMP, RT^{AZTR} only bound 2.7-fold tighter to ATP than RT^{WT} (Table 3), while during D4TMP removal, 2.7-fold weaker binding was observed (Table 4), showing no consistent trend.

Effects of TAMs on the Inhibition of Removal by the Next Correct Nucleotide. It has been shown that the next correct incoming nucleotide can inhibit chain terminator removal most likely by causing RT to translocate to a position one nucleotide past the chain terminator, no longer allowing for cleavage of the newly formed phosphodiester bond (*53*). Consistent with previous data (*54*), AZTMP was found to be 50-fold less sensitive to inhibition by the next correct nucleotide than D4TMP during both pyrophosphorolysis and ATP-mediated removal by either RT^{WT} or RT^{AZTR} (Table 5 and Figure 4A). These results illustrate RT's sensitivity to nucleotide analogue structural features during removal (discussed further below).

Decreased sensitivity to the next correct nucleotide may also serve as a mechanism for increasing resistance of RT mutants by allowing them to more efficiently remove chain terminators in a cellular environment where the next deoxynucleotide is present. Residues 215 and 219 have been implicated in altering the interaction with the incoming nucleotide through biochemical (*53*), molecular modeling (*65*), and crystallographic (*66*, *67*) findings. Data presented here show changes in the interaction of RT^{AZTR} with the chain terminator as well as the incoming next correct nucleotide during removal. The decreased level of inhibition of the next correct nucleotide on PP_i-mediated removal of D4TMP by RT^{AZTR} may illustrate how, in the absence of large changes in rate or binding of PP_i, PP_ilysis may play a role in resistance. Although sequence-dependent differences were apparent, decreased sensitivity due to mutations present in RT^{AZTR} during ATP-mediated removal of D4TMP and AZTMP were also obtained. These data are consistent with results showing a decreased level of inhibition by the next correct nucleotide during ATP-mediated removal of AZTMP and ddAMP in response to TAMs by others (*53*). Taken together, these results suggest that a decreased level of inhibition by the next correct nucleotide may allow mutant RT to utilize the more rapid process of PP_i-mediated removal to enhance resistance as well as minimize inhibition of ATP-mediated removal in a cellular environment.

Structure−Activity Relationships for the Removal of Cytidine Analogues. The removal of structurally distinct cytidine analogues showed that like D4TMP chain-terminated primers, D4CMP chain-terminated primers are also prone to efficient removal by RT^{AZTR} with either PP_i or ATP. From these results, a structure−activity relationship begins to emerge for ribose ring modifications which are more prone to RT^{AZTR} removal. Kinetic assays have shown the planar unsaturated analogues D4TMP, D4CMP, and CBVMP are subject to efficient removal. In a recent report, we have hypothesized that the correlation between abacavir resistance and TAMs may be due to CBVMP forming strong hydrophobic interactions in the active site, causing it to remain in a position competent for removal (*39*). Both D4TMP and D4CMP may also form strong $\pi-\pi$ stacking interactions with Tyr115 and be more likely to occupy the RT active site in a favorable position for removal to take place. AZTMP, however, does not have the same potential for hydrophobic interactions within the active site, but its large azido group may allow for many electrostatic and hydrogen bonding contacts within the 3′-hydroxyl pocket of the active site. These could act to improve positioning for the removal reaction as well as anchor AZTMP in the active site, disfavoring translocation and inhibition by the next correct nucleotide. The potential for these interactions is evident in the recently reported crystal structure of RT with AZTMP

in the nucleotide binding site (*68*). It has also been hypothesized that the azido group sterically hinders the next incoming nucleotide (*52*) and this and/or tight binding may cause AZTMP chain-terminated primers to be superior substrates for removal and less sensitive to inhibition by the next incoming dNTP.

Increased Selectivity by RT^{AZTR} during Incorporation into a Model Initiation System. Similar to previous studies on RNA-dependent incorporation, a slight increase in selectivity was seen for RT^{AZTR} in our model initiation system (*27*, *28*). The similarities between the kinetic constants determined with the full $tRNA_3^{Lys}$ sequence and those determined with an 18-mer RNA (*27*) suggest that interactions with the additional nucleotide sequence do not play a role in resistance. It is possible that other factors present in the initiation complex, including modified nucleotides present in natural $tRNA_3^{lys}$, the virally encoded nucleocapsid protein (*69*−*71*), or the phosphorylated state of RT (*72*), may modulate AZT resistance. Further in-depth studies may yield interesting information about the initiation process and its potential role in NRTI resistance. Data showing the inefficient removal of chain terminators during RNA-directed processes illustrate the potential importance of even slight selectivity advantages in allowing resistant virus to replicate in the presence of nucleotide analogues.

CONCLUSIONS

Our studies have shown that AZT resistance mutations cause a more effective positioning of substrates for the catalysis of ATP-mediated removal. It was also found that a decreased level of interactions with the next correct nucleotide during D4TMP removal by RT^{AZTR} may illustrate how PP_i can play a role in resistance despite no marked increase in the pyrophosphorylitic rate of removal. Decreased sensitivity to the next correct nucleotide was also shown to serve as a potential mechanism for an increased level of ATP-mediated removal. Because of RT's weakened ability to remove nucleotide analogues during RNA-directed processes, the slight differences in selectivity noted during AZTMP incorporation might be an important factor in enhancing resistance. Indeed, our data illustrate that increased selectivity, PP_ilysis, and ATP-mediated removal may all contribute to the overall magnitude of resistance observed in a cellular system. An initial structure−activity relationship suggests that nucleotide analogues with the potential to form strong interactions with the RT active site may be more prone to resistance both by being better substrates for the removal reaction and perhaps by having their removal less inhibited by the next correct incoming nucleotide. A deeper understanding of the effects of nucleotide features on removal may allow for the rational design of new and more effective agents against resistant HIV.

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