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# The Enzymatic Basis for Thymidine Analogue Resistance in HIV-1

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## Abstract

**Mutations at six sites in HIV-1 reverse transcriptase (RT) that have long been associated with resistance to zidovudine (AZT) are now referred to as thymidine analogue mutations (TAMs) because they help the virus escape from stavudine (d4T) as well. These mutations include M41L, D67N, K70R, L210W, T215F or Y and K219Q. Studies on the molecular mechanism of resistance have recently focused on the enhanced primer unblocking activity conferred by these mutations - i.e. the mutant enzyme has increased ability to remove a chain terminator after it has been incorporated. Removal is accomplished by transfer of the chain terminator to an acceptor that may be pyrophosphate or nucleoside di- or triphosphate. The intracellular acceptor substrate for this reaction has not been determined. TAMs are clustered at some distance from the dNTP-binding site in HIV-1 RT, but they are well positioned to interact with a nucleoside triphosphate substrate for the unblocking reaction. RTs containing TAMs have elevated unblocking activity against most chain terminators; however, in culture-based assays for drug susceptibility, the mutant viruses are highly resistant to AZT and only minimally resistant to other nucleosides. Addition of micromolar concentrations of natural dNTPs to the *in vitro* reaction mixtures results in better agreement with the *in vivo* assays. *In vitro* experiments have shown that the removal of chain-terminating nucleotides such as ddAMP or d4TMP is inhibited by micromolar concentrations of the next complementary dNTP, whereas removal of AZTMP is relatively insensitive to this inhibition. The intracellular concentrations of dNTPs in the cells used in culture-based drug susceptibility assays are likely to be high enough to prevent the removal of most chain-terminating nucleotides other than AZTMP. A full understanding of the mechanism of resistance conferred by these mutations will require the definition of the contributions of each residue to nucleotide binding and discrimination, primer unblocking activity, and viral replication fitness.**

## Key words

**Reverse transcriptase. Thymidine analogue mutations (TAMs). Drug resistance. Pyrophosphorolysis.**

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## Introduction

Antiviral therapy depends on our ability to exploit the sometimes subtle biochemical differences between a virus and its host. Even when an antiviral agent is identified that suppresses viral replication without being extremely toxic, the virus can often circumvent the therapy by acquiring mutations that reduce its susceptibility to the agent. HIV is able to develop drug resistance rapidly because of the high rate of virus replication in an infected individual and the low fidelity of the viral replication machinery. Rapid selection of drug resistance mutants may be the most important limitation on the success of antiretroviral therapies against HIV. Recent developments have begun to shed light on the molecular mechanisms involved.

Monotherapy with 3'-azidothymidine (AZT, zidovudine) results in a 5 to 10-fold drop in viral load in previously untreated HIV-infected patients. This is followed by a return to near baseline over 6 to 12 months, at which time a mutant population of virus predominates that is resistant to AZT in culture-based assays (phenotypic resistance). Resistance is usually accompanied by selection of mutations at six sites in the gene for reverse transcriptase (RT)[1-5]. The mutations are M41L, D67N, K70R, L210W, T215Y or F, and K219Q. High-level resistance to AZT and the presence of mutations at codons 41 or 215 confer increased risk of disease progression and death in HIV-1 infected patients, even when adjusted for other factors predictive of disease progression[6,7].

The number of AZT-resistance mutations present in a virus isolate correlates with the level of resistance[8]. In cross-sectional studies of AZT-treated patients, mutations are observed most frequently at residue 215[8-10], suggesting that this site plays a central role in AZT resistance. In fact, the mutation at residue 70 usually appears first followed by the other mutations in a defined order[11]. These observations led to the concept of primary resistance mutations - i.e., mutations that can confer resistance by themselves (K70R and T215Y or F), and secondary mutations - i.e., those that usually appear when one of the primary mutations is already present (M41L, D67N, L210W and K219Q). The clinical utility of this distinction has been questioned recently and an expert panel established by the International AIDS Society has recommended that the distinction should be dropped for purposes of clinical management[12]. Nonetheless, the ordered appearance and disappearance of these mutations provides potentially useful information about their interactions. M41L and L210W only exert their effects when T215Y or F is already present[13], suggesting that these secondary mutations may cooperate with the 215 mutation to fine-tune the resistance mechanism, or they may act indirectly by compensating for deficiencies in replication caused by the 215 mutation. Of course, the mutations may operate at both levels. A full understanding of the molecular mechanisms of drug resistance will depend on sorting out the interactions among all of the mutations that contribute to the resistance phenotype.

## Selection of AZT-resistance mutations by therapy with other nucleosides

Over the past few years, evidence has been accumulating that "AZT-resistance mutations" can be selected by therapy with 2',3'-didehydrodideoxythymidine (d4T, stavudine)[14-25] and that the presence of AZT-resistance mutations has a negative impact on the benefit derived from d4T therapy[25-29]. AZT-resistance mutations have also been reported after prolonged therapy with ddI in the absence of AZT[30-32]. As a result, this group of mutations has been renamed thymidine analogue mutations (TAMs) and, more recently, "multi-NRTI-resistance mutations" or just NAMs (nucleoside-associated mutations), to reflect the fact that they may also contribute to resistance to non-thymidine nucleosides[12].

These results appear to be in conflict with experiments indicating that AZT-resistance mutations are highly specific for AZT, with little cross-resistance for even such closely related nucleosides as d4T[14,33]. With more extensive data and improved standardization of the phenotypic resistance assays, it has been possible to detect limited cross-resistance[25]; however, the increase in $IC_{50}$ for d4T is only 3 to 4-fold for mutant combinations that have 50 to 100-fold increases in the $IC_{50}$ for AZT.

## The search for a biochemical basis of resistance

AZT is converted to AZT triphosphate (AZTTP) by cellular enzymes. The triphosphate is recognized by the viral RT as an analogue of dTTP and incorporated into the growing DNA chain, resulting in chain termination and inhibition of viral replication. Based on experience with other resistance mechanisms, it was anticipated that resistance to AZT would involve the selection of a mutant enzyme that could discriminate against AZTTP. This was highly plausible because a 3'-azido group in place of the natural 3'-hydroxil group represents a substantial structural change that could readily form the basis for increased discrimination; however, structural and enzymatic data on RT containing the AZT-resistance mutations, accumulated over several years at distinct laboratories, have provided little support for this type of mechanism[1,34-38]. Using pre-steady-state kinetic analysis, Kerr and Anderson[39] have reported a small but significant increase in discrimination against AZTTP by an AZT-resistant RT. This difference was seen when synthesis was monitored on a RNA template and was not observed using a DNA template of the same sequence. Vaccaro and Anderson[40] reported a somewhat greater ability to discriminate against AZTTP when the mutant RT was used to extend an RNA primer on an RNA template - a reaction that occurs during the initiation of minus-strand synthesis. These authors have pointed out that a small difference in discrimination against AZTMP at each A position on the genome may be multiplied into a much larger difference when the entire viral genome is replicated, and that other factors not reproduced in the *in vitro* assays may mag-

nify the differences observed between the mutant and wild-type enzymes to account for the greater than 100-fold resistance seen for these mutants *in vivo.* However, these explanations do not apply to other well-known nucleoside resistance mutations where discrimination occurs *in vitro* that is quantitatively similar to that seen *in vivo.* The L74V (ddI resistance) mutation produces a mutant RT that favors dATP over ddATP in *in vitro* assays[35,36,41]. RT containing the M184V (3TC resistance) mutation exhibits a high level of discrimination against 3'-thiacytidine triphosphate (3TCTP) in favor of dCTP[38,42,43]. In addition, the Q151M (multinucleoside-resistance) mutant RT discriminates against several chain-terminating nucleotides including AZTTP *in vitro*[44]. The difficulty in reproducing the nucleoside-resistance phenotype is somehow unique for AZT-resistance mutations, suggesting that additional factors must be at work.

The residues that are altered in AZT-resistant RT have been located in the 3-dimensional structures[45-50]. Residues 210, 215 and 219 are in the "palm" domain and 41, 67 and 70 are in the "fingers" domain, but all are sufficiently far from the active site of the enzyme that direct contact with 3' substituents on the incoming nucleotide is unlikely. It is possible that the mutations exert their effects at a distance by inducing conformational changes in the protein. A structure for AZT-resistant RT has been determined that showed long range conformational changes that repositioned the active site aspartate residues, providing a mechanism capable of explaining increased discrimination by this enzyme[49]. The structure of the RT•primer-template•dNTP complex reported by Huang, et al.[50] shows that binding of nucleotide substrate induces a substantial conformational change in RT. The "fingertips" bend inward toward the palm by about 20° bringing a number of residues together to form the dNTP binding site. The "fingers" residues, including D67 and K70, are positioned closer to the dNTP binding site but direct contact that could lead to increased dNTP discrimination is still unlikely. Figure 1 shows the positions of primer, template, bound dTTP and the amino acids corresponding to codons 41, 67, 70, 210, 215 and 219 in the Huang structure. The left panel shows a side view and the right panel shows a view looking down the phosphate chain toward the deoxyribonucleoside portion of dTTP. Direct contact between residues 70 or 215 and the γ-phosphate of dTTP may be possible but contact with the 3' position of dTTP is unlikely. Interactions have been proposed between the residues at positions 41 and 215 and the residues that form the dNTP-binding site that could result in increased substrate discrimination[50].

Taken together, the kinetic and structural analysis of the mutant RTs are insufficient to prove the hypothesis that increased discrimination against AZTTP incorporation is the sole or primary mechanism for resistance.

## AZT resistance mutations enhance the primer unblocking activity of RT

Recently, several laboratories have proposed that AZT-resistance mutants may differ from wild-type in their ability to remove AZTMP from blocked primers[51-53]. RT lacks 3' exonuclease activity[54];however, removal may occur through transfer of the terminal AZTMP to pyrophosphate (PPi) (pyrophosphorolysis, the reversal of polymerization)[51,52] or to a

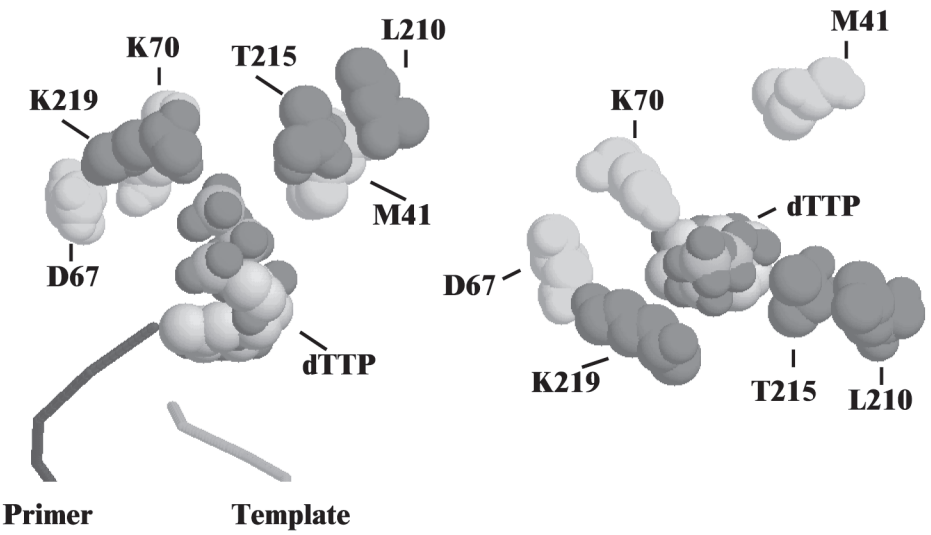


**Figure 1.** *Location in the ternary complex of wild-type HIV-1 RT of the residues substituted in thymidine analogue mutations. The six TAM residues are shown as space-filling models and identified in the figure. Residues in the "fingers" domain are light colored and those in the "palm" domain are dark. The primer and template are shown as rods corresponding to their DNA backbones. The bound dTTP is shown as a ball-and-stick model. The figure was produced with Protein Explorer (http://www.umass.edu/microbio/chime/explorer) using the structure coordinates (1RTD) of Huang, et al.[50] retrieved from the Brookhaven Protein Data Base. Left panel, side view. Right panel, top view looking down the phosphate chain toward the deoxynucleoside portion of dTTP. The primer and template are omitted in the right panel for simplification.*

nucleoside di- or triphosphate[53] (Fig. 2). Significant increases in either or both of these unblocking reactions have been reported for AZT-resistant RT[52,55-62].

The question of whether the PPi and nucleotide-dependent reactions both play a role in resistance has not been resolved since some investigators report that AZT-resistant RT has increased PPi-dependent unblocking activity[52,56-58,61,62] and others report that the PPi-dependent reaction is not increased[37,55,59,60,63]. In contrast, there is general agreement that the nucleotide-dependent reaction is increased for the mutant enzymes[55,58-62]. Circumstantial evidence for the importance of a nucleotide substrate for this reaction *in vivo* is provided by the locations of the AZT-resistance mutations in the 3-dimensional structure of HIV-1 RT (see below). Since ATP is present at higher concentrations than other nucleotides in most cells[64], it would be the most likely nucleotide to participate in this reaction *in vivo*.

## A structural rationale for resistance by AZT-resistance mutants

The evidence that primer-unblocking activity of HIV-1 RT plays a role in resistance has stimulated renewed inspection of the crystal structures of the enzyme. The covalently trapped catalytic complex of Huang, et al.[50] corresponds to a stable substrate complex that has been captured just prior to the polymerization reaction, but the structure can equally be viewed as the product complex resulting from PPi-dependent pyrophosphorolysis of a DNA chain ending with dTMP. The nucleotide-dependent removal reaction is analogous to the PPi-dependent reaction and it is reasonable to assume that the dinucleoside polyphosphate product would occupy an analogous position on the enzyme. For ATP-dependent removal of dTMP, the product would be $Ap_4dT$, in which ATP is joined to dTMP, and the dTMP moiety would be bound in the dNTP binding site on the enzyme, connected by a chain of four phosphates to the adenosine from ATP. The residues altered in AZT-resistance mutants are positioned to interact with the AMP moiety of the $Ap_4dT$ product[55,60].

As shown in Fig. 1, the residues that are substituted in the AZT-resistance mutations lie in a cluster positioned just above the dNTP binding site in the enzyme. This corresponds to an indentation or pocket in the surface of the enzyme reaching in towards the active site of the enzyme. While none of these residues is likely to make direct contact with a 3' substituent on the incoming nucleotide, they are well positioned to make contact with a nucleotide substrate for the removal reaction.

Lacey and Larder[65] compared mutants of HIV-1 with various amino acids substituted at codon 215 and showed that tyrosine and phenylalanine gave maximal resistance, tryptophan gave low level resistance, and other amino acids gave no increase in resistance over wild-type. From this it can be concluded that the introduction of an aromatic side chain at residue 215 is important for the resistance phenotype. Boyer, et al.[60] modeled ATP into the Huang, et al. structure and showed that the adenine base could make direct contact with an aromatic side chain introduced at position 215. Virus containing only the

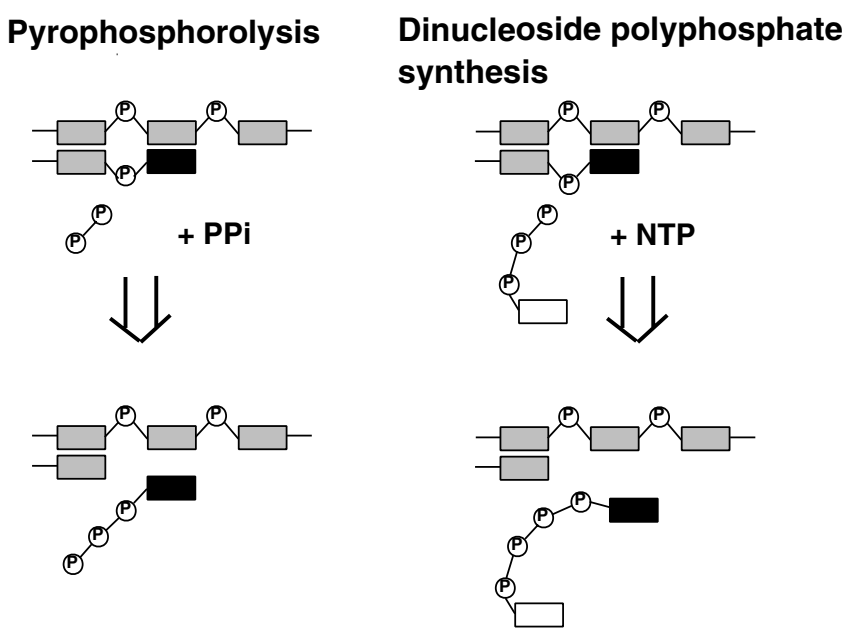


**Figure 2.** *Removal of a chain-terminating nucleotide by transfer to PPi or to a nucleoside triphosphate. A chain-terminating nucleotide (dark rectangle) is removed from a blocked DNA chain by transfer to PPi (left panel) to form the triphosphate or to a nucleoside triphosphate such as ATP (right panel) to form the dinucleoside tetraphosphate. The open rectangle can be any ribo- or deoxyribonucleoside. The shaded rectangles are natural deoxyribonucleosides in the primer and template chains. Adapted from Meyer, et al.[53].*

T215Y mutation show modest AZT resistance *in vivo*[66] and a modest increase in *in vitro* primer unblocking activity[60,62,67]. The introduction of additional TAMs dramatically enhances both activities[9,60-62,66,67].

The view that TAMs enhance the binding site for the nucleotide substrate for the primer unblocking reaction, receives additional support from the observations of Meyer, et al.[53,55]. These authors have shown that, while nucleoside di- and triphosphates are about equally efficient as substrates for the unblocking reaction with wild-type RT, unblocking activity is enhanced with the mutant enzyme only when nucleoside triphosphates are used as substrate. The product of the ATP-dependent removal of ddNMP from the 3' position on the primer is $Ap_4ddN$, whereas the product of the ADP-dependent reaction is $Ap_3ddN$. In either case the ddN portion of the product would be formed in the dNTP binding site on the enzyme and would retain an affinity for that site. The position of the adenosine moiety on the enzyme would be determined by the length of the phosphate chain connecting the two portions of the dinucleoside polyphosphate. When ATP is the substrate, the adenosine moiety will be positioned so that it can interact with an aromatic group at residue 215[60]. When ADP is the substrate, the adenosine moiety will not be close enough to interact with that residue.

Based on this model, structural explanations are possible to explain the effects of some of the other TAMs. Enzyme containing only the K70R and D67N mutations have 4 to 5-fold increased nucleotide-dependent removal activity[55]. The K70R mutation may adjust the position of the positive charge to facilitate an interaction with the additional phosphate residue derived from ATP, which would not be present if ADP or PPi were the substrate. Mutation D67N disrupts a salt bridge between D67 and K219 in the wild-type enzyme and may provide increased access for nucleotide attack at the primer terminus[60]. The M41L mutation cooperates with T215Y both *in vivo*[66] and *in vitro*[67]; however, the basis for this interaction is not evident from the crystal structure. The residues are too far apart for simultaneous interaction with the nucleotide substrate for the removal reaction, and other possible interactions that would alter unblocking activity or replication activity are not obvious.

M41L and K70R are antagonistic *in vivo*. They very rarely occur together in patients[10,68] and a constructed HIV-1 variant with the two mutations is replication deficient[69], which may suggest that M41L plays a role in a replication function of RT. The replication deficiency disappears when the 215 mutation is also present[69], which may help explain why, during the evolution of AZT resistance in an infected patient, the K70R disappears at about the time that M41L and T215Y appear, and why the K70R mutation can reappear later. These observations serve to emphasize that many of the interactions between these mutations are not understood and that effects on replication (viral fitness) are also an important part of the equation.

## Selective resistance to AZT

*In vitro* measurement of primer unblocking activity and *in vivo* culture-based phenotypic resistance assays appear to be in conflict when it comes to defining the specificity of AZT resistance. The mutant RTs show approximately the same enhancement of unblocking activity on primers terminated with a wide variety of chain terminators[55,63,70]. In contrast, an HIV isolate containing multiple TAMs may have 50 to 100-fold decreased susceptibility to AZT, while susceptibility to other nucleoside drugs is only minimally reduced. This discrepancy can be at least partly resolved by carrying out the *in vitro* reaction in the presence of physiological concentrations of dNTPs[55,60,61,63,71]. Under these conditions, the mutant enzyme loses the ability to remove chain terminators such as ddAMP or d4TMP but retains the ability to remove AZTMP.

This phenomenon relies on the fact that RT that is bound to a chain-terminated primer-template can bind the next complementary dNTP on the template and attempt to carry out the polymerization reaction[72]. When this happens, RT forms a tight non-productive complex with the dNTP and primer-template, the presence of the chain terminator at the 3' end of the primer prevents the formation of a phosphodiester bond, and the enzyme is locked in a dead-end complex and is prevented from carrying out the unblocking reaction. An azido group in the 3' position of the primer interferes with the binding of the incoming dNTP, and dead-end complex formation is inhibited[55,60,63,72]. As a result, the removal of AZTMP is at least 50-fold less sensitive to inhibition by the next complementary dNTP, and the block to elongation is removed under physiological conditions, while blocks by other chain terminators are not removed. For the measurement of phenotypic drug sensitivity, mitogen-stimulated peripheral blood mononuclear cells[14,16,73] or transformed human cell lines[33,74-77] are used. In either case, the intracellular dNTP pools are high due to mitogenic stimulation, and the removal of most chain terminators except AZTMP will be inhibited. Since resistance depends on the removal activity, the drug resistance profile obtained in these assays shows high-level resistance to AZT and minimal resistance to other chain terminators.

In patients carrying virus with TAMs, cell populations are likely to exist, at least transiently, that do not have high dNTP pools. This would lead to the unmasking of an unblocking reaction for chain terminators that are not removed in rapidly dividing cells and would provide an explanation for the selection of these mutations by drugs such as d4T and ddI. This would also explain why the clinical benefit is less if TAMs are present at the onset of therapy with the other nucleosides.

## Conclusions

The ability of a chain terminating nucleotide to block HIV-1 replication depends on its activation and incorporation into viral DNA during reverse

transcription and also on the extent to which it can be removed by the primer unblocking activity of HIV-1 RT. The selection of resistance mutations is a function of the ability of specific changes in the sequence of RT to avoid incorporation of the activated form of the drug (discrimination), remove the chain terminating nucleotide after it has been incorporated (primer unblocking), and support viral DNA synthesis using natural nucleotide substrates (fitness). Increased primer unblocking activity plays a major role in the resistance to AZT and d4T by thymidine analogue mutations; however, it is necessary to investigate each of these parameters to define the contribution from each of the mutations.

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