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Review

Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs)

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Abstract. Nucleoside reverse transcriptase inhibitors (NRTIs), such as 3′-azido-3′-deoxythymidine, 2′,3′-dideoxyinosine and 2′,3′-dideoxy-3′-thiacytidine, are effective inhibitors of human immunodeficiency type 1 (HIV-1) replication. NRTIs are deoxynucleoside triphosphate analogs, but lack a free 3′-hydroxyl group. Once NRTIs are incorporated into the nascent viral DNA, in reactions catalyzed by HIV-1 reverse transcriptase (RT), further viral DNA synthesis is effectively terminated. NRTIs should therefore represent the ideal antiviral agent. Unfortunately, HIV-1 inevitably develops resistance to these inhibitors, and this resistance correlates with mutations in RT. To date, three phenotypic mechanisms have been identified or proposed to account for HIV-1 RT resistance to NRTIs. These mechanisms include alterations of RT discrimination between NRTIs and the analogous dNTP (direct effects on NRTI binding and/or incorporation), alterations in RT-template/primer interactions, which may influence subsequent NRTI incorporation, and enhanced removal of the chain-terminating residue from the 3′ end of the primer. These different resistance phenotypes seem to correlate with different sets of mutations in RT. This review discusses the relationship between HIV-1 drug resistance genotype and phenotype, in relation to our current knowledge of HIV-1 RT structure.

Key words. Human immunodeficiency virus type 1; reverse transcriptase; nucleoside reverse transcriptase inhibitors; DNA polymerization; chain termination; antiviral drug resistance; phosphorolysis; pyrophosphorolysis.

Introduction

Retroviruses such as the human immunodeficiency virus (HIV) are RNA viruses that replicate through a double-strand DNA intermediate. This novel viral replication cycle requires that retroviruses carry a specific enzyme, reverse transcriptase (RT), since there are no cellular enzymes that can convert single-strand RNA into double-strand DNA. RT is a DNA polymerase that can copy both DNA templates (like cellular enzymes) and RNA templates (unlike cellular enzymes). HIV RT differs from cellular DNA polymerases in two additional respects. First, HIV RT readily utilizes many chemically altered analogs of the normal deoxynucleoside triphosphate (dNTP) DNA polymerase substrates. Second, HIV RT lacks a formal ′proofreading′ activity. These characteristics are important from a pharmaceutical focus, and direct the use of nucleoside analog inhibitors as anti-HIV pharmaceuticals. As of January 2000, six of the current FDA-approved anti-HIV drugs are nucleoside reverse transcriptase inhibitors (NRTIs) [1].

NRTIs are analogs of the normal dNTP substrates of DNA polymerases, with important modifications (fig. 1). The 2′,3′-dideoxynucleosides such as ddC and ddI

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lack a 3′-OH on the sugar, whereas other analogs such as 3′-azido-3′-deoxythymidine (AZT) have the 3′-OH replaced by other functional groups that do not allow primer extension. NRTIs require intracellular metabolic transformation for antiviral activity, namely conversion to the triphosphate, a process catalyzed by cellular kinases [2].

After conversion to the active triphosphate, NRTIs must compete with the natural dNTPs both for recognition by RT as a substrate (binding) and for incorporation into the nascent viral DNA chain (catalysis). NRTIs thus inhibit RT-catalyzed proviral DNA synthesis by two mechanisms [3]. First, they are competitive inhibitors for binding and/or catalytic incorporation with respect to the analogous dNTP substrate. Second, they terminate further viral DNA synthesis, due to lack of a 3′-OH group. Chain termination is the principal mechanism of NRTI antiviral action.

NRTIs should be the 'ideal' anti-HIV therapeutics. Each HIV virion carries only two copies of genomic RNA. There are about 20,000 nucleotide incorporation events catalyzed by RT during the synthesis of complete viral DNA, thus providing about 5000 chances for chain termination by any given NRTI. Since HIV-1 RT lacks a formal proofreading activity (i.e. some formal mechanism to identify and excise inappropriate nucleotide incorporation), a single NRTI incorporation event should suffice to quell viral DNA synthesis. In reality, NRTIs are less potent inhibitors of HIV replication than might be expected; reasons for this will be discussed later. In addition, although NRTI therapy is initially quite effective in reducing viral load in HIV-1-infected individuals, the viral burden inevitably rebounds despite continued therapy, due to the appearance of drug-resistant strains of HIV. Numerous mutations in HIV-1 RT have been identified in NRTI-resistant HIV strains (table 1) [4, 5].

The simplest mechanism for resistance would be one of discrimination, i.e. some mechanism for RT to exclude the NRTI, while retaining the ability to recognize the analogous natural dNTP substrate. However, this discrimination is actually somewhat of a problem, since in

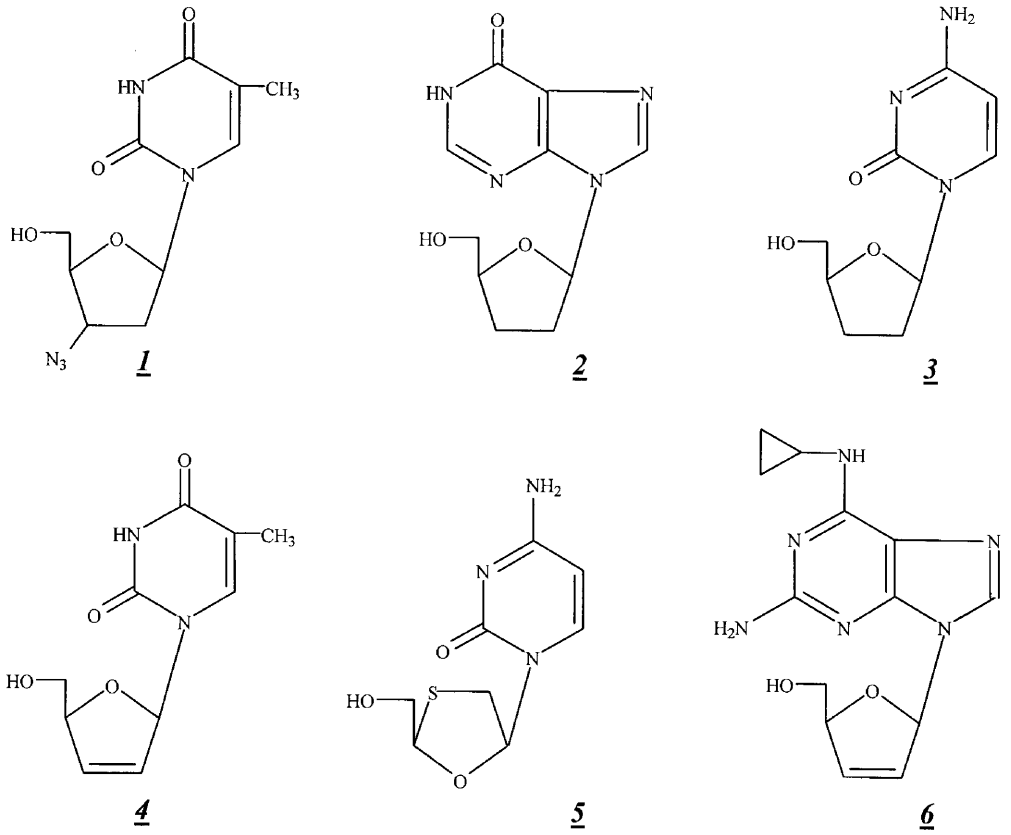

Figure 1. Structures of current clinically-used nucleoside reverse transcriptase inhibitors (NRTI). AZT (1), ddI (2), ddC (3), d4T (4), 3TC (5), abacavir (6).

Table 1. Mutations in HIV-1 RT correlated with resistance to NRTI.

ddN	RT residue											
	41	65	67	69	70	74	75	151*	184	210	215	219
AZT†	M41L		D67N		K70R					L210W	T215Y/F	K219Q
ddC		K65R		T69D			V75T		M184V			
3TC		K65R			K70E				M184V			
ddI (ddA)		K65R				L74V	V75T					
d4T				68↓↓70‡			V75T					
Abacavir		K65R				L74V			M184V			

* The mutation Q151M appears in patients with resistance to multiple ddN.
† High-level AZT resistance requires the presence of two or more mutations.
‡ Two-amino acid insertion mutants are not specific for d4T resistance, but were first identified in patients with d4T resistance. These insertion mutations are found in multidrug resistant HIV-1, generally over a background of AZT-resistance mutations.

Table 2. Resistance phenotypes associated with NRTI-resistance mutations in HIV-1 RT.

Mutation	Resistance conferred	NRTI-resistance phenotype
K65R	ddC, ddI, 3TC, PMEA	discrimination
T69-S-S/G-K70*	multidrug resistance	uncertain (may be combination of discrimination and phosphorolysis)
L74V	ddI, ddC	T/P repositioning
V75T	ddC, d4T	uncertain
E89G	ddG	T/P repositioning
Q151M	multidrug resistance	discrimination
M184I/V	3TC	discrimination (also negative effect on phosphorolysis);T/P repositioning may also play a role
D67N/K70R/T215F(Y)/Q219K	AZT	phosphorolysis
M41L/T215Y	AZT	phosphorolysis†
L210W	AZT	uncertain

* AZT-resistance mutations are required in addition to insertion mutations to provide multi-drug resistance.
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many cases this requires that RT must selectively ignore a structurally less rich compound (NRTI, lacking the 3′-OH) in favor of the structurally more complex dNTP analog. In addition, our understanding of the molecular aspects of NRTI resistance has been complicated by the complex patterns of mutations required for resistance to some NRTIs such as AZT [6].

To date, three mechanisms have been proposed to account for the molecular basis of the NRTI resistance phenotype. These mechanisms apply to different stages of NRTI inhibition, and include (i) Selective alterations in NRTI binding and/or incorporation (i.e. discrimination), (ii) template/primer (T/P) repositioning, which then influences NRTI incorporation and (iii) phosphorolytic removal of an incorporated chain-terminating NRTI residue from the 3′-end of the nascent viral DNA. Correlations of these phenotypes with specific mutations in RT and the NRTIs affected are summarized in table 2.

HIV-1 RT structure and function

The HIV-1 RT gene encodes a 66-kDa protein; however, the presumed biologically relevant form of HIV-1 RT is a heterodimer comprising of subunits of 66 and 51 kDa (termed p66 and p51) [7]. The p51 subunit is produced during viral assembly and maturation via HIV-1 protease-mediated cleavage of the C-terminal domain of a p66 subunit. The structure of the HIV-1 RT heterodimer is illustrated in fig. 2.

The overall shape of the p66 subunit has been likened to that of a ‘right hand’ [8], with the major subdomains of the polymerase domain of p66 appropriately termed fingers (residues 1–85, 118–155), palm (86–117, 156–237) and thumb (238–318) (fig. 2). The DNA polymerase catalytic aspartate residues (D110, D185, D186) are in the palm subdomain. In addition, the p66 has two additional major subdomains, the ‘connection’ (residues 319–426) and the C-terminal ribonuclease H (RNase

H) (427–565) domains. The latter subdomain is missing in the p51 subunit. Whereas the overall folding of the subdomains is similar in both p66 and p51 subunits, the spatial arrangement of the subdomains differs markedly [8, 9]. The p66 subunit adopts an 'open', catalytically competent conformation that can 'grasp' a nucleic acid template, whereas the p51 subunit is in a 'closed' conformation. The p51 subunit is considered to play a largely structural role, although it may also be important in interacting with the transfer RNA $(tRNA)^{Lys3}$ primer used for the initiation of HIV-1 DNA synthesis [8].

Mutations associated with NRTI resistance occur primarily in the fingers and the palm subdomains of RT (table 1, fig. 2). Because of the nature of HIV-1 RT heterodimer formation, NRTI resistance mutations obviously occur in both p66 and p51 subunits. However, only those in the p66 subunit are generally considered to have phenotypic consequences. Whereas one report has hypothesized that mutations in the p51 subunit may also contribute to the resistance phenotype [10], there are no biochemical data to support this conjecture.

The conversion of HIV-1 genomic RNA into double-strand viral DNA is a complex process, yet all chemical steps are catalyzed by RT. This requires RT to be multifunctional, with two types of DNA polymerase activity, RNA-dependent DNA polymerase (RDDP) to synthesize a DNA strand copy of the viral genomic RNA template and DNA-dependent DNA polymerase

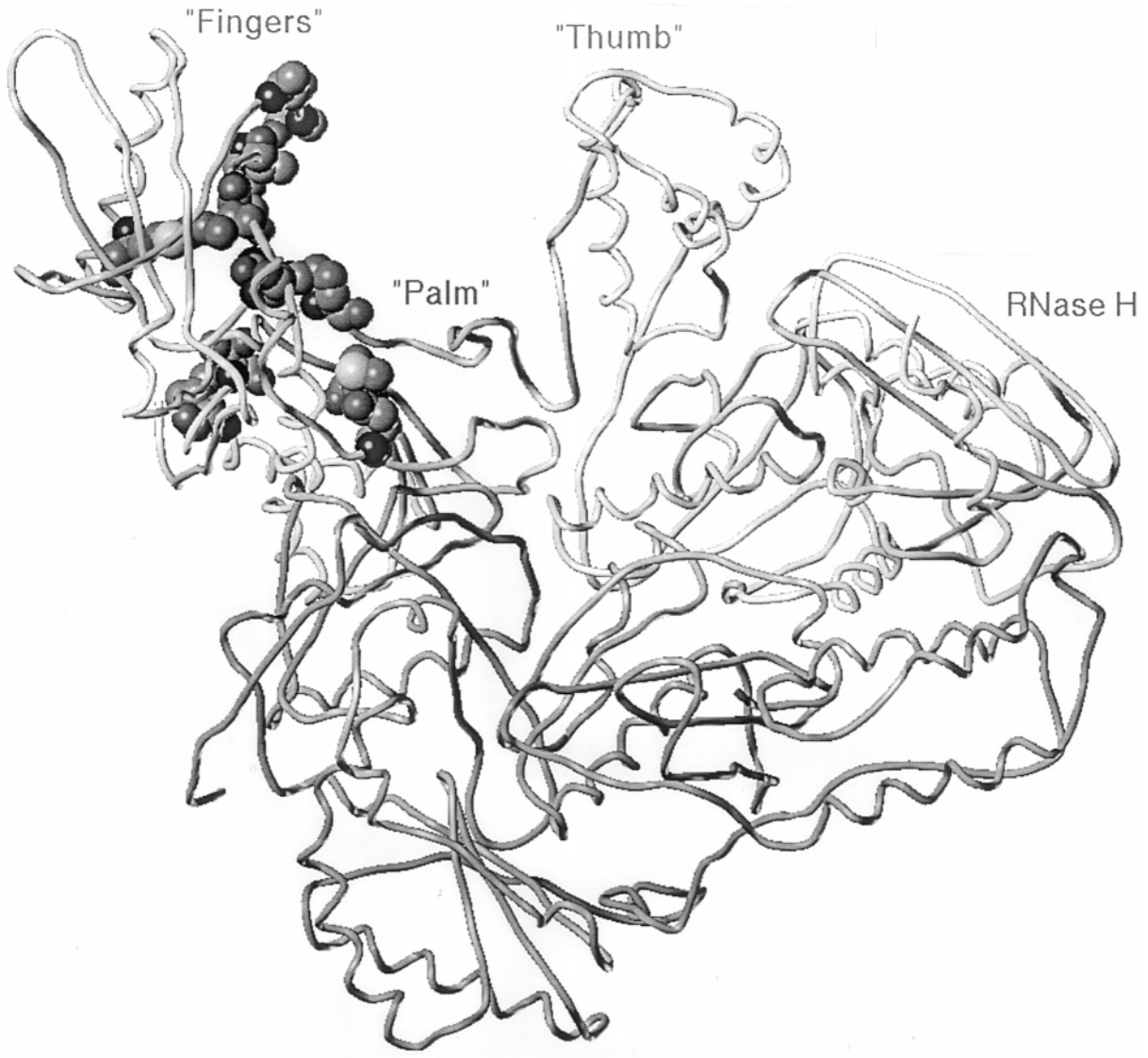

Figure 2. Structure of the HIV-1 RT heterodimer showing locations of residues mutated in NRTI resistance. The crystal coordinates used to generate this figure are Brookhaven Protein Data Bank (PDB) 2HMI [19].

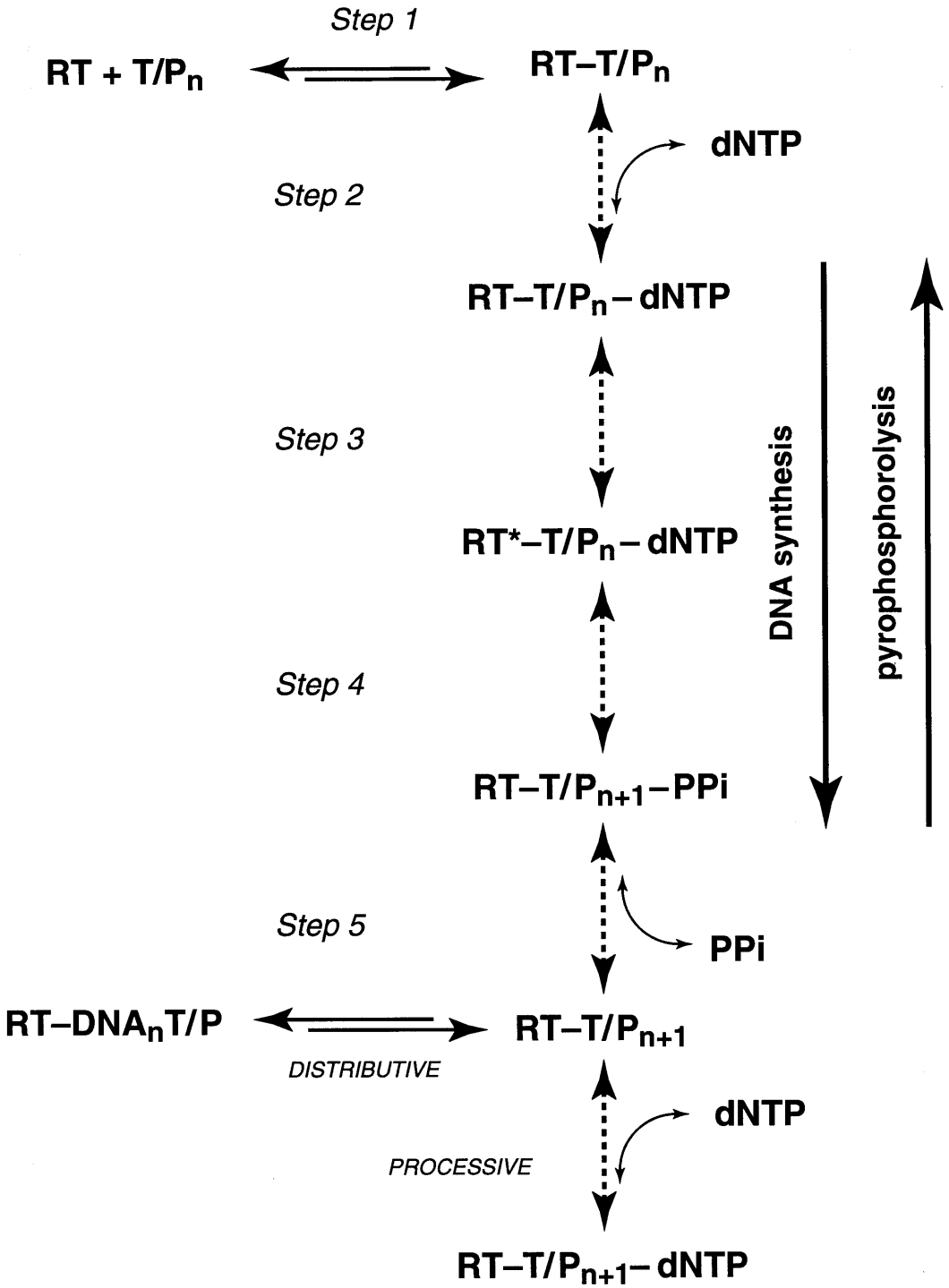

Figure 3. Reaction mechanism for RT-catalyzed DNA synthesis, showing the multiple mechanistic forms of RT involved, and the relationship between forward reaction DNA synthesis and reverse reaction pyrophosphorolysis.

(DDDP) to complete the synthesis of double-strand viral DNA. As well, RT possesses an intrinsic RNase H activity, to degrade the genomic RNA component of the DNA/RNA duplex intermediate formed during RT RDDP synthesis. NRTIs are directed against RT RDDP and DDDP.

HIV-1 RT DNA synthesis follows an ordered 'bi bi' mechanism [11–14] involving several RT mechanistic species. Free RT first binds the template/primer (T/P) to form a tight RT-T/P_n binary complex (fig. 3, step 1). This is followed by the binding of dNTP, to form the RT-T/P_n-dNTP ternary complex (step 2, fig. 3). Binding of dNTP appears to be a two-stage process [15]. The initial interaction may be nonselective, with each of the four dNTPs binding with relatively similar affinities. The second stage is more selective, and involves the correct positioning of only that dNTP complementary to the template base. The selectivity involved in positioning and incorporating the correct nucleotide is primarily controlled by the free energy of base pairing with the complementary template nucleotide base. Since NRTIs and the analogous dNTPs have identical base structures, in most cases RT has little opportunity to readily effect discrimination between natural substrates and inhibitors at this stage.

Formation of the initial RT-T/P_n-dNTP induces a rate-limiting change in protein conformation to form a very tight ternary complex, RT*-T/P_n-dNTP (step 3, fig. 3). The formation of this ternary complex allows the critical transition state to be reached, enabling nucleophilic attack by the 3′-OH primer terminus on the α-phosphate of the bound dNTP. This results in phosphodiester-bond formation and extension of the viral DNA strand by one nucleotide, along with formation of the RT-T/P_{n+1}-PPi ternary pyrophosphate complex (step 4). The PPi product then dissociates, leaving the RT-T/P_{n+1} binary complex, which is kinetically indistinguishable from the RT-T/P_n binary complex. RT can then either dissociate from the newly elongated T/P_{n+1} (distributive mode of polymerization) or translocate along the bound template, bind the next complementary dNTP and continue DNA synthesis (processive mode of polymerization). The key kinetic steps that define the incorporation of dNTP (and the analogous NRTIs) are the nucleotide binding event (step 2), the rate-limiting conformational change (step 3) and the efficiency of incorporation (step 4).

Crystal structures for three of the RT mechanistic forms have been solved. These include substrate-free enzyme (RT) [16, 17], the RT-T/P binary complex [18, 19] and an RT*-T/P-dNTP ternary complex [20]. The 'missing' structures in terms of the RT reaction pathway include the RT-T/P-PPi product ternary complex. Also missing is that complex resulting from the initial binding of the dNTP (RT-T/P-dNTP). However, such a structure has been solved for another DNA polymerase [21]. These structural data have provided considerable insight into the conformational changes associated with DNA polymerization, the molecular structure of the RT active site, and the relative position of certain critical residues during catalysis, all of which are important for understanding the molecular basis of HIV-1 RT resistance to NRTIs.

Comparison of the structures of free RT with the RT-T/P binary complex shows that interaction of RT with the nucleic acid induces a conformational rotation of the p66 thumb subdomain [18, 19]. Comparison of the structures of the RT-T/P binary complex with the RT*-T/P-dNTP ternary complex reveals that dNTP binding induces further conformational changes in RT [20]. In particular, parts of the fingers subdomain rotate inwards toward the palm subdomain and the polymerase active site, effectively 'clamping' the dNTP into the active site region. Two fingers residues make important contacts with the dNTP substrate. The ε-amino of K65 and the guanidinium group of R72 interact with the γ- and α-phosphates of the bound dNTP substrate, respectively (fig. 4). As seen in tables 1 and 2, the K65R mutation arises in HIV-1 resistance to a number of different NRTIs. The dNTP triphosphate moeity also interacts with the main-chain amide-NH of residues D113 and A114, and with the Mg^{2+} divalent metal ion cofactor bound to the polymerase active site aspartates (D110, D185, D186).

The divalent metal ions facilitate the nucleophilic attack by the 3′-OH of the primer terminus on the α-phosphorous of the incoming dNTP, and stabilize the transition state [22]. Only two of the active site aspartates (D110, D185) are coordinated to Mg^{2+} in the HIV-1 RT ternary complex [20]. The third aspartate (D186) may be involved in positioning the primer terminus by interacting with the 3′-OH of the primer terminal nucleotide (this functional group is absent in the ternary complex). The sugar 3′-OH of the dNTP projects into a small pocket formed by the side chains of D113, A114, Y115, F116 and Q151. Interestingly, the 3′-OH of the nucleotide forms a hydrogen bond with the main-chain -NH of Y115. Since all NRTIs are missing the 3′-OH, this interaction may be of significance in understanding the kinetic efficiency of NRTI incorporation into the growing viral DNA chain.

Mechanisms of HIV-1 resistance to NRTIs

Discrimination-resistance due to impaired NRTI binding and/or incorporation

A large number of mutations in HIV-1 RT have been identified in resistance to NRTIs (tables 1 and 2). In a few cases, such as the AZT resistance and certain multidrug resistance phenotypes, multiple mutations are needed to observe high phenotypic resistance. For the most part, however, single point mutations suffice to confer resistance to individual NRTI.

Point mutations such as K65R, T69D, Q151M and M184V/I lead to alterations in affinity of RT for spe-

cific NRTIs with little or no change in affinity for the corresponding dNTP substrate. These mutations are located in, or close to, the dNTP substrate binding site [20], and may therefore affect the initial binding and/or the subsequent positioning of the NRTI for catalysis in a manner such that the mutant RT is able to discriminate between the NRTI and the analogous dNTP substrate. As mentioned previously, discrimination between NRTIs and analogous dNTPs requires that RT must selectively ignore the structurally less rich NRTI in favor of the structurally more complex dNTP analog. It is important to note that wild-type RT is generally less efficient at catalyzing the incorporation of bound ddNTP into the nascent DNA compared with the corresponding dNTP substrate [13, 23]. Two examples will be used to illustrate how RT is able to carry out this discrimination.

K65R. The first example concerns the K65R mutation. As seen in table 1, this mutation confers some level of resistance to a variety of NRTIs, but is primarily recognized for viral cross-resistance to 3TC, ddC and ddI (ddA), as well as to PMEA [24–27]. In vitro studies with purified recombinant RT show that the K65R HIV-1 RT exhibits an eight-fold decrease in the affinity for ddCTP and ddATP, compared with only a twofold decrease in K_M for dCTP and dATP [24, 28]. K65 is in the highly flexible $\beta 3$–$\beta 4$ loop in the fingers domain of the p66 subunit of RT, and the ε-amino of K65 interacts with the γ-phosphate of the bound dNTP substrate [20]. Molecular modeling studies indicate that the K65R

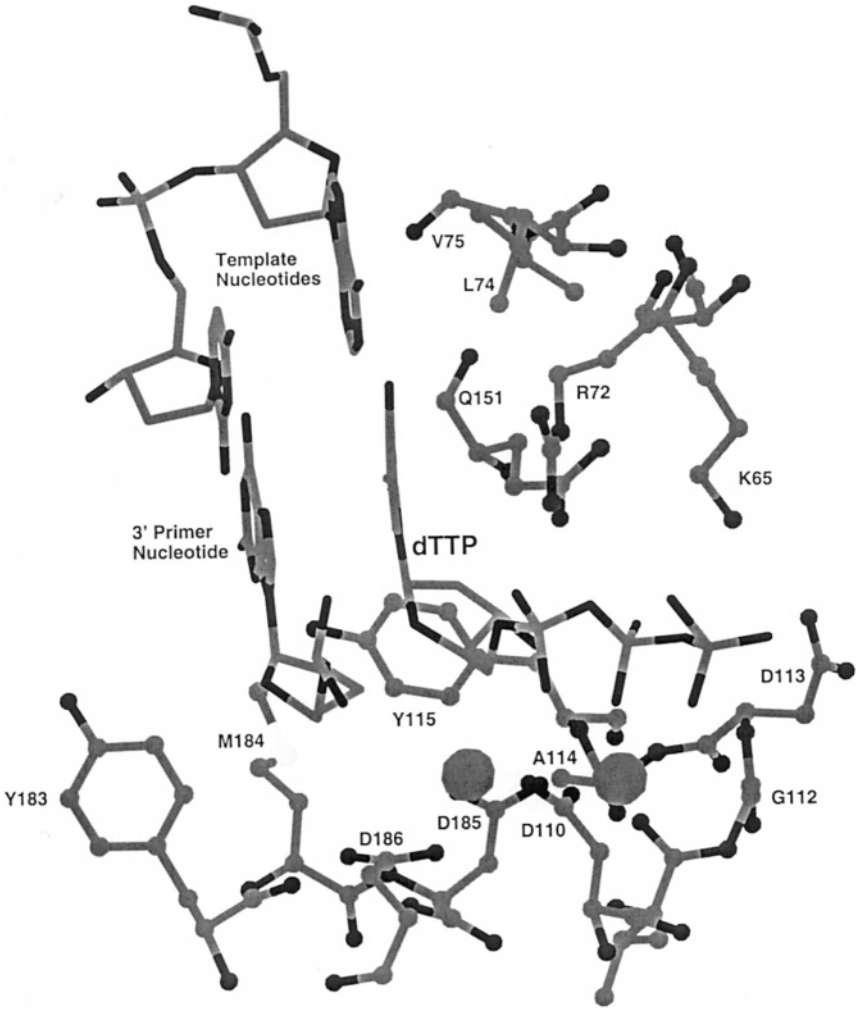

Figure 4. View of the dNTP binding pocket in the RT*-T/P-dNTP ternary complex. The figure was created on Sybyl 6.5 using PDB coordinates 1RTD [20]. The RT residues shown are those that interact with the dNTP substrate (K65, R72, G112, D113, A114, Y115, Q151), the primer 3′-terminal nucleotide (Y183, M184), the complementary template nucleotide (L74, V75) and the catalytic aspartate triad (D110, D185, D186) with coordinated metals.

substitution induces packing rearrangements of the nucleotide substrate, leading to subtle differences in orientation of the phosphate backbone [28]. This leads to displacement of the base component of the nucleotide, which may hinder the positioning of the base for hydrogen bonding to the complementary template base. But why is this effect more pronounced for the NRTI than the analogous dNTP? As previously discussed, the 3′-OH of the dNTP substrate makes significant interactions with the '3′-OH' binding pocket in RT, and in particular to residue Q151. These interactions may facilitate proper positioning of the dNTP base to allow base pairing with the template. NRTI lack a 3′-OH, and are therefore unable to contact the '3′-OH' pocket on the enzyme. The absence of this positioning interaction makes it more difficult for the NRTI base to hydrogen-bond to the template, and the RT-bound NRTI is therefore more likely to adopt an orientation in the polymerase active site that is unfavorable for catalysis. Thus, the absence of the 3′-OH positioning interaction in NRTI binding, coupled with the subtle displacement of the NRTI base arising from the K65R substitution, can account for the selective decrease in RT affinity for ddCTP and ddATP relative to the dNTP substrates.

The slightly decreased affinity of the K65R mutant for dATP and dCTP further increases the already existing bias of RT against NRTI (in this case ddCTP and ddATP) incorporation into the nascent viral DNA, resulting in a readily observable resistance phenotype. The K65R mutant RT possesses an increased processivity in DNA synthesis relative to wild-type enzyme [29]. This may compensate for the modest loss of affinity for dCTP and dATP exhibited by the mutant enzyme, thereby enabling normal replication kinetics.

It is intriguing that the K65R mutation appears to confer selective resistance to some, but not all, NRTIs. For example, we demonstrated that whereas the K65R mutation provides cross-resistance to 3TC, ddC and ddI (ddA), the mutant is not resistant to either ddG or ddT. While the precise mechanism for this selective resistance is not yet known, a clue is obtained from crystal studies of the dNTP complexes with another DNA polymerase, the Klenow fragment of *Thermus aquaticus* DNA polymerase I [30]. The binding of all the dNTP is due in part to interaction of the γ- and α-phosphates of the dNTP with two basic amino acid residues (analogous to K65 and R72 of HIV-1 RT). However, the base components of the dNTPs occupy different positions in the bound structures. The base rings of dCTP and dATP occupy similar positions in the bound structure, and have few contacts with protein residues. The base rings of dGTP and dTTP also occupy similar regions of space as well, but these differ from the regions occupied by the bases of dCTP and dATP. The base components of dGTP and dTTP make significant additional contacts with surrounding enzyme residues, which may stabilize the positioning of these dNTPs for incorporation. We propose that a similar situation may exist for RT. The selective resistance to ddCTP and ddATP conferred by the K65R mutation results from (i) the loss of the 3′-OH pocket interaction, and (ii) the lack of additional base-stabilizing contacts with surrounding RT residues. This results in an unacceptable flexibility in ddCTP and ddATP binding such that correct positioning for catalytic incorporation is impaired. In contrast, the flexibility of ddGTP and ddTTP binding is reduced due to additional contacts of the base ring with RT residues. These NRTIs are more readily positioned for incorporation into the growing DNA chain.

We have recently compared the properties of recombinant RTs with a variety of amino acid substitutions at K65 [28]. Whereas the K65R mutation confers resistance only to specific NRTIs, other mutations such as K65A and K65Q result in broad resistance to all NRTIs (i.e. multidrug resistance). So why does HIV not generate these mutations? These mutant RTs have severe impairments in the incorporation of the natural dNTP substrates as well. Our modeling experiments [28] suggest that only enzymes with K or R at position 65 are able to make significant contacts with the γ-phosphate of the dNTP. The loss of this contact in the other position 65 mutants prevents the proper positioning of the dNTP for catalytic incorporation. Thus, HIV-1 is severely curtailed in the choice of mutations at this position.

M184V (and M184I). The second example of discrimination as a resistance phenotype concerns the point mutation M184V (sometimes M184I) that confers high-level resistance to 3TC [31, 32]. M184 is located in the YMDD motif, which is highly conserved among retroviral RTs. In HIV-1 RT, this motif contains two of the three catalytic aspartates of the DNA polymerase active site [16–20]. The M184V RT is about 140-fold less efficient in incorporating 3TCTP into the nascent viral DNA compared with the wild-type enzyme [33]. This decreased incorporation efficiency results from steric hindrance that leads to decreased binding and/or inappropriate positioning of the 3TCTP for catalysis. In the RT*-T/P-dNTP ternary complex of wild-type RT [20], the side chain of M184 contacts the sugar and the base of the 3′ nucleotide in the primer. Molecular modeling experiments [20, 34] show that substitution of M184 with an amino acid with a β-branched side chain (isoleucine or valine) results in an additional contact with the dNTP sugar moiety (fig. 5). The side chain of a β-branched amino acid at position 184 makes inappropriate contact with the sulfur of the sugar oxathiolane ring of 3TCTP, preventing proper positioning of 3TCTP for catalysis, consistent with the data of Feng and Anderson [33]. The analogous substrate dCTP does

not have a sulfur in its sugar ring, and no steric hindrance of dCTP binding is noted. In contrast, Krebs et al. [23], while demonstrating that the M184V mutant RT shows a markedly reduced efficiency of incorporation of 3TCMP into viral DNA, found no differences in binding affinity of the wild-type and M184V mutant enzymes for 3TCTP. These observations differ from those of Feng and Anderson, and may support the T/P repositioning model (see below). Thus, the precise mechanism by which the M184V/I mutations confer resistance to 3TC is still not completely certain. However, we feel that steric hindrance preventing correct positioning of 3TCTP in the RT active site likely plays a major role in this resistance mechanism.

Template/primer repositioning

Boyer et al. [35] showed that wild-type RT was inhibited by ddITP when the template extension was four nucleotides or more beyond the 3′ end of the primer, but was resistant to ddITP when the template extension was three nucleotides or fewer [35]. In contrast, ddITP resistance shown by the L74V and E89G mutant enzymes was independent of template extension length. The RT-T/P binary complex crystal structure [18] suggested that residues 74 and 89 contacted the DNA T/P; the recently solved RT*-T/P-dNTP ternary complex [20] shows that L74 interacts with the template nucleotide that is base-paired to the incoming dNTP. Boyer et al. [35] therefore proposed that the L74V and E89G mutations (and perhaps others) confer resistance to NRTI as a result of repositioning of the T/P in the RT-T/P complex, which in turn alters the ability of the enzyme to select for, or reject an incoming dNTP. While there is no structural evidence for T/P repositioning in the L74V or E89G mutants, comparison of the M184I RT-T/P binary complex with the wild type RT-T/P binary complex reveals some alterations in the position of the T/P in the M184I mutant RT structure [34]. As discussed above, the data of Krebs et al. [23] provide circumstantial support for the T/P repositioning model in 3TC resistance. Nonetheless, the relevance of T/P repositioning in phenotypic resistance to NRTI is uncertain. How could this repositioning selectively alter incorporation of ddNTP, without also dramatically affecting incorporation of the corresponding dNTP substrate? In addition, it is not certain whether the shift in T/P position noted in the M184I RT-T/P binary complex would be retained in the subsequent RT-T/P-dNTP ternary complex. Finally, as discussed above, the phenotype for 3TC resistance is satisfactorily explained by the steric conflict between the oxathiolane ring of 3TCTP and the side chain of the branched β-amino acid. Therefore, there does not appear to be a need to invoke T/P repositioning to explain this resistance.

Template/primer repositioning may, however, play a role in the decreased DNA synthesis processivity associated with the L74V mutation for ddI resistance [36]. However, this altered processivity does not necessarily contribute to the resistance phenotype. Whereas L74 interacts with the template nucleotide that is base-paired to the incoming dNTP molecule, it is also proximal to Q151 and R72, two residues that make significant contacts with the bound dNTP [20]. Thus the L74V mutation may alter ddNTP binding and/or catalysis by altering the packing rearrangements of ddNTP through its interactions with the aforementioned residues.

Phosphorolytic removal of incorporated chain-terminating NRTI—the novel mechanism of AZT resistance

The efforts of investigators to elucidate the biochemical phenotype for HIV-1 resistance to AZT was complicated by the absence of any measurable effect on inhibition of RT by AZTTP in vitro, despite the more than 200-fold resistance to AZT demonstrated in cell culture [37, 38]. AZT resistance correlates with multiple mutations in RT, including M41L, D67N, K70R, L210W, T215Y/F and Q219K [39–42] (table 1). The extent of AZT resistance is related to the combination of mutations present. In general, two or more of these mutations are normally needed in RT to show high-level AZT resistance [6, 38]. The structure of the AZT-resistant substrate-free RT containing the D67N/K70R/T215F/K219Q mutations suggested that the T215F and K219Q mutations might induce long-range conformational changes that impact on the active site aspartate residues [43]. Examination of the RT*-T/P-dNTP ternary complex shows that AZT resistance mutations all cluster around the dNTP binding pocket, suggesting a direct influence on AZT binding. However, detailed kinetic analyses were unable to detect significant differences between the AZT-resistant RT and wild-type RT for the incorporation of AZTTP into the DNA chain [23, 44, 45].

We and others recently proposed that the AZT resistance phenotype is related to increased rates of phosphorolytic removal of chain-terminating AZT from the 3′-terminus of the primer [46–49]. This removal, which may be facilitated by the enhanced binding of AZT-resistant RT to an AZT-MP-terminated primer [50], may be accomplished by two mechanisms that use different substrates to carry out the phosphorolysis reaction. In the first, we showed that the AZT-resistant mutant RT exhibits an increased rate of RT-catalyzed pyrophosphorolysis (the reverse reaction of DNA polymerization; fig. 3). The substrate for pyrophosphorolysis is PPi, which is the normal product of DNA synthesis and

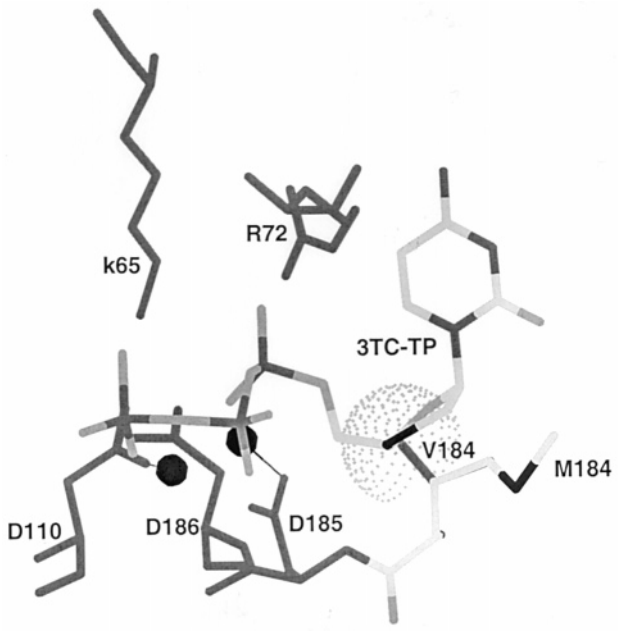

Figure 5. Molecular model of the proposed steric conflict between 3TC and M184V. The model was created with Sybyl 6.5 using the Kollman-All-Atom force field for energy minimization. A Connolly dot surface is provided for the side chain–CH_3 of V184, to illustrate the steric overlap with the oxathiolane sugar ring of 3TCTP. This overlap is not present with the wild-type M184 residue.

is also present at an intracellular concentration of about 150 μM [51]. RT-catalyzed pyrophosphorolysis occurs readily at these intracellular levels of pyrophosphate (PPi), and leads to removal of the chain-terminating AZT from the 3′-end of the primer [46, 48]. The reaction mechanism for pyrophosphorolysis is provided in figure 3, and presented schematically in figure 6. In this reaction, the RT-T/P complex binds pyrophosphate, which carries out the nucleophilic attack on the phosphodiester bond between the last two nucleotides of the primer. This results in the release of the chain-terminating 3′-nucleotide, with the concomitant appearance of a free 3′-OH on the primer. Continued forward direction DNA synthesis can now occur.

We demonstrated that it is the D67N/K70R mutations in the AZT-resistant RT that lead to the increase in the rate of RT-catalyzed pyrophosphorolytic cleavage of chain-terminating AZT [46, 48]. However, these mutations also increase the overall back reaction of RT DNA polymerization, resulting in increased removal of normal nucleotides as well. This would hinder facile viral DNA synthesis and confer a replication deficit to AZT-resistant HIV-1. However, the increased rate of the reverse (pyrophosphorolysis) reaction is compensated by increased DNA synthesis processivity. The latter phenotype is due to the T215F/K219Q mutations [46, 48]. The net result of these multiple mutations in RT is the facile synthesis of full-length viral DNA in the presence of AZT.

Meyer et al. have shown that HIV-1 RT also possesses an interesting capacity to carry out ribonucleotide-dependent phosphorolysis [47]. This reaction excises the terminal nucleotide from the 3′ end of the primer with the concomitant formation of a dinucleoside tetraphosphate (fig. 6); if this nucleotide is a chain terminator such as AZT, this results in the appearance of a free 3′-OH on the primer, enabling DNA synthesis to resume. The ribonucleotide-dependent phosphorolysis reaction is inhibited by the dNTP complementary to the next position on the template, suggesting competition between dinucleoside polyphosphate synthesis and DNA polymerization. The AZT-resistant mutant RT is more efficient at carrying out this reaction than wild-type RT, suggesting that ribonucleotide-dependent phosphorolysis may also constitute a phenotypic mechanism for HIV-1 RT resistance to AZT [49]. In this model, the D67N/K70R mutations in the AZT-quad mutant RT lead to elevated primer unblocking activity, whereas the T215F/K219Q mutations are implicated in reduced inhibition of the phosphorolysis reaction by the next complementary dNTP.

It is important to realize that the chemistry involved in pyrophosphorolysis and the ribonucleotide-dependent phosphorolysis reactions is identical (fig. 6). In both cases, the removal of the chain-terminating AZT results from nucleophilic attack of polyphosphate (PPi, or the γ-phosphate of ATP) on the phosphodiester bond between the last two nucleotides of the primer, resulting in removal of the 3′-terminal AZT, either as AZTTP (from pyrophosphorolysis) or as an adenosine-3′-azido, -3′-deoxythymidine-5′-5′-tetraphosphate (from ribonucleotide-dependent phosphorolysis).

It is not yet known whether pyrophosphorolysis or ribonucleotide-dependent phosphorolysis is the predominant biological phenotype for AZT resistance. There are advantages and disadvantages associated with both phenotypic mechanisms. Enzymatic reactions are intrinsically reversible, and pyrophosphorolysis provides the simplest mechanism of chain-terminating nucleotide removal. Pyrophosphate is the normal product of DNA polymerization, and PPi readily binds to RT at concentrations close to the intracellular PPi levels [52, 53]. Indeed, it is possible that the same PPi formed during AZT incorporation may serve to immediately excise the chain terminator. The increased affinity of AZT-resistant RT for both PPi [46] and AZT-terminated T/P [50] is consistent with such a scenario. The disadvantage of the pyrophosphorolysis mechanism is that the reaction regenerates AZTTP, which may then

be reincorporated into the viral DNA. However, AZTTP binding to AZT-resistant RT is significantly diminished in the presence of physiological intracellular levels of PPi [46, 48]; this reduced binding may play a role in diminishing the reincorporation of the AZTTP. In contrast to PPi, HIV-1 RT has only a very low affinity for ribonucleotides such as ATP [47]. Furthermore, in order to initiate the primer unblocking activity, ribonucleotide triphosphates such as ATP would need to bind to RT in a very different manner than the normal dNTP substrate, in order to allow attack by the γ-phosphate of ATP on the phosphodiester bond between the last two nucleotides of the viral DNA (fig. 6). Indeed, it might be envisioned that ATP binds in an orientation 'backwards' from that of the normal dNTP substrate in order for the γ-phosphate of ATP to be in proximity to the phosphodiester bond between the last two nucleotides of the primer strand, to enable the ribonucleotide-dependent phosphorolysis reaction to proceed. The advantage of the ribonucleotide-dependent phosphorolysis mechanism is that the phosphorolytic removal of AZT results in the formation of dinucleoside tetraphosphate which, unlike the AZTTP product of pyrophosphorolysis, cannot be reincorporated into the viral DNA.

Regardless of which mechanism predominates in AZT resistance in vivo, in our hands both mechanisms are equally effective in removing AZT from chain-terminated primers in vitro [54]. In fairness, however, it must be mentioned that Meyer et al. contend that they are unable to observe differences in pyrophosphorolytic activity between wild-type and AZT-resistant RT, but are readily able to identify differences in the ATP-dependent phosphorolytic activity of these same enzymes [49]. This is a rather surprising observation, given the identity of the chemistry implicit in these two mechanisms.

Mutations antagonistic to the AZT resistance phenotype

Several reports have shown that certain mutations, when added to an AZT-resistant genetic background, reverse HIV-1 resistance to AZT [55–66]. The first mutation reported to restore sensitivity to AZT-resistant virus strains to AZT was the L74V mutation that appeared under ddI drug pressure [55]. Subsequently, the mutations K65R and M184I/V, associated with PMEA and 3TC resistance, respectively, and Y181C and L100I, associated with resistance to nonnucleoside RT inhibitors, were also found to suppress AZT resistance [56–60]. Phosphonoformic acid (PFA), a pyrophosphate analog, selects for a variety of resistance mutations, several of which markedly increase HIV-1 viral sensitivity to AZT both for wild-type virus and importantly for AZT-resistant virus [61–66].

We recently found that the introduction of the A114S PFA-resistance mutation into a background of the D67N/K70R/T215F/K219Q mutations in RT (that confer high-level AZT resistance) results in a dramatic diminution in the rate of pyrophosphorolysis catalyzed by the AZT-resistant enzyme, and lowers the DNA synthesis processivity carried out by the mutant RT [54]. Importantly, the ability of the D67N/K70R/T215F/K219Q + A114S mutant to remove AZT from a chain-terminated primer was completely eliminated. Both pyrophosphorolysis and ribonucleotide-dependent phosphorolysis reactions were affected by the A114S mutation. We propose that the ability of the A114S mutation to completely suppress the phosphorolytic removal of chain-terminating AZT accounts for the resensitization of AZT-resistant HIV-1 to AZT, despite the continued presence of the NRTI-resistance mutations.

Interestingly, the A114S mutation results in a 20-fold decreased affinity of RT for AZTTP; both wild-type and the D67N/K70R/T215F/K219Q AZT-resistant RT are equally affected [54]. This decreased affinity for AZTTP would be expected to confer AZT resistance. In fact, as discussed above, the A114S mutation restores sensitivity to AZT. This finding further highlights the importance of phosphorolysis as the primary AZT-resistance phenotype.

We have recently extended these findings to show that the M184V mutation also dramatically reduces the ability of RT to carry out phosphorolysis reactions [67]. When present in a background of AZT resistance, the M184V completely eliminates the enhanced phosphorolytic activity of the D67N/K70R/T215F/K219Q RT. This observation may account for the success of AZT + 3TC combination therapy in the treatment of HIV infection. Although we have not yet characterized the effects of the K65R, L74V, L100I and Y181C 'AZT-resensitizing' mutations, we predict that these will also affect the ability of AZT-resistant RT to catalyze phosphorolytic removal of chain-terminating AZT.

HIV-1 multidrug resistance

The mutation Q151M in HIV-1 RT is associated with multidrug resistance [68]. This mutation alone appears to confer 10-fold resistance to AZT, 20-fold resistance to ddC and 5-fold resistance to ddI in cell culture virus replication assays. As previously discussed, Q151 is a key residue in the dNTP binding pocket. Therefore, resistance due to the Q151M mutation may arise as a result of disruptions of the hydrogen-bonding network involving Q151 and the side chain of R72, the main-

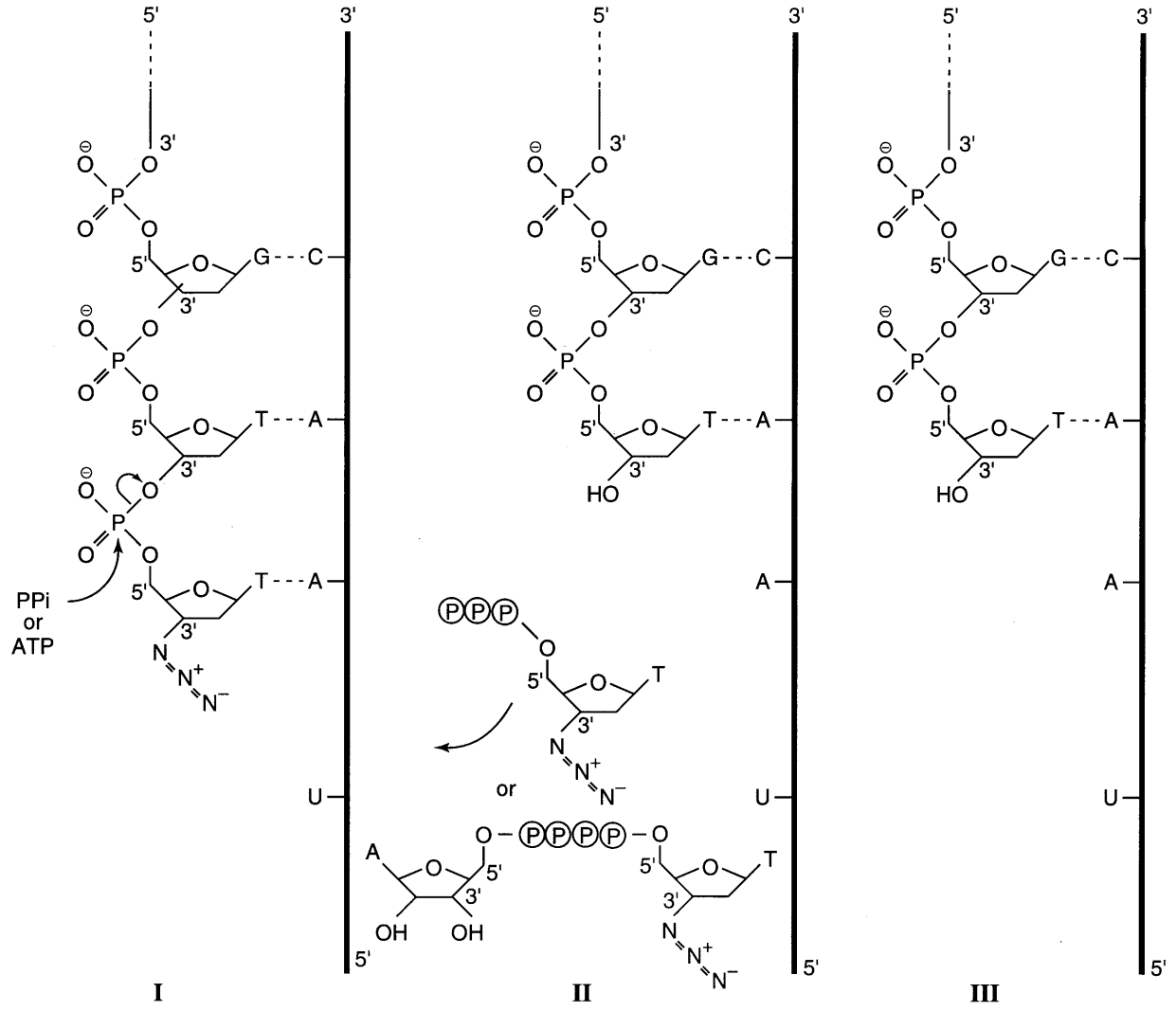

Figure 6. The role of PPi and ATP in removing 3′-terminal AZT from a chain-terminated primer. Panel I illustrates the nucleophilic attack by PPi or the γ-phosphate of ATP on the 3′–5′-phosphodiester bond between primer 3′-terminal AZT and the penultimate primer nucleotide. This reaction leads to the removal of the terminal AZT (panel II) either as AZTTP or as an adenosine-AZT 5′,5′-tetraphosphate, leaving a nucleotide with a free 3′-OH at the primer 3′-terminus (panel III). Viral DNA synthesis may now proceed.

chain carbonyl of R73, and the 3′-OH and the α-phosphate of the dNTP. The Q151M mutation is generally accompanied by mutations at four neighboring residues (A62V, V75I, F77L and F116Y) [68]. These additional mutations may reinforce the effects of the Q151M primary mutation by increasing the observed level of resistance and by improving the RT-catalyzed DNA polymerase activity, which is impaired by the Q151M mutation. For the most part, the phenotype(s) involved in multidrug resistance conferred by Q151M are not yet characterized. However, since Q151 is an integral component of the 3′-OH pocket for dNTP binding, altered discrimination between NRTI and the analogous dNTP may be a factor. In this regard, Ueno et al. [69] showed that multidrug resistant RT (with the A62V, V75I, F77L, F116Y, Q151M mutations) has 65-fold resistance to AZTTP, 12-fold resistance to ddCTP, 9-fold resistance to ddATP and 3-fold resistance to ddGTP compared with wild-type enzyme. They concluded that the mutations resulted in altered recognition of these inhibitors.

HIV-1 multidrug resistance is also associated with insertion mutations that provide two additional amino acid residues between RT residues 68 and 70 (T69S-(S-S)-K70 or T69S-(S-G)-K70). Boyer et al. [70] have suggested that these insertions simply provide a discrimination phenotype that provides low-level resistance to ddITP. However, why should HIV-1 need to

provide a two-amino-acid insertion to provide a four-fold resistance to ddI, when the L74V mutation provides a similar resistance profile? We think the answer is more complex. A recent study showed that the insertion mutations provide multidrug resistance mainly when they are present in a background of AZT resistance mutations [71]. Our experiments show that the enhanced phosphorolysis associated with the D67N/K70R/T215F/K219Q RT is relatively specific for AZT-terminated DNA [46]. Perhaps the insertions, which arise in the same flexible $\beta 3$–$\beta 4$ loop in the fingers domain as many other NRTI resistance mutations, broaden the specificity for phosphorolytic removal of chain-terminating NRTIs, thereby providing multidrug resistance. Also of interest is the observation that patients receiving d4T and ddI combination therapy develop AZT-resistance mutations [72]. This may indicate that under certain conditions the phosphorolysis phenotype associated with AZT resistance may also be important for resistance to other NRTIs.

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