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UNDERSTANDING THE MOLECULAR MECHANISM OF DRUG RESISTANCE OF ANTI-HIV NUCLEOSIDES BY MOLECULAR MODELING

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1. ABSTRACT

Nucleoside-resistant isolates have been identified in patients receiving antiretroviral nucleoside drugs. The different resistance phenotypes seem to correlate with different sets of mutations in reverse transcriptase (RT), and the effect of individual set of mutations on resistance to a specific NRTI can only be presumed by kinetic studies and building up the enzyme active site by molecular modeling studies. However, the understanding how mutations affect RT structure and function, and the ensuing loss of potent antiviral activities of certain NRTIs have not been demonstrated in conjunction with their binding modes, which would provide invaluable insight into the design of more effective NRTIs active against the mutant RTs. This review discusses our recent efforts to assess the structural adjustment resulting from mutations and the accompanying energetic consequences based on the assumption that mutation may either deform the active site conformation through structural realignment or destabilize inhibitor binding.

2. INTRODUCTION

Viruses, contrary to bacteria and parasites, meet their metabolic needs by using both their host's and their own enzymes. The fact that the viral enzymes are often quite similar to their cellular equivalents is one of the reasons that make the development of effective, nontoxic drugs against viral infections a rather challenging task. One way to overcome this problem has been to identify, within viral enzymes, those which have properties not shared by the host counterparts. These enzymes could then be selective targets for agents which impair the viral replication without harming the host.

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 multifunctional enzyme that has two types of DNA polymerase activity, RNA-dependent DNA polymerase (RDDP, unlike cellular enzymes) to synthesize a DNA strand copy of the viral genomic RNA template and DNA-dependent DNA polymerase (DDDP, like cellular enzymes) to complete the synthesis of double-strand viral DNA. Additionally, RT possesses an intrinsic RNaseH activity to degrade the genomic RNA component of the DNA/RNA duplex intermediate formed during RT RDDP synthesis. As a

result of the key role of HIV-1 RT in virus replication, it has been a major target for the development of many anti-AIDS drugs. Examples include the nucleoside/nucleotide inhibitors (NRTIs/NtRTIs): AZT (1), 3TC (2), ddI (3), ddC (4), d4T (5), abacavir (6), tenofovir disoproxil (7), as well as the non-nucleoside inhibitors (NNRTIs), nevirapine (8), delavirdine (9) and efavirenz (10). However, even in the context of the multi-drug combination therapy now widely in use, the mutability and resultant adaptability of HIV-1 RT present a major challenge to the design of effective antiviral strategies because many initially potent drugs lose efficacy over time.

In the case of the NNRTIs, most of the resistance mutations associated with the p66 subunit are located proximal to the drug-binding site, resulting in direct steric perturbations. In contrast, until the recent publication of the crystal structure of a covalently trapped catalytic complex of HIV-1 RT (11), some aspects of the structural basis for the mechanisms of resistance for NRTI drugs have largely remained a puzzle because many of the mutation sites that give resistance are distal (between 10-20 Å) to the putative nucleoside triphosphate binding site on the enzyme. In addition, this work (11) reveals that there is a conformational change of the so-called fingers and thumb domains of the p66 subunit of HIV-1 RT that, together with a loop movement, results in a more 'closed' conformation of the central cleft of p66, instead of the 'open' conformation seen in previous HIV-1 RT crystal structures. The presence of this unanticipated conformation places certain NRTI drug resistance mutations much closer to the nucleoside triphosphate binding site of the enzyme than previously predicted, thereby providing a simple steric explanation for their mechanism of resistance. However, the structure of the wild type enzyme complexed with the natural substrate (dNTP) fails to delineate the mechanism of resistance conferred by mutation in the amino acid residues of the enzyme to many structurally different NRTIs. In addition, in certain cases such as AZT-resistant mutants, simple combination of the mutations relatively far away from the dNTP binding site does not provide any structural information of resistance. Therefore, even though there is a critical need for a comprehensive understanding of the molecular mechanism of anti-HIV drug resistance by mutant RTs, lack of crystallographic and structural information of the mutant RTs complexed with DNA duplex as well as NRTI triphosphate has prevented detailed investigations. As an aid to understanding how mutations affect RT structure and function, several molecular modeling studies have been undertaken on RT modified at specific residues (12). Presumably, mutation may either deform the active site conformation through structural realignment or destabilize inhibitor binding. Therefore, under the given limitations of the computational method, assessment of the structural adjustment resulting from these local changes and the accompanying energetic consequences may provide insight into the design of more effective NRTIs. This review discusses our recent efforts to correlate the structure with enzyme function in the HIV-1 RT, which may lead to the understanding of the NRTI drug resistance conferred by mutations.

3. INHIBITION OF HIV-1 RT BY NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NRTIS)

HIV-1 RT differs from cellular DNA polymerases in two respects. First, HIV RT readily utilizes many chemically altered analogues of the natural deoxynucleoside triphosphate (dNTP). Second, HIV RT lacks a formal 'proofreading' activity (13). These characteristics are important for the drug design view point for HIV. NRTIs are analogues of the normal deoxynucleoside substrates of DNA polymerases. The 2',3'-dideoxynucleosides such as ddC and ddI lack a 3'-OH on the sugar, whereas other analogues such as 3'-azido-3'-deoxythymidine (AZT) have the 3'-OH replaced by other functional groups that do not allow the primer extension. Efficiency of the inhibition of HIV-1 RT is dependent on four major factors: (i) the efficiency of cellular uptake of the analogue, (ii) the efficiency of the initial phosphorylation by cellular kinases, (iii) the level of competitive inhibition with the natural substrate, and (iv) the efficiency of triphosphate binding to RT and subsequent incorporation into the growing viral DNA (14).

3.1. Cellular Uptake

Most nucleoside analogues, such as ddC and 3TC, enter cells by active transport (15). In contrast, a more efficient mechanism, i.e., passive or nonfacilitated diffusion, has been observed for entry of AZT and ddI (16). Phosphorylation of nucleoside analogues precedes antiviral activity in HIV-infected cells.

3.2. Phosphorylation

NRTIs require intracellular metabolic transformation for antiviral activity, namely conversion to the triphosphate, a process catalyzed by cellular kinases. The first phosphorylation step, often considered as the rate limiting, is dependent on cytosolic thymidine kinase 1 (TK1), cytosolic deoxycytidine kinase (dCK), mitochondrial thymidine kinase 2 (TK2) or mitochondrial deoxyguanosine kinase (dGK), depending on the nucleoside considered (17). Conversion of AZT to AZT 5'-monophosphate (AZT-MP) by thymidine kinase occurred with about the same efficiency as synthesis of TMP by the same enzyme, but the process and kinetics of phosphorylation were not identical for other thymidine analogues (18). The pathways of ddA and ddI phosphorylation are more complex and less well understood than those of either AZT or ddC phosphorylation (19). ddI can be aminated by adenosine deaminase to form ddA, or it may be phosphorylated by a cytosolic 5'-nucleotidase to form ddIMP (20, 21). All ddIMP, generated by the exogenous addition of ddI or ddA to cells, is eventually aminated and phosphorylated to ddATP by adenosine deaminase and purine nucleoside kinases, respectively (19, 20). In contrast, many beta-L-cytidine analogues such as 3TC (22), FTC (22) and L-dC (23) are substrates of dCK regardless of the nature of the pentose. In addition to the activation of cytosine nucleoside derivatives, human dCK also catalyzes the phosphorylation

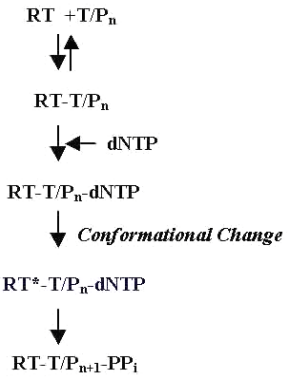


Figure 1. Reaction mechanism for RT-catalyzed DNA synthesis.

of D- and L-enantiomers of beta-dA, beta-araA, and beta-dG with enantioselectivities favoring the unnatural L-enantiomer for adenosine derivatives and the natural D-enantiomer for 2'-deoxyguanosine (24). In the case of the carbocyclic analogues of deoxyguanosine, the enzyme (dGK) catalyzes the phosphorylation of both enantiomers with a preference for the L-antipode (25).

3.3. Competitive Inhibition and Incorporation into the Growing Viral DNA

After conversion to the active triphosphate, NRTIs must compete with the natural dNTPs, both for recognition by RT as a substrate (binding) and for subsequent incorporation into the nascent viral DNA chain (catalysis). NRTIs thus inhibit RT-catalyzed proviral DNA synthesis by two mechanisms (26). 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. Even though the chain termination is the principal mechanism of NRTI antiviral action, both mechanisms are thought to be required for the inhibition of HIV replication in host cells (14, 27). HIV-1 RT DNA synthesis follows an ordered 'bi-bi' mechanism involving several RT mechanistic species (figure 1) (28-31). Free RT first binds to the template/primer (T/P) to form a tight RT-T/P_n binary complex, which is followed by the binding of dNTP, to form the RT-T/P_n-dNTP ternary complex (figure 1). Binding of dNTP appears to be a two-stage process (32). 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 specific 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 affect the 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 (figure 1). The formation of this ternary complex allows the critical transition state to be reached, enabling a nucleophilic attack by the 3'-OH primer terminus on the alpha-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}-PP_i ternary complex (figure 1). The PP_i 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 RT/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). Competitive inhibition by ddNTP results from stronger binding of RT to ddNTP than to dNTP, and, therefore, the strength of a competitive inhibitor of RT is directly related to the ratio of its K_i (inhibition constant or enzyme-inhibition dissociation constant) for ddNTP to its K_m (Michaelis constant, a measure of dissociation) for dNTP. In RT reactions employing homopolymeric template-primer (T/P), the K_i for AZT-TP was nearly 10-fold less than the K_m for TTP, making this analogue a potent competitive inhibitor of RT (33-35). With the possible exception of the acyclic analogue phosphonylmethoxyethyladenine (36), nearly all ddNTP having anti-RT activities were shown to be competitive inhibitors of HIV-1 RT: ddCTP, 3TC-TP and ddATP (K_i/K_m <10) were relatively poor competitive inhibitors of HIV-1 RT compared with AZT-TP, carbovir-TP and ddGTP (K_i/K_m>19). Nevertheless, competitive inhibition by NRTIs was not sufficient to inactivate HIV-1 RT (14, 37).

Chain termination of HIV-1 reverse transcription has been observed with nearly all ddNTP studied in cell-free RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP) reactions (35, 38-41). Incorporation of ddNMP by RT and subsequent chain termination are preceded by a NRTI-TP binding event. The key kinetic steps that define the incorporation of dNTP (and NRTIs) are the nucleotide binding event, the rate-limiting conformational change, the efficiency of incorporation and RT-T/P_{n+1} dissociation constant (42).

4. HIV-1 RT STRUCTURE AND FUNCTION

Crystal structures for three of the RT mechanistic forms have been solved, which include substrate-free enzyme (RT) (43-44), the RT-T/P binary complex (45-46) and an RT*-T/P-dNTP ternary complex (11). 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 resistance to NRTIs.

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 51kDa (termed p66 and p51) (47). The p51 subunit is produced during viral assembly and maturation via HIV-1

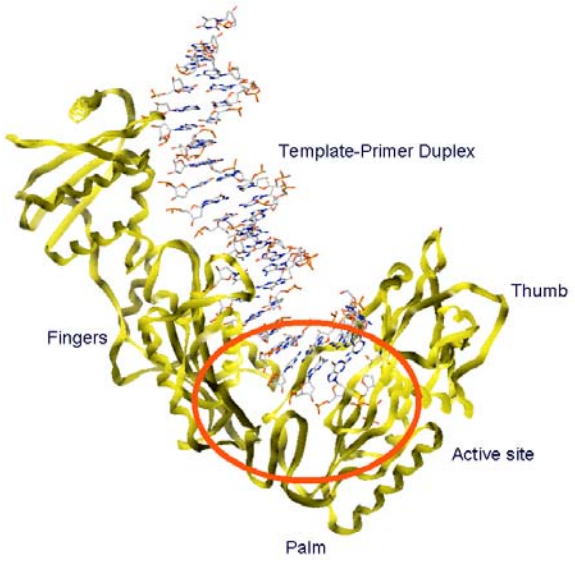


Figure 2. Three dimensional structure of HIV-1 RT•T/P•TTP ternary complex

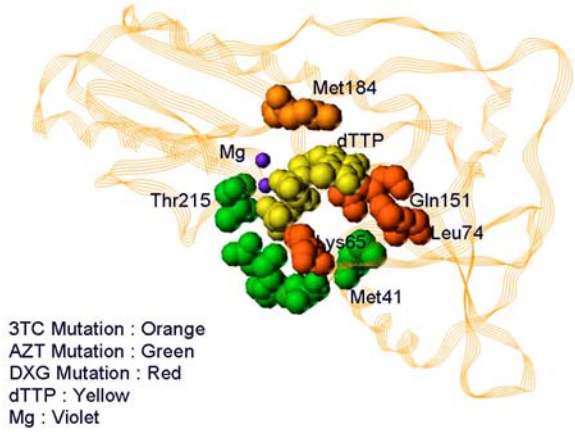


Figure 3. Mutations in HIV-1 RT resistant to NRTIs.

protease-mediated cleavage of the C-terminal domain of a p66 subunit. The first crystal structure of HIV-1 RT revealed that the p66 subunit was arranged with a right-handed fingers (residues 1-85)-palm (residues 86-117, 156-237)-thumb (residues 238-318) domain structure containing a cleft that is similar to that first observed for the Klenow fragment of PolI (48) (figure 2). The DNA polymerase catalytic aspartate residues (D110, D185 and 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) 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 (49-50). 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) primer used for the initiation of HIV-1 DNA synthesis (49). However, only the palm domains of these two proteins have similar three-dimensional folds. The RT structure revealed that nevirapine, a NNRTI, can bind in the palm domain close but not overlapping to the putative dNTP binding site. This and further RT-inhibitor complex structures were able to explain the NNRTI resistance mutations (51-52), which were clustered around the drug binding pocket. However, the NRTI mutations that had been reported at that time appeared to be positioned on both the fingers and palm domains, at some distance from the presumed dNTP site (figure 3).

An RT structure with a bound double-stranded DNA template-primer together with a Fab fragment confirmed the previous 'open' RT p66 conformation (45). However, 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. As the complex reaches the transition state (RT^*-T/P-dNTP ternary complex) by binding dNTP, further conformational changes in RT are induced (11). 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. Therefore in order for the enzyme-ligand complex to be catalytically active, there must be some conformational changes in RT leading to a tight binding of the substrate. Two fingers residues make important contacts with the dNTP substrate. The epsilon-amino group of Lys65 and the guanidinium group of Arg72 interact with the alpha-and beta-phosphates of the bound dNTP substrate, respectively (figure 4a). The dNTP triphosphate moiety also interacts with the main-chain amide-NH of residues Asp113 and Ala114 (figure 4b), and with the divalent metal ion cofactor (Mg^{2+}) bound to the polymerase active site aspartates (Asp110, Asp185 and Asp186) (figure 5). The divalent metal ions facilitate the nucleophilic attack by the 3'-OH of the primer terminus on the alpha-phosphorous of the incoming dNTP, and stabilize the transition state (53). Only two of the active site aspartates (Asp110 and Asp185) are coordinated to Mg^{2+} in the HIV-1 RT ternary complex (figure 5) (11). The third aspartate (Asp186) may be involved in positioning the primer terminus by interacting with the 3'-OH of the primer terminal nucleotide, which projects into a small pocket formed by the side chains of Asp113, Ala114, Tyr115, Phe116 and Gln151 (figure 4b). Interestingly, the 3'-OH of the nucleotide forms hydrogen bonds with the backbone –NH of Tyr115 and triphosphate moiety of the dNTPs (figure 4b), which also plays an important role accommodating the dNTP in HIV mutant RT.

5. MECHANISM OF HIV-1 RESISTANCE TO NRTI

The low fidelity of HIV-1 RT and the high rate of replication of HIV result in mutations at a frequency of about 10^{-4} per cycle (37). Mutant viruses, many of which are replication defective (54), are continuously produced by $CD4^+$ cells that contain stably integrated proviral DNA. This heterogeneity allows for early selection of replication-competent viruses by antiviral drugs, permitting the emergence of drug-resistant forms (55-60). Nucleoside-resistant isolates have been identified in patients receiving

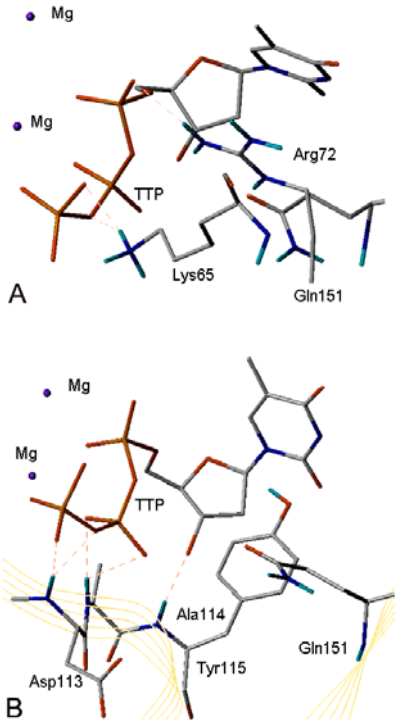


Figure 4. Active site of HIV-1 RT.

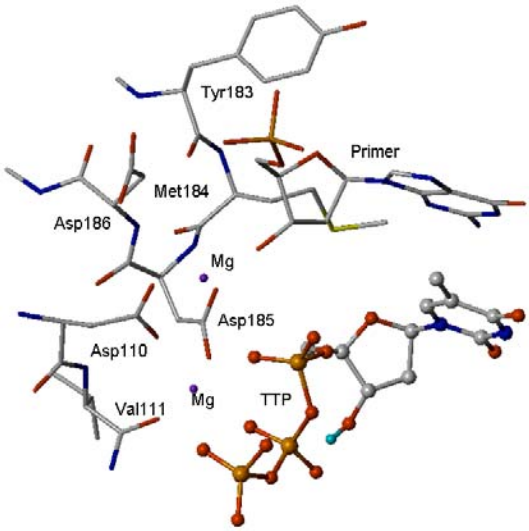


Figure 5. YMDD motif and Mg-coordination geometry of HIV-1 RT.

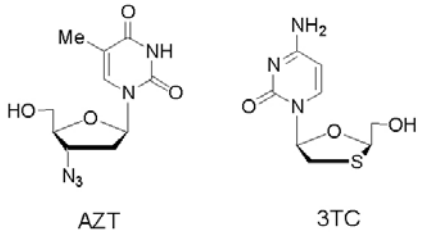


Figure 6. Structures of AZT and 3TC.

antiretroviral nucleoside drugs (61-63). In addition, resistant variants can be generated through tissue culture passage of drug-sensitive strains in the presence of increasing drug concentrations (62, 64-67). Cloning and sequencing of nucleoside-resistant isolates have identified mutations in RT that are responsible for the resistance phenotype, as confirmed by site-directed mutagenesis of wild-type HIV clones (table 1).

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. Point mutations such as K65R, T69D, Q151M and M184V/I lead to alterations in affinity of RT for specific 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 (11), and may therefore affect the initial binding and/or the subsequent positioning of the NRTI for catalysis in such a manner that the mutant RT is able to discriminate between the NRTI and the analogous dNTP substrate. However, 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 (68-69).

In the absence of structural data for NRTI-resistant RTs, three mechanisms have been proposed to account for the molecular basis of the NRTI resistance phenotype. These include selective alterations in NRTI binding and/or incorporation, template/primer (T/P) repositioning and NTP-mediated as well as phosphorolytic removal of an incorporated chain-terminating NRTI residue from the 3'-end of the nascent viral DNA (70). The different resistance phenotypes seem to correlate with different sets of mutations in RT, and the effect of individual set of mutations on resistance to a specific NRTI can only be presumed by kinetic studies and building up the enzyme active site by molecular modeling studies. However, the understanding how mutations affect RT structure and function, and the ensuing loss of potent antiviral activities of certain NRTIs have not been demonstrated in conjunction with their binding modes, which would provide invaluable insight into the design of more effective NRTIs active against the mutant RTs.

Under the assumption that mutation may either deform the active site conformation through structural realignment or could destabilize inhibitor binding, we tried to assess the structural adjustment resulting from mutations and the accompanying energetic consequences.

6. MOLECULAR MODELING STUDY OF MUTANT RT

Understanding the mechanisms involved in HIV resistance to 3TC and AZT (figure 6), particularly in the context of combination treatment, is of enormous practical importance, since both compounds are important components of many currently employed drug regimens. For the past several years, we have tackled this challenging

Table 1. Fold resistances in mutant HIV-1 RT associated with NRTI resistance

Mutation	Resistance Conferred (Fold Resistance)
M41L/T215Y	AZT (60-70)
D67N/K70R/T215Y/K219Q	AZT (120)
M41L/D67N/K70R/T215Y	AZT (180)
K65R	ddC (4-10), ddI (4-10), 3TC (17), Abacavir (3), DXG (5.6)
L74V	ddI (5-10), ddC (5-10), Abacavir (4), DXG (3.5)
M184I/V	3TC (>1,000), ddC (2-5), Abacavir (2-5)
V75T	ddC, ddI, d4T (7)
Q151M	Multidrug resistance, Appears in patients with resistance to multiple ddN
K65R/Q151M	DXG (>20)

question by comparing the wild-type and computer-generated mutant HIV-1 RT complexed with DNA duplex as well as various NRTI-TP in the energy-minimized states (71-72). The comparison of the binding modes of AZTTP, 3TCTP and various NRTI-TPs which are known to be active against the mutant RTs provided interesting insights to the combat against the NRTI-resistant RTs.

6.1. Computational Methods

All molecular modeling of the enzyme-substrate complexes was carried out using SYBYL 6.6 (Tripos Inc. St. Louis, MO), and the Monte Carlo conformational search (73) was performed by Macromodel 7.0 (Schrödinger, Inc).

6.1.1. Enzyme Sites

The enzyme site of the enzyme-ligand complex was constructed based on the X-ray structure of the covalently trapped catalytic complex of HIV-1 RT with TTP and primer-template duplex (PDB entry 1rtd) (11). Given the large size of the HIV-1 RT, calculations of the entire protein-ligand complex were not practical. Therefore, a model of the NRTI binding site was constructed which consisted of residues between Lys1 and Pro243 in the p66 subunit. Hydrogen atoms were added, and the clipped residues (Lys1 and Pro243) were capped with acetyl groups. The template-primer duplex had to be truncated as well. Interestingly, 5:2 (template:primer, ratio of bases) duplex was not stable enough presumably because of the insufficient base stacking interaction to maintain the duplex structure. Thus, a 7:4 (template:primer) duplex, which was shown to be stable enough to give a reliable result was used for the modeling studies (74). The missing 3'-OH group in G22 [the 3'-end (n^{th}) nucleotide in the primer strand] in the original X-ray coordinates (1rtd) (11) was added, so that it could properly coordinate the adjacent Mg atom. Three clipped residues in template and primer strands [G3, C19(template)/ G9(primer)] were capped with methyl groups (72).

6.1.2. Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

The initial Cartesian coordinates for each inhibitor were generated based on the X-ray coordinates [AZT (75) and ddC (76)] or conformational analysis (75) of the modified crystal structures [3TC from FTC (77)/ L-d4FC and L-3'Fd4C from d4C (78)]. The X-ray coordinates of D-dioxolane cytosine (79) were used as a template for construction of DXG structure, whose heterocyclic base moiety, cytosine, was modified to guanine before docking into the enzyme. Also, the heterocyclic moiety of $n+1^{th}$ nucleotide in template overhang was modified to the base complementary to the incoming NRTIs. The enzyme site and the inhibitor were merged to form a catalytic complex and then the NRTI triphosphate was situated in such an orientation that it could be paired with its complementary base in the template strand by adjusting the torsional angles to those found in the X-ray structure (11).

6.1.3. Method

Gästeiger-Hückel charge (80-81) was given to the enzyme-ligand complex with formal charges (+2) to two Mg atoms in the active site. Then, Kollman-All-Atom charges (82-83) were loaded to enzyme site from the biopolymer option in SYBYL 6.6 (Tripos Inc. St. Louis, MO). Fluorine parameters were obtained from the literature (84-85) and MM2. In order to eliminate local strains resulting from merging inhibitors and/or point mutations, residues inside 6 Å from the merged inhibitors and mutated residues were annealed (72) until energy change from one iteration to the next was less than 0.05 kcal/mol (hot region: 6 Å , interesting region : 12 Å). The annealed enzyme-inhibitor complexes were minimized by using Kollman-All-Atom Force Field (86) until iteration number reached 5,000.

6.1.4. Calculation of Relative Binding Energy (E_{rel})

The binding affinities were estimated by means of the binding energy differences (relative binding energy, E_{rel}) between the NRTI triphosphate-RT complex and the corresponding natural substrate, 2'-deoxynucleoside triphosphates (dNTPs)-RT complex. The binding energy (E_b) of each nucleoside triphosphate was calculated by the Equation 1.

E_b = E(ligand) + E(enzyme) – E(enzyme-inhibitor complex) ……..…. (Eq 1)

Where, E_b = binding energy of the NRTI triphosphate or dNTP to RT

E(enzyme) = energy of RT-DNA duplex complex

E(ligand) = energy of NRTI triphosphate or dNTP

E(enzyme-ligand complex) = energy of RT-DNA duplex-NRTI triphosphate ternary complex or RT-DNA duplex-dNTP ternary complex

The relative binding energy (E_{rel}) of the NRTI triphsophate was calculated by the difference of the binding

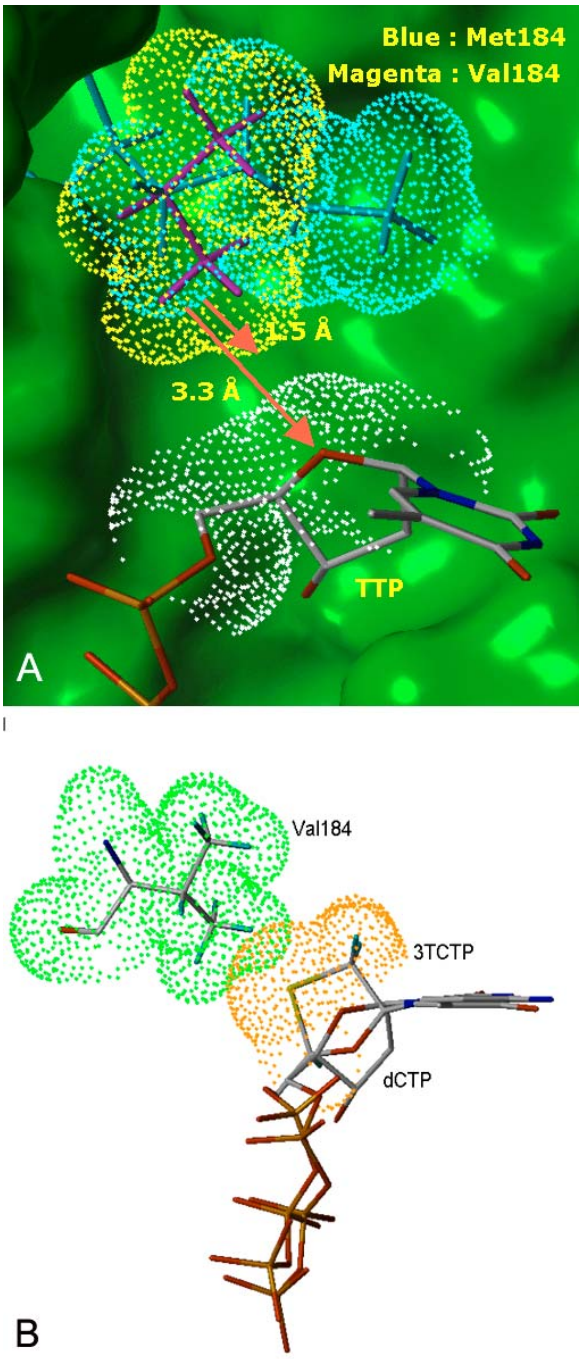


Figure 7. Active sites of M184V RT and wild-type RT.

energy between NRTI triphosphate and the corresponding dNTP (Eq 2).

$$E_{rel} = E_b(\text{NRTI triphosphate}) - E_b(\text{dNTP}) \quad \text{(Eq 2)}$$

Therefore, a favorable binding affinity would be represented by a large positive E_{rel} and a poor binding NRTI triphosphate would have a small or negative E_{rel}.

6.1.5. Monte Carlo Conformational Search

The Monte Carlo conformational search of D-dioxolane thymine was performed in 5,000-step, in the presence of GB/SA water model using MM3 force field (Macromodel) (74).

6.2. M184V RT (3TC Resistance)

The M184V mutation in RT that confers high-level resistance to 3TC is located in the YMDD motif that constitutes a portion of the polymerase-active site of RT and other polymerases (87). Mutated RT that contains the M184V mutation can discriminate between 3TC-TP and dCTP, in such a way that the incorporation rates of 3TC-MP are 20- to 100-fold less than with the wild-type enzyme (88-91). Using the M184I RT/DNA and wild-type RT/DNA crystal structures, Sarafianos *et al.* (92) have modeled the interactions of dCTP and 3TCTP at the active site of the M184I enzyme, assuming similar binding of the triphosphate moieties and similar base-pairing interactions of the incoming inhibitors with a modeled template overhang. The predicted position of the nucleotide with respect to the active site YIDD motif in this model is strongly supported by the structure of the polymerase active site in the ternary HIV-1 RT/DNA/dTTP complex (11). The 3TCTP and dCTP nucleoside ring configurations are enantiomeric, so that alignment of the triphosphates and bases would cause the nucleoside ring of the beta-L-inhibitor to project 1.5-2.0 Å further toward residue 184 than would the ribose ring of dCTP. Therefore, the most significant difference between the interactions of wild-type HIV-1 RT and the 3TC-resistant mutant HIV-1 RT (M184V RT) with ribose and beta-L-oxathiolane nucleoside inhibitors would be a very close contact between the C-gamma-2 methyl of Val and the protruding oxathiolane ring. This unfavorably close contact (2.5 Å in M184I vs. 3.9 Å in wild-type) would result in severe steric hindrance, preventing proper positioning of 3TCTP for catalysis, consistent with the data of Feng and Anderson. A recent pre-steady-state kinetic analysis of the D- and L- isomeric 3TC compounds as well as the corresponding 2',3'-dideoxy and natural nucleoside triphosphate provided a mechanistic basis for understanding the inhibition of RT by 3TC (89). Thus, the maximum rate of incorporation (kpol) followed the decreasing order of dCTP > ddCTP > (+)-BCH-189 (D- counterpart of 3TC) > 3TC while the Kd values determined for the DNA/RNA primer-template followed the decreasing order of 3TC ≅ (+)-BCH-189 ≅ ddCTP > dCTP. The corresponding efficiency of incorporation followed the decreasing order of dCTP > ddCTP > (+)-BCH-189 > 3TC, which suggests that perturbations on the ribose ring of cytidine analogues (C→S) decrease the rate and efficiency of incorporation but enhance the binding affinity. Our computer-generated three dimensional structure of M184V RT shows almost no difference from the wild-type (WT) RT even at the active site because by simply rotating its side chain to the open NRTI binding site the mutated residue (Val184) can acquire stable conformation. As a result, the deoxy ribose sugar moiety of TTP is in closer proximity to Val184 (3.3 Å) but not enough to be sterically hindered (figure 7a).

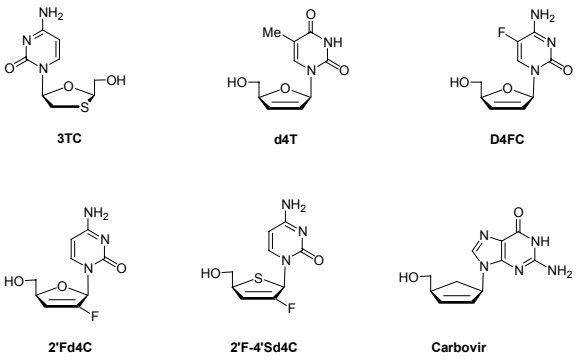


Figure 8. Structures of 2',3'-unsaturated NRTIs.

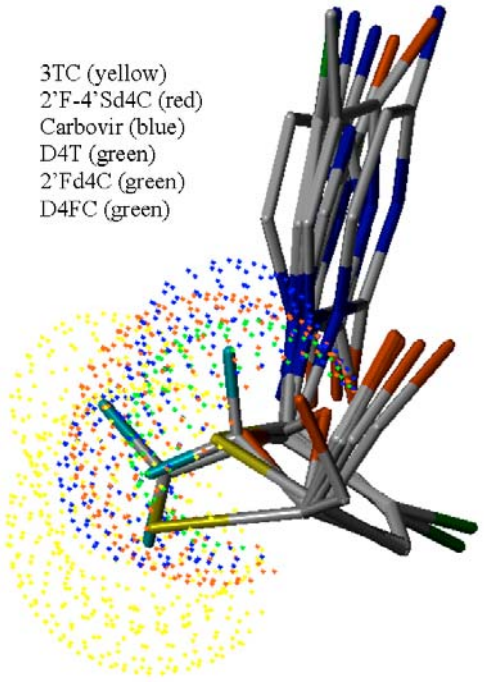


Figure 9. Superimposed structures of 3TC/D4T/ 2'F4'Sd4C/2'Fd4C/Carbovir/D4FC.

However, even though the natural substrate such as TTP can manage to fit into the active site without unfavorable steric hindrance, it is obvious that the approach of the sterically demanding Val184 into the NRTI binding site significantly limits the conformation of the sugar moiety of the NRTI, which can be confirmed by the binding mode of 3TC triphosphate (figure 7a). For the sake of proper base pairing with the complementary base in the template strand, the unnatural L-configuration of 3TC locates its oxathiolane sugar moiety at the upper open space of the NRTI binding site in the WT RT, where Met184 contributes to the binding of 3TCTP by an extended van der Waals contact to the oxathiolane sugar moiety of 3TCTP. However, in M184V RT, 3TCTP cannot be accommodated into the binding site, which is already occupied by the side chain of Val184 (figure 7b). In this context, it is noteworthy that, to date, no L-configured nucleoside has been reported to have antiviral activity against the M184V RT.

Concerns about the development of viral resistance to 3TC (93-94) prompted the discovery of structurally related nucleosides with activity against HIV isolates containing common 3TC resistance mutations. Until now, three classes of NRTIs have been reported to be active against M184V RT, which include dioxolane/oxathiolane [DAPD (95)/dOTC (96)], acyclic (Tenofovir disoproxil) (97) and 2',3'-unsaturated nucleosides [d4T (98), d4FC (99), D-2',3'-unsaturated-2'-fluorocytidine (2'Fd4C) (100)].

6.2.1. 2',3'-Unsaturated (d4) Nucleosides (73)

Among these, the 2',3'-unsaturated nucleosides including 2'-fluorine substitution (101) have been studied to provide the structure-activity relationships against M184V RT, where D-2',3'-unsaturated cytidine analogs show potent activity against M184V RT (101) but the isosteric replacement of 4'-oxygen with a sulfur atom (D-2',3'-unsaturated 2'-fluoro-4'-thiocytidine, 2'F-4'Sd4C) (102) develops significant cross-resistance (table 2).

On the other hand, abacavir, which has a 4'-CH_2 group instead of an oxygen atom, exhibits only a 2- to 5-fold increase to M184V RT (table 2) (99), suggesting that the 4'-substituent in the 2',3'-substituted nucleosides is critical in determining the cross-resistance to the M184V RT. Thus, it was of interest to find out how the 2',3'-unsaturated moiety and its substitution contribute to the binding mode of the unsaturated nucleosides at the active site of M184V RT.

In order to investigate the binding mode of each NRTI to RT, various 2',3'-unsaturated NRTI structures (figure 8), obtained from the quantum mechanical ab initio calculations using RHF/3-21G* basis in Spartan 5.1.1, were superimposed and compared.

3TC has the largest van der Waals radius of the sugar moiety which is directly projected to Val184 in the HIV RT. The van der Waals radii of 4'-CH_2 in carbovir and 4'-sulfur in 2'F-4'Sd4C are similar, and the 4'-oxygen atoms in D4T, D4C and 2'Fd4C has the smallest van der Waals radius (figure 9). As the 4'-substituent of the sugar moiety of NRTI triphosphate is in close contact to Val184, it might be possible to correlate the cross-resistance of NRTIs to M184V RT with the relative sizes of the 4'-substituents. D4T, D4FC and 2'Fd4C with an oxygen atom at the 4'-position are still active or more active against M184V RT than against WT RT, while ABC with 4'-CH_2 substituent has little cross-resistance to M184V RT. However, different resistance profiles of 2'F-4'Sd4C and ABC, which have the 4'-substituents similar in size, deserved more investigation.

Comparison of the minimized structures of ternary complexes (NRTI-DNA-WT RT) shows several interesting features: (a) Every 2',3'-unsaturated nucleoside studied shows their 2',3'-double bond at close proximity (~ 3.5 Å) with right orientation to the aromatic side chain of Tyr115, which can be

Table 2. *In vitro* antiviral activity (EC_{50}, microM) of the NRTIs against WT and M184V RT

	WT	M184V	FI
3TC	0.042	>50	>1,200
D4T	0.45	0.5	1.1
D4FC	0.18	0.14	0.8
2'Fd4C	8.0 [a]	1.8 [a]	0.23
2'F-4'Sd4C	5.0[a]	125[a]	25
ABC	5.3	15.5	2.9

[a] EC_{90} in microM

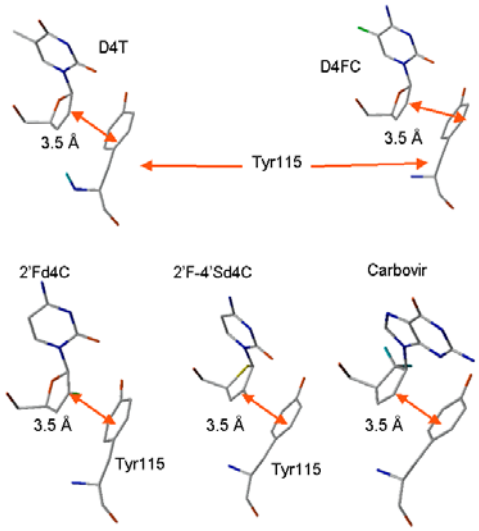


Figure 10. Hydrophobic pi–pi interaction.

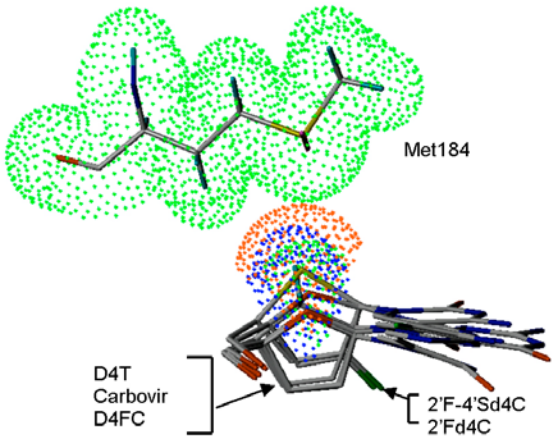


Figure 11. 4'-Oxygen in 2'Fd4C (green) vs 4'-sulfur atom in 2'F-4'Sd4C (red).

understood as a hydrophobic pi–pi interaction (figure 10). This pi–pi interaction strengthens the binding between the HIV RT and the triphosphates, and may partially explain the significant anti-HIV activity of the d4Ns against the M184V mutant by the pi–pi interaction. (b) The 2'-fluoro 2',3'-unsaturated nucleosides (2'Fd4C and 2'F-4'Sd4C) show their sugar moieties slightly rotated to locate their 4'-substituents closer to Met184 (figure 11). This conformational difference is believed to be the result of the possible steric hindrance between the 2'-fluorine atom and the adjacent amino acid (Gln151). This conformational change was manageable when the 4'-substituent was an oxygen atom (2'Fd4C), but the 4'-sulfur atom (2'F-4'Sd4C), which has larger van der Waals radius than oxygen, cannot avoid the unfavorable steric hindrance with the side chain of Val184, resulting in high cross-resistance to M184V RT (figure 11). However, the 4'-CH_2 group in carbovir is not close enough to Val184 because there is no 2'-fluoro substituent (figure 12). Therefore, the steric hindrance between the sugar moiety of carbovir and Val184, if any, should not be significant.

Potent anti-HIV activity of the fluorinated 2',3'-unsaturated (d4) nucleosides prompted extensive structure-activity relationship studies of D- and L- 2'-fluoro-d4 nucleosides (101-102), 2'-fluoro-4'-thio nucleosides (102-103), 3'-fluoro-d4 nucleosides (104-105) and 3'-fluoro-4'-thio nucleosides (106-107) (figure 13). Unfortunately, in spite of the remarkable anti-HIV activity against the wild-type RT, no L-nucleoside was found to be active against M184V RT, and the molecular modeling studies showed that the 2'- or 3'-fluorinated d4 L-nucleoside also cannot avoid the steric hindrance with Val184 (101, 104-106). The large binding pocket of the wild type HIV-1 RT was found to provide enough space for L-nucleosides to bind at the active site, and it is noteworthy that the potent anti-HIV activity of L-3'-Fd4C correlates with its high relative binding energy by virtue of the participation of the 3'-fluorine atom in the hydrogen bonding with the amide backbone of Asp185 (figure 14a) (105). This favorable binding mode, however, cannot be maintained when the triphosphate of the L-3'-fluoro 2',3'-unsaturated nucleoside binds to the active site of M184V RT because the bulky side chain of Val184 occupies the space needed for binding and pushes the 3'-fluorine atom of L-3'-Fd4CTP away from the amide backbone of Asp185, out of hydrogen bonding distance, resulting in poor binding energy and thereby cross-resistance (figure 14b) (105). Large van der Waals radius of the sulfur atom at the 4'-position of the sugar moiety was also found to result in the steric hindrance with Val184. Thus, the M184V RT was found to be insensitive to fluorinated 4'-thio d4 nucleoside (102).

Interestingly, even though the binding mode of D-3'-Fd4CTP to the wild-type RT is indistinguishable from that of D-2'-Fd4CTP due to their conformational similarity, the low anti-HIV activity of D-3'-Fd4C is exceptional in this series (106). Presumably, D-3'-Fd4C is not easily metabolized to its monophosphate by cellular kinases such as deoxycytidine kinase (dCK) because of the unfavorable interaction of the 3'-fluorine with the enzyme active site. Among the 2'- or 3'-fluorinated 2',3'-unsaturated nucleosides, therefore, only D-2'-Fd4C maintains its high activity against M184V RT (101).

Table 3. Antiviral activities of D-dioxolane nucleosides against M184V RT

	3TC		D-Dioxolane T		DXG		D-FDOC	
	EC_{50} (microM)	FI	EC_{50}	FI	EC_{50} (microM)	FI	EC_{50} (microM)	FI
WT	0.041	--	0.200	--	0.21	--	0.045	--
M184V	>50	>1,200	0.088	0.44	0.44	2.1	0.030	0.67

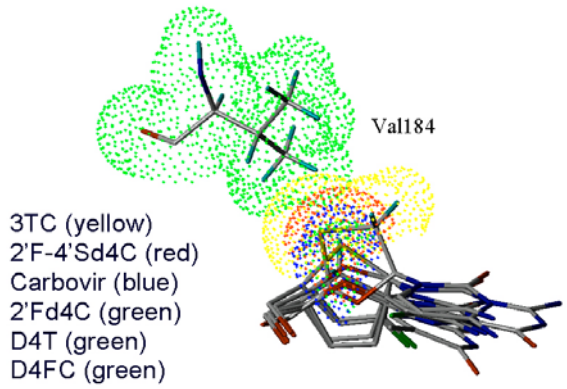


Figure 12. 4'-CH2 in carbovir is not close to Val184 because there is no 2'-fluorine.

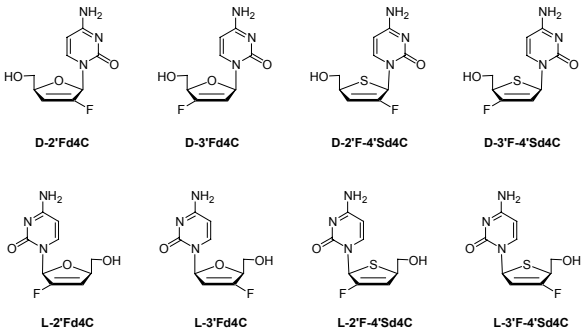


Figure 13. 2'- or 3'-fluorinated 2',3'-unsaturated nucleosides.

6.2.2. D-Dioxolane Nucleosides (108)

Since the discovery of D-dioxolane guanosine and its prodrug, D-dioxolane 2,6-diaminopurine (DAPD), which are active against AZT- as well as 3TC-resistant mutants (109-110), nucleosides which have dioxolane sugar moiety have been extensively studied in the pursuit of new drug candidates for anti-HIV therapy. Among the series of nucleosides, thymidine, 5-fluorocytidine and guanidine analogues (figure 15) showed potent anti-HIV activity against 3TC-resistant mutant RT (table 3). In this context, it is of interest to understand the role of the dioxolane moiety as well as the heterocyclic bases for the anti-HIV activity of these nucleosides against 3TC-resistant mutant RT. The steric pressure on the active site of RT conferred by the M184V mutation not only blocks the space where the sugar moiety of the L-nucleoside resides, but significantly limits the conformation of D-nucleosides. As was seen in our previous study, the D-2'-fluoro-4'-thio-2',3'-unsaturated nucleoside is a good example in this category because the cytidine nucleoside, potent against WT RT, shows high cross-resistance to M184V RT (102). As its 4'-oxa analogue, the D-2'-fluoro-2',3'-unsaturated nucleoside, is known to maintain its antiviral activity against M184V RT, the characteristic mechanism of resistance of D-2'-fluoro-4'-thio-2',3'-unsaturated nucleoside is quite interesting. The energy-minimized structure of the D-2'-fluoro-4'-thio cytidine/RT complex shows that, in spite of the same conformation of D-2'-fluoro-4'-thio-2',3'-unsaturated cytidine as that of its 4'-oxa derivative, the steric clash with Val184 caused by the longer C-S bond as well as the larger van der Waals radius of the 4'-sulfur atom result in a destabilization of the active site (102). Molecular modeling studies indicate that, unlike other nucleosides cross-resistant to M184V RT, the D-dioxolane nucleosides are able to escape the stress of Val184 by adjusting their conformation with no energy cost. Also, the 3'-oxygen atom of D-dioxolane sugar moiety is critical in locating the nucleoside triphosphate away from Val184 by interacting with the active site residues of RT. In every minimized structure of D-dioxolane nucleoside triphosphate/RT complexes, the 3'-oxygen atom of D-dioxolane sugar moiety was always found to be in specific interaction with the nearby enzyme residues regardless of its conformation (figure 16), and the conformational analysis showed that the interconversion of the dioxolane's conformation from one to another can happen without significant loss of energy (figure 17). The specific interaction between dioxolane sugar moiety and the enzyme residues effectively holds the nucleoside triphosphate out of the clashing distance with Val184 (figure 16c). In this context, it is interesting that, in the crystal structure of TTP bound to the WT RT, the 3'-OH of TTP is in specific interaction with its own triphsophate moiety as well as the nearby enzyme residue Tyr115 (figure 18a) (11). As Tyr115 is located on the other side of the active site of RT from Val184, it is possible that the 2'-deoxy sugar moiety of TTP can stay away from Val184 by virtue of the interaction between 3'-OH and Tyr115. Also, AZT, which has the same conformation as TTP, does not experience any cross-resistance to M184V RT (111) because the 3'-azido group of AZT is in the right position to interact with Tyr115 (figure 18b) (72). On the other hand, moderate cross-resistance of ddI and ddC (66), which do not have any functionality inside their sugar ring to interact with the enzyme residues in a specific manner, to M184V RT supports our claim.

6.3. M41L/D67N/K70R/T215Y (AZT-Resistance) (72)

Tissue culture and clinical data have revealed that multiple mutations in the "fingers" and "palm" subdomains of RT are required to confer resistance to AZT (69). Thus, AZT resistance correlates with multiple mutations in RT, including M41L, D67N, K70R, L210W, T215Y/F and K219Q (56, 112-114). The extent of AZT resistance is

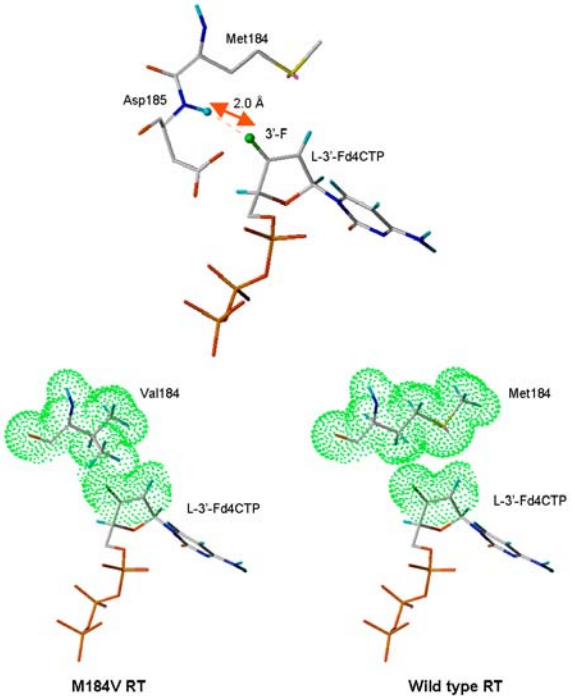


Figure 14. (a) 3'-F is hydrogen-bonded to the amide backbone of Asp185 in L-3'-Fd4CTP (b) Steric hindrance of L-3'-Fd4CTP with the bulky side chain of Val184 in M184V RT (left).

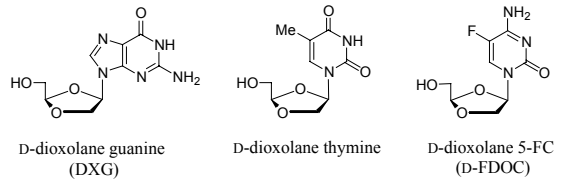


Figure 15. Structures of D-dioxolane nucleosides.

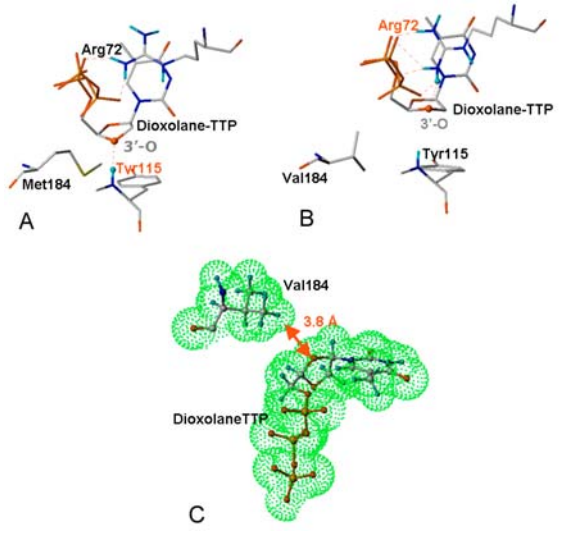


Figure 16. D-dioxolane thymine triphosphates-RT complexes with (a) WT RT, (b) M184V RT. (c) The dioxolane sugar moieties stay away from Val184.

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 (68-69). 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 (91, 115-116). Recently, several groups have proposed that AZT resistance can involve the phosphorolytic removal of the chain-terminating AZT from the 3'-terminus of the primer after it has been incorporated into viral DNA (117-121). This removal, which may be facilitated by the enhanced binding of AZT-resistant RT to an AZT-MP-terminated primer, may be accomplished by two mechanisms that use different substrates to carry out the phosphorolysis reaction. In the first, the AZT-resistant mutant RT exhibits an increased rate of RT-catalyzed pyrophosphorolysis (the reverse reaction of DNA polymerization) (119-120,122-123). The substrate for pyrophosphorolysis is PPi, which is the normal product of DNA synthesis and is also present at an intracellular concentration of about 150 microM (122). 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 (119-120). However, the weakness of the pyrophosphorolysis mechanism is that the reaction regenerates AZTTP, which may then be reincorporated into the viral DNA. In addition, there is a report which failed to observe differences in pyrophosphorolytic activity between wild-type and AZT-resistant RT (123). Meyer *et al*. have shown that HIV-1 RT also possesses an interesting capacity to carry out ribonucleotide-dependent phosphorolysis (dinucleotide polyphosphate synthesis) (123). This reaction, by the same chemistry involved in pyrophosphorolysis, excises the terminal nucleotide from the 3'-end of the primer with the concomitant formation of a dinucleoside tetraphosphate. 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. However, in contrast to PPi, HIV-1 RT has only a very low affinity for ribonucleotides such as ATP (122). Furthermore, it should be envisioned that ATP binds in an orientation 'backward' from that of the normal dNTP substrate in order for the gamma-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 (71). On the other hand, it is not clear how the AZT-resistant mutant RT acquires the enhanced affinity to AZT-MP-terminated primer. Thus, even though the phosphorolytic removal of nucleoside chain terminators by pyrophosphate acceptor molecules (PPi or ATP) has been proposed as a mechanism of AZT resistance, the several limitations of the mechanism mentioned above questions another possible route for the resistance conferred by mutations.

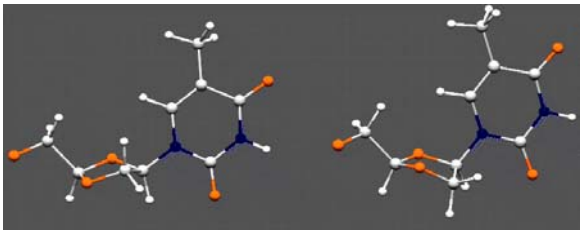


Figure 17. Two different conformations of D-dioxolane thymine: (a) 3'-exo (b) 3'-endo.

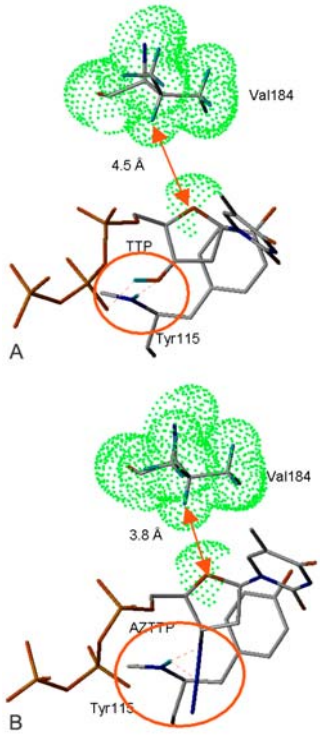


Figure 18. AZT (b), which has the same conformation as TTP (a), does not experience any cross-resistance to M184V RT. The 3'-azido group of AZT is in the right position to interact with Tyr115 to hold the nucleoside triphosphate away from Val184.

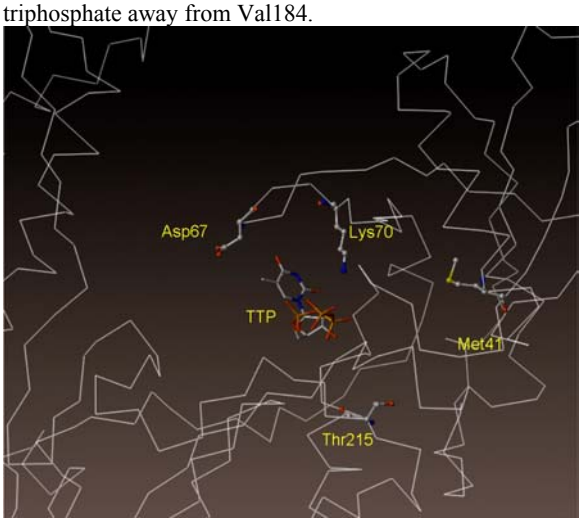


Figure 19. Mutation sites of AZT-resistant RT surrounds dNTP-binding site.

In this regard, the crystal structure of the AZT-resistant substrate-free RT containing the D67N/K70R/T215F/K219Q mutations (51) draw our attention because it suggests that the T215F and K219Q mutations might induce long-range conformational changes that impact on the active site aspartate residues, which is likely to provide a subtle method of introducing discrimination when the drug molecule mimics a substrate. As confirmed by many studies, TTP and AZT-TP have virtually identical conformations when bound to RT (123). Therefore, unlike 3TC, the scope for mutating contact residues is limited strictly because significant changes are likely to be incompatible with the maintenance of an active enzyme (51). The mutations sites 41, 67, 70 and 215 are surrounding the dNTP binding site, but do not contact the dNTP directly (figure 19).

Among the four mutation sites, only 41 is in close distance from the bottom half of the so-called 3'-OH pocket composed of Asp113, Ala114, Tyr115 (figure 19 and figure 20). Since Lys70 is in direct contact to dNTP, the mutation to arginine, which has a short side chain, may fail to interact with the gamma-phosphate of dNTP. The remaining two residues, 67 and 215, are relatively far away from the dNTP site, and the effect of the mutation in these two amino acid residues is interesting. Our energy-minimization study of quadruple mutant RT (M41L/ D67N/ K70R/ T215Y) complexed with TTP shows that the mutation does not change the conformations of dNTP and the 3'-OH pocket (Asp113, Ala114 and Tyr115) of RT (figure 20a). The mutations M41L and T215Y do not seem to accompany significant conformational change either (figure 20a). However, 67 and 70 show significant conformational differences between WT and mutant (figure 20a).

Taken together, unlike the 3'-OH pocket, the coordination geometry of the two Mg^{2+} ions by Asp185, Asp110 and Val111 show significant movement (figure 20b). Therefore, of conformational interest is how these changes affect the binding event of AZTTP at the dNTP binding site of RT. The calculated relative binding energies (E_{rel}) of AZTTP and DXGTP complexed with WT as well as AZT-resistant RT shows that AZT is a potent competitive inhibitor against WT RT, but its affinity to the mutant RT is significantly decreased (table 4). Interestingly, DXGTP, which is known to maintain its antiviral activity against the AZT-resistant RT, shows only a moderate decrease in the relative binding energy to the mutant RT (table 4). Therefore, it seems that the significant conformational change in the Mg^{2+} coordination geometry conferred by the combination of mutations selectively affect the binding of AZTTP.

The mutations at 41 and 215 create small conformational changes around their positions, and several residues such as Glu40, Glu44, Lys46, Gly112 and Ser117 experience conformational changes, which propagate ultimately to the three catalytic residues Asp110, Asp185 and Asp186 through Asp110, Val111, Gly112 and Asp113 (figure 20b). However, this conformational change affects the binding mode of nucleoside triphosphates at the active

Table 4. AZT- resistant RT: correlation of binding energy (E_{rel}) with activity (EC_{50})

	AZT			DXG		
	EC_{50} (microM)	**Fold Increase (EC_{50})**	**E_{rel} *(kcal/mol)***	**EC_{50} (microM)**	**Fold Increase (EC_{50})**	**E_{rel} *(kcal/mol)***
WT	0.0041	--	146.4	0.21	--	12.0
M41L/D67N/ K70R/T215Y	0.082	20	73.4	0.24	1.1	8.0

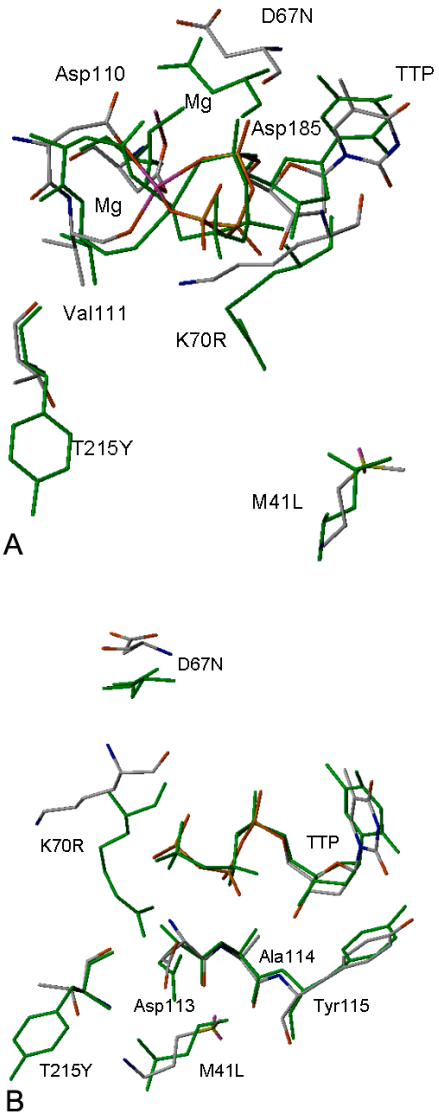


Figure 20. Energy-minimized structure of AZT-resistant RT complexed with TTP (Atom type: Wild type, green: mutant).

site in quite a different way. A close comparison with TTP binding to the AZT-resistant mutant enzyme revealed an interesting change in the conformation of the enzyme-ligand complex mediated by the interaction between the triphosphate moiety of AZTTP and the Arg70 residue of RT (figure 21a). The lack of 3'-OH in AZTTP as well as the mutation K70R result in a shift of the entire triphosphate moiety outward from the binding site (0.8 Å). As a result, the Arg70, which interacts with the gamma-phosphate, shifts significantly (~ 3 Å) out of the binding pocket in order to maintain its hydrogen bonding to the gamma-phosphate (figure 21a). This movement of Arg70 exerts significant effects to the three dimensional structure of the enzyme-ligand (AZTTP) complex (figure 21a): the hydrogen bond between Arg70 and Asp113 is not effective any more and the movement of Arg70 unfolds the palm domain resulting in a loose and ineffective binding mode. Therefore, a slight change caused by the binding of the AZTTP moiety propagates to Arg70, resulting in a significant overall change in enzyme-ligand complex without deforming the "3'-OH pocket", which emulates essentially for the natural substrate (TTP) binding to the mutant RT.

6.3.1. D-Dioxolane nucleosides

The favorable binding of DXGTP to the quadruple AZT mutant-RT can be explained by two factors. As was in the quadruple mutant-AZTTP complex, the conformational change originated from 41 and 215 propagates to the three catalytic residues Asp110, Asp185 and Asp186 (figure 21b). In addition, as Asp67 mutates to Asn67, three residues Asn67, Lys219 and Asp110 form a salt bridge, which shifts Asp110 1.2 Å out of the binding site (figure 21b). Interestingly, this shift can be found in the binding mode of none of other nucleoside triphosphates (AZTTP, TTP and dGTP) to the same mutant enzyme. Therefore, the unique binding mode of DXGTP deserves more attention. Due to the lack of a 3'-OH in DXGTP sugar moiety, DXGTP fails to stabilize its beta-phosphate by hydrogen bonding. As a result, an adjacent residue, Lys65, moves toward the hydrogen bonding distance to the beta-phosphate. However, it should be noted that in AZTTP binding mode, the movement resides in Arg70 not in Lys65 (figure 21a), which requires further explanation. Compared to the AZTTP binding, the interaction between Arg72 and the triphosphate moiety may be the critical factor in determining the residue which should move. In DXGTP binding, Arg72 rotates toward the triphosphate to form hydrogen bonds with both alpha-and beta-phosphates. As a result, the triphosphate moiety has a more contracted conformation compared with that of AZTTP binding, and the gamma-phosphate does not move out significantly. The conformational change in Lys65 propagates to Asn67 through Arg70, Thr69 and Ser68. As the conformation of Asn67 changes, the adjacent residue Lys219 pulls down to form a stable salt bridge among Asn67, Lys219 and Asp110 (figure 21b). The combined effect of propagation of conformational change and salt bridge formation results

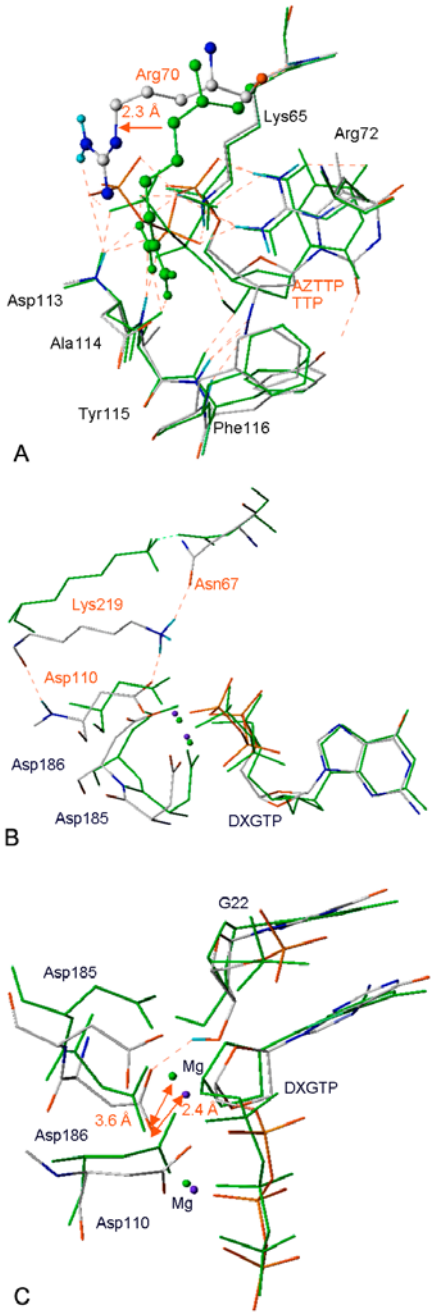


Figure 21. Comparison of AZT-resistant mutant complexed with AZTTP and DXGTP. (Green: Mutant RT/dNTP complex, Atom type: Mutant RT/ NRTI-TP complex)

in conformational changes in three catalytic aspartate residues (Asp110, Asp185, and Asp186), which improves the chelation of the two Mg^{2+} atoms (figure 21c). The two Mg^{2+}, one catalytic and the other nucleotide-binding are believed to facilitate an S_N2 nucleophilic attack by the 3'-OH of the primer terminus on the alpha-phosphorus of the incoming dNTP and to stabilize the negative charge in the transition state. Therefore, changes in conformation as well as binding mode of the three conserved Asp residues could contribute to the alignment of Mg atoms at the transition state and an increase in the polymerization rate or processivity. Another change followed by a salt bridge formation is the distance between fingers and palm domains. Fingers and palm domains move 1.1 Å and 0.4 Å toward each other to result in a decreased distance and therefore a tight binding state.

6.4. K65R Mutant RT (72)

Several ddN resistance mutations arise in the flexible beta3-beta4 loop situated in the fingers domain of the p66 subunit of RT (124). Five residues, that form part of this loop (Ile63, Lys64, Lys65, Tyr71 and Arg72), are conserved in all retroviral RTs (68) and among those, two fingers residues make important contacts with the dNTP substrate. The epsilon-amino group of Lys65 and the guanidinium group of Arg72 make salt bridges with the gamma- and alpha- phosphates of the bound dNTP substrate, respectively. These interactions help positioning the dNTP for base-pairing with the complementary template residue as well as for catalysis of phosphodiester bond formation with the 3'-OH of the primer terminal nucleotide. While the available structural data have provided considerable information on the structure of RT as well as the relevant amino acid residues critical for catalysis, it has not provided the molecular mechanism how mutations on RT influence the dNTP binding, thereby the anti-HIV potency. Even though this mutant is very rare, DXG experiences modest cross-resistance, which is well correlated with the decrease in its relative binding energy (E_{rel}) to the mutant enzyme, K65R RT (table 5). This decrease in binding energy can be explained by the outward rotation of Arg72 from the binding site in DXGTP binding mode (figure 22b). In WT RT/DXGTP complex (figure 22b), unlike the WT RT/dGTP complex (figure 22a) where the inward movement of Arg72 is not allowed because of the 3'-beta-hydrogen atom of the deoxyribose sugar ring, Arg72 is significantly involved in bridging the DXGTP and Gln151 by the formation of multiple hydrogen bondings. As a result, DXGTP has a very tight binding mode in WT RT. In K65R RT, however, as the longer and bulkier side chain of Arg65 occupies the binding site, the nearby Arg72 is pushed away from the binding site. This conformational change, initiated by the movement of Arg65 toward the binding site, results in the loss of hydrogen bonds between Arg72 and DXGTP dioxolane moiety and Gln151 and thereby a loose complex (figure 22d).

6.5. L74V Mutant RT (72)

In the structure of RT-DNA-dNTP (11), Leu74 is not in a position to directly affect dNTP binding. However, it does interact with the template nucleotide that is base-paired to the incoming dNTP and is also proximal to Gln151, which is a key residue in the dNTP-binding pocket. The L74V mutation has been thought to reduce sensitivity to ddI and ddC by altering template position and/or by affecting the orientation of Arg72 and Gln151 side chains (35, 125). Leu74 upholds the template nucleotide by hydrophobic interactions so that the template nucleotide could be base-paired with the incoming dNTP. Therefore, the mutation of this residue from leucine to valine results in the misalignment of the template residue

Table 5. DXG-resistant RTs : correlation of binding energy (E_{rel}) with activity (EC_{50})

	DXG		
	EC_{50} (microM)	**Fold Increase (EC_{50})**	**E_{rel} *(kcal/mol)***
WT	2.19	--	12.0
K65R	14.0	6.4	-37.1
L74V	6.3	2.9	0.2
K65R/ Q151M	>50.0	22.8	-44.4

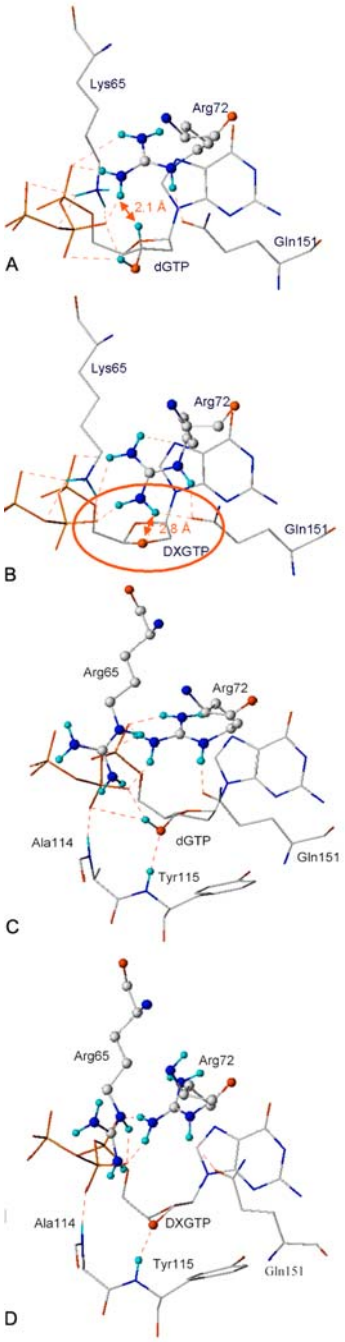


Figure 22. (a) WT RT/dGTP complex, (b) WT RT/DXGTP complex, (c) K65R/dGTP complex, (d) K65R/DXGTP complex.)

for base pairing and a significant conformational change in adjacent residues such as Gln151 and Arg72 (figure 23b).

In DXGTP binding, the combined effect of the misalignment of base pairing and the conformational change in Arg72 result in the downward shift of the dioxolane ring and the concurrent deformation of the active site (figure 24a), which is reflected on the decreased relative binding energy of DXGTP to L74V RT (table 5). As a result, the triphosphate moiety of DXGTP adopts a new conformation, which hampers the formation of optimal geometry for S_N2-type polymerization reaction (figure 24b): the distance between the nucleophile (3'-OH at the end of primer strand) and the reaction center (phosphorous atom at the alpha-phosphate moiety) is lengthened (3.66 Å vs 3.42 Å in WT) and the angle among the nucleophile, reaction center and the leaving group (beta-and gamma-phosphates) (160.4^o) deviates from that of optimal value (177.0^o in WT) (table 6).

6.6. K65R/Q151M RT (72)

In the crystal structure of the ternary complex, Gln151 interacts with residues that either directly interact with dNTP (e. g. Arg72, which hydrogen bonds with dNTP) or affect the position of important structural elements (e. g. the main chain carbonyl oxygen of residue 73 is hydrogen-bonded to Gln151, bridging the palm and fingers subdomains) (11). The Q151M mutation might therefore engender multidrug resistance to AZT, d4T and ddC/ddI by inducing both direct and indirect changes at the dNTP-binding pocket (table 1) (126-127). A site-directed mutagenesis study found that a combination mutation of K65R and Q151M was fully resistant to DXGTP and this mutant exhibits a significant decrease in antiviral activity of DXGTP (table 5). The decreased relative binding energy (E_{rel}, table 5) and the minimized RT-DXGTP structure shows two important features which this mutation invokes at the dNTP binding site. The bulky Met151 pushes neighboring Arg65 away from the binding site (figure 25). In dGTP binding (figure 25a), the pushed Arg65 also rotates itself in order to prevent another steric hindrance with the adjacent residue Lys70. However, in DXGTP binding (figure 25b), Arg65 is heavily involved in the stabilization of beta-phosphate and can neither move out nor rotate to prevent the unfavorable steric hindrance with Met151 and Lys70, which results in a high-energy binding state (figure 25b). In addition to that, as with the L74V mutation, the geometry for S_N2 type polymerization reaction is significantly deformed as a result of conformational change in both Arg65 and the triphosphate moiety in DXGTP: the nucleophile and the reaction center are separated by 4.40 Å, and the angle around the reaction

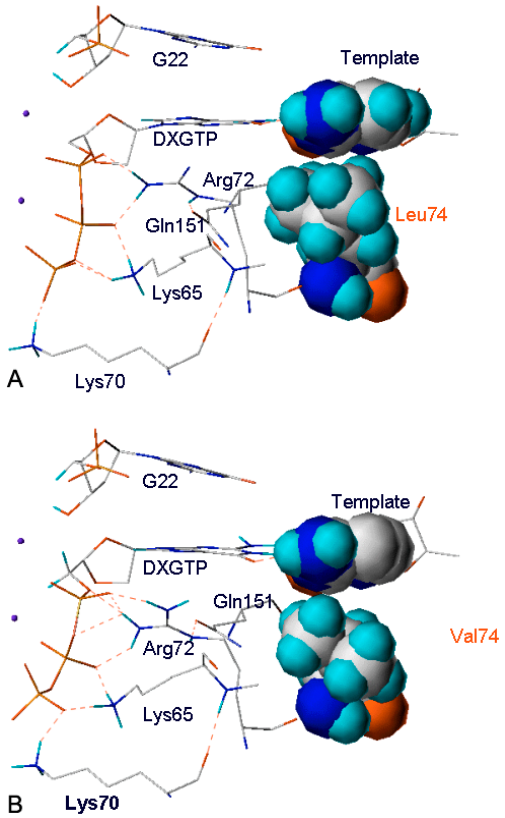


Figure 23. (a) WT RT/DXGTP, (b) L74V RT/ DXGTP

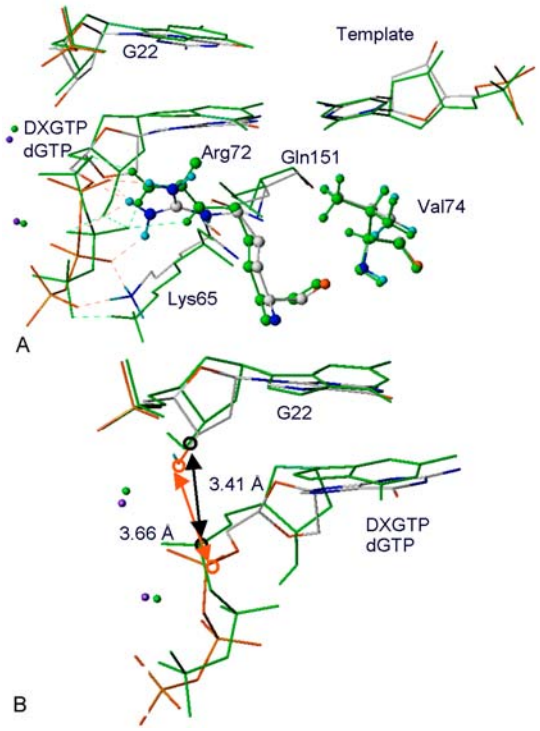
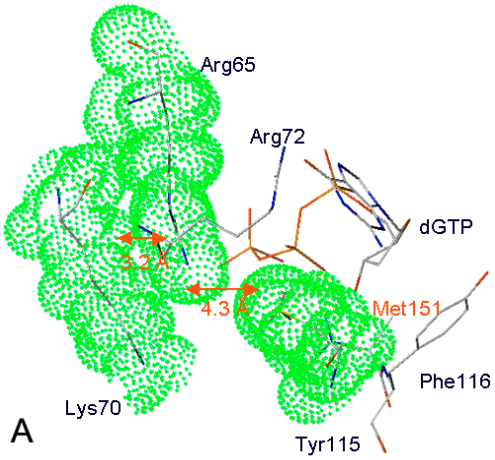


Figure 24. Deformation of the active site and S_N2 type polymerization geometry. (Green: dGTP, Atom type: DXGTP)



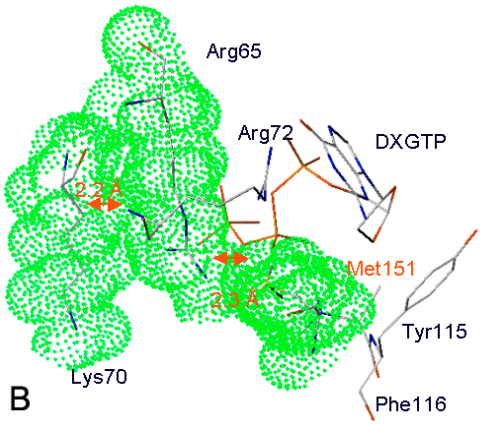


Figure 25. K65R/L74V mutant complex with (a) dGTP and (b) DXGTP.

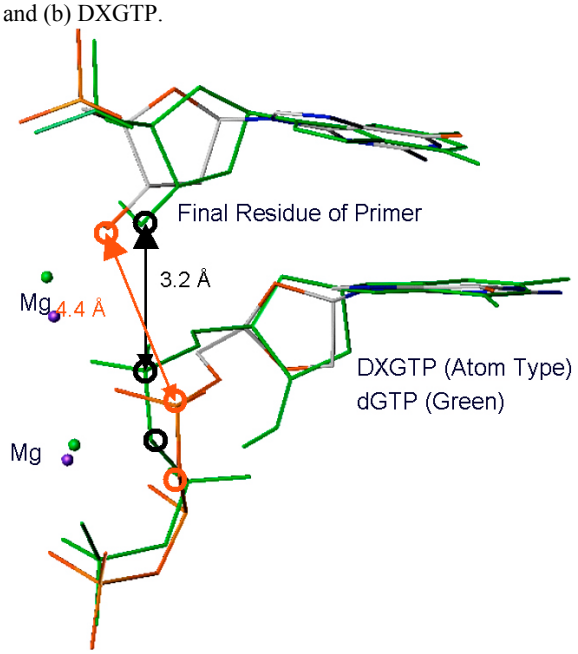


Figure 26. K65R/L74V RT complex. SN2 type polymerization geometry.

Table 6. Comparison of geometry for S_N2 type nucleophilic attack.

	Wild-type		K65R		L74V		K65R/Q151M	
	DXG	dGTP	DXG	dGTP	DXG	dGTP	DXG	dGTP
Distance[a] (Å)	3.42	3.23	3.36	3.32	3.66	3.41	4.40	3.37
Angle[b] (degree)	177.0	174.9	177.3	176.0	160.4	173.6	158.8	176.4

[a] Distance between the nucleophile (3'-oxygen of the terminal residue in primer strand) and the reaction center (alpha phosphorous atom in NRTI triphosphates), [b] Angle among the nucleophile, reaction center and the leaving group (alpha and beta phosphates in NRTI triphosphate)

center (158.8°) deviates significantly from that of the WT (table 6) (figure 26). Therefore, the incorporation of DXGTP into the growing DNA chain in K65R/Q151M mutant enzyme can be expected to be more difficult than in any other mutant enzymes studied.

7. ACKNOWLEDGMENTS

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