Molecular Insights into the Mechanisms of HIV-1 Reverse Transcriptase Resistance to Nucleoside Analogs

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Abstract: The causative agent of acquired immunodeficiency syndrome, HIV-1, depends on one of its enzymes, reverse transcriptase, to copy its single stranded RNA genome into a double stranded DNA nucleic acid suitable for integration in the host cell genome. In the last two decades, the advances in the knowledge of the kinetic mechanism of reverse transcription and in the determination of the crystallographic structures for the complexes of the enzyme with substrates and products were huge. However, all of this knowledge resulted in the design of RT inhibitors for which the virus, after a short period of exposure, becomes less susceptible, due to the development of resistance. The development of resistance is caused by the high frequency of viral mutation and the toxicity of those same drugs. Therefore, a closer look at all the available information might shed some light into this subject and help to develop new strategies to overcome the lack of long term clinical efficiency of these drugs. Here, we present a critical atomic level study of all the mutations that have been detected and reported so far, as a reaction of the enzyme to counteract the action of the inhibitors.

Keywords: HIV-1, reverse-transcriptase, NRTIs, resistance mutations.

INTRODUCTION

Reverse transcriptase, RT, is a key enzyme in the life cycle of HIV-1. The RT enzyme has three distinct catalytic activities that enable it to convert the single-stranded RNA genome of HIV-1 into double-stranded DNA suitable for integration in the host cell genome [1]: 1) RNA-template dependent polymerisation; 2) RNase H cleavage of the RNA; and 3) DNA-template dependent polymerization.

The enzyme is a heterodimer of 66-kDa (p66) and 51-kDa (p51) subunits. The former is derived from p66 by proteolysis of the C-terminal region [2]. Both subunits have four common domains termed palm, fingers, thumb and connection due to their resemblance of the structures to a hand [3]. P66 has also at the C-terminal portion a fifth domain, the RNase H domain. Only p66 has a functional polymerase active site. The function of p51 appears to be only structural [4]. These features are illustrated on Fig. **1**.

RT mediated polymerization begins with the enzyme's thumb subdomain in the 'closed' conformation. Binding of the template-primer promotes an open thumb conformation so that the enzyme can accommodate the nucleic acid (Fig. **2**). After the formation of the RT.p/t complex, the dNTP substrate binds in a two step process. The initial step in dNTP binding forms a ternary complex (RT.p/t.dNTP), in which the incoming dNTP base pairs with the corresponding nucleotide in the template. The crystallographic structure of this ternary complex has not been determined yet, however, it is believed to have the fingers in an open configuration.

Afterwards, in a second step, a conformational change occurs, whose kinetic studies have assigned to be the rate-limiting step [5-11]. With the determination of the crystallographic structure of the ternary complex [12], it was proposed that the mentioned conformational change was the movement of the fingers subdomain, which originates an active closed state (Fig. **2**). In this conformation, the active site residues become optimally aligned with respect to the bound dNTP so that the chemical step can occur. The primer terminus is positioned at the priming site (P-site), and the incoming nucleotide binds at the nucleotide binding site (N-site) (Fig. **2**). Nucleophilic attack by the 3'-OH of the primer terminus to the α-phosphate of the incoming nucleotide results in pyrophosphate release (PPi) and the formation of a new phosphodiester bond. A new conformational change should then be triggered, moving the fingers domain back to the open conformation.

In processive synthesis, the p/t is then translocated to the P site and new catalytic cycle begins.

Nucleoside reverse transcription inhibitors (NRTIS), such as zidovudine (AZT), staduvine (d4T), zalcitabine (ddC), didanosine (ddI), lamivudine (3TC), abacavir (ABC) and tenofovir (PMPA) are currently used clinically to inhibit retroviral replication (Fig. **3**). After conversion to the active triphosphate forms, analogs compete with the natural nucleotides (dNTPs) for binding and incorporation in the nascent viral chain. However until now, for each RT inhibitor used in the treatment of AIDS, the virus developed resistance mechanisms. This is due to the error prone nature of RT, which is at least in part a consequence of the absence of a 3'→ 5' proofreading activity [13, 14]. Furthermore, the long-term use of the approved drugs is also limited by toxicity. A detailed understanding of the resistance mechanisms of RT to NRTIs should facilitate the development of more effective drugs.

It is known that all the RT mutations that are caused by NRTIs promote the development of resistance by at least two mechanisms: the discrimination of the nucleotide analog or the repair of the analog terminated chain [15-18]. With the current knowledge of the kinetic mechanism of DNA polymerization, of the mutational patterns after drug

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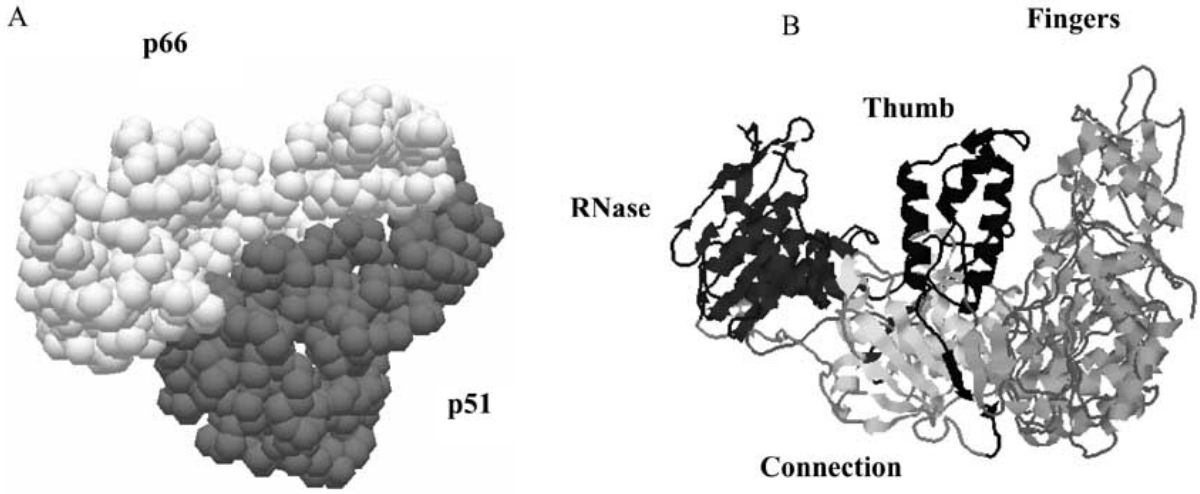

Fig. (1). A. RT is a heterodimer of the p51 (red) and p66 (blue) subunits. B. The monomers are subdivided in several domains termed palm, fingers, thumb and connection. p66 has also a RNase domain.

administration, and with the determined crystallographic structures of RT unliganded [19-25], with bound p/t [24, 26-28] and with bound p/t and dNTP or NRTIs [12, 29-30] some conclusions have been already drawn [16, 17, 24, 29]. In this article we review and discuss the proposed mechanisms of RT-NRTI resistance. We also propose alternative hypotheses based on the available structural and kinetic data.

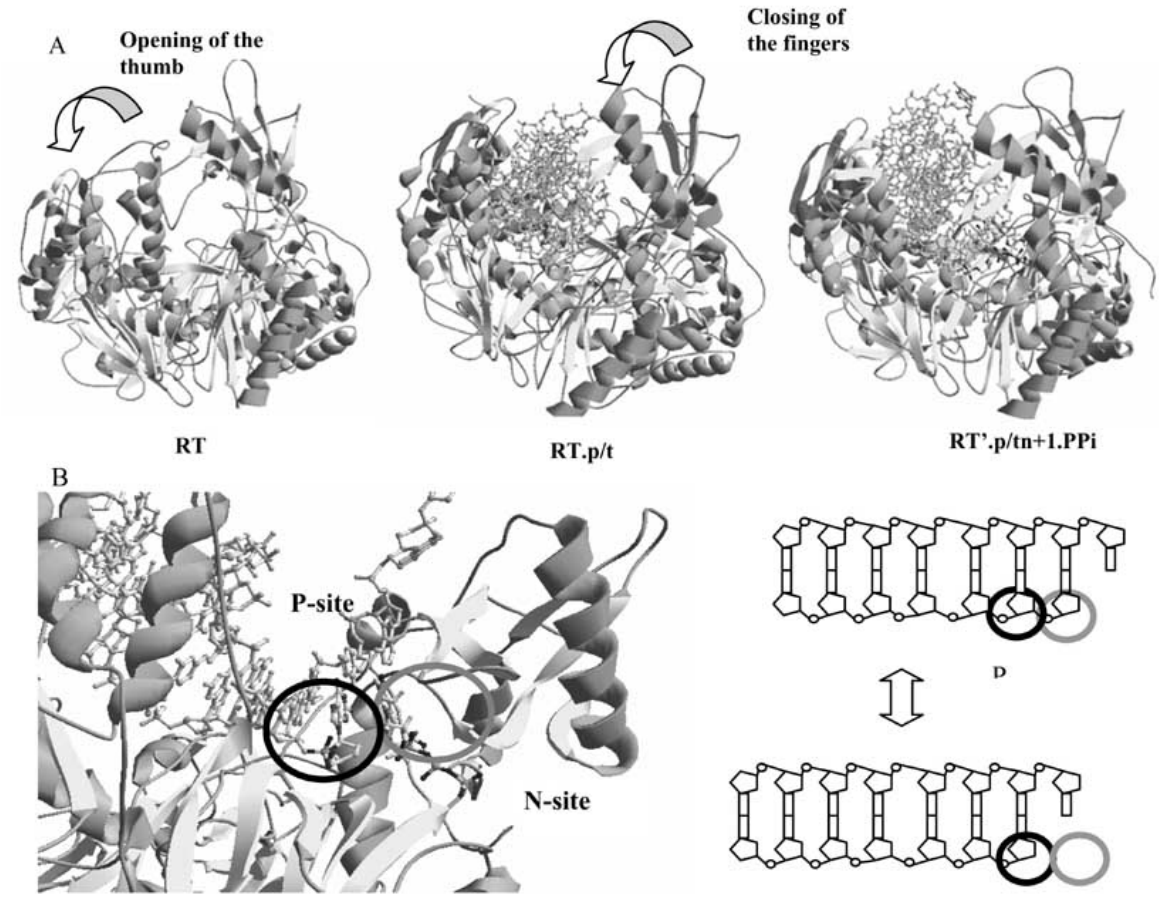

Fig. (2). A. Scheme of the conformational changes on the enzyme during RT mediated polymerization. B. (left) The incoming nucleotide first base pairs with the corresponding template base at the N-site. (right) In processive synthesis, after the chemical step, translocation occurs leaving the incorporated nucleotide at the P-site.

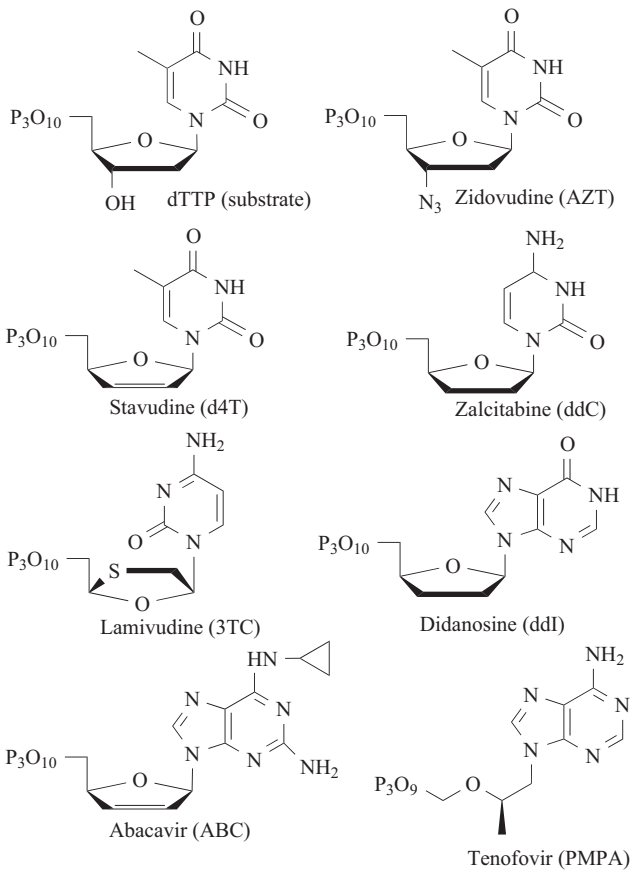

Fig. (3). Structure of the dTTP and of the currently approved NRTIs.

1. DISCRIMINATION

Discrimination is reflected in the efficiency of incorporation of the NRTI relatively to the normal substrate. It can be achieved either through selective decreased binding of the analog (reflected by an increase in the binding equilibrium constant, K_d) or at the catalytic step of incorporation of the analog into viral DNA (reflected by a decrease of *k*pol value, the catalytic constant of incorporation of the nucleotide analog into DNA).

Table 1. Mutations that Affect Reverse Transcriptase Susceptibility to NRTIs

Drug	RT resistance Mutations
3TC	E44A/D, V118I, M184I/V
d4T	E44A/D, V118I, L210W, V75T, TAMs[a], Q151M resistance complex[b]
AZT	E44A/D, V118I, L210W, TAMs[a], Q151M resistance complex[b]
ddC	K65R, T69D, M184V, Q151M resistance complex[b]
ddI	K65R, L74V, Q151M resistance complex[b]
PMPA	K65R, TAMs[a]
abacavir	K65R, L74V, Y115F, M184V, TAMs[a]

[a]TAMs-M41L, D67N, K70R, L210W, T215Y/F, K219Q.
[b]Q151M resistance complex- Q151M, A62V, V75I, F77L, F116Y.

1.1. Mutations that Affect the K_d

1.1.1. M184V

Resistance to lamivudine is associated, initially, with the substitution of isoleucine for methionine at position 184. This mutation, however, is rapidly replaced by the variant M184V [31-33]. This residue is located on the highly conserved YMDD motif that contains two of the three catalytic active site aspartates.

The wild type enzyme does not incorporate lamivudine monophosphate in DNA efficiently, but the presence of M184V increases discrimination. This increase was attributed to the 77- increased K_d measured for the binding of lamivudine triphosphate to the RT.p/t complex [34].

At a molecular level, the reason that has been pointed out to explain the resistance to this NRTI, was based on the analysis of a X-ray structure of the RT.p/t complex with the M184V mutation (pdb code 1J5O) [24] and was attributed to steric conflict between the oxithiolane ring of the drug and the beta-branched side chain of isoleucine or valine, which perturbs inhibitor binding leading to a reduction of incorporation of the same [24].

However, the superposition of the X-ray structure 1J5O with the structure of the wild type RT.p/t complex (pdb code 2HMI) in which we have docked lamivudine, clearly shows that the substitution of methionine for a residue which has a smaller side chain and is beta-branched, like valine or isoleucine, interferes with the primer and not with the drug (see Fig. **4**).

The hypothetical movement of the primer must be responsible for the increase in K_d with the drug. It is possible that lamivudine cannot be able to accommodate in the modified (by the movement of the primer) binding site.

The M184V mutation also confers resistance to ddNTPs and abacavir, which have the normal sugar configuration, which emphasizes that the steric clash should not involve the oxithiolane ring, as previously proposed.

1.2. Mutations that Affect the *k*pol

1.2.1. Q151M

One particular interesting mutation is Q151M. This substitution is identified in patients who develop multiple dideoxynucleotide resistance mutations. The Q151M is generally the first of a set of substitutions involving also A62V, V75I, F77L and F116Y [36].

By itself, Q151M confers low level resistance to AZT, ddI, ddC and d4T [36-38]. A62V, V75I, F77L and F116Y do not affect drug susceptibility by themselves, but the combination with Q151M results in up to more that 100 fold resistance to AZT, ddI, ddC and d4T, and low cross resistance to 3TC [37, 39, 40].

The atomic level explanation for the resistance conferred by this mutation is currently unknown; the observation of the determined structure of the RT.p/t.dNTP complex, 1RTD [12], seems not to give any clue about its origin. However, the crystallographic structure 1RTD has a low resolution (3.20 Å), and hence some side chain conformations might not be quite correct. This could be the case of Q151. The polar nature of this amino acid side chain should bring it

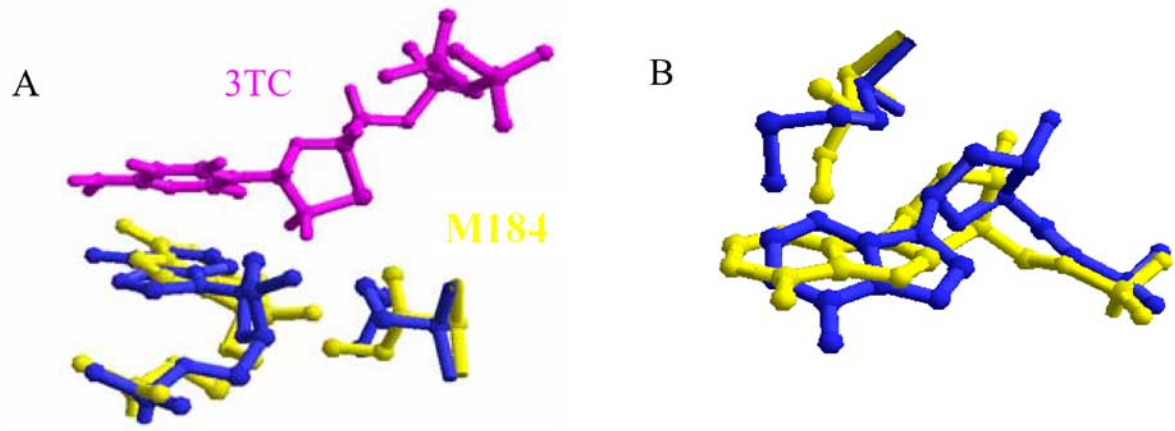

Fig. (4). A. Superposition of the last nucleotide of the primer and residue 184 of the structures 2HMI (blue) in which we docked l amivudine (violet) and 1J5O (M184I, yellow). The substitution of a beta-branched aminoacid for methionine seems to displace the primer from its normal position instead of colliding with the drug. B. Another view in which the displacement of the last nucleotide of the primer is more obvious.

more closely to the incoming nucleotide to successfully contribute to its stabilization. Furthermore, and although the incoming nucleotide is correctly base paired with the template, the position of the substrate is not the correct one for catalysis. Several interactions are necessary to correctly position the dTTP. One of them is the intramolecular bridge between the 3'OH of the incoming nucleotide and the oxygen of the beta-phosphate (see Fig. **5**), which has been inferred to be essential for catalysis [16]. Another could be provided by Q151. Thus Q151 could contribute to the correct positioning of the incoming nucleotide relatively to the primer, possibly by forming a second hydrogen bridge with the 3'-OH of the incoming nucleotide. When the substitution of this glutamine for methionine occurs, catalysis should be impaired. However, the synthesis should continue because the intramolecular bridge is still formed.

Resistance emerges for AZT, ddI, ddC and d4T because these NRTIs lack a 3'OH intermolecular bridge, and hence none of these interactions is formed. After the Q151M substitution the mutations A62V, V75I, F77L and F116Y generally follow, and this combination results in high resistance. These nonpolar and aromatic amino acids are in the region of the fingers domain that is next to the template primer at the N site.

The noncovalent interactions in double stranded DNA chains are provided by hydrogen bridges between the complementary bases and the stacking interactions between adjacent bases. In the N site we have the primer terminus and single stranded template, and hence the mentioned residues (i.e. Q151, A62, V75, F77 and F116) could create a hydrophobic cavity at the N site that accounts for the correct positioning of t/p.

The misalignment of the p/t adding to the mentioned incorrect positioning of the incoming nucleotide should result in high level resistance.

1.2.2. V75T

When d4T is given as the sole drug in cultured HIV infected cells the mutation V75T occurs [41]. The level of resistance to this drug is low; in fact, the most important d4T mutations are AZT mutations [42].

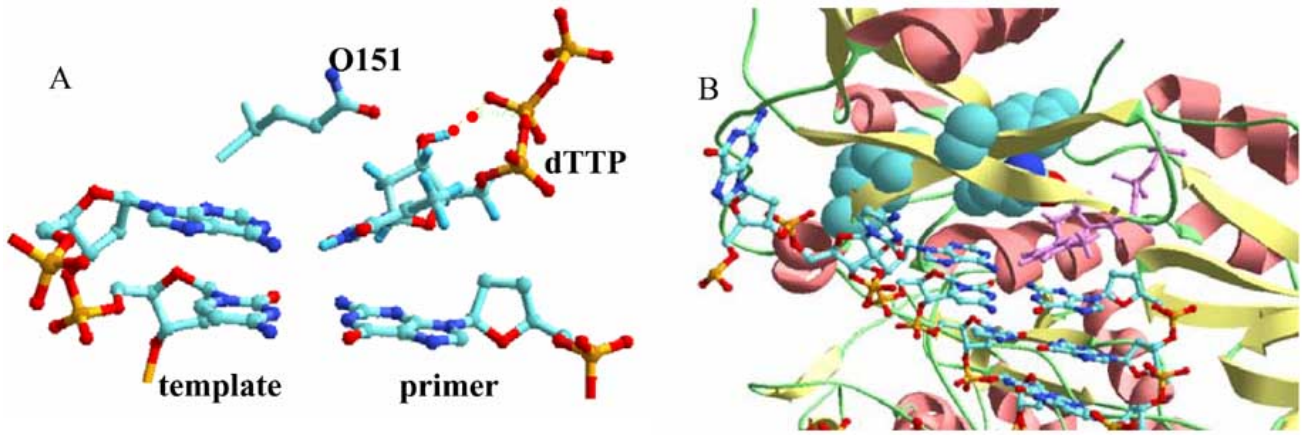

Fig. (5). The active site of reverse transcriptase. The primer/template and incoming nucleotide are in stick representation. Glutamine151 is also in sitck representation. A. dTTP as the incoming nucleotide. The intramolecular bridge within the nucleotide is shown. B. Localization of the amino acids of the Q151M resistance complex (in vdW representation).

However, it has been proven that this particular substitution discriminates 3.6 fold d4T relatively to dTTP and increases the RT mediated repair of d4T by pyrophosphate [16].

No atomic level explanation was given to the resistance conferred by this mutation. However, in the 1RTD structure, the 75 residue is in close proximity with Q151 (Fig. **6**). After the substitution of V75 to threonine, the repulsion between the carboxylate of the threonine side chain and the carboxylate of the Q151 backbone could change the orientation of Q151, and hence disrupt the stabilizing interaction between this residue and the incoming nucleotide.

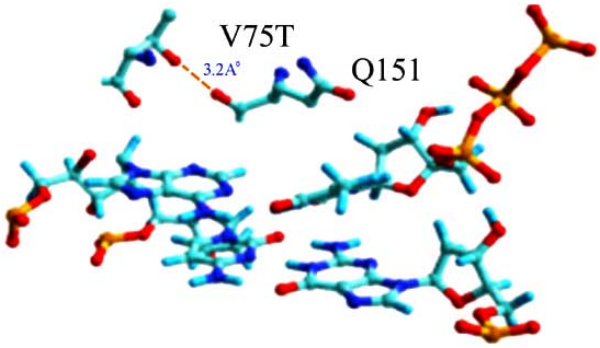

Fig. (6). Hypothetical repulsion between the side chain of theonine 75 and glutamine 151. The V75T mutation was performed with spdbv package [35].

1.2.3. K65R

The K65R mutation is principally associated with resistance to abacavir, ddI, ddC and PMPA [36, 39, 43]. The level of resistance provided by K65R to ddNTPS is moderate (< 12 fold) [15, 44]. In the crystal structure of the RT.p/t.dTTP complex, K65 side-chain is close to an oxygen atom in the gama-phosphate of the incoming nucleotide [12].

The resistance conferred by this substitution to ddNTPs was explained in a previous study to be the result of the absence of the essential intramolecular bond between the absent 3'OH and one of the beta-phosphate oxygens of the NRTIs. It was proposed that in the absence of such bond, catalysis was impaired irrespectively of which amino acid is present at position 65. When K65R and the normal substrate are present the intramolecular bond of the substrate stabilizes the position of the alpha-phosphate. When both the intramolecular bond is absent (ddNTPS) and K65R is present, K65R further displaces the alpha-phosphate from a correct alignment for catalysis [17].

However, if catalysis was impaired irrespectively of which amino acid was at position 65, there should not be a reason for the enzyme to select that particular mutation during the administration of such NRTIs. In fact, although lysine and arginine are similar amino acids, both being positively charged, the different sized and branched side chain of arginine and the presence of three possible hydrogen donors contrarily to only one in lysine may account for a different pattern of hydrogen bridges. Because the NRTIs do not have the 3'OH group to establish the intramolecular H-bond, one of the beta phosphate oxygens could engage in a hydrogen bridge with arginine, and this interaction should further displace the drug from the correct alignment for catalysis (Fig. **7**).

Furthermore, a hydrogen bridge between the gama-phosphate and lysine 65 seems important for the correct positioning of the incoming nucleotide at the N site.

1.2.4. L74V

L74V is the most common resistance mutation associated with ddI [45] (Kozal *et al*, 1994). It is also associated to low level resistance to abacavir, but such level is increased in the presence of M184V, K65R and Y115F [46]. It is also associated with the development of resistance for dioxolane guanosine (DXG) a new prodrug currently in clinical trials [47]. These nucleotide analogs have in common purine bases (tenofovir has an adenine as the base but lacks the ribose moiety and hence should have more flexibility/mobility).

L74 is localized in the fingers domain and in the proximity of the template strand.

The molecular basis for the involvement of this mutation in resistance to drugs with a purine as a base has not been possible to explain. It seems that the substitution of a gama-branched amino acid for a beta-branched amino acid like valine creates more space for the pyrimidine base of the template, but this extra space is not sufficient for a purine (Fig. **8**). If the incoming nucleotide is one of the mentioned NRTIs, the referred movement of the template base to which

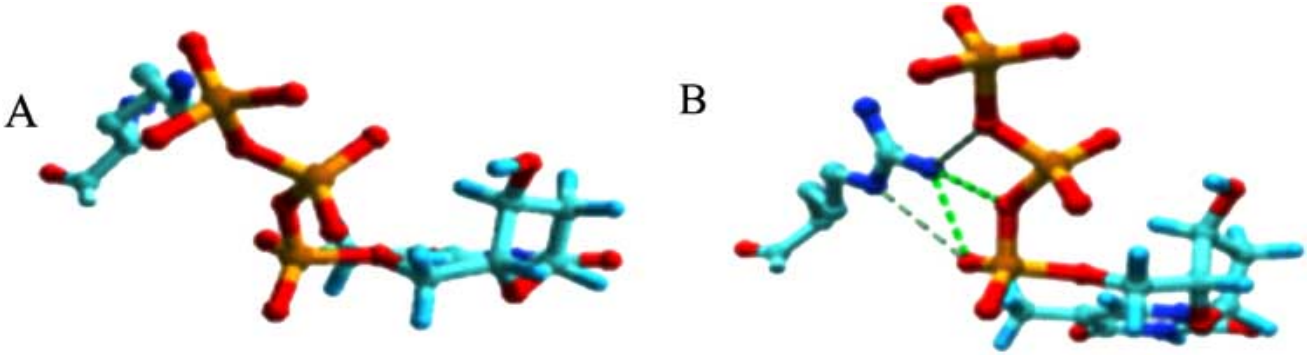

Fig. (7). A. In wt RT lysine 65 establishes a hydrogen bridge with one of the oxygens of the gama-phosphate. B. When the substitution of lysine65 for arginine occurs, different hydrogen bridges can be formed. The K65R mutation was performed with the spdbv program [35].

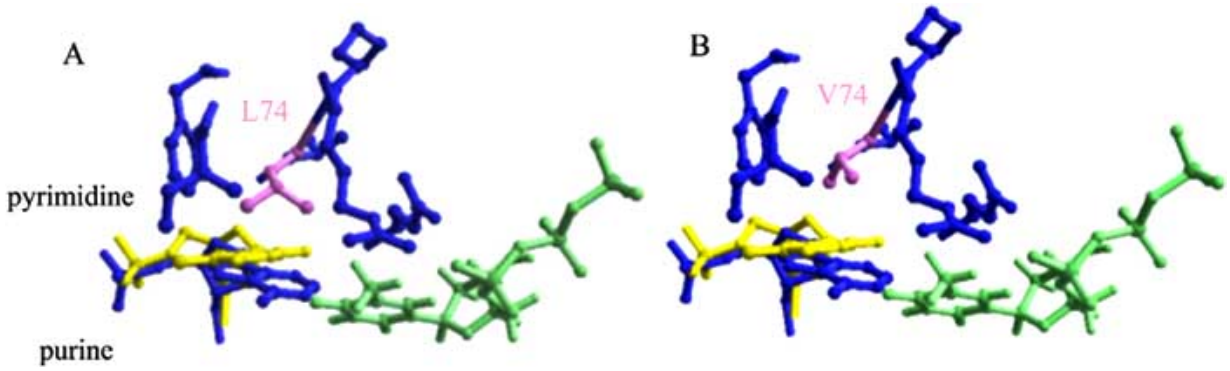

Fig. (8). A. Superimposition at the N site of RT of a template strand purine (blue) and pyrimidine (yellow). The L74 is also represented in violet. B. The L74V mutation could create more space for the template strand pyrimidine base but not for a purine .

it base pairs and the lack of hydrogen bridges at the missing 3'OH would result in the development of resistance.

2. REPAIR OF THE ANALOG-TERMINATED CHAIN

2.1.TAMs

The TAMs comprise a group of 6 point mutations, M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E [14, 48] that were initially described in association with resistance to AZT [48-52]. Subsequent research has also implicated these mutations in diminished susceptibility to d4T as well as moderate levels of resistance to other NRTIs, including abacavir, ddI, PMPA, and ddC, depending on the specific mutational pattern present [14, 48-51]. Generally, two or more of these substitutions including T215F/Y are needed in RT to acquire high level resistance to AZT [53].

The excision mechanism is the reversal of polymerization and involves nucleophilic attack by a pyrophosphate donor (adenine triphosphate or PPi) on the blocked primer, which permits DNA synthesis to continue [54, 55]. ATP is believed to be the main pyrophosphate donor *in vivo* [55-63].

In the case of AZT, it was proposed that excision could only occur if the blocked primer was at the nucleotide binding site (N-site). If the dideoxy-terminated primer was at the P-site, it could form a stable dead end ternary complex with the incoming nucleotide preventing excision. The azide group interfered with the formation of a stable dead-end complex because of steric interactions between it and the incoming dTTP [29, 64]. This would displace the translocation equilibrium towards the N-site increasing excision.

However, excision also occurs for d4T and this group only has a hydrogen attached to the 3'carbon, which excludes, in this case, the possibility of steric clash with the incoming nucleotide. Other (presently unknown) molecular interactions should be involved in this phenomenon.

In the crystal structure of RT.t/p.dTTP [12] the TAMs are far away from the active site. Residues 215 and 219 are 10 Å away and residues 67 and 70 are in a different domain more than 20 Å away. For residue 215, it was proposed that aromatic interactions of tyrosine or phenylalanine side chains with the adenine ring of the ATP actually contribute to improve ATP binding [29, 64]. Regarding the mutations in the fingers (67, 70), they appear to destabilize the ternary complex leading to enhanced excision [58, 60, 65, 66] (Fig. **9**).

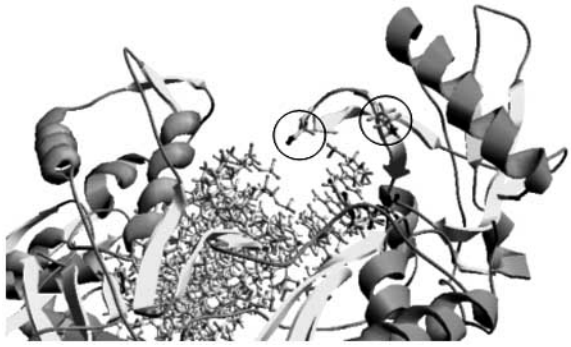

Fig. (9). Amino acids in the fingers domain that belong to the TAMs.

CONCLUSION

The use of an analog of a nucleotide, without the 3´OH required for elongation, as a drug in the treatment of AIDS seemed a good idea at the very beginning. However, the emergence of resistance by RT to the approved drugs allied to their long term toxicity calls for better drugs.

Competition between the normal substrate and the drug results in mutations that favor dNTP addition. Depending on the inhibitor, different mutations can be selected by the virus, which affect the mechanism of nucleotide addition at different steps.

NRTI incorporation is affected by the Q151 resistance complex, as well as V75T, K65R and L74V mutations. Excision is affected by the TAMs. Binding has been proposed to be affected by the M184V mutation. Interestingly enough, the presence of some discrimination mutations, e.g. K65R and L74V, may be antagonized by the earlier presence of excision mutations – the TAMs.

The correct positioning for incorporation should depend on the interactions that the incoming nucleotide establishes with the surrounding residues. Similarly, nucleotide addition or excision also depends on the interactions of the incoming nucleotide (dNTP or NRTI) with the RT.p/t complex.

Therefore, the question we should ask is: Which interactions between the RT.p/t complex and the incoming nucleotide are fundamental for catalysis?

When we are able to answer this question we will be able to design new and better inhibitors.

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