Insertions and deletions in HIV-1 reverse transcriptase: Consequences for drug resistance and viral fitness

Luis Menéndez-Arias*, Tania Matamoros, and Clara E. Cases-González

Centro de Biología Molecular "Severo Ochoa", Consejo Superior de Investigaciones Científicas – Universidad Autónoma de Madrid, 28049 Madrid, Spain

* To whom correspondence should be addressed at Centro de Biología Molecular "Severo Ochoa", Consejo Superior de Investigaciones Científicas – Universidad Autónoma de Madrid, 28049 Madrid, Spain; Tel.: +34 914978477; Fax: +34 914974799; E-mail: lmenendez@cbm.uam.es

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is an important target of drugs fighting HIV infection. The introduction of potent antiretroviral therapies based on the use of RT inhibitors and/or protease inhibitors has been an important achievement towards the control of AIDS. However, the development of drug resistance constitutes a major hurdle towards long-term efficacy of those therapies. With the increasing complexity of the antiretroviral regimens, novel mutational patterns conferring high-level resistance to nucleoside and nonnucleoside RT inhibitors have been identified in viral isolates. Among them, insertions and deletions in the β3-β4 hairpin-loop-coding region of HIV-1 RT have been identified in heavily-treated patients. Insertions of one, two or several residues appear to have a significant impact on nucleoside analogue resistance. The frequently found combination of a dipeptide insertion and thymidine analogue resistance mutations (*i.e*. T215Y) in the viral RT confers an ATP-dependent phosphorolytic activity that facilitates the removal of the inhibitor from primers terminated with zidovudine or stavudine. Furthermore, this mechanism appears to be relevant for resistance mediated by one amino acid-deletions appearing in combination with thymidine analogue resistance mutations. However, in other sequence contexts (*i.e*. in the presence of Q151M), the effects of the deletion are not fully understood. Drugs targeting the excision repair mechanism could be an important aid in the fight against multinucleoside-resistant HIV isolates bearing complex mutational patterns in their RT-coding region.

Keywords: HIV, reverse transcriptase, antiretroviral therapy, drug resistance, nucleoside analogues, zidovudine, excision repair

INTRODUCTION

According to the December 2004 UNAIDS report on the global situation of AIDS [1], there were around 40 million people infected with the human immunodeficiency virus (HIV) around the world. It was also estimated that more than 3 million people died of AIDS in 2004, while another 5 million people were newly infected with HIV in the same year. Seventy percent of the HIV-infected people in the world live in sub-Saharian Africa. In Western Europe, an estimated 610,000 people are living with HIV, and in North America, this figure goes up to 1,000,000. Currently, there are around 20 drugs licensed for treatment of HIV infection. These drugs are compounds targeting viral enzymes such as the reverse transcriptase (RT) or the protease, or interfering with virus entry by inhibiting the step involving fusion of the viral envelope and the cell membrane [2-5]. Since the introduction in the mid-90s of highly active antiretroviral therapies (HAART), mortality due to HIV infection has decreased in those countries where the population has access to antiretroviral treatments. In a significant number of patients receiving antiretroviral therapy, AIDS has become a chronic albeit incurable disease [6-8]. Currently prescribed drug cocktails (HAART) are usually a combination of three or more antiretroviral drugs, including either one or two nucleoside/nucleotide RT inhibitors, one nonnucleoside RT inhibitor (NNRTI) and/or one protease inhibitor, which sometimes can be complemented by a fusion inhibitor (enfuvirtide). Most HAART-treated patients show undetectable levels of plasma virus (<50 RNA copies/ml) within a few months after the start of therapy. Despite the success of potent combination therapies, the development of HIV-1 drug resistance during the antiretroviral treatment remains a major cause of therapy failure in patients adherent to treatment.

Since the discovery of zidovudine (AZT; β-D-(+)-3´-azido-3´-deoxythymidine) as an effective antiretroviral agent against HIV [9] and its approval for clinical use in March 1987, RT inhibitors have been extensively used for treatment of HIV infection. The loss of the therapeutic effect of AZT as a result of the acquisition of resistance was first recognized by Larder *et al*. [10], who demonstrated that HIV isolates from patients with advanced HIV

disease became less sensitive to the drug during the course of treatment. Interestingly, other nucleoside RT inhibitors such as didanosine (ddI; β-D-(+)-2′,3′-dideoxyinosine), zalcitabine (ddC; β-D-(+)-2′,3′-dideoxycytidine) and stavudine (d4T; β-D-(+)-2′,3′-dideoxy-2′,3′-didehydrothymidine) [11, 12] were soon developed into antiretroviral drugs and licensed for clinical use in October 1991, June 1992 and June 1994, respectively. Combination therapies involving the use of AZT plus ddI or ddC led to selection of drug-resistant HIV isolates with unusual combinations of amino acid substitutions in their RTs (*i.e.* A62V, V75I, F77L, F116Y and Q151M) [13]. With the introduction of HAART, an increasing complexity in the mutational patterns found in HIV type 1 (HIV-1) RT was observed. In this scenario, insertions and deletions in the RT-coding region were found to be associated with resistance in HIV isolates from patients that did not respond to therapy.

HIV-1 RT STRUCTURE AND INHIBITION

HIV-1 RT catalyzes the synthesis of double-stranded proviral DNA using the single-stranded viral RNA as template. The RT is a multifunctional enzyme that has RNA-dependent and DNA-dependent DNA polymerase activity in addition to an endonuclease (RNase H) activity that degrades RNA-DNA intermediates formed during proviral synthesis. Unlike eukaryotic DNA polymerases, retroviral RTs lack a proofreading activity and show a relatively high error rate (for recent reviews, see [14, 15]), which together with the HIV-1's high rate of replication contribute to the high variability of the virus. The mature RT is a heterodimer composed of two subunits of 66 and 51 kDa, which are designated as p66 and p51, respectively.

Crystal structures of HIV-1 RT have revealed that both subunits contain four common subdomains, termed the "fingers", "palm", "thumb" and "connection" [16] (Figure 1). The 66-kDa polypeptide has an extra C-terminal domain spanning the last 120 residues, which provides the RNase H activity. The overall folding of the subdomains is similar in p66 and

p51, but their spatial arrangements are rather different. Highly conserved regions in the fingers and palm subdomains of the 66-kDa subunit, together with two α-helices of the thumb subdomain, act as a clamp that positions the template-primer complex relative to the active site of the polymerase [18, 19]. The active site of the enzyme resides within the 66 kDa subunit that contains the catalytic aspartic acid residues (Asp-110, Asp-185 and Asp-186). Other amino acids in their vicinity (*i.e*. Lys-65, Arg-72, Asp-113, Ala-114, Tyr-115 and Gln-151) are involved in interactions with the incoming dNTP [17]. The comparison of binary and ternary complexes of HIV-1 RT shows that parts of the fingers subdomain rotate towards the palm subdomain and the 3´ end of the primer [20]. As a result, the tips of the fingers formed by a hairpin loop connecting β strands 3 and 4 get close to the dNTP binding site, and facilitate hydrogen bond interactions between the side-chains of Lys-65 and Arg-72, and the incoming dNTP (Figure 1).

Approved antiretroviral drugs targeting the RT include seven nucleoside analogue inhibitors: zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine [3TC; ß-L-(–)-2´,3´-dideoxy-3´-thyacytidine], abacavir [(–)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol] and emtricitabine [FTC; ß-L-(–)-2´,3´-dideoxy-5-fluoro-3´-thyacytidine], one acyclic nucleoside phosphonate: tenofovir [*R*-9-[2-(phosphonomethoxy)propyl]adenine], and three NNRTIs (nevirapine, delavirdine and efavirenz) [for recent reviews, see refs. 5, 21-23]. Nucleoside analogue inhibitors and acyclic nucleoside phosphonates must be phosphorylated to their triphosphate form to act as competitive inhibitors (or alternate substrates) of HIV-1 RT. These inhibitors are chain terminators, which after incorporation into the growing DNA chain are not further elongated due to the lack of a hydroxyl group in the 3´ position of their ribose moiety. NNRTIs bind to a hydrophobic pocket, located 10-15 Å away from the active site of the polymerase [for recent reviews, see refs. 24-26]. Binding of a NNRTI apparently blocks the chemical steps of polymerization [27, 28], possibly by affecting the conformational changes required for polymerase catalysis [19; and reviewed in ref. 29].

PATHWAYS TOWARDS THE ACQUISITION OF RESISTANCE TO RT INHIBITORS

Resistance to RT inhibitors can be achieved through the accumulation of one or more mutations in viral RT-coding region. Single-nucleotide mutations conferring resistance to RT inhibitors have been identified in lamivudine-resistant strains, and are frequently associated with resistance to NNRTIs. These mutations render amino acid substitutions in the viral RT that decrease the enzyme's ability to bind the inhibitor. For example, M184V confers resistance to lamivudine (3TC) by decreasing the catalytic efficiency of incorporation of 3TC-triphosphate by the mutant polymerase [30-32]. In the case of resistance to nevirapine and other NNRTIs, there are several amino acid substitutions that by themselves can confer high-level resistance to the drug. Examples are K103N, V106A, Y181C, Y181I, Y188L, Y188H, G190A or G190S, which confer resistance to nevirapine. Interestingly, the amino acid substitution K103N which arises from a transition mutation at codon 103 confers resistance to all three approved NNRTIs [2, 25, 33; and references therein]. Those amino acid changes have a destabilizing effect on inhibitor binding through the loss of hydrophobic (*i.e.* Y181C) or electrostatic interactions (*i.e.* K103N).

High-level resistance to nucleoside RT inhibitors is frequently achieved through the accumulation of two or more amino acid substitutions in the viral enzyme. For example, combinations of mutations, such as M41L, D67N, K70R, L210W, T215F or T215Y, and K219E or K219Q are frequently observed in HIV isolates from patients under therapy with AZT [reviewed in ref. 34]. Mutations associated with resistance to AZT are also observed in the clinical setting during treatment with d4T [35-37], and have been designated as "thymidine analogue resistance mutations" (TAMs). Cross-reactivity between nucleoside analogues due to the presence of TAMs has also been reported for abacavir, based on clinical evidence and phenotypic data [38-40].

AZT was the first antiretroviral drug to be used in AIDS therapy and the only one available from 1987 until 1991. Therefore, AZT monotherapy led to the emergence of HIV isolates containing TAMs and having different degrees of resistance. Novel drugs such as ddC, ddI and d4T allowed the use of combination therapies that selected for viruses containing alternative drug resistance patterns. For example, the simultaneous treatment with AZT and ddI rendered viral isolates with reduced sensitivity to AZT, ddC, ddI, dideoxyguanosine and d4T [13, 41-43]. The resistant viruses contained the substitutions A62V, V75I, F77L, F116Y and Q151M, with the side-chains of Phe-116 and Gln-151 forming part of the nucleotide binding site of HIV-1 RT. The mechanism of resistance mediated by the Q151M complex involves a selective reduction in the catalytic rate constant k_{pol} of incorporation of the analogue into DNA, as demonstrated with various RT inhibitors [44-46].

HIV-1 RTs WITH INSERTIONS IN THE FINGERS SUBDOMAIN

With the increasing complexity of the antiretroviral regimens and the natural variability of HIV-1, novel and unusual mutational patterns have been found in drug-resistant isolates. Thus, in 1997, De Antoni *et al*. [47] reported on the identification of a two-amino-acid insertion between residues 69 and 70 of the RT, in virus isolated from one patient undergoing treatment with ddI and hydroxyurea. The insertion (Ser-Ser) was detected after only 12 weeks of therapy and was apparently caused by a six-nucleotide duplication in the viral genome, occurring after a C→G mutation at the second base of codon 69. After 24 weeks of therapy, the inserted AGTAGT sequence changed to AGTGGT, which translates to Ser-Gly. Further reports on the presence of dipeptide insertions (*i.e.* Ser-Ser, Ser-Gly, or Ser-Ala) in the fingers subdomain of HIV-1 RT of virus isolated from heavily-treated patients revealed the presence of other amino acid substitutions in the viral RT, which were related to drug resistance [48-52]. Most of those patients had been previously treated with AZT and other nucleoside analogues, and emergence of the dipeptide insertion was related to combination

therapies. Sequence alignments showing representative amino acid and nucleotide sequences of insertion-containing RTs are given in Figure 2.

The prevalences of the dipeptide insertions in HIV-infected patients ranged from 0.5 to 2.7%. The lowest figures (< 1 %) were usually obtained with populations including all patients subjected to HIV protease and RT genotyping [48, 59-62]. When the study population was restricted to those patients who did not respond to the antiretroviral therapy, the prevalence of the dipeptide insertion increased to 1.9 – 2.7 % [53, 54, 63, 64]. The large screening studies also revealed the presence of one-amino acid insertions between codons 69 and 70 of the RT in viral isolates from treated patients [53, 64]. Amino acids constituting the one-amino-acid insertion were Asp, Asn, Gly, Ser and Thr, which appeared with or without the T69S substitution [50, 51, 53, 54, 64, 65]. Except for one subtype A viral isolate carrying a T69SQS insertion [64], so far all of the viral isolates carrying one- or two-amino-acid insertions have been classified as subtype B.

Larger insertions in the fingers subdomain are relatively uncommon in clinical HIV isolates. The first evidence of their occurrence was reported by Sato *et al*. [57] who found an in-frame 33-nucleotide insertion mutation in the RT-coding region of an HIV-1 subtype E (CRF01_AE) variant isolated from a patient who had not responded to treatment with nucleoside analogue RT inhibitors. The origin of the insert (sequence given in Figure 2) was uncertain, since no homology was found with any viral or human sequences. A 5-amino-acid insertion (TRVMG) resulting from the translocation of the first 15 bases of the envelope gene has also been reported [53, 66]. Viral isolates containing the insertion showed high-level resistance to AZT, d4T, ddI, 3TC and abacavir in phenotypic assays [53, 66]. More recently, van der Hoek *et al*. [58] reported on the presence of an 8-amino-acid insert (STGKKDST) in a patient treated with AZT and ddC. Apparently, the 24-nucleotide insert resulted from a partial duplication of local sequences and the acquisition of a sequence segment of an unknown origin. Phenotypic studies carried out with constructs derived from the insertion-containing

viral isolate showed that the 8-amino-acid insert augments nucleoside analogue resistance [58].

Other large insertions found in clinical isolates and involving residues in the fingers subdomain of the RT, include a 5-amino acid insertion associated with the amino acid substitution T69D (T69DRKGSE) [58], an 8-amino-acid insertion (T69TTEGKKDST) [53], and the duplication of the sequence KGSNR at positions 66-70 [67]. Viral isolates carrying the insertions T69DRKGSE or T69TTEGKKDST displayed high-level resistance to AZT in phenotypic assays, while the KGSNR duplication had a small impact on nucleoside analogue resistance. In addition, HIV-1 strains carrying a one or two amino acid insert between codons 102 and 103 of the viral RT have been recently found in clinical isolates [68]. Those inserts, that occur at the NNRTI binding pocket, were shown to have an influence nevirapine and efavirenz resistance.

The number of HIV-1 genotypes having insertions in the region encoding for the β3-β4 hairpin loop of the RT has been steadily increasing. Although Ser-Gly, Ser-Ser and Ser-Ala appear as the most prevalent, dipeptide sequences found at positions 69-70 of the RT show a high degree of variability, as illustrated in Figures 2 and 3. Since insertions are usually found in viral strains isolated from heavily-treated patients, it is not surprising that they are usually found in combination with drug resistance-related mutations, particularly with TAMs such as M41L, L210W or T215Y. T215Y/F appears to be strongly associated with the insertions T69SSS, T69SSG, T69SSA, T69SST and T69SVT (>95% of the insertion-containing sequences have Tyr or Phe at position 215). In contrast, the insertion T69SVG was rarely associated with TAMs in HIV clinical isolates (Figure 3), although recombinant viruses showed high-level resistance to AZT and other nucleoside analogues in phenotypic assays [53, 56, 72].

ROLE OF THE INSERTIONS IN THE MECHANISM OF AZT RESISTANCE

HIV-1 isolates with insertion-containing RTs showed high-level resistance to AZT, and moderate levels of resistance to other nucleoside RT inhibitors, such as d4T, ddI, ddC, abacavir and tenofovir, in phenotypic assays [48, 50, 55, 59, 77-79]. Mutations conferring nucleoside analogue resistance act either by (i) interfering with the ability of HIV-1 RT to incorporate the triphosphate form of the drugs [80, 81; and references therein], or (ii) increasing the removal of the 3´-terminal chain-terminating inhibitors from blocked DNA primers in the presence of physiological concentrations of pyrophosphate (PPi) or ATP [82, 83; and reviewed in ref. 84] (Figure 4).

Removal of chain-terminating nucleoside analogues through phosphorolysis arises as the major AZT resistance mechanism in virus harboring the RT substitutions D67N, K70R, T215F/Y and K219Q/E [82, 83]. The mutant polymerases showed similar kinetics of nucleotide incorporation and inhibitor sensitivity to the wild-type RT in *in vitro* assays [87-90]. In addition, the comparison of the crystal structures of the AZT-resistant RTs bearing mutations M41L and T215Y [91] or D67N, K70R, T215F and K219Q [92] and the wild-type RT revealed only minor conformational changes and subtle differences in the orientation of specific side chains. Other studies suggested that AZT-resistant RTs were more processive [93], or were able to bind AZT-terminated primers more tightly than the wild-type RT [94]. However, significant differences between mutant and wild-type enzymes were observed only in assays measuring the RT's ability to remove AZT-monophosphate from the 3´-end of terminated primers, in the presence of a PPi donor [82, 83]. In these assays, the largest differences were obtained when a ribonucleoside-triphosphate (typically 1 – 5 mM ATP) was used as the PPi donor in the reaction [83].

The role of a dipeptide insertion (Ser-Ser) in the mechanism of resistance to AZT has been studied by using a recombinant RT derived from a clinical isolate obtained from a heavily-treated patient, who did not respond to antiretroviral therapy [55]. Apart from the insertion, the studied RT contained a number of mutations related to drug resistance (M41L,

A62V, T69S, K70R, V108I, V118I, Y181C, M184I, L210W, T215Y and G333E), as well as other amino acid substitutions including polymorphisms (K43E, K104R, D123E, I135T, S162A, K172R, V179I, G196E, Q197K, Q207E, L214F, H221Y, L283I, I293V, E297T, L301M, D324E, Q334H, G359S, A371V, T376A, K390R, E404D, N460D, K461R, V466A, P468S, L469I, N471D, L491S, N519S and Q524E). As reported for other insertion-containing HIV strains, recombinant HIV having the RT described above showed high-level resistance to AZT and moderate levels of resistance to other nucleoside RT inhibitors such as d4T, ddC and ddI [55]. These studies showed that AZT resistance could not be attributable to differences in nucleotide specificity or processivity between the mutant and the wild-type RT [55]. However, in comparison with the wild-type enzyme, the RT containing the insertion had an increased ability to remove the 3´-terminal nucleotide from AZT-terminated primers in the presence of physiological concentrations of ATP (typically within the range of 0.8 – 4 mM) [55, 95]. The increased ATP-dependent phosphorolytic activity was also observed with dideoxythymidine-terminated primers.

Interestingly, the ATP-mediated excision activity of the RT was reduced by about 3-fold, when the insertion was removed from the sequence while maintaining mutations T69S and K70R [55]. These results were recently confirmed by deleting the dipeptide insertion in an RT, which derived from a different clinical isolate that contained a T69SSS insertion plus additional mutations including TAMs M41L, L210W and T215Y [79]. In contrast with those observations, introducing the insertion in an otherwise wild-type sequence background did not confer significant ATP-dependent phosphorolytic activity [55, 96-98]. It has been shown that when the insertions T69SSS or T69SSG were introduced in combination with T215Y in a wild-type HIV-1 RT, the resulting enzyme showed small but significant ATP-dependent phosphorolytic activity on primers terminated with AZT [99, 100]. On the other hand, substituting Thr, Ser or Asn for Tyr-215 in an insertion-containing multinucleoside-resistant HIV-1 RT led to the loss of the enzyme's ability to remove AZT-monophosphate from blocked primers in the presence of ATP, an effect that correlated with the loss of resistance to AZT

found with those mutants in phenotypic assays [100]. Altogether, these data demonstrate that Tyr-215 is critical for the RT's excision activity.

T215F/Y and other TAMs such as M41L or L210W are located outside the dNTP binding site of the RT [17]. Molecular modeling and crystallographic studies suggest that those residues are involved in ATP binding through hydrophobic interactions between their side-chains and the incoming ribonucleotide [101, 102]. In agreement with this proposal, mutational studies carried out with RTs having 1, 2 or more TAMs have shown that D67N and K70R in the fingers subdomain, together with T215Y were necessary to obtain significant levels of ATP-dependent phosphorolytic activity [83, 103, 104]. In addition, the double-mutant M41L/T215Y also showed significant levels of ATP-dependent phosphorolytic activity on dideoxyadenosine-terminated primers [105]. The dipeptide insertion in RTs derived from clinical isolates is usually accompanied by 2, 3 or more TAMs. The ATP-dependent phosphorolytic activity of these enzymes is much higher than the activity reported for mutants containing the insertion plus mutations T69S and T215Y (*i.e.* T69SSS/T215Y).

Additional TAMs and other mutations in the polymerase domain of HIV-1 RT are likely to be the most relevant for the ATP-mediated excision activity. However, a recent report showed that mutations impairing RNase H activity, such as H539N or D549N, could enhance thymidine analogue resistance [106]. Since nucleotide excision is expected to occur when the inhibitor moiety of the blocked primer is located at the dNTP binding site (for recent reviews, see [81, 84]), reduced RNase H activity is likely to shift the equilibrium towards nucleotide excision [106]. These data suggest that in multidrug-resistant clinical isolates, which accumulate a relatively large number of mutations, potential long-distance interactions could modulate the efficiency of the rescue reaction.

CROSS-REACTIVITY WITH OTHER NUCLEOSIDE ANALOGUES

AZT-terminated primers are excellent substrates of the ATP-mediated excision reaction catalyzed by insertion-containing RTs, but other nucleoside analogues can also be excised from blocked primers through the same mechanism. Available data indicate that thymidine analogues (*i.e.* AZT, d4T and dideoxythymidine) are the best substrates of the reaction [95, 99], followed by tenofovir [79], while dideoxyadenosine is also removed albeit poorly in comparison with AZT [98, 99]. In contrast, cytidine analogues (*i.e.* ddC- and 3TC-monophosphates) are poor substrates of the reaction [95, 99]. In agreement with those observations, HIV-1 strains bearing an insertion-containing RT in which Tyr-215 was replaced by Thr, Ser or Asn were found to be sensitive to AZT and d4T, while remaining resistant to ddI, ddC and 3TC [100, 107].

Biochemical data suggest that the insertion has a relatively small effect on AZT-triphosphate *versus* dTTP or ddCTP *versus* dCTP discrimination [55, 95], but nucleotide selectivity has not been studied in detail with other inhibitors. Thus, phenotypic assays showed that recombinant HIV-1 carrying the dipeptide insertions Ser-Ser or Ser-Gly, together with only 2 or 3 additional mutations (*i.e*. T69S and T215Y, or T69S, L210W and T215Y) were resistant to lamivudine [48, 77]. Since primers terminated with 3TC-monophosphate are poor substrates of the excision reaction [95], it is still possible that the insertion plays a role in 3TC-triphosphate *versus* dCTP discrimination.

AZT-resistant RTs bearing mutations D67N, K70R, T215F or T215Y and K219Q, K219E or K219N also showed a preference for thymidine analogues at the 3´ end of the terminated primer [83, 103]. However, RTs bearing the combination D67N/K70R/T215Y displayed very low ATP-dependent removal activity on primers terminated with tenofovir [108]. The catalytic efficiency of the removal reaction can vary several hundred-fold in different sequence contexts and is strongly affected not only by the nature of the base pair at the 3´-primer terminus but also by the six base pairs upstream of it [109]. The upstream sequence

has a relatively smaller influence on excision of primers terminated with thymidine analogues than on primers terminated with other dideoxynucleosides [109].

ATP-mediated excision reactions can be inhibited by the next complementary dNTP [83, 103], due to the formation of a "dead end complex" constituted by the RT, a template bound to a blocked primer and the next complementary dNTP [86] (Figure 4). The removal of AZT is not inhibited at physiological dNTP concentrations (IC_{50} > 0.25 mM). However, excision of d4T-, dideoxythymidine- and dideoxyadenosine-monophosphates can be inhibited *in vitro* at concentrations of the next complementary dNTP within a 0.5 to 25 µM range [95, 103]. Estimates of dNTP concentrations inside the cell depend on assumptions about cell volume, and show large variations even when determinations are made with the same type of cells. Reported values range from 0.2 µM to 24.5 µM in resting human lymphocytes, whereas in activated human lymphocytes these concentrations are 2 to 10 times higher, depending on the assay conditions and the dNTP analyzed [110-112]. The highest dNTP levels have been reported for established cell lines (*i.e*. H9, U937 promonocytes, CEM lymphoblasts, or SupT1 cells) [112-115], where nucleotide concentrations range from 50 to 300 µM. Under those conditions, rescue of d4T-terminated primers should be inhibited. The lack of cross-reactivity between AZT and d4T in phenotypic drug susceptibility assays [116-119] can be attributed to the use of either mitogen-stimulated peripheral blood lymphocytes or transformed human cell lines to optimize virus replication. It should be noted that cross-resistance between AZT and d4T is frequently observed *in vivo* in patients undergoing treatment with nucleoside analogues [35-37, 75].

DIPEPTIDE INSERTIONS AND THEIR IMPACT ON VIRAL FITNESS

Studies following the intrahost evolution and dynamics of a multidrug-resistant HIV-1, containing an insertion of two amino acids (Ser-Ser or Ser-Gly) and several amino acid changes within the RT-coding region showed that after termination of therapy, the insertion

mutants were quickly replaced by wild-type viruses [70, 71]. The analysis of the quasispecies found at the time of appearance of the dipeptide insertion revealed three sequence patterns in insertion mutants: (i) the presence of the insertion of two amino acids between codons 69 and 70, together with amino acid changes at adjacent positions (*i.e.* T69S, K70A or K70T), (ii) a T215Y change, and (iii) amino acid substitutions at position 67 (*i.e*. D67S, D67T, etc.) [71]. While T215Y was conserved in all insertion clones over time, the wild-type Asp at position 67 was changed into Asn in the majority of the early insertion mutants and quickly replaced by Ser, which became predominant very soon. In fact, the amino acid substitution D67N, which is related to AZT resistance [34], is rarely associated with dipeptide insertions in the HIV-1 RT (Figure 3).

In the absence of antiretroviral therapy, HIV strains containing drug resistance mutations have reduced fitness compared with the wild-type virus [for a recent review, see ref. 120]. The relative fitness of multidrug-resistant HIV strains containing the dipeptide insertion has been estimated at 66 to 84 % compared with the wild-type virus [71, 107, 121]. Dual infection/competition experiments revealed that in the presence of low concentrations of AZT, removal of the two serine residues in the multidrug resistant isolate does not cause a detrimental effect on the replication capacity of the virus [121]. Furthermore, in the absence of drug, RT insertions improve the fitness of viruses carrying a number of accompanying mutations associated with resistance to multiple nucleoside analogues (*i.e*. M41L, L210W, T215Y, etc.). However, the insertion by itself renders a virus that replicates poorly [121]. The presence of Tyr-215 together with the insertion in the RT of a wild-type subtype B virus (*i.e*. BH10) confers some resistance to AZT and d4T, while decreasing the viral replication capacity [107].

The amino acid change T215Y results from a two-nucleotide substitution, and its reversion implies the emergence of variants having Ser or Asn at that position. In multinucleoside-resistant RTs bearing a dipeptide insertion, AZT and d4T resensitization was acquired through the substitution of Asn, Ser or Thr for Tyr-215 [100, 107]. The

corresponding revertant HIV-1 strains showed slightly increased replication capacity [107]. These observations suggest that *in vivo*, HIV isolates harboring the insertion-containing RT (and lacking Tyr-215) could be selected in the absence of antiretroviral therapy. This situation would be similar to that observed with HIV strains bearing the classical AZT resistance mutations (*i.e.*, M41L, D67N, L210W, T215Y, etc.), selected during treatment with nucleoside analogues, or transmitted from an infected individual. It has been observed that in the absence of drugs, those variants are eventually replaced by AZT-susceptible revertants that contain ununsual residues at codon 215, such as Asp, Asn, Cys or Ser [122-124].

However, the emergence of revertant HIV strains with insertions in their RT has not been demonstrated in infected patients. This could be explained by the low prevalence of the insertion in HIV-treated patients, but also because the insertion appears in heavily-treated individuals, which may be also infected with other drug-susceptible variants whose selection is favored in the absence of antiretroviral drugs [70, 71].

While Tyr-215 plays a clear role in AZT and d4T resistance, the effects of substituting Asn for Asp-67 are not so clear. Neither viral fitness nor drug susceptibility appear to be significantly affected by introducing the D67N substitution in the insertion-containing RTs [100, 107]. The low prevalence of D67N in viruses harboring dipeptide insertions (Figure 3) and its low stability in the viral population [71] suggest that selection against this mutation could operate in conditions different from those established in cell culture (*i.e.*, low dNTP concentrations), although these issues have not been specifically addressed so far.

INHIBITION OF THE ATP-MEDIATED EXCISION REACTION

Inhibitors of the excision reaction would be of great help in antiretroviral therapy, not only for treating those patients that do not respond to drug therapy with RT inhibitors, but also in the long term, assuming the increasing prevalence of TAMs in the infected population. In principle, there are several possible strategies to interfere with the excision reaction.

First, by using compounds that interfere with ATP binding. This approach would benefit from the low ATP-binding affinity determined for excision-proficient RTs (the K_d for ATP was estimated to be around 0.3 – 1.8 mM, depending on the enzyme and the template-primer used) [125]. However, the lack of information on the precise boundaries of the ATP binding site, as well as the relatively low specificity of the excision reaction [85] limit the development of these inhibitors. Nevertheless, bisphosphonate inhibitors specifically targeting the RT-catalyzed ATP-dependent and PPi-dependent excision of AZT-monophosphate *in vitro* have been recently described [126]. One of these compounds, designated as BPH-218, showed an IC_{50} of 2 µM for the excision reaction, while having minimal effect on the DNA polymerase activity of HIV-1 RT.

The second approach would be the development of nucleotide RT inhibitors that could block DNA synthesis but would be resistant to excision. Potential candidates include methanocarbathymidines in their North conformation [127]. The corresponding triphosphate derivative of this compound does not block DNA synthesis at the point of incorporation, but only after a few additional normal dNTPs have been added to the DNA. Experiments with purified excision-proficient HIV-1 RT mutants revealed that methanocarbathymidine-terminated primers are relatively resistant to excision in comparison with those terminated with AZT. However, wild-type HIV-1 RT shows significant ATP-dependent phosphorolytic activity on primers terminated with methanocarbathymidines [127]. A major caveat of these compounds is that they are poorly phosphorylated in mammalian cells, hampering their development into potent antiretroviral drugs. In contrast, α-boranophosphate or α-thiophosphate derivatives of AZT and d4T are effective RT inhibitors and good substrates of nucleotide diphosphate kinases [128, 129]. Recently reported data show that primers terminated with phosphorothioates such as 3´-azido-3´-deoxythymidine-5´-O-(1-thiotriphosphate)-monophosphate are inefficiently excised by AZT-resistant RTs, including the multidrug-resistant insertion-containing RT [130]. Despite showing some promise, there are

problems of intracellular delivery and toxicity that need to be addressed before these compounds could be tested *in vivo*.

A third strategy relates to the use of analogues of the dinucleoside tetraphosphate product of the excision reaction. These compounds could serve either as inhibitors of the excision reaction and/or as inhibitors of the forward reaction (by providing simultaneous binding at the dNTP and the ATP binding sites) [for a review, see ref. 131]. Dinucleoside polyphosphates are present in the cytosol of all cells at concentrations ranging from 0.05 to 1 µM, but they are also secreted into extracellular spaces where they can affect vascular tone and blood circulation, platelet disaggregation, neurotransmission, activation of glycogen breakdown and of phospholipase, regulation of neutrophil function, etc... [for reviews, see refs. 132, 133]. Hydrolysis-resistant analogues of diadenosine 5',5'''-P^1,P^4-tetraphosphate (AppppA) have been tested as anti-thrombotic agents [134-136]. Recently, it has been reported that *in vitro* AZT resistance mutations, alone or in combination with T69SAG, confer increased susceptibility to inhibition by dinucleoside polyphosphates containing chain-terminating nucleoside analogues (*i.e*. ddN$p_4$ddN) [137].

EFFECTS ON DRUG RESISTANCE AND VIRAL FITNESS OF DELETIONS IN THE β3-β4 HAIRPIN LOOP OF HIV-1 RT

The first report on an HIV-1 RT deletion was published in 1998 and described a three-nucleotide deletion between residues 67 and 69, found in a clinical isolate [49]. This deletion was observed in a heavily-treated patient and did not disappear after HAART treatment with protease inhibitors administered in combination with AZT and 3TC or d4T and 3TC. These regimens selected for T215F, but at the first time point where the deletion was detected, the only observed mutations were T69G (assuming the deletion of codon 67), M184V, T215L and K219E [54].

Three-nucleotide deletions in the β3-β4 hairpin loop are less frequently observed than the dipeptide insertions referred in previous sections of this article [53, 54, 61, 138-141]. In a large study involving 2,152 RT sequences collected from HIV-infected patients treated wtih nucleoside RT inhibitors, authors found 4 deletion-containing sequences (estimated prevalence of 0.2 %) [53]. In all four cases, the deletion of codon 67 (Δ67) was associated with a T69G mutation and 0-3 TAMs, and in three out of four cases, the mutation M184V was also present. Phenotypic assays showed high-level resistance to 3TC when M184V was present, and to AZT in the clone harboring 3 TAMs (L210W, T215F and K219E) [53]. Other mutational patterns found in clinical isolates that confer phenotypic resistance to AZT contained the deletion Δ67 and T69G in combination with TAMs such as K70R, T215F and K219E [138] (Figure 5).

Another study monitoring the emergence of the Δ67 deletion has shown that the deletion appears within a background of TAMs (*i.e*. D67N, K70R, T215F), and arises in combination with T69G while mutation D67N is lost [146]. The deletion was found to be associated with a large increase in resistance to AZT, when T69G, K70R, T215F and K219Q were present [146]. Interestingly, T69G by itself did not affect nucleoside analogue sensitivity [147, 148]. However, within a sequence background containing TAMs, it conferred some resistance at the expense of a viral fitness loss. The development of the Δ67 deletion compensates for the loss of viral replication capacity, rendering a virus that replicates efficiently while showing high-level resistance to AZT [147]. In the absence of TAMs, the combination of the Δ67 and T69G confers some resistance to 3TC, d4T and abacavir, although impairing viral replication to some extent [149]. In this context, L74I may compensate for this fitness loss.

The recombinant HIV-1 RT containing the Δ67 complex of mutations (M41L/Δ67/T69G/K70R/L74I/K103N/T215Y/K219Q) showed significant nucleoside analogue excision activity on primers terminated with AZT, d4T and tenofovir [150]. Since this RT

contains 4 TAMs these findings are not surprising, and further studies will be necessary to assess the contribution of the deletion to the excision activity. However, an interesting observation of those studies is the increased excision activity on AZT-, d4T- and tenofovir-terminated primers, observed in reactions carried out in the presence of PPi, suggesting that PPi could play a role in resistance mediated by the Δ67 complex of mutations.

A three-nucleotide deletion in the β3-β4 hairpin loop accompanied by amino acid substitutions of the Q151M complex has been identified as a distinct mutational pattern found occasionally in clinical isolates [138, 142, 151] (Figure 5). Nucleotide sequence alignments suggest that for these viruses the deletion occurs at codons 69 or 70. These isolates show different degrees of resistance to nucleoside analogues, particularly when several mutations of the Q151M complex are present (*i.e*. V75I, F77L, F116Y, Q151M) [142]. However and quite surprisingly, some of them showed high-level resistance to nevirapine and other NNRTIs [138, 142, 151], a property that was observed even in those variants lacking drug resistance mutations specific for NNRTIs [138, 151]. The molecular mechanism underlying nevirapine resistance mediated by the Δ69/Δ70 complex of mutations has not been elucidated. Also, the evolutionary pathways leading to the selection of deletions at codons 69 or 70 are not known, and further studies will be necessary to identify the relevant sequence contexts favoring the emergence of the deletion.

CONCLUSION AND PERSPECTIVES

With the increasing complexity of the antiretroviral drug treatments and a larger population of HIV-infected patients under therapy, insertion- and deletion-containing RTs are likely to become more prevalent in the coming years. These enzymes are usually resistant to multiple nucleoside analogues. Current evidence suggests that a large portion of those isolates contain one or more TAMs, and become nucleoside-analogue resistant thorough the acquisition of an ATP-dependent phosphorolytic activity that acts predominantly on primers

terminated with thymidine analogues (*i.e*. AZT or d4T). The development of novel inhibitors blocking the excision activity would be very helpful to complement current treatments with RT inhibitors. Recent efforts on PPi-binding molecules, non-excisable nucleotide analogues and dinucleoside polyphosphates are amongst the compounds which will probably attain more attention in the future.

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ABBREVIATIONS

HIV = Human immunodeficiency virus

RT = Reverse transcriptase

HAART = Highly active antiretroviral therapy

NNRTI = Nonnucleoside RT inhibitor

AZT = Zidovudine, β-D-(+)-3´-azido-3´-deoxythymidine

ddI = Didanosine, β-D-(+)-2´,3´-dideoxyinosine

ddC = Zalcitabine, β-D-(+)-2´,3´-dideoxycytidine

d4T = Stavudine, β-D-(+)-2´,3´-dideoxy-2´,3´-didehydrothymidine

3TC = Lamivudine, ß-L-(–)-2´,3´-dideoxy-3´-thyacytidine

TAM = Thymidine analogue resistance mutation

PPi = Pyrophosphate

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transcriptase residue 69 conferring selective high-level resistance to nevirapine. AIDS Res Human Retrovir 2001; 17: 1293-6.

LEGENDS TO FIGURES

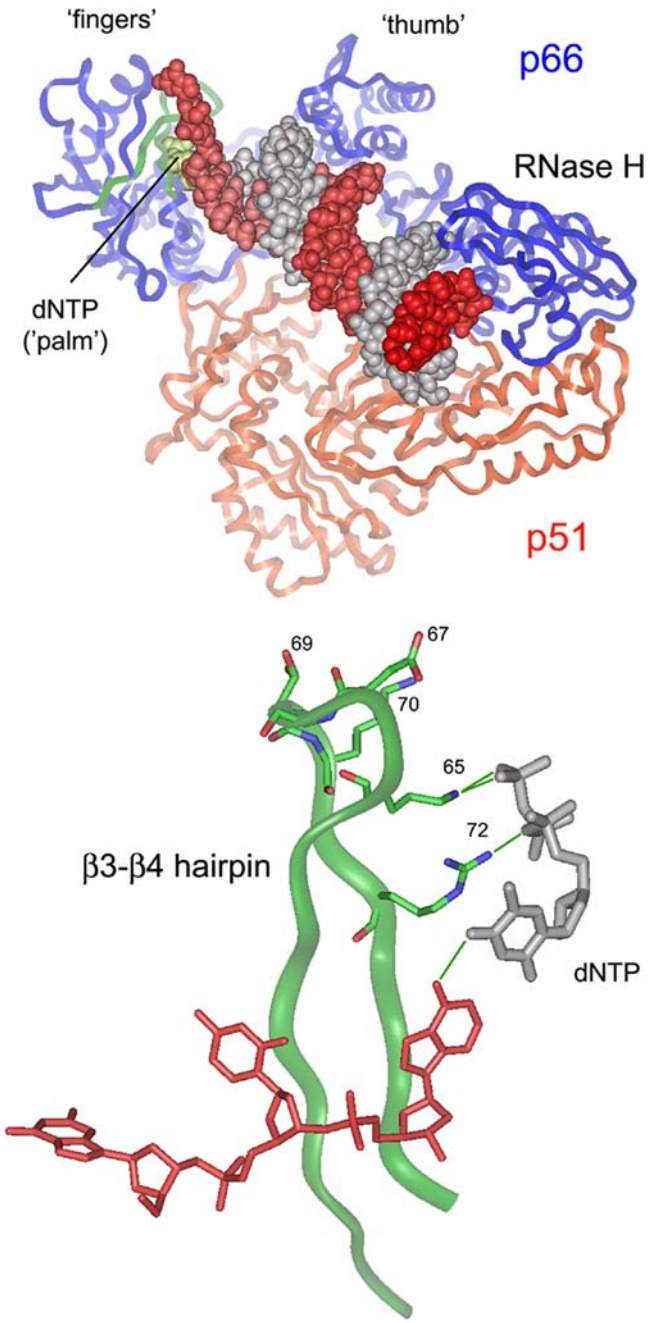
Fig. (1). Crystal structure of HIV-1 RT. The structure of the ternary complex of HIV-1 RT, a DNA-DNA template-primer and an incoming dNTP [17] is shown above. Ribbon diagrams are used to represent the structures of p66 (blue) and p51 (red), with the β3-β4 hairpin loop of p66 shown in green. A closer view of the β3-β4 hairpin loop with the location of relevant residues involved in interactions with the incoming dNTP, as well as the position of side-chains at the tip of the hairpin are given below. The template overhang is shown in red using a stick representation.

Fig. (2). Sequence alignments of residues 61 – 77 of HIV-1 RTs with insertions in the β3-β4 hairpin loop. Nucleotide sequence duplications are shown underlined. GenBank accession numbers for each sequence are indicated on the right. Clinical data and phenotypic resistance of the corresponding viral isolates are given in refs. 48 (AF096881 and AF096894), 53 (AF315240, AF315248, AF315255, AF315256, AF315262, and AF315268 – AF315271), 54 (AF311159, AF311162, AF311173, AF311177, and AF311179), 55 (AF304024), 56 (AF186771), 57 (AB053087) and 58 (AY877314).

Fig. (3). Drug resistance-related mutations within the polymerase domain of HIV-1 RT, associated with insertions of 1 or 2 amino acids between codons 69 and 70. Reported values represent the number of sequences where the mutation is found, relative to the total number of sequences containing information for the indicated residue. Amino acid substitutions shown are those that occur at codons displaying the highest variability (mutated in >15% of all RT sequences containing insertions at positions 69-70) [47-51, 53-56, 59, 60, 63, 64, 69-76].

Fig. (4). Schematic representation of the AZT-monophosphate excision reaction with several PPi donors. The highest catalytic efficiencies of the phosphorolytic reaction are obtained with PPi, although differences between wild-type and AZT-resistant strains are relatively small [82]. Other PPi donors (*i.e*. nucleoside-diphosphate and -triphosphates) that are able to excise AZT-monophosphate from blocked primers at millimolar concentrations are also shown [85]. $AMPCH_2PP$ and $AMPPCH_2P$ are ATP analogues with methylene groups instead of phosphodiester bonds in the α,β- and β,γ-linkages, respectively. The excision reaction can be inhibited by dNTPs complementary to template position +1, through the formation of a "dead end complex" [86].

Fig. (5). Amino acid sequences at positions 64 – 71 of HIV-1 RTs with one-amino-acid deletions, found in clinical isolates . The wild-type sequence is given above and conserved residues are encircled. The accompanying drug-resistance-related mutations in each case are given on the right. Sequences have been collected from GenBank and were obtained from references [40, 53, 142-145].


'fingers'
'thumb'
p66
RNase H
dNTP
('palm')
p51
69
67
70
65
72
β3-β4 hairpin
dNTP

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61 62 63 64 65 66 67 68 69 -  -  70 71 72 73 74 75 76 77
F  V  I  R  K  K  E  S  A  S  G  K  W  R  K  V  V  D  F        AF096894
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F  A  I  K  K  K  D  S  S  S  G  K  W  R  K  L  V  D  F        AF315240
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F  A  I  R  K  K  E  S  S  S  S  K  W  R  K  L  V  D  F        AF311177
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F  V  I  K  K  K  D  S  S  S  S  R  W  R  K  L  V  D  F        AF311162
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F  V  I  K  K  K  D  S  S  S  S  R  W  R  K  L  V  D  F        AF304024
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F  A  I  K  K  K  E  T  S  S  A  K  W  R  K  L  V  D  F        AF096881
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F  X  I  K  K  K  E  S  S  E  S  K  W  R  K  L  V  D  F        AF311173
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61 62 63 64 65 66 67 68 69 -  70 71 72 73 74 75 76 77
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Insertions	Number of sequences	Amino acid substitutions													References
		M41L	A62V	D67E	D67G/S/Q/K	S68G/T/N/L/Y	K70R	K70A/Q/T/W	M184I/V	L210W	L210F	T215Y	T215F	T215I	
T69SSG	49	20/48	18/48	14/49	11/49	2/49	5/49	3/49	18/48	20/48	0/48	45/48	1/48	0/48	[47-51, 53, 54, 60, 63, 64, 69-72]
T69SSS	42	29/40	16/40	10/42	4/42	0/42	8/42	0/42	28/40	24/40	1/40	38/40	2/40	0/40	[47-49, 51, 53-55, 59, 60, 63, 64, 70, 73-75]
T69SSA	28	23/28	11/28	3/28	1/28	2/28	0/28	0/28	6/28	14/28	0/28	25/28	1/28	0/28	[48, 49, 53, 54, 60, 64, 69]
T69SST	17	17/17	7/17	8/17	0/17	0/17	0/17	0/17	9/17	12/17	0/17	17/17	0/17	0/17	[53, 54, 64]
T69SVG	8	1/8	0/8	1/8	2/8	0/8	0/8	1/8	2/8	2/8	0/8	1/8	0/8	2/8	[53, 54, 56, 63, 72]
T69SVT	8	8/8	5/8	5/8	0/8	1/8	0/8	0/8	2/8	7/8	0/8	8/8	0/8	0/8	[50, 53, 54]
T69ASG	6	6/6	3/6	3/6	0/6	0/6	0/6	0/6	4/6	6/6	0/6	6/6	0/6	0/6	[48, 54, 64]
T69AVG	4	4/4	0/4	0/4	1/4	4/4	0/4	0/4	1/4	4/4	0/4	4/4	0/4	0/4	[53, 64]
T69ASA	3	3/3	1/3	0/3	0/3	0/3	0/3	0/3	1/3	2/3	0/3	3/3	0/3	0/3	[60, 64]
T69AVA	3	3/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	3/3	0/3	3/3	0/3	0/3	[64]
T69SES	3	0/3	2/3	2/3	0/3	0/3	1/3	0/3	0/3	2/3	0/3	3/3	0/3	0/3	[53, 54, 76]
T69SQS	3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	3/3	0/3	0/3	3/3	0/3	[64]
T69STT	3	3/3	0/3	1/3	1/3	1/3	1/3	0/3	1/3	2/3	0/3	2/3	1/3	0/3	[53, 60]
T69SCA	2	2/2	0/2	0/2	1/2	1/2	0/2	0/2	0/2	1/2	0/2	1/2	1/2	0/2	[53, 64]
T69SCT	2	2/2	0/2	0/2	2/2	0/2	0/2	0/2	2/2	1/2	0/2	2/2	0/2	0/2	[53]
T69SMT	2	0/2	0/2	2/2	0/2	0/2	0/2	0/2	0/2	2/2	0/2	2/2	0/2	0/2	[49, 53]
T69STG	2	0/2	0/2	0/2	1/2	0/2	1/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	[54, 72]
T69SVA	2	2/2	2/2	0/2	0/2	0/2	2/2	0/2	1/2	0/2	0/2	0/2	0/2	0/2	[53, 76]
T69SVS	2	0/2	2/2	0/2	0/2	0/2	2/2	0/2	2/2	0/2	0/2	0/2	2/2	0/2	[53, 76]
T69ASS	1	1/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	1/1	0/1	0/1	[64]
T69SIG	1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1	[53]
T69SSC	1	0/1	0/1	1/1	0/1	1/1	0/1	0/1	0/1	1/1	0/1	1/1	0/1	0/1	[53]
T69SSK	1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	1/1	0/1	1/1	0/1	0/1	[53]
T69STS	1	0/1	0/1	1/1	0/1	0/1	1/1	0/1	0/1	1/1	0/1	1/1	0/1	0/1	[48]
T69TTR	1	0/1	0/1	0/1	1/1	0/1	0/1	1/1	1/1	0/1	0/1	0/1	1/1	0/1	[49]
T69SD	7	0/7	1/7	0/7	7/7	6/7	6/7	0/7	2/7	0/7	0/7	0/7	5/7	0/7	[54, 64]
T69TT	6	0/6	0/6	0/6	4/6	0/6	6/6	0/6	4/6	0/6	0/6	4/6	0/6	0/6	[53, 54, 65]
T69TD	2	2/2	0/2	0/2	2/2	2/2	2/2	0/2	2/2	0/2	0/2	0/2	2/2	0/2	[51]
T69SG	1	0/1	0/1	1/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	1/1	0/1	[53]
T69ST	1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	[65]
Total	212	131/209	72/209	53/212	39/212	20/212	35/212	5/212	90/209	108/209	1/209	169/209	20/209	2/209	

Figure 3

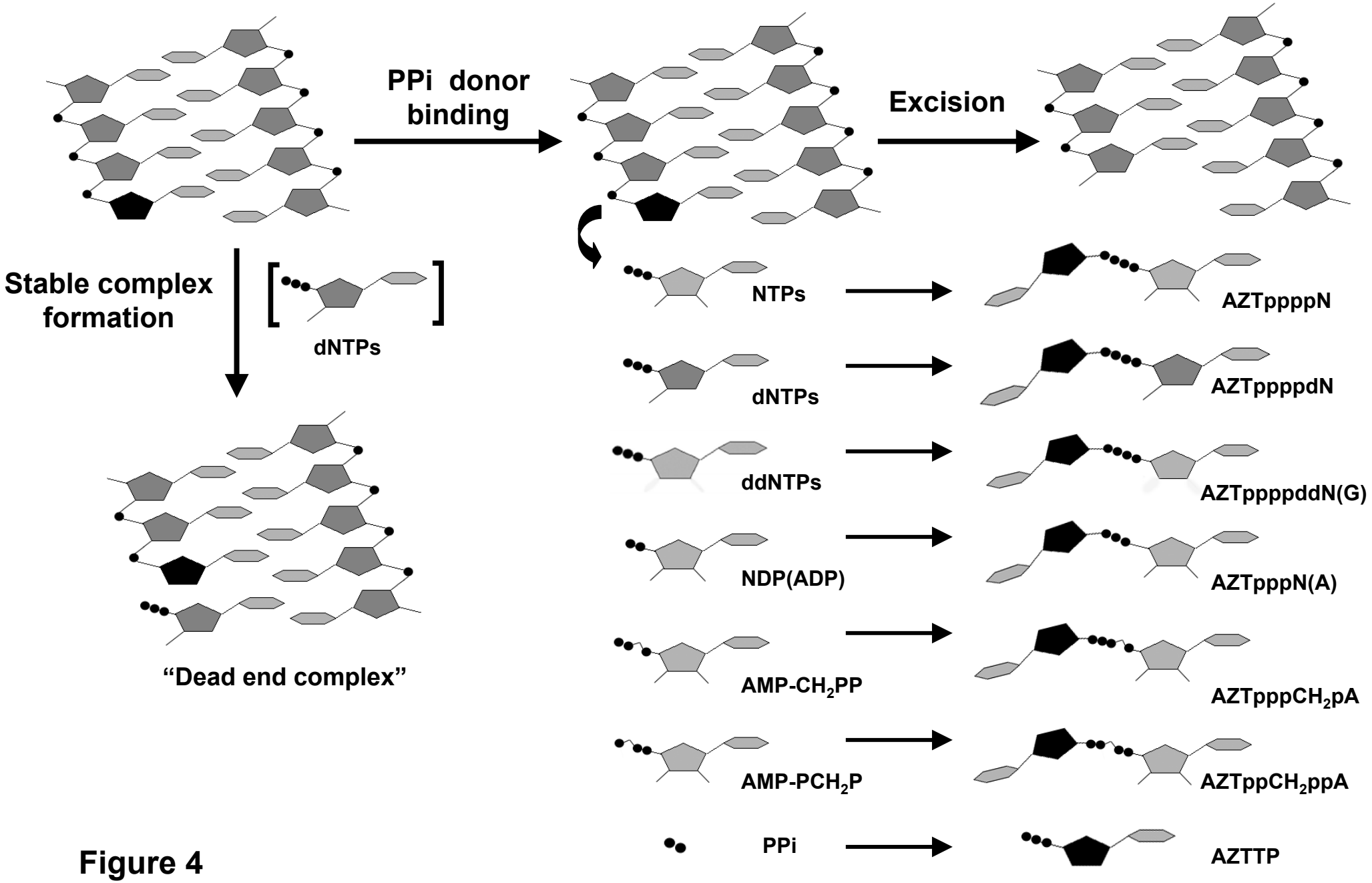
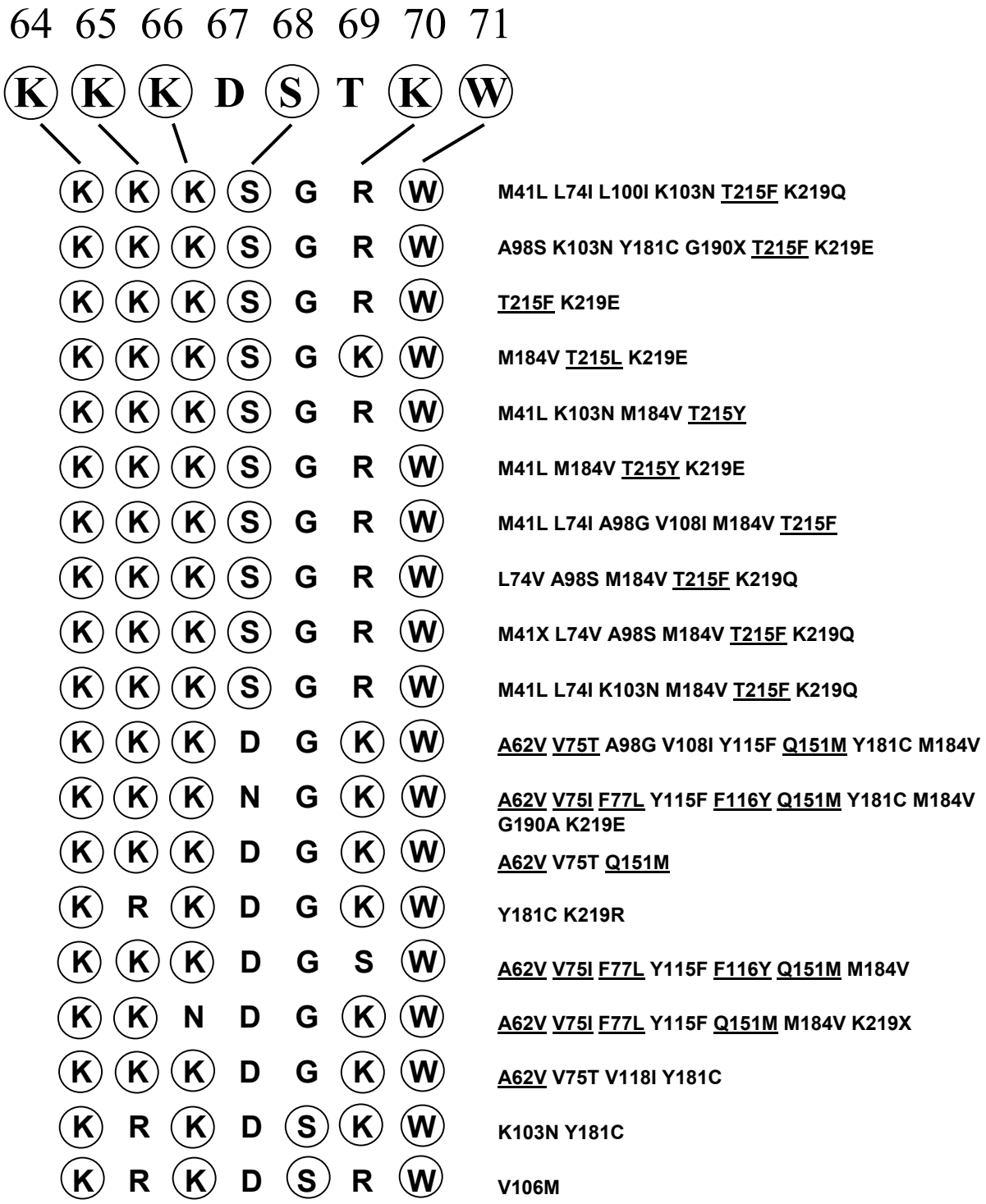

PPi donor binding
Excision
Stable complex formation
dNTPs
"Dead end complex"
NTPs
AZTppppN
dNTPs
AZTppppdN
ddNTPs
AZTppppddN(G)
NDP(ADP)
AZTpppN(A)
AMP-CH2PP
AZTpppCH2pA
AMP-PCH2P
AZTppCH2ppA
PPi
AZTTP

Figure 4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K K K S G R W L74V A98S M184V T215F K219Q
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K K K D G K W A62V V75T A98G V108I Y115F Q151M Y181C M184V
K K K N G K W A62V V75I F77L Y115F F116Y Q151M Y181C M184V G190A K219E
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K R K D G K W Y181C K219R
K K K D G S W A62V V75I F77L Y115F F116Y Q151M M184V
K K N D G K W A62V V75I F77L Y115F Q151M M184V K219X
K K K D G K W A62V V75T V118I Y181C
K R K D S K W K103N Y181C
K R K D S R W V106M

Figure 5