Nucleoside analogues play a key role in the fight against HIV-1. Unfortunately, under therapeutic pressure, HIV-1 inevitably develops resistance to these inhibitors. This resistance correlates with specific \textit{pol} gene mutations giving rise to specific substitutions in reverse transcriptase that are responsible for the loss of efficacy of the corresponding analogue. This work is an overview of the molecular mechanisms of HIV-1 drug resistance as judged by the analysis of chemical reactions at play at the reverse transcriptase active site. One class of mechanism involves nucleotide analogue discrimination either at the binding step or at the catalytic step, the latter being by far the most common mechanism. The other class of mechanism involves repair of the analogue-terminated DNA chain. The mechanisms were elucidated using purified reverse transcriptase and biochemical assays aimed at correlating resistant HIV-1 phenotypes to enzymatic data. The elucidation of these molecular mechanisms of drug-resistant reverse transcriptase is important for effective and rational combination therapies as well as for the conception of second-generation drugs that do not confer nucleotide resistance to reverse transcriptase or are active against pre-existing resistant viruses.

Boulbaba Selmi, Jérôme Deval, Joëlle Boretto and Bruno Canard*

Centre National de la Recherche Scientifique et Université d'Aix-Marseille I and II, UMR 6098, Architecture et Fonction des Macromolécules Biologiques, ESIL-Case 925, 163 avenue de Luminy, 13288 Marseille cedex 9, France

*Corresponding author: Tel: +33 491 828 644; Fax: +33 491 828 646; E-mail: bruno@afmb.cnrs-mrs.fr

**Antiviral Therapy**

Nucleoside analogue binding, catalysis and primer unblocking in the mechanisms of HIV-1 reverse transcriptase-mediated resistance to nucleoside analogues

Nucleoside analogues play a key role in the fight against HIV-1. Unfortunately, under therapeutic pressure, HIV-1 inevitably develops resistance to these inhibitors. This resistance correlates with specific \textit{pol} gene mutations giving rise to specific substitutions in reverse transcriptase that are responsible for the loss of efficacy of the corresponding analogue. This work is an overview of the molecular mechanisms of HIV-1 drug resistance as judged by the analysis of chemical reactions at play at the reverse transcriptase active site. One class of mechanism involves nucleotide analogue discrimination either at the binding step or at the catalytic step, the latter being by far the most common mechanism. The other class of mechanism involves repair of the analogue-terminated DNA chain. The mechanisms were elucidated using purified reverse transcriptase and biochemical assays aimed at correlating resistant HIV-1 phenotypes to enzymatic data. The elucidation of these molecular mechanisms of drug-resistant reverse transcriptase is important for effective and rational combination therapies as well as for the conception of second-generation drugs that do not confer nucleotide resistance to reverse transcriptase or are active against pre-existing resistant viruses.

Introduction

The replication of HIV in infected cells relies on reverse transcriptase (RT), an essential RNA- and DNA-dependent DNA polymerase encoded by the viral \textit{pol} gene. RT synthesizes a double-stranded DNA from the viral (+) RNA genome [1]. HIV RT differs from cellular DNA polymerases in two respects. First, HIV RT utilizes many chemically altered analogues of the natural deoxynucleoside triphosphates (dNTPs) readily. Second, HIV RT lacks a formal ‘proof-reading’ activity (that is, a mechanism to identify and excise inappropriately incorporated nucleotide). These characteristics are important from a pharmaceutical point-of-view and validate the use of nucleoside analogue inhibitors as antiretroviral agents [2]. RT is thus a major target for drugs acting as inhibitors of retroviral replication. Nucleoside reverse transcriptase inhibitors (NRTIs) (Figure 1) are currently used \textit{in vitro}, in clinical trials and in the clinic to inhibit retroviral replication [3,4]. Zidovudine (AZT), stavudine (d4T), zalcitabine (ddC), didanosine (ddI), lamivudine (3TC), abacavir and tenofovir (PMPA) are used in the fight against HIV. After conversion to the active triphosphate, analogues must compete with natural nucleotides (dNTPs) both for recognition by the RT as a substrate (binding) and incorporation into the nascent viral DNA chain (catalysis).

During antiretroviral therapies, the emergence of drug-resistant viruses limits the efficiency of nucleoside drugs. This limitation represents a major cause of failure to control HIV infection. Most drug-resistant viruses isolated from patients treated with nucleoside analogues harbour mutations in the \textit{pol} gene [4]. Remarkably, the set of resistance mutations selected during antiretroviral therapy is specific to the nucleoside drug used (Table 1). When 3TC or dideoxynucleosides are given alone to patients, resistant viruses emerge, and their RTs harbour mainly single amino acid substitutions such as M184V and K65R, or L74V, respectively [3–7]. When zidovudine is given as the sole drug, several patterns of amino acid changes involving M41L/D67N/K70R/L210W/T215F or Y/K219Q give rise to up to a 200-fold AZT resistance [2,3,8].

Much efforts are directed towards the characterization of drug resistance mechanisms. A nucleotide analogue is characterized by its efficiency of incorporation $[k_{pol}/K_{d(dNTP)}]$ into DNA as compared with that of its natural counterpart. $k_{pol}$ is the rate constant for the creation of the phosphodiester bond and $K_{d(dNTP)}$ the equilibrium (or dissociation, or binding) constant of the nucleotide for RT [9]. These constants are generally determined using pre-steady state kinetics [9].
Discrimination of the analogue relative to its natural counterpart dNTP is reflected by the efficiency of incorporation of the analogue into DNA relative to that of the natural nucleotide substrate dNTP. The polymerization reaction (Figure 2) shows that this efficiency is dependent on the binding constant $K_d$(dNTP) of the analogue to RT and the rate constant $k_{pol}$ of the phosphodiester bond formation. Consequently, nucleoside analogue discrimination due to an amino acid substitution in RT can be explained.
by a $K_d$ (dNTP) value increase or a $k_{pol}$ value decrease, or both. Recently, several of these mechanisms have been elucidated at the molecular level. In all cases, the variant (or 'drug-resistant') RT has acquired or enhanced biochemical properties regarding either discrimination of the nucleotide analogue, or repair (named also excision, unblocking) by pyrophosphate (PPi) or adenosine triphosphate (ATP) of the analogue-terminated DNA-chain [3,6,7,10]. This work is an overview of the main molecular mechanisms of RT-mediated resistance due to either nucleotide analogue discrimination, repair of the analogue-terminated DNA chain or the combination of the two previous mechanisms.

**Discrimination: resistance due to impaired NRTI binding or incorporation, or both**

A large number of substitutions in RT have been identified in the course of NRTI resistance studies. A single point mutation is often sufficient to confer resistance to an individual NRTI. Point mutations such as M184V/I, K65R or Q151M lead to alterations in either the affinity of RT for specific NRTIs, as judged by $K_d$ (dNTP), with no significant change in the affinity for the corresponding natural dNTP substrate, or in the catalytic constant $k_{pol}$ of incorporation of the analogue into DNA. These substitutions are located in, or close to, the dNTP substrate-binding site [11]. The mutant RT may, therefore, discriminate between NRTIs and analogous dNTP substrate by affecting (directly or indirectly) the binding constant or the subsequent positioning of the NRTI for catalysis. It is important to note that wild-type RT is generally less efficient in catalyzing the incorporation of the bound ddNTP into the nascent DNA than that of the corresponding dNTP substrate [7,12,13]. Two examples will be used to illustrate how drug-resistant RT is able to carry out this discrimination.

**Lamivudine triphosphate discrimination by M184V RT in vitro:** a $K_d$ (dNTP) increase

Initially, resistance to 3TC is associated with the substitution of isoleucine for methionine at position 184 of HIV-1 RT, which results from a single base change (ATG to ATA). During 3TC therapy, this M184I variant is rapidly replaced by the variant M184V [14–16]. M184V is located in the YMDD motif of RT. This motif is located at the polymerase active site [17] and is highly conserved amongst retroviral RTs. In HIV-1 RT, this motif contains two of the three catalytic aspartates of the DNA polymerase active site [11,18]. Resistance to this analogue develops rapidly in the clinic. Both in vivo and in vitro studies showed that the 3TC-resistant virus (M184V) has a 500- to 1000-fold reduced drug susceptibility [19,20]. Wild-type RT does not incorporate lamivudine monophosphate into DNA efficiently, but the presence of M184V further increases this discrimination [21]. The decrease in the incorporation efficiency observed by M184V RT is primarily due to a 77- and 22-fold increased binding constant $[K_d$ (dNTP)] for the lamivudine triphosphate to the enzyme/DNA (or RNA) complex poised for DNA elongation, respectively [21].

In the absence of a crystal structure of 3TC-resistant RTs in complex with a DNA template/primer and lamivudine triphosphate, the precise details of the mechanism by which the M184V/I mutations confer resistance to 3TC is still not completely clear. Based on the structure of wild-type RT/DNA (template/primer) in complex with deoxythymidine triphosphate (RT/DNA/dTTP) [11], it was demonstrated that the side chain of M184 contacts both the deoxynucleoside triphosphate and the 3’ end of the primer. Crystal
structures of the 3TC-resistant mutant RT (M184I) in both the presence and absence of a DNA/DNA template/primer were determined [22]. Molecular modelling and crystallographic experiments [11,22] show that substitution of M184 with an amino acid having a β-branched side chain (isoleucine or valine) results in additional contact with lamivudine triphosphate. The side chain of the β-branched amino acid at position 184 makes a contact with the oxathiolane ring of lamivudine triphosphate, the latter having a pseudo-ribose of L conformation. This contact disrupts the proper alignment of the inhibitor and prevents proper positioning of lamivudine triphosphate in the RT active site for catalysis, consistent with the previously published data [23]. With natural dNTP substrates having a ribose under a D configuration, no steric hindrance is noted. However, other authors have found that relative to wild-type RT, M184V RT exhibits no difference in its ability to bind lamivudine triphosphate [13] and deoxynucleoside triphosphate [13,24]. The already very slow rate of incorporation of lamivudine monophosphate seen with wild-type RT was further reduced (by a factor of 23 and 36 with DNA/DNA primer/template and DNA/RNA primer/template, respectively) for M184V RT. It is, therefore, possible that lamivudine triphosphate discrimination at the RT active site proceeds by a mixed mechanism involving a poor binding of the inhibitor that translates into a decrease in its incorporation rate.

Discrimination of dideoxynucleoside triphosphate by a decreased \( k_{\text{pol}} \)

When 2',3'-dideoxynucleosides (ddNs) are given to patients, dideoxynucleoside resistance emerges and is mainly correlated with single amino acid substitutions such as L74V or K65R [3–5,25]. Q151M is also such a substitution, but additional substitutions add up to yield highly resistant viruses. In vitro, the levels of

---

Figure 2. DNA synthesis by HIV-1 reverse transcriptase

RT, reverse transcriptase; T/Pn, DNA primer annealed to a template (RNA or DNA); dNTP, nucleotide or analogue triphosphate; PPi, pyrophosphate. The complex RT-T/Pn represents RT in complex with the template/primer. N site, nucleotide binding site; P site, priming site. After each deoxynucleoside monophosphate insertion, one molecule of PPi is produced. Under physiological conditions, the pyrophosphorolysis (reverse of polymerization) does not compete with the polymerization reaction due to the importance of the affinity of RT for the deoxynucleoside triphosphate (\( K_{d(\text{dNTP})} \) relative to PPi (\( K_{\text{ppi}} \))).
Resistance provided by L74V and K65R are moderate (<12-fold), whereas Q151M and its associated other mutations provide much higher resistance levels (10- to >100-fold) [3,4]. The K65R mutation is found infrequently on viral isolates from patients treated with ddl and ddC [26,27]. However, its clinical relevance is increasing, since K65R has been identified in a significant number of viral isolates resistant to three recent antiretroviral drugs (R)-9-(2-phosphonylmethoxypropyl) adenine (PMPA), β-D-dioxolane-guanosine (DXG) and abacavir (1592U89) [4,28,29]. K65R is thus a multidrug resistance substitution and understanding the mechanism by which K65R confers drug resistance might help in designing new drugs either eliciting no resistance or active against drug-resistant viruses.

K65R RT has been extensively studied in vivo and in vitro [25,30–32], but a detailed molecular mechanism incriminating Kp(dNTP), kpol or repair of the dideoxynucleoside monophosphate-terminated primer was still lacking until recently. From the crystal structure, it was long-known that the lysine 65 side-chain contacts an oxygen atom in the γ-phosphate of the incoming nucleotide at the RT active site. Mutational analysis of lysine 65 and molecular modelling of this substitution in the RT active site have suggested that K65R alters the binding affinity of the dideoxynucleoside triphosphate substrates [33]. However, since dideoxynucleoside triphosphate and dideoxynucleoside triphosphate are identical at their γ-phosphate position, it was unclear how selectivity for 3'-OH was achieved. The molecular mechanism of dideoxynucleoside resistance conferred by the K65R RT has been elucidated [7]. It was shown that K65R RT exhibits resistance to both dideoxyadenosine triphosphate and zalcitabine triphosphate using a kpol-based discrimination mechanism. Based on the examination of the crystal structure of the RT-DNA-dNTP complex, it was interpreted that the presence of an intramolecular hydrogen bond between the 3'-OH and one oxygen atom of the β-phosphate of the incoming nucleotide is critical for an efficient catalytic step. This intramolecular interaction represents the missing link to explain how dideoxynucleoside triphosphates are discriminated up to 14-fold better by K65R RT than by wild-type RT [7]. A satisfactory view of the mechanism can be elaborated when one represents this intramolecular bond as ‘dominant’ relative to the amino acid present at position 65. In the absence of such an intramolecular bond, as it is the case for ddNTPs, catalysis is selectively impaired irrespective to which amino acid is present at position 65 (dominant effect). When K65R is present, the presence of the intramolecular bond stabilizes the position of the α-phosphate and has little effect on catalysis. When both the intramolecular bond is absent (ddNTP at the active site) and K65R is present, K65R further displaces the α-phosphate from a correct alignment required for efficient catalysis. This mechanism is in fact relevant to other drug resistance mutations for which no molecular mechanisms had been demonstrated until recently, such as multidrug resistance substitutions involving Q151M.

The Q151M substitution is identified in patients who develop multiple dideoxynucleoside resistance (MDNR) mutations. It is generally found with four other substitutions (A62V, V75I, F77L and F116Y) [34]. By itself, Q151M confers low-level resistance to AZT, ddl, ddC and d4T [34–36]. In contrast, the A64V, V75I, F77L and F116Y mutations do not affect drug susceptibility by themselves, but their co-occurrence with Q151M results in high-level (up to >100-fold) resistance to AZT, ddl, ddC, d4T and low cross resistance to 3TC [35,37,38] due to an altered recognition of these inhibitors at the RT active site.

The mechanisms have been elucidated at the molecular level and a selective drop in kpol could be evidenced [39]. Again, the presence of M151 disrupts a critical hydrogen bond network involving the nucleotide ribose and the leaving pyrophosphate group (Figure 3). This disruption promotes a decreased catalytic rate-constant specifically to dideoxynucleoside triphosphates, because the latter become unable to align stably and activate their reactive centres [39].

Zidovudine resistance: repair of the zidovudine monophosphate-terminated primer by RT carrying the zidovudine resistance substitutions

Resistance to AZT is acquired through the selection of specific substitutions such as M41L/D67N/K70R/L210W/T215F or Y/K219Q in the RT molecule (Table 1) [8,40]. The extent of zidovudine resistance is related to the combination of mutations present. Generally, two or more of these substitutions including T215F/Y are needed in RT to acquire high-level AZT resistance [41]. The elucidation of the biochemical phenotype for HIV-1 resistance to AZT was complicated by the absence of any measurable effect on inhibition of DNA synthesis by zidovudine triphosphate in vitro, despite a more than 200-fold resistance to zidovudine demonstrated in HIV-infected cell culture [13,41,42]. The examination of the crystal structure of AZT-resistant RT [11,43] showed that residues 215 and 219 are ~10 Å from the conserved active site aspartate residues, whereas residues 67 and 70 are in different domain ~20 Å away. Structurally, the effect of 215 and 219 mutations is quite different from that of changes at residues 67 and 70. They cause
A concerted movement of the structure, extending to the polymerase active site \([43]\). It was shown that AZT-resistant RT binds more specifically to zidovudine monophosphate-terminated DNA chain than wild-type, suggesting that AZT resistance might be an RT-mediated repair mechanism \([44]\). Subsequently, it was shown that one AZT resistance mechanism involves repair of the zidovudine monophosphate-terminated primer by either pyrophosphate \([45]\) or adenosine triphosphate \([46]\) to catalyze the formation of zidovudine triphosphate or dinucleoside tetraphosphate on the terminal phosphodiester bond of the zidovudine monophosphate-terminated DNA primer (Figure 4A). It was shown that binding of the next complementary deoxynucleoside triphosphate to HIV-1 RT and the nucleoside analogue monophosphate-terminated primer results in formation of dead-end complex (DEC) that is stable enough to inhibit the dinucleoside polyphosphate (AppppddN) synthesis (Figure 4B) \([46]\). The next correct nucleotide binds to the nucleotide binding site (named N site) while the chain-terminating analogue has translocated to the P site \([47]\). The DEC is less readily made with AZT-resistant RT than with wild-type RT. Thus, AZT-resistant RT has a greater ability to repair the zidovudine monophosphate-terminated primer than wild-type RT, accounting for AZT resistance.

The chemistry involved in pyrophosphorolysis and the ribonucleotide-dependent phosphorolysis reactions is similar. In both cases, the removal of the chain-terminating zidovudine monophosphate results from the nucleophilic attack of either pyrophosphate or the \(\gamma\)-phosphate of adenosine triphosphate on the terminal phosphodiester bond of the zidovudine monophosphate-terminated DNA primer (Figure 4A). It is clear that both wild-type and AZT-resistant RTs can carry out an excision reaction \textit{in vitro} using either pyrophosphate or nucleoside triphosphate as pyrophosphate donors. Therefore, an important question remains: what is the pyrophosphate donor \textit{in vivo}? Although pyrophosphate-mediated repair is much more efficient than nucleoside triphosphate-mediated repair, AZT resistance substitutions significantly increase repair of the zidovudine monophosphate-terminated DNA by nucleoside triphosphate but not by pyrophosphate \([46]\). At the virus level, the selective advantage of the ribonucleotide-dependent phosphorolysis mechanism over pyrophosphate-mediated repair is that the phosphorolytic removal of zidovudine monophosphate results in the formation of dinucleotide tetraphosphate (AppppAZT), which, unlike the zidovudine triphosphate product of pyrophosphorolysis, cannot be re-incorporated into the viral DNA. Recent data suggests that AZT resistance substitutions enhance the binding of adenosine triphosphate to the zidovudine monophosphate-terminated primer/template/RT complex \([47–49]\).

In patients subjected to prolonged AZT therapy with or followed by administration of other nucleoside analogues, an insertion of two amino acids (often ser-ser, ser-gly or ser-ala) between residues 69 and 70 of HIV-1 RT has been recently described \([50–52]\). The insertion appears to be associated with multiple amino acid substitutions, including AZT-resistant substitutions such as T215Y. The comparison of the catalytic properties and the inhibitor susceptibility of wild-type RT and the AZT-resistant RT carrying a dipeptide (ser-ser) insertion between codon 69 and 70 (SS RT) have demonstrated that in presence of adenosine triphosphate, the SS RT has an increased ability to remove the 3’-terminal nucleotide from zidovudine monophosphate-terminated primers \([49,53]\). Recombinant virus carrying the insertion-containing
RT (SS RT) showed reduced susceptibility to all nucleoside RT inhibitors in clinical use, particularly to AZT [50]. This study supports well the work previously published [46], showing the importance of ribonucleotide-dependent phosphorolysis as a general AZT resistance mechanism.

The most physiologically relevant pyrophosphate donor for removal of chain terminators in vivo is currently unresolved. The physiological concentrations of adenosine triphosphate are within the range of the $K_m$ for adenosine triphosphate (0.7–4.3 mM), suggesting that adenosine triphosphate is a likely pyrophosphate donor for the chain terminator removal reaction in vivo [48,49,54]. It was shown that the specificity of the excision reaction for a zidovudine monophosphate-terminated primer is not directly due to substitutions that confer resistance, but depends instead on the overall structure of the region around the HIV-1 RT polymerase active site and on its interactions with the azido group of AZT [47]. Thus, the steric constraints involving the azido group of AZT cause the end of a zidovudine

**Figure 4. DNA synthesis by HIV-1 reverse transcriptase inhibition and its inhibition by nucleoside analogue**

**A**. Chain termination by a nucleotide inhibitor. The analogue-terminated primer is repaired by pyrophosphorolysis (PPi or ATP) to yield ddNTP or dNppppA, respectively, and the DNA synthesis resumes. The chemistry involved in the repair reaction by pyrophosphorolysis (PPi) and the ribonucleotide-dependent phosphorolysis (ATP) reactions is similar. B. The drawing shows the stable dead-end complex that forms when HIV RT has incorporated a nucleoside 5'-monophosphate analogue into DNA, translocated to the priming site (P) and bound the incoming dNTP.
monophosphate-terminated primer to preferentially reside at the nucleotide binding site, a position that favours the repair reaction.

**A mixed molecular mechanism for stavudine resistance: stavudine triphosphate discrimination and repair of the stavudine monophosphate-terminated primer**

In the clinic, d4T occupies a very special place amongst nucleoside analogue drugs. Indeed, it has the seemingly unique property of eliciting very moderate and ill-defined resistance. A single substitution, though, seems to be specifically associated to the use of d4T. When d4T is given as the sole drug in cultured cells infected by HIV-1, RT bearing a valine to threonine substitution at position 75 can be selected [55]. This substitution confers a two- to threefold increase in IC50 for d4T to HIV-1 and this level of resistance is generally considered close to the limit of significance. Moderate (≤10-fold) resistance to d4T is observed in the clinic as well as in cultured cells infected by HIV-1 [55,56]. The most important d4T resistance mutations are in fact AZT resistance mutations [56]. In the clinic, the V75T substitution is observed at low frequency (up to 10%) following d4T therapy, the highest frequency being on AZT-naive patients [57]. In the case of V75T, Lennérstrand et al. found that adenosine triphosphate had no effect on the repair of stavudine monophosphate-terminated primer [58]. It was concluded that stavudine triphosphate might be discriminated against deoxythymidine triphosphate, or in other words that the V75T substitution might be involved in a decreased binding of RT to stavudine triphosphate, independently to the repair reaction mediated by adenosine triphosphate. A number of questions have been addressed in order to use the full therapeutic potential of d4T. Why does d4T elicit only a moderate (≤10-fold) resistance as compared with AZT (>100-fold)? What is the molecular mechanism by which V75T confers d4T resistance?

To answer to the first question, it has been proposed that the planar sugar ring of d4T mimicks a transition state intermediate that may be responsible for the remarkable incorporation properties by RT of stavudine monophosphate into DNA [59]. Indeed, stavudine 5'-triphosphate is not discriminated against at the RT active site [60]. The second question has been addressed and the molecular mechanism of d4T resistance was elucidated recently [6]. Structural analysis of HIV-1 RT showed that the valine 75 is located at the basis of the fingers subdomain between the template contact point and the nucleotide binding pocket. V75T RT discriminates 3.6-fold stavudine triphosphate relative to deoxythymidine triphosphate, as judged by pre-steady state kinetics of incorporation of a single nucleotide into DNA. V75T also increases the RT-mediated repair of the stavudine monophosphate-terminated DNA by pyrophosphate but not by adenosine triphosphate. V75T recombinant virus was three- to fourfold d4T-resistant and three-fold resistant to phosphonofomeric acid (PFA) relative to wild-type, confirming that the pyrophosphate traffic is affected in V75T RT. Thus, it was proposed that V75T defines a type of amino acid change conferring resistance to nucleoside analogues by a mixed mechanism involving both nucleotide selectivity and pyrophosphate-mediated repair [6].

**Is the mechanism of resistance predictable?**

Several questions relevant to future drug design can be asked. For a given analogue, can we predict what type of mechanism is going to be selected? and why? It is fairly understandable that resistance to dideoxynucleoside triphosphate might not be achieved at the RT active site by an increase of Kd(dNTP). Indeed, it is hard to envisage how an amino acid substitution in resistant RT would sensor the absence of the 3'-OH and promote a decreased affinity. Therefore, it is logical that dideoxynucleoside triphosphate resistance mutations involve a decreased Kd with no change in Kd(dNTP). This decrease in Kd is achieved through the perturbation of the alignment of the catalytic centres provided by the presence of an intramolecular hydrogen bond between the 3'-OH and the leaving pyrophosphate (Figure 3) [7,39]. It is thus safe to propose that any nucleoside drug having a ribose moiety (or equivalent) smaller that the natural ribose, but no such intramolecular bond, will elicit resistance by the same mechanism. Consistent with this hypothesis, the main tenofovir resistance mutation is K65R [28]. It will be interesting to determine by which mechanism dioxolane guanosine (DXG) will elicit resistance, and if our hypothesis is valid, the dioxolane guanosine triphosphate will be discriminated at the RT active site by a selective drop in Kd. Conversely, the oxathiolane ring of 3TC is larger than the natural ribose and the M184V substitution involves a decreased binding affinity as judged by increased Kd(dNTP). In the same vein, it is also logical that decreased binding affinities lead to decreased catalytic rate constant. The other way around is unlikely: there is no example yet of nucleotides exhibiting fast incorporation rate while being loosely bound.

Isel et al. have proposed that resistance could be predicted based on the biochemical properties pre-existing on RT [61]. Wild-type RT is able to repair the zidovudine monophosphate-terminated primer and
AZT resistance mutations increase this propensity. The case of Q151M substitutions promoting both dideoxynucleoside and AZT resistance is instructive. It indicates that zidovudine triphosphate can still be discriminated at the RT active site and that increasing discrimination of both zidovudine triphosphate and dideoxynucleoside triphosphate is selected in an easier manner than selecting substitutions promoting repair of the dideoxynucleoside monophosphate-terminated primer. The case of V75T is illustrating nicely this discussion because it lies exactly at the crossroads of our propositions. In one hand, the 3′,3′-double bond. As a consequence of both this acidity and the planar sugar ring, this proton has the possibility to hydrogen bond weakly to one oxygen of the leaving pyrophosphate, as it was demonstrated structurally [62]. Logically, d4T is discriminated by V75T RT. On the other hand, pyrophosphate-mediated repair of a stavudine monophosphate-terminated primer is occurring quite efficiently with wild-type RT, and V75T increases this repair activity. Pyrophosphate-mediated repair cannot be enhanced indefinitely in drug-resistant RT because it is the reversal of the polymerization reaction. V75T viruses may represent the highest possible enhancement of the pyrophosphorolysis activity without impairing viral fitness [6].

How to counteract or overcome nucleoside analogue resistance?

Having these mechanisms characterized, new generation analogues should either: (1) Have an decreased $K_{p,dd}$ at the RT active site. To do so, one should logically increase the area/surface of the analogue to promote a tighter binding to RT. In so doing, a resistance amino acid substitution might unfortunately appear to counteract this increase in affinity; (2) Have bona fide incorporation properties, as in, have an increased incorporation rate. Such analogues, α-boranophosphate analogues, have been described [7,39,62]. Indeed, they exhibit astonishing suppression properties whenever $k_{p,dd}$ is at the origin of drug resistance [7,39]. The presence of the borane group restores a catalytic rate constant close to that of a natural deoxynucleoside triphosphate substrate, overcoming up to 150-fold resistance; (3) Have chemical attributes that decrease or suppress the unblocking reaction. The boranophosphate nucleoside shows a modest effect (two- to ninefold effect) in that respect [62]. The inability of RT to excise tenofovir-terminated primer might be the first relevant example of this interesting property [63].

Conclusion

Although there are now combination therapies for HIV-1 that are reasonably effective, the emergence of drug-resistant virus remains a serious problem. The different molecular mechanisms show that resistance to nucleoside analogues could be related to the altered biochemical properties of RT. The knowledge of these molecular mechanisms of RT resistance is crucial for the design of new anti-HIV chemotherapeutic agents that may prevent or reverse resistance to nucleoside analogues, or both. They are also useful for therapy combinations by directing the use of analogues with complementary properties. For example, both the M184V and K65R substitutions suppress the repair activity of AZT-resistant RT (D67N/K70R/T215F/K219Q). Thus, we can understand why combinations AZT+3TC and AZT+dideoxynucleoside (dideoxyadenosine or ddC) are effective: the RT containing the D67N/K70R/T215F/K219Q+M184V and D67N/K70R/T215F/K219Q+K65R substitutions are sensitive to AZT but resistant to 3TC and dideoxynucleosides, respectively [3,14,29]. In the same vein, it has been reported that substitutions in RT associated with HIV-1 resistance to PFA resensitize AZT-resistant virus to AZT [64–67]. Since phosphonoformic acid is a pyrophosphate analogue, it was suspected that mutations conferring PFA resistance might affect the enhanced pyrophosphorolytic activity of AZT-resistant RT. Indeed, the introduction of the A114S substitution into a background of AZT resistance (D67N/K70R/T215F/K219Q) eliminates the increased phosphorolytic removal of chain-terminating zidovudine monophosphate. Both pyrophosphorolysis and nucleotide-dependent phosphorolysis are impaired by A114S [68]. A114S allows zidovudine triphosphate to act again as a stable chain terminator of viral DNA synthesis, thereby restoring antiviral activity of the drug against AZT-resistant viruses [68]. A class of non-nucleoside RT inhibitors (NNRTIs), such as nevirapine, inhibits HIV-1 by a different mechanism than chain termination [69,70]. In contrast to AZT resistance, high-level resistance to nevirapine can be conferred by a single mutation at codon 181 (Y to C) [71]. While conferring resistance to NNRTIs, however, the Y181C substitution in a AZT resistance background (D67N/K70R/T215F/K219Q) significantly suppresses AZT resistance [71,72], but the precise mechanism is not yet known. The detailed understanding of such molecular mechanisms certainly guide the design of second-generation drugs that do not confer resistance and are active against pre-existing HIV-1-resistant viruses.
Acknowledgements

We thank Luis Menéndez-Arias and Barbara Selisko for critical reading of the manuscript.

References


29. Bazmi HZ, Hammond JL, Cavaletti SC, Chu CK, Schinazi RF & Mellors JW. In vitro selection of mutations in the human immunodeficiency virus type 1 reverse transcriptase

©2000 International Medical Press
that decrease susceptibility to (3')-β-D-ribofuranosyl-α-D-3-deoxythymidine. Antimicrobial Agents & Chemotherapy 2000; 44:1783–1788.


41. Lacey SF, Reardon JE, Furicine ES, Kunkel TA, Bebenek K, Eckert KA, Kemp SD & Larder BA. Biochemical studies on the reverse transcriptase and RNase H activities from human immunodeficiency virus type 1 reverse transcriptase strains resistant to 3'-azido-3'-deoxythymidine. Journal of Biological Chemistry 1992; 267:15789–15794.


63. Naeger LK, Margot NA & Miller MD. Tenofovir (PMPA) is less susceptible to pyrophosphorolysis and nucleotide-dependent chain-terminator removal than zidovudine or stavudine. Nucleosides Nucleotides Nucleic Acids 2001; 20b:35–639.


