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# Review

# Nucleoside and nucleotide inhibitors of HIV-1 replication

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**Abstract.** HIV-1 reverse transcriptase (RT) is one of the main targets for antiviral therapy. Two classes of RT inhibitors can be distinguished: those that are nucleoside or nucleotide analogues (sharing the common NRTIs abbreviation) and those that are not. This review focuses on the NRTIs, which are highly efficient in slowing down viral replication and are used in combination regimens. Unfortunately, the current inhibitors do not completely suppress viral replication and due to the high capacity of adaptation of HIV, allow the selection of drug-resistant viruses. Resistance mechanisms to NRTIs have been extensively investigated and can be divided into two types: improved discrimination of a nucleotide analogue relative to the natural substrate or increased phosphorolytic cleavage of an analogue-blocked primer. This knowledge is important both for the development of new drugs designed to target resistant strains and for the development of new antiviral strategies. The NRTIs currently in clinical trials and new developments in this area are also reviewed.

**Key words.** NRTI; resistance; reverse transcriptase; HIV-1; AIDS; drug; chain termination; DNA synthesis.

## Introduction: HIV-1 reverse transcriptase as a drug target

In 1983, human immunodeficiency virus type 1 (HIV-1) was identified as the causative agent of the acquired immune deficiency syndrome (AIDS) [1, 2]. Since then, the reverse transcriptase (RT) encoded by this retrovirus has become one of the major targets for the development of antiviral drugs. RT is an asymmetric heterodimer (p66/p51) harbouring an RNA- and DNA-dependent DNA polymerase activity that catalyses the reverse transcription of the viral genomic RNA into a double-stranded proviral DNA, as well as an RNase H activity [for review see ref. [3]. Both catalytic activities are carried by p66, while p51 plays a structural role [4–6].

The p51/p66 heterodimer results from the maturation of a p66/p66 homodimer. The p66 subunit folds into five separate subdomains: the RNase H domain, which is absent in p51 and corresponds to the C-terminal part of p66, and four subdomains forming the polymerase domain [4–6]. As with other polymerases, the resemblance of this latter domain to a right hand resulted in the naming of the subdomains as 'fingers', 'palm' and 'thumb' [6]. The fourth polymerase subdomain, called 'connection', joins the polymerase and the RNase H domains [6].

Even though the four polymerase subdomains of p66 and p51 share a very similar tertiary structure, their relative positionings are different, so that the polymerase domain of p51 and p66 are quite different. Unlike p66, p51 has no cleft, and the residues involved in the polymerase activity are buried [4–6].

Antiviral treatments (http://www.niaid.nih.gov/daids/dtpdb/fdadrug.htm) combine RT inhibitors and protease inhibitors (PIs) and have recently been supplemented with an inhibitor of viral and cellular membrane fusion, T20 [7, 8]. Two classes of RT inhibitors can be distinguished: those that are nucleosides analogues (NRTIs) (fig. 1) and those that are not (NNRTIs). NRTIs are competitive inhibitors with respect to the natural deoxynucleotide triphosphates (dNTPs), whereas NNRTIs are allosteric non-

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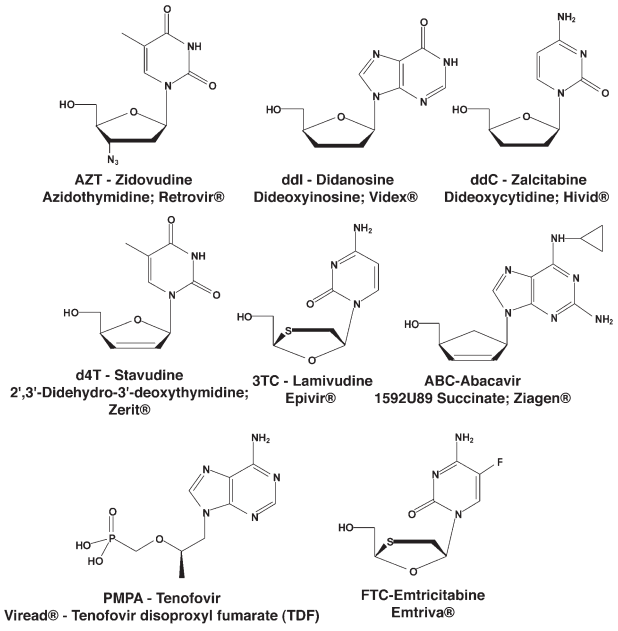



Figure 1. Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) approved by the Food and Drug Administration.

competitive inhibitors [9]. NNRTIs bind to a hydrophobic pocket adjacent to the polymerase active site on the p66 subunit and induce structural modifications that decrease the nucleotide incorporation rate [9] by displacement of the catalytic aspartate residues [10].

## The nucleoside and nucleotide RT inhibitors

Zidovudine (AZT), a thymidine analogue with an azido group in place of the hydroxyl group at the 3′ position of the ribose (fig. 1) was the first drug approved by the FDA, in 1987. This molecule displayed excellent antiviral activity in cell culture assays [11]. It was followed by the approval of the dideoxynucleosides didanosine (ddI) and zalcitabine (ddC) [12, 13], by compounds with a double bond between positions 2′ and 3′ of the dideoxyribose, stavudine (d4T) and abacavir (ABC) and, more recently, by molecules with a $\beta$-L-oxathiolane ring system instead of the ribose ring of canonical nucleosides, lamivudine (3TC) and emtricitabine (FTC) (fig. 1). The family of FDA-approved NRTIs was extended in 2001 to a nucleotide analogue, tenofovir disoproxil fumarate (TDF). TDF is an acyclic nucleoside phosphonate diester analogue of adenosine, requiring an initial diester hydrolysis to generate tenofovir (PMPA) (fig. 1).

The NRTIs are administered as unphosphorylated prodrugs that are consequently recognized by cellular kinases after their penetration into the host cell. Nucleoside analogues must be tri-phosphorylated [14, 15], while TDF, which already possesses one phosphonate group, must be di-phosphorylated [16]. In addition, ddI and ABC undergo a cellular activation step that converts them into ddAMP [17] and CBVMP, respectively [18]. Consequently, the intracellular concentration of the active NRTI depends on the rate of prodrug activation. Generally, the first phosphorylation step is the slowest, except for AZT, where the nucleoside monophosphate phosphorylation step is limiting [19–21].

The tri-phosphorylated NRTIs bind to the polymerase active site of RT (figs. 2A, 3). Once incorporated into the growing DNA chain, they act as chain terminators because they lack a 3′ hydroxyl group on the NRTI ribose or pseudo-ribose moiety. This prevents 3′-5′ phosphodiester bond formation, blocking further extension of the DNA (fig. 3).

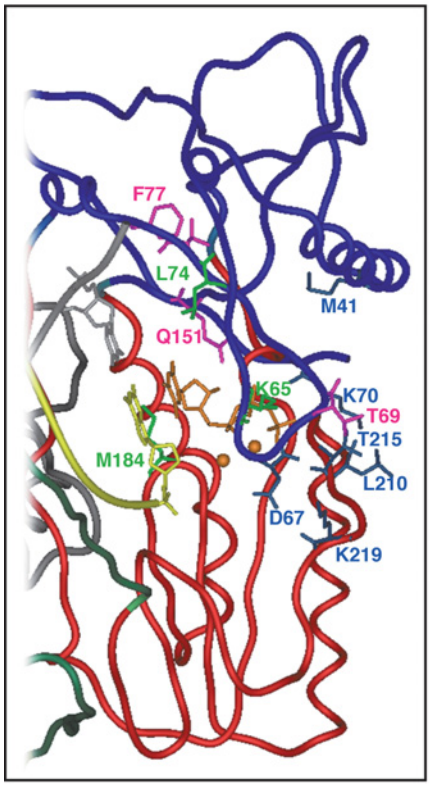
## Emergence of resistance mutations

Base substitutions associated with increased resistance to the drugs used were rapidly observed in the *pol* gene encoding the RT of patients treated with NRTIs [for a review, see ref. 22, and for a compilation of all the known RT mutations, see ref. 23] (fig. 2B). The rapid emergence and selection of resistance mutations is explained by the low fidelity of HIV-1 RT [24–26], the high level of HIV-1 replication [27] and the high rate of RT-mediated recombination [28, 29]. The threshold of significance in terms of drug resistance *in vivo* is reached when three- to fourfold resistance arises. Drug-resistant variants have in most cases a better viral fitness under conditions of drug pressure, but their overall rate of replication in the absence of drug is generally lower than wild-type (WT) viruses [30].

Early clinical observations revealed that patients undergoing AZT mono-therapy for 6 months or more displayed highly reduced sensitivity to this drug [31], while remaining sensitive to ddI and ddC [31, 32]. High-level resistance towards AZT was associated with mutations M41L, D67N, K70R, L210W, T215Y/F and K219Q (fig. 2A), but most AZT-resistant HIV-1 isolates contain only a subset of these mutations [33–37]. The same set of mutations were found in resistant virus isolated from cell cultures treated with AZT [38, 39]. Interestingly, they were also observed in isolates of patients under d4T therapy (fig. 2B) [40–44] or in combination regimens of AZT or d4T with other NRTIs [36, 37, 40–42, 44–46]. This set of six mutations providing resistance towards AZT and d4T was therefore named the thymidine-associated mutations (TAMs). TAMs also occur in about 10% of individuals receiving ddI mono-therapy [47, 48].

The TAMs are located both in the fingers and in the palm subdomains of RT, at the front of the nucleotide-binding pocket (fig. 2A). T215Y is the main mutation observed *in vivo* and is considered as the most important for the resistance phenotype [36, 49, 50]. It causes intermediate

**A**




**B**

| NRTI | Position of mutated residues in HIV-1 RT | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 41 | 65 | 67 | 69 + insertion | 70 | 74 | 75 | 151 | 184 | 210 | 215 | 219 |
| AZT | M41L | * | D67N | T69D | K70R | * | V75T | Q151M | * | L210W | T215F | K219Q |
| d4T | M41L | K65R | D67N | T69D | K70R | | V75T | Q151M | * | L210W | T215F | K219Q |
| TDF | M41L | K65R | D67N | T69D | | | V75T | Q151M | * | L210W | T215F | K219Q |
| ABC | M41L | K65R | D67N | T69D | | L74V | V75T | Q151M | M184V | L210W | T215F | K219Q |
| ddI | M41L | K65R | D67N | T69D | | L74V | V75T | Q151M | M184V | L210W | T215F | K219Q |
| ddC | M41L | K65R | D67N | T69D | K70R | L74V | V75T | Q151M | M184V | L210W | T215F | K219Q |
| 3TC | M41L | K65R | | T69D | K70R | | | Q151M | M184V | L210W | T215F | |
| FTC | M41L | K65R | | T69D | | | V75T | Q151M | M184V | L210W | T215F | |

High-level resistance
Intermediate resistance
Low-level resistance
Contributes to resistance
No resistance
Hypersusceptibility
X000X Main mutations associated to the NRTI resistance
X000X Less frequent mutation

Figure 2. The main NRTI resistance mutations. (*A*) Localization of the main NRTI resistance mutations. The palm and finger subdomains of HIV-1 RT are in red and blue, respectively. The resistance mutations are marked: green for resistance to ddI, ddC and 3TC, dark blue for resistance to AZT and pink for cross-resistance to AZT and ddI or ddC. The primer/template backbone is in yellow/grey, with only the base pair near the active site shown in balls an sticks; the incoming dTTP and the $Mg^{2+}$ ions are in orange. (*B*) NRTIs, their associated resistance mutations and their contribution to resistance.

(10- to 15-fold) resistance to AZT. K70R and T215Y are antagonist in their effect on AZT resistance and only occur together if other TAMs are present as well [51, 52]. Four or more TAMs trigger >100-fold resistance to AZT, contribute to cross-resistance to other NRTIs [53–55] and induce 5- to 7-fold resistance to ABC [56] and 2- to 5-fold resistance to d4T [57, 58], ddI [59], ddC and tenofovir [60–63] (fig. 2B). These mutations cause low-level resistance to 3TC that, despite reduced responses, do not compromise the effectiveness of 3TC, which is therefore often present in tri-therapies, even in patients bearing multiple TAMs [64, 65].

Upon 3TC mono-therapy, the first mutation to appear is M184I [66–69], associated with high levels of resistance. Because of the low enzymatic efficiency of the M184I RT, mutation M184V, which confers >100-fold resistance to 3TC [51, 66, 68, 70–72], as well as to FTC [67, 68] is rapidly selected both *in vitro* and *in vivo* [73]. The same mutation is also often observed during ABC mono-therapy [56, 68, 74] (fig. 2B).

Mutation at position 74 (mostly L74V) is frequently found in patients receiving ddI mono-therapy [48, 68, 75, 76] and also occurs during ABC mono-therapy [56, 74]. In the former case, L74V is associated with other mutations (mainly M184V) [77]. The L74V mutation causes virologic failure primarily in patients receiving ddI [75, 76, 78], but not ABC mono-therapy and prevents antiviral activity when ddI is used for intensification [79]. The L74V mutation also confers 2- to 5-fold resistance to ddC [75] (fig. 2B).

Mutation K65R emerges after mono-therapy with ddI [48, 80], ddC [80, 81] or ABC [74], and during tenofovir intensification [60]. K65R is also frequently selected in cell culture by ABC [77], tenofovir [60] and d4T [82], and its incidence increased recently, mainly in patients receiving tri-therapies excluding AZT [83]. K65R confers intermediate resistance levels to ddI [84], ddC [80, 84, 85], 3TC [84–86], d4T [82], tenofovir [60, 87] and ABC when associated with other mutations [77] (fig. 2B).

Position T69 is the most commonly mutated site after the TAMs and M184V. Initially, T69D was only associated with ddC resistance [88], but was subsequently found after treatment with each of the NRTIs. Mutation at position 69 is associated with intermediate level resistance to

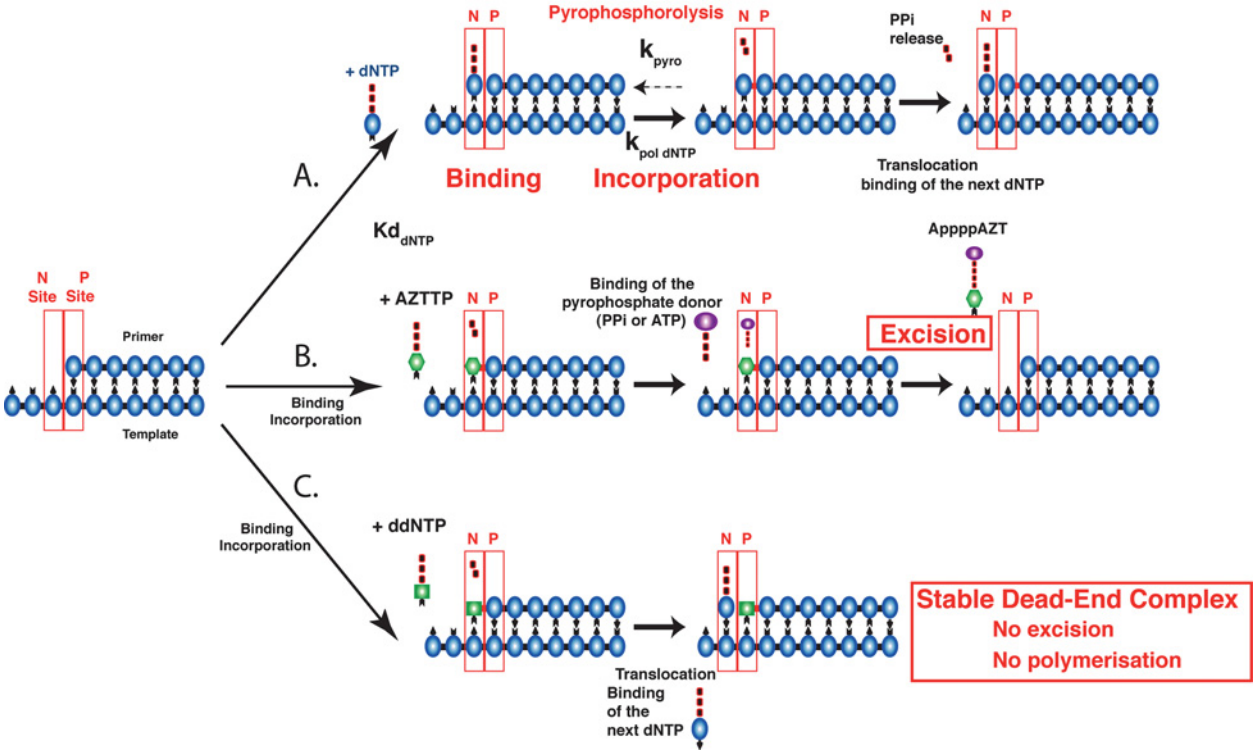



Figure 3. Schematic representation of the polymerization and excision reactions carried out by HIV-1 RT. N and P correspond to the N (nucleotide) and P (priming) sites of RT (see text). (*A*) Incorporation of a natural cognate dNTP. The end of the primer is in the P site and the incoming dNTP binds to the N site. Its $\alpha$-phosphate is then joined to the growing DNA chain and PPi is released. Translocation moves the end of the primer into the P site and a new dNTP can bind to the end site for the cycle to continue. The efficiency of incorporation of a dNTP or any dNTP analogue depends on its affinity for the primer/template-RT complex ($K_d$) and on the incorporation rate ($k_{pol}$). (*B*) Model for the excision reaction. When AZTMP is incorporated into the DNA, the next incoming dNTP cannot bind due to steric crowding of the azido group. The AZTMP-blocked primer resides primarily in the N site and the pyrophosphate donor (PPi or ATP) can bind, AZTMP is removed and forms a dinucleotide tetraphosphate, AZTppppA. (*C*) Dead-end complex (DEC) formation. When a chain terminator other than AZTMP is incorporated, translocation of the end of the primer to the P site occurs and the next cognate nucleotide binds to the N site. Because of the lack of a 3′ OH group at the end of the primer, the complex is stalled, forming a so-called DEC.

ddI [89], and also contributes to resistance to each of the NRTIs in the context of TAMs [60, 90–93]. In about 2% of heavily treated HIV-1-infected patients [94] having received prolonged AZT treatment followed by administration of other NRTIs, RT has a dipeptide insertion (SA, SG or mainly SS) between position 69 and 70, associated with other amino acid substitutions such as T69S or T215Y and other TAMs [94–96]. Individually, the dipeptide insertions confer only a low level of resistance to each of the NRTIs, but in the context of T215Y and other TAMs, high levels of resistance to AZT and moderate levels to d4T, ddC, ddI and tenofovir are achieved [95–99] (fig. 2B).

Mutation at position 75 (V75T) develops in response to increasing concentrations of d4T as the sole drug in cultured cells infected by HIV-1 [49]. In the clinic, V75T occurs at a 10% frequency [44, 100], mainly in AZT-naïve patients [101]. V75T causes intermediate level (5-fold) resistance to d4T, ddI, ddC and FTC [49] (fig. 2B).

The aim of the initial antiviral treatment for newly infected patients is to decrease as quickly as possible the viral load to an undetectable level. All associations of NRTIs, NNRTIs and PIs are not possible, due to either insufficient efficacy (ABC+TDF+3TC), high induced toxicity (d4T+ddI), antagonist effects (AZT+d4T) or synergistic toxicity (efavirenz+nevirapine) [65]. Commonly used multi-therapies combine two NRTIs with one NNRTI or one PI. Table 1 summarizes recommended, conceivable and contraindicated combinations [65].

Several studies have shown that combining two NRTIs with one NNRTI was as efficient as the combination of two NRTIs with one PI. However, poor adherence to treatments with NNRTIs rapidly enables the selection of mutations conferring resistance to the entire class, whereas PIs rarely generate mutations rendering the HIV-1 protease resistant to all PIs [65]. Only one multitherapy based on a combination of three NRTIs (AZT, 3TC and ABC) is efficient enough to be prescribed (Trizivir).

Table 1. Recommended and contraindicated drug associations for an initial antiretroviral treatment [adapted from ref. 65].

| **Recommended associations** | | | | |
|---|---|---|---|---|
| 2 NRTIs | + | 1 NNRTI | or | 1 IP (+ ritonavir) |
| AZT[1] or<br>TDF or<br>ddI or<br>ABC[2] | + 3TC1 or<br>FTC | efavirenz or<br>nevirapine[2] | | fosamprenavir or<br>indinavir or<br>lopinavir or<br>saquinavir |
| **Conceivable choices** | | | | |
| d4T + 3TC + [1 NNRTI or 1PI]<br>AZT + ddI + [1 NNRTI or 1 PI] | | no advantages, more constraints and less well tolerated | | |
| AZT + 3TC + ABC | | risks of a poorer virologic reponse and hypersensitization to ABC | | |
| 2 NRTIs + nelfinavir | | necessity for a very good adherence and administration with a meal | | |
| **Contraindicated associations or treatments** | | | | |
| Monotherapy<br>Bitherapy of NRTIs | | insufficient efficacy with compounds available | | |
| Tritherapy of NRTIs:<br>ABC + TDF + 3TC<br>TDF + ddI + 3TC<br>d4T + ddI+ 3TC<br>d4T + ddI + ABC | | rapid selection of resistant viruses | | |
| d4T + ddI<br>d4T + ddC<br>ddI + ddC | | unfavourable advantages/risks ratio<br>risks of high toxicity | | |
| treatment including AZT + d4T | | antagonistic effect | | |
| treatment including 3TC + FTC | | no advantages expected, molecules with the same pattern of resistance | | |
| Efavirenz in pregnant women | | teratogenicity | | |
| Efavirenz + nevirapine | | no benefits, synergistic toxicity | | |
| Ritonavir at full dose, as the only PI[3] | | bad tolerance | | |

[1] AZT and 3TC association is the most studied combination of two NRTIs.
[2] ABC and nevirapine association is contraindicated.
[3] Ritonavir is commonly administrated with others PIs to increase their potency.

## Resistance mechanisms

Mutations associated with resistance to NRTIs reside primarily in the palm and fingers subdomain of RT and give rise to two types of resistance mechanism [for reviews, see refs. 102, 103]: (i) improved discrimination during polymerization and at the active site of the mutated drug-resistant RT of a dNTP analogue relative to the natural dNTP; (ii) increased phosphorolytic cleavage of an analogue-blocked primer. The latter resistance mechanism is mainly due to the TAMs.

### Resistance by discrimination during incorporation

The incorporation efficiency of a dNTP analogue into DNA, and hence its DNA termination potency, relies on two factors: (i) competition of the dNTP analogue with its natural counterpart for binding to the RT active site, reflected by their respective dissociation constant ($K_d$); (ii) subsequent incorporation into the DNA with a favourable catalytic rate constant ($k_{pol}$) (see fig. 3). The NRTI-induced mutations either interfere with NRTI binding and/or with its incorporation rate into the growing DNA chain.

**Mutation M184I/V.** This mutation is located in the palm subdomain of HIV-1 RT, within the highly conserved catalytic motif YMDD, which interacts with the 3′ end of the primer, the incoming dNTP and the metal cofactors [104] (fig. 2A). The crystal structure of a DNA/DNA-M184I RT complex revealed that replacement of the Met side chain by either Val or Ile causes steric hindrance with the sulphur atom present in the ribose ring of 3TC and FTC [105]. Most kinetic studies have shown that such struc-

tural conflict results in decreased affinity of these dNTP analogues for the mutated enzyme [106–108].
The M184V mutation is also associated with increased fidelity [109–112] and/or decreased processivity [113, 114], related to a significant decrease in the viral replication capacity compared to the WT. Accordingly, reversal of M184V was observed in patients interrupting 3TC treatment [115], indicating that this mutation reduces viral fitness. The impact of M184V on viral replication is also related to the activation state of the cells: it is more pronounced when dNTP pools are smaller [116]. Importantly, the increased fidelity does not limit the ability of HIV to develop new mutations under more diverse drug pressure [for review, see ref. 117]. In addition, M184V RT is impaired in the by-passing of some rate-limiting steps during reverse transcription involving RNA-primed DNA synthesis, i.e. initiation of (–)strand DNA synthesis (from the $tRNA_3^{Lys}$) and initiation of (+)strand DNA synthesis (from the polypurine tract (PPT) sequence) [118]. Release of the same enzyme from strong pausing sites was also found to be a major obstacle for efficient DNA synthesis [118].

**Mutation L74V**. In the case of L74V, which weakens viral replication in the absence of drug [75, 118–120], resistance was explained by altered substrate and inhibitor recognition by the mutant enzyme [78]. Enzymatic studies reported a decreased processivity [120] and reduced incorporation of the dNTP analogue. L74V was also found to improve RT fidelity [121] and similarly to M184V, it affects initiation of reverse transcription and release from pausing sites [118]. These effects were even more pronounced in the double mutant L74V+M184V [118]. Interestingly, L74V induces hypersensitivity to AZT [75, 86] (fig. 2B), and is rarely found with AZT/ddI dual-nucleoside therapy, making it an advantageous combination. Indeed, L74V alone or associated with other TAMs inhibits primer rescue of AZT-blocked primers [122, 123] (see below).

**Mutation K65R.** Position 65 of HIV-1 RT is located in the flexible $\beta$3-$\beta$4 loop in the fingers subdomain (fig. 2A). *In vitro* studies have reported contradictory increased [124], decreased [125] or unchanged [81] processivity of K65R RT compared to the WT RT, depending on the nucleotide concentration used. Pre-steady-state kinetics showed that mutation K65R reduces the incorporation of ddI and ddC, compared to the natural dNTPs, by affecting $k_{pol}$ [126]. The increased discrimination between dNTPs and ddNTPs, which share the same $\gamma$-phosphate, cannot be explained by the interaction of the side chain of K65 with an oxygen of the $\gamma$-phosphate of the incoming dNTP [104]. Rather, an intramolecular hydrogen bond between the 3′-OH of the ribose and an oxygen of the $\beta$-phosphate was found to be crucial for polymerization. In such a context, the effect of the K65R mutation is to amplify the negative impact associated with the lack of the 3′-OH on ddNTPs [126]. K65R RT also displays improved fidelity compared to WT RT [127].
K65R also induces moderate resistance to tenofovir [60, 62] due to a reduced $k_{pol}$ of tenofovir-PP [128]. However, the combination of K65R with M184V restores sensitivity to this drug [60, 62, 125], due to a further reduction in incorporation of the natural substrate dATP [128]. Hence, the reduced fitness of the double-mutant virus is explained by the general reduction in incorporation of the natural dNTPs by the corresponding mutated RT as well as reduced processivity.
In the case of mutations K65R and L74V, both selected by ddI, an 84-fold loss in the catalytic rate constant of the double-mutated RT compared to the WT also accounts for the poor ability to use natural dNTPs and the resulting poor viral fitness [129] and explains why K65R and L74V are never selected together in the clinic [48].
Finally, clinical data revealed that K65R hypersensitizes HIV-1 to AZT [55, 95, 130] and does not develop in patients receiving AZT-containing regimens [131]. Accordingly, in a more recent clinical study, a low frequency of K65R mutation emerged, in treatment-experienced patients, but only for patients without detectable TAMs at baseline, suggesting a functional and/or structural incompatibility for the two substitutions within the same RT. Intriguingly, even more recent studies suggested that TAMs (and even L74V) and K65R can occur together on single viral backbones, with an incidence higher for patients with an initial absence of TAMs [132–134]. In the absence of biochemical data, obtained from purified recombinant RTs bearing various TAMs in combinations with K65R, and *in vitro* replication studies of the fitness of the corresponding viruses, interpretation of these results remains very speculative.
All these data demonstrate the need to understand the mechanisms underlying drug resistance and viral fitness and their impact on each other. Indeed, the above-cited studies also clearly show that selection of certain drug-resistant variants may actually be beneficial for treatment.

## TAM-associated resistance by nucleotide analogue excision

**Pyrophosphorolysis and ATP-mediated removal of AZT.** The TAM-associated mechanism of resistance to AZT remained long unexplained. Indeed, the small increase in AZT discrimination between the WT and AZT-resistant RT ($^{AZTR}$RT) could not account for the >100-fold resistance patterns observed in patients [135]. In addition, WT and AZT-resistant virions were found to be similar in their endogenous reverse transcription patterns [136]. However, TAMs were shown to confer resistance to

AZT in an *Escherichia coli* strain in which the DNA Pol I was replaced by the HIV-1 RT, indicating that no eukaryotic factor was involved in the resistance mechanism [137].

Clues about the resistance mechanism came from different experiments. First, HIV-1 RT, which lacks 3′ exonuclease proofreading activity [24], is, however, capable of pyrophosphorolysis (PPi lysis), the reverse reaction of polymerization, releasing an unblocked, extensible DNA chain and the analogue triphosphate [138, 139] (fig. 3). $^{AZTR}$RT was also reported to bind AZTMP-terminated primers more tightly than WT RT does [140] and, at the same time, AZT resistance mutations lead to enhanced excision of AZT from the nascent DNA by PPi lysis [141–143], although this observation was not confirmed by other groups [144, 145]. Finally, $^{AZTR}$RT was shown to unblock efficiently AZT-terminated primers by transfer of the chain terminator to a nucleoside tri-phosphate, most likely ATP *in vivo* [145–148], in a reaction similar to PPi lysis and named ATP lysis [143, 145, 149, 150]. This reaction yields a 5′-5′ dinucleoside tetraphosphate, AZTppppA, [149] (fig. 3) and is very inefficient with WT RT [145, 151].

**Effect of the next complementary nucleotide and formation of a dead-end complex.** In the absence of the next correct nucleotide to be incorporated into the growing DNA chain, $^{AZTR}$RT excises not only AZT [145, 150] but also other nucleotide analogues, and the excision rate follows the trend AZT>d4T>>ddC>ABC>3TC>ddI> tenofovir [152]. However, the addition of physiological concentrations of the next complementary dNTP reduces ATP-mediated removal of all analogues except AZT and ABC [145, 150, 152]. These data help to explain why the TAMs confer higher levels of resistance to AZT than to d4T or ddI. They also explain why, despite the selection of TAMs during d4T treatment, there is no resistance detected in conventional *in vitro* phenotypic drug susceptibility assays that use cell lines with high dNTP pools.

The inhibition of the excision reaction by the next incoming dNTP was explained by formation of a stable quaternary P/T-RT-dNTP complex called the ‘dead-end complex’ (DEC) (fig. 3). The stability of the DEC depends on the nature of the chain terminator, the AZTMP-terminated complex forming the least stable DEC, and there is a direct correlation between the stability of the DEC and the inhibition of the PPi- or ATP-dependent removal reaction by the incoming dNTP [145, 150, 153].

**A model for the excision reaction (fig. 3).** On the basis of the above biochemical and structural data, a model for the ATP-mediated NRTI excision by $^{AZTR}$RT and its inhibition by the next complementary dNTP was developed [154]. Central to this model is the possible positioning of the 3′ end of the primer in two distinct positions in the polymerase active site (fig. 3). In most crystal structures, the 3′ end of the primer is located in the so-called P (priming) site [5, 155]. In that configuration, the next incoming nucleotide can bind to the N (nucleotide-binding) site [104], and translocation to the P site occurs after the chemical step of dNTP incorporation, probably after PPi release [154]. Recent site-specific footprinting experiments on pre- and post-translocational complexes showed that the 3′ end of the primer is in dynamic equilibrium between the N and P sites [156]. According to the model, the excision reaction occurs only when the 3′ end of the primer is positioned in the N site. The crystal structures of frozen pre- and post-translocated complexes, where an AZTMP-terminated primer strand is cross-linked to the RT, have also been solved [157]. Docking of the next incoming dNTP into the X-ray structure of the post-translocated AZTMP-terminated complex suggests that steric crowding due to the azido group impairs formation of a stable DEC [157], in agreement with the biochemical data. Thus, the amount of N complex should be increased, favouring excision. Experiments showing that AZT excision was enhanced in cross-linked pre-translocated complexes compared to post-translocated complexes confirmed this hypothesis [158]. Interestingly, the rather specific AZTMP excision is mainly provided by the nature of the dNTP analogue, rather than by the TAMs [154]. However, site-specific footprinting revealed that the mutations favouring primer unblocking increase the fraction of primer residing at the N site [156] and recent data showed that only pyrimidine 3′-azido nucleosides and not purine 3′-azido nucleosides are excised efficiently by $^{AZTR}$RT [159].

**Open questions.** Early biochemical experiments showed that D67N and K70R were the mutations most responsible for rescue of chain-terminated primers [141, 145]. More recent structural and modelling studies suggested that T215Y increases the affinity of the $^{AZTR}$RT for ATP [154, 160] resulting in higher levels of ATP lysis. Experiments showing that Tyr215 allows the establishment of $\pi$-$\pi$ interactions with ATP [147] further supported this idea.

However, recent mechanistic [151, 161] and biochemical data [162] challenged that model. Indeed, pre-steady state kinetics performed by the group of K. Anderson [151] and by us [M. Rigourd and R. Marquet, unpublished data] showed that (i) PPi lysis is more efficient than ATP lysis to remove AZT and other dNTP analogues, both with WT RTs and $^{AZTR}$RTs and (ii) ATP is not bound more tightly by $^{AZTR}$RT. Instead, it seems that TAMs are involved in effective positioning of the ATP for the catalytic attack of the terminating dNMP, via Tyr215 [161] and possibly via residues 67 and 70 [151]. The role of the latter residues was recently confirmed by the finding that 67N/70R/219Q RT displayed higher rates of primer unblocking than 41L/210W/215Y RT [123].

Interestingly, several groups have shown that rescue of a chain-terminated primer can effectively occur by PPi lysis, with WT RT, thus conferring an innate resistance towards dNTP analogues [139, 144, 153, 163]. Remarkably, the NRTIs that frequently select TAMs are those that are the most effectively pyrophosphorolysed by WT RT in the presence of the next incoming dNTP, indicating that TAMs do not confer new properties to the enzyme, but rather exacerbate pre-existing features [153]. In view of these results and the above-mentioned data on $^{AZTR}$RT and ATP affinity, the reason why resistance emerges using ATP-mediated primer unblocking is still not clear.
Recently, the levels of the potential endogenous acceptors for the unblocking reaction, mainly ATP and PPi, were precisely measured in H9 cells, macrophages and unstimulated and activated T cells [148]. ATP was present at similar levels in all cases (1.3–2.7 mM), consistent with previous literature data. The levels of PPi, however, were found to be much lower than expected, 7–15 μM in unstimulated cells, reaching 55–79 μM in activated cells. At the latter PPi concentrations, in conditions where the bulk of the viral replication and hence selection for AZT resistance mutations seem most likely to occur, PPi lysis prevailed, with similar rates of excision for WTRT and $^{AZTR}$RTs. This suggests that selection of AZT resistance mutations and enhanced ATP lysis by $^{AZTR}$RT is only manifested in an environment where the PPi concentration is low.

**Other factors involved in efficiency of primer rescue.**

1. The primer template sequences.
The DNA or RNA nature of the P/T is also critical for the rescue reaction. Indeed, removal is less efficient during RNA-directed processes [151, 164], and even totally abolished during the $tRNA_3^{Lys}$-primed initiation phase of reverse transcription [164]. In addition, not all AZTMP or other dNTP analogue incorporation sites are repaired with the same efficiency [147, 153, 160, 163, 165]. Accordingly, rescue of AZTMP-blocked primers was shown to be sequence dependent [156, 166], due to a selective decrease in the amount of pre-translocation complex [156]. In addition, ddTMP- or AZTMP-terminated complexes are less influenced by sequence-specific interactions than ddA-, ddC- or ddG-terminated ones [167]. The major sequence determinants affecting removal efficiency are located at the 3′ primer terminus and within the six base pairs upstream of it [167]. These determinants must interact with RT in a sequence-dependent manner that affects the removal reaction.

2. The RNase H activity of HIV-1 RT.
The influence of HIV-1 RT RNase H activity itself and/or potential mutations in the RNase H subdomain associated with drug therapy are potentially strongly underestimated, given that genotypic analysis for clinical drug resistance usually does not include this sequence.
Once a chain terminator like AZT or d4T is incorporated, there is competition between primer repair and RNase H activity, the latter potentially leading to a premature dissociation of the newly synthesised DNA from the RNA template that would block replication. In agreement with this hypothesis, mutations reducing the RNase H activity lead to increased AZT and d4T resistance, most likely by increasing the time available for excision [168]. These mutations have no influence on the resistance to 3TC or efavirez that do no involve excision [168]. Simultaneous RNase H mutations and TAMs increased resistance to NRTIs in a synergistic manner [168]. Importantly, mutations reducing RNase H activity were detected in AZT- and d4T-treated patients [168].

3. Synergy between NRTIs and NNRTIs.
It is well documented that combination therapies using the two classes of RT inhibitors exert a synergistic inhibition on HIV replication. Early mechanistic studies demonstrated that the natural dNTPs and NNRTIs can bind simultaneously to their respective sites [9] and suggested communication between the two sites. Synergy between NRTIs and NNRTIs was confirmed by experiments showing that NNRTIs inhibit the ATP-mediated removal of several dNTP analogues through an effect on both the catalytic rate of the chemical step of ATP-mediated hydrolysis and the binding of ATP to the RT, resulting in an overall decrease in the efficiency of removal [169–171]. More recently, it was suggested that inhibition of the basal phosphorolytic activity of WT RT by NNRTIs may also account for the synergy found in antiviral assays [172].

4. Other suppressive mutations involved in AZT resistance.
Mutations Y181C/I, located in the hydrophobic pocket adjacent to the polymerase active site, confer resistance to several NNRTIs [173]. Even though mutation Y181C was found early on to partially suppress AZT resistance in the context of TAM-bearing RTs [174], the mechanism was elucidated only recently. Desensitization is apparently due to neither increased discrimination nor enhanced DEC formation, but rather to reduced ATP binding, resulting in a less efficient ATP-mediated unblocking reaction [175]. Finally, mutation Y181I alone confers cross-resistance to d4T only, by increasing both discrimination against d4TTP and its ATP-dependent excision [176].
Viruses highly resistant to AZT isolated from patients undergoing long-term antiretroviral therapy contain, in addition to the usual set of AZT resistance mutations, a deletion at position 67 (named the Δ67 complex) [89]. RTs of such viruses are highly efficient in excising

AZTMP, at a lower ATP concentration compared to other $^{AZTR}$RTs [177]. They are, however, less efficient in excising a broad spectrum of NRTIs compared to standard $^{AZTR}$RTs, but still more efficient than WT RT. Such Δ67 viruses would therefore replicate reasonably well in quiescent cells, even in the presence of AZT.

## Cross-road between the two resistance mechanisms

**TAMs associated with other point mutations.** The M184V mutation, in the context of some of the TAMs, namely M41L/T215Y, decreases susceptibility to ddI, ddC and ABC, but resensitizes the virus to AZT, d4T and tenofovir [92, 178–181]. This is due to inhibition of AZTMP excision by ATP lysis, which occurs only in the context of the TAMs, and not in a WT background [143, 160]. However, with four or more AZT resistance mutations, resensitization to AZT is overcome [55, 68]. Another clinical benefit of mutation M184V is the later emergence of TAMs [182]. Finally, M184V associated with T215Y interacts synergistically to decrease RT processivity [141]. The combination of M184V/T215Y +K219Q reverses that effect and enhances processivity even more than in the WT [183].

Inhibition of the rescue of AZT-blocked primers by ATP lysis was also observed when the L74V mutation was present either alone or in combination with TAMs [122, 123], and it was established that this effect was not due to changes in the ratios of pre- and post-translocational complexes [122].

**Mutation V75T.** Pre-steady-state experiments showed that V75T has a direct effect on dNTP selectivity by discriminating about threefold d4TTP relative to dTTP, but also increased the polymerization rate by facilitating translocation along single-stranded templates. The modest increase in discrimination is further potentiated by an increased rescue of d4TMP-terminated primers by PPi, but not ATP lysis [184]. Position V75 is therefore at the cross-road of the two mechanisms involved in drug resistance.

**Multi-drug resistance.** Most of the single resistance mutations are associated with resistance to specific RT inhibitors. However, there is also wide cross-resistance between anti-retroviral drugs of the same family and even of different classes. Combination therapies often give rise to multiple ddNTP-resistant RTs that mainly fall into two categories: (i) the Q151M mutation, where RT remains susceptible to tenofovir and 3TC [60, 61] and (ii) insertion mutations at position 69, where viruses have reduced susceptibility to all currently approved NRTIs, including tenofovir.

1. The Q151M complex

One of the multiple ddNTP-resistant viruses carries a set of five mutations (Q151M/A62V/V75I/F77L/F116Y) that reduce considerably sensitivity to AZT, ddI, ddC and d4T *in vivo* [185, 186] (fig. 2B). Amongst these point mutations, Q151M, a two-base-pair substitution close to the first nucleotide of the single-stranded region of the template [104, 187] (fig. 2A), is a key mutation [188, 189] which appears first during the course of the treatment, in 5% of the patients receiving dual ddI/AZT or ddI/d4T therapy [40, 44, 94, 190, 191], but rarely with 3TC-containing regimens. Q151M causes an intermediate level of resistance to AZT, ddI, ddC, d4T [188, 192–194] and ABC [194] and, when followed by the four other mutations, generates viruses with significant resistance to the previously cited ddNTP analogues and low-level resistance to 3TC and tenofovir [61, 178]. Steady-state kinetics pointed to altered recognition of the incoming dNTP as the mechanism of drug resistance [146, 195]. Furthermore, pre-steady-state kinetics on the Q151M RT, as well as the RT bearing all five resistance mutations, showed that the binding constant of the dNTP analogue remains unchanged while a selective decrease of the $k_{pol}$ for phosphodiester bond formation explains improved discrimination [196]. Mutation Q151M was also associated with increased RT fidelity [197].

2. Mutation/insertion at position 69

In the absence of TAMs, RTs with SG and AG, but not SS insertions at position 69 have increased unblocking activity but all have decreased sensitivity to dNTP inhibition [167, 198]. In the context of the TAMs T215Y and M41L, insertions at position 69 induce increased AZT excision via ATP lysis [167, 198–200] by destabilizing the DEC, so that positioning of the nucleotide analogue at the N site is favoured [199]. Efficient excision of d4T also occurs in this context, even in the presence of high concentrations of the next complementary dNTPs [167, 199]. In addition, the enzyme also removes, at a lower rate compared to AZT or d4T, the other ddNTPs and dNTP analogues much more efficiently than the WT RT or RT bearing the classical TAMs [199, 200]. Resistance is further increased in this mutant by a reduced incorporation of 3TC, and to a lesser extend ddI [199]. Indeed, when associated with TAMs, the T69S-SS mutation generates resistance to all NRTIs, including tenofovir [200]. A slight increase in tenofovir discrimination does not account for the resistance observed, which is due to enhanced ATP lysis compared to T69S+TAMs- or WT RTs. In the case of the T69S-SS +TAMs RT, the next incoming dNTP hardly inhibits the unblocking reaction [200]. Recently, an eight-amino-acid insertion at position 69 has been reported, in the context of the TAMs, for a patient receiving an AZT and ddC combination therapy [201]. Such a mutant enzyme has increased resistance to a wide range of drugs

and the size and sequence of the inserted sequence influences the level of resistance to dNTP analogues.
A single-amino-acid insertion mutation together with T69S/T was recently selected in association with Q151M +F116Y [202]. The clinical consequences of such mutations have yet to be determined but modelling experiments suggested that there would be no steric clash between the side chains of the mutated residues, implying that such RTs should be functional.

3. Mutation Q145M/L
Recently, a new mutation, Q145M/L, located in the nucleotide-binding pocket, was reported in patients failing therapy [203], with a frequency comparable to the insertion mutation. This mutation appeared to be associated with resistance to both NRTIs and NNRTIs. Because of its proximity to position 151, it was suggested that Q145 might also be involved in dNTP discrimination. Assays of HIV-1 recombinant strains showed that Met and Leu were the only changes at this position that confer drug resistance to NRTIs and NNRTIs [204]. Steady-state kinetics studies of the recombinant RTs bearing these mutations revealed a strong loss of catalytic efficiency and significant resistance to AZT and efavirenz [204].

## New nucleoside analogue inhibitors

### Nucleoside RT inhibitors in clinical development

In addition to the previously described FDA-approved anti-HIV NRTIs, several new nucleoside analogues have been designed and developed to improve safety and efficacy profiles and to minimize cross-resistance. The following paragraphs summarize the data relative to the compounds currently in clinical trials: amdoxovir, reverset, elvucitabine, racivir, AVX754, alovudine, DOT, MIV-210 and KP1212 (fig. 4). Additional information is available in older excellent reviews [205, 206] and on the web (www.thebody.com/tag/articles/pipeline.html; http://aidsinfo.nih.gov/drugs/drugtype.asp?type=2; http://www.aidsmap.com/en/docs/ux/drugs.asp?new=1&section=C92B1B00–7482–449B-B50B-931A8014FBF2)

**Amdoxovir, DAPD, (2R,4R)-4-(2,6-diaminopurin-9-yl)-1,3-dioxolane-2-methanol or (–)-*β*-D-2,6-diaminopurine dioxolane.** Amdoxovir, currently in phase II clinical trials, is a purine nucleoside analogue designed to be a more soluble and bioavailable prodrug of the anti-HIV agent (–)-*β*-D-dioxolane-guanine (DXG) [207]. It is rapidly absorbed and deaminated *in vivo* by adenosine deaminase to generate DXG [208], which is phosphorylated intracellularly to yield the active metabolite DXG-5′-triphosphate (DXG-TP). DXG-TP is incorporated approximately 17-fold more slowly than the natural substrate dGTP [208] and acts as a chain terminator [209]. *In vitro,* DXG demonstrated an anti-HIV-1 activity greater than that of d4T, ddI and adefovir, comparable with that of 3TC and ABC, but lower than that of AZT and FTC [210].

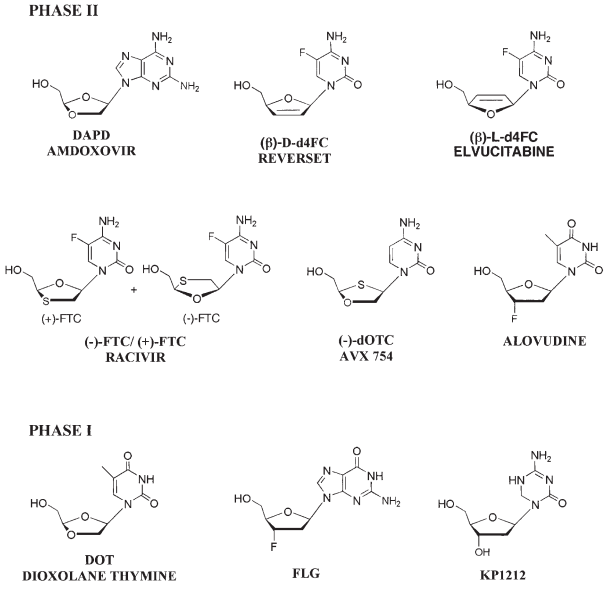



Figure 4. Nucleoside RT inhibitors in clinical development.

*In vitro* analysis indicated that HIV-1 strains resistant to AZT, 3TC, ddI, ddC, ABC, NNRTIs and PIs were still susceptible to DXG [211–213]. Viruses containing the multi-drug resistance-associated mutations G333E and the SS insertion between codons 69 and 70 were also sensitive to DXG [210]. There is some evidence that the wide activity spectra of amdoxovir against resistant HIV strains is related to its unique structure, with an oxygen atom in the 3′ position of the sugar ring [214]. *In vitro,* DAPD/DXG demonstrated synergistic antiviral activity with AZT, 3TC and nevirapine [211], as well as enfuvirtide (fuzeon) [215], and mycophenolic acid [216]. A phase I/II study is currently comparing the effect of concurrent administration of amdoxovir and the fusion inhibitor fuzeon to current regimens in HIV-infected patients (http://www.retroconference.org/Search_Abstract_2005/Default.aspx). On the other hand, viruses containing an L74V mutation or a K65R and Q151M double mutation were fully resistant to DXG, whereas viruses containing K65R or Q151M mutation alone were only moderately resistant [210, 211]. Under amdoxovir therapy, HIV-1 developed the K65R and L74V mutations [212].
Long-term toxicology studies indicated that high doses of amdoxovir were associated with lenticular opacities in monkeys and obstructive nephropathy in rats due to the limited aqueous solubility of amdoxovir/DXG. In humans, amdoxovir is generally well tolerated and the most frequently occurring adverse effects reported were head-

ache, pain, nausea, diarrhoea, skin rash, abdominal pain, malaise and tooth disorder. Kidney problems were not seen in phase I clinical trials, but in one of the pilot studies, five patients had to stop the drug because of eye problems (http://www.retroconference.org/Search_Abstract_2005/Default.aspx) and clinical development has been put on hold.

**Reverset, RVT, D-D4FC, 2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine, DPC 817.** Reverset, formerly known as DPC 817, is currently in phase IIb clinical trials under a FDA-approved Investigational New Drug (IND) protocol and two pivotal phase III studies should start in the second half of 2005. Reverset is a cytidine nucleoside analogue with potent anti-HIV activity in treatment-naïve and -experienced patients [217]. After phosphorylation by cellular kinases, the triphosphate analogue inhibits HIV RT by competing with the natural substrate (dCTP) and inducing chain termination with a more than 40-fold decrease in viral load [218]. One hypothesis to explain the potency of reverset against HIV-1 was that the fluorine atom on the heterocyclic base increased the overall efficiency of nucleotide incorporation during viral DNA synthesis [219]. Reverset has a long half-life in human whole blood. However, it is unstable in acidic medium; so, it should be given with an antiacid agent or in a buffered solution for oral administration [220].

*In vitro* cell culture experiments suggested that the antiviral effects of reverset are additive and in some cases synergistic with PIs, NNRTIs and NRTIs [221]. Reverset is active against HIV-1 strains resistant to AZT, 3TC, d4T and tenofovir [218], as well as strains coresistant to AZT and 3TC and several strains bearing double and triple mutations [220]. Activity against 3TC-resistant strains was also reported in the SCID-hu Thy/Liv mouse model [222]. Reverset is also active against viruses with TAMs, such as the 215 mutation, but not against the multinucleoside resistance mutations Q151M or 69SS [223]. In addition, reverset selects for the K65R mutation, which confers 5.3- to 8.7-fold resistance to this nucleoside analogue [223]. The *in vitro* results were confirmed by phase IIa clinical trials in treatment-experienced patients: by adding reverset to an NRTI (including 3TC or tenofovir) failing regimen, viral load was reduced by at least 0.7–0.8 $\log_{10}$ copies/ml (http://www.hivandhepatitis.com/2004icr/icaac2004/docs/1108/1108_c.html).

In phase I studies, reverset was well tolerated in all patients with no significant adverse effects [224]. In phase IIa clinical trials, the most common side effects were cold symptoms, headache and fatigue with no known raised lactate levels or mitochondrial toxicity [218]. To date, the only noticeable adverse effect is a higher incidence of asymptomatic hyperlipasaemia (a marker of pancreatic inflammation) in patients also receiving ddI, a known problem when other NRTIs were used in combination with ddI.

**Elvucitabine, ACH-126,443, L-d4FC, 2′,3′-dideoxy-2′, 3′-didehydro-$\beta$-L-5-fluorocytidine.** Elvucitabine is currently in late phase II studies. This $\beta$-L-nucleoside analogue is the mirror image of reverset and was designed to improve the activity observed with 3TC. Once phosphorylated intracellularly by cytoplasmic deoxycytidine kinase, L-d4FC-TP acts as a chain terminator and has 10- to 20-fold greater antiviral activity compared to 3TC, probably because of better pharmacokinetic parameters. Indeed, elvucitabine has excellent oral bioavailability and an intracellular half-life that exceeds 24 h [225].

*In vitro*, elvucitabine did not show loss of sensitivity against clinical strains of HIV with known nucleoside and non-nucleoside resistance. Elvucitabine exhibits synergistic activity with d4T or AZT, and additive activity with ddC or ddI [225]. In *in vitro* resistance studies, two mutations simultaneously emerged in RT, M184I and D237E, conferring ~tenfold resistance to elvucitabine (http://www.mediscover.net/journals.cfm; session 1, Abstract 5). The rapid switch of M184I to M184V described with 3TC was not observed in the case of elvucitabine. On the other hand, the D237E mutation has not been reported previously. A preliminary cross-resistance study indicated that this mutant was cross-resistant to 3TC but not to other NRTIs tested. In a phase II study, elvucitabine demonstrated potent antiviral activity in HIV-infected patients with resistance to 3TC and to other NRTIs, NNRTIs and PIs (http://www.mediscover.net/journals.cfm; session 1, abstract 2).

As expected from the unnatural L configuration, *in vitro* studies indicated that elvucitabine was not a substrate for the mitochondrial deoxypyrimidine nucleoside kinase and no inhibitory effect was observed on mitochondrial DNA synthesis at concentrations up to 10 mM [226]. In addition, elvucitabine reduced the level of mitochondrial damage caused by d4T when the two drugs were combined. However, preliminary toxicity studies indicated that elvucitabine induced serious, but reversible, bone marrow suppression [227]. Several patients experienced myelosuppression while taking elvucitabine at doses of 50 or 100 mg/day and mild headache and gastrointestinal distress were also reported. These studies were halted and experiences with lower doses are expected.

**Racivir, (+/–) 2′,3′-dideoxy-3′-thia-5-fluorocytidine, RCV, (+/–)FTC.** Racivir is currently in phase II clinical study. This oxathiolane drug is a 50:50 mixture of emtricitabine (–)FTC and its positive enantiomer (+)FTC. Racivir has potent *in vitro* activity against HIV [206, 228] and in a SCID mouse model for HIV, it was more potent than either 3TC or (–)FTC in reducing viral load in blood [229].

In a phase I/II dosing and efficacy study, racivir was administered in combination with d4T and efavirenz [230]. After 2 weeks, this regimen resulted in a viral load reduction greater than 20-fold and HIV RNA levels remained suppressed for more than 2 weeks following the end of treatment. There was no evidence that coadministration of d4T and efavirenz had an adverse effect on the pharmacokinetics of racivir (http://www.retroconference.org/2003/cd/Abstract/552.htm). Racivir is currently being studied in HIV-infected patients for activity against 3TC resistant mutants. In early studies, single and multiple doses of racivir appeared to be well tolerated with excellent oral bioavailability.

**AVX754, (–)-dOTC, (–)-2′-deoxy-3′-oxa-4′-thiocytidine.** AVX754, previously called SPD754, is in phase II clinical trials under IND status. This compound is the successor to BCH-10652, the racemic mixture of (+)and (–)-dOTC, whose development was suspended in 1999 when severe toxicity was found in monkeys. Activity of AVX754 was tested in a phase IIa trial in HIV-infected treatment-naive patients. AVX754 showed a statistically significant, greater than 25-fold decrease in viral load (http://hivandhepatitis.com/2003icr/2ndias/documents/071803l.html). Additionally, AVX754 is able to penetrate the cerebrospinal fluid, potentially flushing out hidden pools of the virus from this HIV 'sanctuary'.

In test tubes, the drug is active against AZT, 3TC and other NRTI-resistant viruses [231, 232] with a twofold increase in the mean IC50 when key mutations at codons 184 and 215 were present (http://www.mediscover.net/journals.cfm; session 1, abstract 3). Passaging experiments have shown that resistance is slow to develop *in vitro* in comparison with 3TC, and is associated with K65R, V75I and M184V mutations, causing cross-resistance to 3TC [233]. However, in phase II clinical trials, AVX754 does not select for known mutations associated with resistance to NRTIs (http://www.retroconferenc.org/2004/cd/Abstract/526.htm). Pharmacokinetic studies indicated that concentrations of AVX754, 3TC and 3TC-TP were unaffected by coadministration of the two drugs but intracellular AVX754-TP concentrations were reduced approximately six fold (http://www.retroconference.org/2004/cd/Abstract/138.htm). This antagonism contraindicates the coadministration of SPD754 with 3TC, but it can be used as second line therapy when 3TC is no longer effective.

The safety profile of AVX754 was studied in Cynomolgus monkeys, and the (–) enantiomer AVX754 had a more favourable safety profile and is lacking the serious toxicity seen with the racemate BCH-10652. At 1000 mg/kg per day, only minimal mucocutaneous hyperpigmentation, mild gastrointestinal effects and minimal changes in red blood cell parameters were observed (http://www.retroconference.org/2004/cd/Abstract/527.htm). In humans, AVX754 seems well tolerated at all doses tested and no side effect was reported (http://www.hivandhepatitis.com/2003icr/2ndias/documents/072503b.html#anew).

**Alovudine, MIV-310, FLT, 3′-deoxy-3′-fluoro-β-D-thymidine.** The nucleoside analogue alovudine, close in structure to AZT, has reached phase II clinical trials. Alovudine is very potent against HIV variants highly resistant to AZT [234, 235]. *In vitro*, alovudine demonstrated good activity against NRTI multi-resistant HIV strains [236]. In addition, a clinical study in individuals with TAMs and detectable viral load showed that adding alovudine at low doses (7.5 mg once daily) was effective in reducing viral loads by over 1 $\log_{10}$ copies/ml. The greatest reduction was seen for individuals who did not receive d4T (1.88 $\log_{10}$). In patients who received concurrent d4T, a smaller reduction was seen (0.54 $\log_{10}$) suggesting that alovudine should not be combined with this NRTI [237, 238].

Side effects of alovudine are related to bone marrow damage, which includes liver toxicity and anaemia. Sundseth et al. [239] reported that FLT caused extensive DNA fragmentation and induced apoptosis in CEM cells, providing a possible mechanism for its toxicity. In early clinical trials, the most common side effects reported were anaemia and neutropaenia. However, despite no serious side effects in a phase II study, clinical development of alovudine has been put on hold.

**DOT, (2R,4R)-4-(thymidin-1-yl)-1,3-dioxolane-2-methanol or (–)-β-D-thymidine dioxolane.** DOT, a dioxolane-thymidine nucleoside analogue developed following the discovery of DXG activity, is in phase I clinical trials. Recent preliminary pharmacokinetic studies indicated that the bioavailability of DOT in monkeys and rats is close to 100% [240]. *In vitro*, DOT is active against WT HIV-1 and it is the first thymidine-kinase-activated nucleoside that is significantly active against all of the commonly found NRTI-resistant HIV-1 mutants [241]. DOT was found to be highly active against strains carrying the mutations D67N/K70R/T215Y/K219Q, K65R and M184V [242]. According to molecular modelling studies on DXG and DOT [214], the functional 3′-oxygen atom of the dioxolane moiety seems to play a significant role by mimicking the 3′-OH group of the natural substrate and stabilizing binding between the mutant M184V RT and the incoming dNTP.

**MIV-210: prodrug of FLG, 2′,3′-dideoxy-3′-fluoro-β-D-guanosine.** MIV-210 is in phase I clinical trials. When administered to healthy volunteers, it demonstrated a very good oral bioavailability and achieved high blood plasma levels of FLG (www.medivir.se/OuterFrameEng.asp.).

In cell culture, MIV-210 retains activity against HIV resistant to all anti-HIV agents in the clinic, including 3TC (www.medivir.se/OuterFrameEng.asp) [243]. Its activity against variants with TAMs was the same as against WT viruses. However, it was not effective against viruses with the Q151M mutation or T69S double insertions. Two variants, with modifications at positions 35 and 133, emerged from *in vitro* resistance induction studies. These mutants were resistant to MIV-210, and cross-resistant to 3TC, but not to AZT.

**KP1461: prodrug of KP1212, 5,6-dihydro-5-aza-2′-deoxycytidine.** KP1461, a 2′-deoxy-5-azacytidine analogue with a non-planar heterocyclic base, is currently under phase I clinical trials. Contrary to the other NRTIs (approved or under development), KP1212 does not inhibit HIV replication by blocking proviral DNA synthesis. This compound uses a different strategy and treats viral infections through lethal mutagenesis, an approach first developed by Loeb et al. [244] (see below). KP-1212 is incorporated randomly into the replicating viral genome, and increases the mutation rate of HIV by mispairing, resulting in defective viruses. This compound seems to have the appropriate safety and efficacy profiles to be used as an anti-HIV-1 agent, with an $EC_{50}$ of 10 nM and $CC_{50} \geq 1$ mM. There was no evidence of cross-resistance with HIV strains resistant to approved drugs or evidence of development of new resistant strains when using KP1212. Additionally, HIV strains treated with KP1212 show increased sensitivity towards KP1212 itself and AZT [245].

During the phase Ia trial, no consistent toxicity was noted. In addition, KP1212 does not increase the mutation frequency of a cellular gene (HGPT) in Chinese hamster ovary cells and male lymphoblasts, and it does not demonstrate evidence of significant genotoxicity or mitochondrial toxicity [245, 246]. However, a recent study suggested that even if exonucleases are able to remove KP1212 from cellular DNA, it may possibly present mitochondrial toxicity [247].

## New classes of anti-HIV nucleoside analogues

With the emergence of HIV strains resistant and/or cross-resistant to nearly all anti-retroviral regimens (NRTIs, NNRTIs and PIs), novel therapy approaches have to be considered. In our opinion, two interesting strategies have been reported in the field of anti-HIV nucleoside analogues: delayed polymerization arrest and selective viral mutagenesis.

**Delayed polymerization arrest.** In the context of the resistance to NRTIs described above, the development of nucleoside analogues without modification of the 3′ position of the ribose that are able to induce delayed polymerization arrest is quite attractive. As the compounds are very different from all FDA-approved NRTIs, little cross-resistance is expected. In addition, as they are not immediate chain terminators, they should be protected from phosphorolytic excision, which eliminates only the last nucleotide incorporated (fig. 5).

Several years ago, nucleoside analogues with a 3′-OH unmodified function were reported. Maag et al. [248] described 4′-azido-thymidine (4′-AZT) as a potent anti-HIV-1 agent *in vitro*, and Sugimoto et al. [249] and Kodama et al. [250] reported a series of 4′-ethynyl-2′-deoxynucleosides with activity against WT and multidrug-resistant HIV-1 strains and HIV-2. Even though the exact mechanism of action of these compounds has not yet been elucidated, several experiments strongly suggested that 4′-ethynyl-nucleoside analogues belong to the NRTI family [250]. In addition, Chen et al. [251] reported that viral DNA chain elongation is blocked after the incorporation of two consecutive 4′-AZT-MP molecules or two 4-AZT-MP separated by one natural 2′-deoxynucleoside-MP, possibly because of the structural distortion of the growing primer.

Toxicity studies indicated that 4′-substituted nucleosides are generally poor substrates for cellular DNA polymerases [250]. However, polymerases a and b incorporate a single 4′-AZT-MP molecule in host cell DNA without any effect on DNA elongation [251]. We can therefore suspect that this compound may produce long-term toxicity even if immediate cytotoxic effects are not detected.

In our opinion, 4′-substituted-2′-deoxy-nucleoside analogues are the precursors of a promising new class of anti-HIV agents: the delayed chain terminators. Like all first-generation molecules, they need improvements, particularly in their toxicity profiles, but they could lead to interesting progress in anti-HIV therapy.

It should be noted that some FDA-approved drugs targeting viruses other than HIV act by a mechanism of delayed termination. This is the case for the anti-VZV DNA polymerase inhibitor brivudin (BVDU) and the broad-spectrum anti-DNA virus DNA polymerase inhibitor cidofovir (HPMPC) [252]: BVDU is incorporated internally in the growing DNA chain thus leading to a reduced integrity and functioning of viral DNA, and HPMPC creates distortions in the nascent DNA following two consecutive incorporations.

Recently, Boyer and colleagues opted for another strategy to induce delayed chain termination. Close to the catalytic site, the DNA duplex is in the A form and the sugar moiety of the nucleotides is in the north (N) conformation [4, 253] The incoming nucleotide also adopts the N conformation [4]. However, six to seven nucleotides from the polymerase site, the DNA duplex undergoes a transition toward the B form, and the sugar rings of the nucleotides adopt the south (S) conformation [4, 253]. Thus, Boyer et al. [254] synthesized methanocarba-2′-deoxynucleosides locked in the N

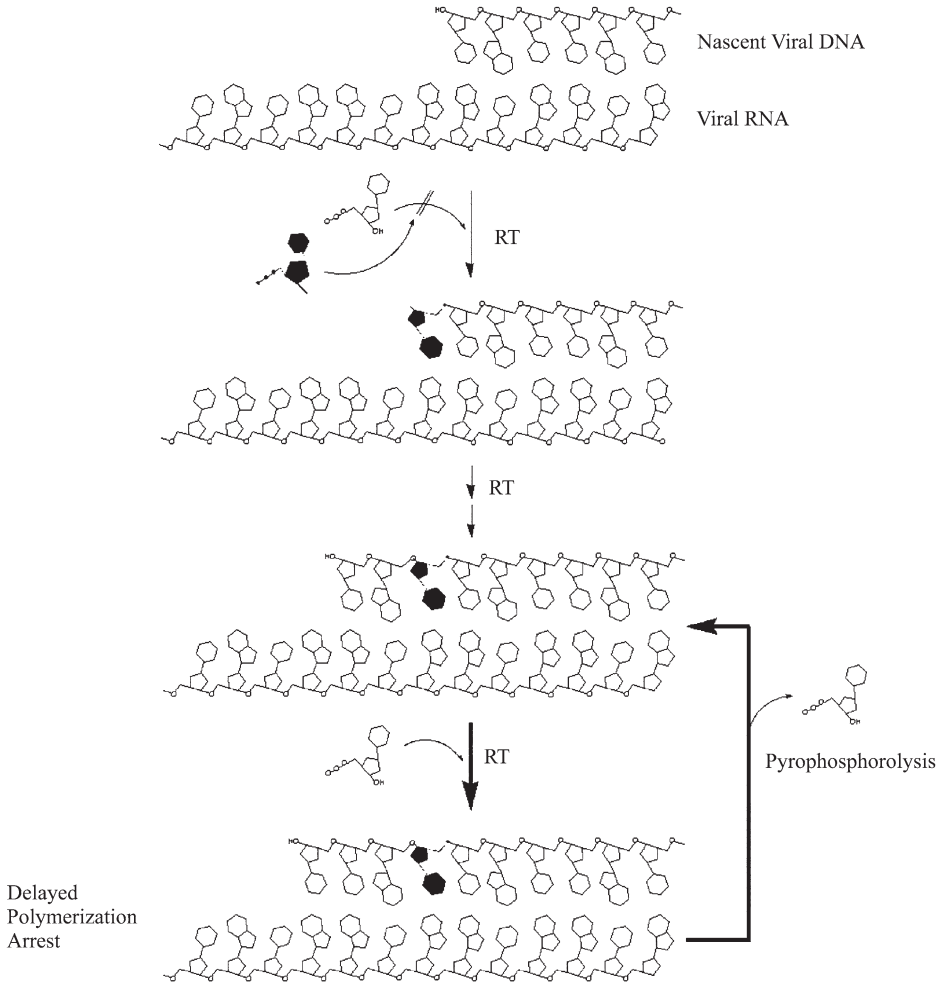



Figure 5. Inhibition of reverse transcription by 'delayed polymerization arrest' . The tri-phosphate dNTP analogue (in black) is incorporated into the growing DNA chain. Due to its non-modified 3′OH group on the ribose moiety, it allows incorporation of the next complementary nucleotides and induces a delayed polymerization arrest, most likely due to structural alterations of the primer/template duplex.

or S conformation. The N-locked nucleoside analogues were efficiently incorporated by RT and induced delayed chain termination, while the S-locked nucleoside analogues did not [254]. However, there is a drawback inherent to this strategy, since cellular kinases, unlike DNA polymerase, have a strong preference for substrates in the S conformation. Therefore, the N-locked methanocarba-2′-deoxynucleosides displayed anti-HIV activity only in cells expressing the thymidine kinase from the herpes simplex virus, which has a relaxed specificity [254].

**Selective viral mutagenesis or lethal mutagenesis.** In infected individuals, HIV mutates at a very high rate, approximately a million times greater than cellular DNA genome. While this genetic diversity allows viral subpopulations to escape conventional antiviral therapies, most of these mutations are probably lethal or crippling, which explains why most viral particles are not infectious. Selective viral mutagenesis (SVM) or lethal mutagenesis [246, 255, 256] relies on the poor fidelity of HIV-1 RT. Rather than causing chain termination and attempting to immediately halt viral replication, as with conventional NRTIs, the purpose of SVM agents is to be incorporated into the viral genome during replication and, by mispairing, to introduce mutations into the HIV genome to the point where the virus cannot replicate further [257]. The slight increase in mutations affects all viral proteins and cumulatively, over a number of replication cycles, is lethal to the virus. Since host cell DNA polymerization is quite faithful, with DNA repair processes able to eliminate errors in double-stranded DNA, the cellular toxicity should be minimal. DNA mutagenic nucleosides are currently being screened for activity against HIV, but they may have activity against hepatitis B and smallpox virus as well. In addition, there is some evidence that the anti-HCV drug ribavirin is an RNA virus mutagen [258–260].

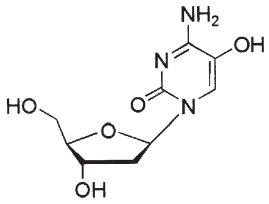



Figure 6. Structure of 5-hydroxy-deoxycytidine, a mutagenic nucleoside.

Loeb et al. [244] initially tested the hypothesis of lethal mutagenesis by culturing HIV-infected cells in the presence of several mutagenic deoxyribonucleoside analogues. In the presence of 5-hydroxy-deoxycytidine (fig. 6), they noted a sudden and dramatic loss of viral replication after sequential passages of HIV in human CEM cells. However, the concentration of 5-OH-dC required for abolition of viral infection (0.5–1.0 mM) was likely to be prohibitive and other mutagenic nucleosides were screened. More recently, Koronis Pharmaceuticals reported a novel SVM nucleoside with the appropriate safety and efficacy profiles, KP1212 (fig. 4). KP1212 and its prodrug KP1461 have the requisites for a new generation of antiretroviral drugs, including lack of cross-resistance with HIV strains resistant to approved drugs. This promising compound is under phase I clinical trials (see above).

## Conclusions

NRTIs are one of the major classes of inhibitors used in all combination therapies for the treatment of HIV-1-infected patients. Even though the current inhibitors considerably slow down the progression of the disease, selection of drug-resistant viral strains can cause drug failure. Considerable progress has been made in understanding the mechanisms of resistance of HIV-1 RT towards NRTIs at the molecular level. This knowledge is essential for several purposes. It allows the best combination of drugs to be selected, both to avoid antagonist effects, and to take advantage of synergistic activities. It is also crucial for the design of new compounds with better toxicity profiles and which can target resistant viruses. To this end, new nucleoside analogues are being developed, some of which are already in clinical trials. In addition, new strategies for inhibiting HIV-1 with nucleotide analogues are investigated. Some nucleoside analogues possessing a 3′ hydroxyl group induce 'delayed' polymerization arrest. These compounds are very different from all FDA-approved NRTIs, therefore little cross-resistance is expected. In addition, as they are not immediate chain terminators, they should be immune from phosphorolytic excision. A second new class of NRTIs is designed to be incorporated into the viral genome during replication and, by mispairing, introduce mutations into the HIV genome to the point where the virus cannot replicate further. This strategy of 'lethal mutagenesis' should be favourable in terms of cellular toxicity since host cell DNA polymerases possess proofreading activities. One such compound in currently under phase I clinical trial.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