The results of Table 5 support this speculation. In spite of more sites being detected under positive selection for between-host evolution for the recent group than for the early group, none of those sites was in the V1–V5 regions, suggesting that some mutations in these epitopes, although needed for a new infection in the 1980s, may have become fixed in the population in the 2000s. In a recent study, Kawashima et al. [43] demonstrated strong evidence of HIV adaptation to HLA at a population level. The authors pointed out that the process of viral adaptation might alter currently established HLA associations with slow disease progression to AIDS. Although *pol* and *gag* genes were analyzed in their study, it appears sensible to expect similar adaptive evolution to occur on the *env* gene as well. Collection of more clinical data may shed light on this issue.

While the size of our data for the early samples is relatively small, our search in the HIV Sequence Database [44] did not locate any full-length *env* genes collected in the 1980s from the Japanese population. Instead, we found 21 partial sequences covering V2, V3, C2 and part of C3 regions of the *env* gene. These sequences were sampled from two Japanese subjects in 1988: subject 9 (AB002885-AB002894) and subject 20 (AB002922-AB002932) [45]. Subject 9 was infected through HIV contaminated blood products during 1983–1985 and subject 20 was infected through sexual transmission in 1986. Those partial sequences were aligned with the same region of our 142 full-length *env* genes using ClustalW to form a new dataset of 163 sequences, each of 693 bp. This V2–V3 dataset was analyzed in the same way as the dataset for the full-length *env* genes. The phylogeny was reconstructed using the NJ method, which was further used to detect positive selection using the branch-site and extended clade models. The reconstructed phylogenetic tree showed that the sequences obtained from each subject most tightly clustered together and were divergent from those of other subjects (Fig. S1). The codon-based analysis produced similar results to those from the full-length dataset. For example, the branch-site model suggested that the positive selection pressure along the 2000s-between branches ($\omega_6 = 3.462$) was significantly weaker than for 1980s-between ($\omega_5 = 7.124$), with $2\Delta\ell = 6.24$. However, the V2–V3 dataset is less informative due to its smaller size (231 codons vs. 926 codons in the full-length dataset), and did not lead to significant results in some tests (results not shown).

The present study also highlights the utility of the branch-site and extended clade models in analysis of viral samples when the viral gene may be under different positive selection pressures during different time periods or in different viral populations. By using pre-specified branch types, to represent viral evolution within hosts and transmission between hosts, we can estimate ω ratios for different branch types and test potential differences in selective pressure during different periods of viral evolution. Notably, the extended clade model appears to be considerably useful to detect positive selection in the case that multiple viral lineages in the phylogeny may have been evolving under different selective pressures. In our study, the model performed well in both analysis of the full-length gene and the V2–V3 region datasets.

Materials and Methods

Viral samples

All the blood samples were preserved in the National Institute of Infectious Diseases (NIID) in Tokyo. Transmission routes and sampling times were available for all subjects but detailed clinical information was unavailable for the subjects collected in 1980s (Table 1). All subjects were Japanese and did not receive anti-HIV therapy. Both PBMC and blood plasma were available for early samples (continuously stored at -130°C), whereas blood plasma only was available for recent samples. This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of the NIID. All patients provided written informed consent for the collection of samples and subsequent analysis.

Sequencing and phylogenetic analysis

Proviral DNA was extracted from PBMC using the QIAamp DNA Blood Mini Kit. Viral RNA was extracted from blood plasma using the MagNa Pure Compact Nucleic Acid Isolation Kit I (Roche Applied Science). RNA products were used for cDNA synthesis, using Superscript II RT Kit and random hexamer (Invitrogen).

DNA products were sequenced using the Single-Genome Sequencing (SGS) method (25). For full-length *env* gene amplification, they were PCR amplified using a set of primers (Env-6116F: 5′ - gcaatagttgtgtggwcyatag -3′ and Env-1M: 5′ - tagcccttccagtccccc-cttttctttta - 3′) followed by nested amplification (Env-SF1: 5′ - ctaa-tagaaagagcagaagacagtgg -3′ and Env-PR2: 5′ - gctsccttrtaagt-cattggtct -3′). The PCR products sequenced with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems).

To identify the subtypes of the viral samples sequenced in this study, we used Subtype Reference Alignments [18]. We also tested for recombination using the program RIP 3.0 (Recombinant Identification Program) available at the same Website. Moreover, since the infection time of some subjects was unknown (Table 1), we used WebPSSM [46] to predict the co-receptor usage, as it has been known that CCR5-using viruses are important during early stages of the HIV infection, whereas CXCR4-using viruses emerge later in the progression to AIDS [47]. The default option, x4r5 matrix, was used for the prediction.

The sequences were aligned using ClustalW. First, the 142 nucleotide sequences were translated into amino acid sequences using MEGA4, which were aligned using ClustalW implemented in MEGA4. Then the aligned amino acid sequences were used to construct the nucleotide alignment. There are 926 codons (2778 bp) in the alignment. We used the software FindModel [48] to find the appropriate substitution model. Models GTR+G, HKY+G, K80+G and TrN+G were suggested to have better AIC scores and likelihoods. Then we reconstructed the phylogenetic tree using the ML method (PhyML) [21] under GTR+G and the NJ method (MEGA4) [19] under K80+G [20] (the GTR+G model is not available in MEGA4). As the resultant trees were similar, we used the NJ tree for further analysis. However, the ML tree under the GTR+G model was used to duplicate the analyses to confirm the robustness of our results to possible topological errors.

Inferring the genetic diversity of the envelope gene

To investigate the diversity change, we inferred within- and between-host mean diversities for the 1980s and the 2000s, respectively, using the nucleotide diversity, π, implemented in MEGA4 under the K80+G model again. As the sequences are highly similar, the choice of the substitution model makes little difference to the distance calculation. For Setup Data, the viral sequences obtained from the same subject were grouped as one subpopulation. Then, the within-host diversity was calculated by Mean Diversity within Subpopulations, whereas the between-host diversity was calculated by Mean Interpopulational Diversity.

Detecting temporal changes of positive selection pressure using codon models

We used codon models of coding sequence evolution to detect positive selection operating on the HIV-1 *env* gene. In particular, we are interested in differences in positive selection pressure on the

virus between the 1980s and the 2000s, and between within-host evolution and between-host evolution. We use the branch-site model [23,49] to detect such temporal changes in positive selection pressure, as implemented in the CODEML program in the PAML package [25]. The likelihood ratio test compares the branch-site model A with a null model that fixes $\omega_2 = 1$, with one degree of freedom used. This phylogeny-based analysis assumes that there is no recombination with the sequence so that all sites in the sequence are related by the same phylogenetic tree and that the substitution process has been stationary. The codon frequencies are described using the F3×4 model, which uses the nucleotide frequencies at the three codon positions to describe the codon usage, with 9 free parameters used. The frequency parameters are estimated using the frequencies observed in the data. The branch-site model makes rather stringent assumptions about the selective pressures acting on the foreground and background branches. Simulations, however, suggest that the test is quite robust if the real selective scheme is far more complex [23].

The assumption, made in the branch-site model, of no positive selection on the background branches and of only two branch types may be too restrictive for the HIV-1 *env* genes. Thus we extend the clade model C of Bielawski and Yang [26] to allow for more than two branch types. Clade model C was developed to detect divergent evolution in two clades (two branch types): with site class 0 to include conserved sites with $\omega_0<1$, class 1 to be neutral sites with $\omega_1 = 1$ and class 2 represent sites undergoing divergent evolution with different selective pressures (ω_2 and ω_3) in the two clades. We extend this model to allow for more than two branch types, which have different rate ratios ω_2 and ω_3, ..., for site class 2, where $0<\omega_2, \omega_3, \ldots<\infty$. The model thus accounts for conserved sites, nearly neutral sites, as well as positive-selection sites that are under different levels of selection among the branch types. We have also extended the Bayes empirical Bayes (BEB) method to calculate the posterior probabilities for the site classes when there are more than two branch types [24]. The extended clade model has been implemented in the CODEML program in the PAML package [25]. For the analysis of our data, we used five branch types: 1980s-within, 2000s-within, 1980s-between, and 2000s-between, with all other branches grouped into one branch type.

Supporting Information

Figure S1 Unrooted phylogenetic tree of 163 V2–V3 region sequences from 16 Japanese subjects. The tree was constructed by using the neighbour-joining method (MEGA4). The 2 subjects found in the database are indicated as "S_88JP9" and "S_88JP20" respectively. The red represents viral samples from the 1980s group while the blue represents those from the 2000s group.
(TIF)

Figure S2 The foreground branches in the different analyses under the branch-site model. The tree topology is the same as in Fig. 1, but different foreground branches are assumed in four analysis: (a) within-1980s (red), (b) within-2000s (blue), (c) between-1980s (red), and (d) between-2000s (blue).
(TIF)

Table S1 Log-likelihood values and parameter estimates under the branch-site models using the Fcodon model (CodonFreq = 3).
(DOC)

Table S2 Log-likelihood values and parameter estimates under the clade model using the Fcodon model (CodonFreq = 3).
(DOC)

Table S3 Log-likelihood values and parameter estimates under the branch-site models using GTR+G model (CodonFreq = 2).
(DOC)

Table S4 Log-likelihood values and parameter estimates under the clade model using GTR+G model (CodonFreq = 2).
(DOC)

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Author Contributions

Conceived and designed the experiments: IY WS FR ZY HT. Performed the experiments: IY JS WS. Analyzed the data: IY FR ZY HT. Contributed reagents/materials/analysis tools: IY JS WS ZY FR HT. Wrote the paper: FR ZY IY WS JS.

References

1. Crum-Cianflone N, Eberly L, Zhang Y, Ganesan A, Weintrob A, et al. (2009) Is HIV becoming more virulent? Initial CD4 cell counts among HIV seroconverters during the course of the HIV epidemic: 1985–2007. Clin Infect Dis 48: 1285–1292.
2. Dorrucci M, Phillips AN, Longo B, Rezza G (2005) Changes over time in post-seroconversion CD4 cell counts in the Italian HIV-Seroconversion Study: 1985–2002. Aids 19: 331–335.
3. Dorrucci M, Rezza G, Porter K, Phillips A (2007) Temporal trends in postseroconversion CD4 cell count and HIV load: the Concerted Action on Seroconversion to AIDS and Death in Europe Collaboration, 1985–2002. J Infect Dis 195: 525–534.
4. Gali Y, Berkhout B, Vanham G, Bakker M, Back NK, et al. (2007) Survey of the temporal changes in HIV-1 replicative fitness in the Amsterdam Cohort. Virology 364: 140–146.
5. Muller V, Maggiolo F, Suter F, Ladisa N, De Luca A, et al. (2009) Increasing clinical virulence in two decades of the Italian HIV epidemic. PLoS Pathog 5: e1000454.
6. Vanhems P, Lambert J, Guerra M, Hirschel B, Allard R (1999) Association between the rate of CD4+ T cell decrease and the year of human immunodeficiency virus (HIV) type 1 seroconversion among persons enrolled in the Swiss HIV cohort study. J Infect Dis 180: 1803–1808.
7. Herbeck JT, Gottlieb GS, Li X, Hu Z, Detels R, et al. (2008) Lack of evidence for changing virulence of HIV-1 in North America. PLoS One 3: e1525.
8. Muller V, Ledergerber B, Perrin L, Klimkait T, Furrer H, et al. (2006) Stable virulence levels in the HIV epidemic of Switzerland over two decades. Aids 20: 889–894.
9. Arien KK, Troyer RM, Gali Y, Colebunders RL, Arts EJ, et al. (2005) Replicative fitness of historical and recent HIV-1 isolates suggests HIV-1 attenuation over time. Aids 19: 1555–1564.
10. Keet IP, Veugelers PJ, Koot M, de Weerd MH, Roos MT, et al. (1996) Temporal trends of the natural history of HIV-1 infection following seroconversion between 1984 and 1993. Aids 10: 1601–1602.
11. Arien KK, Vanham G, Arts EJ (2007) Is HIV-1 evolving to a less virulent form in humans? Nat Rev Microbiol 5: 141–151.
12. Holmes EC, Zhang LQ, Simmonds P, Ludlam CA, Brown AJ (1992) Convergent and divergent sequence evolution in the surface envelope glycoprotein of human immunodeficiency virus type 1 within a single infected patient. Proc Natl Acad Sci U S A 89: 4835–4839.
13. Leitner T, Kumar S, Albert J (1997) Tempo and mode of nucleotide substitutions in gag and env gene fragments in human immunodeficiency virus type 1 populations with a known transmission history. J Virol 71: 4761–4770.
14. Korber B, Muldoon M, Theiler J, Gao F, Gupta R, et al. (2000) Timing the ancestor of the HIV-1 pandemic strains. Science 288: 1789–1796.
15. Ross HA, Rodrigo AG (2002) Immune-mediated positive selection drives human immunodeficiency virus type 1 molecular variation and predicts disease duration. J Virol 76: 11715–11720.
16. Rambaut A, Posada D, Crandall KA, Holmes EC (2004) The causes and consequences of HIV evolution. Nat Rev Genet 5: 52–61.
17. Nickle DC, Heath L, Jensen MA, Gilbert PB, Mullins JI, et al. (2007) HIV-specific probabilistic models of protein evolution. PLoS ONE 2: e503.

18. Leitner TBK, Marcus Daniels, Charles Calef, Brian Foley (2005) HIV-1 Subtype and Circulating Recombinant Form (CRF) Reference Sequences, 2005. Los Alamos National Laboratory, Los Alamos, NM.
19. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.
20. Kimura M (1980) A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J Mol Evol 16: 111–120.
21. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704.
22. Yang Z, Nielsen R (2002) Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. Mol Biol Evol 19: 908–917.
23. Zhang J, Nielsen R, Yang Z (2005) Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. Mol Biol Evol 22: 2472–2479.
24. Yang Z, Wong WS, Nielsen R (2005) Bayes empirical bayes inference of amino acid sites under positive selection. Mol Biol Evol 22: 1107–1118.
25. Yang Z (2007) PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol 24: 1586–1591.
26. Bielawski JP, Yang Z (2004) A maximum likelihood method for detecting functional divergence at individual codon sites, with application to gene family evolution. J Mol Evol 59: 121–132.
27. Bielawski JP, Yang Z (2003) Maximum likelihood methods for detecting adaptive evolution after gene duplication. J Struct Funct Genomics 3: 201–212.
28. HIV Molecular Immunology Database website. Available: http://www.hiv.lanl.gov/content/immunology/. Accessed: 2010 March 3.
29. Tokunaga K, Ishikawa Y, Ogawa A, Wang H, Mitsunaga S, et al. (1997) Sequence-based association analysis of HLA class I and II alleles in Japanese supports conservation of common haplotypes. Immunogenetics 46: 199–205.
30. Yap VB, Lindsay H, Easteal S, Huttley G (2010) Estimates of the effect of natural selection on protein-coding content. Mol Biol Evol 27: 726–734.
31. Goldman N, Yang Z (1994) A codon-based model of nucleotide substitution for protein-coding DNA sequences. Mol Biol Evol 11: 725–736.
32. Goudsmit (1998) Viral Sex: The Nature of AIDS OUP USA; New Ed edition.
33. Yamaguchi Y, Gojobori T (1997) Evolutionary mechanisms and population dynamics of the third variable envelope region of HIV within single hosts. Proc Natl Acad Sci USA 94: 1264–1269.
34. Yamaguchi-Kabata Y, Gojobori T (2000) Reevaluation of amino acid variability of the human immunodeficiency virus type 1 gp120 envelope glycoprotein and prediction of new discontinuous epitopes. J Virol 74: 4335–4350.
35. Kupfer B, Sing T, Schuffler P, Hall R, Kurz R, et al. (2007) Fifteen years of env C2V3C3 evolution in six individuals infected clonally with human immunodeficiency virus type 1. J Med Virol 79: 1629–1639.
36. Draenert R, Allen TM, Liu Y, Wrin T, Chappey C, et al. (2006) Constraints on HIV-1 evolution and immunodominance revealed in monozygotic adult twins infected with the same virus. J Exp Med 203: 529–539.
37. Ohnishi Y, Tanaka T, Yamada R, Suematsu K, Minami M, et al. (2000) Identification of 187 single nucleotide polymorphisms (SNPs) among 41 candidate genes for ischemic heart disease in the Japanese population. Hum Genet 106: 288–292.
38. Yamada R, Tanaka T, Ohnishi Y, Suematsu K, Minami M, et al. (2000) Identification of 142 single nucleotide polymorphisms in 41 candidate genes for rheumatoid arthritis in the Japanese population. Hum Genet 106: 293–297.
39. Haga H, Yamada R, Ohnishi Y, Nakamura Y, Tanaka T (2002) Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190,562 genetic variations in the human genome. Single-nucleotide polymorphism. J Hum Genet 47: 605–610.
40. Kruglyak L, Nickerson DA (2001) Variation is the spice of life. Nat Genet 27: 234–236.
41. Friedrich TC, Dodds EJ, Yant LJ, Vojnov L, Rudersdorf R, et al. (2004) Reversion of CTL escape-variant immunodeficiency viruses in vivo. Nat Med 10: 275–281.
42. Knipe D, Howley PM Fields Virology: Lippincott Williams & Wilkins; Fifth Edition, Volume one, page 331–333.
43. Kawashima Y, Pfafferott K, Frater J, Matthews P, Payne R, et al. (2009) Adaptation of HIV-1 to human leukocyte antigen class I. Nature 458(7238): 641–645.
44. HIV Sequence Database website. Available: http://www.hiv.lanl.gov/. Accessed 2010 Jul 5.
45. Shioda T, Oka S, Xin X, Liu H, Harukuni R, et al. (1997) In vivo sequence variability of human immunodeficiency virus type 1 envelope gp120: association of V2 extension with slow disease progression. J Virol 71: 4871–4881.
46. Jensen MA, Li FS, van 't Wout AB, Nickle DC, Shriner D, et al. (2003) Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences. J Virol 77: 13376–13388.
47. Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR (1997) The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc Natl Acad Sci U S A 94: 1925–1930.
48. FindModel website. Available: http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html. Accessed: 2010 Sep 18.
49. Yang Z, Rannala B (2005) Branch-length prior influences Bayesian posterior probability of phylogeny. Syst Biol 54: 455–470.

Review
Future Microbiology

Clinical significance of HIV reverse-transcriptase inhibitor-resistance mutations

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In this article, we summarize recent knowledge on drug-resistance mutations within HIV reverse transcriptase (RT). Several large-scale HIV-1 genotypic analyses have revealed that the most prevalent nucleos(t)ide analog RT inhibitor (NRTI)-resistance mutation is M184V/I followed by a series of thymidine analog-associated mutations: M41L, D67N, K70R, L210W, T215Y/F and K219Q/E. Among non-nucleoside RT inhibitor (NNRTI)-resistance mutations, K103N was frequently observed, followed by Y181C and G190A. Interestingly, V106M was identified in HIV-1 subtype C as a subtype-specific multi-NNRTI-resistance mutation. Regarding mutations in the HIV-1 RT C-terminal region, including the connection subdomain and RNase H domain, their clinical impacts are still controversial, although their effects on NRTI and NNRTI resistance have been confirmed *in vitro*. In HIV-2 infections, the high prevalence of the Q151M mutation associated with multi-NRTI resistance has been frequently observed.

More than 25 years have passed since the discovery of HIV-1 and HIV-2 as the pathogens of AIDS [1–4]. According to the latest report from the United Nations Programme on HIV/AIDS, the number of HIV infections worldwide in 2008 was estimated at 33.4 million, with 2.7 million new infections and 2.0 million HIV-related deaths [201]. The data clearly indicate that HIV/AIDS still remains a major worldwide health issue.

In the absence of an effective vaccine, the only hope for HIV-infected individuals has been antiretroviral chemotherapy. The prognosis of HIV-infected patients has been greatly improved by the advent of HAART, a combination therapy of at least three antiretrovirals [5,6]. However, the virus has not been eradicated owing to the persistence of latently infected $CD4^+$ T cells, which are recognized as a viral reservoir [7–9]. Therefore, after HAART is initiated, therapy should be continued throughout the patient's lifetime to maintain undetectable viral replication [10]. However, lifelong treatment is a challenge owing to obstacles, such as adverse effects of HAART and the emergence of drug-resistant viruses.

In this article, we focus on the first developed class of antiretroviral drugs and the backbone of HAART, HIV reverse transcriptase (RT) inhibitors. To date, 11 RT inhibitors are available, of which the latest (approved in 2008) is etravirine (Table 1). We review the recent knowledge on drug-resistance mutations within HIV RT and discuss their clinical significance, molecular mechanisms and differences in HIV-1 subtypes or in HIV-2 infections.

Major characteristics of HIV-1 RT

HIV-1, a plus-strand RNA virus, is a member of lentiviruses within the *Retroviridae* family. After the viruses attach and enter into targeted $CD4^+$ cells, viral genomic ssRNAs are converted to dsDNAs by HIV-1 RT through reverse transcription, the processes of RNA-dependent DNA polymerization, template RNA digestion and DNA-dependent DNA polymerization.

HIV-1 RT is well characterized as an error-prone DNA polymerase owing to the lack of exonucleolytic proofreading activity [11,12], and the HIV-1 mutation rate has been estimated at 1.4×10^{-5}–5.4×10^{-5} mutations/base pair/cycle in cell assay systems [13–17]. In addition to this high mutation rate, the robust production of viral particles, at least 1.03×10^{10} virions/day/individual, enables HIV-1 to generate sufficient genetic diversity to evade host immune responses and antiretroviral drug pressure [18,19]. HIV-1 RT is a heterodimer enzyme with two related subunits, p66 and p51, produced from gag-pol polyproteins through proteolytic cleavages (Figure 1) [20–22]. Whereas p66 (residues 1–560) contains both a DNA polymerase domain and an RNase H domain and exhibits enzymatic activities, p51 (residues 1–440) contains only

Keywords

▪ antiretroviral ▪ drug resistance ▪ HIV-1 ▪ HIV-2 ▪ inhibitor ▪ mutation ▪ reverse transcriptase ▪ review ▪ subtype

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Table 1. Approved antiretroviral drugs for treatment in HIV infections[†].

Generic name	Trade name	Approval (year)	Note
NRTIs			
Zidovudine	Retrovir®	1987	
Didanosine	Videx	1991	
Zalcitabine	Hivid	1992	Discontinued in 2006
Stavudine	Zerit	1994	
Lamivudine	Epivir	1995	
Abacavir	Ziagen	1998	
Tenofovir	Viread®	2001	Supplied as a prodrug, tenofovir disoproxil fumarate
Emtricitabine	Emtriva®	2003	
NNRTIs			
Nevirapine	Viramune®	1996	
Delavirdine	Rescriptor	1997	
Efavirenz	Sustiva®, Stocrin	1998	
Etravirine	Intelence®	2008	
Protease inhibitors			
Saquinavir	Invirase	1995	
Indinavir	Crixivan®	1996	
Ritonavir	Norvir	1996	
Nelfinavir	Viracept®	1997	
Amprenavir	Agenerase®	1999	Discontinued in 2007
Lopinavir/ritonavir	Kaletra®, Aluvia®	2000	
Atazanavir	Reyataz®	2003	
Fosamprenavir	Lexiva®, Telzir	2003	Prodrug of amprenavir
Tipranavir	Aptivus®	2005	
Darunavir	Prezista®	2006	
Integrase inhibitors			
Raltegravir	Isentress®	2007	
Fusion & entry inhibitors			
Enfuvirtide	Fuzeon®	2003	
Maraviroc	Selzentry, Celsentri®	2007	

[†]Drugs combined with NRTIs or NRTIs/NNRTIs are not listed.
NNRTIs: Non-nucleoside reverse-transcriptase inhibitors; NRTIs: Nucleos(t)ide analog reverse-transcriptase inhibitors.

the DNA polymerase domain and functions as a structural component. Specifically, the DNA polymerase domain contains four subdomains: fingers, palm, thumb and connection (Figure 1). The fingers, palm and thumb subdomains of p66 form a large cleft for binding to a template-primer, the palm subdomain of p66 has the DNA polymerase catalytic site defined as a triad of aspartic acid residues at positions 110, 185 and 186 [20–22], and the outer part of the fingers subdomain (fingertips) functions as a crucial part of the deoxynucleotide triphosphate (dNTP)-binding site [22].

Since the discovery of HIV-1, RT has been recognized a key target for developing antiretroviral therapy (ART). All 12 approved RT inhibitors belong to one of two classes, nucleos(t)ide analog RT inhibitors (NRTIs) or non-nucleoside RT inhibitors (NNRTIs). The two classes of RT inhibitors differ distinctly in the mechanisms by which they inhibit RT and in inducible patterns of drug-resistance amino acid mutations.

NRTIs & their mechanism of RT inhibition

Many of the eight NRTIs developed from 1987 to 2003 are still used today. They are zidovudine [23], didanosine [24], zalcitabine [24], stavudine [25], lamivudine [26], abacavir [27], tenofovir [28] and emtricitabine [29] (Figure 2). Among these RT inhibitors, seven are nucleoside analogs, whereas tenofovir is a nucleotide analog RT inhibitor. This drug has a unique acyclic structure linked with a phosphate group, and thus requires only two phosphorylation steps for conversion to its active 5′-triphosphate form [28]. Today, zalcitabine is not available, and a few

drugs are less frequently used; however, most are recommended as a backbone combination of two NRTIs in the latest first-line regimens [202–204].

As for the mechanism by which NRTIs inhibit RT, the drugs are first metabolized inside cells and then converted to their active 5′-triphosphate forms, which compete with the natural substrates (dNTPs) for HIV RT and inhibit its DNA polymerization [30–32]. In addition, after the drugs are incorporated into a growing viral DNA, they function as a chain terminator owing to lack of a 3′-hydroxyl group (present in natural dNTPs) necessary for further DNA synthesis (Figure 2) [32].

NRTI-resistance mutations & their mechanism of drug resistance

In 1989, drug-resistant HIV-1 was isolated from patients treated with zidovudine [33,34], leading to identification of the first cluster of drug-resistance amino acid mutations (D67N, K70R, T215F/Y and K219Q) in RT [35]. Thereafter, no antiretroviral has been found to successfully avoid the emergence of drug-resistant variants. According to the latest list of drug-resistance mutations for HIV clinical practitioners [36], 16 amino acid positions associated with NRTI resistance are mapped in the 3D structure of HIV-1 RT (Figure 3). Interestingly, all 16 amino acid positions are located in the fingers and palm subdomains of RT, which surround the incoming nucleotide [22].

The most prevalent NRTI-resistance mutation recently identified in large-scale analyses of drug-resistant viral genotypes was the M184V/I mutation (>38%, M184V predominant) [37–40]. This high prevalence of the M184V/I mutation in therapy-failure cases was explained by the frequent first-line therapy use of lamivudine, emtricitabine and abacavir (drugs corresponding to the M184V/I mutation). In addition, acquisition of the M184V mutation alone is sufficient for the virus to exhibit high-level resistance to both lamivudine and emtricitabine [41–43]. The M184V/I mutation was first identified in the course of viral passages with lamivudine or emtricitabine, and simultaneously characterized as the mutation that restored susceptibility of zidovudine-resistant variants to zidovudine [43,44]. While the M184V-mediated zidovudine resensitization was confirmed *in vivo* during 6 months of zidovudine plus lamivudine therapy [41], the resensitization was transiently observed in most zidovudine-experienced patients (69%, 20/29) during 1 year of combination therapy [45]. Furthermore, M184V-containing RT was shown in most biochemical studies to have drastically reduced binding activity to lamivudine- or emtricitabine-5′-triphosphate [46–49]. Concerning the molecular mechanism of resistance, structural studies show that the M184V/I mutation creates steric hindrance that interferes with lamivudine-5′-triphosphate binding to the active site of RT [22,50].

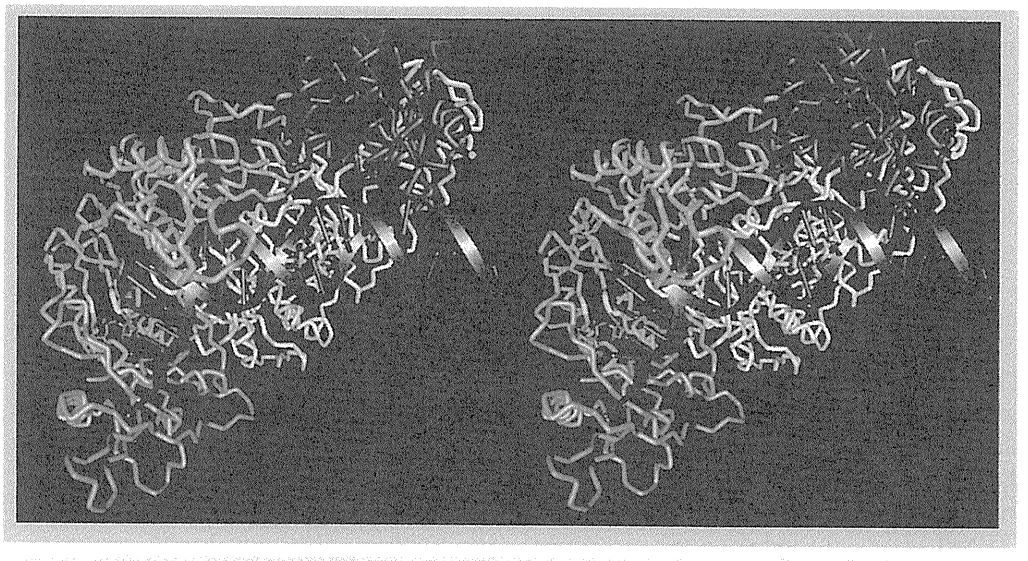

Figure 1. Stereogram for the 3D structure of HIV-1 reverse transcriptase in complex with a DNA template-primer and dTTP. The p66 subunit of HIV-1 reverse transcriptase has two domains: a DNA polymerase domain (with fingers, palm, thumb and connection subdomains) and an RNase H domain. The four subdomains and the RNase H domain are shown in blue, green, light purple, orange and brown, respectively. The p51 subunit is shown in white. The DNA template-primer and dTTP are shown in dark gray and magenta, respectively. A triad of aspartic acid residues at positions 110, 185 and 186 within the DNA polymerase catalytic site are shown in yellow.
dTTP: Deoxythymidine-5′-triphosphate.
This figure was prepared from the Protein Data Bank ID code 1RTD [22].

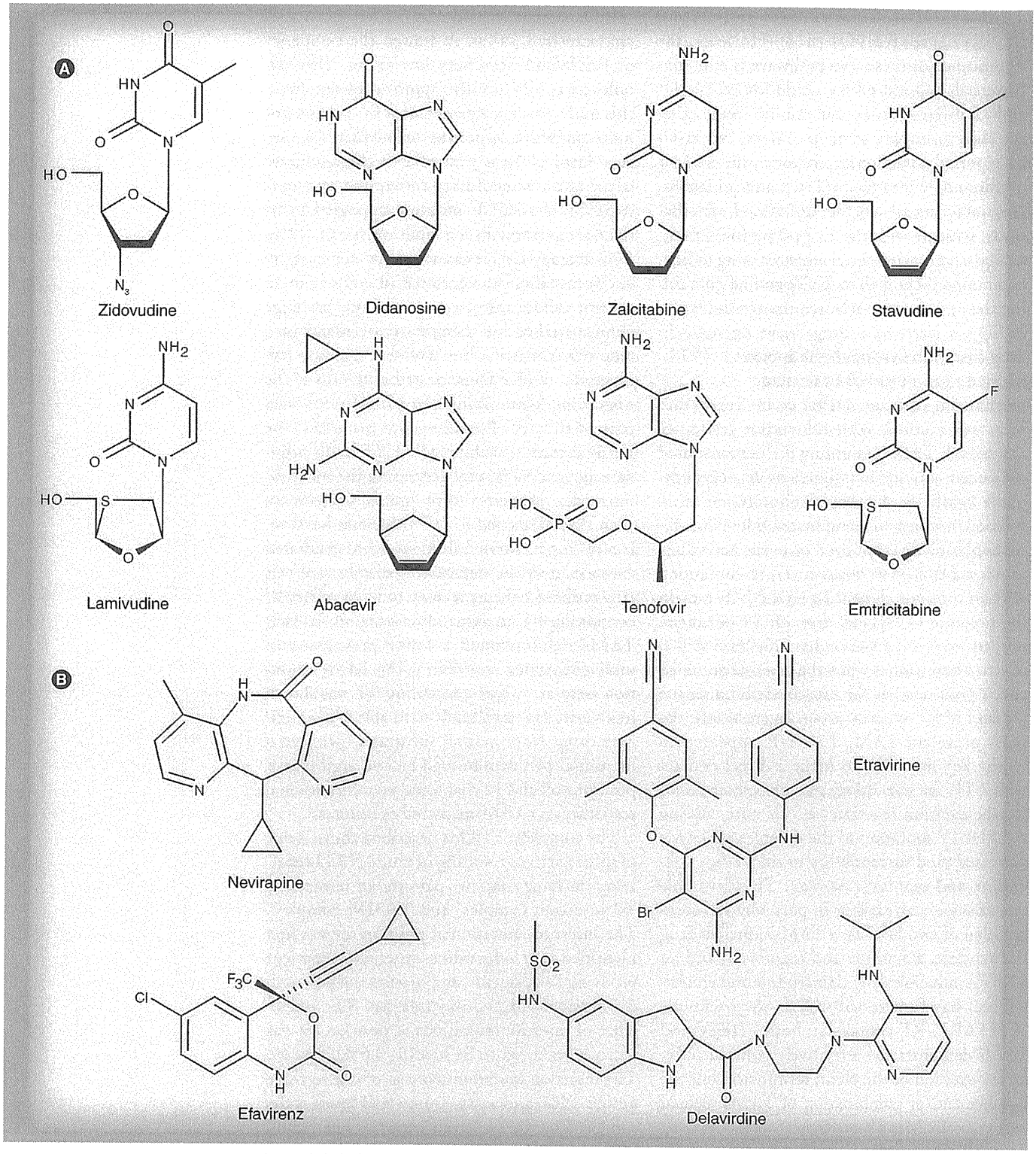

Figure 2. HIV-1 reverse-transcriptase inhibitors. (A) Structures of eight nucleos(t)ide analog reverse-transcriptase inhibitors are shown. Tenofovir is the only nucleotide analog, and the others are nucleoside analogs. **(B)** Structures of four non-nucleoside reverse-transcriptase inhibitors are shown.

The second most prevalent NRTI-resistance mutation in drug-resistant viral genotypes was identified as a series of thymidine analog-associated mutations (TAMs): M41L, D67N, K70R, L210W, T215Y/F and K219Q/E [37–40]. The prevalence of TAMs ranges from 19 to 42% [37], 7–20% [38] and 13–25% [40], and T215Y/F commonly shows the highest prevalence. Focusing on the T215 mutation patterns, several studies proposed two different TAM pathways:

TAM-1 (M41L+L210W+T215Y) and TAM-2 (K70R+T215F+K219Q) [51–55]. However, the significance of these two pathways is still controversial, and indeed the definition of TAM-1 and -2 differs among the studies. Regarding the clinical impact of the pathways, two studies reported a relationship between the TAM-1 profile and poor virological responses to tenofovir-containing salvage therapy [53,54]. In another study, patients with the TAM-2 profile demonstrated a better virological response to stavudine-containing therapy than to zidovudine-containing therapy [55]. Importantly, the accumulation of TAMs leads to a progressive decrease in drug susceptibility for all the approved NRTIs, referred to as multi-NRTI resistance [56,57]. The mechanism of drug resistance by TAMs has been well studied, with zidovudine resistance as a model. TAM-containing RT demonstrated enhanced activity for dinucleoside polyphosphate synthesis, a pyrophosphorolysis-related reaction in which an incorporated zidovudine-5′-monophosphate is removed from the end of terminated DNA [58,59] (Figure 4). TAM-containing RT can continue elongating viral DNA, even in the presence of NRTIs, through a mechanism termed 'excision'. Excision has only been shown in structural studies when the chain-terminated end is positioned at the nucleotide-binding site (N site) of RT (Figure 4) [60,61]. Interestingly, the most prevalent TAM, T215Y/F, appeared to be the key mutation to make a direct contact with ATP, the most likely pyrophosphate donor for the excision reaction [58]. Of note, adding the M184V mutation to the complex of TAMs increased viral susceptibility to zidovudine, stavudine and tenofovir [56,62,63]. This favorable interference may explain in part why the combination of two NRTIs, a TAM-inducible drug (zidovudine, stavudine and tenofovir), and an M184V-inducible drug (lamivudine and emtricitabine) has functioned well as the backbone of HAART. RT containing both M184V and TAMs demonstrated a relatively reduced activity for excision of the chain terminator, but the number and/or combination of TAMs seemed to impact the M184V-mediated increase in drug susceptibility [64–66].

Most of the other NRTI-resistance mutations were identified as low prevalence (<5%) [37–40]. Among these, the K65R mutation (prevalence: 2.5–4.9%) should be noted owing to its interesting characteristics. K65R was first identified as a mutation conferring resistance to didanosine, zalcitabine, lamivudine [67,68], and its wide spectrum of cross-resistance to all approved NRTIs except for zidovudine has been well characterized [69–73]. Although the frequency of K65R had been very low among therapy-failure cases before the approval of tenofovir, this mutation was recently reconfirmed as a primary mutation conferring tenofovir resistance. In a large clinical trial of 299 therapy-naive patients on a tenofovir/lamivudine/efavirenz regimen, the K65R mutation appeared to be the only pathway to tenofovir resistance (17%, 8/47 therapy-failure cases) [74]. By contrast, no K65R mutation was detected in any of the 12 therapy-failure cases reported in another large clinical trial of 258 therapy-naive patients on a tenofovir/emtricitabine/efavirenz regimen [75]. Since RT residue 65 is located at the tip of the fingers subdomain, which functions as a crucial part of the dNTP-binding site (Figure 3C), the K65R mutation enables HIV-1 RT to discriminate against NRTIs by decreasing the incorporation rate of their 5′-triphosphate forms more than that of natural dNTP substrates [49,76–79]. Interestingly, K65R inhibited TAM-mediated excision, and the decreased excision rate was responsible for the hypersusceptibility of K65R-containing RT to zidovudine [73,79–82]. In fact, TAMs demonstrated a lower prevalence in viral genotypes containing the K65R mutation [40,82–86]. K65R-containing RT was shown in a recent structural study to be able to discriminate drugs from natural substrates owing to a molecular platform formed by two arginines at positions 65 and 72 that force a conformational restriction on TAM-mediated excision [87].

The complex of TAMs described above is one of three patterns resulting in multi-NRTI resistance; the remaining two patterns are termed the '69 insertion complex' and 'Q151M complex'. The insertion mutation at position 69 was first identified in a zidovudine-experienced patient receiving subsequent combination therapy with didanosine and hydroxyurea [88]. The prevalence of insertion mutations at position 69 was recently reported to be low (0–0.6%) [37,38,40]. The insertion mutations consist of one to three amino acids, and a two-amino acid insert is the most frequently observed pattern. The region with the 69th amino acid, the tip of the fingers subdomain, appears to be structurally vulnerable for acquiring insertion mutations. Interestingly, higher numbers of insertions have been reported, ranging from four to 11 amino acids [89,90]. Usually, the insertion mutation accompanies other NRTI-resistance mutations, such as M41L, A62V, K70R, L210W, T215Y/F and K219Q/E. This 69 insertion complex

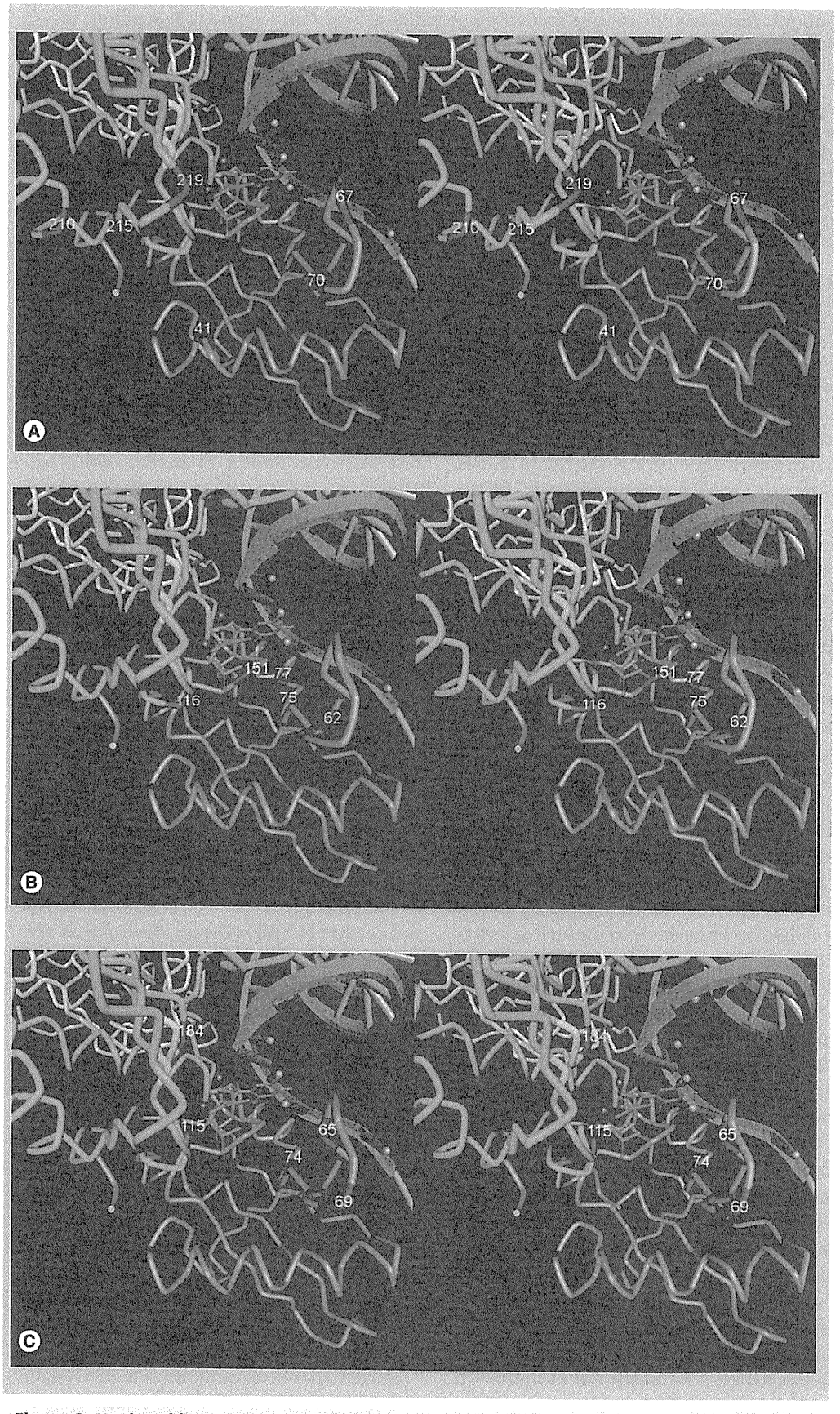

Figure 3. Nucleos(t)ide analog reverse-transcriptase inhibitor-resistance amino acid positions in the 3D structure of HIV-1 reverse transcriptase. See legend on next page.

Figure 3. Nucleos(t)ide reverse-transcriptase inhibitor-resistance amino acid positions in the 3D structure of HIV-1 reverse transcriptase. A total of 16 amino acid positions associated with nucleos(t)ide reverse-transcriptase inhibitor resistance [36] are shown in red. Two magnesium ions necessary for DNA polymerization are shown as tiny brown balls near the deoxythymidine-5′-triphosphate (dTTP; magenta). The fingers and palm subdomains of the p66 subunit are shown in blue and green wire-frame, respectively. A triad of aspartic acid residues at positions 110, 185 and 186 within the DNA polymerase catalytic site are shown in yellow. The DNA template-primer is shown in dark gray. **(A)** Amino acid positions of thymidine analog-associated mutations (TAMs): M41L, D67N, K70R, L210W, T215Y/F and K219Q/E. **(B)** Amino acid positions for the multinucleoside reverse-transcriptase inhibitor-resistance complex of Q151M with A62V, V75I, F77L and F116Y mutations. **(C)** Amino acid positions for K65R, 69-insertion, L74V, Y115F and M184V/I mutations.

confers cross-resistance to all approved NRTIs [57,62,91–95]. RT with the 69 insertion complex had a decreased binding or incorporation rate for some NRTI triphosphate forms, with simultaneously enhanced activity for removing a chain terminator from the end of DNA [64,96–100]. Therefore, the key mutation essential for ATP-mediated excision, T215Y/F, was found in most viruses with the 69 insertion complex (97%) [96].

A complex of the Q151M mutation with four other mutations (A62V, V75I, F77L and F116Y) was first identified in a patient receiving long-term therapy with an alternating regimen of zidovudine and zalcitabine [101]. Soon after this identification, Q151M complex-mediated cross-resistance to zidovudine, didanosine, zalcitabine and stavudine was well characterized [102,103]. The Q151M complex alone still retained moderate susceptibility to lamivudine, emtricitabine and tenofovir (<2.7-fold change), but the Q151M complex combined with the K65R mutation dramatically decreased susceptibility to drugs (>13.5-fold change) [57,63]. In fact, although the Q151M mutation had a low prevalence (0.9–1.4%) [37,38,40], it tended to emerge more frequently in the viral genotype with the K65R mutation [82,84–86]. Concerning the mechanism of multi-NRTI resistance, the Q151M complex is unlike the other two patterns (TAMs and 69 insertion complexes) because it does not rely on ATP-mediated excision of chain terminators [64,104]. The Q151M complex did not change RT binding affinities for NRTI triphosphate forms, but selectively reduced NRTI incorporation rates at the catalytic step [104,105]. To date, the crystal structure of RT with either the Q151M or the 69 insertion complex has not been published; such a structure would provide valuable information for understanding the molecular mechanism of multi-NRTI resistance.

NNRTIs & their mechanism of RT inhibition

The name of NNRTIs clearly indicates that they do not have a typical nucleoside analog structure and show a diversity of chemical structures (Figure 2). Four NNRTIs were developed between 1996 and 2008: nevirapine [106], delavirdine [107], efavirenz [108] and etravirine [109]. In the latest clinical guidelines, nevirapine or efavirenz is recommended as a part of first-line regimens [202–204]. On the other hand, delavirdine is rarely used owing to inconvenient dosing (three times daily) and a relatively inferior efficacy in viral suppression [203]. Although little clinical evidence supports the use of etravirine in first-line therapy, the efficacy of etravirine-containing HAART on therapy-naive patients is currently being assessed in randomized clinical trials.

The NNRTIs differ from NRTIs in their mechanism of RT inhibition. First, NNRTIs require no intracellular metabolism [106–109]. Second, they bind directly to a hydrophobic pocket near the catalytic site of HIV-1 RT, also referred to as the 'NNRTI-binding pocket' [20,110–112]. Third, NNRTIs block the chemical reaction step of DNA polymerization [113]. Although further details remain to be elucidated for the molecular mechanism of RT inhibition by NNRTIs, structural studies explain that NNRTIs distort the catalytic site by shifting the three key aspartic acid residues at positions 110, 185 and 186 [114], and that NNRTIs restrict the flexibility of a key loop between positions 183 and 186 (referred to as 'the YMDD loop'), and prevent the catalytic site from attaining the metal-binding conformation necessary for DNA polymerization [115].

NNRTI-resistance mutations & their mechanism of drug resistance

The latest list of drug-resistance mutations for HIV clinical practitioners [36] indicates that NNRTI resistance is associated with 14 amino acid positions mapped in the 3D structure of HIV-1 RT. As shown in Figure 5, all 14 amino acid positions surround the NNRTI-binding site. A total of 13 positions are in the palm subdomain of the p66 subunit, whereas the remaining position (138) is in the p51 subunit. Recent large-scale analyses of drug-resistant viral genotypes

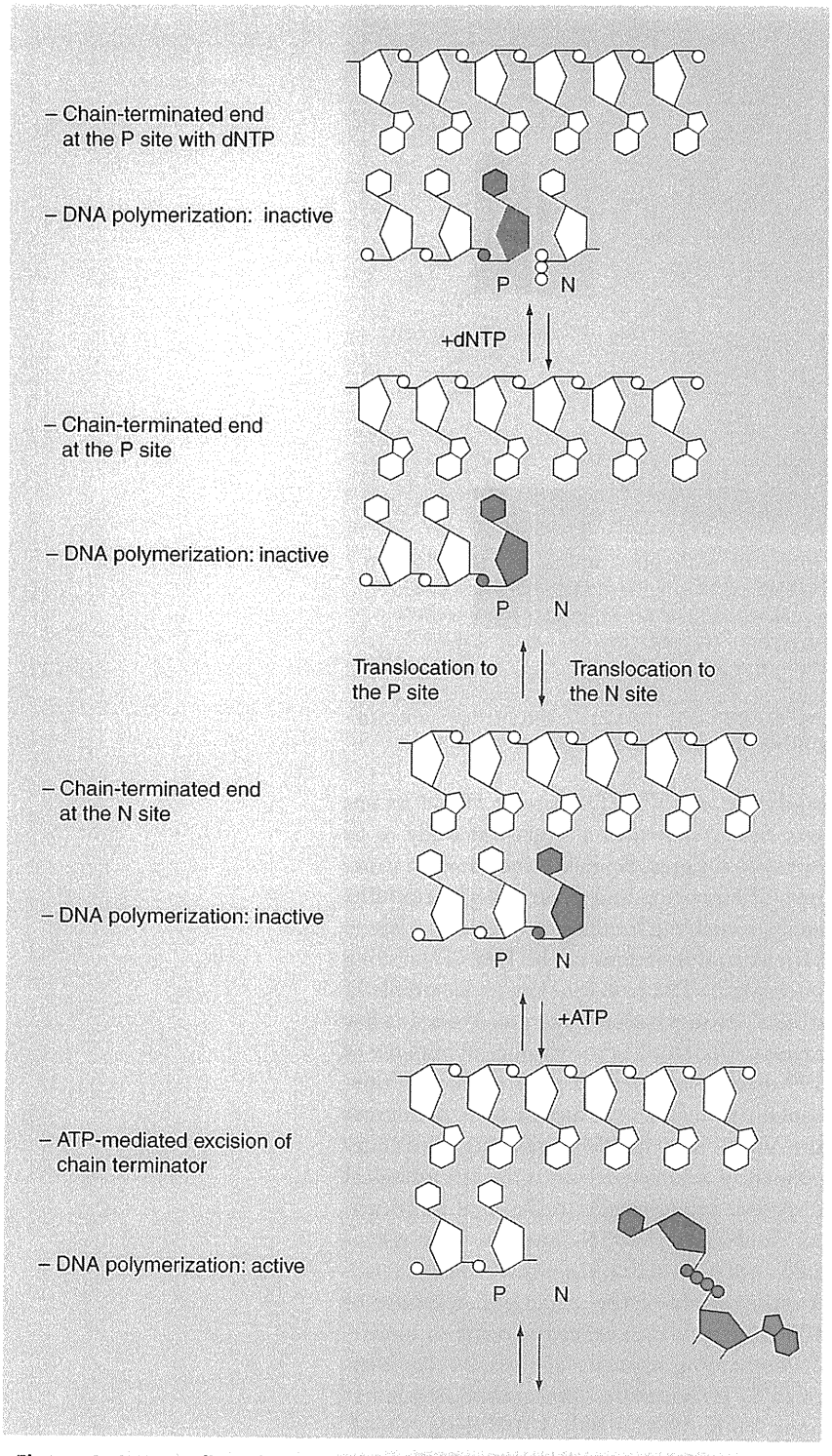

Figure 4. ATP-mediated chain-terminator excision. Thymidine analog-associated mutation-containing reverse transcriptase has enhanced activity for dinucleoside polyphosphate synthesis, in which an incorporated nucleos(t)ide analog reverse-transcriptase inhibitor monophosphate (red) is removed from the end of terminated DNA (bottom). The most likely pyrophosphate donor for the excision reaction is adenosine-5′-triphosphate (ATP; blue). After removing the nucleos(t)ide analog reverse-transcriptase inhibitor monophosphate, the mode of DNA polymerization becomes active again (bottom). The P site and N site of HIV-1 reverse transcriptase are shown as purple and green boxes, respectively.
dNTP: Deoxynucleotide triphosphate; N site: Nucleotide-binding site; P site: Priming site.

identified three prevalent NNRTI-resistance mutations: K103N (prevalence: 43–57%), Y181C (17–27%) and G190A (17–20%) [37,38,116].

The highest prevalence of the K103N mutation is likely explained by four observations: first, nevirapine and efavirenz are frequently used in first-line therapy [202–204]; second, the mutation alone confers high-level resistance to both nevirapine and efavirenz (>19-fold change) [116,117]; third, K103N does not have any major incompatibility with other RT inhibitor-resistance mutations; and fourth, K103N has only minor effects on viral replication fitness [118]. K103N-containing RT had slightly decreased RNase H activity [118], but no substantial changes in nucleotide binding affinity and the catalytic rate of nucleotide incorporation were observed [119,120]. These biochemical data help to explain the little interference of the K103N mutation with viral replication fitness. In a structural study of K103N-containing RT in complex with efavirenz, the K103N mutation was shown to induce rearrangement of the NNRTI-binding pocket, thus altering some contacts between efavirenz and the NNRTI-binding pocket [111]. In another structural study of unliganded K103N-containing RT, the NNRTI-binding pocket of mutated RT was shown to form a new network of hydrogen bonds with the potential to stabilize the closed form of the NNRTI-binding pocket and decrease the rate of NNRTI-binding to RT [121].

Either the Y181C or G190A mutation alone confer high-level resistance to nevirapine (>41-fold change), but low- or intermediate-level resistance to efavirenz (1.1- to 6.8-fold change) [116,117]. This difference could explain the finding that both Y181C and G190A mutations were more frequently found in therapy-failure cases treated with nevirapine-containing regimens than in those treated with efavirenz-containing regimens [122,123]. Interestingly, the Y181C mutation was shown to restore the susceptibility of zidovudine-resistant variants to zidovudine *in vitro* [124]. Furthermore, a clinical study clearly demonstrated that nevirapine–zidovudine combination therapy prevented the emergence of the Y181C mutation and increased that of other nevirapine-resistance mutations at positions 103, 106, 188 and 190 [125], suggesting severe incompatibility between the Y181C mutation and TAMs. This incompatibility was confirmed at the enzyme level; introducing the Y181C mutation to TAMs did not change nucleotide selectivity but diminished ATP-mediated excision of zidovudine-5′-monophosphate from the end of terminated DNA [126]. Y181C-containing RT had dramatically

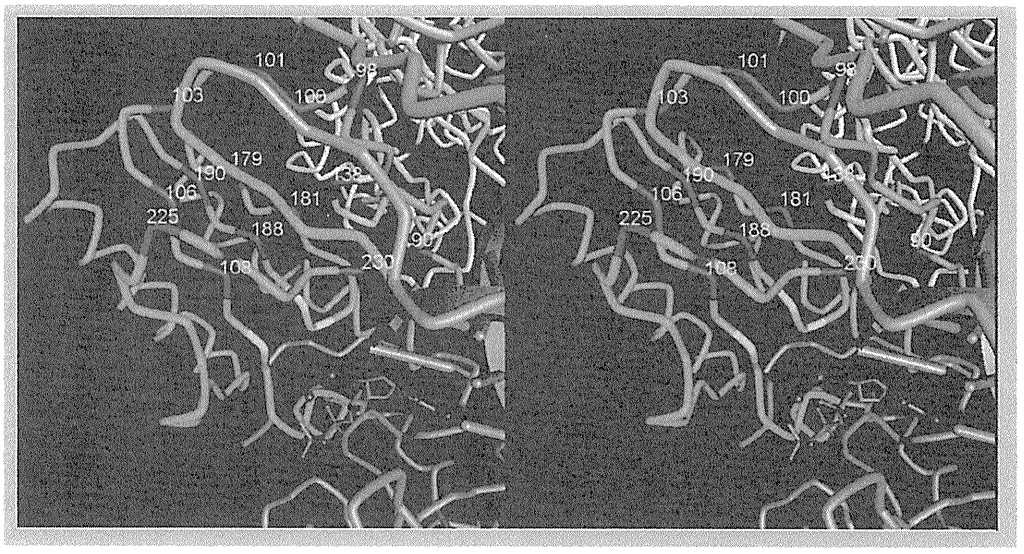

Figure 5. Non-nucleoside reverse-transcriptase inhibitor-resistance amino acid positions in the 3D structure of HIV-1 reverse transcriptase. A total of 14 amino acid positions associated with non-nucleoside reverse-transcriptase inhibitor resistance [36] are shown in red. Among these positions, 13 are in the palm subdomain (green wire-frame) of the p66 subunit, whereas the remaining position (138) is in the p51 subunit (white wire-frame). Two magnesium ions necessary for DNA polymerization are shown as tiny brown balls near the deoxythymidine-5′-triphosphate (dTTP; magenta). A triad of aspartic acid residues at positions 110, 185 and 186 within the DNA polymerase catalytic site are shown in yellow. The DNA template-primer is shown in dark gray.

lower affinity for nevirapine (>132-fold change) but still retained DNA polymerization activity similar to that of wild-type RT [127]. In addition, Y181C-containing RT had slightly decreased RNase H activity [118]. These biochemical data help to explain the little interference of the Y181C mutation with viral replication fitness [118,128]. A structural study demonstrated that the main contribution of the Y181C mutation to nevirapine and efavirenz resistance is the loss of interaction between the amino acid at position 181 and the drugs [129]. Concerning the G190A mutation, no reports were found for severe incompatibility with other RT inhibitor-resistance mutations. G190A-containing RT did not differ significantly in nucleotide binding and incorporation kinetics [120], but it had lower RNase H activity than wild-type, Y181C-containing and K103N-containing RTs [118]. These biochemical data are highly consistent with the relatively low *in vitro* replication fitness of G190A-mutated virus [118]. As the crystal structure of G190A-containing RT has not been published, the molecular mechanisms by which G190A confers resistance to approved NNRTIs are still unknown. In contrast to other previously approved NNRTIs, etravirine demonstrated potent antiretroviral activity against most clinical and laboratorial NNRTI-resistant HIV-1 variants [109,130], and a high genetic barrier to developing resistance *in vitro* [130]. The potent antiretroviral activity of etravirine against both wild-type and NNRTI-resistant HIV-1 strains was clearly shown in a structural study to be owing to the great flexibility and compact structure of etravirine and other diarylpyrimidine analogs, enabling the drug to access and adapt to various conformations of the NNRTI-binding pocket [112]. The etravirine-resistance profile in clinical settings has been examined in only a few reports. One study of the virological response of 199 therapy-experienced patients to etravirine-containing regimens at month 2 [131] found that the Y181V and E138A mutations were independently associated with a lower virological response. Interestingly, in the final multivariate model, the K103N mutation was associated with a favorable virological response [131]. Another study of the virological response of 406 treatment-experienced patients to etravirine-containing regimens at week 24 [132] identified 17 etravirine-resistance-associated mutations: V90I, A98G, L100I, K101P/E/H, V106I, E138A, V179F/D/T, Y181I/V/C, G190S/A and M230L. These mutations were used to construct a weighted genotypic scoring algorithm for the virological response to etravirine [132]. Of note, the K103N mutation did not affect virological response to etravirine in this study [132]. The fact that Y181C and G190A mutations were frequently found in nevirapine-containing regimens and the K103N mutation in efavirenz-containing regimens [122,123] allows us to speculate that

efavirenz is a better choice than nevirapine in current first-line therapy from the viewpoint of using etravirine in salvage therapies.

Mutations in the C-terminal region of HIV-1 RT

The acquisition of RT-inhibitor resistance has recently been discussed in several publications in terms of the potential contribution of amino acid mutations in the C-terminal region of HIV-1 RT (the thumb and connection subdomains and the RNase H domain). However, their clinical significance is poorly understood at the moment. A recent clinical study reported that the presence of at least one of 13 mutations in the connection subdomain (E312Q, Y318F, G333D/E, G335C/D, N348I, A360I/V, V365I, A371V, A376S and E399G) was significantly associated with poor virological response to therapy (viral load decrease: <1 $\log_{10}$ copies/ml at week 24) in a step-wise multivariate analysis ($p = 0.045$) [133]. By contrast, another recent clinical study reported no significant association in adjusted Cox regression models between six mutations tested in the connection domain (N348I, R356K, R358K, A360V, A371V and A376S) and virological therapy-failure (viral load: >50 copies/ml) [134].

At present, the best studied mutation in the connection subdomain is N348I. This mutation was first identified as one of several mutations (E312Q, G335C/D, N348I, A360I/V, V365I and A376S) that contributed to enhancing zidovudine resistance in the presence of TAMs [135]. Interestingly, the N348I mutation was identified again as a unique mutation with the potential to develop dual-class resistance to NRTIs (zidovudine and didanosine) and NNRTIs (nevirapine, efavirenz and delavirdine) in the absence and presence of well-known RT inhibitor-resistance mutations, TAMs and K103N [136,137]. Furthermore, a subsequent *in vitro* study recently reported that the N348I mutation, in combination with Y181C and three TAMs, enhanced the level of resistance to etravirine and tenofovir, respectively [138].

Regarding its mechanism of NRTI resistance, the N348I mutation was proposed in recent studies to reduce the RNase H activity of RT and prolong the half-life of viral template RNA, providing more time to excise zidovudine-5′-monophosphate from the end of terminated DNA [135,136]. Interestingly, another study noted the RNase H-independent contribution of double mutations, N348I and A360V, in combination with TAMs, to the excision of zidovudine-5′-monophosphate [139]. Concerning the mechanism of NNRTI resistance, one group proposed that the lower RNase H activity provided more time for the NNRTI to dissociate from a polymerization-incompetent NNRTI/RT/template-primer complex, leading to more efficient resumption of viral DNA synthesis [140]. On the other hand, another group proposed that the N348I mutation counteracted the removal of primer mediated by RNase H and enhanced by NNRTIs during initiation of plus-strand DNA synthesis, especially in the presence of nevirapine [141].

HIV-1 subtype-specific differences in genetic barrier to RT-inhibitor resistance

Among the four genetic HIV-1 groups (M, N, O and P) [142–144], HIV-1 group M is responsible for the global epidemic of HIV/AIDS. In HIV-1 group M, nine subtypes (A, B, C, D, F, G, H, J and K) have been identified to date, along with 48 circulating recombinant forms (CRFs; inter-subtype recombinant strains) [205]. Until now, the majority of information on HIV-1 drug resistance has been obtained from HIV-1 subtype B, the predominant subtype in developed countries. However, subtype B is responsible for only 10% of HIV-1 infections in the worldwide pandemic [145]. On the other hand, the most prevalent HIV-1 worldwide is subtype C (50%, predominant in South Africa, India and Ethiopia) followed by subtype A (12%, predominant in Eastern Europe and Central Asia), subtype G (6%, predominant in West Africa), and subtype D (3%, predominant in North Africa and the Middle East) [145]. In addition, 18% of HIV-1 infections worldwide are from recombinant forms (predominant in West Africa, South, East and Southeast Asia) [145].

At this moment, there is no consensus about the clinical impact of HIV-1 subtype on the virological response to ART. No evidence of different virological response (change in viral load) at 24 and 48 weeks was found in a European network study of 108 children infected with different subtypes (A, B, C, D, F, G, H, CRF01_AE and CRF02_AG) [146]. Similarly, 172 patients infected with subtypes A, B, C, D, G and CRF01_AE were followed for 6 months by a Swedish study group, and their virological responses (viral load <500 copies/ml) were not associated with HIV-1 subtype in a univariate analysis, but were associated with ethnicity [147]. By contrast, in a Brazilian study, 84 patients infected with subtype B were found to acquire primary NRTI- and protease inhibitor-resistance mutations more frequently than 52 patients infected subtype C, and that significant differences were observed from 4 years

after initiating ART [148]. However, this study also noted no significant difference between subtype B and C infections in average time for viral rebound (15.6 and 19 months, respectively) [148]. Similarly, a recent large scale study in the UK (2116 patients infected with subtypes A, B, C, D, CRF02_AG and others) reported that patients infected with subtype C and A showed more rapid suppression of viral load (<50 copies/ml) than those infected with subtype B within 12 months (hazard ratios: 1.16, p = 0.04 and 1.35, p = 0.02, respectively) [149]. In addition, virological rebounds (viral load >1000 copies/ml) were more rapid in patients infected with subtype C (hazard ratio, 1.40; p = 0.05) than in those infected with subtype B [149]. On the other hand, another UK study reported no significant difference between patients (n = 374) infected with subtypes A, B, C, D and CRF02_AG in the time for viral suppression (viral load <400 copies/ml) after initiation of HAART [150]. However, that study reported a significantly higher rate of virological rebound (viral load >400 copies/ml) in patients infected with subtype D (70%) than in those infected with A (35%), B (45%), C (34%) and CRF02_AG (44%) at 6 months after initial viral load suppression [150].

Subtype-dependent differences in genetic barriers to acquiring RT-inhibitor resistance could be a candidate that affects the virological response to ART. Among NNRTI-resistance mutations, V106M in subtype C was the first identified subtype-specific mutation that conferred multi-NNRTI resistance to efavirenz, nevirapine, and delavirdine [151]. Although both wild-type subtype C and B strains generally possess valine at position 106, subtype C and B strains have different codon usages, such as 'GTG' and 'GTA', respectively. To obtain the V106M mutation, subtype C strains need one transition, GTG (valine) to ATG (methionine), whereas subtype B strains require at least two transitions, GTA (valine) to ATG (methionine) [151]. Therefore, subtype B strains tend to acquire alternative NNRTI-resistance amino acid mutations, V106A/I, through one transition; GTA (valine) to GCA (alanine), and GTA (valine) to ATA (isoleucine). Thus far, no other resistance mutation has been identified whose vulnerability to acquiring resistance can be explained by nucleotide substitution number or type. However, several subtype-dependent barriers to resistance acquisition have been reported, (e.g., V108I in subtype G, Q151M in subtypes D and F, L210W in subtypes C, F, G and CRF02_AG, and P225H in subtype A [152,153]). On the other hand, in an *in vitro* study, subtype C strains were found to acquire K65R, a NRTI-resistance mutation, more rapidly than subtype B strains under pressure from tenofovir, suggesting the need for careful attention to the efficacy of tenofovir-containing regimens in subtype C infections [154]. However, the difference between subtype C and B strains was unlikely due to the number and type of nucleotide substitutions because both strains generally require the same transition: AAG (lysine) to AGG (arginine) in subtype C, and AAA (lysine) to AGA (arginine) in subtype B. Interestingly, a subsequent *in vitro* study demonstrated that the adenine hexamer between codons 63–65 in the RT-coding region of subtype C enabled HIV-1 RT to pause at codon 65 during plus-strand DNA synthesis [155], suggesting that misaligned DNA synthesis enhanced nucleotide substitutions, which led to a high prevalence of K65R mutation [155–157]. In addition, the A98S mutation is considered a subtype C-specific nevirapine-resistance mutation by one expert panel on HIV-1 drug resistance [206]. However, as the A98S mutation was also reported in Israel as a frequently observed natural polymorphism (prevalence of 25%) in subtype C [158], further studies are needed to clarify the impact of this mutation in nevirapine resistance.

Characteristics of NRTI-resistance pathways in HIV-2

In contrast to the global pandemic of HIV-1, the distribution of HIV-2 has been mainly restricted to the original endemic area of West Africa and several related European and Asian countries, with the number of HIV-2 infections worldwide estimated at one or two million. Among the eight genetic HIV-2 groups (A–H), groups A and B appear to be the major groups circulating in endemic areas [159–163]. Recently, the first CRF of HIV-2, CRF01_AB, was identified [164]. HIV-2 has been characterized as less pathogenic than HIV-1, with lower rates of AIDS development and mortality [165–169]. The plasma viral load in HIV-2 infection was significantly lower than that in HIV-1 infection, but the same plasma viral load resulted in a similar rate of $CD4^+$ T-cell decline either in HIV-1 or HIV-2 infection [170]. As antiretroviral drugs have primarily been developed against HIV-1, several approved drugs are less effective against HIV-2. In particular, all four approved NNRTIs were demonstrated to be less effective for HIV-2 strains *in vitro* [109,171,172], meaning limited treatment options for HIV-2-infected patients. In addition, optimal antiretroviral regimens for HIV-2-infected patients have not been well established owing to the lack of randomized

clinical trials [173]. These insufficient therapeutic preparations for HIV-2-infected patients are the major reasons for their poorer responses to ART compared with HIV-1-infected patients [174–177].

HIV-2 and HIV-1 RT amino acid sequences have approximately 60% identity [178]. However, their overall 3D structures appear similar, suggesting that amino acids at positions 101, 106, 138, 181, 188, and 190 known as NNRTI-resistance loci, are in an unfavorable pattern for NNRTI binding (Table 2) [179]. In addition, five HIV-2-specific amino acids, 69N, 75I, 210N, 215S and 219E, at the positions known as NRTI-resistance loci (Table 2), suggest that these amino acid differences affect the drug-resistance pathways of HIV-2 RT.

Clinical studies on HIV-2-infected patients treated with ART demonstrated both the high prevalence of the Q151M mutation associated with multi-NRTI resistance (9–50% in NRTI-experienced patients) and the low prevalence of TAMs identified in HIV-1-infected patients [180–188]. A structural comparison of HIV-2 RT with HIV-1 RT found important differences between their DNA polymerization domains, especially in the ATP-binding site in the HIV-1 RT [189]. In fact, HIV-2 RT demonstrated markedly less efficient activity in excising zidovudine-5′-monophosphate from the end of terminated DNA [189]. These findings suggest that the main mechanism of NRTI resistance in HIV-2 RT is based on its enhanced ability to discriminate between NRTI triphosphate forms and natural dNTP substrates rather than the ATP-mediated excision of chain terminators. In addition, a recent site-directed mutagenesis study clearly demonstrated that the Q151M mutation alone was sufficient for HIV-2 RT to exhibit multi-NRTI resistance to zidovudine, didanosine, stavudine and emtricitabine, and that Q151M combined with the other two prevalent mutations, K65R and M184V, conferred resistance to all currently available NRTIs [190].

Conclusion

Although HIV has not been eradicated by even the most potent combination ART, the prognosis of HIV/AIDS cases has been improved by recent progress in antiretrovirals, such as enhanced antiviral potency, better pharmacokinetics and higher genetic barriers to resistance acquisition. However, no antiretroviral has successfully avoided the emergence of drug-resistant variants, as the high capacity of HIV to generate escape variants overcomes the pressure of antiretroviral drugs. Therefore, drug-resistance mutation patterns have been changing with the introduction of new antiretrovirals [191]. Thus, developing antiretrovirals against current prevalent drug-resistant variants is still important to prepare sufficient treatment options for second- and third-line, as well as further therapies.

Future perspective

Etravirine, the latest NNRTI to be approved, demonstrates potent antiretroviral activity against prevalent NNRTI-resistant viruses with the K103N mutation. Therefore, etravirine is expected to be a key drug in salvage therapy for efavirenz- or nevirapine-resistant cases. At this moment, limited information is available on the clinical profile of etravirine resistance, and further findings from several ongoing clinical studies are awaited. In addition, a new investigational drug, rilpivirine (TMC-278), has been submitted for approval to the US FDA. Like etravirine, rilpivirine is a diarylpyrimidine compound showing potent antiretroviral activity against 62% of NNRTI-resistant clinical isolates *in vitro* [192]. As etravirine and rilpivirine possess similar chemical structures, both new antiretrovirals showed cross-resistance in *in vitro* drug-resistance analyses [192]. Since these two new NNRTIs appear to have a high genetic barrier to resistance acquisition, improved outcomes are also expected in first treatment of therapy-naive patients. Another new NNRTI, lersivirine (UK-453,061), a pyrazole compound, is in Phase I and II studies [207]. Interestingly, lersivirine was shown *in vitro* to have a unique resistance profile containing two key mutations, V108I and F227L [193]. Lersivirine also showed improved antiretroviral activity against NNRTI-resistant clinical isolates *in vitro* [193].

In NRTI resistance, the most prevalent current mutations are M184V/I and TAMs. Although antiretrovirals have been continuously developed against the prevalent NRTI-resistant variants, no investigational NRTI is currently in any active Phase III clinical trials. On the other hand, the molecular basis of zidovudine-5′-monophosphate excision by TAM-containing HIV-1 RT has been clarified in detail in a very recent structural study [194]. Importantly, this study provides the crystal structure of TAM-containing HIV-1 RT in a complex with a DNA template-primer and an excision product, zidovudine adenosine dinucleoside tetraphosphate. As suggested by the authors, appropriately designed mimics of the excision product are expected to become potent inhibitors of TAM-containing HIV-1 RT [194].

The development of new classes of antiretroviral drugs, such as integrase inhibitors and CCR5-antagonistic entry inhibitors, has opened the possibility of considering regimens without NRTIs (Table 1). An ongoing Phase IV pilot study, TERCETO, is evaluating the safety and efficacy of a maraviroc plus raltegravir plus darunavir/ritonavir regimen in HIV-1-infected patients with triple-class resistance to NRTIs, NNRTIs and at least one protease inhibitor (except for darunavir) [207]. Eventually, such a regimen can also be considered in therapy-naive patients to avoid the adverse effects of RT inhibitors.

Recently, knowledge has been rapidly accumulating on drug-resistance-associated mutations in the C-terminal region of HIV-1 RT. Although information on their clinical significance is limited, more detailed information will hopefully be available in the near future. At present, full-length RT genotyping is not involved in general drug-resistance testing. However, when a consensus is reached on the clinical significance of mutations in the C-terminal region of HIV-1 RT, full-length RT genotyping will also be required in routine drug-resistance testing.

The number of HIV-infected individuals receiving ART in low- and middle-income countries increased to approximately 4 million at the end of 2008 (tenfold from 2003) through improved access to antiretroviral drugs, an effort that is driving a decline in HIV-related mortality in these countries [201]. However, a drawback of improved access in these countries may be more drug-resistant cases. As non-B subtypes are major circulating HIV-1 strains in these countries, it is important to obtain more detailed information on the clinical significance of HIV-1 subtypes and on the efficacy of current ART, including in HIV-2-infected individuals.

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Table 2. HIV-2 consensus amino acids at positions known in HIV-1 drug resistance.

Sequence type	Sequence name	Amino acid positions associated with NRTI resistance																Amino acid positions associated with NNRTI resistance									
		41	*62*	*65*	*67*	*69*	*70*	*74*	*75*	*77*	*115*	*116*	*151*	*184*	*210*	*215*	*219*	*90*	*98*	*100*	*101*	*103*	*106*	*108*	*138*	*179*	*181*	*188*	*190*	*225*	*230*
Reference	HIV-1 HXB2	M	A	K	D	T	K	L	V	F	Y	F	Q	M	L	T	K	V	A	L	K	K	V	V	E	V	Y	Y	G	P	M
Consensus†	HIV-1 group M	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	HIV-2 group A	–	–	–	–	N	–	–	**I**	–	–	–	–	–	N	S	**E**	**I**	–	–	A	–	**I**	–	**A**	–	**I**	**I**	**L**	**A**	–
	HIV-2 group B	–	–	–	–	N	–	–	**I**	–	–	–	–	–	N	S	**E**	–	–	–	A	–	**I**	–	**A**	–/**T**	**I**	**I**	**L**	**A**	–
	HIV-2 CRF01_AB	–	–	–	–	N	–	–	**I**	–	–	–	–	–	N	S	**E**	–	–	–	A	R	**I**	–	**A**	**T**	**I**	**I**	**L**	**A**	–

Drug-resistance amino acids known in HIV-1 [36] are shown in bold. Amino acids that are the same as those of the reference HIV-1 HXB2 are shown as bars.
†Consensus amino acid sequences were prepared through the Consensus Maker in the Los Alamos HIV sequence database [205].
CRF: Circulating recombinant form; NNRTI: Non-nucleoside reverse-transcriptase inhibitor; NRTI: Nucleos(t)ide analog reverse-transcriptase inhibitor.

Executive summary

Nucleos(t)ide analog reverse-transcriptase inhibitors & their mechanism of reverse-transcriptase inhibition

- Eight nucleos(t)ide analog reverse-transcriptase (RT) inhibitors (NRTIs) were developed from 1987 to 2003. NRTIs, in a combination of two NRTIs, are recommended as a backbone of the latest first-line regimens.
- NRTIs are converted to their active 5′-triphosphate forms inside cells and compete with dNTPs for HIV RT. In addition, as NRTIs lack a 3′-hydroxyl group, they function as a chain terminator and inhibit reverse transcription.

NRTI-resistance mutations & their mechanism of drug resistance

- M184V/I is the most prevalent mutation. M184V alone confers high-level resistance to both lamivudine and emtricitabine.
- A series of thymidine analog-associated mutations (TAMs) is the second most prevalent mutation. Accumulation of TAMs leads to multi-NRTI resistance. TAM-containing RT demonstrates enhanced excision activity.
- The prevalence of the other most NRTI-resistance mutations is less than 5%.
- K65R is a primary mutation in tenofovir resistance. K65R decreases TAM-mediated excision activity.

NNRTIs & their mechanism of RT inhibition

- Four non-nucleoside RT inhibitors (NNRTIs) were developed between 1996 and 2008. Nevirapine and efavirenz are recommended as key drugs in the latest first-line regimens.
- NNRTIs bind to a hydrophobic pocket near the catalytic site of HIV-1 RT and inactivate enzymatic activity.

NNRTI-resistance mutations & their mechanism of drug resistance

- The most prevalent NNRTI-resistance mutations are K103N, Y181C and G190A. Y181C and G190A were found more frequently in nevirapine-containing regimens, whereas more K103N was found in efavirenz-containing regimens.
- Etravirine, the latest approved NNRTI, shows potent antiretroviral activity against most nevirapine- and efavirenz-resistant HIV-1 strains, and demonstrates a high genetic barrier to resistance acquisition *in vitro*.
- The NNRTI-resistance mutation patterns allow us to speculate that efavirenz is a better choice than nevirapine in current first-line therapy from the viewpoint of using etravirine in salvage therapies.

Mutations in the C-terminal region of HIV-1 RT

- The clinical significance of mutations in the C-terminal region of HIV-1 RT is still controversial.

HIV-1 subtype-specific differences in genetic barrier to RT-inhibitor resistance

- To date, there is no consensus about the clinical impact of HIV-1 subtypes on virological response to ART.

Characteristics of NRTI-resistance pathways in HIV-2

- NNRTIs are less effective against HIV-2 than in HIV-1.
- Q151M has a high prevalence in HIV-2-infected patients treated with ART.
- Q151M+K65R/M184V confer resistance to all currently available NRTIs.

Conclusion

- Further research efforts should be undertaken to develop new antiretrovirals effective against multiclass-resistant viruses.

Future perspective

- Etravirine is expected to improve the outcomes of ART regardless of pre-exposure to other antiretrovirals.
- Rilpivirine (TMC-278) has been recently submitted to the US FDA for approval.
- Appropriately designed mimics of excision products are expected to become potent inhibitors for TAM-containing HIV-1 RT.
- Whether or not to involve the C-terminal region of RT in routine drug-resistance testing is still controversial. More information is needed to make this decision.
- It is important to obtain more information on the efficacy of current ART against HIV-1 subtypes, including HIV-2 infections.

Bibliography

Papers of special note have been highlighted as:
- ▪ of interest
- ▪▪ of considerable interest

1. Gallo RC, Sarin PS, Gelmann EP *et al.*: Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* 220, 865–867 (1983).
2. Barré-Sinoussi F, Chermann JC, Rey F *et al.*: Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 220, 868–871 (1983).
3. Kanki PJ, Barin F, M'Boup S *et al.*: New human T-lymphotropic retrovirus related to simian T-lymphotropic virus type III (STLV-IIIAGM). *Science* 232, 238–243 (1986).
4. Clavel F, Guétard D, Brun-Vézinet F *et al.*: Isolation of a new human retrovirus from West African patients with AIDS. *Science* 233, 343–346 (1986).
5. Collier AC, Coombs RW, Schoenfeld DA *et al.*: Treatment of human immunodeficiency virus infection with saquinavir, zidovudine, and zalcitabine. AIDS Clinical Trials Group. *N. Engl. J. Med.* 334, 1011–1017 (1996).
6. D'Aquila RT, Hughes MD, Johnson VA *et al.*: Nevirapine, zidovudine, and didanosine compared with zidovudine and didanosine in patients with HIV-1 infection. A randomized, double-blind, placebo-controlled trial. National Institute of Allergy and Infectious Diseases AIDS Clinical Trials Group Protocol 241 Investigators. *Ann. Intern. Med.* 124, 1019–1030 (1996).
7. Chun TW, Stuyver L, Mizell SB *et al.*: Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc. Natl Acad. Sci. USA* 94, 13193–13197 (1997).

8. Wong JK, Hezareh M, Günthard HF *et al.*: Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* 278, 1291–1295 (1997).
9. Finzi D, Hermankova M, Pierson T *et al.*: Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* 278, 1295–1300 (1997).
10. El-Sadr WM, Lundgren JD, Neaton JD *et al.*: CD4+ count-guided interruption of antiretroviral treatment. *N. Engl. J. Med.* 355, 2283–2296 (2006).
11. Preston BD, Poiesz BJ, Loeb LA: Fidelity of HIV-1 reverse transcriptase. *Science* 242, 1168–1171 (1988).
12. Roberts JD, Bebenek K, Kunkel TA: The accuracy of reverse transcriptase from HIV-1. *Science* 242, 1171–1173 (1988).
13. Mansky LM, Temin HM: Lower *in vivo* mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. *J. Virol.* 69, 5087–5094 (1995).
14. Mansky LM: Forward mutation rate of human immunodeficiency virus type 1 in a T lymphoid cell line. *AIDS Res. Hum. Retroviruses* 12, 307–314 (1996).
15. Gao F, Chen Y, Levy DN, Conway JA, Kepler TB, Hui H: Unselected mutations in the human immunodeficiency virus type 1 genome are mostly nonsynonymous and often deleterious. *J. Virol.* 78, 2426–2433 (2004).
16. Huang KJ, Wooley DP: A new cell-based assay for measuring the forward mutation rate of HIV-1. *J. Virol. Methods* 124, 95–104 (2005).
17. Abram ME, Ferris AL, Shao W, Alvord WG, Hughes SH: Nature, position, and frequency of mutations made in a single cycle of HIV-1 replication. *J. Virol.* 84, 9864–9878 (2010).
18. Coffin JM: HIV population dynamics *in vivo*: implications for genetic variation, pathogenesis, and therapy. *Science* 267, 483–489 (1995).
19. Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD: HIV-1 dynamics *in vivo*: virion clearance rate, infected cell life-span, and viral generation time. *Science* 271, 1582–1586 (1996).
20. Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA: Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* 256, 1783–1790 (1992).
21. Jacobo-Molina A, Ding J, Nanni RG *et al.*: Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc. Natl Acad. Sci. USA* 90, 6320–6324 (1993).
22. Huang H, Chopra R, Verdine GL, Harrison SC: Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science* 282, 1669–1675 (1998).
23. Mitsuya H, Weinhold KJ, Furman PA *et al.*: 3'-azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/ lymphadenopathy-associated virus *in vitro*. *Proc. Natl Acad. Sci. USA* 82, 7096–7100 (1985).
24. Mitsuya H, Broder S: Inhibition of the *in vitro* infectivity and cytopathic effect of human T-lymphotrophic virus type III/ lymphadenopathy-associated virus (HTLV-III/LAV) by 2',3'-dideoxynucleosides. *Proc. Natl Acad. Sci. USA* 83, 1911–1915 (1986).
25. Baba M, Pauwels R, Herdewijn P, De Clercq E, Desmyter J, Vandeputte M: Both 2',3'-dideoxythymidine and its 2',3'-unsaturated derivative (2',3'-dideoxythymidinene) are potent and selective inhibitors of human immunodeficiency virus replication *in vitro*. *Biochem. Biophys. Res. Commun.* 142, 128–134 (1987).
26. Soudeyns H, Yao XI, Gao Q *et al.*: Anti-human immunodeficiency virus type 1 activity and *in vitro* toxicity of 2'-deoxy-3'-thiacytidine (BCH-189), a novel heterocyclic nucleoside analog. *Antimicrob. Agents Chemother.* 35, 1386–1390 (1991).
27. Daluge SM, Good SS, Faletto MB *et al.*: 1592U89, a novel carbocyclic nucleoside analog with potent, selective anti-human immunodeficiency virus activity. *Antimicrob. Agents Chemother.* 41, 1082–1093 (1997).
28. Balzarini J, Holy A, Jindrich J *et al.*: Differential antiherpesvirus and antiretrovirus effects of the (S) and (R) enantiomers of acyclic nucleoside phosphonates: potent and selective *in vitro* and *in vivo* antiretrovirus activities of (R)-9-(2-phosphonomethoxypropyl)-2,6-diaminopurine. *Antimicrob. Agents Chemother.* 37, 332–338 (1993).
29. Schinazi RF, McMillan A, Cannon D *et al.*: Selective inhibition of human immunodeficiency viruses by racemates and enantiomers of cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl] cytosine. *Antimicrob. Agents Chemother.* 36, 2423–2431 (1992).
30. Furman PA, Fyfe JA, St Clair MH *et al.*: Phosphorylation of 3'-azido-3'-deoxythymidine and selective interaction of the 5'-triphosphate with human immunodeficiency virus reverse transcriptase. *Proc. Natl Acad. Sci. USA* 83, 8333–8337 (1986).
31. Cheng YC, Dutschman GE, Bastow KF, Sarngadharan MG, Ting RY: Human immunodeficiency virus reverse transcriptase. General properties and its interactions with nucleoside triphosphate analogs. *J. Biol. Chem.* 262, 2187–2189 (1987).
32. St Clair MH, Richards CA, Spector T *et al.*: 3'-azido-3'-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. *Antimicrob. Agents Chemother.* 31, 1972–1977 (1987).
33. Larder BA, Darby G, Richman DD: HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. *Science* 243, 1731–1734 (1989).
34. Rooke R, Tremblay M, Soudeyns H *et al.*: Isolation of drug-resistant variants of HIV-1 from patients on long-term zidovudine therapy. Canadian Zidovudine Multi-Centre Study Group. *AIDS* 3, 411–415 (1989).
35. Larder BA, Kemp SD: Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* 246, 1155–1158 (1989).
36. Johnson VA, Brun-Vézinet F, Clotet B *et al.*: Update of the drug resistance mutations in HIV-1: December 2009. *Top. HIV Med.* 17, 138–145 (2009).
37. Shahriar R, Rhee S-Y, Liu TF *et al.*: Nonpolymorphic human immunodeficiency virus type 1 protease and reverse transcriptase treatment-selected mutations. *Antimicrob. Agents Chemother.* 53, 4869–4878 (2009).

▪ **Recent large-scale analysis of non-polymorphic HIV-1 mutations selected by antiretroviral therapy.**

38. Barth RE, van der Loeff MFS, Schuurman R, Hoepelman AIM, Wensing AMJ: Virological follow-up of adult patients in antiretroviral treatment programmes in sub-Saharan Africa: a systematic review. *Lancet Infect. Dis.* 10, 155–166 (2010).

▪▪ **Recent large-scale analysis of virological outcomes and drug-resistance profiles in sub-Saharan Africa. Importantly, almost all the subjects enrolled in the analysis were infected with non-B HIV-1 subtypes (99%).**

39. Gupta RK, Hill A, Sawyer AW *et al.*: Virological monitoring and resistance to first-line highly active antiretroviral therapy in adults infected with HIV-1 treated under WHO guidelines: a systematic review and meta-analysis. *Lancet Infect. Dis.* 9, 409–417 (2009).

40. McColl DJ, Chappey C, Parkin NT, Miller MD: Prevalence, genotypic associations and phenotypic characterization of K65R, L74V and other HIV-1 RT resistance mutations in a commercial database. *Antivir. Ther.* 13, 189–197 (2008).

▪ **Recent large-scale analysis of the nucleos(t)ide analog reverse-transcriptase inhibitor (NRTI)-resistance profile of HIV-1. This paper provides important information on interference between NRTI-resistance mutations.**

41. Larder BA, Kemp SD, Harrigan PR: Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. *Science* 269, 696–699 (1995).

42. Kavlick MF, Shirasaka T, Kojima E *et al.*: Genotypic and phenotypic characterization of HIV-1 isolated from patients receiving (-)-2′,3′-dideoxy-3′-thiacytidine. *Antiviral Res.* 28, 133–146 (1995).

43. Tisdale M, Kemp SD, Parry NR, Larder BA: Rapid *in vitro* selection of human immunodeficiency virus type 1 resistant to 3′-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. *Proc. Natl Acad. Sci. USA* 90, 5653–5656 (1993).

44. Boucher CA, Cammack N, Schipper P *et al.*: High-level resistance to (-) enantiomeric 2′-deoxy-3′-thiacytidine *in vitro* is due to one amino acid substitution in the catalytic site of human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* 37, 2231–2234 (1993).

45. Masquelier B, Descamps D, Carrière I *et al.*: Zidovudine resensitization and dual HIV-1 resistance to zidovudine and lamivudine in the δ lamivudine roll-over study. *Antivir. Ther.* 4, 69–77 (1999).

46. Wilson JE, Aulabaugh A, Caligan B *et al.*: Human immunodeficiency virus type-1 reverse transcriptase. Contribution of Met-184 to binding of nucleoside 5'-triphosphate. *J. Biol. Chem.* 271, 13656–13662 (1996).

47. Krebs R, Immendörfer U, Thrall SH, Wöhrl BM, Goody RS: Single-step kinetics of HIV-1 reverse transcriptase mutants responsible for virus resistance to nucleoside inhibitors zidovudine and 3-TC. *Biochemistry* 36, 10292–10300 (1997).

48. Feng JY, Anderson KS: Mechanistic studies examining the efficiency and fidelity of DNA synthesis by the 3TC-resistant mutant (184V) of HIV-1 reverse transcriptase. *Biochemistry* 38, 9440–9448 (1999).

49. Deval J, White KL, Miller MD *et al.*: Mechanistic basis for reduced viral and enzymatic fitness of HIV-1 reverse transcriptase containing both K65R and M184V mutations. *J. Biol. Chem.* 279, 509–516 (2004).

50. Sarafianos SG, Das K, Clark AD Jr *et al.*: Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance with β-branched amino acids. *Proc. Natl Acad. Sci. USA* 96, 10027–10032 (1999).

51. Hanna GJ, Johnson VA, Kuritzkes DR *et al.*: Patterns of resistance mutations selected by treatment of human immunodeficiency virus type 1 infection with zidovudine, didanosine, and nevirapine. *J. Infect. Dis.* 181, 904–911 (2000).

52. Marcelin A-G, Delaugerre C, Wirden M *et al.*: Thymidine analogue reverse transcriptase inhibitors resistance mutations profiles and association to other nucleoside reverse transcriptase inhibitors resistance mutations observed in the context of virological failure. *J. Med. Virol.* 72, 162–165 (2004).

53. Miller MD, Margot N, Lu B *et al.*: Genotypic and phenotypic predictors of the magnitude of response to tenofovir disoproxil fumarate treatment in antiretroviral-experienced patients. *J. Infect. Dis.* 189, 837–846 (2004).

54. Masquelier B, Tamalet C, Montès B *et al.*: Genotypic determinants of the virological response to tenofovir disoproxil fumarate in nucleoside reverse transcriptase inhibitor-experienced patients. *Antivir. Ther.* 9, 315–323 (2004).

55. Cozzi-Lepri A, Ruiz L, Loveday C *et al.*: Thymidine analogue mutation profiles: factors associated with acquiring specific profiles and their impact on the virological response to therapy. *Antivir. Ther.* 10, 791–802 (2005).

56. Whitcomb JM, Parkin NT, Chappey C, Hellmann NS, Petropoulos CJ: Broad nucleoside reverse-transcriptase inhibitor cross-resistance in human immunodeficiency virus type 1 clinical isolates. *J. Infect. Dis.* 188, 992–1000 (2003).

▪▪ **Provides outstanding data to understand the impact of TAM accumulation on multi-NRTI resistance in both the presence and absence of the M184V/I mutation.**

57. Ross LL, Parkin N, Gerondelis P *et al.*: Differential impact of thymidine analogue mutations on emtricitabine and lamivudine susceptibility. *J. Acquir. Immune Defic. Syndr.* 43, 567–570 (2006).

58. Boyer PL, Sarafianos SG, Arnold E, Hughes SH: Selective excision of AZTMP by drug-resistant human immunodeficiency virus reverse transcriptase. *J. Virol.* 75, 4832–4842 (2001).

59. Meyer PR, Matsuura SE, So AG, Scott WA: Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. *Proc. Natl Acad. Sci. USA* 95, 13471–13476 (1998).

60. Sarafianos SG, Clark AD Jr, Das K *et al.*: Structures of HIV-1 reverse transcriptase with pre- and post-translocation AZTMP-terminated DNA. *EMBO J.* 21, 6614–6624 (2002).

61. Tuske S, Sarafianos SG, Clark AD Jr *et al.*: Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. *Nat. Struct. Mol. Biol.* 11, 469–474 (2004).

62. Miller MD, Margot NA, Hertogs K, Larder B, Miller V: Antiviral activity of tenofovir (PMPA) against nucleoside-resistant clinical HIV samples. *Nucleosides Nucleotides Nucleic Acids* 20, 1025–1028 (2001).

63. Wolf K, Walter H, Beerenwinkel N *et al.*: Tenofovir resistance and resensitization. *Antimicrob. Agents Chemother.* 47, 3478–3484 (2003).

64. Lennerstrand J, Hertogs K, Stammers DK, Larder BA: Correlation between viral resistance to zidovudine and resistance at the reverse transcriptase level for a panel of human immunodeficiency virus type 1 mutants. *J. Virol.* 75, 7202–7205 (2001).

65. Naeger LK, Margot NA, Miller MD: Increased drug susceptibility of HIV-1 reverse transcriptase mutants containing M184V and zidovudine-associated mutations: analysis of enzyme processivity, chain-terminator removal and viral replication. *Antivir. Ther.* 6, 115–126 (2001).

66. Boyer PL, Sarafianos SG, Arnold E, Hughes SH: The M184V mutation reduces the selective excision of zidovudine 5′-monophosphate (AZTMP) by the reverse transcriptase of human immunodeficiency virus type 1. *J. Virol.* 76, 3248–3256 (2002).

67. Gu Z, Gao Q, Fang H *et al.*: Identification of a mutation at codon 65 in the IKKK motif of reverse transcriptase that encodes human immunodeficiency virus resistance to 2′,3′-dideoxycytidine and 2′,3′-dideoxy-3′-thiacytidine. *Antimicrob. Agents Chemother.* 38, 275–281 (1994).

68. Zhang D, Caliendo AM, Eron JJ *et al.*: Resistance to 2′,3′-dideoxycytidine conferred by a mutation in codon 65 of the human immunodeficiency virus type 1 reverse transcriptase. *Antimicrob. Agents Chemother.* 38, 282–287 (1994).

69. Tisdale M, Alnadaf T, Cousens D: Combination of mutations in human immunodeficiency virus type 1 reverse transcriptase required for resistance to the carbocyclic nucleoside 1592U89. *Antimicrob. Agents Chemother.* 41, 1094–1098 (1997).

70. García-Lerma JG, MacInnes H, Bennett D *et al.*: A novel genetic pathway of human immunodeficiency virus type 1 resistance to stavudine mediated by the K65R mutation. *J. Virol.* 77, 5685–5693 (2003).

71. Srinivas RV, Fridland A: Antiviral activities of 9-R-2-phosphonomethoxypropyl adenine (PMPA) and bis(isopropyloxymethylcarbonyl) PMPA against various drug-resistant human immunodeficiency virus strains. *Antimicrob. Agents Chemother.* 42, 1484–1487 (1998).

72. Wainberg MA, Miller MD, Quan Y *et al.*: *In vitro* selection and characterization of HIV-1 with reduced susceptibility to PMPA. *Antivir. Ther.* 4, 87–94 (1999).

73. White KL, Margot NA, Ly JK *et al.*: A combination of decreased NRTI incorporation and decreased excision determines the resistance profile of HIV-1 K65R RT. *AIDS* 19, 1751–1760 (2005).

74. Gallant JE, Staszewski S, Pozniak AL *et al.*: Efficacy and safety of tenofovir DF vs stavudine in combination therapy in antiretroviral-naive patients: a 3-year randomized trial. *JAMA* 292, 191–201 (2004).

75. Gallant JE, DeJesus E, Arribas JR *et al.*: Tenofovir DF, emtricitabine, and efavirenz vs. zidovudine, lamivudine, and efavirenz for HIV. *N. Engl. J. Med.* 354, 251–260 (2006).

76. Gu Z, Fletcher RS, Arts EJ, Wainberg MA, Parniak MA: The K65R mutant reverse transcriptase of HIV-1 cross-resistant to 2′,3′-dideoxycytidine, 2′,3′-dideoxy-3′-thiacytidine, and 2′,3′-dideoxyinosine shows reduced sensitivity to specific dideoxynucleoside triphosphate inhibitors *in vitro*. *J. Biol. Chem.* 269, 28118–28122 (1994).

77. Gu Z, Arts EJ, Parniak MA, Wainberg MA: Mutated K65R recombinant reverse transcriptase of human immunodeficiency virus type 1 shows diminished chain termination in the presence of 2′,3′-dideoxycytidine 5′-triphosphate and other drugs. *Proc. Natl Acad. Sci. USA* 92, 2760–2764 (1995).

78. Selmi B, Boretto J, Sarfati SR, Guerreiro C, Canard B: Mechanism-based suppression of dideoxynucleotide resistance by K65R human immunodeficiency virus reverse transcriptase using an α-boranophosphate nucleoside analogue. *J. Biol. Chem.* 276, 48466–48472 (2001).

79. Sluis-Cremer N, Sheen C-W, Zelina S, Torres PSA, Parikh UM, Mellors JW: Molecular mechanism by which the K70E mutation in human immunodeficiency virus type 1 reverse transcriptase confers resistance to nucleoside reverse transcriptase inhibitors. *Antimicrob. Agents Chemother.* 51, 48–53 (2007).

80. White KL, Chen JM, Feng JY *et al.*: The K65R reverse transcriptase mutation in HIV-1 reverses the excision phenotype of zidovudine resistance mutations. *Antivir. Ther.* 11, 155–163 (2006).

81. Parikh UM, Zelina S, Sluis-Cremer N, Mellors JW: Molecular mechanisms of bidirectional antagonism between K65R and thymidine analog mutations in HIV-1 reverse transcriptase. *AIDS* 21, 1405–1414 (2007).

82. Parikh UM, Bacheler L, Koontz D, Mellors JW: The K65R mutation in human immunodeficiency virus type 1 reverse transcriptase exhibits bidirectional phenotypic antagonism with thymidine analog mutations. *J. Virol.* 80, 4971–4977 (2006).

83. Parikh UM, Barnas DC, Faruki H, Mellors JW: Antagonism between the HIV-1 reverse-transcriptase mutation K65R and thymidine-analogue mutations at the genomic level. *J. Infect. Dis.* 194, 651–660 (2006).

84. Winston A, Mandalia S, Pillay D, Gazzard B, Pozniak A: The prevalence and determinants of the K65R mutation in HIV-1 reverse transcriptase in tenofovir-naive patients. *AIDS* 16, 2087–2089 (2002).

85. Valer L, Martín-Carbonero L, de Mendoza C, Corral A, Soriano V: Predictors of selection of K65R: tenofovir use and lack of thymidine analogue mutations. *AIDS* 18, 2094–2096 (2004).

86. Segondy M, Montes B: Prevalence and conditions of selection of the K65R mutation in the reverse transcriptase gene of HIV-1. *J. Acquir. Immune Defic. Syndr.* 38, 110–111 (2005).

87. Das K, Bandwar RP, White KL *et al.*: Structural basis for the role of the K65R mutation in HIV-1 reverse transcriptase polymerization, excision antagonism, and tenofovir resistance. *J. Biol. Chem.* 284, 35092–35100 (2009).

88. De Antoni A, Foli A, Lisziewicz J, Lori F: Mutations in the pol gene of human immunodeficiency virus type 1 in infected patients receiving didanosine and hydroxyurea combination therapy. *J. Infect. Dis.* 176, 899–903 (1997).

89. Menéndez-Arias L, Matamoros T, Cases-González CE: Insertions and deletions in HIV-1 reverse transcriptase: consequences for drug resistance and viral fitness. *Curr. Pharm. Des.* 12, 1811–1825 (2006).

90. Eggink D, Huigen MCDG, Boucher CAB, Götte M, Nijhuis M: Insertions in the β3-β4 loop of reverse transcriptase of human immunodeficiency virus type 1 and their mechanism of action, influence on drug susceptibility and viral replication capacity. *Antiviral Res.* 75, 93–103 (2007).

91. Winters MA, Coolley KL, Girard YA *et al.*: A 6-basepair insert in the reverse transcriptase gene of human immunodeficiency virus type 1 confers resistance to multiple nucleoside inhibitors. *J. Clin. Invest.* 102, 1769–1775 (1998).

92. Sugiura W, Matsuda M, Matsuda Z *et al.*: Identification of insertion mutations in HIV-1 reverse transcriptase causing multiple drug resistance to nucleoside analogue reverse transcriptase inhibitors. *J. Hum. Virol.* 2, 146–153 (1999).

93. de Jong JJ, Goudsmit J, Lukashov VV *et al.*: Insertion of two amino acids combined with changes in reverse transcriptase containing tyrosine-215 of HIV-1 resistant to multiple nucleoside analogs. *AIDS* 13, 75–80 (1999).

94. Larder BA, Bloor S, Kemp SD *et al.*: A family of insertion mutations between codons 67 and 70 of human immunodeficiency virus type 1 reverse transcriptase confer multinucleoside analog resistance. *Antimicrob. Agents Chemother.* 43, 1961–1967 (1999).

95. Rakik A, Ait-Khaled M, Griffin P, Thomas TA, Tisdale M, Kleim JP: A novel genotype encoding a single amino acid insertion and five other substitutions between residues 64 and 74 of the HIV-1 reverse transcriptase confers high-level cross-resistance to nucleoside reverse transcriptase inhibitors. Abacavir CNA2007 International Study Group. *J. Acquir. Immune Defic. Syndr.* 22, 139–145 (1999).

96. Mas A, Parera M, Briones C *et al.*: Role of a dipeptide insertion between codons 69 and 70 of HIV-1 reverse transcriptase in the mechanism of AZT resistance. *EMBO J.* 19, 5752–5761 (2000).

97. Boyer PL, Sarafianos SG, Arnold E, Hughes SH: Nucleoside analog resistance caused by insertions in the fingers of human immunodeficiency virus type 1 reverse transcriptase involves ATP-mediated excision. *J. Virol.* 76, 9143–9151 (2002).

98. Mas A, Vázquez-Álvarez BM, Domingo E, Menéndez-Arias L: Multidrug-resistant HIV-1 reverse transcriptase: involvement of ribonucleotide-dependent phosphorolysis in cross-resistance to nucleoside analogue inhibitors. *J. Mol. Biol.* 323, 181–197 (2002).

99. Meyer PR, Lennerstrand J, Matsuura SE, Larder BA, Scott WA: Effects of dipeptide insertions between codons 69 and 70 of human immunodeficiency virus type 1 reverse transcriptase on primer unblocking, deoxynucleoside triphosphate inhibition, and DNA chain elongation. *J. Virol.* 77, 3871–3877 (2003).

100. White KL, Chen JM, Margot NA *et al.*: Molecular mechanisms of tenofovir resistance conferred by human immunodeficiency virus type 1 reverse transcriptase containing a diserine insertion after residue 69 and multiple thymidine analog-associated mutations. *Antimicrob. Agents Chemother.* 48, 992–1003 (2004).