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Review

Molecular basis of human immunodeficiency virus type 1 drug resistance: Overview and recent developments

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ABSTRACT

The introduction of potent combination therapies in the mid-90s had a tremendous effect on AIDS mortality. However, drug resistance has been a major factor contributing to antiretroviral therapy failure. Currently, there are 26 drugs approved for treating human immunodeficiency virus (HIV) infections, although some of them are no longer prescribed. Most of the available antiretroviral drugs target HIV genome replication (i.e. reverse transcriptase inhibitors) and viral maturation (i.e. viral protease inhibitors). Other drugs in clinical use include a viral coreceptor antagonist (maraviroc), a fusion inhibitor (enfuvirtide) and two viral integrase inhibitors (raltegravir and elvitegravir). Elvitegravir and the nonnucleoside reverse transcriptase inhibitor rilpivirine have been the most recent additions to the antiretroviral drug armamentarium. An overview of the molecular mechanisms involved in antiretroviral drug resistance and the role of drug resistance-associated mutations was previously presented (Menéndez-Arias, L., 2010. Molecular basis of human immunodeficiency virus drug resistance: an update. Antiviral Res. 85, 210–231). This article provides now an updated review that covers currently approved drugs, new experimental agents (e.g. neutralizing antibodies) and selected drugs in preclinical or early clinical development (e.g. experimental integrase inhibitors). Special attention is dedicated to recent research on resistance to reverse transcriptase and integrase inhibitors. In addition, recently discovered interactions between HIV and host proteins and novel strategies to block HIV assembly or viral entry emerge as promising alternatives for the development of effective antiretroviral treatments.

Contents

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1. Introduction

The human immunodeficiency virus (HIV) is the causative agent of the acquired immunodeficiency syndrome (AIDS). More than 30 million people have died of AIDS-related diseases and currently, there are about 34 million people worldwide infected with HIV (UNAIDS, 2012). In 2011, the number of new infections was estimated around 2.5 million, while the disease caused 1.7 million deaths. No effective vaccine or cure is currently available. However, thirty years after the identification of the AIDS virus, there has been significant progress towards understanding its biology and the pathogenesis of the disease that resulted in the implementation of effective therapies that control HIV propagation.

These treatments, usually referred to as highly active antiretroviral therapy (HAART), consist of combinations of three or more antiretroviral drugs. Commonly prescribed HAART regimens include two nucleoside reverse transcriptase inhibitors (NRTIs) in combination with one nonnucleoside reverse transcriptase inhibitor (NNRTI), a protease inhibitor or an integrase inhibitor. AIDS morbidity and mortality have decreased significantly in developed countries since the introduction of HAART in the mid-90s. Drug adherence, tolerability and long-term toxicity constituted major limitations to their clinical use. However, improvements in potency, safety and dosage simplification have alleviated those problems. Nonetheless, the acquisition and transmission of HIV drug resistance still poses a major risk to the success of antiretroviral therapies.

HIV has a high mutation rate ($\sim 10^{-4}$ to 10^{-5} mutations per nucleotide and cycle of replication) and a high frequency of recombination (for a review, see Menéndez-Arias, 2009). In addition, although individuals are usually infected with only a few original clones (Keele et al., 2008), it has been estimated that virus production can go up to 10^{10} virions per day in untreated patients (Coffin, 1995; Perelson et al., 1996), therefore generating viral quasispecies composed of many HIV variants, that could include drug-resistant strains. Selection of resistance to antiretroviral drugs depends on many factors, including a genetic barrier and the viral fitness of the mutated strains selected under drug pressure (Götte, 2012). The genetic barrier is defined as the minimal number of mutations required to overcome drug selection pressure. Independent factors, such as the fitness of the resistant strains that could depend on the environmental conditions (e.g. host cell factors, drug bioavailability) contribute to a more general 'resistance barrier' (Menéndez-Arias et al., 2003; Wargo and Kurath, 2012).

Drug resistance can be acquired through drug selection pressure or transmitted from person to person. The prevalence of transmitted HIV-1 drug resistance for any class of antiretroviral drug has been estimated at around 8–20% of the untreated population (Weinstock et al., 2004; Wensing et al., 2005; Jayaraman et al., 2006; Hattori et al., 2010; Wheeler et al., 2010; Truong et al., 2011), although recent reports from studies carried out in Europe and the US showed a decline during the last decade (Miller et al., 2012; UK Collaborative Group on HIV Drug Resistance, 2012). Nonetheless, this decline appears to be small, particularly for the prevalence of NRTI resistance mutations (Vercauteren et al., 2009; UK Collaborative Group on HIV Drug Resistance, 2012). Transmitted drug resistance also poses a serious risk for the preventive use of antiretroviral drug-based microbicides. Development of resistance to these drugs is a matter of concern since it might limit therapeutic options for individuals who become infected while taking microbicides (Shattock and Rosenberg, 2012).

At present, there are 26 drugs licensed for treatment of HIV infection (De Clercq, 2009) (Table 1). These compounds target different steps of the viral life cycle: (i) viral entry (e.g. coreceptor antagonists and fusion inhibitors); (ii) reverse transcription (NRTIs and NNRTIs); (iii) integration (inhibitors of the viral integrase); and (iv) viral maturation (protease inhibitors). Powerful combinations of these antiretroviral drugs have shown considerable success in controlling HIV infection, but understanding the basis of drug resistance is important to improve current therapies. In a previous article we provided an overview on the molecular mechanisms involved in the acquisition of drug resistance to approved and investigational drugs available at that time (Menéndez-Arias, 2010). Now, we summarize current knowledge on this topic, while adding further insights coming from recent research particularly on recently approved drugs such as rilpivirine or elvitegravir, and new developments towards understanding interactions involving secondary mutations related to resistance emerging with current therapies.

2. HIV-1 RT structure

HIV type 1 (HIV-1) and HIV type 2 (HIV-2) package two copies of a single-stranded RNA genome within each viral particle. All HIV genomes contain three major genes, arranged in the order 5′-*gag-pol-env*-3′, and a series of genes encoding for accessory and regulatory proteins that in the case of HIV-1 are *vif*, *vpr*, *vpu*, *tat*, *rev* and *nef* (Fig. 1a). The viral RT is the enzyme responsible for the conversion of the single-stranded RNA genome into a double-stranded DNA that can eventually integrate in the genome of the infected cell. The RT is a multifunctional enzyme with DNA polymerase (both DNA- and RNA-dependent) and endonuclease (RNase H) activities (for a recent review, see Le Grice, 2012). The HIV-1 RT is a heterodimer composed of subunits of 560 and 440 residues, referred to as p66 and p51, respectively (Fig. 1b). Both subunits share the same amino acid sequence, but p51 lacks the RNase H domain that extends from residue 441 to residue 560 of p66.

Crystal structures revealed that the p66 subunit of the HIV-1 RT and prokaryotic and eukaryotic DNA polymerases shared a similar fold, with subdomains designated as 'fingers' (residues 1–85 and 118–155), 'palm' (residues 86–117 and 156–236) and 'thumb' (residues 237–318), as well as a 'connection' subdomain (residues 319–426) that joins the DNA polymerase and the RNase H domains. The overall arrangement of subdomains is similar in both RT subunits, but their positions relative to each other are different in p66 and p51 (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993; Huang et al., 1998). The p66 subunit contains a nucleic acid bind-

ing cleft that is missing in p51, as well as active-site carboxylates (Asp^{110}, Asp^{185} and Asp^{186}), that bind the magnesium ions required for DNA polymerase catalysis (Mendieta et al., 2008). The crystal structure of the ternary complex of HIV-1 RT bound to double-stranded DNA and an incoming dNTP (Huang et al., 1998) showed that Lys^{65}, Arg^{72}, Asp^{113}, Ala^{114}, Tyr^{115} and Gln^{151} interact with the nucleotide, while Leu^{74}, Pro^{157}, Phe^{160}, Tyr^{183} and Met^{184} could indirectly affect dNTP binding (Fig. 1c).

RT inhibitors in clinical use can be classified as NRTIs or NNRTIs. NRTIs are nucleoside or nucleotide analogues that inside the cell are converted into triphosphate derivatives that compete with natural dNTPs for incorporation in the nascent DNA chain (for a review, see Menéndez-Arias, 2008). DNA elongation is then blocked due to the lack of a 3′-OH in the ribose ring. NNRTIs are small hydrophobic compounds that bind at a hydrophobic pocket adjacent to the polymerase active site in the RT p66 subunit (for recent reviews, see Ren and Stammers, 2008; Menéndez-Arias et al., 2011).

3. Approved NRTIs and molecular mechanisms of resistance

NRTIs constitute the backbone of current antiretroviral therapies. The active metabolites of these drugs act as competitive inhibitors (or alternate substrates) of HIV-1 RT. In their active triphosphate form, zidovudine (AZT; β-D-(+)-3′-azido-3′-deoxythymidine) and stavudine (d4T, β-D-(+)-2′,3′-didehydro-2′,3′-dideoxythymidine) are dTTP competitors; while lamivudine (3TC, β-L-(−)-2′,3′-dideoxy-3′-thiacytidine) and emtricitabine (FTC, β-L-(−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine) compete with dCTP in nucleotide incorporation reactions. Other NRTIs, such as didanosine (ddI; β-D-(+)-2′,3′-dideoxyinosine) or abacavir ((−)-(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol) are converted to triphosphorylated nucleoside analogues that compete with dATP and dGTP, respectively. On the other hand, tenofovir is an acyclic nucleoside phosphonate (*R*-9-(2-phosphonomethoxypropyl)adenine) which is administered as an esterase-sensitive prodrug (Fig. 2). During RT-catalyzed

Table 1
Currently available antiretroviral drugs.

Name	Brand name	Approval date (US)	Manufacturer
Reverse transcriptase inhibitors [nucleos(t)ide analogues]			
Abacavir	Ziagen	12/1998	GlaxoSmithKline
Didanosine	Videx	10/1991	Bristol-Myers Squibb
	Videx EC (enteric-coated)	10/2000	Bristol-Myers Squibb
Emtricitabine	Emtriva	07/2003	Gilead Sciences
Lamivudine	Epivir	11/1995	GlaxoSmithKline
Stavudine	Zerit	06/1994	Bristol-Myers Squibb
Tenofovir disoproxil fumarate (DF)	Viread	10/2001	Gilead Sciences
Zalcitabine	Hivid	06/1992	(sales and distribution discontinued since 2007)
Zidovudine	Retrovir	03/1987	GlaxoSmithKline
Reverse transcriptase inhibitors [fixed-dose combinations of nucleos(t)ide analogues]			
Abacavir/Lamivudine	Epzicom, *Kivexa*	08/2004	GlaxoSmithKline
Abacavir/Lamivudine/Zidovudine	Trizivir	11/2000	GlaxoSmithKline
Emtricitabine/Tenofovir DF	Truvada	08/2004	Gilead Sciences
Lamivudine/Zidovudine	Combivir	09/1997	GlaxoSmithKline
Reverse transcriptase inhibitors (nonnucleosides)			
Delavirdine	Rescriptor	04/1997	Pfizer
Efavirenz	Sustiva, *Stocrin*	09/1998	Bristol-Myers Squibb/Merck Sharp & Dohme
Etravirine	Intelence	01/2008	Janssen Pharmaceuticals Inc.
Nevirapine	Viramune	06/1996	Boehringer Ingelheim
Rilpivirine	Edurant	05/2011	Janssen Pharmaceuticals Inc.
Reverse transcriptase inhibitors (fixed-dose combinations of both types of inhibitors)			
Efavirenz/Emtricitabine/Tenofovir DF	Atripla	07/2006	Bristol-Myers Squibb, Gilead Sciences
Emtricitabine/Rilpivirine/Tenofovir DF	Complera, *Eviplera*[a]	08/2011	Gilead Sciences
Protease inhibitors			
Atazanavir	Reyataz	06/2003	Bristol-Myers Squibb
Darunavir	Prezista	06/2006	Janssen Pharmaceuticals Inc.
Fosamprenavir	Lexiva, *Telzir*	10/2003	GlaxoSmithKline
Indinavir	Crixivan	03/1996	Merck
Lopinavir/ritonavir[b]	Kaletra, *Aluvia*	09/2000	Abbott Laboratories
Nelfinavir	Viracept	03/1997	Agouron Pharmaceuticals
Ritonavir	Norvir	03/1996	Abbott Laboratories
Saquinavir	Invirase	12/1995	Hoffmann-La Roche
Tipranavir	Aptivus	06/2005	Boehringer Ingelheim
Integrase inhibitors			
Raltegravir	Isentress	10/2007	Merck
Fixed-dose combinations of reverse transcriptase and integrase inhibitors			
Elvitegravir[c]/Cobicistat[d]/Emtricitabine/Tenofovir DF	Stribild	08/2012	Gilead Sciences
Fusion inhibitors			
Enfuvirtide	Fuzeon	03/2003	Hoffmann-La Roche, Trimeris
CCR5 antagonists			
Maraviroc	Selzentry, *Celsentri*	08/2007	Pfizer

Brand names in italics are those used in Europe (Kivexa, Stocrin, Eviplera, Telzir, Celsentri) or in the developing world (Aluvia). Information on Food and Drug Administration approved anti-HIV medication was taken from http://aidsinfo.nih.gov/contentfiles/ApprovedMedstoTreatHIV_FS_en.pdf (last accessed on January 2013).

[a] http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/002312/WC500118802.pdf

[b] Contains a sub-therapeutic dose of ritonavir. This drug is a potent inhibitor of cytochrome P450 3A4 (Sham et al., 1998) and helps to maintain the blood levels of lopinavir relatively high.

[c] Elvitegravir has been approved only for use as a component of Stribild.

[d] Cobicistat is a pharmacokinetic enhancer that is used to prolong the effect of elvitegravir.

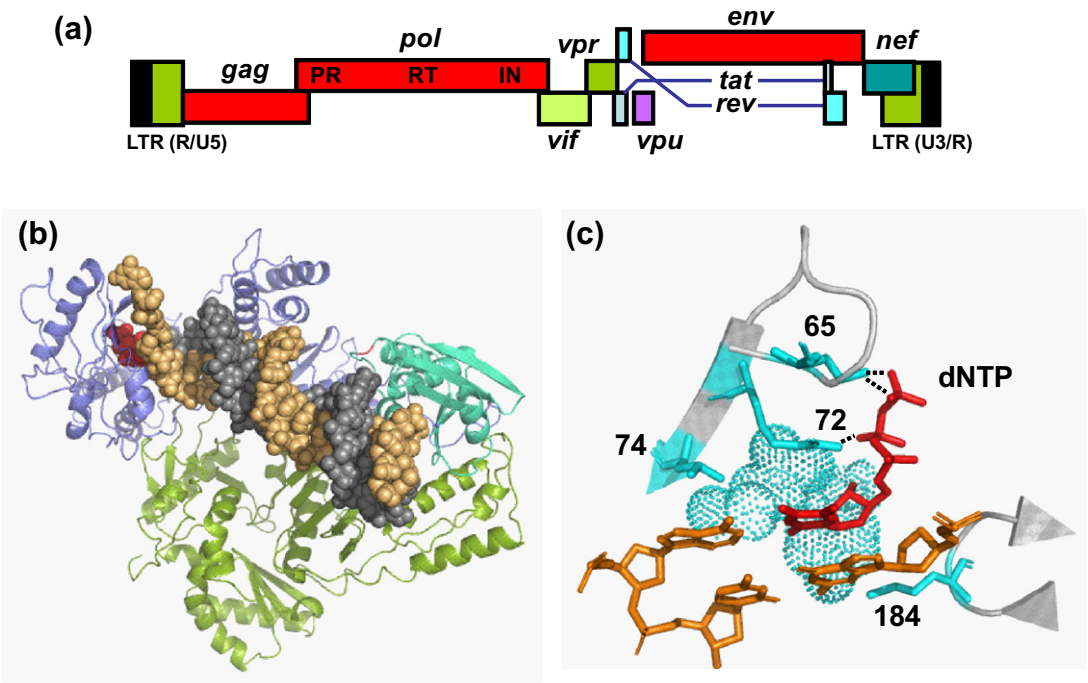


Fig. 1. HIV-1 genome organization and RT structure. (a) Major (*gag, pol, env*) and accessory and regulatory genes (*vif, vpr, vpu, tat, rev, nef*) in the HIV-1 genome. Antiretroviral drug targets such as the protease (PR), the reverse transcriptase (RT) and the integrase (IN) are encoded within the *pol* gene. (b) Crystal structure of the ternary complex of HIV-1 RT (ribbon representation with the p66 DNA polymerase domain in purple, the p66 RNase H domain in dark green, and p51 in light green), double-stranded DNA (brown and orange spheres) and an incoming nucleotide (red spheres). (c) The nucleotide binding site with the side chains of Lys^{65} and Arg^{72} making hydrogen bonds with the phosphate groups of the incoming nucleotide. Van der Waals surface of the side-chains of Tyr^{115} and Gln^{151} are shown in blue. Stick representations are used to show the location of Lys^{65}, Arg^{72}, Leu^{74} and Met^{184} (blue) and template/primer nucleotides (orange). Atomic coordinates were obtained from PDB file 1RTD (Huang et al., 1998).

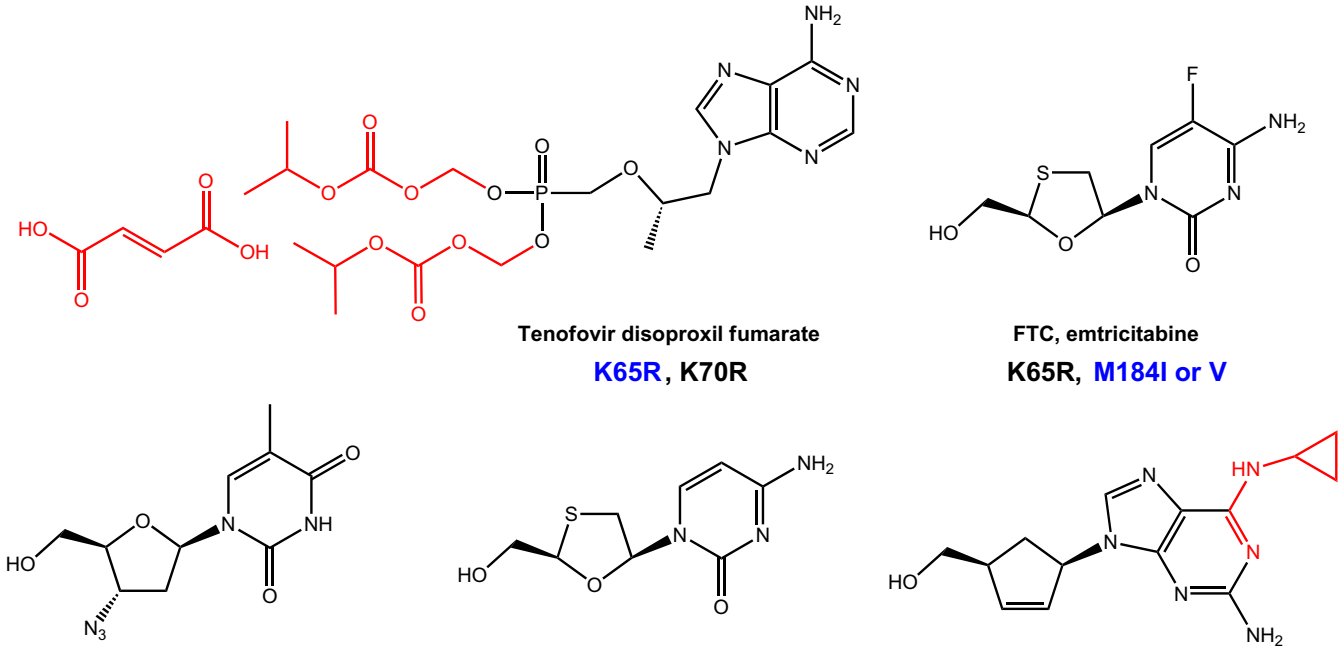


Fig. 2. Chemical structures of nucleoside analogues used as part of available fixed-dose combinations. Atoms modified during the conversion to metabolically relevant derivatives are indicated in red. Drug resistance associated mutations for each drug are given below. The mutations with the largest impact on resistance are shown in blue. Combinations of TAMs associated with AZT resistance are also responsible for cross-resistance with abacavir and tenofovir. The Q151M complex (A62 V/V75I/F77L/F116Y/Q151M) confers resistance to all shown inhibitors except tenofovir.

DNA polymerization, tenofovir-diphosphate competes with dATP for binding and incorporation into the nascent DNA chain.

There are two major molecular mechanisms by which HIV-1 becomes resistant to NRTIs. First, NRTI-associated resistance mutations can increase the ability of HIV-1 RT to discriminate against the triphosphate derivatives of NRTIs. Classical examples of mutations acting through this mechanism are M184I and M184V. These amino acid substitutions confer high-level resistance to lamivudine (3TC) and emtricitabine (FTC). The presence of a β-branched amino acid (usually Val or Ile) at position 184 interferes with binding of 3TC- or FTC-triphosphate due in part to a steric clash with the oxathiolane ring of the inhibitor (Sarafianos et al., 1999; Gao et al., 2000). Other amino acid substitutions affecting residues of the dNTP binding site or its vicinity (e.g. K65R, K70E, V75I, etc.) also confer resistance by affecting nucleotide discrimination.

An alternative mechanism of resistance to NRTIs involves the acquisition of mutations such as M41L, D67N, K70R, L210W, T215F or T215Y, and K219E or K219Q, also known as 'thymidine analogue resistance mutations' (TAMs) because they usually emerge under treatment with AZT or d4T. TAM-containing RTs are able to excise 3′-terminal chain-terminators from blocked DNA primers, through phosphorolysis mediated by a pyrophosphate donor, which is probably ATP, under physiological conditions (Meyer et al., 1999) (Fig. 3). Biochemical studies have demonstrated that AZT, d4T and tenofovir are good substrates of the excision reaction, while cytidine analogues are removed very inefficiently (for a review, see Menéndez-Arias, 2008).

3.1. Mutational patterns associated with nucleotide selectivity

Several amino acid substitutions affecting residues of the dNTP binding site in HIV-1 RT have been associated with resistance to NRTIs. Examples are K65R, K70E, L74V, Y115F and M184I or M184V. These amino acid substitutions have an impact on the catalytic parameters of nucleotide incorporation, as determined preferentially by pre-steady-state kinetics (for reviews, see Menéndez-Arias, 2008, 2010).

K65R has been identified as a relevant mutation conferring resistance to tenofovir in treated patients (Margot et al., 2002). Lys^{65} interacts with the γ-phosphate of the incoming dNTP (Huang et al., 1998). HIV-1 RTs bearing Arg^{65} instead of Lys showed a decreased nucleotide incorporation rate (k_{pol}) compared with the wild-type enzyme (Selmi et al., 2001; Deval et al., 2004, 2005; Barrioluengo et al., 2011), in agreement with their lower fitness in viral replication assays (Deval et al., 2004; Cong et al., 2007; Svarovskaia et al., 2008). The K65R RT is able to discriminate against several NRTIs (tenofovir, ddI, abacavir, 3TC, FTC and zalcitabine) by having an even slower incorporation rate than the natural substrates (Deval et al., 2004; Sluis-Cremer et al., 2007; Das et al., 2009). For example, the dATP incorporation rate (k_{pol}) of K65R RT is about 4.5 times smaller than the rate obtained with the wild-type enzyme. However, the k_{pol} for tenofovir-diphosphate or ddATP is >23 times smaller in the case of the K65R mutant. On the other hand, no significant changes were observed in the binding affinity (K_D) (Das et al., 2009).

Crystal structures of the HIV-1 RT mutant K65R cross-linked to double-stranded DNA and in complex with tenofovir-diphosphate or dATP showed that the substitution had a minor effect on nucleotide binding interaction. However, the guanidinium moiety of Arg^{65} has an enhanced stacking interaction with its equivalent group in Arg^{72}. This interaction restricts the conformational adaptability of the RT polymerase active site and causes a decrease in the rate of nucleotide incorporation (Das et al., 2009).

Several studies have suggested that K65R develops more quickly in HIV-1 subtype C than in HIV-1 subtype B variants (Doualla-Bell et al., 2006; Invernizzi et al., 2009; Sunpath et al., 2012). DNA synthesis assays carried out with subtype C templates produce more K65R-containing transcripts than subtype B templates. The predisposition of subtype C HIV-1 toward acquiring this mutation appears to be related to template usage. RT codon 65 is part of a polyadenylated sequence. In subtype C viruses, there is an intrinsic difficulty in synthesizing polyadenylated sequences that leads to pausing at codon 65, thereby facilitating the selection of K65R under drug pressure (Coutsinos et al., 2009, 2011; Invernizzi et al., 2009).

M184I and M184V are mutations that decrease viral replication capacity, particularly in the presence of low dNTP concentrations (Back et al., 1996; Back and Berkhout, 1997; Wei et al., 2003; Van Cor-Hosmer et al., 2010). Although both mutations are selected during treatment with cytidine analogues (i.e. 3TC or FTC), the prevalence of mutations at position 184 was significantly lower in patients who received FTC and tenofovir disoproxil fumarate (tenofovir-DF) than in those who received 3TC and tenofovir-DF (Drogan et al., 2010; Marcelin et al., 2012). The presence of a β-branched amino acid (Val or Ile) at position 184 interferes with 3TC- or FTC-triphosphate binding due to a steric clash with the NRTI (Sarafianos et al., 1999).

The Q151M complex (including mutations A62V, V75I, F77L, F116Y and Q151M) confers multi-NRTI resistance through improved discrimination against triphosphate derivatives of nucleoside analogues. Q151M and accompanying mutations produce a significant reduction of the catalytic rate constants (k_{pol}) of incorporation of AZTTP, d4TTP, ddATP, ddCTP or carbovir-TP relative to their natural substrate equivalents (dTTP, dATP, dCTP, etc.) (De-

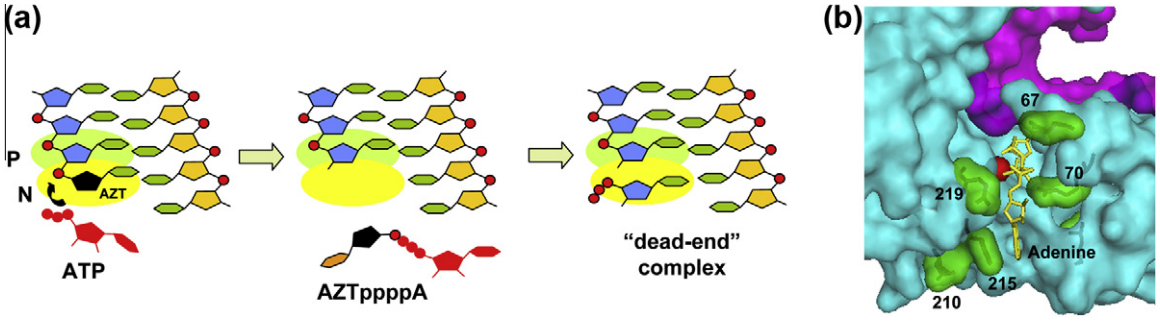


Fig. 3. Molecular basis of the excision reaction. (a) Schematic representation of the ATP-mediated excision reaction leading to the formation of a dinucleoside tetraphosphate product. On the right, the "dead-end" complex that inhibits the reaction is formed when the 3′-OH of the primer occupies the P site, while the complementary dNTP binds to the N site. (b) Crystal structure of AZT-resistant HIV-1 RT (M41L/D67N/K70R/T215Y/K219Q) bound to DNA–DNA template–primer (surface in magenta), and the AZT adenosine dinucleoside triphosphate product (shown in yellow with a stick representation). The red sphere represents one Mg^{2+} cation. The surface of p66 is shown in cyan, except for residues Asn^{67}, Arg^{70}, Leu^{210}, Tyr^{215} and Gln^{219} that are shown in green. Atomic coordinates were taken from PDB file 3KLE (Tu et al., 2010).

val et al., 2002, 2005; Ray et al., 2002; Frangeul et al., 2008). Modeling studies suggest that the decrease in the k_{pol} value results from the loss of hydrogen bonding interactions affecting the 3′-OH of the ribose of the dNTP (Deval et al., 2002). The side chain of Gln^{151} is part of the dNTP binding site and interacts with the nucleotide substrate (Huang et al., 1998), while accompanying mutations have a subtle effect on the catalytic efficiency of the RT and improve viral fitness. Selection of Q151M is relatively difficult because this amino acid substitution requires two nucleotide changes and potential intermediates (i.e. Q151L or Q151K) are rarely observed *in vivo* due to their reduced replication capacity.

Several mutations have been associated with the emergence of the Q151M mutational pattern. Thus, S68G and M230I were shown to improve the replicative capacity of Q151L-containing viruses (Matsumi et al., 2003). Interestingly, a few mutations around positions 67–72 (at the β3–β4 hairpin loop) have been found associated with the Q151M complex. Examples are T69N, K70G, K70Q or the deletion of Thr^{69} (Hachiya et al., 2011; Kisic et al., 2011; Mbisa et al., 2011). Virological and biochemical analysis have shown that K70Q adds high-level tenofovir resistance, while increasing phenotypic viral susceptibility to AZT, ddI, d4T, 3TC and abacavir (Hachiya et al., 2011). Pre-steady-state kinetic analysis of recombinant enzymes demonstrated that the Gln^{70} decreases binding of tenofovir-diphosphate and reduces the incorporation of the inhibitor in the nascent DNA chain.

3.2. Resistance mediated by phosphorolytic excision of NRTIs

Resistance to AZT and other NRTIs is often mediated by TAMs. These amino acid substitutions confer resistance by increasing the ability of the HIV-1 RT to excise 3′-terminal chain-terminator inhibitors from blocked DNA primers through phosphorolysis mediated by ATP or pyrophosphate (Arion et al., 1998; Meyer et al., 1998, 1999; for recent reviews, see Menéndez-Arias, 2008, 2010). Available evidence suggests that ATP is the pyrophosphate donor under physiological conditions.

Primers terminated with thymidine analogues (AZT or d4T) or tenofovir are the best substrates of the excision reaction. If the DNA is terminated with AZT-monophosphate, the product of the ATP-dependent excision reaction is a dinucleoside tetraphosphate derivative (AZTppppA) (Fig. 3a). The efficiency of the excision reaction also depends on the specific template–primer sequence (Meyer et al., 2004), and studies carried out with tenofovir-blocked primers indicate that excision is more efficient on the polypurine tract (PPT), than on oligonucleotides containing the primer binding site (PBS) required for the initiation of reverse transcription (Iyidogan and Anderson, 2012).

The excision reaction can be inhibited by the next complementary nucleotide (Meyer et al., 1999, 2000) due to the formation of a "dead-end complex" (Tong et al., 1997). In these complexes, the blocked primer would be in a translocated position with the NRTI moiety in the P site. Meanwhile, the RT nucleotide binding site would be occupied by the complementary dNTP (Marchand and Götte, 2003; Marchand et al., 2007). Excision of AZT-monophosphate is not inhibited at physiological dNTP concentrations (IC_{50} > 250 μM), but removal of tenofovir, as well as d4T- and ddA-monophosphates can be inhibited at low concentrations of the next complementary dNTP (Meyer et al., 2000; Mas et al., 2002; White et al., 2004). Structural models suggest that excision could only occur if the blocking NRTI remains in a pre-translocated status.

A recent report describing the crystal structures of wild-type and mutant M41L/D67N/K70R/T215Y/K219Q RT in their unliganded forms, as well as complexed with AZT-terminated DNA/DNA, and with double-stranded DNA (dsDNA) and AZTppppA has provided valuable insights into the excision mechanism (Tu et al., 2010). The analysis of those structures revealed that in the complex of mutant RT/dsDNA/AZTppppA, the AZTTP portion of AZTppppA and the dTTP molecule in the ternary complex of HIV-1 RT/dsDNA/dTTP (Huang et al., 1998) are topologically equivalent. The adenine in the AZTppppA molecule makes π–π stacking interactions with the side chain of Tyr^{215}, while the guanidinium group of Arg^{70} makes hydrogen bonds with the ribose ring and the phosphate group of the AMP moiety (Fig. 3b). None of these interactions occur with the wild-type RT. In equivalent complexes of this enzyme, the AZTTP portion of AZTppppA occupies a pocket defined by Lys^{219}, Lys^{220}, His^{221} and Leu^{228}, away from Thr^{215}. The relevance of mutations K70R and T215Y is further supported by their early appearance under suboptimal antiretroviral therapy with thymidine analogues (Jeeninga et al., 2001).

3.2.1. Clusters of TAMs and associated mutations

Genotypic analysis of viral isolates from patients treated with thymidine analogues reveals two major mutational clusters of TAMs: (i) TAM1: M41L, L210W and T215Y, and (ii) TAM2: D67N, K70R and K219E or Q, and sometimes T215F. TAM1 mutations confer higher levels of AZT resistance and are responsible for extensive cross-resistance to other NRTIs. In addition, in subtype B and C HIV-1 variants, the TAM1 combination of mutations seems to be the fittest (Armstrong et al., 2009). Key mutations in the TAM1 pathway are M41L and T215Y, while in the TAM2 complex D67N and K70R are needed to achieve significant ATP-dependent phosphorolytic activity (Meyer et al., 1999). Accumulation of TAMs increases with exposure to AZT or d4T (Cozzi-Lepri et al., 2009).

Although TAMs are the key mutations for acquisition of resistance through the excision mechanism, a number of secondary mutations are frequently associated with TAM1 or TAM2 clusters in HIV-1 subtype B isolates. Examples are E40F, K43E or Q or N, E44A or D, K64H, V118I, K122E, I135T, E203D or K, H208Y, L214F, D218E, K223E or Q, L228H or R and R284K (Stürmer et al., 2003; Lu et al., 2005; Svicher et al., 2006; Nebbia et al., 2007; Puertas et al., 2009; Betancor et al., 2012; Melikian et al., 2012). Phe^{40}, Glu^{43}, Glu^{122}, Tyr^{208}, Phe^{214} and Lys^{284} seem to associate with the TAM1 cluster. Studies with recombinant HIV-1 (group M subtype B) have shown that the amino acid changes K43E, Q207D, L214F and R284K increase the viral replication capacity in the presence of TAMs (Lu et al., 2005; Huigen et al., 2008; Puertas et al., 2009; Betancor et al., 2012). Although authors have speculated with the possibility of an effect on the catalytic activity of the RT mediated by those secondary mutations, formal demonstration has been accomplished only for mutation R284K (Betancor et al., 2012). R284K increases viral replication capacity of HIV-1 bearing RT mutations M41L/L210W/T215Y in the absence and in the presence of AZT or tenofovir. The R284K mutation does not affect excision, but improves the nucleotide incorporation catalytic efficiency and diminishes the RT's RNase H activity. Unblocked primers are more efficiently extended by the M41L/L210W/T215Y/R284K RT than by mutant M41L/L210W/T215Y (Betancor et al., 2012).

RT residues in the thumb-connection subdomains as well as in the RNase H domain can modulate AZT susceptibility by altering the balance between excision and template RNA degradation (Nikolenko et al., 2005; Delviks-Frankenberry et al., 2007; Nikolenko et al., 2007; Yap et al., 2007). Lower RNase H activity results in increased stability of the complex formed by the chain-terminated DNA primer and the RNA template, and therefore, a longer residence time of its NRTI moiety at the dNTP binding site. In this scenario, AZT is amenable to excision and resistance is enhanced (Brehm et al., 2008; Delviks-Frankenberry et al., 2008; Ehteshami et al., 2008a). Factors promoting the emergence of HIV-1 RT connection subdomain mutations and their effect on antiretroviral therapy are largely undetermined. N348I, R356K, R358K, A360V and A371V have a higher prevalence in the treated

population than in naïve individuals (von Wyl et al., 2010a), and selection of N348I occurs frequently during subtype C HIV-1 infection in patients treated with nevirapine-containing regimens (Brehm et al., 2012). Co-occurrence of M184V and N348I has been observed in patients infected with subtype B HIV-1, and receiving AZT and 3TC (von Wyl et al., 2010a). In these viruses, N348I restores partially the reduced RT processivity and AZT excision activity associated with the M184V mutation (von Wyl et al., 2010b).

RT thumb subdomain mutations that associate with TAMs and appear during treatment with nucleoside analogues are R284K (Cane et al., 2007; Waters et al., 2009; von Wyl et al., 2010a; Betancor et al., 2012) and the cluster $Pro^{272}/Arg^{277}/Thr^{286}$. This cluster is more prevalent than $Ala^{272}/Lys^{277}/Ala^{286}$ in patients failing treatment with abacavir/d4T-containing regimens (Garriga et al., 2009). Selected thumb subdomain mutations were found to increase viral fitness in the presence of TAM1 mutations (Betancor et al., 2010, 2012).

N348I confers decreased susceptibility to AZT (Yap et al., 2007; Lengruber et al., 2011). This mutation as well as A360V and Q509L increase chain-terminated primer rescue with RNA/DNA complexes, but not with DNA/DNA template–primers (Nikolenko et al., 2007; Yap et al., 2007; Brehm et al., 2008; Delviks-Frankenberry et al., 2008; Ehteshami et al., 2008a; Hachiya et al., 2008; Radzio et al., 2010). These mutations, as well as others found in the connection and RNase H domains of the RT (e.g. RNase H primer grip mutations such as G335C or D, V365I, A376S, A400T, etc.) (Delviks-Frankenberry et al., 2007, 2008, 2009) had an impact on RNase H activity either by decreasing its specific activity or by altering RNase H secondary cleavage kinetics. N348I in the RT p51 subunit is responsible for the lower frequency of secondary RNase H cleavages shown by this mutant (Radzio and Sluis-Cremer, 2011). Thr^{400} is found in isolates from naïve patients infected with CRF01_AE strains, and TAM-containing strains of this clade exhibit higher levels of resistance than their homologues in HIV-1 subtype B (Delviks-Frankenberry et al., 2009).

G333D promotes resistance to AZT in the presence of TAMs without affecting RNase H activity. However, increased excision was detected with both RNA/DNA and DNA/DNA substrates (Zelina et al., 2008). The RT thumb subdomain polymorphisms $Pro^{272}/Arg^{277}/Thr^{286}$ enhance NRTI excision and further extension of the unblocked primer, but only on RNA/DNA complexes (Betancor et al., 2010). Unlike in the case of N348I, these effects are independent of the RNase H activity of the RT and can be explained by the higher affinity for RNA/DNA complexes shown by the RT containing the $Pro^{272}/Arg^{277}/Thr^{286}$ cluster in comparison with enzymes having $Ala^{272}/Lys^{277}/Ala^{286}$ (Betancor et al., 2010).

3.2.2. Insertions and deletions in the β3–β4 hairpin loop as constituents of a mutational complex with excision activity

HIV-1 RTs with dipeptide insertions (usually Ser–Ser, Ser–Gly or Ser–Ala) at positions 69–70 were initially reported in the late 90s in heavily-treated patients (for a review, see Menéndez-Arias et al., 2006). The ATP-dependent excision activity of those enzymes was very high and removal of the insertion together with the amino acid substitution T69S (i.e. T69SSS) increased the viral susceptibility to AZT while decreasing the phosphorolytic activity of the viral polymerase (Mas et al., 2000). Further studies demonstrated that T69SSS together with T215Y confer significant excision activity on AZT- and d4T-terminated primers and low-level resistance to AZT in phenotypic assays carried out with recombinant HIV-1 (Boyer et al., 2002; Matamoros et al., 2004). Mutational analyses have shown that M41L, A62V and in a lesser extent K70R contribute to the high-level resistance to AZT, by increasing the ATP-mediated excision activity of the RT (Cases-González et al., 2007).

RT deletions are rarely found in clinical samples. Three-nucleotide deletions at codons around positions 67–70 have been identified in virus isolated from treated patients and showing high-level resistance to AZT and other NRTIs (Menéndez-Arias et al., 2006). Genotypic analysis of HIV-1 *pol* sequences from treated patients showed two major mutational patterns associated with single amino acid deletions. The Δ67 complex (i.e. Δ67/T69G/K70R) is usually found together with TAMs, while the deletion of codon 69 (Δ69) is usually associated with mutations of the Q151M complex (Menéndez-Arias et al., 2006; Villena et al., 2007; Kisic et al., 2011). Biochemical and virological studies demonstrate that the complex Δ67/T69G/K70R confers significant ATP-dependent excision activity on primers terminated with AZT or d4T, while increasing the phosphorolytic activity shown by the double-mutant M41L/T215Y (Kisic et al., 2011). These observations were consistent with AZT susceptibility measurements carried out with the corresponding mutant HIV-1 (Kisic et al., 2011). Interestingly, the deletion of codon 69 alone or in combination with S68G/K70G decreased the RT's ability to rescue primers terminated with thymidine analogues, conferring hypersusceptibility to AZT (Kisic et al., 2008, 2011). These effects were more pronounced when the K219E mutation was present. Molecular dynamics studies predict that in the Δ69 RT, the side-chain of Lys^{70} locates away from the putative pyrophosphate binding site, and therefore its participation in the excision reaction is unlikely (Kisic et al., 2011).

3.2.3. Antagonists of the excision reaction

Mutations that antagonize the effects of TAMs in excision and NRTI susceptibility fall into three categories: (i) amino acid changes that affect the dNTP binding site, such as K65R (White et al., 2005, 2006), L74V (Frankel et al., 2005; Miranda et al., 2005), V75I (Matamoros et al., 2009), A114S (Arion et al., 2000), F160Y (Hammond et al., 2001), M184V (Götte et al., 2000), and the deletion of codon 69 (Kisic et al., 2011); (ii) foscarnet resistance mutations other than those affecting residues of the nucleotide binding site (e.g. K65R, A114T and F160Y): W88G, E89K, L92I, S117T, S156A, Q161L and M164V (Tachedjian et al., 1996; Arion et al., 2000; Bazmi et al., 2000; Hammond et al., 2001; Meyer et al., 2003); and (iii) mutations affecting NNRTI binding.

Biochemical studies showing the mechanism of suppression for the NNRTI resistance mutation Y181C (Selmi et al., 2003) and the polymorphism R172K (Hachiya et al., 2012) have been described in detail. Structural analysis shows that Lys^{172} affects interactions between α-helix E (where the residue is located) and the active site β9-strand that includes both the conserved Tyr^{183}-Met^{184}-Asp^{185}-Asp^{186} (YMDD) catalytic loop and the NNRTI binding pocket. R172K confers hypersusceptibility to nevirapine and efavirenz. Other NNRTI resistance-related amino acid changes that could have an antagonistic effect on TAM-mediated excision are L100I, V108I and G190S, although in those cases, evidence of their suppressive effect on NRTI resistance is restricted to viral AZT susceptibility assays with HIV-1 clones (Lawyer et al., 2011; reviewed in Menéndez-Arias, 2008). In contrast, I132M is a rare NNRTI resistance mutation that decreases viral susceptibility to AZT, 3TC and tenofovir by interfering with the RT's ability to discriminate between the natural dNTP and the corresponding NRTI in its triphosphate form (Ambrose et al., 2009).

Suppression of AZT resistance by antagonistic mutations could result from changes in the conformation of the β3–β4 hairpin loop and the architecture of the dNTP binding site, but also from long-range interactions between the RNase H domain and the polymerase active site that could be influenced by changes in the NNRTI binding site. Foscarnet facilitates the formation of stable pretranslocational complexes (Marchand et al., 2007). Mutations that favor the pretranslocated status (e.g. A62V) are theoretically more susceptible to foscarnet inhibition (Scarth et al., 2011). In contrast, the concentrations of foscarnet which are needed to form a pretranslocational complex are considerably higher for the E89K RT

than for the wild-type enzyme (Marchand et al., 2007). E89K is a mutation that produces a >10-fold reduction of ATP-mediated excision by TAM-containing RTs.

Connection subdomain mutations could play a relevant role in suppressing the antagonistic effect of some mutations, such as M184V (Kemp et al., 1998). In TAM-containing RTs, the antagonistic effect of M184V on excision of AZT-monophosphate is more pronounced on an RNA template than on a DNA template with the same sequence (Acosta-Hoyos et al., 2012). These differences in template usage are mitigated by inactivating the RNase H. Decreased excision seems to be related to an increase cleavage of the RNA template at position −7 relative to the primer terminus. However, the mechanism involved could be different for other mutations in the dNTP binding site. Thus, K65R suppresses the excision activity of the RT to the same extent on either an RNA or a DNA template (Acosta-Hoyos et al., 2012).

3.3. NRTIs in preclinical development and potential mechanisms of resistance

Newly developed NRTIs have been designed to improve safety and efficacy profiles and to minimize drug resistance. Most of them show significant activity against HIV-1 strains containing commonly found resistance mutations such as M184V or TAMs. One example is apricitabine, a (−) enantiomer of 2′-deoxy-3′-oxa-4′-thiocytidine (also known as BCH-10618, AVX754 or SPD754). HIV-1 harboring RTs with mutations such as L74V, M184V or combinations of TAMs including M41L and T215F or Y were susceptible to the drug, although resistance developed slowly and led to the selection of K65R, V75I or M184V in separate experiments (Gu et al., 2006; Oliveira et al., 2009; reviewed in Cahn and Wainberg, 2010). Relatively high levels of resistance to apricitabine (>10-fold increase of the IC_{50}) were observed for viruses having the RT mutations Q151M and Q151M/M184V (Gu et al., 2006).

Selection experiments carried out with the HIV-1_{LAI} strain in the presence of β-D-2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine (dexelvucitabine or Reverset) led to the identification of the deletion of Ser^{68} as the major change conferring a 10- to 30-fold increase in resistance to dexelvucitabine (Schinazi et al., 2011). In some experiments, the deletion was found in combination with K65R. As in the case of apricitabine, dexelvucitabine resistance is acquired through a discrimination mechanism.

EFdA (4′-Ethynyl-2-fluoro-2′-deoxyadenosine) is a potent NRTI that inhibits HIV-1 replication in primary peripheral blood mononuclear cells with an EC_{50} of 50 pM, about 400 times more potent than AZT. After being incorporated into the DNA chain, the EFdA-monophosphate inhibits primer translocation (Michailidis et al., 2009). EFdA retains significant potency against a broad range of clinically important drug-resistant isolates, including K65R, L74V, M41L/T215Y or the Q151M complex (Kawamoto et al., 2008). However, M184V contributes to EFdA resistance particularly in combination with P119S and T165A (Kawamoto et al., 2008; Yang et al., 2009).

GS-9148 is a phosphonate nucleotide analogue RT inhibitor (i.e. an adenosine derivative with a 2′,3′-dihydrofuran ring structure that contains a 2′-fluoro group), active against RTs bearing single mutations such as K65R, L74V or M184V, as well as combinations of 6 TAMs (Boojamra et al., 2008; Cihlar et al., 2008). Available as a prodrug (GS-9131), GS-9148 acts as a competitive inhibitor of RT with respect to dATP (Cihlar et al., 2008). Crystal structures of GS-9148 diphosphate bound to RT/dsDNA showed that the phosphonate has a similar binding mode as dATP, although its dihydrofuran ring makes more interactions with the aromatic side chain of Tyr^{115} and was found in close proximity to Gln^{151} (Lansdon et al., 2010a). Interestingly, GS-9148 selects for the very rare Q151L that compromises binding of the diphosphate derivative to RT. Models suggest that the 2′-fluoro group of GS-9148 causes steric hindrance with the side chain of the Q151L mutant. GS-9148 resistance is achieved through a discrimination mechanism (Scarth et al., 2011).

4. NNRTI resistance

NNRTIs are small hydrophobic molecules (usually of less than 600 Da) that act as allosteric inhibitors of HIV-1 RT. The NNRTI binding pocket is about 10 Å away from the DNA polymerase active site and is formed by residues of the p66 (Leu^{100}, Lys^{101}, Lys^{103}, Val^{106}, Thr^{107}, Val^{108}, Val^{179}, Tyr^{181}, Tyr^{188}, Val^{189}, Gly^{190}, Phe^{227}, Trp^{229}, Leu^{234}, Pro^{236} and Tyr^{318}) and p51 (Glu^{138}) subunits (Kohlstaedt et al., 1992). At present, there are five NNRTIs approved for clinical treatment of HIV-1 infection: nevirapine, delavirdine,

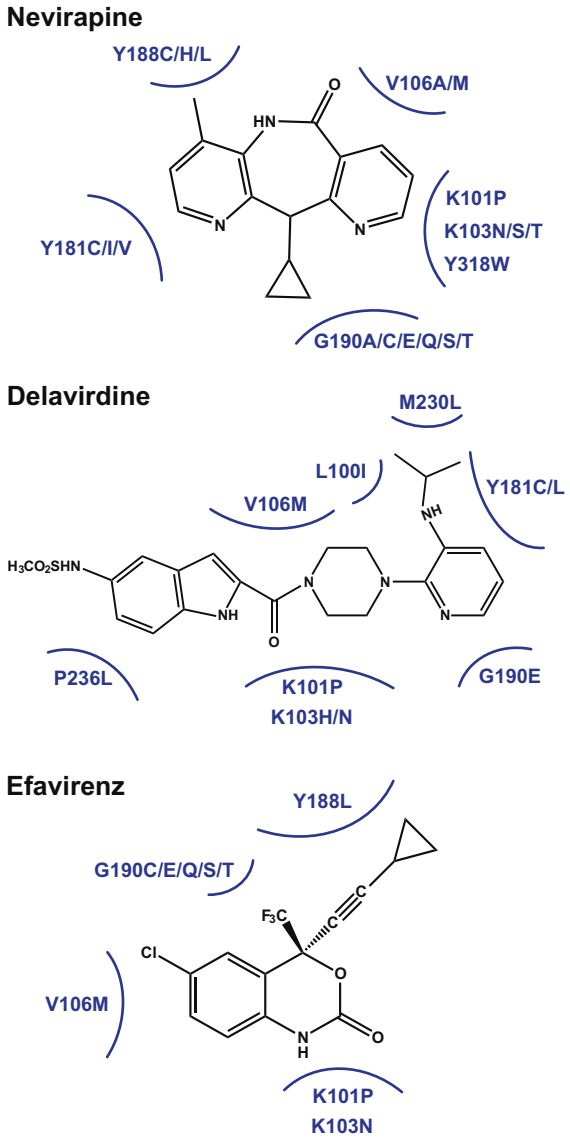


Fig. 4. Chemical structures of nevirapine, delavirdine and efavirenz and amino acid substitutions that by themselves confer a >40-fold increase in the inhibitory concentration (IC_{50}) relative to the wild-type virus in phenotypic assays using recombinant HIV-1 strains. Relative positions of amino acid residues and NNRTI structures were obtained from the corresponding crystal structures of RT/NNRTI complexes (for a list of PDB coordinates, see Menéndez-Arias et al., 2011). NNRTI susceptibility data on the effects of single amino acid substitutions were taken from Menéndez-Arias, 2012. Reproduced from Menéndez-Arias et al. (2011), with permission from Elsevier©.

efavirenz, etravirine and rilpivirine. First generation inhibitors such as nevirapine, delavirdine or efavirenz have a relatively low genetic barrier to resistance (Fig. 4).

Pre-steady-state and steady-state kinetic analyses suggested that NNRTI binding inhibits the chemical step of polymerization (Rittinger et al., 1995; Spence et al., 1995). However, others have shown that a conformational step preceding chemical catalysis is critical in NNRTI inhibition (Xia et al., 2007). Structural studies have shown that upon NNRTI binding, aromatic side-chains of Tyr^{181} and Tyr^{188} swivel out of the binding pocket while the primer grip region (β12–β13–β14 strands) moves away from the DNA polymerase catalytic triad (β6–β10–β9 strands) to create space for the antiretroviral drug (reviewed in Paris et al., 2009). Single amino acid substitutions that confer resistance either to nevirapine or efavirenz can eliminate important stabilizing stacking interactions (e.g. Y181C), alter the conformation or size of the NNRTI binding pocket (e.g. Y188L), or block inhibitor access to the binding site (e.g. K103N).

Several mechanisms have been proposed to explain inhibition by NNRTIs: (i) restriction of thumb mobility (known as the "molecular arthritis" mechanism) (Kohlstaedt et al., 1992), (ii) distortion of the catalytic triad that limits the conformational flexibility of the YMDD loop (Ren et al., 1995), (iii) repositioning of the primer grip involving large displacements (of about 4 Å) (Das et al., 1996), and (iv) loosening of the clamp formed by the RT thumb and fingers subdomains (Liu et al., 2008). Crystal structures of ternary complexes of RT–DNA–nevirapine and RT–DNA–AZTTP and their comparison with the binary complex (RT–DNA) have shown that AZTTP and nevirapine cannot bind the RT simultaneously (Das et al., 2012). In the ternary complex, nevirapine is surrounded by three walls formed by β-strands 12, 13 and 14, β-strands 6, 9 and 10, and the 100–105 loop of p66 plus Glu^{138} of p51. Nevirapine binding opens the NNRTI binding pocket and shifts the 3′ end of the DNA primer by 5.5 Å. Loop mutations (K101P, K103N, and others) facilitate exit of the NNRTI from the pocket, allowing the primer grip to position the nucleic acid in a catalytically relevant mode, while shifting the equilibrium towards the RT–DNA binary complex conformation (Das et al., 2012). The ternary complex containing nevirapine is unlikely to accommodate ordered binding of dNTP in a catalytically competent mode. A distorted dNTP binding site may also decrease binding of the excision substrates ATP or pyrophosphate.

4.1. Resistance to diarylpyrimidines (DAPY)

Next-generation NNRTIs include the recently approved drugs etravirine and rilpivirine (Fig. 5). These inhibitors adopt a horseshoe shape in the binding pocket (Das et al., 2004, 2008; Lansdon et al., 2010b). DAPYs show high specificity and low toxicity. Unlike nevirapine, efavirenz and other first-generation NNRTIs, DAPY series compounds can bind the NNRTI binding pocket in different conformations. Thus, torsional flexibility ("wiggling") of these drugs can generate many conformational variants, while their compact design allows their repositioning and reorientation ("jiggling") (Das et al., 2004).

The central pyrimidine ring of etravirine locates between Leu^{100} and Val^{179} and establishes a key hydrogen bond with Lys^{101}. Residues Val^{106}, Pro^{225}, Phe^{227}, Leu^{234}, Pro^{236} and Tyr^{318} form a pocket that accommodates the benzonitrile moiety of etravirine, with its dimethylcyanophenyl group oriented towards Tyr^{188}, Phe^{227} and Trp^{229} (Das et al., 2004; Lansdon et al., 2010b) (Fig. 5). Etravirine resistance has been associated to 20 different mutations, based on published *in vitro* drug susceptibility data and clinical studies. These mutations are: V90I, A98G, L100I, K101E or H or P, V106I, E138A or K or G or Q, V179D or F or T, Y181C or I or V, G190A or S and M230L (Andries et al., 2004; Das et al., 2004; Tambuyzer et al., 2009). Underlined substitutions have been associated with a 25% reduction in viral load in phase III clinical trials (Vingerhoets et al., 2010).

These observations suggested a higher genetic barrier to resistance for DAPY compounds than for first-generation NNRTIs. However, mutations affecting Glu^{138} (e.g. E138Q, E138K, E138R and E138S) were shown to confer low-level resistance to etravirine in phenotypic assays, and similar findings were reported for K101P, K101E, Y181C and M230L (Andries et al., 2004; Marcelin et al., 2010; Xu et al., 2010; Tambuyzer et al., 2011). Other mutations at position 181 (e.g. Y181I and Y181V) conferred more than 10-fold decreased susceptibility to the drug (Azijn et al., 2010). At least two amino acid substitutions are required *in vitro* to confer high-level resistance to etravirine. Examples are V179F/Y181C, V179F/Y181I or Y181I/M230L (Azijn et al., 2010; Javanbakht et al., 2010). E138K has been frequently observed as the first selected mutation in HIV-1 subtype B, C or CRF02_AG clinical isolates passaged *in vitro* in the presence of etravirine (Asahchop et al., 2011).

However, E138K was not selected when the viruses grown in tissue culture contained the Y181C mutation (Xu et al., 2012). RTs having both mutations showed decreased DNA polymerase activity. The addition of E138K to Y181C increased etravirine susceptibility in comparison to the Y181C mutant.

The genetic barrier and resistance profile of rilpivirine (formerly known as TMC278) is similar to that described above for etravirine. Rilpivirine binds HIV-1 RT in a similar but not identical conformation in comparison with etravirine (Lansdon et al., 2010b). Rilpivirine-resistant strains selected *in vitro* contained combinations of V90I, L100I, K101E, V106A or I, V108I, E138G or K or Q or R, V179F or I, Y181C or I, V189I, G190E, H221Y, F227C and M230I or L (Azijn et al., 2010). However, phase III clinical trials (ECHO and THRIVE) showed that E138K and M184I were the most frequent mutations to emerge in patients failing treatment with rilpivirine (combined with tenofovir/emtricitabine) (Cohen et al., 2011; Molina et al., 2011).

In vitro studies demonstrate that in the absence of NNRTI resistance mutations, E138K emerges as the major resistance mutation for both rilpivirine and etravirine (Asahchop et al., 2012). By itself, E138K has a modest effect on rilpivirine resistance. However, it enhances rilpivirine resistance when combined with M184I (Hu and Kuritzkes, 2011; Kulkarni et al., 2012). E138K improves the viral replication capacity of HIV-1 bearing RT mutations M184I or M184V (Xu et al., 2011). Biochemical studies have shown that M184I (in p66) impairs dNTP binding and decreases the DNA polymerase catalytic efficiency of the RT. However, Lys^{138} in both subunits or in p51 alone abrogated the negative effect of M184I by improving dNTP binding (Singh et al., 2012). Reduced rilpivirine susceptibility can be attributed to the presence of Lys^{138} in p51 that alters the ratio of rilpivirine dissociation to rilpivirine association. This is probably due to the disruption of the salt bridge involving Glu^{138} (in p51) and Lys^{101} (in p66). Structural models predict that Ile^{184} affects the positioning of Tyr^{183} in the active site, while Lys^{138} restores Tyr^{183} to a wild-type conformation (Singh et al., 2012).

4.2. Connection subdomain mutations and their impact on NNRTI resistance

Although routine genotypic analysis of HIV-1 isolates in infected patients is usually restricted to residues 1–250 of the RT, recent reports have demonstrated that mutations in the connection subdomain can impact resistance to RT inhibitors (for a review, see Menéndez-Arias et al., 2011). Tyr^{318} is part of NNRTI binding site and mutations such as Y1318F or Y318W confer resistance to nevirapine and delavirdine. Other amino acid substitutions such as N348I, T369I or V, and A376S were shown to confer low-level

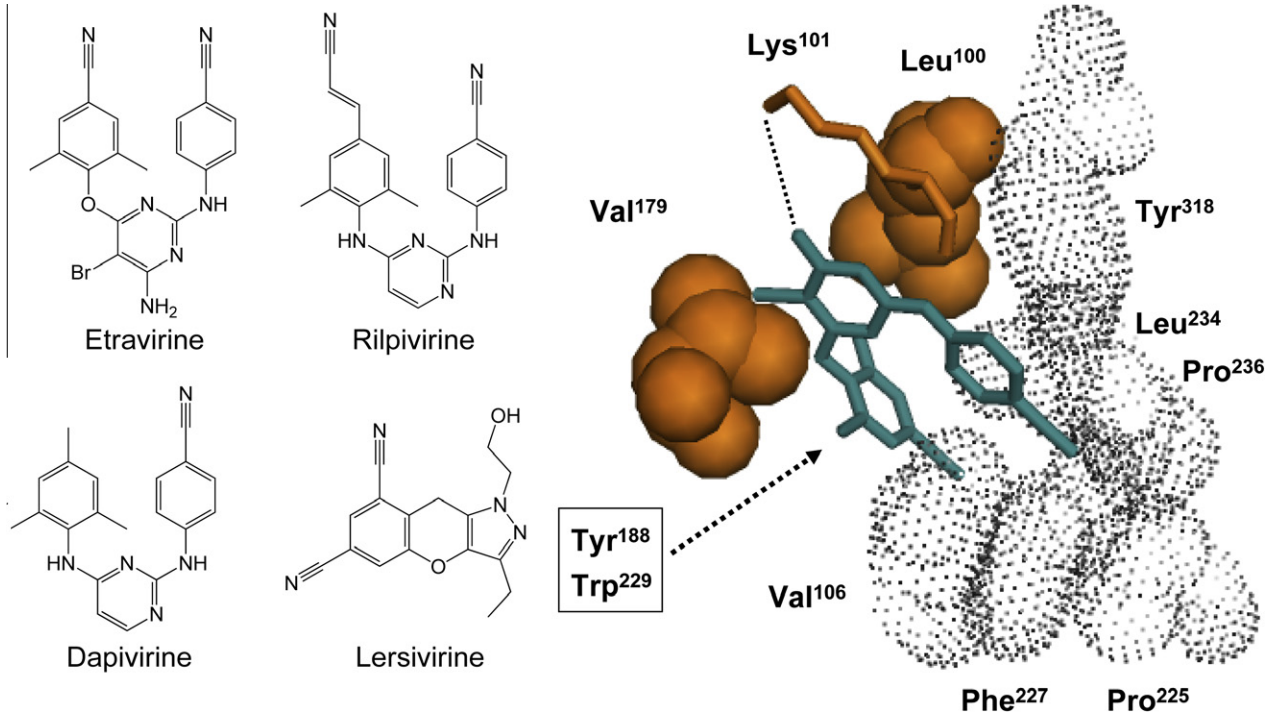


Fig. 5. Chemical structures of DAPYs (etravirine, rilpivirine and dapivirine) and lersivirine. Relevant interactions of etravirine and its binding pocket in HIV-1 RT are shown on the right. Atomic coordinates were taken from PDB file 3MEC (Lansdon et al., 2010b).

resistance to nevirapine and other NNRTIs. Selection of N348I occurs frequently in patients receiving nevirapine-containing regimens (Yap et al., 2007; Brehm et al., 2012), although an increased prevalence has also been reported under efavirenz therapy (Price et al., 2010). N348I appears early in response to antiretroviral therapy (Yap et al., 2007).

In all tested HIV-1 subtypes, N348I confers decreased susceptibility to nevirapine and to a lesser extent to other NNRTIs (Gupta et al., 2010; Sluis-Cremer et al., 2010; McCormick et al., 2011; Brehm et al., 2012). *In vitro* nevirapine resistance is caused by a reduction in the inhibitor binding affinity for the RT (Schuckmann et al., 2010). This behavior has been attributed to the specific influence of the mutation on the initiation of plus-strand DNA synthesis (Biondi et al., 2010). In addition, N348I diminishes RNase H activity, probably as a result of alterations in template–primer binding. Long-range interactions between RT subdomains relevant for nucleotide incorporation could be affected by the mutation both in the presence or absence of NNRTI binding.

A376S confers low-level resistance to nevirapine in phenotypic assays. Although Ala^{376} is away from the NNRTI binding site, its replacement by Ser affects nevirapine affinity, probably as a consequence of an alteration in the RT's ability to bind the template–primer (Paredes et al., 2011). Recently published structural studies suggest that connection subdomain mutations favor the RT polymerization-competent state because they may prevent the conformational change that is needed to accommodate the RNA/DNA structure in a position suitable for RNase H cleavage (Lapkouski et al., 2013).

4.3. Resistance to NNRTIs in preclinical development

In principle, novel NNRTIs should be effective on HIV-1 strains resistant to approved drugs. Lersivirine (UK-453,061) is a pyrazole derivative that binds the RT in a conformation different from that shown by nevirapine or efavirenz, characterized by the rotation of Tyr^{181}. Lersivirine is active against HIV-1 strains bearing single amino acid substitutions in the RT such as L100I, K101E, K103N, V106A, V108I, E138K, Y181C, Y181I, M184V, Y188C, F227L, E233V, L234I, and P236L (Corbau et al., 2010). High-level *in vitro* resistance to lersivirine is conferred by F227C as well as the double-mutants V106A/F227L and Y181I/Y188L (Corbau et al., 2010). Mutations found in patients failing therapy with lersivirine include K101E, V106M, V108I, Y188H, H221Y, F227C or L, and L234I (Vernazza et al., 2013).

Other NNRTIs in preclinical development that show excellent potency against K103N and Y181C mutants are difluoromethylbenzoxazole (DFMB) pyrimidine thioether derivatives (Boyer et al., 2011), MK-6186 (Lu et al., 2012), RO-0335 (Javanbakht et al., 2010), and dapivirine (TMC120) (Fig. 5), a DAPY analogue, developed to be used as a vaginal microbicide (Schader et al., 2012). However, high-level resistance to dapivirine is conferred by mutations L100I/K103N and K103N/Y181C (Fletcher et al., 2009), and other mutations such as L100I, K101E, V106I, E138K, V179I, Y181C and G190A have been selected *in vitro* in the presence of the drug (Schader et al., 2012). Not surprisingly, the mutational profile of dapivirine resembles the ones described for etravirine and rilpivirine.

4.4. Resistance to nucleotide-competing RT inhibitors (NcRTIs)

In addition to classical NNRTIs, indolopyridinones represent a novel class of inhibitors that inhibit HIV-1 RT by competing with the natural dNTPs through binding the DNA polymerase active site during reverse transcription (i.e. nucleotide-competing RT inhibitors) (Jochmans et al., 2006; Zhang et al., 2006). The prototype compound INDOPY-1 (or VRX-413638) is active against NNRTI-resistant strains (Zhang et al., 2006; Jegede et al., 2011). Intermediate resistance to INDOPY-1 is conferred by amino acid substitutions affecting the nucleotide binding site such as M184V or Y115F. However, double-mutants Y115F/M184V show >100-fold increased resistance in comparison with the wild-type enzyme (Ehteshami et al., 2008b). In contrast, K65R confers hypersuscepti-

bility to the inhibitor and mitigates the inhibitory effects mediated by M184V (Ehteshami et al., 2008b). Additional mutations related to NRTI resistance have been occasionally selected in HIV-1 cultured in the presence of indolopyridinones. Examples are M41L, A62T or V, S68N, G112S and V118I (Zhang et al., 2006).

5. Resistance to protease inhibitors

The formation of mature HIV-1 virions with conical-shaped capsids results from the proteolytic processing of the precursor polyproteins Gag and Gag–Pol by a virally-encoded aspartyl protease. The protease cleaves Gag into the structural proteins matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), p6 and two small spacer peptides designated as SP1 (p2) and SP2 (p1). Gag–Pol cleavage renders structural proteins as well as the viral enzymes protease, RT and integrase. The HIV-1 protease is a homodimer composed of subunits of 99 amino acids. It is an aspartyl protease with the conserved sequence Asp^{25}–Thr^{26}–Gly^{27} in both subunits. Asp^{25} residues in both subunits share an acidic proton and interact with water in the absence of substrate or inhibitor. Each protease subunit contains a flap region (residues 42–58) constituted by a flexible β-hairpin loop. These loops close down upon binding of substrate or inhibitors of the HIV-1 protease that adopt an extended conformation in their binding site (Wlodawer and Vondrasek, 1998). The side chains of the substrate lie in subsites S4–S3′ formed by protease residues. Interactions between protease and inhibitors (or natural substrates) are mostly hydrophobic. Hydrogen bonds between β-sheets at the N- and C-termini of each subunit seem to be important for protease dimerization.

Currently, there are six protease inhibitors recommended for treatment of HIV infection: darunavir, lopinavir, atazanavir, fosamprenavir, saquinavir and tipranavir (Fig. 6). The first three are the most widely used, but in all cases the recommended prescription involves ritonavir-boosting (only atazanavir could be used without boosting in some conditions) (for a review, see Hull and Montaner, 2011). Ritonavir inactivates cytochrome P450 3A4 (CYP3A4), and increases plasma concentrations of other protease inhibitors oxidized by CYP3A4. Clinical trials evaluating ritonavir-boosted atazanavir, lopinavir or darunavir as single agents have been carried out in virologically suppressed patients. These therapies were not better than current HAART combinations, but clinical trials confirmed their powerful antiviral effects.

Development of first-generation inhibitors of HIV-1 protease (e.g. saquinavir) was facilitated by knowledge of inhibitors of other aspartic proteases such as renin. Renin inhibitors had been previously used for the treatment of hypertension. All approved protease inhibitors bind in the active site of the enzyme, and except for tipranavir, all are peptidomimetics. Since the approval of saquinavir in 1995, protease inhibitors played an important role in HAART regimens. However, their efficiency was limited by their toxicity, relatively high pill burden, cost, and the usual risk of drug resistance. Better inhibitors have been developed by maximizing interactions with the HIV protease active site, including the establishment of extensive hydrogen bonding networks. These inhibitors show enhanced binding affinity, significant activity against drug-resistant protease variants, and a high genetic barrier to the development of resistance (Ali et al., 2010; Ghosh et al., 2012). For example, the wild-type HIV-1 protease inhibitory constant for darunavir has been estimated around 14 pM. This inhibitor showed significant activity against HIV-1 isolates resistant to first generation protease inhibitors, such as saquinavir, ritonavir, indinavir or nelfinavir (reviewed in Ghosh et al., 2012). Usually, darunavir-resistant viruses accumulate more than ten amino acid changes in their protease-coding region (Koh et al., 2010).

There are many mutations conferring resistance to protease inhibitors. By themselves, primary resistance mutations reduce susceptibility to one or more protease inhibitors while affecting residues of the substrate-binding pocket or its vicinity (Fig. 7). Examples are D30N, V32I, L33F, M46I or L, I47A or V, G48V, I50L or V, V82A or F or L or S or T, and I84A or V (Johnson et al.,

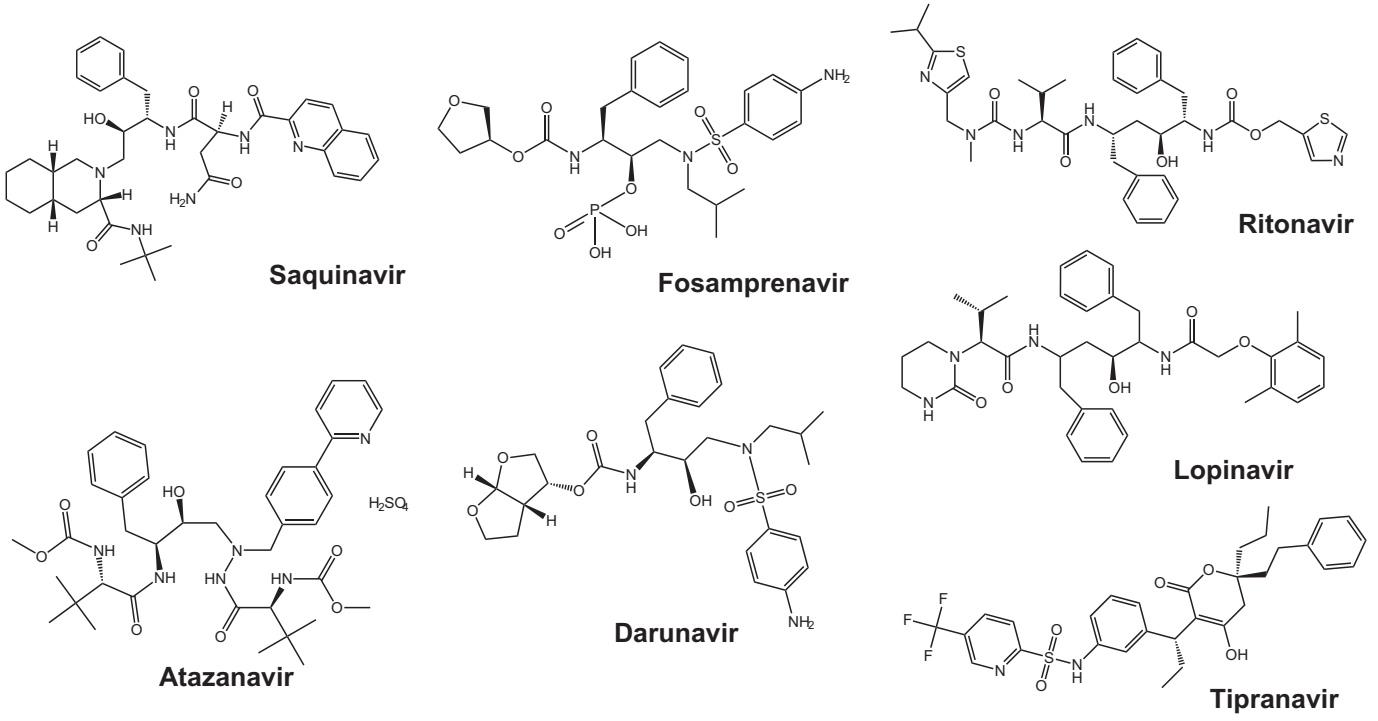


Fig. 6. Chemical structures of currently used protease inhibitors. Ritonavir is co-administered at a low dose in order to maintain high-level plasma concentrations of the protease inhibitors.

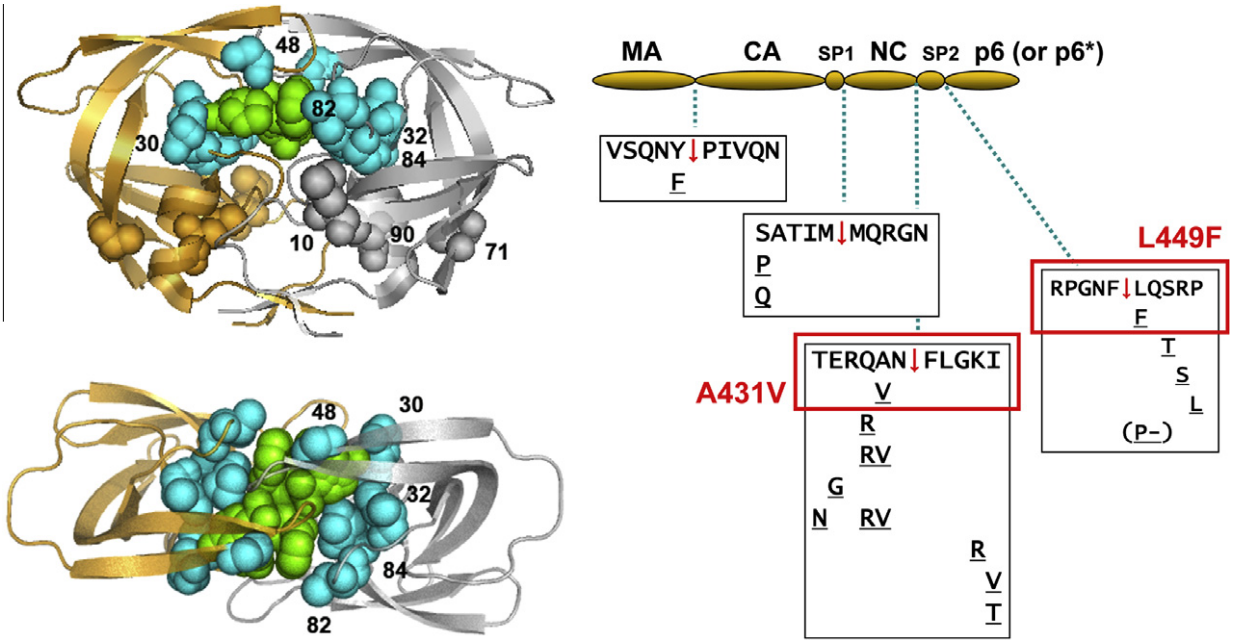


Fig. 7. HIV-1 protease structure complexed with darunavir and amino acid substitutions in Gag cleavage sites related to protease inhibitor resistance. The polypeptide chains in HIV-1 protease are represented as ribbon diagrams (gold and silver). Darunavir is shown in green with a CPK model. Blue spheres represent residues involved in major drug resistance-associated mutations. Gold and silver spheres are used to indicate the location of residues involved in secondary mutations and affecting protease stability. The lower view is taken from above and shows the relative location of the flaps. Atomic coordinates were taken from PDB file 3QOZ. Gag cleavage sites with amino acid substitutions related to the emergence of protease inhibitor resistance are boxed. Most frequent mutations at cleavage sites are shown in red boxes (for a recent review, see Fun et al., 2012).

2011). Among them, V32I, G48V, V82F and I84A or V have been associated with decreased susceptibility to several protease inhibitors (Rhee et al., 2010). Major resistance mutations affect hydrophobic, van der Waals and/or electrostatic interactions at the expense of a reduction in catalytic efficiency that implies a loss of viral fitness.

Secondary mutations are generally selected later in protease inhibitor treatment and occur at codons that encode amino acids out of the enzyme active site. These amino acid substitutions compensate for impaired protease function by increasing the stability or the activity of the enzyme. For example N88D compensates for impaired replication caused by D30N, particularly if L90M is present (Sugiura et al., 2002). The loss of stability caused by L90M (that affects the protease dimer interface), or V82A or I84V can be compensated by distal mutations such as L10I and A71V, as determined by differential scanning calorimetry (Chang and Torbett, 2011). Compared to darunavir, tipranavir shows a lower genetic barrier against the development of HIV-1 resistance. In HIV-1 group M-subtype B clones, two mutations (I54V and V82T) are sufficient to confer reduced susceptibility to the drug. However, in subtype C HIV-1, these two changes did not affect tipranavir susceptibility (Aoki et al., 2012). Tipranavir inhibits the proteolytic activity and blocks the dimerization of the HIV-1 protease subunits. The presence of Met^{24} in HIV-1 subtype B protease compromises dimerization inhibition by tipranavir, particularly after the acquisition of the secondary mutation E34D. The resulting viruses show significant resistance to the drug (Aoki et al., 2012).

In addition to compensatory mutations within the protease-coding region, other molecular mechanisms can also contribute to fitness recovery during therapy with protease inhibitors. Thus, mutations at Gag cleavage sites (particularly NC/p1 and p1/p6) can facilitate polyprotein processing. For example, A431V (TERQAN/FLGKI → TERQVN/FLGKI, NC/p1 site) and L449F (RPGNF/LQSRP → RPGNF/FQSRP, p1/p6 site) are selected *in vivo* during treatment with protease inhibitors (Fig. 7), often associated with major resistance mutations such as D30N, I50L or I84V (Kolli et al., 2009; Larrouy et al., 2011; Kožišek et al., 2012; reviewed in Fun et al., 2012). The substitution L449F can also enhance *pol* expression by 3- to 11-fold, by modifying the frameshift signal found at the 3′ end of *gag* (Doyon et al., 1998). Several amino acid substitutions outside the cleavage sites of Gag have been selected in the presence of protease inhibitors. Most of those changes have been found in MA and CA (Gatanaga et al., 2002; Aoki et al., 2009; Koh et al., 2010; Parry et al., 2011). The mechanism by which those mutations increase viral fitness is not clear, although mutations in CA such as H219Q and I223V could contribute to increase the viral replication capacity by reducing cyclophilin A binding (Koh et al., 2010). An alternative explanation is that those changes could make cleavage sites more accessible to the viral protease. In addition to increase frameshifting, mutations in the transframe protein (p6*) could affect autoproteolytic processing of the HIV-1 protease, and restore the enzymatic activity of dimerization-deficient enzymes (Dautin et al., 2003).

Recently approved protease inhibitors show very high genetic barriers to resistance. High-level resistance to ritonavir-boosted lopinavir or darunavir usually requires a minimum of three to four mutations, although it is not unusual to find HIV-1 isolates showing decreased susceptibility to darunavir or tipranavir with 20–30 mutations in the protease-coding region (Rhee et al., 2010). Protease inhibitors in preclinical development include darunavir-related compounds with potent activity that are effective on protease–inhibitor resistant strains and select for mutations rarely found in patients treated with currently prescribed antiretroviral drugs. Examples are GRL-1398 that selects for several mutations including A28S (Ide et al., 2011), or TMC310911 that selects for mutations at codon 41 in the protease-coding region (Dierynck et al., 2011).

6. Resistance to integrase inhibitors

Retroviral integrases catalyze the insertion of the proviral DNA generated during reverse transcription into the host chromosome.

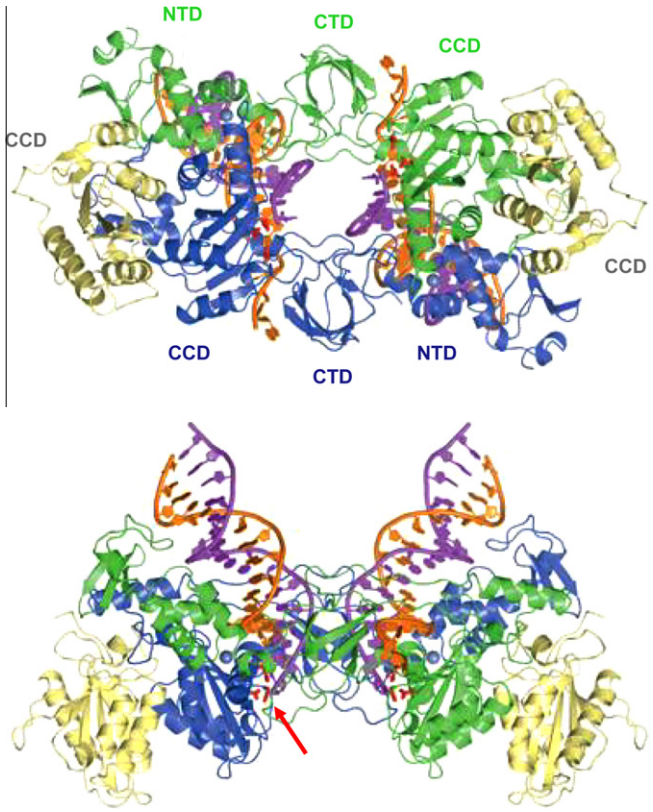


Fig. 8. Structure of the PFV intasome. Side and front views showing the PFV integrase tetramer bound to viral DNA. Side-chains of catalytic residues are indicated in red using a stick representation (see arrows). N-terminal domain, catalytic core and C-terminal domains are indicated with the corresponding abbreviations: NTD, CCD and CTD, respectively. Adapted by permission of Macmillan Publisher Limited: Nature© (Hare et al., 2010a).

This process occurs in two reactions. First, the integrase cleaves a dinucleotide from each viral DNA terminus (i.e. long terminal repeat (LTR)) to produce reactive CpA 3′-hydroxyl ends (3′-end processing), and then the 3′-end processed DNA is covalently linked to the host DNA in a process known as strand transfer (for recent reviews, see Delelis et al., 2008; Craigie and Bushman, 2012).

HIV-1 integrase is a 32-kDa protein (288 amino acids) that contains three domains (N- and C-terminal domains and the catalytic core). The three domains are required for integration. The catalytic core (residues 51–212) contains the DDE motif formed by the active site residues (Asp^{64}, Asp^{116}, Glu^{152}) that coordinate with two divalent metal cations (Mg^{2+} or Mn^{2+}) required for 3′-end processing and strand transfer. The N-terminal domain consists of a bundle of α-helices coordinated with a single Zn^{2+} ion. The C-terminal domain binds DNA non-specifically and is important for integrase tetramerization. The integrase exists as a monomer, dimer and higher oligomers in solution, and multimerization is essential for its catalytic activity. The determination of the crystal structure of prototype foamy virus (PFV) integrase in complex with viral DNA (Fig. 8) has been a major breakthrough towards understanding integrase function (Hare et al., 2010a; Maertens et al., 2010). PFV is a *Spumavirus* that was originally isolated from a nasopharyngeal carcinoma from a Kenyan patient, and is closely related to simian foamy viruses found in chimpanzees (Linial, 2007). The PFV intasome is a homotetramer of integrase assembled on viral DNA ends. Protein–protein and protein–DNA interactions are responsible for holding together the tetrameric structure. In the tetrameric structure, two N- and two C-terminal domains of integrase monomers appear disordered and do not participate in the tetramerization interface. Their function is not known.

Clinically used integrase inhibitors (Fig. 9) bind to the catalytic core of the enyme and target the strand transfer step of integration (Hazuda et al., 2000; for a recent review see Mouscadet et al., 2010). In 2007, raltegravir became the first approved integrase inhibitor, and in 2012 elvitegravir was approved as a component

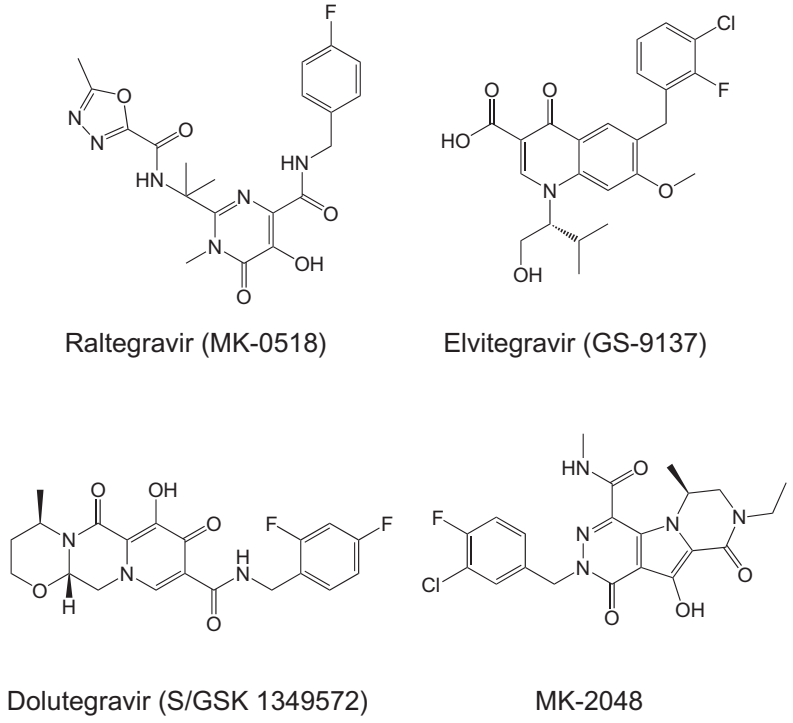


Fig. 9. Chemical structures of integrase inhibitors.

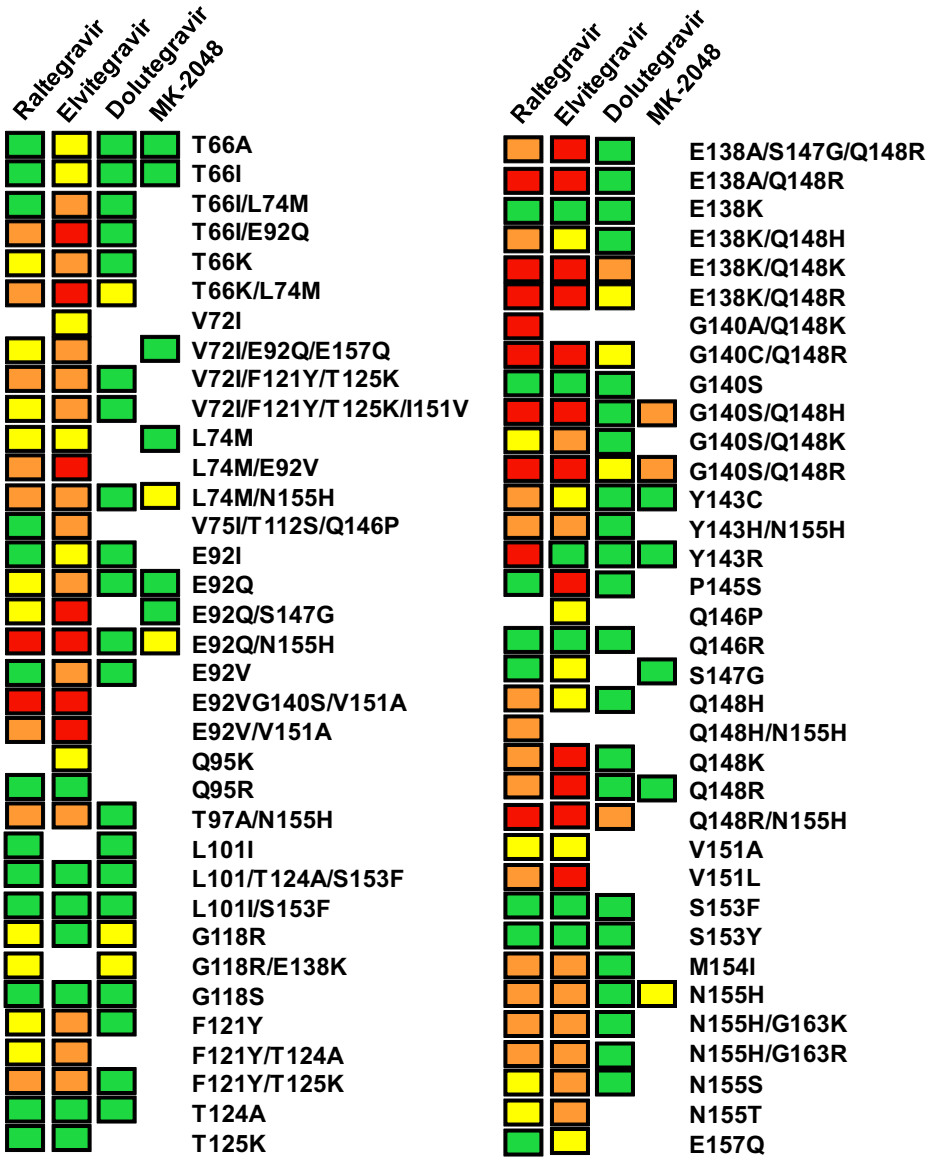


Fig. 10. Summary of phenotypic drug susceptibility data obtained with HIV-1 with amino acid substitutions in the integrase. High-level (>100-fold increase of the IC_{50} for the inhibitor), moderate (10- to 100-fold increase) and low-level (3- to 10-fold increase) resistance is indicated in red, orange and yellow, respectively. Drug susceptibility data were taken from Kobayashi et al., 2008; Shimura et al., 2008; Fransen et al., 2009; Jones et al, 2009; Nakahara et al., 2009; Ceccherini-Silberstein et al., 2010; Delelis et al., 2010; and Goethals et al., 2010 (raltegravir and elvitegravir); Kobayashi et al., 2011a (dolutegravir); and Van Wesenbeeck et al., 2011 (MK-2048). Data for G118R/E138K integrase mutants were taken from Kobayashi et al., 2011b.

of a daily single-tablet coformultation (known as Quad or Stribild) that also contained emtricitabine, tenofovir disoproxil fumarate and cobicistat (DeJesus et al., 2012; Sax et al., 2012) (Table 1). Cobicistat is a pharmacoenhancer (booster) for drugs that are metabolized by cytochrome P450 3A (CYP3A) enzymes. It is more effective and selective than ritonavir and it does not inhibit HIV-1 replication and propagation. Therefore, it eliminates the risk of selection of protease inhibitor resistance mutations, associated with ritonavir boosting. Second-generation integrase strand transfer inhibitors such as dolutegravir (S/GSK 1349572) are now in advanced clinical trials (for a recent review, see Wainberg et al., 2012).

6.1. Mutational pathways selected under therapy with raltegravir or elvitegravir

Raltegravir is an integrase strand transfer inhibitor that targets the catalytic core domain of the enzyme. Mutations affecting Tyr^{143}, Gln^{148} or Asn^{155}, usually in combination with one or more additional changes at positions in their vicinity are frequently observed after virological failure to raltegravir therapy. Clinical studies have demonstrated two major mutational pathways: (i) N155H (sometimes associated with L74M, E92Q, T97A, G136R or V151I), and (ii) the substitution of Lys, Arg or His for Gln^{148}, usually accompanied with E138K or with G140A or G140S. The two pathways are mutually exclusive and non-overlapping (Malet et al., 2008: Fransen et al., 2009). A third resistance pathway involves the selection of Y143C or Y143R, sometimes associated with L74A or I, E92Q, I203M and S230R (Cooper et al., 2008; Malet et al., 2008; Sichtig et al., 2009). The most common mutational patterns found in raltegravir-treated patients are G140S/Q148H and G140S/Q148R. Both combinations confer high-level resistance to the inhibitor in phenotypic assays (Kobayashi et al., 2008; Fransen et al., 2012) (Fig. 10). G140S confers lower levels of resistance than Q148H or Q148R, but increases viral fitness in the presence of Q148H or Q148K (Delelis et al., 2009; Fransen et al., 2009). N155H variants are frequently selected early in raltegravir therapy but they are

subsequently replaced by variants containing other primary mutations (e.g. Q148H, K or R) after the acquisition of secondary mutations (Malet et al., 2008; Quercia et al., 2009). That shift is a consequence of the lower replication capacity of variants carrying 155 pathway mutations (Fransen et al., 2012).

In comparison with raltegravir, elvitegravir (formerly known as GS-9137) shows a similar resistance mutation profile. The major raltegravir resistance-associated mutations at positions 140, 148 and 155, as well as associated accessory mutations have also been selected with elvitegravir in cell culture and in patients (Goethals et al., 2008; Blanco et al., 2011; Taiwo et al., 2011). Despite those similarities, amino acid substitutions at positions 66 and 92 seem to have a different impact depending on the drug. Thus, T66I does not affect raltegravir susceptibility but confers resistance to elvitegravir (Fig. 10). Other changes at this position (e.g. T66K or T66R) have a larger impact on resistance to elvitegravir than to raltegravir. In addition, the effects of E92Q on elvitegravir resistance are also more pronounced than on raltegravir (Shimura et al., 2008). Clinical studies have shown that E138K and S147G may also appear in elvitegravir-treated patients as secondary mutations (Winters et al., 2012). Interestingly, HIV-1 variants containing T66I/Q95K/Q146P/S147G (with or without E138K) have been selected *in vitro* in the presence of high concentrations of elvitegravir (Shimura et al., 2008).

6.2. Mechanisms of action of raltegravir and elvitegravir and molecular basis of drug resistance

Structural studies of PFV intasome/drug complexes have shown that raltegravir binding blocks access of target DNA to the integrase active site. In addition, the inhibitor produces a displacement of the 3′ end of the viral DNA relative to the integrase active site. These conclusions can be extended to the HIV-1 integrase, based on the amino acid sequence similarity of both enzymes (Krishnan et al., 2010). Most of the resistance mutations lie within the catalytic core. Glu^{92}, Gly^{140}, Gln^{148} and Asn^{155} are located near the three acidic residues that form the catalytic triad (Fig. 11), with Gln^{148} interacting with the 5′ end of the viral DNA (Johnson et al., 2006). Tyr^{143} is slightly outside the active site, but most likely, its side-chain makes π–π stacking interactions with raltegravir (Hare et al., 2010b). It has been shown that integrase inhibitor resistance mutations act by increasing the k_{off} for raltegravir and elvitegravir. Thus, Q148H, N155H and Y143R increase the k_{off} for raltegravir, but only the first two mutations affect the dissociation of elvitegravir from integrase–DNA complexes (Hightower et al., 2011). These results correlate well with phenotypic susceptibility data and suggest that the dissociation rate is an important factor that determines the efficiency of integrase inhibitors (Copeland et al., 2006).

6.3. Resistance to dolutegravir and other integrase inhibitors in preclinical development

Dolutegravir (formerly known as S/GSK 1349572) is currently in phase III clinical trials (Fig. 9). The results of the SPRING-2 study comparing the safety and efficacy of dolutegravir and raltegravir in treatment-naïve patients did not reveal major differences between both drugs (Raffi et al., 2013). *In vitro*, dolutegravir has shown efficacy on raltegravir- and elvitegravir-resistant clinical isolates of HIV-1 containing mutations Y143R, Q148K, N155H, and G140S/Q148H (Kobayashi et al., 2011a). Dolutegravir shows a slower dissociation rate (k_{off}) from wild-type integrase–DNA complexes in comparison with raltegravir and elvitegravir (Hightower et al., 2011). Prolonged binding of dolutegravir was also observed with complexes containing the characteristic resistance mutations of raltegravir and elvitegravir (e.g. E92Q, G140S, Y143C, H or R, Q148H, K or R, and N155H) (Hightower et al., 2011). HIV-1_{IIIB} clones bearing mutations T124A/S153Y and L101I/T124A/S153F were selected *in vitro* in MT-2 cells grown in the presence of the inhibitor. However, the effects of those mutations on drug susceptibility were not significant as measured in phenotypic assays (Kobayashi et al., 2011a). Further studies have shown that L101I, T124A and other HIV-1 integrase polymorphisms have a minor effect on dolutegravir susceptibity (Vavro et al., 2013).

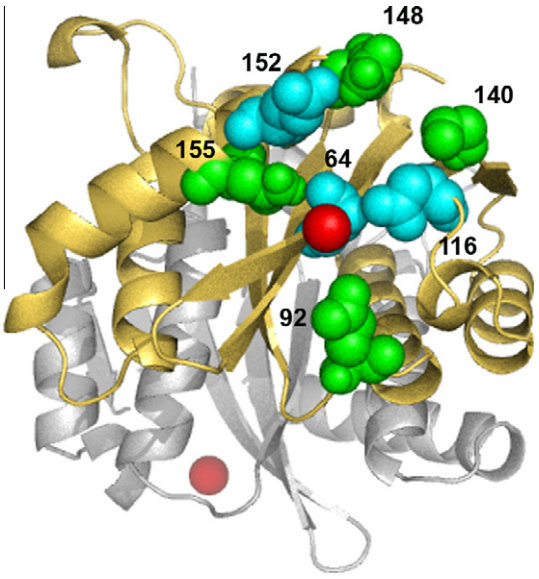


Fig. 11. Structure of the catalytic core of HIV-1 integrase showing the location of amino acids relevant for drug resistance. Gold and silver ribbons represent subunits of the integrase structure. The catalytic triad (Asp^{64}, Asp^{116}, Glu^{152}) is represented using dark blue spheres. Green CPK models are used to show the location of the side-chains of Glu^{92}, Gly^{140}, Gln^{148} and Asn^{155}. Mg^{2+} ions are shown in red. Atomic coordinates were taken from PDB file 1BIU (Goldgur et al., 1998).

Selection experiments carried out in cord blood monocytic cells using HIV-1 clones of subtypes B, C and A/G yielded the R263K mutation (Quashie et al., 2012a). This mutation confers low-level resistance to dolutegravir and decreased integration in cell culture without altering reverse transcription (Quashie et al., 2012a). Arg^{263} is not located in the catalytic domain of the integrase, but in its C-terminal region. The role of the C-terminal domain has not been clearly established although it may contribute to viral DNA binding and could have a role in the nuclear import of the HIV integrase. R263K has also been identified as a secondary mutation in selection studies carried out with elvitegravir and confers low-level resistance to this inhibitor (Margot et al., 2012).

MK-2048 (Fig. 9) is another second-generation integrase inhibitor active against raltegravir- and elvitegravir-resistant strains, although the N155H mutation conferred some resistance to the drug (Goethals et al., 2011). Selection studies in cell culture revealed a novel mutational pattern dominated by the combination G118R/E138K (Bar-Magen et al., 2010). Gly^{118} is a highly conserved residue in retroviral integrases, and its replacement could affect the active site geometry. Despite the favorable resistance profile, the development of MK-2048 has been discontinued due to its poor pharmacokinetic profile. Other HIV-1 integrase inhibitors in pre-clinical development include strand transfer inhibitors (e.g. quinoline derivatives, S/GSK-1265744 and MK-0536) and inhibitors of the 3′ processing reaction (e.g. BI-C) (for recent reviews, see Pendri et al., 2011; Quashie et al., 2012b). BI-C is a precursor of BI 224436, a compound that interferes with the interaction between integrase and the chromatin targeting LEDGF/p75 protein,

decreasing 3′-processing and viral replication. L102F appears to be a major resistance mutation associated with decreased susceptibility to these compounds, and was selected together with Y99H, H171T and N22K in resistant strains (Fenwick et al., 2011). Y99H and other mutations such as A128T and A129T were previously identified in selection experiments carried out with 2-(quinolin-3-yl)acetic acid derivatives that inhibit the interaction of HIV-1 integrase with LEDGF/p75 (Christ et al., 2010).

7. Resistance to inhibitors of viral entry

A series of cell-attachment factors (e.g. heparan sulfate proteoglycans, α4β7 integrin, DC-SIGN and gangliosides) play a role in HIV-1 infection by facilitating the interaction of the virion and the host cell. However, HIV-1 infection occurs after binding of the viral envelope glycoprotein (gp120) to the primary cellular receptor CD4 and then to a cellular coreceptor (for recent reviews, see Klasse, 2012; Pollakis and Paxton, 2012; Wilen et al., 2012). In HIV-1, major coreceptors are CCR5 and CXCR4, which are members of the seven-transmembrane G protein-coupled receptor superfamily, and can be blocked by natural ligands (MIP-1α, MIP-1β and RANTES for CCR5, and SDF-1 for CXCR4) (Pollakis and Paxton, 2012). MIP-1α, MIP-1β, RANTES and SDF-1 are human chemokines whose systematic names are CCL3, CCL4, CCL5 and CXCL12, respectively (Zlotnik and Yoshie, 2012). Coreceptor binding triggers fusion of the viral and host cell membranes with the participation of the viral transmembrane glycoprotein gp41 (for a recent review, see Blumenthal et al., 2012).

Drugs targeting viral entry include attachment inhibitors (e.g. zintevir, chicoric acid derivatives, dextran sulfate, cyanovirin and lectins), CD4 binding inhibitors (e.g. azaindole derivatives such as BMS-378806 or BMS-599793), monoclonal antibodies against CD4, the coreceptor or the gp120/gp41 complex (e.g. ibalizumab or IgG1b12), CXCR4 and CCR5 antagonists (e.g. bicyclams such as AMD3100, maraviroc, vicriviroc and cenicriviroc), and fusion inhibitors (e.g. enfuvirtide, albuvirtide and sifuvirtide) (for reviews, see Menéndez-Arias and Esté, 2004; Esté and Telenti, 2007; Melby and Westby, 2009). Despite considerable research in this area, only maraviroc (Fig. 12) and enfuvirtide have been approved for clinical use. In the case of enfuvirtide, the need for twice-daily subcutaneous injections, and its high cost have limited its use to "salvage" therapy in patients with multidrug-resistant HIV.

7.1. Resistance to maraviroc and other CCR5 antagonists

Maraviroc (formerly known as UK-427,857) binds to a pocket formed by the transmembrane helices of CCR5. Therefore, unlike other antiretroviral drugs, it targets a host protein. Single amino acid substitutions in CCR5, such as W86A, Y108A, Y108F, I198A, Y251A, Y251F and E283A were shown to produce a >25-fold decrease in maraviroc binding (Labrecque et al., 2011). HIV-1 gp120 interacts with the N-terminal region and the second extracellular loop of CCR5. Maraviroc inhibits HIV-1 entry by altering the conformation of the CCR5 extracellular loops. In the absence of maraviroc, all HIV-1 isolates (either resistant or susceptible to the drug) are able to interact efficiently with CCR5. However, in the presence of the drug, the interaction is affected by conformational changes occurring at the N terminus of CCR5 and at residues His^{88} and His^{181} (in extracellular loops 1 and 2, respectively) (Roche et al., 2011).

Maraviroc has potent antiviral activity against all CCR5-tropic HIV-1 strains, including primary isolates from various clades (Dorr et al., 2005). Clinical efficacy of maraviroc has been demonstrated in clinical trials (Gulick et al., 2008; Fätkenheuer et al., 2008). HIV-1 may escape from maraviroc treatment by utilizing CXCR4 coreceptors (Westby et al., 2006). In addition, maraviroc resistance can be developed through the acquisition of mutations in the *env* gene that allow HIV-1 to continue using the CCR5 coreceptors, even in the presence of bound maraviroc (Westby et al., 2007). Critical residues for viral resistance to maraviroc locate at the V3 loop of gp120, although the V4 loop can modulate the effects of V3 loop mutations (Tilton et al., 2010; Berro et al., 2012).

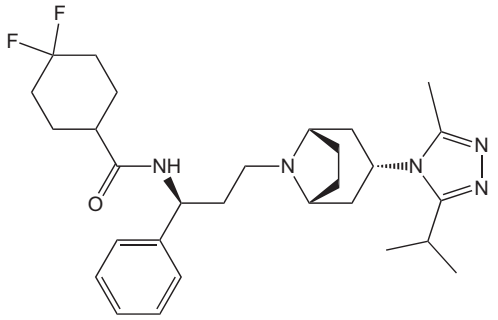


Fig. 12. Chemical structure of maraviroc.

In vitro selection experiments carried out with CCR5-tropic clinical isolates have demonstrated that the most relevant residues for maraviroc resistance are Ala^{316} and Ile^{323} in the V3 loop of HIV-1 gp120 (Westby et al., 2007). However, resistant isolates bearing a different set of mutations in the V3 loop have also been identified (Tilton et al., 2010; Yuan et al., 2011). The maraviroc resistance-associated mutation I323V alters the secondary structure of the V3 loop and as a consequence, it modifies the buried surface area of the V3 loop–CCR5 N terminus interface (Roche et al., 2011). An alternative route towards the development of maraviroc resistance involves the acquisition of the N425K mutation in the C4 region of gp120 (Ratcliff et al., 2013). Structural modeling suggests that this mutation has a major impact on CD4 interactions.

CCR5 antagonists with a similar mechanism of action than maraviroc are vicriviroc, aplaviroc, cenicriviroc (formerly TAK-652), SCH-C, AD101, TAK-779 and TD-0680 (reviewed in Hertje et al., 2010). Mutational patterns obtained in selection experiments with those drugs led to the identification of resistance mutations in the V3 loop of HIV-1 gp120. For example, cenicriviroc selects for T306K and Q309E (Baba et al., 2007), TAK-779 selects for I304V, H305N, I306M, F312L and E317D (Yusa et al., 2005), and several combinations of V3 loop mutations have been associated with resistance to vicriviroc (Marozsan et al., 2005; Tsibris et al., 2008; Ogert et al., 2010; Putcharoen et al., 2012; Tsibris et al., 2012). Despite having a common molecular target (i.e. the V3 loop of gp120), small molecule CCR5 inhibitors may trigger the evolution of distinct resistance patterns in V3, as recently shown for maraviroc in comparison with vicriviroc (Berro et al., 2012). On the other hand, vicriviroc resistance has also been associated with a series of amino acid substitutions occurring in the fusion peptide of HIV-1 gp41 (i.e. the substitution of the sequence GIVAVILG for GIGAMFLG) (Anastassopoulou et al., 2009). This cluster of gp41 mutations was responsible for vicriviroc resistance without affecting coreceptor usage. Evidence of this alternative V3-independent resistance pathway is consistent with the existence of at least two forms of CCR5 with different affinities for their antagonists.

Another approach to block the coreceptor involves the usage of monoclonal antibodies that bind the extracellular loop 2 of CCR5, such as MAb3952 (Jekle et al., 2010). MAb3952-resistant viruses are CCR5-tropic, but use the N-terminus of CCR5 as their target. Mutations associated with HIV-1 resistance to MAb3952 are most frequently found in gp120 (at regions C1, C2, V2 and V3), but also in gp41.

7.2. Neutralizing antibodies and passive immunization

In general, HIV-1 primary strains are resistant to neutralization by antibodies. Studies of passive immunization with conventional anti-HIV antibodies have been disappointing, due to the high mutation rates of HIV-1 that allowed the quick selection of resistant variants. However, those studies were limited to a handful of neutralizing antibodies. Examples of neutralizing antibodies are IgG1b12 (also known as b12), VRC01 and 2G12 directed towards gp120, and 2F5 and 4E10 which recognize the transmembrane glycoprotein gp41 (reviewed in Burton et al., 2004; Mascola and Montefiori, 2010) (Fig. 13). IgG1b12 and VRC01 recognize epitopes overlapping the CD4-binding site of gp120. Resistance to IgG1b12 is conferred by the amino acid substitutions D182N and P365L (Mo et al., 1997). However, Asn^{460} (in the V5 region of gp120) is the most relevant residue in VRC01 susceptibility. A steric effect due to the presence of the long side-chain of Asn^{460} and an enhancing effect of glycosylation are probably responsible for the viral escape from VRC01 (Guo et al., 2012). The observed changes that emerge in the gp120 V5 region did not affect HIV-1 susceptibility to IgG1b12 or to ibalizumab (a monoclonal antibody that binds CD4).

The recent discovery of a plethora of potent broadly neutralizing monoclonal antibodies in HIV-infected individuals provided new grounds for renewed optimism towards the design of antibody-based vaccines and opened new possibilities for immunotherapeutic approaches aimed at treating established HIV-1 infections (Pejchal et al., 2011; Walker et al., 2011). Newly discovered neutralizing antibodies recognize the CD4 binding site, the HIV glycan shield or hydrophobic regions in the gp120/gp41 complex close to the virus membrane (for a review, see Burton et al., 2012). Recent work carried out in HIV-infected humanize mice (i.e. mice with a human hematopoietic system) has demonstrated that passive immunotherapy can be potent *in vivo* (Klein et al., 2012). A combination of five broadly neutralizing antibodies targeting different epitopes in the gp120/gp41 complex was given subcutaneously once or twice weekly for as long as one month. Viral load in mice blood dropped below detectable levels, and this control of HIV viremia continued for 60 days after treatment cessation. This study constitutes a proof-of-principle of the usage of cocktails of neutralizing antibodies to suppress viral replication. Nevertheless, clinical trials in humans are needed to evaluate its real value.

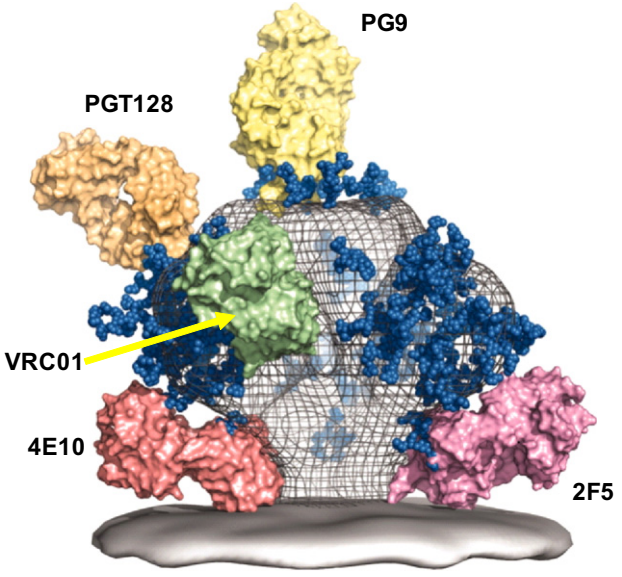


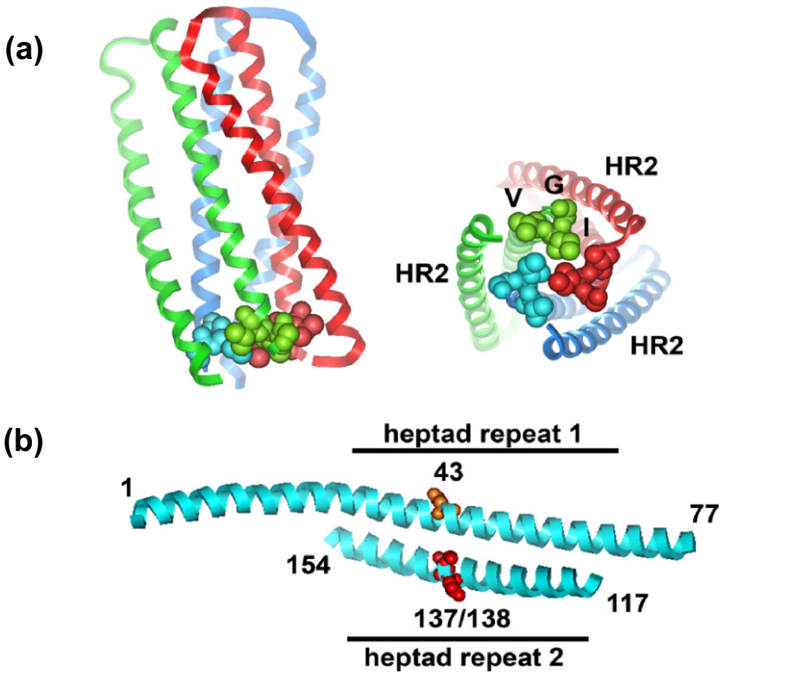
Fig. 13. HIV-1 envelope spike (gp120/gp41) model derived from cryoelectron microscopy with bound broadly neutralizing antibodies. Green Fabs bind at or around the CD4 receptor binding site. Carbohydrates (in blue) were modeled based on the structure of the YU2 gp120 core. Anti-Fabs shown are PG9 (PDB file 3U4E), PGT128 (3TYG), VRC01 (3NGB), 4E10 (2FX7) and 2F5 (2F5B). Adapted with permission from the American Association for the Advancement of Science© (Burton et al., 2012).

7.3. Fusion inhibitors: mechanisms of resistance to enfuvirtide

The HIV-1 transmembrane glycoprotein (TM, gp41) mediates fusion between the viral and host cell membranes. HIV-1 gp41 is a trimer composed of 345 amino acid polypeptides, each of them having an N-terminal ectodomain (172 amino acids), a transmembrane region (21 amino acids) and a long cytoplasmic domain of around 150 residues (Menéndez-Arias and Esté, 2004; Roux and Taylor, 2007). The ectodomain contains two helical regions (heptad repeats HR1 and HR2) that in the process of membrane fusion form a six-helix bundle structure (Fig. 14a). This conformational rearrangement brings the virus and cell membranes in close proximity, leading to fusion pore formation and membrane fusion.

Enfuvirtide, a peptide of 36 amino acids that derives from the C-terminal region of HR2 was the first drug that validated the fusion process as a druggable target (reviewed in Matthews et al., 2004; Eggink et al., 2010). Amino acid substitutions at positions 36–38 of gp41 (in the HR1 region) were shown to confer resistance to enfuvirtide both *in vitro* and *in vivo* (Rimsky et al., 1998; Wei et al., 2002). The amino acid sequences DIV (in wild-type virus), SIV, GIV and GIM have been found in drug-susceptible virus, while enfuvirtide-resistant strains contain SIM, DIM or DTV (Rimsky et al., 1998) (Fig. 14a). Single mutations such as I37K, V38D, V38E and V38G were shown to confer >100-fold increased resistance to enfuvirtide in phenotypic assays (Nameki et al., 2005; Eggink et al., 2009). In addition, combinations of two mutations conferring high-level resistance to enfuvirtide are: G36D/N42T, G36V/N42D, I37M/N43D, V38A/N42D, V38A/N42T, V38A/L44D, V38E/N42S and Q41R/N43D (Menzo et al., 2004; Mink et al., 2005; reviewed in Greenberg and Cammack, 2004).

The negative impact on viral fitness of enfuvirtide-resistance mutations can be compensated by different mechanisms. First, secondary mutations in the heptad repeat HR2 can increase the stability of the six-helix bundle. An example is S138A that compensates for impaired fusion kinetics of HIV-1 variants carrying primary mutations abrogating enfuvirtide binding such as N43D (Xu et al., 2005; Izumi et al., 2009; Ray et al., 2009) (Fig. 14b). A similar mechanism of action has been described for N125D, a mutation that compensates for the fitness loss produced by enfuvirtide-resistance mutations such as Q40H or Q56R (Ray et al., 2009). On the other hand, the double-mutant V36A/N126K showed increased fitness in comparison with V36A, but it was unable to infect cells in the absence of drug. Mechanistic studies suggest that both mutations make HIV-1 hyperfusogenic by accelerating the formation of the six-helix bundle, an event that is delayed in the presence of enfuvirtide (Baldwin and Berkhout, 2008). A second mechanism that enhances HIV-1 replication in the presence of enfuvirtide resistance mutations involves the emergence of synonymous mutations at gp41 residues Gln^{41} (CAG to CAA) and Leu^{44} (UUG to CUG) (Ueno et al., 2009). These changes occur in the stem-loop III of the HIV-1 Rev responsive element (RRE). RRE is essential for transporting non- and singly spliced viral RNA to the cytoplasm from the nucleus. In heavily treated patients, sequencing of gp41 and Rev revealed a cluster of amino acid substitutions in Rev (E57A and N86S) and in enfuvirtide-resistant gp41 (Q40H and L45M). The emergence of E57A in Rev correlated with an increase in viremia and a concomitant decrease of the CD4 cell count (Svicher et al., 2009). In addition to those mechanisms, it should be noted that the conformation and structure of viral coreceptors



(c)

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                72                                          29
HR1          VALIRAQLQKIGWVTLQLHQQAEIARLLNNQQQVIGSLLQRAQ
HR2                WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFNIS
                        117                                 154

Enfuvirtide                  YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF
Tifuvirtide                WQEWEQKITALLEQAQIQQEKNEYELQKLDKWASLWEWF
T2635              TTWEAWDRAIAEYAARIEALIRAAQEQQEKNEAALREL
Sifuvirtide          WIEWEREISNYTNQIYEILTESQNQQDRNEKDLLE

C34                  WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL
T-649                WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLEL
CP621-652    QIWNNMTWMEWDREINNYTSLIHSLIEESQNQQEKNEQ
CP32M        VEWNEMTWMEWDREINNYTSLIHSLIEESQNQQEKNEQ
SC34                 WXEWDRKIEEYTKKIKKLIEESQEQQEKNEKELK
SC34EK               WXEWDRKIEEYTKKIEELIKKSQEQQEKNEKELK
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Fig. 14. Structure of the HIV-1 gp41 ectodomain and peptide sequences of fusion inhibitors. (a) Structural location of major residues involved in resistance to enfuvirtide in the trimer of hairpins motif (heptad repeat 1 (HR1) – linker – heptad repeat 2 (HR2)) forming the HIV-1 gp41 ectodomain. Left and right views show side and bottom views. Residues in HR1 involved in resistance (Gly[36], Ile[37] and Val[38]) are represented with a CPK model. Atomic coordinates were taken from the PDB file 1F23 (Liu et al., 2001). (b) Postfusion state of the assembly of HR1 and HR2 in gp41 (PDB file 1ENV, Weissenhorn et al., 1997), showing the location of Asn[43], Asn[137] and Ser[138]. N137K and S138A compensate for fitness defects caused by the enfuvirtide resistance mutation N43D. (c) Amino acid sequences of enfuvirtide and novel fusion inhibitors that mimick in part the HR2 structure. X (in the SC34 and SC34EK sequences) represents norleucine. Adapted from Menéndez-Arias (2009) and reproduced with permission from Elsevier©.

could also modulate enfuvirtide susceptibility (Reeves et al., 2002, 2004).

7.4. Resistance to fusion inhibitors in preclinical development

Peptides derived from amino acid sequences overlapping HR2 or adjacent to that motif are represented by T-649, tifuvirtide (T-1249), T2635 and sifuvirtide (FS0101) (for a review, see Eggink et al., 2010) (Fig. 14c). Resistance to these second- and third-generation fusion inhibitors is often mediated by the same or similar mutations as those described for enfuvirtide. This has been demonstrated for tifuvirtide that selects for resistant variants accumulating mutations at positions 33, 36, 38, 43 and 45, as well as A50V, with compensatory mutations in HR2 (e.g. N126K and S138A) (Melby et al., 2007). Although enfuvirtide resistance-associated mutations V38E and V38R were selected *in vitro* in the presence of tifuvirtide, mutations not previously involved in resistance such as Q79E and K90E were also identified in some of the selected clones, and were shown to confer some resistance to tifuvirtide (Eggink et al., 2008). Nevertheless, a potent synergistic activity against laboratory-adapted and primary HIV-1 strains has been observed *in vitro* by combining enfuvirtide and tifuvirtide (Pan et al., 2009). Both inhibitors are susceptible to proteolytic degradation in the serum. Stabilization of the α-helical structure by creating salt bridges between the turns of the helix has led to the design of third-generation inhibitors such as sifuvirtide, T2635 and CP32M (Fig. 14c).

Sifuvirtide has a 93% α-helical content and compared with enfuvirtide, it has a 5 times longer half-life. Sifuvirtide is more potent than enfuvirtide against wild-type HIV-1 and shows efficacy against enfuvirtide-resistant strains bearing mutations such as V38A/N42D, V38A/N42T, V38E/N42S, V38M, V38M/N43T, N42T/N43K, N43D or N43D/A50V (He et al., 2008; Wang et al., 2009; Covens et al., 2010; Liu et al., 2011; Yao et al., 2012a). However, single-amino acid substitutions such as I37T, V38A, Q41K or R and N43K decreased sifuvirtide susceptibility by 5-fold (Liu et al., 2011). Low-level resistance to T2635 is conferred by single mutations in HIV-1 gp41, including A6V, Q66R, K77E, Q79E, K90E,

T94N, N126K and K154Q, while double-mutants containing Q66R and N113D, E, K or R showed moderate resistance to the inhibitor (Eggink et al., 2011). This inhibitor shows little cross-reactivity with enfuvirtide.

Two additional peptides (CP32M and CP621–652) have been designed based on the amino acid sequence QIWNNMT, located upstream of HR2 (Chong et al., 2012; Yao et al., 2012b). These compounds were found to be potent inhibitors of enfuvirtide-resistant strains, although substituting Ala for Met^{115} or Ala for Thr^{116} in gp41 confers resistance to CP621–652 in cell fusion and virus entry assays (Chong et al., 2012). Another approach towards the design of better fusion inhibitors includes the introduction of electrostatic constraints as in peptides SC34 and SC34EK (Fig. 14c). These inhibitors showed 5-fold enhanced activity as compared with the reference C34 and a relatively high genetic barrier. However, mutations D36G and N126K were quickly selected *in vitro* in the presence of either SC34 or SC34EK (Shimura et al., 2010).

A rather unique fusion inhibitor has been isolated from human hemofiltrates and identified as an antiviral peptide of 20 amino acids, resulting from degradation of the serine protease inhibitor α1-antitrypsin (Münch et al., 2007). Unlike the fusion inhibitors described above, this virus-inhibitory peptide (VIRIP) interacts with the fusion peptide of HIV-1 gp41 (i.e. residues 1–16 of the glycoprotein), preventing membrane insertion and subsequent fusion. Resistance to a more stable VIRIP derivative (i.e. VIR-353) has been associated with the simultaneous presence of two mutations in gp120 (A433T and V489I) and an additional change in gp41 (V59I). VIRIP and VIR-353 were both active against enfuvirtide-resistant strains (Gonzalez et al., 2011; González-Ortega et al., 2011).

8. Final remarks

Recently approved anti-HIV drugs combine antiviral efficacy with a relatively high genetic barrier against the development of resistance (Table 2). At the same time, improved bioavailability and pharmacokinetics has allowed the simplification of dosing regimens. The success of currently prescribed combination therapies has shifted the clinical approach to HIV disease to a point where major efforts in basic research and the pharmaceutical industry are directed towards a cure (i.e. HIV eradication) or the development of new more economical drugs and formulations to be used as preventive therapies. However, we should not forget that a significant number of patients remain with limited available therapies due to HIV resistance, and we cannot predict if current drugs will retain efficacy for many years (side effects and resistance, particularly transmitted resistance remain as major threats to current therapies).

In this scenario, we still need to exploit other druggable viral targets. Interesting examples of drugs in development acting on viral life cycle steps different from those targeted by drugs described in this review include bevirimat, a betulinic acid derivative that interferes with the proteolytic processing of Gag and renders virions with abnormal capsids (reviewed in Adamson and Freed, 2008; Martin et al., 2008). Resistance to bevirimat was extensively discussed in my previous review (Menéndez-Arias, 2010), and is mainly conferred by mutations around the CA-p2 cleavage site of the Gag polyprotein (Li et al., 2003; Zhou et al., 2004; Adamson et al., 2010; Fun et al., 2011). Other small molecules as well as peptide derivatives have been shown to inhibit interactions between capsid monomers, thereby interfering with HIV-1 assembly (for a recent review, see Bocanegra et al., 2012). One of these molecules (i.e. PF-46396) showed a similar mechanism of action to that of bevirimat, but in addition to mutations around the CA-p2 cleavage site, it selected for amino acid substitutions in the HIV-1 CA protein (specifically at Ile^{201} and the major homology region, MHR, than includes residues 154–167) (Waki et al., 2012).

Even in the case of well-studied enzymes, such as the HIV-1 RT, current drugs are directed exclusively towards the DNA polymerase domain, and so far, compounds inhibiting the RNase H activity have not advanced to clinical trials. Vinylogous urea derivatives and other drugs have shown efficacy in inhibiting RNase H activity *in vitro* (Chung et al., 2010; reviewed in Beilhartz and Götte, 2010).

Finally, recent discoveries in HIV cell biology such as the interaction between the viral integrase and the host cell protein LEDGF/

Table 2
Potency and genetic barrier of antiretroviral drugs.

Potency (log change in viral load)[a]	Genetic barrier to resistance		
	1 mutation	2–3 mutations	>3 mutations
⩽1 log		Zidovudine Stavudine	
>1 log to <3 log	Abacavir[b] Didanosine[b] Emtricitabine Lamivudine Nevirapine Tenofovir[b]	r/Atazanavir[c]	Maraviroc r/Tipranavir
⩾3 log	Efavirenz Elvitegravir Enfuvirtide Etravirine[d] Raltegravir Rilpivirine[d]		r/Darunavir r/Lopinavir

[a] The antiviral activity has been estimated based on the viral load changes obtained with the drug used in monotherapy, or obtained after comparing treatments with or without the drug (Tang and Shafer, 2012; and references therein). Additional data for rilpivirine and elvitegravir were obtained from recent clinical trials (Hughes et al., 2009; Cohen et al., 2011; Molina et al., 2011; DeJesus et al., 2012; Sax et al., 2012). The genetic barrier to resistance is here defined as the minimal number of mutations required to develop significant resistance to the drug.

[b] The presence of only one amino acid substitution (K65R in the case of tenofovir, and L74V in the case of abacavir or didanosine) is sufficient to recommend switching therapy. However, resistance to abacavir, didanosine and tenofovir could also result from the accumulation of 2 or more TAMs.

[c] r/ is used to indicate that the drug has been administered with a low dose of ritonavir.

[d] E138K and other substitutions at this position are sufficient to confer resistance to these drugs (Asahchop et al., 2012). However, unlike in the case of other approved NNRTIs, high-level resistance requires at least two amino acid substitutions (Azijn et al., 2010; Javanbakht et al., 2010).

p75, and the identification of interacting surfaces between both proteins paved the way to the discovery of integrase inhibitors with a different mechanism of action (Christ et al., 2010, 2012; Hu et al., 2012). Other targets involving interactions between virus and cell proteins include APOBEC and Vif (Kitamura et al., 2012), TRIM5α and the HIV-1 CA protein (Yang et al., 2012), or Vpu and its role in HIV budding, among others. These efforts will be aided by a better understanding of the molecular and cell biology of HIV-1 and its interaction with the infected organism.

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