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Review

Molecular basis of human immunodeficiency virus drug resistance: An update

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ABSTRACT

Antiretroviral therapy has led to a significant decrease in human immunodeficiency virus (HIV)-related mortality. Approved antiretroviral drugs target different steps of the viral life cycle including viral entry (coreceptor antagonists and fusion inhibitors), reverse transcription (nucleoside and non-nucleoside inhibitors of the viral reverse transcriptase), integration (integrase inhibitors) and viral maturation (protease inhibitors). Despite the success of combination therapies, the emergence of drug resistance is still a major factor contributing to therapy failure. Viral resistance is caused by mutations in the HIV genome coding for structural changes in the target proteins that can affect the binding or activity of the antiretroviral drugs. This review provides an overview of the molecular mechanisms involved in the acquisition of resistance to currently used and promising investigational drugs, emphasizing the structural role of drug resistance mutations. The optimization of current antiretroviral drug regimens and the development of new drugs are still challenging issues in HIV chemotherapy.

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Contents

1. Introduction

Human immunodeficiency virus (HIV) is the etiologic agent of the acquired immunodeficiency syndrome (AIDS), a disease that already claimed the lives of more than 25 million people. The global incidence of human immunodeficiency virus (HIV) infection in 2007 was estimated to be approximately 33.2 million people (UNAIDS, 2008). Every day, over 6800 people become infected with HIV and over 5700 persons die from AIDS. There is currently no effective vaccine or cure, although the introduction of potent combination therapies in the mid-1990s has significantly improved the prognosis for infected individuals with access to treatment.

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However, there are important drawbacks related to drug adherence, tolerability and long-term toxicity that limit the efficacy of those treatments. In addition, the high mutation rate of the virus ($\sim 10^{-4}$ to 10^{-5} mutations per nucleotide and cycle of replication) and its high frequency of recombination, together with its rapid turnover in an infected individual (approximately 10^9 viral particles produced daily), promote the emergence of drug-resistant variants that eventually can lead to antiretroviral therapy failure in patients adherent to treatment. Surveys using conventional bulk sequencing in North America and Europe, where the history of antiretroviral therapy is extensive, have shown that transmitted or primary HIV-1 drug resistance is present in 8–20% of the untreated population (Grant et al., 2002; Little et al., 2002; Weinstock et al., 2004; Wensing et al., 2005; Jayaraman et al., 2006).

In March 1987, zidovudine (AZT; β-D-(+)-3′-azido-3′-deoxythymidine) became the first drug to be approved for the treatment of AIDS and HIV infection (Mitsuya et al., 1985). Twenty-two years later, the number of compounds licensed for clinical use has increased up to 25. Currently available antiretroviral drugs target different steps of the HIV life-cycle: (i) viral entry (including coreceptor antagonists and fusion inhibitors); (ii) reverse transcription [i.e. nucleos(t)ide and non-nucleoside reverse transcriptase (RT) inhibitors]; (iii) integration (integrase inhibitors); and (iv) viral maturation (protease inhibitors). Most of the licensed drugs are RT or protease inhibitors, which constitute the backbone of highly active antiretroviral therapy (HAART). HAART regimens include either one or two nucleoside RT inhibitors, one non-nucleoside RT inhibitor and/or one protease inhibitor, and are the standard of care for the treatment of HIV infection. However, the emergence of multi-drug resistant HIV variants has serious clinical implications, and the inclusion of novel drugs targeting other steps in the viral life cycle is becoming essential to ensure the effectiveness of therapy. In this review, I will discuss on the molecular basis of resistance to antiretroviral drugs and the mechanisms that lead to drug-resistant HIV.

2. HIV-1 RT structure and inhibition

All retroviruses contain two copies of genomic RNA. Reverse transcription is a process that involves the conversion of single-stranded viral RNA genome into double-stranded proviral DNA. RTs are multifunctional enzymes possessing RNA- and DNA-dependent DNA polymerase, RNase H, strand transfer and strand displacement activities. HIV type 1 (HIV-1) and HIV type 2 (HIV-2) RTs are heterodimeric enzymes composed of two subunits that derive from proteolytic processing of a large polyprotein precursor, termed Gag-Pol. The mature HIV-1 RT is composed of subunits of 66 and 51 kDa, which are designated as p66 and p51, respectively. Both subunits have identical primary structures, but p51 lacks the 120 residues of the C-terminal region of p66 that form the RNase H domain. Crystal structures of HIV-1 RT showed that both subunits contain four common subdomains, termed 'fingers' (residues 1–85 and 118–155), 'palm' (residues 86–117 and 156–236), 'thumb' (237–318) and 'connection' (319–426) (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993; Huang et al., 1998) (Fig. 1A). The overall folding of the subdomains is similar in p66 and p51, but their positions relative to each other are different in both subunits. The DNA polymerase domain of the RT includes the 'fingers', 'palm' and 'thumb' subdomains, and contains the active-site carboxylates (Asp110, Asp185 and Asp186), that bind two divalent cations (Mg^{2+}) that are required for catalysis (Mendieta et al., 2008). Other residues in their vicinity, such as Lys65, Arg72, Asp113, Ala114, Tyr115 and Gln151 are involved in the interactions with the incoming dNTP, while Leu74, Pro157, Phe160, Tyr183 and Met184 could indirectly affect dNTP binding (Huang et al., 1998) (Fig. 1B).

Approved antiretroviral drugs targeting the DNA polymerase activity of HIV-1 RT can be classified into three major groups: nucleoside analogue inhibitors (zidovudine, lamivudine, stavudine, didanosine, zalcitabine, abacavir and emtricitabine), acyclic nucleoside phosphonates (i.e. tenofovir, which is administered as an esterase-sensitive prodrug) and non-nucleoside RT inhibitors (nevirapine, delavirdine, efavirenz and etravirine). Inside the cell, nucleoside analogue RT inhibitors and acyclic nucleoside phosphonates are phosphorylated to their triphosphate form to act as competitive inhibitors (or alternative substrates) of HIV-1 RT. In contrast, non-nucleoside RT inhibitors bind at a hydrophobic pocket adjacent to the polymerase active site in the p66 subunit of the enzyme (for a review, see Ren and Stammers, 2008). The non-nucleoside RT inhibitor binding site is largely associated with the palm subdomain of p66, although Glu138 from p51, located at the edge of the pocket can interact with certain inhibitors.

3. Molecular mechanisms of resistance to RT inhibitors

Resistance to RT inhibitors can be achieved through the accumulation of one or more mutations in the viral RT-coding region. In general, resistance mutations emerge at the expense of a loss in viral fitness (as determined in the absence of drugs), and sometimes are accompanied by compensatory mutations that improve the viral replication capacity of the virus (for reviews, see Menéndez-Arias et al., 2003; Martinez-Picado and Martínez, 2008). Single-nucleotide mutations conferring resistance to RT inhibitors have been identified in lamivudine-resistant strains, and are frequently associated with resistance to non-nucleoside RT inhibitors. These mutations render amino acid substitutions in the viral RT that decrease the enzyme's ability to bind the inhibitor. For example, M184V that confers high-level resistance to lamivudine [3TC; β-L-(−)-2′,3′-dideoxy-3′-thiacytidine] and emtricitabine [FTC; β-L-(−)-2′,3′-dideoxy-5-fluoro-3′-thiacytidine] is close to the ribose ring of the incoming dNTP. 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). Both mutations M184I and M184V are known to decrease the viral replication capacity, particularly in the presence of low concentrations of dNTP (Back et al., 1996; Wei et al., 2003). Other mutations, such as L74V, K65R, K70E or V75T also confer resistance to nucleoside analogues by influencing nucleotide discrimination (Table 1). In the case of non-nucleoside RT inhibitors, the loss of important interactions, either hydrophobic (e.g. Y181C) or electrostatic (e.g. K103N) plays a key role in the acquisition of resistance to nevirapine (Richman et al., 1994; Havlir et al., 1996) or efavirenz (Young et al., 1995; Bacheler et al., 2000; Shulman et al., 2000), respectively. In contrast to M184I/V, K103N does not affect viral replication capacity as determined in phenotypic assays (Gerondelis et al., 1999; Imamichi et al., 2000a), while Y181C has a relatively minor impact on viral fitness in vivo (Antinori et al., 2001; Collins et al., 2004).

Suboptimal therapies can accelerate the emergence of multidrug-resistant strains through the accumulation of inhibitor-specific mutations. However, combination therapies can also select for other mutational patterns conferring multi-drug resistance. For example, the acquisition of resistance through the Q151M pathway was first observed in virus isolated from patients receiving zidovudine and didanosine (Shirasaka et al., 1995), and had not been previously detected in individuals under zidovudine or didanosine monotherapy. Resistant viruses contained the amino acid changes A62V, V75I, F77L, F116Y and Q151M, and exhibited high-level resistance to zidovudine, didanosine and stavudine, and low-level resistance to abacavir, lamivudine, emtricitabine and tenofovir in phenotypic assays (Shirasaka et al., 1995; Iversen et al., 1996; Kodama et al., 2001; Feng et al., 2005; Smith et al., 2008). Gln151

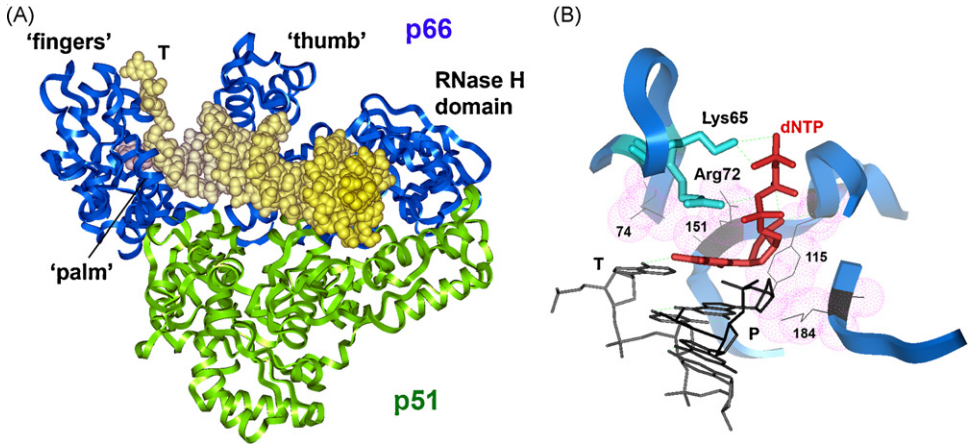

Fig. 1. Crystal structure of the ternary complex of HIV-1 RT, double-stranded DNA and incoming dNTP (A). Ribbon representations of the backbones of the p66 and p51 subunits are shown in blue and green respectively. The incoming dNTP (in purple) is located in the palm subdomain of the p66 subunit. (B) shows the dNTP binding site with the side chains of Lys65 and Arg72 making hydrogen bonds with the phosphate groups of the incoming nucleotide. Van der Waals surface of the side-chains of residues 74 (Leu), 115 (Tyr), 151 (Gln) and 184 (Met) are shown in pink. T and P stand for template and primer, respectively. Atomic coordinates were obtained from PDB file 1RTD (Huang et al., 1998). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

is located in the palm subdomain of the HIV RT and its side-chain interacts with the incoming dNTP, contacting the ribose moiety and the nitrogen base of the incoming dNTP (Huang et al., 1998). Q151M and the accompanying mutations affect the hydrogen bonding network between the incoming dNTP and the enzyme, while enhancing the importance of interactions of the RT with the 3′-OH of the nucleotide (Deval et al., 2002). This allows the mutant enzyme to better discriminate between normal dNTPs, which have the 3′-OH, and nucleoside analogue RT inhibitors, which do not have a 3′-OH (Table 1). Q151M alone confers significant resistance to apricitabine, a deoxycytidine analogue nucleoside RT inhibitor in clinical development (Gu et al., 2006). In vitro studies have shown that K65R and dipeptide insertions between codons 69 and 70 confer some resistance to apricitabine (Frankel et al., 2007;

Table 1
Nucleoside analogue HIV-1 RT inhibitor resistance mutations, whose action is mediated by a nucleotide discrimination mechanism.

Mutation	Nucleoside analogue	Comments
K65R	Tenofovir Didanosine Abacavir Lamivudine Emtricitabine	Lys65 interacts with the γ-phophate of the incoming dNTP (Huang et al., 1998). Nucleotide discrimination mediated by K65R results from the reduction of the k_{pol} for the incorporation of the inhibitor (Selmi et al., 2001; Deval et al., 2004, 2005; Feng et al., 2006; Sluis-Cremer et al., 2007). In the case of tenofovir-diphosphate, the mutation has a minor effect on nucleotide affinity (K_D), but decreases the insertion rate (k_{pol}) of the analogue by >10-fold (Deval et al., 2004; Sluis-Cremer et al., 2007). M184V increases tenofovir susceptibility when the K65R mutation is present (White et al., 2002).
	Zalcitabine	Discrimination against ddCTP appears to be influenced by both K_D and k_{pol} (Selmi et al., 2001).
K70E	Tenofovir	Lys70 is located in the β3–β4 hairpin loop. K70R is a characteristic thymidine analogue resistance mutation. K70E affects primarily nucleotide discrimination, although some effects on excision might be observed. Nucleotide discrimination appears to be related to the lower incorporation rate of the inhibitor (Sluis-Cremer et al., 2007).
L74V	Abacavir Didanosine	Leu74 interacts with the template strand at position +1 (Ding et al., 1998; Huang et al., 1998). L74V has a relatively small impact on nucleotide discrimination against ddATP (the relevant intermediate in ddI resistance). The observed effects appear to be related to differences in the observed k_{pol} for the incorporation of the inhibitor (Deval et al., 2004). A K_D-effect may also affect discrimination for both ddATP and carbovir-triphosphate (the physiologically relevant metabolite of abacavir).
V75I	Acyclovir[a]	Val75 interacts with the template strand at position +1 (Ding et al., 1998; Huang et al., 1998). V75I is an accessory mutation of the multidrug resistance Q151M complex (Shirasaka et al., 1995), and emerges in cell culture under the selective pressure of acyclovir (McMahon et al., 2008). V75I discriminates against acyclovir at the level of catalysis (k_{pol}), while binding of the inhibitor remains largely unaffected (Tchesnokov et al., 2009).
V75T	Stavudine	Improved discrimination against d4T-triphosphate relates to a small decrease in the affinity for the nucleotide analogue (Selmi et al., 2001).
Q151M	Zidovudine Stavudine Didanosine Zalcitabine Abacavir	Gln151 is located in the palm subdomain of the RT, and its side chain contacts the ribose moiety and the nitrogen base of the incoming dNTP (Huang et al., 1998). Nucleotide discrimination mediated by Q151M is a consequence of a reduction in the catalytic rate constant (k_{pol}) of incorporation of the inhibitor (Deval et al., 2002, 2005; Ray et al., 2002a; Frangeul et al., 2008).
M184V	Lamivudine Emtricitabine Abacavir	The side-chain of Met184 contacts the sugar and base of the 3′ nucleotide in the primer, but introducing a β-branched side-chain also creates a contact with the dNTP sugar ring (Huang et al., 1998; Sarafianos et al., 1999). M184V decreases the catalytic efficiency of incorporation (i.e. k_{pol}/K_D) of lamivudine-triphosphate by the mutant enzyme (Krebs et al., 1997; Feng and Anderson, 1999; Deval et al., 2004), but there is no agreement on whether it is a k_{pol}- or a K_D-mediated effect. Both k_{pol} and K_D are responsible for the lower efficiency of incorporation of carbovir-triphosphate relative to dGTP by the mutant M184V (Ray et al., 2002b).

[a] Acyclovir is a guanosine nucleoside analogue that inhibits herpes simplex virus replication. It appears to inhibit HIV replication in patients coinfected with herpes simplex virus. Phosphorylated acyclovir directly inhibits HIV-1 reverse transcriptase, by terminating DNA chain elongation (Lisco et al., 2008).

Southby et al., 2009). However, the drug remains active against lamivudine- and zidovudine-resistant HIV-1 strains (Gu et al., 2006).

3.1. Excision of nucleoside analogue RT inhibitors

Another important mechanism of resistance to nucleoside analogues involves the selective removal of the nucleoside RT inhibitor from the end of the viral DNA after it has been incorporated by the polymerase (Arion et al., 1998; Meyer et al., 1998, 1999; for recent reviews, see Vivet-Boudou et al., 2006; Menéndez-Arias, 2008) (Fig. 2). The relevance of the excision mechanism for zidovudine resistance has been extensively studied. High-level resistance to zidovudine is acquired through the selection of specific amino acid substitutions in HIV-1 RT, which include M41L, D67N, K70R, L210W, T215Y or T215F, and K219E or K219Q (Larder et al., 1989, 1991; Kellam et al., 1992; Harrigan et al., 1996; Hooker et al., 1996), although most zidovudine-resistant isolates contain only a subset of these mutations (Harrigan et al., 1996; Hooker et al., 1996). These mutations are also selected in virus from patients under stavudine therapy (Lin et al., 1994; Izopet et al., 1999; Pellegrin et al., 1999; Coakley et al., 2000), and are commonly designated as TAMs (i.e. thymidine analogue resistance mutations).

The analysis of RT sequences of viral isolates from patients treated with thymidine analogues has revealed that TAMs can be associated in two different clusters. One of them (designated as TAM-1) involves the association of M41L, L210W and T215Y, while excluding K70R. The second (TAM-2) includes mutations D67N, K70R and K219E/Q, and sometimes T215F (Yahi et al., 1999; Hanna et al., 2000; Cozzi-Lepri et al., 2005; Hu et al., 2006; Svicher et al., 2006; Armstrong et al., 2009). TAM-1 combinations confer higher levels of AZT resistance and are responsible for more extensive cross-resistance to other nucleoside RT inhibitors. Met41, Leu210 and Thr215 are located away from the dNTP binding site. Early biochemical studies showed that AZT-resistant RTs bearing a TAM-2 mutational pattern (i.e. D67N/K70R/T215F/K219Q) were able to discriminate between dTTP and AZTTP with the same efficiency as the wild-type enzyme in DNA-dependent DNA polymerization reactions (Lacey et al., 1992; Carroll et al., 1994; Krebs et al., 1997). In addition, there were relatively small differences between the crystal structures of mutant RTs with the combinations D67N/K70R/T215F/K219Q (Ren et al., 1998) or M41L/T215Y (Chamberlain et al., 2002), and the three-dimensional structure of the wild-type enzyme. However, AZT-resistant RTs are able to unblock primers terminated with nucleoside analogues in the presence of a pyrophosphate donor (Arion et al., 1998; Meyer et al., 1998, 1999). Despite the initial controversy about whether ATP or PPi is the pyrophosphate donor in the excision reaction, it seems now clear that ATP is the physiologically relevant compound, rather than PPi, which is the normal product of the DNA polymerization reaction. Molecular modeling suggests that the amino acid substitution T215Y or T215F is selected because Tyr215 or Phe215 can stack with the adenine ring of ATP (Boyer et al., 2001), enhancing the ability of RT to bind ATP (Dharmasena et al., 2007).

Although AZT-terminated primers are excellent substrates of the ATP-mediated excision reaction, other nucleoside analogues can be eliminated from blocked primers through the same mechanism. Available data indicate that thymidine analogues (i.e. AZT, d4T and dideoxythymidine) and tenofovir are the best substrates of the reaction, while cytidine analogues are removed very inefficiently (Meyer et al., 1999, 2000; Boyer et al., 2001; Isel et al., 2001; Mas et al., 2002; Naeger et al., 2002; Ray et al., 2003; White et al., 2006; Marchand et al., 2007a; Sluis-Cremer et al., 2007). Despite being excisable, there is no agreement on the unblocking efficiencies for primers terminated with carbovir-monophosphate (CBVMP) (Naeger et al., 2002; Ray et al., 2002a; White et al., 2006) or dideoxyadenosine-monophosphate (ddAMP) (Isel et al., 2001; Meyer et al., 2002; Naeger et al., 2002). CBVMP and ddAMP are active metabolites of abacavir and didanosine, respectively. It should be noted that excision efficiencies are influenced by the nucleotide sequence context (Meyer et al., 2004).

The excision reaction can be inhibited by the next complementary dNTP (Meyer et al., 1999, 2000), due to the formation of a "dead-end complex" constituted by the RT, a template bound to a blocked primer and the next complementary dNTP (Tong et al., 1997) (Fig. 2). In this complex, the 3′-end of the primer is in a post-translocated configuration (Marchand and Götte, 2003; Marchand et al., 2007b), not accessible to the PPi donor, in agreement with theoretical models based on crystallographic data (Boyer et al., 2001; Sarafianos et al., 2002). The removal of AZT is not inhibited at physiological dNTP concentrations (IC_{50} > 250 μM). However, excision of d4T-, dideoxythymidine-, ddA-monophosphates and tenofovir can be inhibited in vitro at low concentrations of the next complementary dNTP (within 0.5 and 25 μM) (Meyer et al., 2000; Mas et al., 2002; White et al., 2004). These differences could explain the lack of cross-resistance between AZT and d4T in phenotypic drug susceptibility assays (Larder et al., 1990; Lin et al., 1994; Petropoulos et al., 2000; Whitcomb et al., 2002), since these analysis are usually performed in transformed human cells which may contain relatively high dNTP levels (for a review, see Smith and Scott, 2006).

Extensive use of nucleoside analogues in the treatment of HIV-infected individuals has facilitated the emergence of HIV variants containing insertions or deletions in the fingers subdomain of the RT (for recent reviews, see Menéndez-Arias et al., 2006; Eggink

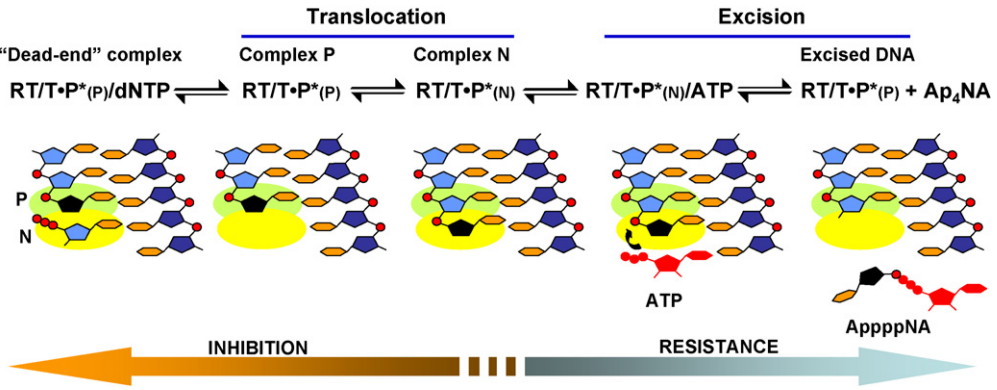

Fig. 2. Simplified models showing the equilibrium between the incorporation of an excisable nucleoside analogue and the subsequent inhibition of DNA polymerization, and its removal from chain-terminated primers by an ATP-dependent phosphorolytic reaction. The inhibition of DNA synthesis due to the formation of a "dead-end complex" (mediated by the incorporation of the dNTP complementary to the template base at position +1) is favoured when the nucleoside analogue moiety of the primer is in the P site. Reprinted from Menéndez-Arias (2008), with permission from Elsevier.

et al., 2007). HIV-1 RT variants with a dipeptide insertion (usually Ser–Ser, Ser–Gly or Ser–Ala) between codons 69 and 70 and additional mutations such as M41L, A62V, T69S, K70R and T215Y showed very high levels of excision activity with primers terminated with AZTMP and d4TMP (Mas et al., 2000; Matamoros et al., 2004; Cases-González et al., 2007). The insertion contributed to increase the ATP-dependent phosphorolytic activity on primers terminated with thymidine analogues, as demonstrated after deleting the Ser–Ser insertion in the heavily mutated sequence background (Mas et al., 2000, 2002). Further studies revealed that the dipeptide insertion, plus T69S and T215Y were sufficient to confer low-level resistance to AZT, and measurable ATP-dependent phosphorolytic activity on primers terminated with AZT or d4T (Boyer et al., 2002; Matamoros et al., 2004). Other mutations such as M41L and A62V and in a lesser extent K70R increased excision activity and thymidine analogue resistance (Cases-González et al., 2007). Based on the crytallographic structure of an HIV-1 RT ternary complex (Huang et al., 1998), it has been proposed that M41L and A62V could have an effect on the conformation of the β3–β4 hairpin loop, and specifically on the position of the side-chain of Arg72 (Cases-González et al., 2007), a residue that appears to be important for PPi binding (Sarafianos et al., 1995).

Unlike in the case of the dipeptide insertions, deletions found in clinical HIV-1 isolates (typically one-amino-acid deletions affecting residues 67 or 69) appear to have a small effect on RT activity and resistance to inhibitors, although they may improve viral fitness (Imamichi et al., 2000b; Villena et al., 2007). Both the deletion of codon 67 in combination with T69G, and the deletion of codon 69 confer some resistance to lamivudine and emtricitabine in phenotypic assays (Imamichi et al., 2001; Villena et al., 2007; Kisic et al., 2008). In comparison with the wild-type enzyme, HIV-1 RT lacking Thr69 showed reduced excision activity on primers terminated with AZTMP (Kisic et al., 2008), thereby providing additional support for the role of the β3–β4 hairpin loop in thymidine analogue resistance.

Phenotypic drug susceptibility assays have demonstrated that mutations such as K65R, L74V, W88G, E89K, L92I, A114S, S117T, S156A, F160Y, Q161L, M164I, Y181C and M184V are able to enhance AZT susceptibility in the presence of TAMs (Tachedjian et al., 1996; Arion et al., 2000; Götte et al., 2000; Hammond et al., 2001; Meyer et al., 2003; Selmi et al., 2003; Miranda et al., 2005; White et al., 2006). Biochemical studies have shown that mutations selected in the presence of foscarnet (i.e. W88G, E89K, A114S, S117T, Q161L and M164I) reduce the ATP-mediated excision activity of TAM-containing RTs, on AZT- and ddA-terminated primers (Arion et al., 2000; Meyer et al., 2003), and similar effects have been reported for mutations such as K65R (White et al., 2005, 2006), L74V (Frankel et al., 2005; Miranda et al., 2005), Y181C (Selmi et al., 2003) or M184V (Götte et al., 2000), that interfere with the removal of AZT, tenofovir and other nucleoside analogues (for a review, see Menéndez-Arias, 2008).

Recent findings have suggested that mutations in the connection subdomain and in the RNase H domain of the RT can significantly amplify resistance to AZT, 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). A reduction of the specific RNase H activity of the viral RT stabilizes the RNA/DNA duplex, giving RT more time to excise the AZT from the terminated primer (Brehm et al., 2008; Delviks-Frankenberry et al., 2008; Ehteshami et al., 2008). The expected increase in resistance to nucleoside analogues has been demonstrated experimentally with HIV variants carrying RTs containing mutations in the RNase H domain (i.e. Q509L, H539N or D549N) (Nikolenko et al., 2005; Brehm et al., 2007, 2008). Mutations in the connection subdomain such as E312Q, G335C/D, N348I, A360I/V, V365I, and A376S, are selected in response to antiviral therapy (Nikolenko et al., 2007; Yap et al., 2007), and these mutations can also enhance AZT resistance in the presence of TAMs (Nikolenko et al., 2007). Most of the selected connection subdomain mutations decrease primary and secondary RNase H cleavages facilitating ATP-mediated excision of AZTMP on RNA templates in comparison with DNA templates (Delviks-Frankenberry et al., 2007, 2008; Yap et al., 2007; Ehteshami et al., 2008). It has been shown that N348I and A360V could also modulate the excision activity of the RT by an RNase H independent mechanism. Both mutations were able to increase the processivity of mutant RTs bearing TAMs, but lacking RNase H activity (Ehteshami et al., 2008). A similar mechanism of action has been proposed for the RNase H mutation Q509L. This mutation affects the RT's ability to bind template-primers with short RNA/DNA duplexes in a polymerase-independent RNase H cleavage mode (Brehm et al., 2008).

3.2. Non-nucleoside RT inhibitor resistance: structural basis and mechanisms

Non-nucleoside RT inhibitors are chemically distinct from nucleosides and do not require intracellular metabolism for activity. There are four drugs approved for clinical use: nevirapine, delavirdine, efavirenz and etravirine. These drugs bind to an allosteric site in the HIV-1 RT and interfere with dNTP incorporation, probably by altering the conformation of residues in the vicinity of the active site (Xia et al., 2007), rather than having a direct effect on phosphodiester bond formation (Spence et al., 1995). In this regard, structural studies have demonstrated that non-nucleoside RT inhibitor binding affects the conformation of the catalytic carboxylates that bind the metal cofactors (Mg^{2+} cations) and causes a shift in the position of the "primer grip" (Ding et al., 1997; Das et al., 2007), that could slow down the conformational changes required for the alignment of substrates, that precedes phosphodiester bond formation.

Stabilization of non-nucleoside RT inhibitor binding is achieved by (i) stacking interactions between the aromatic rings of the inhibitors and the side-chains of Tyr181, Tyr188, Trp229 and Tyr318, (ii) electrostatic forces (particularly significant for Lys101, Lys103 and the p51 residue, Glu138), (iii) van der Waals interactions with Leu100, Val106, Val179, Tyr181, Gly190, Trp229, Leu234 and Tyr318; and (iv) hydrogen bonds between the non-nucleoside RT inhibitor and the main chain of the RT.

Single amino acid substitutions (e.g. Y181C, K103N, G190A, etc.) that arise from single-nucleotide changes are often sufficient to confer high-level resistance to nevirapine, delavirdine and other non-nucleoside RT inhibitors. Resistance mutations are clustered around the non-nucleoside RT inhibitor binding pocket, which is formed upon binding of the antiretroviral drug (Fig. 3). Structural studies showed that nevirapine and other first-generation non-nucleoside RT inhibitors adopt a 'two-ring' binding mode, where the planes of the rings are separated by ∼120° (Kohlstaedt et al., 1992; Ren et al., 1995). Tyr181 and Tyr188 play an important role in stabilizing nevirapine binding through stacking interactions between their aromatic side-chains and the pyridine groups of the inhibitor. Not surprisingly, non conservative amino acid substitutions at residues 181 (i.e. Y181C or Y181I) or 188 (i.e. Y188C, Y188I, Y188L or Y188H) confer high-level resistance to the inhibitor.

Second-generation inhibitors, such as efavirenz, were designed to minimize the contribution of stacking interactions in their binding to the viral RT (Young et al., 1995; Ren et al., 2000, 2001). Upon binding, efavirenz makes direct or water-mediated hydrogen bonds with the protein backbone of Lys101 and Lys103, while Pro236 moves inwards to form a hydrogen bond between its carbonyl group and the NH of Lys103 (Ren et al., 2000). K103N confers resistance to efavirenz, but also to nevirapine and delavirdine. Lys103 is located at the rim of the non-nucleoside inhibitor binding pocket with its

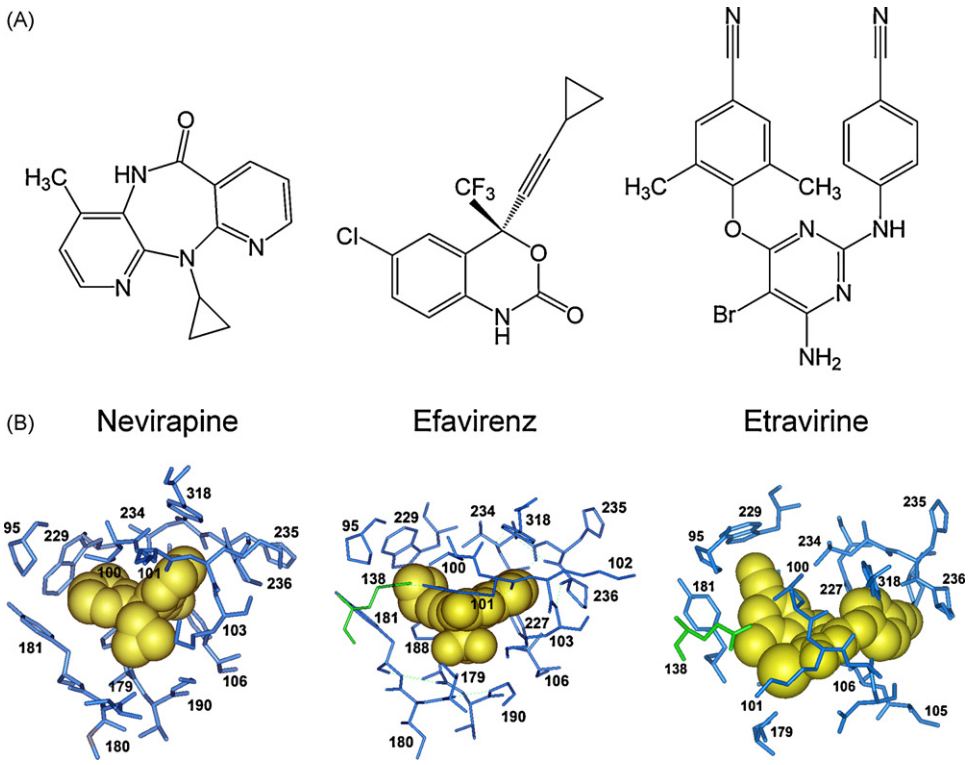

Fig. 3. Structure of the non-nucleoside RT inhibitor binding pocket of the HIV-1 RT. (A) Chemical structures of approved non-nucleoside RT inhibitors. (B) Residues at less than 5 Å from the inhibitor are shown with a stick representation (blue for p66 residues and green from p51 residues). The inhibitors are represented in yellow using a Corey–Pauling–Koltun (CPK) model. Atomic coordinates were obtained from PDB files 3HVT (RT/nevirapine complex) (Kohlstaedt et al., 1992); 1FK9 (RT/efavirenz complex) (Ren et al., 2000) and 1SV5 (RT/etravirine complex) (Das et al., 2004). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

side-chain pointing out. Modelling of the K103N change in the crystal structure of unliganded HIV-1 RT shows that a hydrogen bond could be made between the OH group of Tyr188 and the Asn103 NH group. Such an interaction is likely to stabilize the apo-RT conformation and hence create an energy barrier to binding non-nucleoside RT inhibitors (for a discussion, see Ren and Stammers, 2008).

Third-generation non-nucleoside RT inhibitors have been selected to avoid cross-reactivity with nevirapine, delavirdine and efavirenz. Etravirine, formerly TMC-125, is the latest approved non-nucleoside RT inhibitor and shows an increased genetic barrier in comparison with first- and second-generation non-nucleoside inhibitors (Andries et al., 2004). Structural studies have shown that etravirine, as well as other diarylpyrimidine (DAPY) analogues can adapt to changes in the non-nucleoside RT inhibitor binding-pocket in several ways: (i) DAPY analogues can bind in at least two conformationally distinct modes; (ii) within a given binding mode, torsional flexibility ("wiggling") of DAPY analogues permits access to numerous conformational variants; and (iii) the compact design of the DAPY analogues permits significant repositioning and reorientation (translation and rotation) within the pocket ("jiggling") (Das et al., 2004). Such adaptations appear to be critical for potency against wild-type and a wide range of drug-resistant mutant HIV-1 RTs.

Etravirine was found to be effective against virus containing combinations of frequent drug resistance mutations such as K101E/K103N or K103N/Y181C (Andries et al., 2004). Etravirine-resistant strains have been selected after passage in cell culture (Vingerhoets et al., 2005). Previously identified resistance mutations such as L100I, Y181C, G190E, M230L or Y318F, as well as novel mutations such as V179I or V179F were observed in etravirine-resistant isolates. However, high-level resistance to etravirine was attained through the accumulation of at least two drug resistance mutations in comparison with the wild-type strain

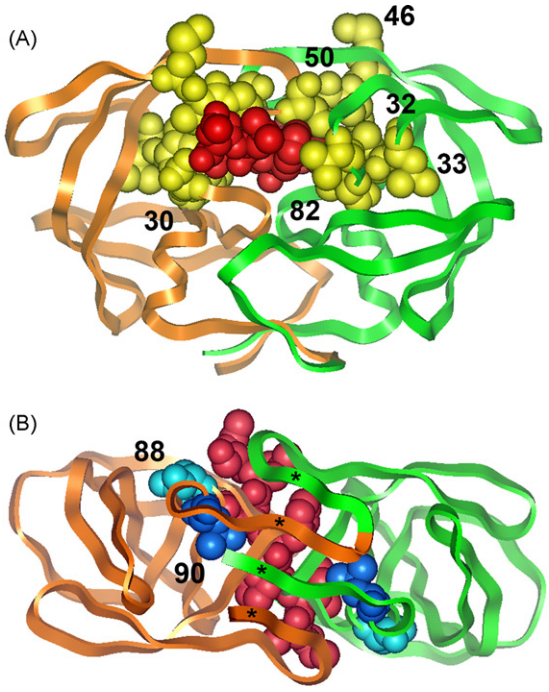

Fig. 4. Crystallographic structures of HIV-1 protease complexed with an inhibitor. (A) The two polypeptide chains of the protease are represented as ribbon diagrams (orange and green). The inhibitor is shown in red using a CPK model. Yellow spheres represent residues that are involved in the acquisition of major drug resistance mutations. (B) Bottom view of the HIV-1 protease/inhibitor complex showing the β-sheet structure involved in the stabilization of the homodimer and the location of Asn88 and Leu90 (pale and dark blue CPK models). Atomic coordinates were taken from PDB file 7HVP (Swain et al., 1990). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(i.e. Y181C/V179F, L100I/K103N/Y181C, Y181C/G190E/T386A, E40K/V179F/Y181C/M230L, L100I/K103N/Y181C/E194G, and V179F/Y181C/L214F/M230L) (Das et al., 2004; Vingerhoets et al., 2005). Clinical studies have shown that decreased virological response to etravirine was associated with the presence of three or more RT DNA polymerase domain mutations (including V90I, A98G, L100I, K101E, K101P, V106I, V179D, V179F, Y181C, Y181I, Y181V, G190A and G190S) at baseline (Vingerhoets et al., 2007). Despite the limited information on the role of connection subdomain mutations in etravirine resistance (e.g. Y318F, N348I, T386A), it has been reported that other mutations such as E399D could also contribute to decrease etravirine susceptibility in clinical isolates (Poveda et al., 2008).

Structural data indicate that single mutations do not prevent etravirine binding but reduce the number of alternative binding configurations available for the inhibitor. Therefore, its relative flexibility restricts the potential for accomodating additional RT mutations. This supports the finding that etravirine resistance requires multiple mutations, and it may provide a rationale for particular resistance-associated combinations of mutations when these affect both wings of RT-bound etravirine, such as Y318F and L234I (Das et al., 2004; Vingerhoets et al., 2005). Further exploitation of these concepts resulted in the development of rilpivirine (TMC278), another DAPY analogue with superior activity profile against wild-type and mutant HIV-1 strains (Janssen et al., 2005; Das et al., 2008).

4. Resistance to protease inhibitors

The proviral DNA is transcribed and translated by cellular enzymes to produce large polypeptide chains that are referred to as polyproteins. The HIV-1 protease cleaves two of these polyproteins (i.e. Gag and Gag-Pol) into smaller functional proteins, thereby allowing the virion to mature. HIV proteases are homodimeric enzymes composed of two polypeptide chains, each containing 99 residues (Fig. 4A). Each monomer contains the conserved sequence Asp–Thr–Gly, which provides the aspartyl group that is necessary for catalysis. High-resolution X-ray and neutron crystallography has provided evidence indicating that only one of the aspartic acid residues in the homodimer is protonated (Adachi et al., 2009). This observation was consistent with the formation of a tetrahedral intermediate of the substrate, where the inner carboxylate oxygen of Asp25 is protonated (Kovalevsky et al., 2007). In addition, both subunits contain a flexible surface β hairpin, known as the 'flap' region (residues 42–58), that closes down upon binding of the inhibitor or the substrate. A four-stranded β sheet formed by the N- and C-termini (residues 1–5 and 95–99, respectively) of each subunit provides a series of hydrogen bonds that appear to be crucial for dimerization (Fig. 4B).

Protease inhibitors are fundamental in HAART regimens. Currently licensed protease inhibitors include saquinavir, ritonavir, indinavir, nelfinavir, amprenavir (and its prodrug, fosamprenavir), lopinavir, atazanavir, tipranavir and darunavir. Except for tipranavir, protease inhibitors used clinically to treat HIV infection are substrate-based compounds that act as competitive inhibitors of the proteolytic reactions needed for viral maturation (Huff and Kahn, 2001; Louis et al., 2007; Menéndez-Arias and Tözsér, 2008). Despite their success in controlling viral replication, the efficiency of protease inhibitors is limited by adverse effects (abnormalities in lipid and glucose metabolism), relatively high number of pills, frequent drug interactions and increasing viral resistance. Pharmacological properties of protease inhibitors have been improved through the coadministration of a small dose of ritonavir together with the inhibitor ('boosting'). This co-formulation has been widely adopted with currently used inhibitors (i.e. lopinavir, darunavir, atazanavir, tipranavir, etc.).

Substrate binds to the HIV protease in an extended β conformation and is anchored by several hydrogen bonds. The enzyme recognizes at least seven substrate residues (four at the N-terminal and three at the C-terminal sides of the cleavage position). Residues 8, 23, 25, 27–30, 32, 47–50, 53, 76, 80–82 and 84 form the substrate binding pocket (Fig. 4A). Substrate-based inhibitors bind in a very similar manner. Interactions between protease and inhibitors are mostly hydrophobic and similar to those involved in enzyme-substrate interactions. Resistance to protease inhibitors is usually achieved through the acquisition of mutations that result in conformational changes in and around the active site that lead to reduced

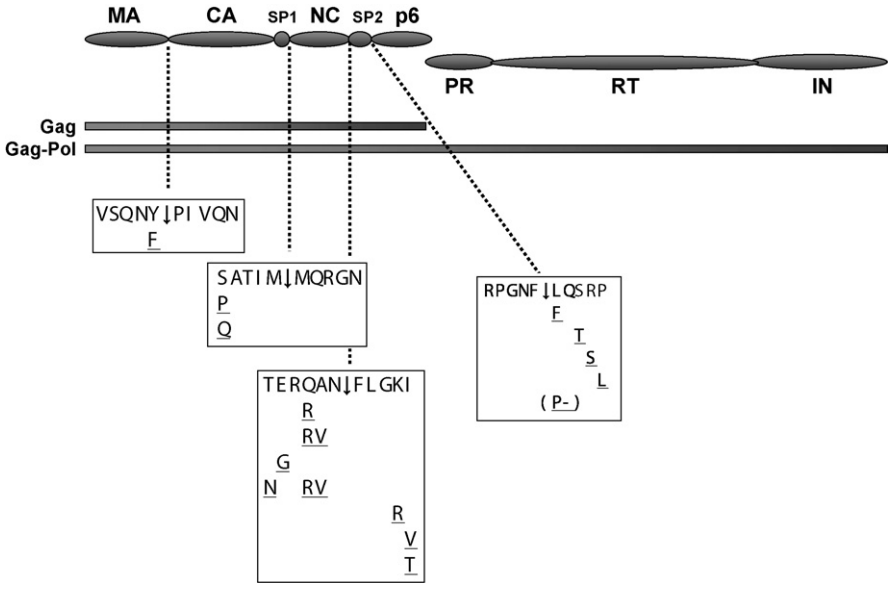

Fig. 5. HIV-1 Gag and Gag-Pol precursor processing sites, indicating the wild-type amino acid sequences around the cleavage sites MA/CA, SP1(p2)/NC, NC/SP2(p1) and SP2(p1)/p6. Amino acid substitutions around the cleavage sites which have been related to the emergence protease inhibitor resistance are shown underlined (Doyon et al., 1996; Zhang et al., 1997; Bally et al., 2000; Maguire et al., 2002; Verheyen et al., 2006; Malet et al., 2007a; reviewed in Mascolini et al., 2008; Menéndez-Arias and Domingo, 2008). Viral protein abbreviations are: MA, matrix protein; CA, capsid protein; NC, nucleocapsid protein; PR, protease; RT, reverse transcriptase; IN, integrase. The amino acid sequences of the spacer peptides SP1 (p2) and SP2 (p1) in the HIV-1 subtype B reference strain HXB2 are AEAMSQVTNSATIM and FLGKIWPSYKGRPGNF, respectively.

inhibitor incorporation or binding. In general, the genetic barrier for protease inhibitor resistance is relatively high, requiring two or more amino acid changes to confer significant resistance. Insertions in the protease-coding region are rare but have become more prevalent in recent years, particularly those located between residues 32 and 42 that probably produce an enlargement of the substrate binding site while impairing flap dynamics (Kožíšek et al., 2008).

Primary resistance mutations affect residues of the substrate-binding pocket or its vicinity. Examples are D30N, V32I, L33F, M46I/L, I47A/V, G48V, I50L/V, V82A/F/L/S/T, and I84V (Johnson et al., 2008). Although these mutations reduce both catalytic activity and viral replication capacity, additional amino acid changes (designated as secondary mutations) compensate for impaired protease function by increasing either the stability or the activity of the enzyme (for a review, see Menéndez-Arias et al., 2003). Thus, saquinavir resistance appears in virus containing the protease mutations G48V and L90M (Jacobsen et al., 1995). G48V exerts a major influence on resistance, while L90M, which is located away from the substrate/inhibitor binding site, contributes to the stability of the HIV protease (Fig. 4B). In a similar way, N88D compensates for impaired replication caused by the nelfinavir resistance-associated mutation D30N, particularly when L90M is present (Sugiura et al., 2002). Unlike primary resistance mutations, several secondary mutations are common polymorphisms which are usually found in drug-naïve patients. Examples are L63P, an HIV-1 subtype B polymorphism, which recovers the viral fitness in a background of multiple combinations of other resistance mutations (Molla et al., 1996; Martinez-Picado et al., 1999), or mutations such as M36I or I93L, which are related to protease inhibitor resistance in HIV-1 subtype B variants (Molla et al., 1996; Markowitz et al., 1998; Patick et al., 1998), but are present in the consensus sequence of wild-type HIV-1 subtype C (Cane et al., 2001; Grossman et al., 2001).

Although compensatory mutations within the protease-coding region increase the catalytic efficiency of the enzyme, there are other molecular mechanisms that lead to fitness recovery during protease inhibitor treatments. Examples are: (i) mutations at Gag cleavage sites (Fig. 5) that increase polyprotein processing (Doyon et al., 1996; Zhang et al., 1997; Pettit et al., 2002), (ii) mutations that affect the frameshift signal between the *gag* and *pol* genes, and lead to an increased expression of *pol* products (Doyon et al., 1998), (iii) mutations outside of the cleavage sites that could affect the conformation of the Gag polyprotein and make the cleavage sites more accessible to the viral protease (Gatanaga et al., 2002; Myint et al., 2004; Aoki et al., 2009; Dam et al., 2009), or (iv) mutations in the transframe polypeptide (p6*), located at the N terminus of the protease, that can restore the enzymatic activity of dimerization-deficient HIV-1 protease variants (Dautin et al., 2003). It is widely accepted that the first protease-mediated cleavage of Gag and Gag-Pol occurs between the spacer peptide p2 and NC (i.e. MA-CA-p2↓NC-p1-p6) and is probably catalyzed by the protease in *trans* (Pettit et al., 2002). However, the carboxyl terminal region of p6* is required for complete activation of the protease and its correct processing is essential to drive temporal and stepwise activation of the enzyme (Ludwig et al., 2008; Tang et al., 2008).

In comparison with saquinavir, ritonavir, indinavir, nelfinavir and amprenavir, novel protease inhibitors (i.e. lopinavir, atazanavir, tipranavir and darunavir) are better pharmacological agents, are devoid of severe side effects and show a favourable resistance profile. These drugs are effective on many strains bearing protease-inhibitor resistance-associated mutations and have a higher genetic barrier. Susceptibility to those drugs is difficult to predict based on genotypic interpretation scores because in most cases the number of mutations that correlated with high-level phenotypic resistance or virological failure was relatively high (often >10 mutations) (De Meyer et al., 2008; Grant et al., 2008; Marcelin et al., 2008). A major goal in designing novel protease inhibitors is to achieve equal potency for wild-type protease and drug-resistant variants, while attaining a high genetic barrier. Targeting the protein backbone has led to exceedingly potent inhibitors such as darunavir (Ghosh et al., 2008), with superb resistance profiles. However, second generation inhibitors have acquired some specificity for HIV-1 strains, and appear to be somewhat less active on HIV-2 proteases (Menéndez-Arias and Tözsér, 2008; Brower et al., 2008).

In an attempt to obtain protease inhibitors with a high genetic barrier, Nijhuis et al. (2007) have shown that in vitro, the substrate analogue RO033-4649, which was effective on many protease inhibitor-resistant HIV-1 strains, selected for viruses having 4–8-fold decreased susceptibility in the absence of mutations in the protease-coding region. Interestingly, full genomic sequence revealed the presence of NC/p1 cleavage site substitutions in the viral Gag polyprotein in all resistant viruses. These mutations enhanced the overall efficiency of polyprotein processing, suggesting a new mechanism by which HIV can become resistant to protease inhibitors.

5. Maturation inhibitors with a novel mechanism of action: mutational patterns and resistance

The clinical success of HIV-1 protease inhibitors validates maturation as an attractive therapeutic target. Non-protease inhibitors acting on maturation are represented by bevirimat, a betulinic acid derivative [3-*O*-(3′,3′-dimethylsuccinyl)betulinic acid], also known as PA-457 (Fig. 6), which inhibits HIV-1 replication in tissue culture and animal model systems, and is currently in phase II clinical trials (Li et al., 2003; reviewed in Temesgen and Feinberg, 2006; Adamson and Freed, 2008; Martin et al., 2008). Bevirimat disrupts the processing of Gag at the CA-p2 (or CA-SP1) cleavage site, leading to the accumulation of the CA-p2 (p25) processing intermediate. HIV-1 virions produced by bevirimat-treated cells display an unusual ultrastructural morphology characterized by the presence of a globular aggregate of electron density acentrically positioned in the particle (Zhou et al., 2004).

Mutations conferring resistance to bevirimat appear at the CA-p2 cleavage site. Examples are KARVL↓AEAMSQ → KARVL↓VEAMSQ (Li et al., 2003), and KARVL↓AEAMSQ → KARVE↓AEAMSQ (Zhou et al., 2004). These mutations rendered HIV-1 less susceptible to the inhibitor when introduced in an infectious clone by site-directed mutagenesis (Zhou et al., 2006). Other mutations in the vicinity of the cleavage site (i.e. at the C-terminus of CA and at the N-terminus of p2) have been detected in vitro after passage of the virus in the presence of the inhibitor. In addition, naturally occurring sequence polymorphisms at positions 6, 7 and 8 in p2 (its amino acid sequence is given in the legend to Fig. 5) have been associated with reduced susceptibility to bevirimat (Van Baelen et al., 2009). The emergence of bevirimat resistance appears to be delayed in the

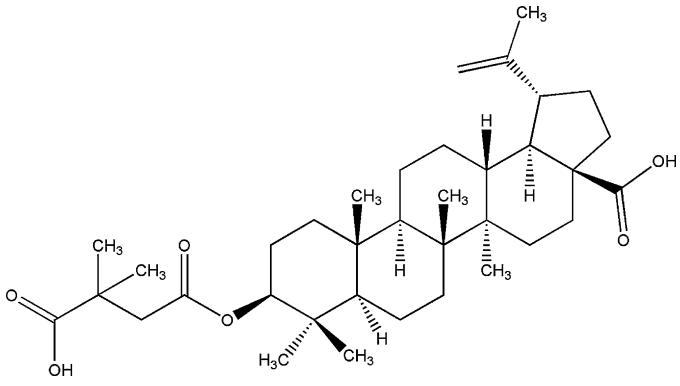

Fig. 6. Chemical structure of bevirimat [3-*O*-(3′,3′-dimethylsuccinyl)betulinic acid].

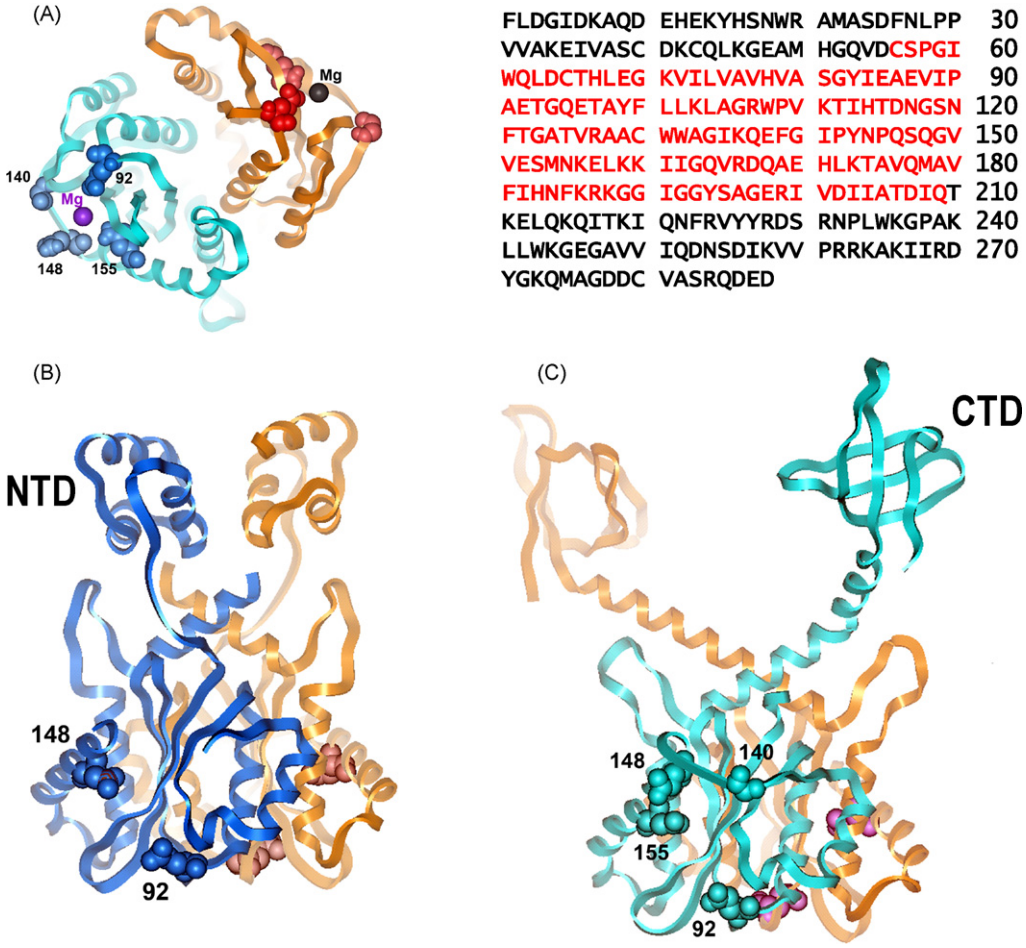

Fig. 7. Crystal structures and amino acid sequence of HIV-1 integrase. (A) Ribbon representation of an HIV-1 integrase catalytic core dimer (residues 56–209) showing the location of residues involved in resistance to integrase inhibitors: Glu92, Gly140, Gln148 and Asn155. Crystal structures of dimeric integrase fragments containing either the N-terminal domain (NTD) and the catalytic core or the C-terminal domain (CTD) and the catalytic core are shown in (B) and (C), respectively. Atomic coordinates for structures shown in panels (A), (B) and (C) were taken from PDB files 1BIU (Goldgur et al., 1998), 1K6Y (Wang et al., 2001), and 1EX4 (Chen et al., 2000), respectively.

presence of protease inhibitor resistance mutations (Adamson et al., 2009), in agreement with previous observations showing their low prevalence in protease inhibitor-experienced patients (Malet et al., 2007b). A3T and A3V (in p2) decreased viral fitness in the absence of drugs, and at suboptimal drug concentrations. The replication defect imposed by A3V was reversed by a second-site change in CA (G225S) (Adamson et al., 2006).

Despite these observations, the involvement of other mechanisms of action cannot be sorted out. There have been reports showing that bevirimat-resistant mutants exhibit enhanced replication and maturation in the presence of the inhibitor (Adamson et al., 2006). In addition, it has been recently reported that the inhibition of HIV-1 virus-like particles by bevirimat can be counteracted by the loss of the zinc binding domain of the viral protein, Vif (DaFonseca et al., 2008). A novel drug blocking maturation by delaying cleavage of CA-p2, and inactive on bevirimat-resistant mutants, designated as vivecon or MPC-9055, has entered phase II clinical trials (Baichwal et al., 2009).

6. Resistance to integrase inhibitors

6.1. Integrase structure and activity

Insertion of the viral genome into host cell DNA by the viral integrase is a necessary step for the propagation of retroviruses. Integrase catalyzes two reactions: 3′-end processing and strand transfer (for reviews, see Asante-Appiah and Skalka, 1999; Li et al., 2006; Delelis et al., 2008). During 3′-end processing, the integrase cleaves a dinucleotide from each viral DNA terminus [i.e. long terminal repeat (LTR)], producing reactive CpA 3′-hydroxyl ends. This reaction takes place within a nucleoprotein complex known as preintegration complex that contains viral and cellular proteins, and is then transported through the nuclear pore into the nucleus. Within the nucleus, the reactive hydroxyl groups are utilized in a nucleophilic attack into the target DNA, by a one-step transesterification reaction that leads to full-site integration. This process is known as strand transfer. Although a dimeric integrase species is required for 3′-end processing, recent evidence showed that tetramerization is required for concerted integration of both viral DNA ends in vitro (Faure et al., 2005; Alian et al., 2009). Although tetramers have been isolated from human cells expressing integrase (Cherepanov et al., 2003), the exact nature of the intracellular integrase complex mediating 3′-end processing and strand transfer reactions remains to be determined.

In HIV-1, the viral integrase is a 32-kDa protein (288 amino acids) that derives from proteolytic cleavage of the Gag-Pol precursor. It has three domains: the catalytic core, the N-terminal domain and the C-terminal domain (Fig. 7). All three domains are required for integration, although the catalytic core (residues 51–212) contains the active site with the conserved DDE motif (Asp64, Asp116, Glu152) that coordinates with the divalent ion cofactor (i.e. Mg^{2+}) required for 3′-end processing and strand trans-

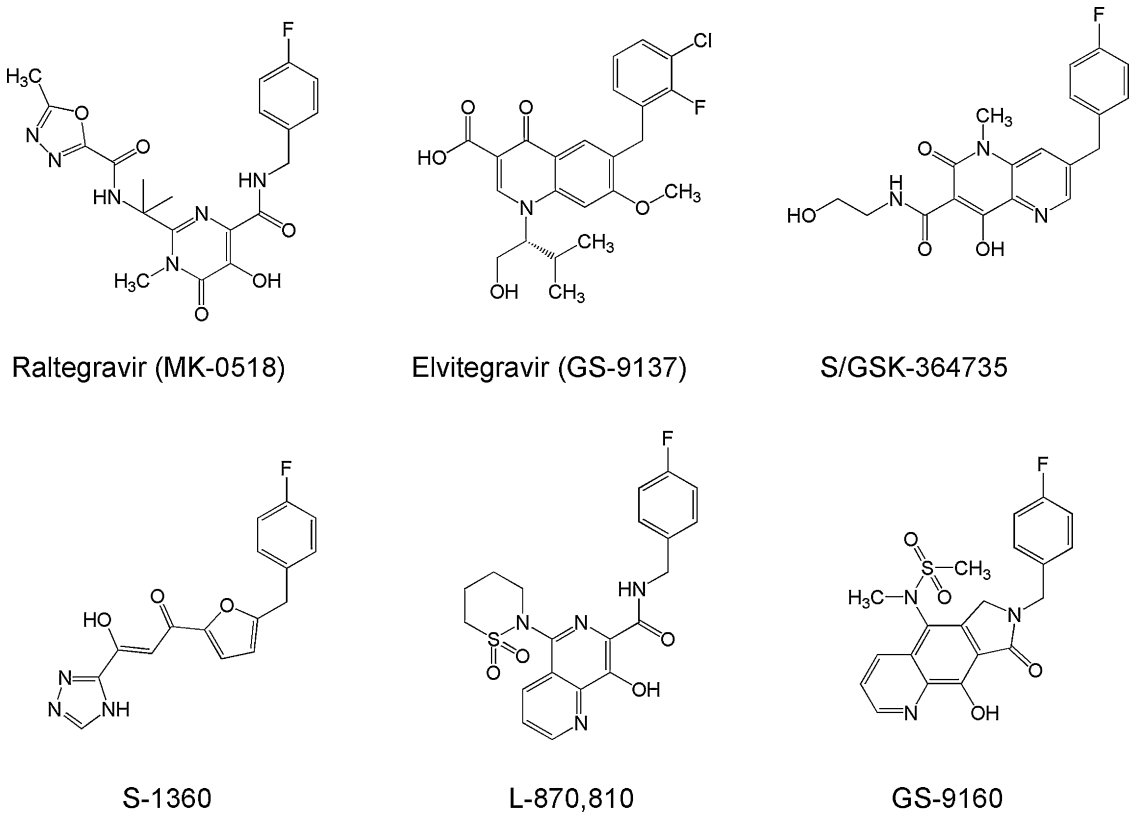

Fig. 8. Chemical structures of integrase inhibitors.

fer. The C-terminal domain confers the ability to bind both viral and host DNA, and the N-terminal domain contains a zinc-finger motif although its function is still unknown. The structure of the full length integrase enzyme has yet to be determined. However, there are available X-ray structures for the HIV-1 integrase catalytic core alone (residues 51–212) (Dyda et al., 1994; Goldgur et al., 1998), as well as integrase fragments containing either the catalytic core and the N-terminal domain (Wang et al., 2001) or the catalytic core and the C-terminal domain (Chen et al., 2000). None of those structures contains DNA, and inhibitor design is limited by the lack of structural information on integrase binding to natural substrates. Some relevant information for drug design has been obtained from the crystal structure of a complex of the integrase catalytic core and 5-CITEP ([1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone]), a β-diketo acid inhibitor (Goldgur et al., 1999). However, in this structure, the number of contacts between 5-CITEP and integrase is relatively small, and it is believed that crystal packing may have a significant influence on the observed binding mode (Chen et al., 2008). The recent determination of the structure of a catalytically active complex of HIV-1 integrase with a viral DNA substrate (Alian et al., 2009) emerges as a promising platform for structural analysis and optimization of drug candidates that target HIV integration.

6.2. Development of integrase inhibitors and identification of resistance mutations

The strand transfer reaction is the target of most integrase inhibitors, including raltegravir, which was licensed for clinical use in 2007 (Fig. 8). The 4-aryl-2,4-diketobutanoic acids (diketo acids) were the first integrase inhibitors with demonstrated antiviral activity, as a consequence of their effect on HIV-1 integration (for recent reviews, see Semenova et al., 2008; Hazuda et al., 2009; Serrao et al., 2009). The most potent compound in this group was L-731,988, which in a cell-based assay was able to inhibit HIV-1 infection with IC_{50}s of 1–2 μM (Espeseth et al., 2000; Hazuda et al., 2000). The amino acid substitution M154I was frequently observed in the viral integrase of isolates selected in cell culture in the presence of the inhibitor. Other mutations such as T66I and S153Y appeared in virus resistant to L-731,988 although the contribution of S153Y was relatively small (Hazuda et al., 2000; Lee and Robinson, 2004). The same mutations were also selected with another diketo acid derivative (i.e. L-708,906) (Hazuda et al., 2000), although later studies lead to the identification of HIV-1 containing mutations T66I/L74M/S230R in the integrase-coding region, which conferred >50-fold reduced susceptibility to the inhibitor (Fikkert et al., 2003). Biochemical studies have demonstrated that some integrase inhibitors (e.g. L-731,988) display a time-dependent inhibition, consistent with a two-step mechanism of binding (Garvey et al., 2009). The first step involves the formation of an initial complex that targets the active site Mg^{2+} cations of integrase, while the second slow step is required for full potency. This slow-binding inhibition mechanism is not observed with the double-mutant T66I/M154I (Garvey et al., 2009).

S-1360, or (*Z*)-1-[5-(4-fluorobenzyl)furan-2-yl]-3-hydroxy-3-(1*H*-1,2,4-triazol-3-yl)propenone was the first integrase inhibitor that entered clinical trials. In cell culture, S-1360 also selected for virus containing mutations T66I and L74M, although additional changes were observed at codons 128, 138, 146, 153, 160, 165 and 201 (Fikkert et al., 2004). Not surprisingly, S-1360-resistant strains were not inhibited by the diketo acid derivative L-708,906. The second inhibitor entering clinical trials was L-870,810, a compound that contains a naphthyridine carboxamide core. The development of L-870,810 into an antiretroviral drug was stopped due to toxicity problems. Interestingly, cross-reaction with diketo acid derivatives was not observed, and high-level resistance to L-870,810 was achieved through the accumulation of integrase mutations V72I, F121Y, T125K and V151I (>50-fold increase in the IC_{50} for the

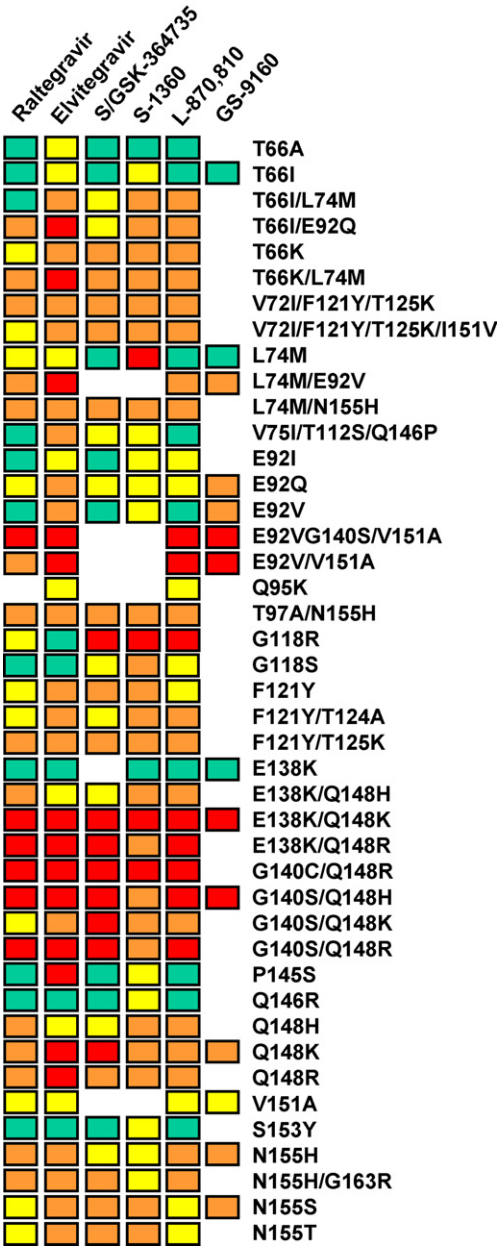

Fig. 9. Summary of phenotypic drug susceptibility data obtained from relevant combinations of amino acid substitutions in the HIV-1 integrase, involved in resistance to integrase inhibitors. High-level (>100-fold increase of the IC_{50} for the inhibitor), moderate (10–100-fold increase) and low-level (3–10-fold increase) resistance is indicated in red, orange and yellow, respectively. Data shown have been taken from Kobayashi et al. (2008), Jones et al. (2009), and Serrao et al. (2009). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

inhibitor compared with a parental wild-type HIV-1 strain) (Hazuda et al., 2004) (Fig. 9). N155S alone produced a 12-fold increase in the IC_{50} for the inhibitor (Hazuda et al., 2004). Other authors have reported the selection of L74M, E92Q and S230N in virus passaged in the presence of L-870,810 (Hombrouck et al., 2008).

6.3. Raltegravir resistance

Raltegravir (formerly known as MK-0518) was the first integrase inhibitor to progress into Phase III clinical trials and became the first one to be approved for clinical use. When combined with an optimized background regimen, virological failure to raltegravir was observed in 23% of treated patients by week 48 (Cooper et al., 2008). Virological failure was generally associated with mutations at one of three residues (Tyr143, Gln148, or Asp155), usually in combination with at least one other mutation. This and other clinical studies revealed two major mutational pathways for the acquisition of raltegravir resistance: (i) N155H (sometimes associated with L74M, E92Q, T97A, G136R and/or V151I), and (ii) Q148K/R/H, usually associated with E138K or with G140A/S (Cooper et al., 2008; Malet et al., 2008; Canducci et al., 2009). E92Q increases the effects of N155H on raltegravir susceptibility when both mutations appear together in same clone (Fransen et al., 2008). Frequently, after the initial emergence of mutation N155H, viral populations containing raltegravir-resistant strains bearing mutations G140S/Q148H become predominant (Miller et al., 2008). In phenotypic assays, N155H decreases viral susceptibility to raltegravir by 14-fold, while a 7–8-fold reduction is observed with mutants E92Q and G140S/Q148H (Malet et al., 2008) (Fig. 9). G140S is a compensatory mutation that mitigates in part the fitness loss produced by Q148H (Delelis et al., 2009; Nakahara et al., 2009). In addition to Q148H, other mutations at the same codon (i.e. Q148R or Q148K) have been found in patients failing raltegravir therapy and conferred significant resistance in phenotypic assays (Cooper et al., 2008; Goethals et al., 2008; Kobayashi et al., 2008; Jones et al., 2009). These observations suggest that the flexible loop extending from residue 140–148 plays a significant role in catalysis (Greenwald et al., 1999), in agreement with theoretical models of inhibitor binding obtained by molecular dynamics (Barreca et al., 2003).

A third pathway leading to the acquisition of raltegravir resistance in patients involves the emergence of a primary mutation Y143C or Y143R, and a series of accessory mutations such as L74A/I, E92Q, T97A, I203M and/or S230R (Cooper et al., 2008; Canducci et al., 2009). It has been suggested that non-conservative substitutions of Tyr143 of integrase could impair transport of the preintegration complex into the nucleus (Tsurutani et al., 2000). However, a detailed analysis of the effects of Y143C or Y143R in the context of raltegravir resistance has not been performed yet.

Raltegravir appears to be effective on HIV-2 strains. Despite the limited information on HIV-2 resistance to raltegravir, there are recent reports showing evidence of the development of N155H (Garrett et al., 2008) or Q148R (Roquebert et al., 2008) in treated patients.

6.4. Resistance to elvitegravir and other integrase inhibitors in development

Similar to raltegravir, elvitegravir has been shown to select for T66I and E92Q, as well as substitutions of amino acids flanking raltegravir-induced substitution sites (Q146P and S147G) (Shimura et al., 2008). Molecular modeling studies based on docking integrase inhibitors in the crystal structure of the catalytic core of the enzyme predict that Thr66, Glu92, Tyr143, Gln148 and Asn155 contribute to raltegravir binding, in agreement with phenotypic drug susceptibility data of mutant HIV carrying amino acid substitutions at those positions (Serrao et al., 2009). Interestingly, similar models obtained for other integrase inhibitors are consistent with extensive cross-reactivity with other integrase inhibitors, including those in clinical development. For example, elvitegravir is expected to interact with Thr66, Glu92, Tyr143, Gln148, and Asp116 and Glu152 of the DDE motif. In addition, Gly140 may also interact with elvitegravir, suggesting that G140S could have a more relevant role for elvitegravir resistance than for raltegravir resistance. Tyr143 and Gln148 as well as the three members of the DDE motif could also contribute to S/GSK-364735 binding (Serrao et al., 2009), thereby explaining its extensive cross-reactivity with raltegravir, S-1340, GS-9160, and other integrase inhibitors (Kobayashi

et al., 2008; Jones et al., 2009; Nakahara et al., 2009) (Fig. 9). These findings underline the need for second generation integrase inhibitors avoiding cross-resistance with raltegravir and other related drugs, with a novel mode of action, or with improved potency and decreased toxicity (Serrao et al., 2009).

7. Resistance to inhibitors of viral entry

HIV entry has become an important pharmacological target. Viral entry could be dissected into four interrelated steps, namely, virus attachment to the cell surface, virus binding to the CD4 receptor, the interaction of the CD4-envelope glycoprotein complex to entry coreceptors, and virus-cell fusion. Over the last twenty years, numerous advances have been made in understanding the molecular mechanisms by which HIV enters CD4+ cells. These advances have lead to the identification of drugs targeting viral entry at different stages (for reviews, see Esté and Telenti, 2007; Melby and Westby, 2009), as well as resistance mutations selected in vitro or in vivo with those inhibitors (for recent reviews, see Menéndez-Arias and Esté, 2004; Briz et al., 2006; Adamson and Freed, 2008). So far, two entry inhibitors (maraviroc and enfuvirtide) have been approved for clinical use, and a few others are at different stages of development.

7.1. Resistance to maraviroc and other CCR5 antagonists

The CD4 receptor was identified as the primary receptor for HIV-1 binding to CD4+ cells (Dalgleish et al., 1984), although later experiments revealed that CD4+ cell binding alone was not sufficient for viral entry into cells (Tersmette et al., 1989). Two different research groups identified CCR5 and CXCR4 as the necessary coreceptors for cellular entry of HIV-1 (Alkhatib et al., 1996; Oberlin et al., 1996). CCR5 and CXCR4 are members of the seven-transmembrane G protein-coupled receptor superfamily, and can be blocked by natural ligands such as MIP-1α, MIP-1β, and RANTES for CCR5 and SDF-1 for CXCR4. These chemokines are natural antagonists that block HIV infection. CCR5 is used by almost all viral isolates found in new and early infections and is present throughout the course of more than 50% of the infections. In addition, natural resistance to HIV infection has been observed in individuals homozygotic for CCR5-Δ32, a non-functional genetic variant of CCR5 that contains a deletion of 32 base pairs (Samson et al., 1996).

Maraviroc (formerly known as UK-427,857) is a selective CCR5 antagonist (Fig. 10), that was developed through an extensive medicinal chemistry program from a lead compound containing a imidazopyridine group (Dorr et al., 2005). Maraviroc demonstrated potent antiviral activity against all CCR5-tropic HIV-1 viruses tested, including 43 primary isolates from different clades and diverse geographic origins. In addition, the drug was active against 200 clinically derived HIV-1 envelope pseudoviruses, half of them derived from virus resistant to existing drug classes (Dorr et al., 2005). However, the percentage of persons infected with virus that is exclusively CCR5 tropic has been shown to decrease with disease progression and with years of antiretroviral experience (from 80 to 90% in antiretroviral-naïve persons to around 50% in antiretroviral-experienced individuals) (Hoffmann, 2007). Screening for clinical trials with maraviroc demonstrated that only 50–60% of the patients for whom at least two antiretroviral regimens failed were infected with R5 HIV-1 only (Melby et al., 2006; Wilkin et al., 2007). In addition, it should be noted that these numbers could be overestimations due to difficulties in detecting X4 strains at baseline.

Maraviroc binds to a pocket formed by the transmembrane helices, while the HIV-1 gp120 interacts with the N-terminus and

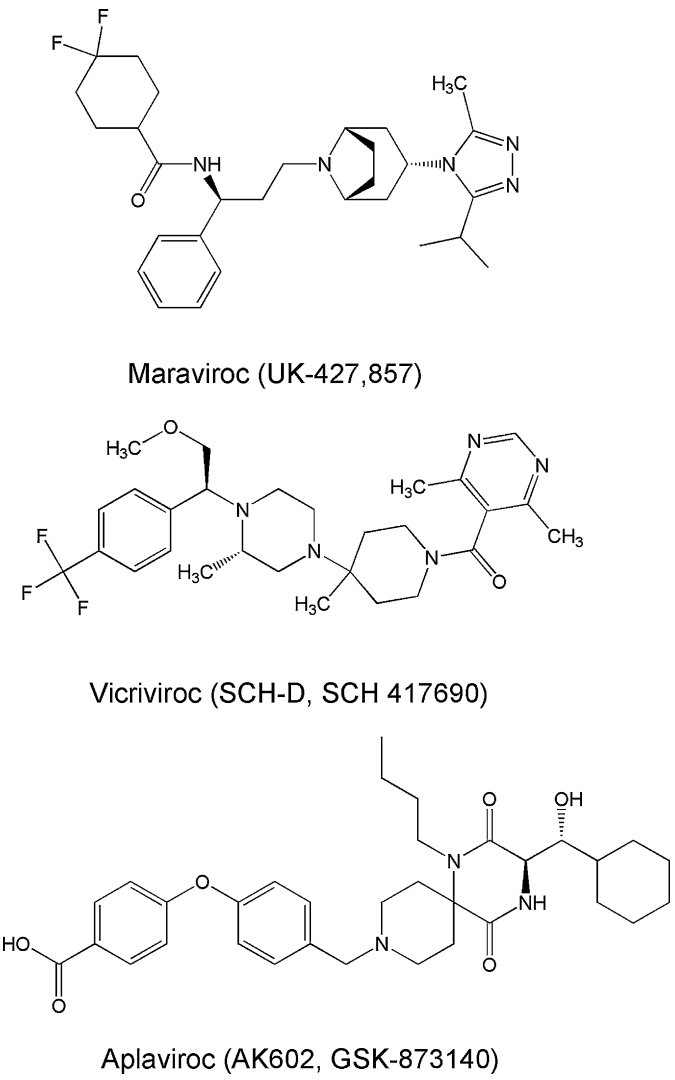

Fig. 10. Chemical structures of maraviroc and related CCR5 antagonists.

the second extracellular loop of CCR5. HIV-1 may escape from maraviroc treatment by utilizing CXCR4 (Westby et al., 2006). Therefore, infection by R5 HIV-1 needs to be demonstrated before initiation of therapy with maraviroc. The most widely used assay for determination of tropism is the Trofile™ assay (Monogram Biosciences) which has the ability to detect minority (CXCR4) variants with the sensitivity of 100% when the percentage of minority variants is at least 10% and with a sensitivity of 85% when the percentage of minority variants is 5% (Whitcomb et al., 2007). At present, genotypic-based tropism determinations appear to be inadequate for prediction of CXCR4 coreceptor usage due to uncertainties in the sequence motifs governing tropism, as well as in their inability to detect minority variants. Ultra-deep sequencing could partially overcome some of these limitations.

In addition, maraviroc resistance can be developed through the acquisition of mutations in the *env* gene that allow HIV-1 to use CXCR4 coreceptors (i.e. a change in tropism), or by mutations that allow HIV-1 to continue using the CCR5 coreceptors, despite blockade by maraviroc (Westby et al., 2007). A change of tropism involves the development of multiple mutations throughout gp160, with resulting lowered replication capacity (i.e. fitness) and less efficient use of coreceptors (Pastore et al., 2004). However, usage of CXCR4 coreceptor has been associated with a more rapid decrease in CD4+ lymphocyte count and faster disease progression (Shankarappa et

al., 1999). In vitro data suggest that coreceptor switch may not be common, since it requires a sequential accumulation of multiple mutations and the stability of many R5 isolates during prolonged exposure to CCR5 antagonists (Pastore et al., 2004, 2006; Baba et al., 2007).

The main mechanism of resistance to maraviroc involves the acquisition of multiple mutations in the *env* gene of HIV-1 that allow the virus to use maraviroc-bound CCR5 coreceptor (Westby et al., 2007). Maraviroc-resistant HIV-1 variants generated by serial passage with CCR5-tropic clinical isolates contained amino acid changes in the variable loops of gp120 V2 (i.e. T163K), V3 (i.e. A316T, I323V) and V4 (i.e. S405A), as well as in the C3 domain (i.e. N355Y), but continued to be phenotypically CCR5-tropic and susceptible to other CCR antagonists such as GW873140, SCH-C and vircriviroc (Westby et al., 2007). Maraviroc-resistant viruses showed incomplete dose-response curves, characterized by a plateau at less than 90% inhibition (i.e. increasing concentrations of maraviroc did not increase the percentage of viral inhibition). These observations suggested that maraviroc-resistant HIV-1 could bind to CCR5 in both its normal conformation and its maraviroc-bound conformation. The height of the plateau would depend of the relative affinity of HIV-1 for inhibitor bound versus free CCR5 (i.e. higher affinities resulted in lower plateaus) (Westby et al., 2007). Key residues for maraviroc resistance were Ala316 and Ile323 in the V3 loop, since reversion of both mutations A316T and I323V restored wild-type susceptibility to the drug. Reversion of either mutation resulted in partially sensitive virus with reduced maximal inhibition.

Another CCR5 antagonist in clinical development is vicriviroc (formerly SCH-D or SCH 417690) (Fig. 10). Like maraviroc, vicriviroc also showed potent, broad-spectrum activity against genetically diverse and drug-resistant HIV-1 isolates (Strizki et al., 2005; Pugach et al., 2007). Its antiviral activity could be enhanced by the coadministration of low doses of rapamycin, a transplant drug that reduces CCR5 density (Heredia et al., 2008). As in the case of maraviroc, phenotypic resistance is associated with an observed plateau in the maximum achievable suppression of viral replication (Watson et al., 2005).

Initial reports showed that vicriviroc-resistant HIV-1 selected after passage in vitro in the presence of the drug contained several mutations in the gp120 variable regions V2 (i.e. D167N, V169M, K171R) and V4 (i.e. N406K), as well as in the C3 domain (i.e. K351E, G354P), but none in the V3 loop (Marozsan et al., 2005). However, other authors have shown that envelopes containing the V3 loop mutations Q315R, A373T, N413T and S437P retained some resistance to vicriviroc (Ogert et al., 2007). In addition, a recently reported study has demonstrated that chimeric envelopes obtained from one patient infected with HIV-1 subtype C, who was experiencing virological failure during a phase IIb study of the drug, showed that changes within the V3 loop were sufficient to confer vicriviroc resistance (Tsibris et al., 2008). These changes included K304R, T306I, F315I, T317R and G318E, although the development of complete phenotypic resistance to the drug was due to the appearance of the S305P mutation. In this sequence context, emergence of vicriviroc resistance was associated with a fitness loss, and vicriviroc-resistant HIV-1 was replaced by vicriviroc-sensitive viruses within 20 weeks of drug discontinuation (Tsibris et al., 2009). A second genetic pathway towards the acquisition of vicriviroc resistance involves the accumulation of amino acid changes in the fusion peptide of gp41 (i.e. the substititution of the sequence GIVAVILG for GIGAMFLG) (Anastassopoulou et al., 2009). It has been suggested that these mutations may alter the conformation of gp120/gp41 complexes but mimicking the consequences of getting the V3 loop changes (i.e. facilitating the interaction between gp120 and the inhibitor-bound CCR5). Escape mutants raised in vitro against CCR5 antagonists (i.e. AD101 or vicriviroc) were found to be resistant to maraviroc as well, suggesting that cross-resistance might be an important consideration relating to the use of CCR5 antagonists (Pugach et al., 2007, 2008).

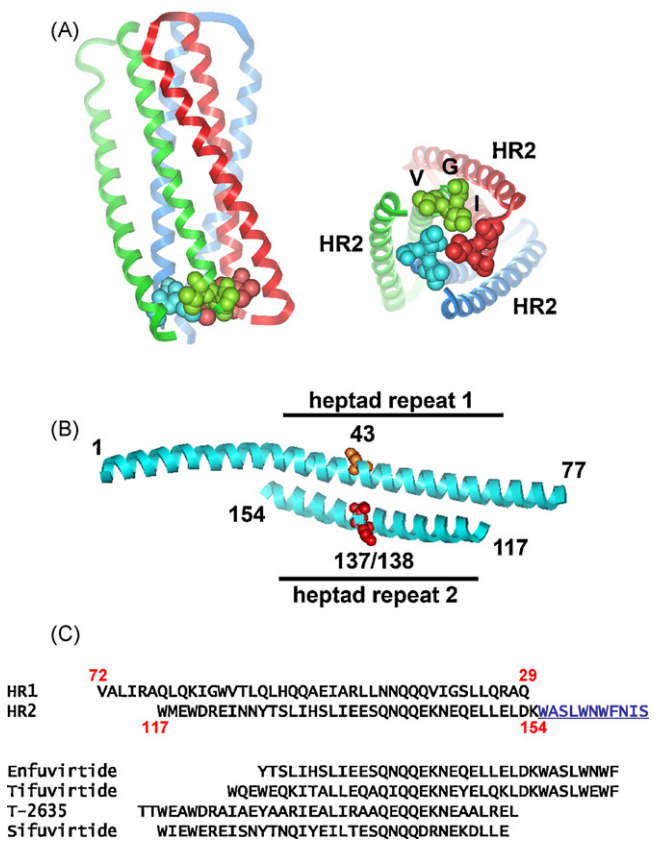

Fig. 11. Structure of the HIV-1 gp41 ectodomain and peptide sequences of fusion inhibitors. (A) Structural location of major residues involved in resistance to enfuvirtide in the trimer of hairpins motif [heptad repeat 1 (HR1) – linker – heptad repeat 2 (HR2)] forming the HIV-1 gp41 ectodomain. Left and right views show side and bottom views. Residues in HR1 involved in resistance (Gly36, Ile37 and Val38) 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 Asn43, Asn137 and Ser138. 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. The underlined sequence corresponds to the linker between HR2 and the transmembrane region of gp41.

Resistance to CXCR4 or CCR5 inhibitors is highly dependent on the HIV-1 envelope backbone, virus heterogeneity and drug efficacy. Different patterns of resistance have been shown for CCR5 and CXCR4 antagonists, depending on the virus and cell culture conditions used. Moreover, virus may switch coreceptor(s) to R5 in the presence of AMD3100 (Esté et al., 1999) or to X4 with maraviroc or vicriviroc (reviewed in Moore and Kuritzkes, 2009) if minor species are already present at initiation of drug treatment. The V3 loop is a major determinant (but not the only one) of coreceptor drug resistance (for both CCR5 and CXCR4 inhibitors). Nevertheless, there are no indications of specific amino acid changes conferring drug-resistance to both types of inhibitors.

7.2. Resistance to enfuvirtide and other fusion inhibitors in clinical development

Current understanding of the molecular basis for the fusion of a viral membrane with a target cell stems from the description of the structures of the influenza virus hemagglutinin in its pre- and post-fusion conformations (reviewed in Eckert and Kim, 2001; Dimitrov, 2004). In HIV-1, after binding of gp120 to the receptors on the surface of the target cell, the viral transmembrane protein (TM, gp41)

mediates fusion between the viral and cellular membranes. The HIV-1 gp41 is a trimer glycoprotein composed of 345 amino acid polypeptides that contain an N-terminal ectodomain (172 amino acids), a transmembrane region (21 amino acids) and a long cytoplasmic domain, comprising around 150 amino acids (for reviews see Menéndez-Arias and Esté, 2004; Roux and Taylor, 2007). The ectodomain contains two helical regions (heptad repeats HR1 and HR2) which in the process of membrane fusion undergo a conformational rearrangement resulting in a six-helix bundle structure (Fig. 11A). This structural transition is thought to bring the virus and cell membranes into close proximity, leading to fusion pore formation and membrane fusion. The trimeric coiled-coils of HR1 peptides or HR2 peptides have been viewed as potential drug targets.

Enfuvirtide (also known as T-20) was the first drug to validate this approach and the only fusion inhibitor approved for clinical use. Enfuvirtide is a synthetic peptide of 36 amino acids that derives from the C-terminal region of HR2 (i.e. residues 127–162). It blocks the formation of the helical bundle through binding to HR1 (Wild et al., 1993; Kilby et al., 1998; reviewed in Matthews et al., 2004). Not surprisingly, enfuvirtide-resistant HIV-1 variants selected in vitro contained mutations at codons 36–38 of gp41, which are located in the heptad repeat HR1. The amino acid sequence of those three residues was DIV (in the wild-type virus), SIV, GIV or GIM in drug-susceptible HIV-1, and SIM, DIM or DTV in drug-resistant variants (Rimsky et al., 1998). The double mutant containing G36S and V38M showed 100-fold increased resistance in comparison with the wild-type virus. Further studies showed that resistance to enfuvirtide could be associated with other substitutions in the vicinity of positions 36–38 (Labrosse et al., 2003; Menzo et al., 2004; Su et al., 2006).

Resistance mutations commonly found in patients treated with enfuvirtide are Q32H, Q32R, G36D, G36S, I37V, V38A, V38M, Q39R, Q40H, N42T, N43D, N43T and N43S (Wei et al., 2002; Marcelin et al., 2004; Menzo et al., 2004; Sista et al., 2004). Other mutations found in vivo were V38G, V38W, V38E, N42H, N42Q and N43Q. Mutations at positions 36 and 38 tend to disappear, while N43D becomes predominant after long-term treatment with the inhibitor (Cabrera et al., 2006). In phenotypic assays, single mutations (e.g. I37K or V38E) and several combinations of two mutations (e.g. G36D/N42T, G36V/N42D, Q41R/N43D, among others) were found to confer high-level resistance to the inhibitor (Wei et al., 2002; Menzo et al., 2004; Nameki et al., 2005; Mink et al., 2005).

The negative impact on viral fitness of resistance mutations within gp41 may also lead to the appearance of secondary mutations in the heptad repeat HR2 that compensate for such defects. Examples are S138A, which appeared in 6 out of 17 patients, later than 8 weeks after the start of treatment with enfuvirtide, and after developing HR1 mutations (Xu et al., 2005). S138A increased the level of resistance by approximately 3-fold over that conferred by the HR1 mutation N43D (Xu et al., 2005), and compensates for the impaired fusion kinetics of HIV-1 variants carrying primary mutations that abrogate enfuvirtide binding (Izumi et al., 2009; Ray et al., 2009). S138A increases the stability of the six-helix bundle, as determined by circular dichroism spectroscopy (Izumi et al., 2009). A similar mechanism of action was observed for N125D, a mutation that compensates for the fitness loss produced by enfuvirtide-resistance mutations Q40H and Q56R (Ray et al., 2009).

A natural baseline polymorphism in HR2 (N137K) was found to be associated with mutations at position 43 during TORO clinical trials (Melby et al., 2007), and was able to rescue full fusion competence of enfuvirtide-resistant HIV-1 bearing N43D (Tolstrup et al., 2007). Structural and biochemical studies revealed that the N43D mutation decreases the thermal stability of the six-helix bundle and causes a significant change in the HR1–HR2 interface, including a loss of HR2 helicity. N137K partially compensates for the loss of stability, probably due to the formation of an ion pair between the side chains of residues 43 and 137 (Bai et al., 2008) (Fig. 11B). Another example of a compensatory effect involves N126K, an HR2 mutation that together with V36A predominated in the viral population after 32 weeks on therapy in a patient failing treatment with enfuvirtide (Baldwin et al., 2004). V36A emerged first and reduced enfuvirtide binding, but at a fitness cost. However, the second-site compensatory mutation was selected in the presence of the inhibitor. In this scenario, the double mutant (V36A/N126K) showed increased fitness, although this virus was unable to infect cells in the absence of the drug. Mechanistic studies suggest that both mutations make HIV-1 hyperfusogenic by accelerating the formation of the six-helix bundle, an event that appears to be delayed when enfuvirtide is present (Baldwin and Berkhout, 2008).

Despite the accumulated evidence discussed above, it should be noted that mutations outside the peptide interaction site could also contribute to decrease baseline susceptibility to enfuvirtide. Notably, the specificity of binding is largely influenced by the conformation and structure of viral coreceptors such as CXCR4 or CCR5 (Derdeyn et al., 2000, 2001). Thus, the amino acid substitution K412D in the bridging sheet region of gp120 reduced the HIV-1 affinity for CCR5, while increasing enfuvirtide susceptibility by 30-fold (Reeves et al., 2002). In another study, it has been reported that, by themselves, mutations in gp120, such as T202G, K421D, I423S, P437A or G441V can reduce CCR5 binding while conferring resistance to enfuvirtide and other related polypeptides (Reeves et al., 2004). Second-generation fusion inhibitors derived from amino acid sequences comprising or adjacent to HR2 are T-649 (Rimsky et al., 1998; Derdeyn et al., 2001), tifuvirtide (T-1249) (Greenberg et al., 2002; Lalezari et al., 2005), T-2635 (Eggink et al., 2008), and sifuvirtide (FS0101) (He et al., 2008a; Wang et al., 2009) (Fig. 11C).

Resistance to tifuvirtide develops through the accumulation of previously characterized enfuvirtide-resistance mutations (L33S, G36R, G36D, V38E, V38R, N43K and L45K), as well as mutations at codon 50 (typically A50V), and sometimes compensatory mutations in HR2 (e.g. N126K and S138A) (Chinnadurai et al., 2005; Melby et al., 2007; Eggink et al., 2008). Although a number of clinical and in vitro-selected enfuvirtide-resistant isolates were inhibited by tifuvirtide (Chinnadurai et al., 2005; Lalezari et al., 2005), its clinical development was halted due to technical difficulties in scaling-up its production. T-2635 and sifuvirtide are similar in size in comparison with enfuvirtide, but lack a C-terminal sequence of about 10 amino acids, while containing an engineered sequence at the N terminus that enhances their helicity and stability (Eggink et al., 2008; He et al., 2008a). Enhancing helicity appears to be a useful strategy to increase potency. Novel inhibitors containing positively and negatively charged residues (e.g. K or E) at positions i and $i+4$ to increase the stability of α-helices by promoting the formation of salt bridges (e.g. CP32M, SC29EK, SC22EK) were highly effective in inhibiting infection by a wide range of primary HIV-1 isolates (He et al., 2008b; Naito et al., 2009). T-2635, sifuvirtide, SC29EK and SC22EK blocked replication of enfuvirtide-resistant HIV-1 strains, including those containing the characteristic resistance mutations selected by the inhibitor (Eggink et al., 2008; He et al., 2008a; Naito et al., 2009; Wang et al., 2009). Despite those findings, partial cross-resistance between sifuvirtide and enfuvirtide has also been demonstrated (Wang et al., 2009). An alternative approach to increase the potency of fusion inhibitors involved the introduction of a cholesterol group attached, via a thioether bond, to the side chain of a cysteine residue added to the C-terminus of C34 (Ingallinella et al., 2009). The resulting compound showed increased antiviral therapy in comparison with second-generation fusion inhibitors such as tifuvirtide, but there is no information about its efficiency on drug-resistant HIV variants.

8. Final remarks

Despite the significant advances in the development of antiretroviral drugs, drug resistance is still a major challenge for new therapies. The genetic barrier to emergent resistance is a property of the drug and its target, and sometimes very subtle chemical changes in the drug can alter that barrier. Minimizing the chemical differences between natural ligands and the drug will undoubtedly raise the genetic barrier. However, this might be difficult to attain in the real world. Developing novel compounds with higher barriers to resistance should continue, but in the expected context of multicomponent therapies, the exploitation of novel and specific HIV targets should play an important role in future research and development. Recent discoveries in HIV cell biology have opened the possibility of exploiting novel targets: (i) APOBEC3G-Vif interaction and function; (ii) LEDGF/p75 as a cofactor of HIV-1 integrase; (iii) TRIM5α as restriction factor for HIV infection; (iv) Gag trafficking and HIV egress; and (v) Vpu and its role in budding, and its interaction with tetherin (for reviews, see Greene et al., 2008; Poeschla, 2008; Waheed and Freed, 2008). However, information about target structures and processes, development of reliable drug screening assays, and the likely interactions with host cell factors may be limiting for a long-term success. In any case, it seems clear that we face a new and challenging era in the discovery of novel HIV therapeutics.

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