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Review

Overcoming HIV drug resistance through rational drug design based on molecular, biochemical, and structural profiles of HIV resistance

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Abstract. There are 20 available drugs for the treatment of human immunodeficiency virus (HIV) infection. With a single exception, all of these drugs inhibit either HIV reverse transcriptase or protease. Reverse transcriptase inhibitors can be further categorized as nucleoside/nucleotide analogs or non-nucleoside reverse transcriptase inhibitors. Resistance that has emerged against all available antiretroviral drugs represents a major challenge in the therapy of HIV infection. Nevertheless, extensive analysis of the molecular and structural mechanisms by which such mutations confer resistance has accumulated over the years. This understanding has driven the development and refinement of novel compounds capable of maintaining antiviral activity against both wild-type and drug-resistant HIV strains. The molecular, biochemical, and structural profiles of reverse transcriptase inhibitor and protease inhibitor resistance are discussed. In addition, how this knowledge has been utilized to generate a new generation of antiviral drugs with activity against drug-resistant HIV is reviewed.

Keywords. Human immunodeficiency virus, drug resistance, reverse transcriptase inhibitor, protease inhibitor.

Introduction

Highly active antiretroviral therapy (HAART) refers to the utilization of a combination of antiviral drugs for treatment of HIV infection, and has been shown to suppress HIV replication while preserving immunologic function. However, during treatment with HAART, inadequate drug concentrations can result from a number of factors including non-adherence, pharmacokinetics, and lack of drug potency. In addition, anatomical sanctuary sites may exist where drug concentrations do not achieve adequate levels despite apparent therapeutic serum drug concentrations. HIV replication can occur in such settings, and the selective pressure of antiretroviral therapy leads to the emergence of HIV harboring drug-resistant mutations. These drug-resistant variants can be generated de novo or, more commonly, arise from the pool of integrated proviruses that existed prior to initiation of therapy. Mutations that confer clinical resistance to each of the 20 available antiretroviral drugs have been documented (Table 1) [1]. As a result, HIV drug resistance has become a major obstacle in the management of HIV infection.

Interpretation of mutational data using commercially available genotypic or phenotypic assays can improve clinical outcomes in patients infected with drug-resistant HIV [2–7]. However, as drug resistance mutations accumulate, predicting what overall effect combinations of

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Table 1. Mutations associated with resistance to currently approved anti-HIV reverse transcriptase or protease inhibitors.

	Mutations conferring high/intermediate resistance									
Nucleoside RT inhibitors (NRTIs)										
Zidovudine (AZT)		NAMS[1],	Q151M complex[2],			69_Ins[3]				
Stavudine (d4T)		NAMS,	Q151M complex,			69_Ins,	K65R			
Tenofovir[4]						69_Ins,	K65R			
Abacavir			Q151M complex,			69_Ins,	K65R,	L74V/I,	M184V	
Didanosine (ddI)			Q151M complex,			69_Ins,	K65R,	L74V/I		
Zalcitibine (ddC)			Q151M complex,			69_Ins,	K65R,	L74V/I,	M184V	
Lamivudine (3TC)			Q151M complex,			69_Ins,	K65R,		M184V	
Emtricitabine (FTC)			Q151M complex,			69_Ins,	K65R,		M184V	
Non-nucleoside RT inhibitors										
Nevirapine	L100I,	K103N,	V106AM,	V108I,	Y181CI,	Y188LCH,	G190A,		M230L	
Delavirdine		K103N,	V106A,		Y181CI,	Y188L			M230L,	P236L
Efavirenz	L100I,	K103N,	V106M,	V108I,		Y188L,	G190AS,	P225H,	M230L	
Protease inhibitors[5]										
Nelfinavir	D30N,		M46IL,			V82ATF,	I84V,	N88S,	L90M	
Saquinavir				G48V,		V82A,	I84V,		L90M	
Indinavir			M46IL,			V82ATFS	I84V,		L90M	
Ritonavir		L33F,	M46IL,			V82ATFS	I84V,		L90M	
fos-Amprenavir[6]			M46IL,	I47V,	I50V,		I84V,		L90M	
Lopinavir		L33F,	M46IL,	I47V,	I50V,	V82ATFS,	I84V,		L90M	
Atazanavir		L33F,	M46I,	G48V,	I50L,	V82A,	I84V,	N88S,	L90M	
Tipranavir		L33F,	M46I,			V82ATFL	I84V,		L90M	

RT, reverse transcriptase.
[1] NAMS, nucleoside analog mutations; include M41L, D67N, K70R, L210W, T215Y/F, K219Q/E/N.
[2] Q151M, complex includes A62V, V75I, F77L, F116Y, Q151M; combination confers multi-NRTI resistance.
[3] 69_Ins insertion (T69S) followed by two or more insertions (SS, SA, SG, others) conferring multi-NRTI resistance.
[4] Nucleotide RT inhibitor.
[5] Usually requires accumulation of multiple mutations to acquire high-level resistance; only primary mutations are listed.
[6] fos-Amprenavir is the prodrug of amprenavir; the latter is no longer manufactured.

mutations might have on antiviral therapy often becomes difficult based on these assays. Understanding of the effects of HIV drug resistance mutations at a molecular level is a critical step in simplifying this complexity.

There are a number of excellent reviews of the mechanisms of HIV drug resistance and their clinical implications [8–14]. Such mechanisms will be discussed, but the ultimate purpose of this paper is to provide examples of how molecular knowledge of drug resistance will aid in designing new generations of antiviral agents with activity against drug-resistant HIV. With the exception of the entry inhibitor, enfuvirtide (T-20), all currently approved HIV drugs target the HIV enzymes reverse transcriptase (RT) or protease, which are responsible for either the conversion of viral RNA to double-stranded DNA, or the processing of immature HIV polyproteins, respectively. Thus, this review will focus on the biochemical and structural knowledge that has accumulated in the understanding of HIV resistance against RT and protease inhibitors (Table 1, Fig. 1). Resistance data for the entry inhibitor, enfuvirtide, is accumulating but will not be discussed in this paper [15–19].

RT inhibitor resistance

HIV RT converts a single-stranded HIV RNA genome into a double-stranded DNA provirus, which can become integrated into the genome of the host cell. Thus, RT is a DNA polymerase capable of using both RNA and DNA as a template. RT exists as a heterodimer consisting of a 66-kDa polypeptide (p66) and a 51-kDa polypeptide (p51) subunit. The p66 subunit is often described as existing in a 'right-hand' conformation, and consists of five domains (Fig. 2a) [20, 21]. The palm domain contains the catalytic site for DNA polymerization. The flexible fingers and thumb domains fold over the nucleic acid forming a cavity where the template:primer (the double-stranded nucleic acid intermediate in the conversion of viral RNA to proviral DNA) is positioned for DNA synthesis. A connecting domain links the polymerase active site of RT to the RNaseH domain. The RNaseH domain contains the catalytic active site for ribonuclease H, which functions to degrade the RNA template once it has been used to direct complementary strand DNA synthesis. The p51 subunit is similar to p66 in terms of primary amino acid sequence, except the carboxyl-terminal RNaseH domain

has been cleaved off by the HIV protease. However, p51 adopts a very different secondary and tertiary conformation from p66, and primarily plays a structural role [20]. The catalytic site for DNA polymerization lies in the palm of RT and consists of three conserved aspartic acid residues: D110, D185, and D186 (Fig. 2b). Conformational changes take place upon positioning of the template:primer complex within the catalytic domain so that the 3′-OH of the primer terminus is near the RT polymerase catalytic site and is ready to accept the incoming dNTP. Upon binding of the dNTP, further conformational changes take place bringing the fingers domain toward the palm, which allows a number of finger residues to form molecular interactions with the just positioned dNTP. The formation of this closed complex conformation is the rate-limiting step in reverse transcription and leads to the attack by the 3′-OH of the primer terminus on the α-phosphate of dNTP [22–24]. The end result is the addition of a dNMP to the primer strand DNA and release of a pyrophosphate end product. After incorporation of dNMP, RT returns to an open conformation and is ready to accept the next nucleotide.

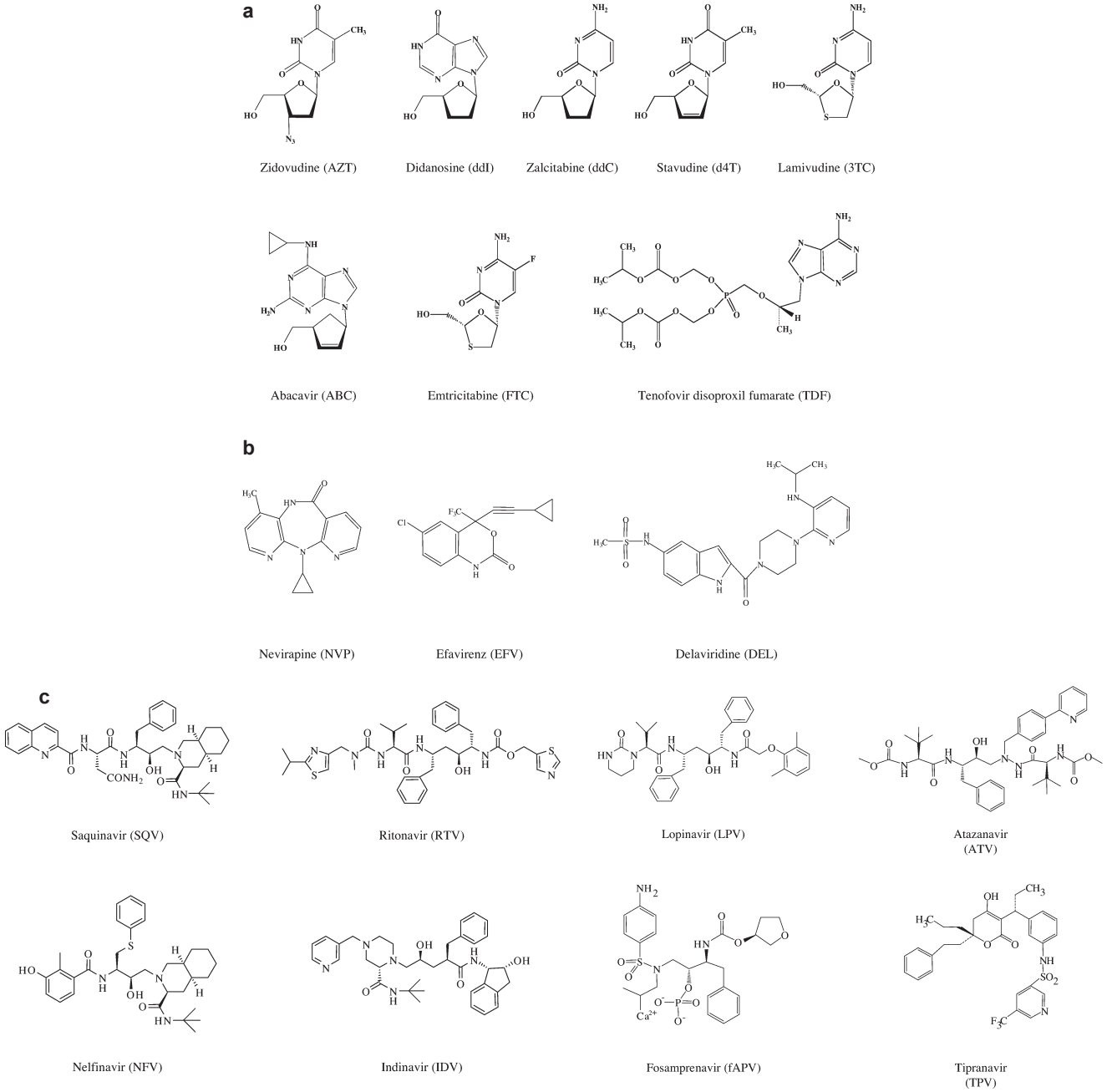


Figure 1. Clinically approved anti-HIV drugs. (*a*) Nucleoside and nucleotide RT inhibitors. (*b*) Non-nucleoside RT inhibitors. (*c*) Protease inhibitors.

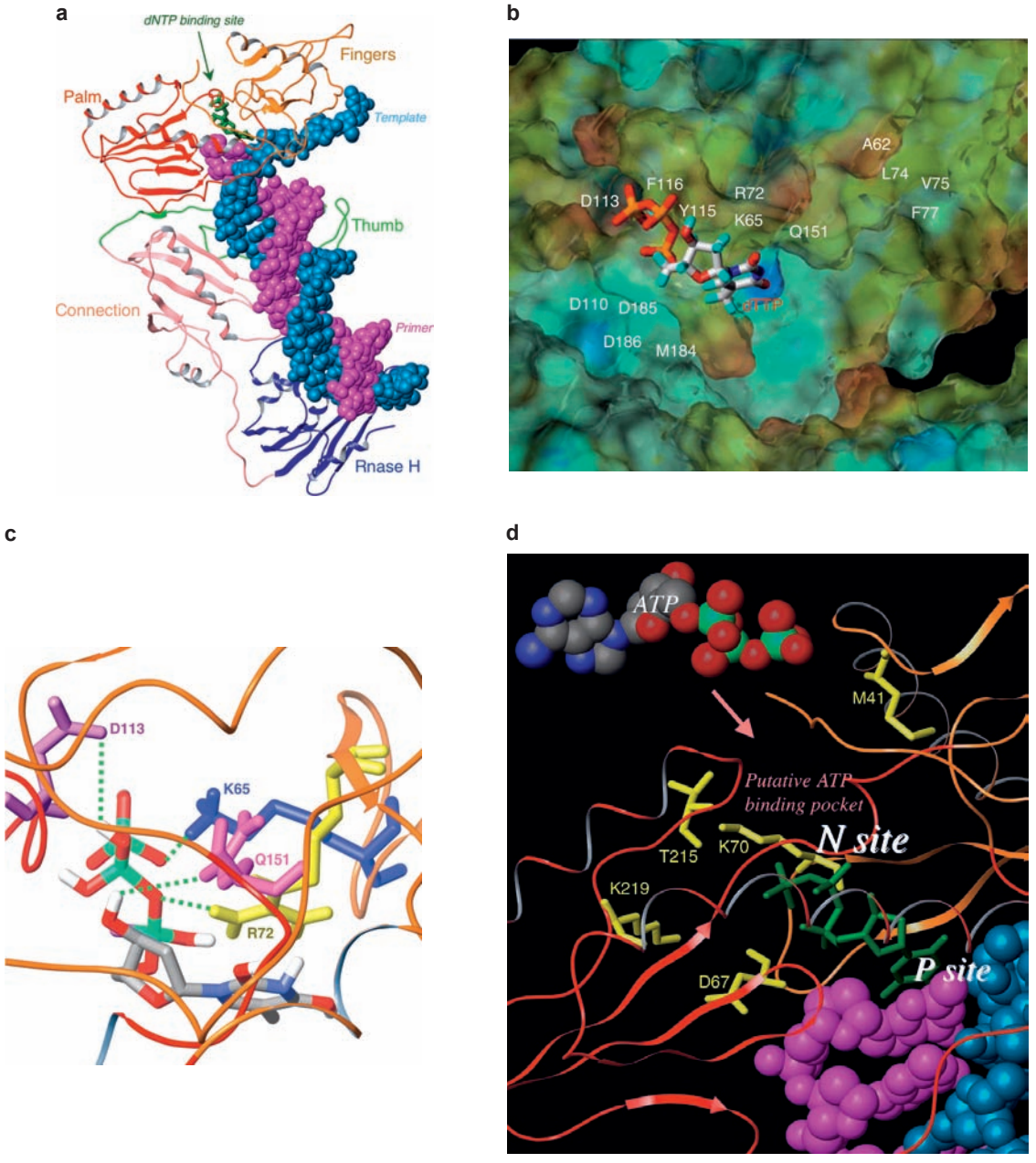


Figure 2. Reverse Transcriptase. (*a*) Molecular model of p66 subunit of RT with template:primer complex and dTTP in the dNTP-binding site (PDB ID: 1RTD). The p55 subunit is not shown. The fingers domain is shown in orange, palm in red, thumb in green, connecting domain in plum, and Rnase H domain in purple. The Figure was generated using Maestro version 7.0 (Schrödinger, LLC, New York). (*b*) Expanded view of the dNTP-binding pocket, demonstrating the Connolly surface around the dNTP-binding site of RT polymerase. The electrostatic potential is mapped onto the surface: the electrostatic potential gradient varies from red, the most positive, to purple, the most negative. D110, D185, and D186 are the conserved catalytic aspartates located in the palm. Amino acid positions K65, L74, M184, and Q151 are associated with primary mutations that cause resistance to nucleoside/nucleotide RT inhibitors (NRTIs) by allowing discrimination between NRTI-TPs and dNTPs. The Figure was generated using Sybyl 7.0 (Tripos). (*c*) The following hydrogen bonds shown between dTTP and RT are important in the positioning in the dNTP-binding pocket: 3′-OH with Q151 (plum), α-phosphate with R72 (yellow), β-phosphate with D113 (bluish purple), and γ-phosphate with K65 (purple). K65 and R72 are in the finger domain and D113 and Q151 are in the palm domain of RT. The Figure was generated using Maestro version 7.0. (*d*) The N ('nucleotide') site, the P ('primer'), and the putative ATP-binding sites are illustrated. The N and P sites represent different positions of the dNTP-binding site before and after translocation, respectively. After a dNTP or NRTI, such as zidovudine (AZT), is incorporated into the growing primer DNA strand at the N site, RT shifts so that the incorporated molecule shifts to the P site (primer strand: magenta spheres; template strand: blue spheres). The N site becomes vacant to accept the next incoming dNTP. Residues associated with thymidine analog mutations (TAMs; M41, D67, K70, T215, K219) surround the putative ATP-binding pocket and are indicated in yellow. The orientation of RT domains with the template:primer is the same as in *a*. The figure was generated using Maestro version 7.0.

Compounds that inhibit HIV RT are categorized as nucleoside reverse transcriptase inhibitors (NRTIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Fig. 1a, b). Historically, NRTIs were the first compounds used clinically to treat HIV infection [25, 26]. These compounds are nucleoside analogs that lack the 3′-OH moiety in their ribose ring, differentiating them from physiological dNTP substrates. Therefore, the mechanism of action for NRTIs includes both competitive inhibition as well as termination of polymerization once the drug is incorporated. NRTI drug resistance was described soon after the introduction of the drugs to medical practice, and our knowledge continues to accumulate [27, 28]. NRTIs must enter cells and become phosphorylated to achieve their active triphosphate form. Tenofovir, a nucleotide RT inhibitor, exists in a monophosphate form and needs only two additional phosphorylation steps. Tenofovir is included in the discussion of NRTIs because of its similar mechanism of action.

Mutations that confer NRTI resistance are scattered among the various domains of RT. Nonetheless, structural analysis indicates that the majority of these mutations occur at amino acid residues involved in positioning of the template:primer complex and dNTP relative to the RT polymerase active site (Fig. 2b and 2c). As a result, these mutations may alter the binding of NRTI-triphosphate (NRTI-TP) to RT in such a manner that allows the enzyme to distinguish (discriminate) the NRTI-TP from the analogous dNTP. A separate group of mutations function to facilitate ATP-mediated excision of an incorporated NRTI (Fig. 2d) [29, 30].

RT resistance due to mutations that distinguish NRTIs from dNTPs

Many NRTI resistance mutations involve changes in amino acids that are responsible for positioning the incoming nucleotide. In a stalled, covalently tethered complex of RT with template:primer containing dTTP in the dNTP-binding site, the 3′-OH of dTTP is oriented into a pocket made up of the side chains of D113, Y115, F116, and Q151, and the peptide backbone of D113 and Y115 (Fig. 2b, c) [23]. The guanidium group of R72 hydrogen bonds with the dNTP α-phosphate, while D113 and K65 donate hydrogen bonds to the dNTP β-phosphate and γ-phosphate, respectively. This differs slightly from a similar structure in which tenofovir is the incoming nucleotide rather than dTTP, where K65 interacts with the α-phosphate rather than the γ-phosphate [31]. Both R72 and K65 are located in the fingers domain.

Q151M is a mutation selected in patients on combination therapy with NRTIs that results in high-level NRTI cross-resistance [32, 33]. Multidrug-resistant HIV strains with Q151M are also found to contain common mutations at amino acid positions A62, V75, F77, and F116 of RT. This group of mutations is referred to as the ‘Q151M complex.’ The loss of the Q151 amide group causes a disruption in the hydrogen bond network necessary for positioning the 3′-OH of the incoming dNTP substrate. Although this may cause relatively subtle differences in the positioning of dNTPs, NRTIs that lack the 3′-OH may be more sensitive to changes in the hydrogen bond network caused by Q151M. This is thought to favor the selective incorporation of dNTP over most NRTIs [34]. In the clinical setting, Q151M appears first, followed by the sequential appearance of mutations A62V, V75I, F77L, and F116Y. V75, F77, as well as Q151, are located within the dNTP-binding pocket close to the ‘template grip.’ This is the region of p66 that is closely associated with dNMPs of the template strand [21, 34]. F116 is also situated close to the dNTP-binding pocket [23]. Together, these mutations appear to allow fine-tuning of RT-containing Q151M, ultimately increasing the level of drug resistance and improving the fitness of this mutant virus [35, 36].

Another primary mutation also located in the region of the incoming dNTP is L74V [37]. L74V confers resistance to NRTIs such as didanosine, zalcitabine, and abacavir [1]. From a molecular standpoint, this mutation may allow discrimination between dNTP and NRTI-TPs by one of two mechanisms. First, L74 contacts the template dNMP that is base-paired to the incoming dNTP [23]. As a result, L74V can affect positioning of the template strand that in turn may affect the discriminatory capacity of RT [38], although the exact mechanism remains unclear. A second mechanism by which L74V may cause NRTI resistance relates to its interaction with Q151 and R72, two amino acids described above that are critical for the formation of the hydrogen bond network necessary to position the incoming dNTP. Thus, L74V may mediate disruption of this hydrogen bond network in a secondary fashion.

K65R is an important mutation found in clinical isolates that confers resistance to several NRTIs including didanosine, zalcitibine, stavudine, abacavir, tenofovir, lamivudine, and emtricitabine [1, 39, 40]. As mentioned above, K65 is located in the fingers domain, and conformational changes initiated by nucleotide binding bring K65 into the proximity of the dNTP-binding site, where it serves to orient the triphosphate moiety of the nucleotide [23, 31]. There are subtle differences in the interaction of K65 with dNTP versus NRTI-TP, and the exact mechanism in which the K65R mutation causes discrimination against a number of NRTI-TPs relative to the natural dNTP substrate is unclear.

M184V and M184I are point mutations that confer high-level resistance to lamivudine and emtricitabine [41, 42]. M184 is located in the highly conserved YMDD motif found in all retroviruses and contributes two of the three catalytic aspartic acid residues of the RT polymerase domain [23, 43]. The β-branched side chains of either va-

line or isoleucine in the M184V or M184I mutants lead to increased contact with the dNTP or NRTI-TP sugar ring. The bulky L-oxathiolane rings of compounds such as lamivudine and emtricitabine make these compounds particularly vulnerable to steric hindrance resulting from these mutations (Fig. 1a). Thus, M184V/I results in high level resistance to lamivudine or emtricitabine [1].

Nucleoside RT resistance due to excision

Zidorudine (AZT) and stavudine, both thymidine analogs, were found to select for a group of mutations that confer high levels of resistance to these drugs and also cross-resistance (albeit at a lower level) to other NRTIs. Referred to as TAMS (thymidine analog mutations) or NAMS (nucleoside analog mutations) in the clinical literature, this group of mutations consists of changes at the following RT amino acid positions: M41L, D67N, K70R, T215Y/F, and K219Q/E/N (Fig. 2d) [27]. Unlike RT mutations that lead to drug discrimination, these mutations do not affect incorporation of AZT-TP [44], but instead lead to increased excision of AZT-monophosphate (AZT-MP) [8, 29, 30]. Excision of either dNMPs or NRTI-MPs can occur, and is mechanistically the reverse event of the normal polymerization reaction. It can be likened to a primitive proofreading function of the enzyme. Due to the increased rate of dNMP excision, NAMs can have a detrimental effect on the catalytic efficiency of RT, and a compensatory increase in processivity of the enzyme with TAMS has been observed to overcome this [29].

Excision can be mediated by either pyrophosphate or ATP, but there are biochemical and structural data to suggest ATP-mediated excision is the primary mechanism *in vivo* [45–47]. NAMs serve to enhance the binding of ATP needed to facilitate excision [46]. In the model proposed by Boyer, an incoming NRTI, such as AZT-TP, can enter the RT catalytic core at the dNTP-binding site (N site), leading to a rotation of the fingers downward (Fig. 2d). It is this closed complex that allows the incorporation of the NRTI-MP into the primer strand of DNA. Incorporation of the NRTI-MP promotes shifting of the enzyme so that the newly incorporated molecule moves to a second position in the active site, the priming site (P site) [48]. The N site is now open and ready to accept the next dNTP or NRTI-TP. ATP-mediated excision of NRTI-MP can occur when the molecule resides in the N site. Once translocation of the incorporated NRTI-MP to the P site occurs, the ATP is no longer in position physically to mediate the excision reaction. However, retro-translocation of the NRTI-MP from the P site back to the N site can occur, placing it in position for potential excision. AZT contains a bulky azido group located at the 3′ position of the ribose ring that is not present on other NRTIs. Thus, the azido group may make AZT more prone to remain anchored in the N site. In addition, once AZT-MP is incorporated into the template strand, and translocation to the P site occurs, the 3′-azido group appears to cause steric constraints that prevent the positioning of a subsequent dNTP into the just vacated N position. This may facilitate retro-translocation of the AZT-MP back to the P site. Either of these mechanisms can lead to the more efficient excision of AZT by RT-containing NAMs, accounting for the higher resistance to AZT mediated by these mutations [46, 47]. A recent paper suggests that the base component of the NRTI is also important in determining whether a drug can be more efficiently excised by RT-containing NAMs [49], and that azido-pyrimidine analogs (such as AZT) are more efficiently excised than azido-purine compounds.

Insertion mutations between positions 69 and 70 of the fingers domain (69-insertions) have been described that result in multidrug-resistant HIV [50]. These mutations are associated with NAMs in clinical isolates [51]. Isolates with 69-insertions and mutations at positions 41 and 215 of RT have an extended range of excisional efficiency, and are capable of excising all NRTI-MPs, not just thymidine analogs such as AZT-MP or stavudine-MP. The mechanism by which this occurs appears to be due to destabilization of the ternary complex consisting of RT, the NRTI-MP terminated primer DNA, and incoming dNTP. The formation of this complex is necessary to initiate conformational closing of the RT active site around the nucleic acid, which must take place before polymerization will occur. After polymerization takes place, a pyrophosphate end product is released followed by translocation. In the wild-type RT, the stability of this complex appears to be greater than what is needed to initiate polymerization and translocation. In RT-containing the 69-insertion mutations, destabilization of the complex leads to a delay in polymerization and translocation. As a result of this delay, NRTI-MP may remain situated in the N site for longer periods, and there is an increased opportunity for ATP-mediated excision to remove the NRTI-MP [52–54].

There has also been evidence to suggest that the activity of RNaseH may also influence drug resistance mediated by NAMs. It should be re-emphasized that RT has two enzymatic activities: DNA polymerization and RNaseH-mediated degradation of RNA template nucleic acid. Although these domains are separated by more than 60 angstroms, their activities are inter-related [55–57]. Degradation of the RNA template plays an important role in template: primer association, which in turn can influence polymerization. How mutations in RNaseH affect current RT inhibitors is unclear. This is largely because our knowledge of clinically relevant drug resistance mutations is based on genotypic analysis of clinical isolates. The current commercial genotyping services only sequence approximately the first 350 amino acids of RT, which excludes a part of the connecting domain and the entire RNaseH do-

main [7, 58, 59]. Recently, a model was proposed in which mutations in RNaseH may increase resistance to NRTIs that are susceptible to NAM-mediated excision, such as AZT [60]. A steady state has been postulated to exist between polymerization of the primer strand and RNaseH-mediated degradation of the RNA template strand. When polymerization and degradation are at equilibrium, the primer and template remain annealed together allowing polymerization to continue. Chain terminators such as NRTIs decrease the rate of polymerization relative to degradation. The relative increase in degradation of the RNA template promotes template:primer complex disassociation leading to termination of DNA synthesis. Mutations in RNaseH may decrease the efficiency of degradation and may allow the template:primer to remain annealed for longer periods, even if polymerization is slowed by an NRTI. This should provide more time for RT with NAMs to excise compounds like AZT, thus decreasing the effectiveness of such chain terminators. In support of this, the RNaseH mutations D549N or H539N have been demonstrated to result in AZT resistance comparable to or greater than an HIV clone containing four NAMS (D67N, K70R, T215Y, K219Q). A clone containing both D549N and the NAMs together reportedly has synergistic resistance to AZT. Drugs that are not removed via the excision mechanism such as lamivudine or efavirenz (an NNRTI) were not affected by the RNaseH mutations studied. The clinical relevance of these RNaseH mutations remains to be demonstrated. Nonetheless, increasing our knowledge of mutations in the RNaseH domain is important not only because of the role they may play in resistance to current RT inhibitors, but also because of the current development of novel RNaseH inhibitors for treatment of HIV infection [61–63].

Designing compounds with activity against NRTI-resistant HIV

RT drug resistance mutations that prevent NRTI binding due to steric conflict have a more pronounced effect on NRTIs with more rigid structures. An example of this is the effect of M184V/I on drugs like lamivudine or emtricitibine, which contain an L-oxathiolane ring. Thus, NRTIs with chemically 'flexible' structures may be more potent against these mutants compared to their more rigid counterparts [64]. For example, tenofovir lacks a deoxyribose ring that is characteristic of natural dNTPs as well as other NRTIs (Fig. 1a). Clinical experience with tenofovir indicates that there is a higher barrier to resistance compared with other NRTIs [65, 66]. The lack of 'handles,' molecular differences between tenofovir and other dNTPs, may make it difficult for RT to discriminate between tenofovir and the natural nucleotide substrate [31]. In addition, the crystal structure of tenofovir in the P site indicates that it is capable of existing in two distinct conformations. This may serve to prevent retrotranslocation to the N site and make tenofovir less prone to resistance due to the NAM mediated excision mechanism. However, this concept of developing flexible NRTIs has only been exploited to a certain degree. Elimination or alteration of the polar interactions that stabilize NRTIs may have a negative effect on drug binding. As will be discussed, this is in contrast to NNRTIs or protease inhibitors, where modifications that affect the hydrophobic interactions between drugs and enzyme is more feasible, as alterations of hydrophobic interactions are not as detrimental to drug binding as a whole.

Alternatively, it may be possible to design drugs that maintain chemical components important for proper positioning of the NRTI in the dNTP-binding pocket. For example, 4′-ethynyl deoxynucleoside (4′-EdN) analogs have been developed which maintain the 3′-OH group and retain similarity to dNTPs in this regard (Fig. 3) [67–69]. The absence of the 3′-OH allows NRTIs to function as chain terminators, but also allows RT to discriminate between these drugs and dNTP. Despite the presence of the 3′-OH moiety, 4′-EdN analogs still appear to function as chain terminators. This may be a result of the R group (4′-ethynyl moiety in this case) attached to the 4′-carbon of the ribose ring exerting steric hindrance around the 3′-OH. As a result, efficient interactions between the 3′-OH and the α-phosphate of the incoming dNTP are prevented. The presence of the 3′-OH may make 4′-EdN congeners less susceptible to mutations that alter the associated hydrogen bond network, as described previously. Indeed, these compounds exert potent activity against HIV containing L74V or Q151M complex mutations. 4′-EdN congeners also reportedly maintain activity against HIV with M41L and T215Y, which impart resistance mediated by the NRTI excision mechanism [68]. However, the M184V/I mutation does appear to bring about steric conflict with some 4′-ethynyl nucleoside analogs, as it does with lamivudine or emtricitabine. Despite this, certain 4′-EdN congeners, such as 4′-EdC, 4′-EdA, 4′-EDAP (diaminopurine), or 4′-EdG, maintain activity against HIV with M184V [68]. Because of the similarity to normal dNTP due to

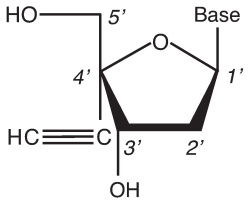


Figure 3. Structure of generic 4′-ethynyl dNTP (4′-EdN). Unlike currently approved NRTIs, these compounds contain the 3′-OH moiety. The 4′-ethynyl is thought to create steric hindrance that prevents the 3′-OH from being used effectively for DNA chain elongation during reverse transcription.

the presence of the 3′-OH, toxicity due to incorporation into cellular or mitochondrial DNA is a concern, but several derivatives reportedly maintain acceptable toxicity profiles.

Designing NRTI inhibitors with activity against HIV with excision-mediated drug resistance (NAMs) is also in progress [70, 71]. One approach is to capitalize on the concept that excision of chain-terminating NRTIs can only take place when the inhibitor lies on the N site of RT [70]. Once translocation of the NRTI-MP to the P site takes place, and a new dNTP enters the just vacated N site, ATP-mediated excision of the NRTI-MP is no longer possible. Normal dNTPs adopt inter-converting forms, described as North and South conformations, but the dNTP must be in the North conformation to be incorporated. However, as chain elongation continues and the dNMP translocates approximately six to seven nucleotides from the polymerase active site, transition to the South conformation is necessary in order to navigate a bend in the DNA imparted by the thumb domain of RT. NRTIs based on a bicyclohexane scaffold were synthesized that are locked in the North conformation [70]. These compounds are adequately incorporated into the primer DNA, and chain elongation occurs since these compounds contain a 3′-OH. However, elongation stalls when the inhibitors fail to transit past the thumb domain. Because this blockade of chain elongation occurs after the inhibitor passes well beyond the N site, excision of the drug by NAM-containing RT is no longer possible. In cell-based assays, these initial compounds had good activity against both HIV with NAMs and wild-type HIV. Unfortunately, compounds conformationally locked in the North position were inefficiently phosphorylated by cellular kinases, limiting their potential as anti-HIV agents [72]. However, dNTPs with 4′-methyl or 4′-ethyl groups similar to those described previously [68] also appear to function as delayed chain terminators and are being further investigated [72].

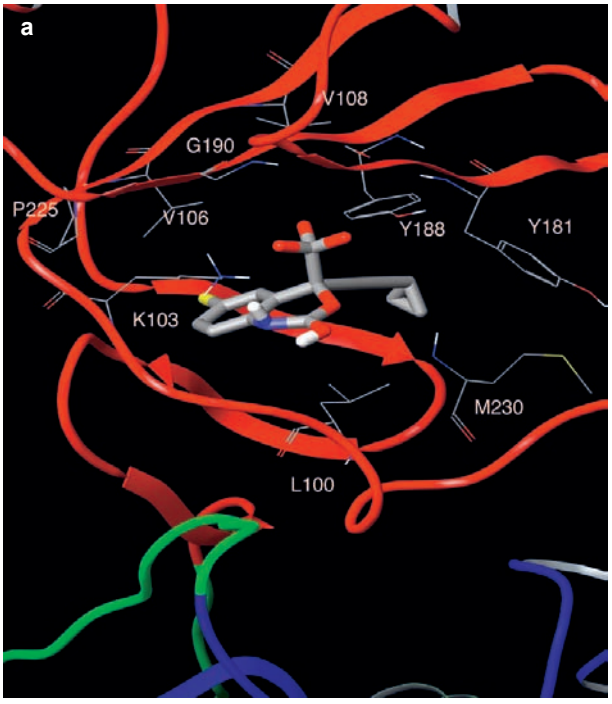
A second proposed approach to combat excision-mediated HIV drug resistance involves the synthesis of compounds based on dinucleoside tetraphosphates [71]. ATP-mediated excision of NRTIs occurs when the γ-phosphate of ATP is oriented in such a way that it can attack the phosphodiester bond between the last two incorporated nucleoside-MPs of the primer DNA (Fig. 2d). In a NAM-containing RT, if the last incorporated molecule is an NRTI such as AZT-MP, it can be excised efficiently by ATP, resulting in a dinucleoside tetraphosphate (3′-AZT-p-p-p-p-adenosine) end product. RT can catalyze this reaction in both the forward and reverse directions such that the dinucleoside tetraphosphate can serve as a donor of dNTP to catalyze RT-mediated DNA polymerization. If an inhibitor can be designed based on a dinucleoside tetraphosphate that is resistant to hydrolysis and contains at least one chain-terminating moiety, it could prevent polymerization while remaining resistant to excision by preventing ATP from entering the ATP-binding site of RT.

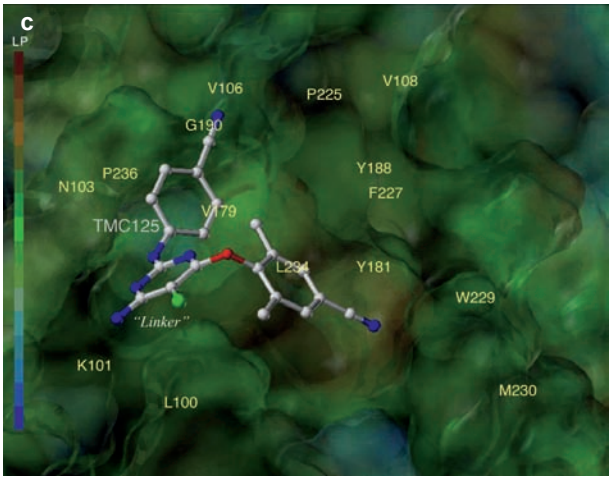
NNRTI resistance

The second class of RT inhibitors in clinical use is the NNRTI class (Fig. 1b) consisting of chemically diverse compounds that bind to a hydrophobic pocket (NNIBP, or non-nucleoside inhibitor-binding pocket) located approximately 10 angstroms away from the RT polymerase active site (Fig. 4a) [73]. NNRTIs do not prevent template:primer or dNTP-binding to RT, but cause misalignment of the template:primer in relation to the catalytic site so that the incoming dNTP cannot be incorporated [21, 74–76]. The first-generation NNRTIs were discovered by screening compounds that inhibited RT activity. Delavirdine, nevirapine, and efavirenz have adequate to strong potency against wild-type HIV strains. However, drug resistance can quickly emerge [73, 77, 78] as a result of mutations at one or more locations in the NNIBP. Common NNRTI resistance mutations include L100I, K103N, V106A, Y181C, Y188L, and G190A, which contribute to drug resistance through different mechanisms (Table 1, Fig. 4a). The structures of early NNRTIs were described as existing in a 'butterfly' conformation, in which two chemical side group 'wings' are attached to a central linker/backbone moiety in a relatively rigid manner [79]. Resistance to these compounds can occur easily by amino acid substitutions that generate steric hindrance and prevent binding of the compounds to the NNIBP. For example, steric hindrance is created when leucine is substituted for the β-branched isoleucine in the L100I mutation, or if a methyl group is introduced in a G190A mutant [80, 81]. Another mechanism of NNRTI resistance involves mutations leading to the loss of aromatic amino acids, such as Y181C and Y188L. This results in the loss of hydrophobic interactions necessary to stabilize the binding of NNRTIs to the NNIBP. A third mechanism of NNRTI resistance involves K103N. This mutation has little effect on inhibitor binding [74, 82]. Instead, the asparagine side chain of the K103N mutant forms a hydrogen bond with the phenoxyl group of tyrosine located at position Y188 of the NNIBP as it exists in the unbound state. The result of this interaction is the formation of a molecular 'gate' that prevents access of drugs in the NNRTI class to the NNIBP. Thus, K103N results in cross-resistance against all three clinically approved NNRTIs.

Development of NNRTIs with activity against NNRTI-resistant HIV

Using knowledge based on the various crystal structures and molecular models of RT, key chemical components





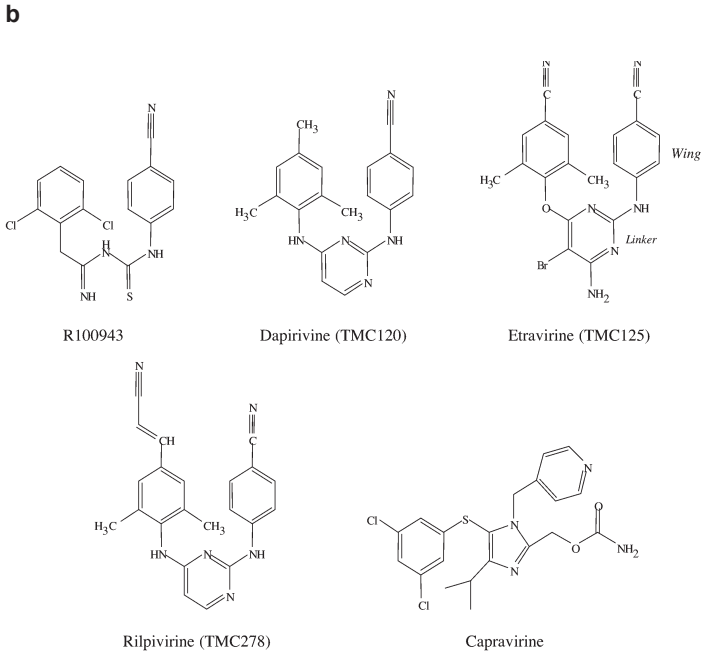


Figure 4. (*a*) The structure of efavirenz crystallized in the NNIBP of RT (PDB ID: 1FK9). Residues associated with NNRTI resistance are indicated. The Figure was generated using Maestro version 7.0. (*b*) Structures of selected experimental NNRTIs. The 'wing' and 'linker' chemical motifs are indicated on etravirine. Chemical modifications of R100943 (imidoylthiourea, ITU) led to the development of the newer-generation NNRTIs that maintain potency against NNRTI-resistant HIV: dapivirine (TMC120, R147681), etravirine (TMC125, R165335) and rilpivirine (R278474). Etravirine and the unrelated NNRTI capravirine (S-1153) are currently in advanced clinical trials. (*c*) The binding of TMC125 to the NNIBP of drug-resistant mutant RT with the K103N mutation is demonstrated (PDB code: 1SV5). The lipophilic nature of the cavity is mapped onto the Connolly surface (brown is most lipophilic and blue is most hydrophilic). The location of alpha carbons of amino acid residues implicated in NNRTI drug resistance is shown. The pyrimidine 'linker' of TMC125 interacts with the long side chain of K103N. When this occurs, K103N can no longer interact with Y188, a process that can lead to the exclusion of the currently clinically available NNRTIs. The figure was generated using Sybyl 7.0 (Tripos).

of NNRTIs have been described [83]. Subsequent modifications to these components led to NNRTIs with improved potency against NNRTI-resistant HIV variants. The first-generation NNRTI, TIBO (tetra-hydro-imidazo-benzo-diazepinone), and a subsequent compound based on TIBO, loviride (α-anilinophenylacetamide, or α-APA), represent two of the initial drugs that were developed using this approach. Chemical modification of α-APA led to the imidoylthiourea (ITU) compounds [84], of which R100943 is representative, that were found to have increased flexibility relative to the α-APA precursors (Fig. 4b). The binding of R100943 to the NNIBP was described as a 'horseshoe' or 'U' conformation to distinguish it from the more rigid binding of the 'butterfly' compounds such as α-APA. Made by modifying the wing portions of the 'butterfly' compounds, R100943 had improved activity against wild-type HIV and certain NNRTI resistant mutants. For example, the methyl group of the side chain of a G190A mutant RT produces steric hindrance that prevents the 'butterfly' NNRTIs from binding to NNIBP. However, the shape of the horseshoe R100943 allowed it to fit into the NNIBP without significantly being affected by G190A. In addition, the torsional flexibility between the wings and linker region of R100943 allows repositioning of R100943 in the NNIBP. This was possible even in HIV containing the drug resistance mutations Y181C and Y188L. Despite these improvements, R100943 did not achieve adequate potency for consideration for clinical trials. However, while attempting to synthesize derivatives of R100943, an unexpected ring closure of the linker portion of R100943 led to a new generation of NNRTIs, the diaryltriazine (DATA) class [85].

This new aromatic triazine ring still allowed flexibility of the wings, but removed redundant torsional flexibility, leading to the development of more entropically stable compounds. One such DATA compound, R120393, was found to bind NNIBP in alternative conformations, filling the elastic NNIBP to a greater extent than previous-generation NNRTIs. Thus, the ability to have multiple modes of binding allows these compounds to maintain activity even in the presence of drug-resistant mutations. Based on this concept, the triazine ring in the linker of the DATA compounds was replaced with a pyrimidine to form the diarylpyrimidine (DAPY) class of NNRTIs. Like the DATA compounds, DAPY NNRTIs could bind NNIBP not only in the horseshoe orientation, but in other configurations as well. This feature was a key element in explaining why DAPY prototypes such as dapivirine (TMC120, R147681) demonstrate increased potency compared with predecessors against NNRTI-resistant strains of HIV (Fig. 4b). A newer DAPY, etravirine (TMC125, R165335), has significant potency against HIV with K103N (Fig. 4b, c). As described above, K103N caused cross-resistance to NNRTIs by interacting with Y188, preventing access of NNRTIs to the NNIBP [86]. The central pyrimidine linker of etravirine may form a direct interaction with the asparagine at position 103 of K103N mutant RT. This may prevent the K103N interaction with Y188 that results in NNRTI resistance.

Further analysis by crystallography and molecular modeling supports the role of conformational flexibility to compensate for the presence of NNRTI resistance mutations. This can take place in the form of 'wiggling' (torsional rotation of the various chemical subgroups of the drug relative to each other), or 'jiggling' (plasticity of the interactions between the drug and its protein-binding site) [86]. Poor absorption prevents dapivirine from being used orally, but it continues to be studied as a topical antimicrobicide. Etravirine is currently in advanced clinical trials [87, 88]. More recently, a new DAPY compound, rilpivirine (R278474), has been synthesized seeking to improve on its dapivirine predecessor (Fig. 4b) [83]. Rilpivirine contains a cyanovinyl group in one of its wing moieties that strengthens an interaction with a conserved tryptophan at position W229. W229 is one of the few amino acids in the NNIBP that results in significant loss of RT activity if mutated [89]. The cyanovinyl modification exploiting the interaction with W229, and the inherent flexibility of DAPY compounds, are likely explanations as to why rilpivirine is 10–20 times more potent against NNRTI-resistant mutants compared with efavirenz, currently the most effective NNRTI.

Another NNRTI in advanced clinical trials that is structurally unrelated to the compounds discussed above is capravirine (S-1153, AG-1549; Fig. 4b) [90–92]. This compound has potent activity against HIV with single resistant mutations such as L100I, Y181C, and V106A. Like DATA and DAPY NNRTIs, the increased flexibility of the capravirine structure is hypothesized to contribute to some of its activity against drug-resistant HIV. In addition, a phenyl group in prototype compounds was substituted with 3,5-dichlorophenyl to generate capravirine. This 3,5-dichlorophenyl non-aromatic moiety appears to allow capravirine to achieve extensive contact with Y181, Y188, and the conserved W229, stabilizing the binding of capravirine even in the presence of mutations Y181C or Y188L. Similar to etravirine, capravirine has activity against HIV harboring K103N as well. Modeling indicates that a hydrogen bond might form between an imidazole nitrogen of capravirine and the asparagine side chain of the K103N mutation, preventing the K103N and Y188 interaction that mediates NNRTI cross-resistance. Finally, capravirine is unique among NNRTIs in that it can form a hydrogen bond network with the main chain backbone of RT. This interaction with the main chain is less affected by the side chain mutations that confer drug resistance against current NNRTIs.

Protease inhibitor resistance

The HIV protease is an aspartyl protease that cleaves the HIV Gag and Gag-Pol polyproteins to generate structural proteins and enzymes of the virus. This processing occurs late in the HIV life cycle during assembly and release from the infected cell, and is an essential step for the formation of mature virus particles. The HIV protease consists of two identical 99-amino-acid subunits, and has an active site that lies at the dimer interface, with each monomer contributing a single catalytic aspartic acid residue (D25 and D25′). The substrate-binding cleft that surrounds the active site contains both hydrophobic and hydrophilic elements (Fig. 5a). Each monomer of the protease also contributes amino acids (positions 45–56) to form a flap that extends over the substrate-binding cleft. The flap must be flexible enough to allow entry and exit of the polypeptide substrates. For each substrate, three to four amino acids located on either side of the peptide bond cleavage site are utilized for binding to the substrate cavity of protease. Protease must cleave the immature HIV polyprotein precursors, Gag and Gag-Pol, in at least nine different cleavage sites for maturation to occur [93–96]. There is very little homology in the primary amino acid sequences of each of these cleavage sites. Instead, substrate specificity appears to be dictated by the secondary structure that remains conserved in each of the different cleavage sites. The amino acid residues immediately upstream of the cleavage site toward the amino terminus (referred to as 'unprimed' amino acids, P1, P2, P3, etc., with each number indicating the amino acid position relative to scissile bond/cleavage site) form a conserved toroidal shape [96, 97]. The substrate amino acids imme-

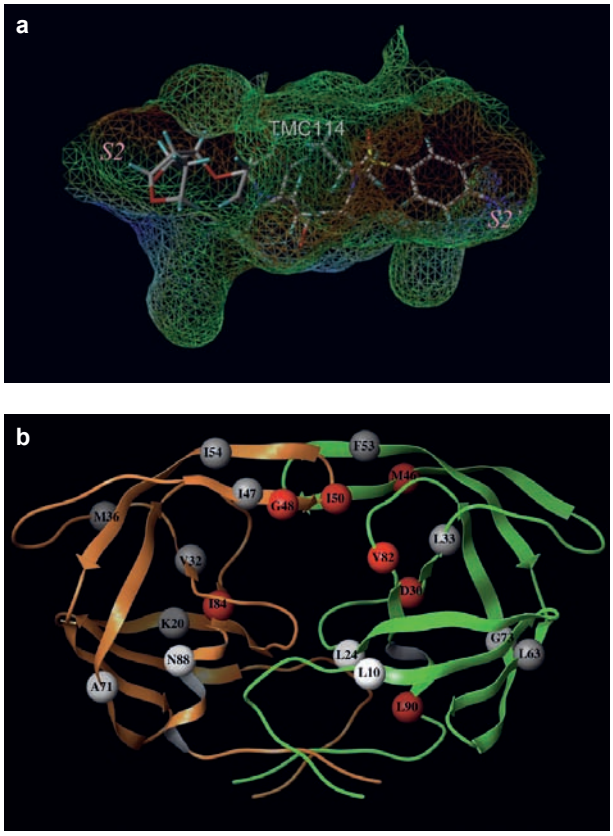


Figure 5. HIV protease. (*a*) The hydrophobic cavity within protease with TMC114 docked (PDB ID 1S6G). The brown and green regions are lipophilic while the blue regions are hydrophilic (determined using MOLCAD). The S2 and S2′ subsites are indicated. The figure was generated using Sybyl 7.0 (Tripos). (*b*) Structure of protease homodimer with positions of amino acid residues associated with clinical resistance to current protease inhibitors. Primary and secondary mutations are indicated with red and white spheres, respectively. The protease monomers are shown as green and orange ribbons. Mutations are shown on only one monomer for clarity. The Figure was generated using Maestro version 7.0.

diately downstream of the cleavage site toward the carboxyl terminus (the 'primed' amino acids P1′, P2′, P3′, etc.) lie in an extended conformation and provide space for water molecules necessary for substrate recognition and/or product release [96]. Seven amino acids, P3–P4′, of the natural peptide substrates of HIV protease form non-covalent interactions with the active site of the enzyme. The corresponding subsites where each of these amino acids lie within the protease cavity are designated S3–S4′ [98].

There are currently eight protease inhibitors (PIs) approved for the treatment of HIV infection (Fig. 1c). All are competitive inhibitors that bind to the protease active site. Drug resistance mutations that surround the active site usually interfere with PI binding and are referred to as primary mutations (Fig. 5b). Nonetheless, because of their location near the substrate-binding cleft, these mutations can affect processing of the natural substrates as well and thus often confer a fitness cost to HIV. Primary mutations that interfere with PI binding, located distant to the active site, have also been documented (i.e. L90M). However, most mutations that are not within the protease substrate-binding cavity do not affect inhibitor binding directly, but serve to compensate for deleterious effects on enzymatic activity caused by primary mutations. These compensatory mutations are referred to as secondary mutations. Additional mutations in the HIV genome have been found that do not lie within the protease enzyme, but are, instead, located near the cleavage sites of Gag substrates. These mutations also appear to be secondary mutations that compensate for the reduced catalytic efficiency caused by primary protease mutations [99–103]. Thus, high-level drug resistance to PIs requires the stepwise accumulation of multiple primary and secondary mutations to generate a protease capable of discriminating inhibitor from natural substrate yet able to maintain adequate catalytic efficiency needed for virus replication.

Protease mutations that allow discrimination of PIs from natural peptide substrates

Each PI tends to select for particular primary mutation(s) ('signature' mutations) and subsequent secondary mutations both *in vitro* and *in vivo* [12]. Nonetheless, many primary mutations are capable of causing cross-resistance to multiple PIs even though they tend only to emerge during therapy with specific inhibitors [104–107]. All PIs are shorter than the natural substrates, and contain hydrophobic moieties that interact with S2-S2′ subsites (Fig. 5a). Thus, although PIs may be chemically unique from each other, they occupy a similar space within the protease-binding cavity, which explains how individual mutations may cause PI cross-resistance.

Structural analysis of primary mutations has formed the basis for our current understanding of PI resistance at the molecular level. One such mutation is V82A, originally described after selection with ritonavir or indinavir [108–110]. This mutation is capable of conferring HIV resistance to a number of PIs, particularly early generation compounds. Crystal structures of protease containing V82A complexed with either natural substrates or a PI were compared (these enzymes also contained D25N, a mutation that inactivated the enzyme to prevent cleavage of the substrate but did not appear to affect hydrogen bonding between protease and ligand) [111]. V82A results in significant changes in the crystal structures of protease complexed to PI, including changes in the flap position and subsequent disruption of hydrogen bonding, as well as the loss of van der Waals interactions between mutant protease and PI. On the other hand, crystal structures between natural substrate peptides complexed to

either wild-type protease or V82A mutant protease have not demonstrated significant changes. Molecular interactions between protease and the longer natural substrates consist mainly of extensive backbone-backbone hydrogen bonds as well as more extensive van der Waals interactions [96, 97, 111]. This suggests that the side chain substitution V82A has little effect on substrate-binding, but has a much greater detrimental effect on PI binding. Others have shown that multidrug-resistant protease with mutations at multiple positions (amino acids L10, M36, M46, I54, L63, A71, V82, I84, and L90) has an expanded active-site cavity [112]. Again, the binding of PIs to this multidrug-resistant protease was noticeably different than binding to wild-type protease. Although the crystal structure of this multidrug-resistant protease with natural substrates was not assessed, this work provides further insight into the structural effects of multiple protease resistance mutations.

To further understand the difference between substrate-binding and PI binding to protease, an analysis of the structures of eight different inhibitors complexed to protease has been conducted [113]. King and colleagues demonstrated that despite the chemical differences of the drugs, all compounds occupied a similar volume within the active-site cavity that is termed the 'inhibitor envelope'. If the inhibitor envelope was compared with the 'substrate envelope,' the space within the protease that is occupied by a natural substrate, the inhibitors protrude from the substrate envelope in very distinct locations. At these positions, PIs may have van der Waals interactions with amino acid positions such as G48, I50, V82, and I84. Mutations at these residues are known to result in PI primary resistance (Table 1, Fig. 5b), and therefore these mutations likely disrupt PI and protease molecular associations. On the other hand, these same mutations have little effect on natural substrates that do not make molecular interactions at these amino acid positions [113].

Development of PIs with activity against drug-resistant HIV

Of the currently approved PIs, amprenavir is the only drug that fits predominantly within the substrate envelope [114]. The mutation profile for amprenavir is also different compared with that for other PIs, providing more evidence that PIs with greater resemblance to natural substrates will be less affected by primary mutations selected by first-generation PIs. However, mimicking the shape of protease substrates is only one element in designing effective PIs. Another key element that differentiates substrate-binding and PI binding is the significant amount of hydrogen bonding between backbone atoms of substrate and protease that is mostly lacking in protease/PI binding. Because mutations of backbone atoms of protease cannot occur, disruption of these bonds is more difficult compared to that of the hydrogen bonds many PIs form between amino acid side chains, which can be affected by substitution mutations. Further development of analogs of amprenavir has successfully exploited these elements and has resulted in certain PIs with significant activity against multidrug-resistant HIV.

Amprenavir contains a tetrahydrofuranyl (THF) urethane moiety that interacts with the S2 region of the protease active site upon drug binding. Replacing THF of amprenavir with a bis-THF component led to the synthesis of two PIs, TMC126 and TMC114 (Fig. 6a) [115–117]. TMC126 differs slightly from amprenavir due to a replacement of 4-aminobenzenesulfonamide with 4-methoxybenezenesulfonamide which interacts with protease at the S2′ subsite, while TMC114 is identical to amprenavir with the exception of the bis-THF. Based on analysis of TMC114, the larger bis-THF rings of TMC126 and TMC114 protrude slightly more from the substrate envelope compared with the THF ring of amprenavir [114]. Despite this, both drugs have increased activity against both wild-type as well as clinical isolates containing multi-PI-resistant mutations [118–120]. At least one explanation for this is the improved hydrogen bond stability with the protease backbone conferred by the bis-THF ring (Fig. 6b) [114, 120, 121]. Although the THF ring of amprenavir is able to form hydrogen bonds with the backbone amides and carboxylate oxygen of D30, bis-THF has an additional hydrogen bond with the main chain atoms of D29 in the S2 subsite of the protease. This extra hydrogen bond may account for a tenfold increase in activity of TMC126 and TMC114 against a panel of clinical isolates with various combinations of protease resistance mutations [120, 122]. Attempts to design compounds capable of further exploiting these critical interactions with the main-chains of D29 and D30 in the S2 subsite are currently underway [123]. On the opposite side of these same inhibitor molecules, it has been reported that the P2′ substituents such as the 4-aminobenzenesulfonamide found on amprenavir and TMC114, or the 4-methoxybenezenesulfonamide of TMC126 also introduce hydrogen bonding with the carboxyl backbone of D30′ in the S2′ subsite (Fig. 6b) [122]. Compounds designed to optimize this interaction have also demonstrated good *in vitro* activity against PI-resistant HIV variants [124]. Maximization of hydrogen bond interactions between the protease backbone and TMC126 or TMC114 results in highly favorable enthalpic contributions that drive inhibitor binding. This differs from first generation PIs (i.e. nelfinavir, saquinavir, and indinavir) that have unfavorable enthalpic interactions with protease. Binding of these PIs to protease was entropically driven as a result of the burial of hydrophobic residues of these compounds. Thus, mimicking the backbone hydrogen bonding of natural substrates in at least two separate subsites of protease has yielded more thermodynamically

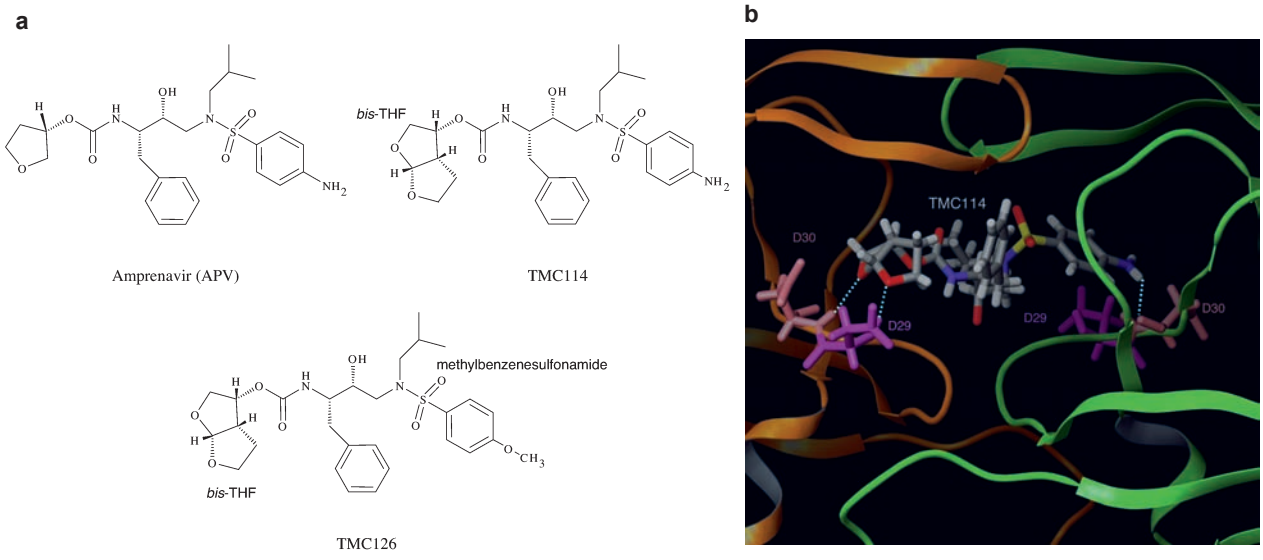


Figure 6. (*a*) Comparative structures of amprenavir, TMC126, and TMC114. The location of the bis-tetrahydrofuranyl (bis-THF) ring and 4-methoxybenezenesulfonamide (4-MBS) of TMC126 are indicated. Designing new PIs with different chemical subgroups at these positions may result in compounds with improved hydrogen bond interactions with protease main chain atoms. (*b*) A model showing the hydrogen bond interaction of TMC114 with D29 (purple) and D30 (pink) in the S2 subsite, and D30′ in the S2′ subsite. The Figure was generated using Maestro version 7.0.

adaptable PIs capable of overcoming protease resistance conferred by amino acid substitutions [114, 125].

As mentioned above, amprenavir selects for a unique pattern of protease resistance mutations compared with first-generation PIs and this holds true for the structurally similar TMC126 and TMC114. TMC126 resistance appears to be mediated by a novel mutation, A28S, along with subsequent acquisition of I50V [118]. Although I50V has been demonstrated to confer primary resistance on amprenavir in clinical isolates, A28S has not been described yet as a common protease resistance mutation, likely because of the effect this mutation has on the catalytic efficiency of protease [118, 126]. Computational modeling analysis does not indicate TMC126 to have interactions with either the backbone or side group of A28, which suggests that the reduction in potency with TMC126 is due to either steric hindrance caused by the larger serine of A28S, or possibly due to unfavorable solvation energy effects during binding [118]. Although the pharmacokinetic properties of TMC126 were not suitable for further clinical development, the related compound TMC114 has significant activity against multi-(PI)-resistant HIV from clinical isolates and favorable pharmacokinetics; TMC114 is currently in advanced clinical trials [119, 120, 127]. Despite the chemical similarities of TMC126 and TMC114, A28S has yet to be described after TMC114 selection [Y. Koh and H. Mitsuya, unpublished data]. The reasons for this are unclear at this point. Instead, *in vitro* resistance with strains harboring R41T and K70E has been observed [119]. Isolates harboring these two mutations were found to have 8- to 10-fold resistance to TMC114, 20-fold resistance to saquinavir, and 6-fold resistance to lopinavir. Otherwise, resistance remained less than 4-fold for all other first-generation PIs. The molecular mechanisms that allow R41T and K70E to confer TMC114 resistance is also currently unknown, as site-directed mutants carrying one or both of these mutations show no reduction in sensitivity to any PI tested [119]. Nonetheless, there appears to be a higher genetic barrier to the emergence of resistance to both TMC126 and TMC114, and both drugs have been shown to maintain potent antiviral activity against multi-PI-resistant strains, suggesting that their unique interactions with HIV protease can provide the framework for developing subsequent generations of PIs. Of note is that a novel bis-THF-containing PI, brecanavir (GW640385), is in pre-clinical trials and appears to have activity against both wild-type and drug-resistant HIV [128]. *In vitro* selection of HIV with brecanavir resulted in the emergence of the novel A28S mutation seen initially with TMC126 [129].

Non-peptidic PIs

Finally, parallel efforts have been made to identify non-peptidic PIs (NPPIs). The recent approval of tipranavir for clinical use in drug-experienced patients has demonstrated the success of structure-based drug design in the development of novel compounds (Fig. 1c). All previously clinically approved PIs were classified as peptidomimetics, due to the fact that they share structural similarity to the tetrahedral intermediate formed during hydrolytic cleavage of a peptide bond of the natural substrate [130]. On the other hand, tipranavir was developed

from a class of compounds known as dihydropyrones that are structurally similar to coumadin and were found to inhibit protease [131]. Traditional peptidomimetics all utilize a ubiquitous water molecule within the protease activity site in order to form hydrogen bonds with the flap domain of the enzyme. A key characteristic of tipranavir and other NPPIs in development is the absence of this water molecule, as seen in crystal structures of NPPIs with protease. Instead, NPPIs contain a suitable chemical moiety that directly forms hydrogen bonds to the flap region without the need for water molecules. This is hypothesized to allow more entropically favorable interactions for the binding of these inhibitors to protease [131].

The unique binding motif and structure of tipranavir has been thought to provide increased flexibility that would allow it to adjust to amino acid changes within the active site [132, 133]. Indeed, tipranavir exerts antiviral activity against a wide range of HIV variants resistant to multiple PIs [132, 134–136]. Nonetheless, resistance to tipranavir can occur, although the acquisition of up to ten mutations is required to achieve high levels of resistance [137]. The mutations L33F and I84V appear to be key substitutions responsible for the development of tipranavir resistance. L33F is a secondary mutation not present in the active site of protease and does not appear to affect tipranavir binding to protease. This mutation has been suggested to protect the protease from autocatalysis [138]. I84V appears to diminish tipranavir binding directly by altering hydrophobic interactions between the drug and this amino acid residue [139]. Despite this, the potent activity of tipranavir against multi-PI-resistant HIV appears to stem from the hydrogen bond network it forms with the most invariant region of the protease active site that includes the catalytic D25, as well as the backbone residues of D29, D30, G48, and I50 [139, 140]. Further development and analysis of NPPIs will continue to refine our knowledge of the molecular mechanisms of resistance and provide us with novel insight for the prevention and treatment of drug-resistant HIV.

Conclusion

Novel anti-HIV agents that target different steps in the HIV replication cycle are currently in pre-clinical trials and will undoubtedly improve our ability to manage HIV infection. However, as has been the case with RT and protease, the development of drug resistance will likely limit the effectiveness of these drugs as well. Thus, a key element in future drug design strategies will be to understand how drug resistance mutations affect the interaction of the drug with its target, and to then develop compounds with the adaptability to inhibit these variants along with wild-type HIV. New-generation RT and protease inhibitors have already shown promise in accomplishing this task, by utilizing knowledge of the molecular, biochemical, structural, and thermodynamic nature of drug resistance. This should serve as a model in the design of more effective anti-HIV therapeutics.

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