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Highly resistant HIV-1 proteases and strategies for their inhibition

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Abstract

The virally encoded protease is an important drug target for AIDS therapy. Despite the potency of the current drugs, infections with resistant viral strains limit the long-term effectiveness of therapy. Highly resistant variants of HIV protease from clinical isolates have different combinations of about 20 mutations and several orders of magnitude worse binding affinity for clinical inhibitors. Strategies are being explored to inhibit these highly resistant mutants. The existing inhibitors can be modified by introducing groups with the potential to form new interactions with conserved protease residues, and the flexible flaps. Alternative strategies are discussed, including designing inhibitors to bind to the open conformation of the protease dimer, and inhibition of the protease-catalyzed processing of the Gag-Pol precursor.

Successful treatment of the HIV/AIDS epidemic is due to a combination of medical, scientific and pharmaceutical efforts in the last three decades [1]. *In lieu* of an effective vaccine, highly active antiretroviral therapy (HAART) with a cocktail of different drugs remains the standard regimen for improved clinical outcomes [2]. The drugs target several essential steps in the viral replication, including virus entry and fusion with the host cell, and the viral enzymes, protease, reverse transcriptase, and integrase. Complications for HAART stem from immune dysfunction, increased risk for non-AIDS related disorders, and side effects from long term large pharmaceutical exposure [3]. The selection of viral strains that are resistant to current inhibitors remains a prominent challenge to successful long-term treatment of HIV infected individuals.

The HIV protease is an important drug target for HIV/AIDS therapy, and its structure and function have been reviewed in [4]. HIV protease performs an essential role in viral maturation by processing specific cleavage sites in the Gag and Gag-Pol precursor polyproteins to release the mature proteins (Figure 1). The protease-catalyzed processing of viral precursors is essential for formation of infectious virus particles. The 99 amino acid aspartic protease forms a homodimer, which is characterized by a central active site cavity

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capped by two flexible flap regions. The dimer presents a closed conformation when bound to substrate or inhibitor, and the flaps open for entry or release of the ligands. The structure-guided strategy for drug design has been extremely successful, resulting in competitive inhibitors that tightly bind in the active site with high affinity by mimicking the transition state of the natural protein substrate. Clinical protease inhibitors (PIs) have significantly improved patient outcomes since their introduction in 1995. Nine PIs have been approved for clinical therapy to date (Table 1). Resistance to inhibitors arises from mutations in HIV protease that alter inhibitor binding site or the subunit-subunit interface of the protease dimer, while still permitting viable levels of hydrolysis of the substrate allowing formation of infectious virus particles. Accumulation of drug resistant mutations can result in highly resistant variants. The two newest PIs, tipranavir and darunavir (DRV), were specifically designed for increased efficacy against resistant mutants. However, no new PIs have been introduced in the clinic since DRV was approved in 2006. The identification of clinically isolated proteases that are highly resistant to DRV and other PIs has sparked renewed interest in developing strategies for inhibition of resistant HIV protease.

Causes of resistance to protease inhibitors

Since the successful debut of the clinical inhibitor saquinavir (SQV) in 1995, viral resistance to protease inhibitors has arisen as an urgent challenge to HAART [5]. Evolution of HIV to acquire resistance to PIs emerges from a combination of several factors. The viral reverse transcriptase lacks a proofreading function; low fidelity transcription drives an increased rate of spontaneous mutagenesis [6]. Selection of resistant strains is increased by loss of adherence to the drug program due to the necessity for long-term use of HAART [7,8]. Patient noncompliance due to drug side-effects, pill-burden, and toxicity has increased viral selection for resistance [9,10]. Primary transmission of resistant strains from HAART treated patients to antiretroviral-naïve patients is also common in middle to low income countries [11,12].

Viral resistance to protease inhibitors evolves initially by mutations in the protease gene. The majority of these mutations associated with resistance are single amino acid substitutions, as illustrated in Table 1. Rarely, insertions have been observed in the protease gene and may play a role in PI resistance [13–15]. Multiple mutations accumulate, and high level resistance may require a combination of 10–20 mutations in the protease. Many different combinations are possible. As resistance evolves, additional mutations occur in the cleavage sites of the polyproteins.

Virus containing resistant protease mutants must still be capable of reproducing *in vivo* by successful processing of the Gag and Gag-Pol precursors in the maturation stage. Several mutations in the precursor cleavage sites have been shown to improve the activity of resistant protease [16,17]. Mutations in the gag cleavage sites of NC-p2 and p2-p6 are commonly associated with drug resistance [18]. Structural modeling suggests that the mutations in the cleavage site act to improve interactions between the substrate and the mutated protease and increase the efficiency of cleavage. Hence, mutations in the Gag and Gag-Pol substrates can compensate for the lower catalytic efficiency of resistant protease mutants [18,19]. This mechanism of resistance can be difficult to detect as the protease

genotype from the patient would not necessarily match the expected PI-resistance profile [20]. Initial mutations in the protease gene co-evolve with compensatory mutations in the cleavage sites of the Gag and Gag-Pol substrates. The result of this network of influences is specific combinations of mutations that confer resistance to PIs but still provide adequate proteolytic activity, viral maturation and production of infectious virus particles.

Mutated residues in HIV PR are classified as either major or minor resistance mutations by their effect in HAART, as summarized in Wensing *et al.* [21]. Minor resistance mutations are assumed to have ancillary roles such as compensation for lower efficiency of proteolysis caused by major mutations. Major resistance mutations tend to confer high levels of resistance to one or multiple inhibitors and develop early in patient treatment. When possible, testing for resistance is performed following diagnosis of HIV infection [22]. For example, specific mutations I47A/V and V32I confer high resistance to lopinavir (LPV) [23–25]. The L76V mutation significantly increases LPV resistance when combined with common partner substitutions M46I, I54V, V82A and L90M [26]. One mutation, I84V, is associated with resistance to all nine currently approved PIs [21].

The 15 sites described for major mutations are distributed between the active site, flap and distal regions of the protease dimer (Figure 2). Patients exhibiting pan-PI-resistance were shown to have convergent evolved patterns of accumulated protease mutations [27]. Different multiple genotypic mutation patterns frequently yield similar phenotypic resistance profiles [28]. A library of laboratory designed recombinant molecular clones with common PI-resistant substitutions is publicly available as representative sequences for studying novel therapeutics for resistant strains [29]. The effects of individual mutations and their combinations are described in the next section.

Diverse mechanisms of resistance

Distinct mechanisms of resistance to PIs have been described. Earlier studies focused on mutants with single substitutions, and the molecular mechanisms contributing to resistance are reviewed in Weber and Agniswamy 2009 [30]. Resistant mutants often demonstrate reduced but viable catalytic activity and defective processing of the polyproteins. Analysis of the changes in structure and activity of the mutants suggested three categories of mutations:

- Single substitutions in active site cavity residues can result in altered drug interactions. This mechanism is a straightforward way of reducing the binding affinity of drugs yet still maintaining catalytic activity. Known mutations using this mechanism include D30N, V32I, I47V, G48V, I50V, Val82 and I84V. These mutations produce small changes in the size and shape of the amino acid side chains that interact with inhibitors. A recent example of this mechanism is a double mutant, G48T/L89M, that displays resistance to SQV via an expanded active site cavity from a defective hydrophobic sliding mechanism [31];
- Mutations can lower dimer stability by interrupting the interactions at the interface between the two subunits in the dimer. The result will be increased dimer dissociation and release of bound inhibitor. This mechanism has been described for

mutants with L24I, I50V and F53L by kinetic and structural studies [30]. HIV protease dimerization is thought to occur in two sequential steps, initial intermolecular interactions at the active site lead to more interactions along the dimer interface [32]. Mutations of Ile50 alter both dimer stability and interactions with PIs [30];

- Distal mutations can cause PI resistance in unique ways. The major mutation of L76V is associated with resistance to DRV, fosamprenavir (APV), indinavir (IDV) and LPV, while conferring susceptibility to the other PIs. Protease bearing the L76V mutation showed low dimer stability and defects in autoprocessing [33]. Analysis of crystal structures showed significant loss of interactions with DRV, while protease interactions were maintained with SQV. Molecular dynamic studies combined with crystal structures suggested L76V contributed to resistance by altering the overall enzyme dynamics [34]. Many distal mutations are classified as minor mutations that modulate the effects of major mutations. One example is G73S, which showed an altered network of interactions leading to the inhibitor binding site [30]. Another indirect effect was observed for the distal mutation N88D, which had altered interactions with water in a conserved region of the protease structure [35]. Changes due to a single distal mutation may be small and difficult to analyze in the absence of major mutations. However, the cooperative influences of distal mutations with active site mutations may be required for high level resistance to multiple drugs [36].

High level resistance from multiple resistance mutations

Multidrug and highly resistant variants often employ a combination of mechanisms that act synergistically to evade inhibition. Several multidrug and highly resistant HIV protease mutants have been identified and characterized in order to elucidate how the mutations affect the structure and function of the enzyme. Several mutants containing different sets of 10–22 substitutions have been investigated by x-ray crystallography and isothermal titration calorimetry, and selected examples are listed in Table 2. The mutants contain one or more major mutations combined with a variety of minor mutations. The binding affinity for DRV decreases by nearly four orders of magnitude in the worst cases, as shown in Table 2. The drastically lower binding affinity for inhibitor suggests that viral strains with these protease variants have lost susceptibility to DRV. Commonly observed structural features of these highly resistant variants include an expanded PI binding site and altered flap dynamics. The structures of the highly resistant mutant PR20 and wild type protease are compared in Figure 3. The different flap conformations highlight the variation observed in the crystal structures. The two closed conformation dimers are complexed with DRV and are similar in overall structure. The open conformation of the flaps is observed in the absence of bound inhibitor. In the absence of inhibitor, PR20 exhibits extremely open conformations of the flaps, and the two flaps in the dimer can exist in asymmetrical conformations, which is atypical for wild type enzyme.

An inactive (D25N) variant of a multidrug resistant clinical isolate deemed MDR769 carrying 10 mutations L10I, M36V, M46L, I54V, I62V, L63P, A71V, V82A, I84V, and L90M has been studied by x-ray crystallography. A variant of MDR769 with V82T displays

11–2600-fold resistance to all nine PIs [37]. Analysis of a 1.3Å resolution crystal structure describes three major differences between MDR769 and wild type protease [38]. First, the flaps of the MDR769 display a ‘wide open’ form as a result of structural rearrangements. Furthermore, the commonly occurring substitution L90M reshapes the drug interactions of the MDR769 active site by a domino effect of perturbations in the 80’s loop, which distorts the S1/S1’ subsites of the binding pocket [39]. The active site is further expanded by approximately 3.0Å via the dual reduction in atom volume from mutations V82A and I84V.

A clinical isolate from a pediatric patient failing HAART that harbors 22 mutations in the protease (T4S, L10V, I13A, K14R, K20I, A22V, L33I, E35D, M36I, N37D, R41K, K43S, G48A, I54V, I66F, H69K, T74S, V82A, I84V, L89I, L90M and T91S) was studied by kinetic and x-ray analysis in complex with DRV [19]. This mutant is highly resistant (>3600-fold increase from wild type) to SQV, RTV, IDV, ATV and NFV, cross-resistant to LPV and APV, and less resistant to DRV. Structural information indicates rearrangements of the flap elbow region leading to loss of direct interactions between protease and DRV. Additionally, V82A/I84V/G48A mutations contribute to the collapse of the 80’s loop by directly affecting inhibitor binding to the S1/S1’ subsites. Furthermore, distal mutations likely induce long-range structural changes altering inhibitor and substrate binding kinetics. The usual loss of catalytic efficiency of highly mutated protease variants was rescued in this case by co-evolution selecting for changes in the Gag-Pol cleavage sites [19]. The accumulated mutations of this protease enable viral replication through both decreased affinity for inhibitor and co-evolution of enzyme and its substrate.

Six clinically derived variants of HIV protease with up to 21 mutations known to be highly resistant to DRV with cross-resistance to several other clinical PIs were characterized extensively [40,41]. Isothermal titration calorimetry (ITC) analysis showed that the six DRV-resistant proteases lose inhibitor binding affinity due to less favorable enthalpy of binding when compared with the wild type enzyme. The analysis of crystal structures of three of these protease mutants complexed with DRV suggested a significant loss of protease-DRV hydrogen bond interactions as the likely explanation for the changes in binding enthalpy.

A different clinical isolate with extremely resistant HIV protease bearing 20 mutations (PR20) has been studied extensively with several different PIs and a substrate analog. This variant exhibits extreme resistance to all clinical PIs of several orders of magnitude [42], and the PR20 dimer dissociates more readily than wild type protease, while the monomer stability is enhanced [43]. Additionally, autocatalytic cleavage of PR20 precursor is unaffected by PIs, in contrast to the inhibition of the wild type precursor autoprocessing by DRV and SQV. To date, the PR20 structure has been solved in the absence of inhibitor or complexed to DRV, SQV, p2-NC peptide analog, APV and two investigational inhibitors GRL0519 and GRL02031 [44,45]. Like other resistant variants of HIV protease, the flaps observed in the different apo-structures of PR20 adopt a more open conformation as shown in Figure 3. The crystal structure of the open conformation of PR20 indicates the higher dissociation constant likely stems from loss of intersubunit contacts between the more flexible flaps. The flexibility of the flaps in PR20 is proposed to arise from structural rearrangements caused by the combination of the M36I/I15V/I33F mutations. Unlike other

highly resistant mutants, PR20 retains the majority of hydrogen bond interactions with clinical inhibitors DRV, SQV and APV [44,45]. Instead, the expanded binding site of PR20 results in loss of hydrophobic interaction with inhibitors. Additionally, the intersubunit interactions involving Arg8/8' and Arg29/29' are perturbed in PR20 by the presence of the L10F mutation; these interactions are important in forming the S2/S2' PI binding subsites. When these mechanisms of resistance are combined with distal mutations that influence the interactions of residues 30–32 with inhibitor, PR20 employs a diverse array of mechanisms to evade inhibition.

Mutant HIV protease variants that have been laboratory selected for resistance to clinical inhibitors such as DRV provide a useful method for studying mechanisms of high resistance to a single drug independent of previous treatment [46]. One of these variants with 14 substitutions, designated P51, is resistant to DRV inhibition of precursor autoprocessing as described for PR20 [42]. The crystal structure of ligand-free P51 with inactive D25N substitution exhibits slightly wider flap separation and loss of intersubunit contacts between the flap tip and Pro81 when compared with ligand-free WT PR [47].

A crystal structure of P51 D25N co-crystallized with DRV (PDB: 4NPT) reveals a unique mode of binding for DRV [47]. DRV binds to the open flaps in an orientation almost perpendicular to the standard mode of binding in the active site. The observed unusual binding of DRV is mainly attributed to V32I/V82I/I84V mutations forming hydrophobic interactions with Val47 and shifted intersubunit contacts from the L24I substitution. The P51-DRV structure yields new insights into possible approaches to target the wide open flaps of resistant mutants.

Taken together, these examples show that significant changes in structure via up to 20 coordinated mutations are required for high level resistance to second generation inhibitors like DRV. Crystallographic analysis of several highly resistant mutants share perturbations in the active site, flap regions, 80's loop, or inter-subunit interactions, which are likely to be mechanisms inducing loss of susceptibility to PIs. In addition, the highly resistant variants are proposed to have increased occupancy of open conformational states relative to wild type enzyme, as assessed by Electron Spin Resonance [48] and Nuclear Magnetic Resonance [49]. These structural and functional insights into HIV PR drug resistant mutants are invaluable in developing novel therapeutics for future AIDS treatment.

Inhibitors designed to bind in the active site

The clinical inhibitors of HIV protease provide a prime example of the success of structure-guided drug design, as discussed in recent reviews [4,50]. The PIs bind in the active site of the protease dimer and compete with substrate binding to the same site, as shown in Figure 4. All the current PIs contain the central hydroxyl group, which mimics the transition state of the reaction. They form interactions with protease similar to those of a peptide substrate, including hydrogen bonds with polar groups like the peptidic amide and carbonyl groups, and hydrophobic groups fitting like amino acid side chains into the protease subsites S2-S2'. The design difficulties can be illustrated by the example of brexnavir, a tyrosyl peptidomimetic with excellent antiviral activity against wild type and resistant virus, which

subsequently failed clinical trials due to its poor metabolic profile. Incorporation of deuterium in an attempt to slow drug metabolism did not improve the pharmacokinetic properties of the compound [51]. The two most recently approved PIs, tipranavir and DRV, were designed to target resistant proteases. Here, the structure-guided design of DRV and the newer antiviral inhibitors will be described to illustrate a viable strategy for targeting resistant protease mutants.

Darunavir (DRV, TMC114, UIC-94017)

The drug DRV was designed with the *bis*-tetrahydrofuranylurethane (THF) moiety at P2 (Figure 5) in order to introduce hydrogen bonds with the conserved main chain atoms of the protease, which cannot be easily altered by mutations, unlike the side chains [52]. The interactions of the *bis*-THF with the protease are illustrated in Figure 6. DRV is effective and well-tolerated in the clinic [53], and shows a high genetic barrier for development of resistance [54]. The effectiveness of DRV is primarily due to its excellent 5–10 pM binding affinity for wild type protease as measured by ITC [55]. In addition, DRV was shown to inhibit protease dimerization [56], and the autoprocessing step, as described in a later section.

The hydrogen bond interactions of DRV are maintained with many resistant mutants, as demonstrated by crystallographic analyses [30]. HIV does evolve resistance to DRV, although generally several mutations are required, as described earlier. Characterized mutants with high level resistance show several orders of magnitude lower affinity for DRV [40–42]. Loss of hydrogen bond interactions with DRV was observed for protease bearing L76V [33], and several resistant mutants isolated from patients with up to 21 substitutions [42,43], while another clinical mutant with 19 mutations showed loss of hydrophobic interactions [45]. These results demonstrate the need for new antiviral inhibitors designed for highly resistant proteases.

Investigational antiviral inhibitors

Collaborative efforts of synthetic chemists, structural biologists, enzymologist and virologists, are needed to design the future-generation protease inhibitors to combat emerging resistant strains. A series of antiviral protease inhibitors with novel chemistry were designed on the DRV scaffold by the strategy of introducing polar interactions with conserved regions of the protease [52]. Structural analysis of protease with resistant mutations has led to an improved strategy for inhibitor design by introducing stronger interactions with the conserved regions around the active site and the flaps. Several compounds introduce new groups at the P2 position of DRV, while modifications of P2' and P1 have also been tested. Selected examples are shown in Figure 5. In the following paragraphs, this strategy will be described for several antiviral compounds with unusual chemistry and potency against tested strains of resistant virus.

Compound GRL-0519 incorporates *tris*-THF instead of the *bis*-THF at the P2 group of DRV, and a methoxy group instead of the amide on the aniline ring at the P2' position [57]. GRL-0519 has almost tenfold better *in vitro* activity than DRV on a wide spectrum of multidrug resistant variants, making this potent inhibitor a prospective candidate for further

studies [53,58]. Crystallographic analysis showed that the *tris*-THF group of GRL-0519 provided additional polar interactions over the *bis*-THF group of DRV. The interactions of P2 with the protease are shown in Figure 6. The third THF ring formed new water-mediated hydrogen bond interactions with the highly conserved protease residues Thr26, Gly27, Asp29 and Arg87 of one subunit and Arg8' of the second subunit. Coordinating these residues at the subunit–subunit interface will tend to stabilize the dimer [57]. These interactions of the inhibitor were maintained in the crystal structures of proteases bearing single substitutions of R8Q, D30N, I50V, I54M and V82A [59]. The loss of hydrophobic interactions with protease containing I50V mutation was consistent with 60-fold worse inhibition of catalytic activity, suggesting this mutation will cause resistance to the GRL-0519 inhibitor. The inhibitor was tested with the highly resistant mutant PR20 [44]. Although the binding affinity of 40 nM as measured by isothermal titration calorimetry is similar to that of DRV, the large *tris*-THF group fits snugly in the enlarged S2 subsite of the mutant, suggesting this strategy is promising for further development.

Another compound developed on the DRV scaffold is GRL-04410, which contains a methoxy substitution at the C4 position of the *bis*-THF moiety within P2 [60]. At the other end, the P2' benzyl has the methoxyl group used in TMC-126, instead of the amino group in DRV. The performance of GRL-04410 surpassed that of DRV with a K_i of 2.9 pM and an IC_{50} of 2.4 nM. A high resolution crystal structure was solved for GRL-04410 in complex with wild type protease. While the DRV-associated backbone hydrogen bonds were preserved, the C4 *bis*-THF methoxy substitution provided a novel C-H...O interaction and a water-mediated hydrogen bond with the amide of Gly 48 in the flap of the protease (Figure 6). By stabilizing the flexible flaps, inhibitors may retain potency against resistant variants showing highly dynamic flaps.

The unique feature of the new antiviral compound GRL-05010 is the *gem*-difluoro addition on the C4 position of *bis*-THF at P2 of DRV. The strategy of introducing fluorine to compounds is employed to increase lipid solubility and improve penetration of the blood–brain barrier [61]. This strategy proved successful in tests on GRL-05010 bearing two fluorines. GRL-05010 has improved lipophilicity and has been shown to penetrate the blood–brain barrier effectively *in vitro*, an infiltration that is not observed with most of the current PIs [62,63]. This antiviral inhibitor demonstrated excellent inhibition of the protease with a K_i of 5.8 pM [63], and antiviral activity with an EC_{50} of 3 nM. Furthermore, the antiviral potency remained in the nanomolar range for DRV-resistant strains [62]. Selection using GRL-05010 resulted in resistance due to M46I and I50V mutations, and to a lesser extent, N38K and M36I [62]. The structure of wild type protease in complex with GRL-05010 was solved at a high resolution, and exhibited similar protease-inhibitor interactions as seen for the DRV complex [63]. Halogen interactions between the two fluorine atoms and the carbonyl oxygen of Gly48 might be responsible for the significant improvement in performance (Figure 6). Altogether, these results suggest that GRL-05010 may overcome resistance by stabilizing the flexible flaps and has potential for eradicating virus reservoirs within the central nervous system.

Larger alterations within the DRV scaffold have also generated novel inhibitors. Qiu *et al.* [64] synthesized DRV-based inhibitors that replaced the *bis*-THF at P2 with an isosorbide

trans-4-hydroxy-L-prolinamide. The isosorbide inhibitors demonstrated a two orders of magnitude improvement in antiviral activity against wild type HIV in comparison to IDV, however, no comparison was reported with DRV and the experimental structure is unknown.

The compound GRL-008 is a derivative of DRV and contains the benzene carboxamide substitution instead of aniline at P2' [65]. The crystal structure in complex with protease exhibits a new water-mediated connection between the NH of the carboxamide and the backbone carbonyl oxygen of Gly48 in the flexible flap [65]. The structure also forms weaker hydrogen bonds to the backbone of conserved residues Asp29, Asp30/30'. A later study revealed that GRL-008 maintained wild-type polar interactions in the active site of a highly mutated protease from a clinical isolate [66]. It is noteworthy that DRV also conserves polar bonding with the same mutant and outperformed GRL-008 in antiviral activity against wild type and resistant strains.

Compound GS-8374 was designed to contain a paraphosphonic acid moiety in the aromatic ring at the P1 position of the TMC-126 parent structure [67]. The structure in complex with protease revealed that the phosphonate group was exposed to the solvent beyond the protein surface. This phosphonate 'solvent anchor' may be responsible for the retention of antiviral activity against resistant variants as measured by ITC. Recently, the structure of GS-8374 was reported in complex with an unusual variant bearing 12 substitutions and a Glu-Glu insertion after Glu35 that is resistant to many drugs [68]. Their analysis suggests that retention of antiviral activity against this mutant is due to the ability of the P1 aromatic phosphonate to rearrange within the expanded cavity walls while remaining anchored to the solvent.

Alternative designs for inhibitors that are not based on DRV/TMC-126 produce distinct binding interactions in the active site of the protease. Another strategy using a pyrrolidine linker was described [69]. C2-symmetric 3,4 disubstituted pyrrolidines with polar benzene carboxamide groups at P2/P2' inhibited wild type protease at low micromolar (0.26–0.48 μM) values. The most potent inhibitor (3*S*,4*S*)-3,4-bis-[4-carbamoyl-benzene-sulfonyl]-(3-methyl-but-2-enyl)-amino]-pyrrolidinium trifluoroacetate engaged the flaps via direct hydrogen bonds between the NH amide of Ile50/50' and the oxygen of the sulfonamides in each symmetrical component, while the pyrrolidine NH interacted with the carboxylate oxygens of the catalytic Asp25/25'. Furthermore, the crystal structures also displayed P2/P2' carboxamides forming direct hydrogen bonds to the main chain atoms of Asp30/30'. Overall, these novel inhibitors offer alternative designs for binding within the active site of the closed conformation of the protease dimer.

Many of these compounds have not yet been evaluated for antiviral efficacy against resistant HIV. The most promising of the tested inhibitors are GRL-0519 with tenfold improved antiviral inhibition *in vitro* against resistant strains of HIV [53,58], and GRL-05010, which retained nanomolar antiviral activity against DRV-resistant virus and showed improved penetration of the blood–brain barrier *in vitro* [62,63]. Further studies are underway on these inhibitors.

Inhibitors targeting alternative binding sites in the protease

Several strategies have been proposed for inhibitors targeting alternative sites, instead of the competitive inhibitors that bind in the active site cavity of the protease. These include inhibitors that bind to the open conformation of the dimer, to the hinge of the flap, or the intersubunit interface. Inhibition of the open conformation of the protease has been proposed from molecular dynamics simulations, such as the report by [70] based on a crystal structure [71]. Interestingly, a unique binding mode was observed for DRV, where the inhibitor bound to the wide open conformation flap of a highly resistant protease mutant (P51) [47].

A different inhibitor was observed bound to the flaps in an open conformation of the protease dimer. Taking advantage of the nontoxic properties of dibenzodiazepines, a derivative was synthesized and crystallized in complex with protease [72]. Two molecules of the inhibitor occupied the protease active site of a semiopen flap conformation. Each individual molecule made one direct backbone hydrogen bond with the carbonyl oxygen of Gly27, while solvent-mediated connections were established with Asp25, Asp29, Asp30 and Ile50. These solvent bridges would normally be observed as direct hydrogen bonds in protease-DRV complexes, thus explaining the relatively weak micromolar potency of the dibenzodiazepine derivative. Two derivatives of the novel inhibitor were connected via an oxalyl link, which improved the inhibition of protease activity to a more practical nanomolar range. Although crystallography attempts were unsuccessful, a docking study suggests that the improved inhibition might be due to the oxalyl oxygens forming direct hydrogen bonds to the catalytic aspartates instead of the indirect interactions mediated by the conserved waters, as well as favorable van der Waals contacts from the bulkier inhibitor.

Two alternative binding sites were identified in a fragment based crystallographic screen of HIV protease: the loop formed by Gly16-Gly17-Gln18, and a shallow groove on the outside of the flap formed by Trp42, Pro44, Met46, Lys55, Val56, Arg57 [73]. Remarkably, DRV was observed bound to an almost identical location of the flap in crystal structures of the highly resistant PR20 mutant [45]. The amino acid residues contributing to this binding groove are not observed as sites for the common resistance mutations (Table 1), which suggests this groove may be a viable target for inhibitor designs.

Blocking dimerization is a potential, alternative strategy to hinder viral replication. DRV was shown to inhibit both dimerization and proteolytic cleavage by binding to both the monomer and dimer [56]. Hence, DRV has several valuable properties in addition to its binding in the active site cavity.

Inhibitors targeting the Gag or Gag-Pol precursors

Viral maturation, which is the protease-catalyzed processing of viral Gag and Gag-Pol precursors to release individual proteins, is a potential target for inhibitors. This essential process in the viral life cycle must occur in a precise order to produce infectious virus [74,75]. An inhibitor of maturation, bevirimat, was developed to target the final processing step for the Gag precursor (Figure 1). Bevirimat binds at the CA-sp1 site, which is the natural substrate for the protease [76,77]. The antiviral potency of bevirimat led to clinical trials, however, high baseline drug resistance due to the existing polymorphisms halted

further development. Mutations in the protease influence the resistance to bevirimat [76]. Resistance mutations were identified around the CA-sp1 cleavage site, at amino acid 201 and the major homology region of CA [78]. New derivatives of bevirimat have been evaluated with limited success to date [79,80].

A critical step in viral maturation is the autoproteolytic cleavage of the protease from its Gag-Pol precursor. Inhibition of this autoprocessing step has been proposed as an alternative target for development, as reviewed by [81]. Two of the current PIs, DRV and SQV, demonstrated significant inhibition of the autoprocessing step that releases mature dimeric protease. DRV and SQV were the most effective of the nine clinical inhibitors in inhibiting precursor autoprocessing at the cleavage site between transframe and the N-terminus of PR [42]. DRV inhibition of this cleavage was reported in mammalian cells [82]. The same two PIs were also shown to be the best inhibitors of proteolysis of the sp1-NC site in Gag-Pol [83], strongly suggesting the unique antiviral properties of these two drugs. In another approach, a monoclonal antibody was found to inhibit autoprocessing at the C-terminus of the protease [84]. The crystal structures of a miniprecursor of HIV-1 protease with four extra N-terminal residues SFNF from the transframe region revealed multiple conformations and unraveling of the N-terminus representing a transient step in autoprocessing [85,86]. These mini-precursor structures provide new targets for design of drugs that may be less susceptible to development of resistance.

Conclusion & future perspective

The rapid mutation of HIV under drug selection to evolve resistant viral strains poses a severe challenge to effective long term AIDS therapy. The virally encoded protease is prone to develop multiple mutations, and highly resistant mutants with around 20 substitutions can show several orders of magnitude worse inhibition by PIs. Strategies to overcome the problem of resistance include the design of competitive inhibitors with very high affinity for wild type enzyme, as exemplified by DRV, inhibitors designed specifically for highly resistant mutants, inhibitors targeting alternative sites on the protease, and compounds binding the precursor substrates of the protease.

Structural and enzymatic studies of highly resistant protease mutants reveal the molecular changes associated with loss of susceptibility to PIs. Mutants with specific combinations of 14–22 amino acids substitutions can show two to four orders of magnitude worse affinity for clinical PIs. Structural analysis reveals the loss of noncovalent interactions of protease with PIs due in part to an expanded binding site. Moreover, the flaps exhibit an increased population of open conformational states. These structural adaptations suggest improved strategies for inhibitor design. First, inhibitors can be designed with larger side groups to fit better in the expanded binding site of the mutants. Second, introducing chemical groups in the inhibitors that form interactions with the flaps will tend to stabilize the protease dimer conformation. This strategy shows promise for new antiviral inhibitors derived on the DRV scaffold. One of these compounds, GRL-05010 includes two fluorides and exhibits improved lipid permeability, which is critical for attacking viral reservoirs in the central nervous system.

Compounds have been identified to bind at alternative sites in the protease structure, such as the interior of the flap in the open conformation or the groove on the outer surface of the closed conformation flap. Little development has been reported for compounds targeting these sites. One problem is that the known compounds are likely to have low affinity for the protease, and may not show effective inhibition of the catalytic activity.

Other potential targets for inhibitors are the rate limiting steps in protease cleavage of its natural substrates, the Gag and Gag-Pol polyprotein precursors. Clinical trials for the maturation inhibitor bevirimat failed due to rapid development of resistant mutations. The cleavage site at the N-terminus of the protease is a promising and little studied target. New structural data for the protease precursor should help in the design of novel inhibitors with affinity for this site. The disadvantage of this target is that the structure of the linker and N-terminal protease residues may have multiple conformations in the precursor.

Overall, the optimal target for inhibitor development is still the active site cavity of the protease dimer. Competitive inhibitors with high affinity for the HIV protease are achievable, and antiviral inhibitors with novel chemistry would be valuable future compounds of HIV/AIDS therapy. The development of new inhibitors specifically designed for the expanded binding site of the highly resistant mutants is an attractive possibility to pursue in future.

Key terms

Highly active antiretroviral therapy	Uses a combination of drugs that target different stages in the viral life cycle. It is also referred to as combined antiretroviral therapy (cART)
Protease inhibitor	Clinically approved antiviral inhibitor of HIV-1 protease
Highly resistant protease mutant	Mutant of HIV protease resulting from drug selection resulting in loss of susceptibility to protease inhibitors by several orders of magnitude as assessed by the binding affinity. A combination of about 20 substitutions is generally observed in highly resistant proteases

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- of interest;
- of considerable interest

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Executive summary

Molecular mechanisms leading to highly resistant protease mutants

- Greater knowledge of the mutations and molecular mechanisms resulting in highly resistant proteases will open new possibilities for inhibitors designed to target resistant HIV. Several studied examples of highly resistant proteases show expanded active site cavities and greater variation in flap conformation.

Competitive inhibitors designed for resistant protease variants

- Inhibitors designed to fit better in the expanded binding site of highly resistant mutants and/or stabilize the dynamic flaps are being evaluated.

Inhibitors targeting precursor proteins

- Autocatalytic cleavage of the HIV protease from the viral Gag-Pol precursor is a critical step in viral replication. Knowledge of the precursor structures involved in protease autoprocessing provides new insights into a key step in viral maturation. Novel inhibitors targeting this step have potential as therapeutic agents that are less susceptible to development of resistance.

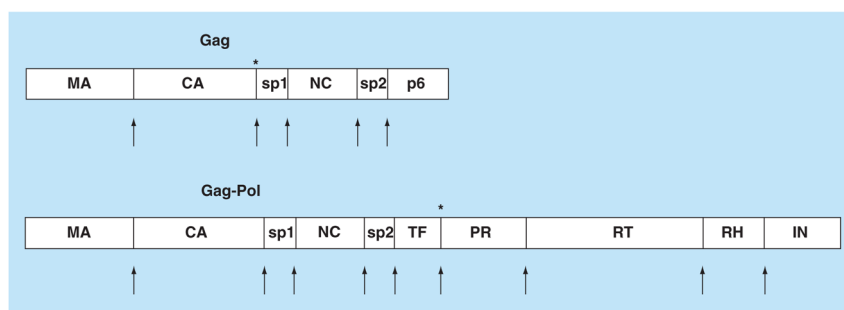


Figure 1. Gag and Gag-Pol precursors

HIV PR processes the Gag and Gag-Pol precursor proteins during the maturation stage of viral replication. PR releases itself and the individual proteins: MA, CA, NC, RT, IN and RH, as well as small, probably unstructured, spacer peptides sp1, sp2 and p6. The arrows indicate the cleavage sites. The maturation inhibitor, Bevirimat, targets the CA-sp1 cleavage site and cleavage of TF-PR at the *N*-terminus of PR is another potential target for novel inhibitors, as indicated by asterisks in Gag and Gag-Pol.

CA: Capsid; IN: Integrase; MA: Matrix; NC: Nucleocapsid; PR: Protease; RH: RNaseH; RT: Reverse transcriptase.

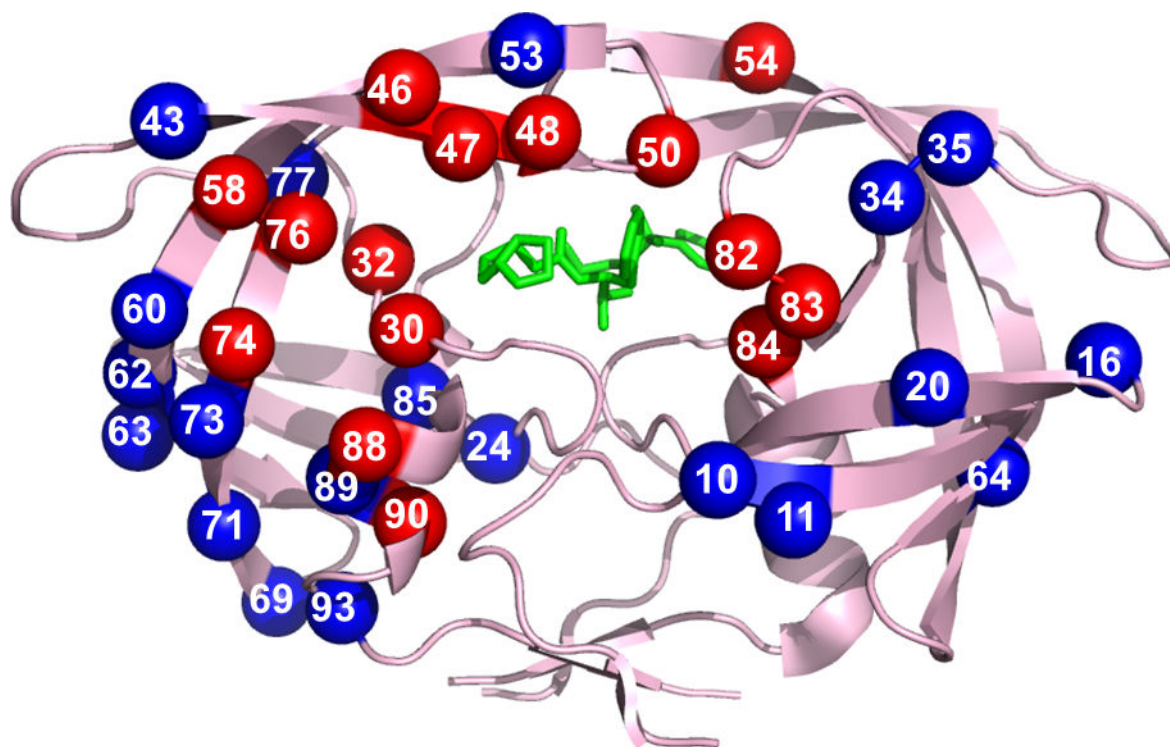


Figure 2. Protease dimer showing sites of resistance mutations

Locations of resistance associated mutations mapped on the HIV protease dimer bound with DRV (PDB: 2IEN). The protease dimer is shown in pink ribbons with DRV in green sticks. Major and minor mutations are distributed among both monomers and shown in red and blue spheres, respectively.

Adapted with permission from [30] (2009); using updated resistance mutations from [21].

For color images please see online www.future-science.com/doi/full/10.4155/FMC.15.44

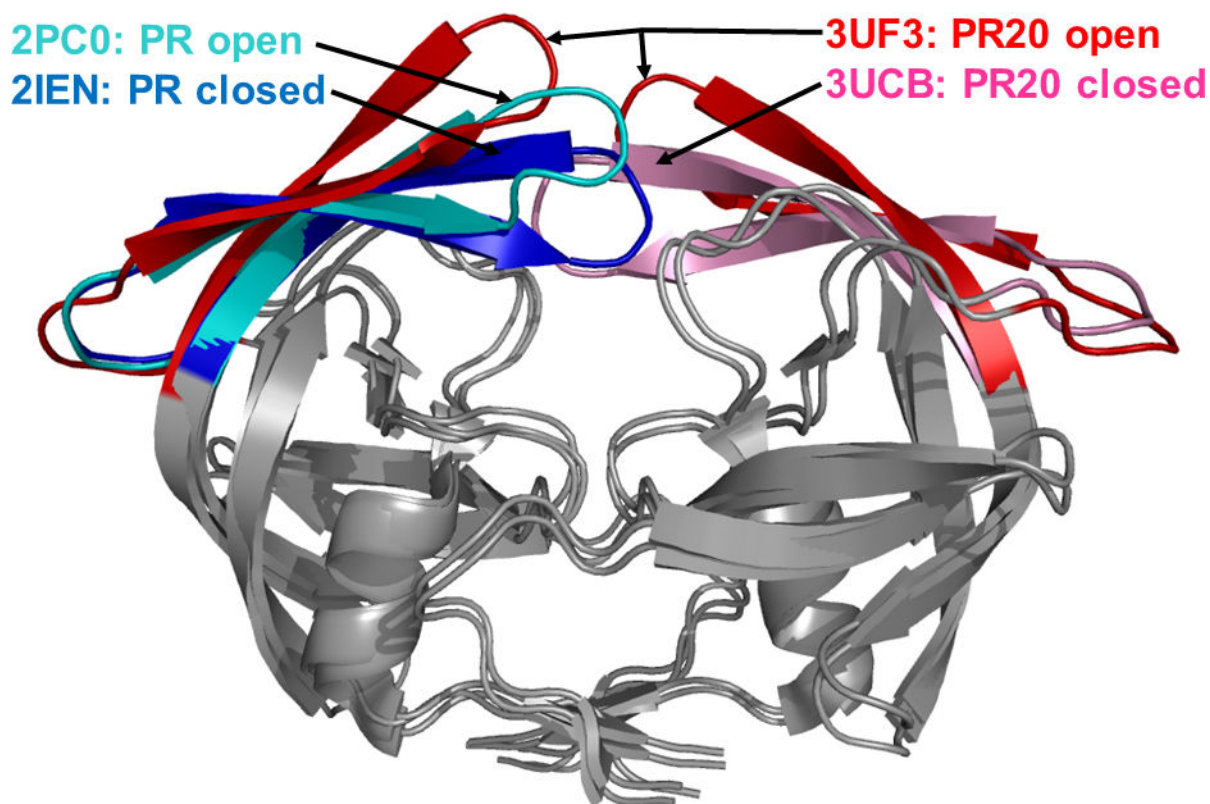


Figure 3. Closed and open conformations of the dimer of wild type protease and highly resistant mutant PR20

Superposition of the crystal structures of PR20 and wild type HIV protease. The dimers are shown in gray, except for the flaps, which are in different colors. The PDB ID is indicated for each structure. The different flap conformations highlight the variation observed in the crystal structures. The two closed conformation dimers are complexed with DRV and are similar in overall structure. The open conformation dimers contain no inhibitor. Open PR20 is shown with the two flaps in the dimer to indicate its atypical flap asymmetry, and the other structures are represented as single flaps.

For color images please see online www.future-science.com/doi/full/10.4155/FMC.15.44

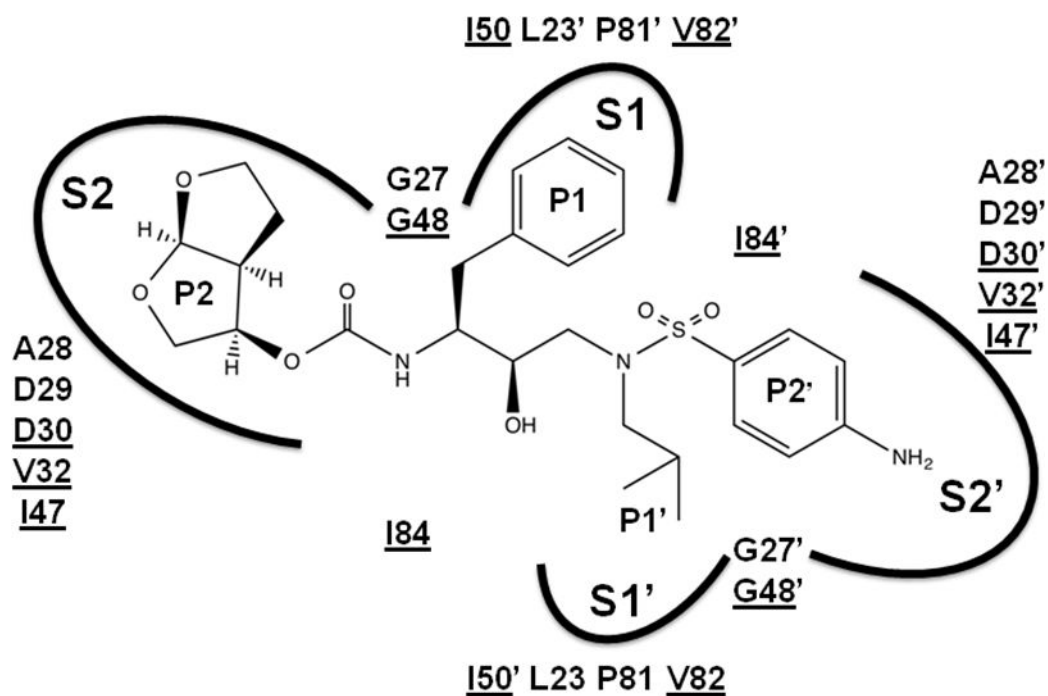


Figure 4. Darunavir in the binding site of HIV protease

Darunavir is shown with groups labeled P2-P2'. The protease binding site is indicated by arcs for subsites S2, S1, S1' and S2' and the amino acid residues contributing to the binding site. Underlined amino acids are mutated in drug resistance.

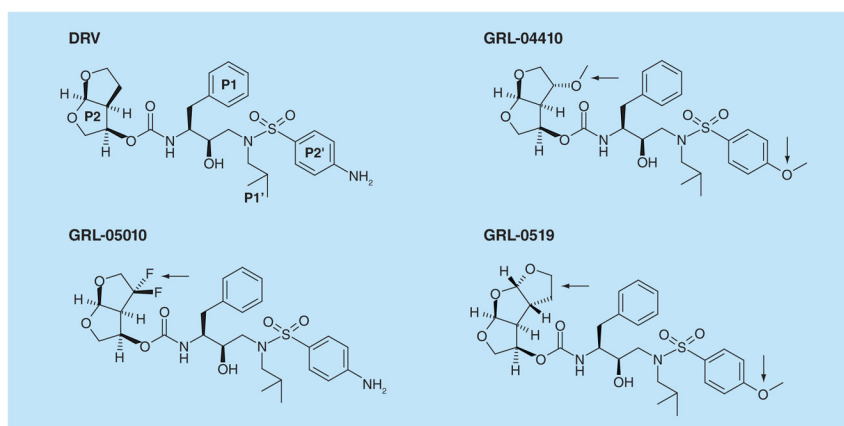


Figure 5. Darunavir and selected investigational inhibitors

Structures are illustrated in similar conformations to facilitate comparison. Darunavir is shown with the P2, P1, P1' and P2' groups labeled. The modifications in the investigational inhibitors are indicated by black arrows. Antiviral inhibitors GRL-04410 and GRL-0519 have a methoxy substitution at P2', while GRL-0519, GRL-04410 and GRL-0510 have *tris*-THF, methoxy and *gem*-difluoro additions at P2, respectively.

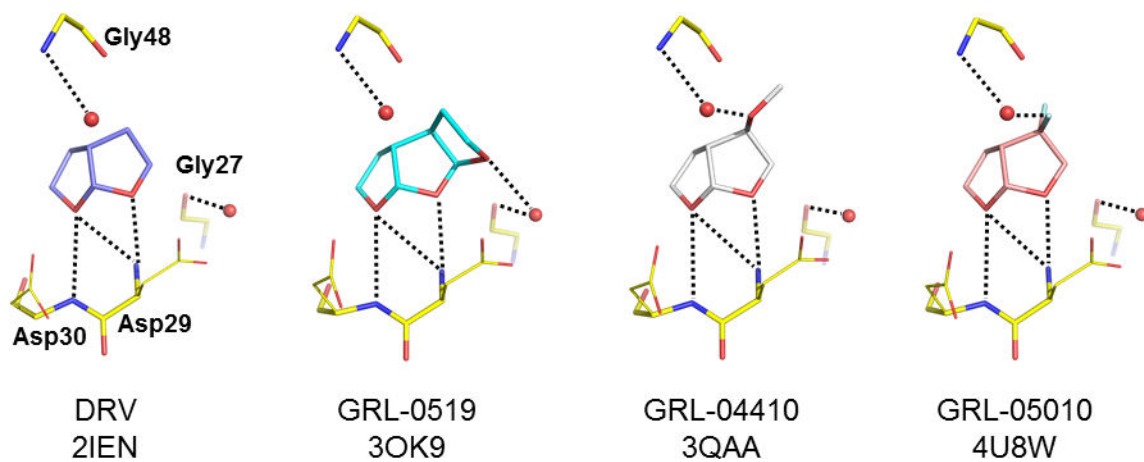


Figure 6. Interactions of HIV protease with P2 group of darunavir and selected inhibitors

The interactions of the P2 bis-THF group of darunavir with protease residues Asp29, Asp30, Gly27 and Gly48, and the same view of the P2 interactions of antiviral inhibitors GRL-0519, GRL-04410 and GRL-05010. The PDB ID is shown for each structure. Water molecules are shown as spheres. Hydrogen bond interactions are indicated by dotted lines.

Table 1

Clinical inhibitors and resistance mutations in HIV-1 protease.

Amino acid	L	V	G	K	L	V	D	30	33	34	36	43	46	47	48	50	53	54	58	60	62	63	64	69	71	73	74	76	77	82	83	84	85	88	89	90	93
Inhibitor																																					
Saquinavir 1995	I				I				<u>Y</u>	V	V						L			V				V	S		I	A	V							<u>M</u>	
	R																							T													
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	10	11	16	20	24	32	30	33	34	36	43	46	47	48	50	53	54	58	60	62	63	64	69	71	73	74	76	77	82	83	84	85	88	89	90	93	
Indinavir 1996	I				M	I	I			I		I	<u>I</u>					V					V	S		V	I	<u>A</u>	<u>Y</u>							M	
	R				R							<u>L</u>												T	A												
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	10				20	24	32			36	46	46	47	50	54	54							71	73	76	77	82	84								<u>90</u>	
Nelfinavir 1997 * w/o RTV	F						<u>N</u>			I	I	I	L										V				I	A	V							<u>M</u>	
	I																						T					F	T							S	
	10					32				46	47	46	47	50	54	54										73	76	82	84								90
Fosamprenavir 1999, 2003	F				I					I	V	<u>Y</u>			L									S		V	A	<u>Y</u>								M	
	I										L				V													F									
	R														M													S									
	V																											T									
	10				20	24	<u>32</u>	34		46	<u>47</u>	46	<u>47</u>	50	53	54						63		71	73	76	82	84								90	
Lopinavir 2000	F				M	I	<u>I</u>	F			I	<u>Y</u>			V	L	V				P		V	S		<u>Y</u>	<u>A</u>	V							M		
	I				R						L	<u>A</u>			L							T						<u>F</u>									
	R														A													<u>T</u>									
	V														M													<u>S</u>									

Amino acid	L	V	G	K	L	V	D	L	E	M	K	M	I	G	I	G	I	F	I	Q	D	I	L	I	H	A	G	T	L	V	V	N	I	I	N	L	L	I	
Location	10	11	16	16	20	24	30	33	34	34	36	43	46	47	48	50	53	54	58	60	62	63	64	69	71	73	74	76	77	82	83	84	85	88	89	90	93		
Atazanavir 2003 *w/ and w/o RTV	I	E	R	I	I	I	I	Q	I	I	I	I	I	I	V	<u>Y</u>	L	L	E	V	E	V	L	L	V	V	C	A	A	A	<u>Y</u>	V	V	<u>S</u>	<u>S</u>	M	L		
	F	M				F	L	L	L	L	L	L	L	V	Y	Y	Y	V				M	M	M	I	S	T	T	T	T	T	T	T	T	T	T	T	M	
	V	I				V	V	V	V	V	V	V	V	M	M	M	T	T	A						L	A	I												
	C	T	V											A	A	A	A	A																					
	10					34	34	36	36	43	46	47	47	54	58	58	58	58	58	58	58	58	58	69	74	74	74	76	82	83	84	84	84	85	88	89	89		
Tipranavir 2005	V					F	I	I	T	L	<u>Y</u>	<u>Y</u>	<u>Y</u>	A	<u>E</u>	A	<u>E</u>	K	<u>P</u>	<u>P</u>	<u>L</u>	<u>D</u>	<u>Y</u>	K	R														
								L	L	V				M	M	V	V																						
	11					33	33					47	47	50	50	54	54																						
Darunavir 2006	I					I	F				<u>Y</u>	<u>Y</u>	<u>Y</u>	<u>M</u>	<u>L</u>	<u>L</u>	<u>L</u>																						

Clinical inhibitors are shown with the year of approval. Major mutations are in **bold** and underlined.

Clinical inhibitors (PIs) and the sites of their drug resistant mutations in HIV protease. PIs are used with ritonavir unless stated.

Adapted from [21].

Table 2

Selected highly resistant protease mutants and Kd for Darunavir.

Protease	Kd DRV (nM)	Relative Kd	Amino acid substitutions
Wild type	0.005	1.0	
PR20 [†]	41.000	8200	Q7K, L10F, I13V, I15V, D30N , V32I , L33F, E35D, M36I, S37N, I47V , I54L , Q58E , I62V, L63P, A71V, I84V , N88D, L89T, L90M
P51 [‡]	37.000	7400	L10I, I15V, K20R, L24I, V32I, I33F, M36I, M46L, I54M , I63P, K70Q, V82I, I84V , L89M
PRdrv1 [§]	15.000	3000	L10I, M36V, M46L, I54V, I62V, L63P, A71V, V82A , I84V , L90M
PRdrv2 [§]	0.750	150	T12V, I13V, I15V, K20M, V32I , L33F, K43T, I54L , K55N, I62V, L63P, A71V, I72V, G73S, V77I, V82L , I84V , L89V, L90M
PRdrv3 [§]	0.004	0.82	I13V, K20R, V32I , L33F, E35D, M36I, R41K, K43T, I47V , I54M , I62V, L63V, A71V, I72T, G73S, T74P , V82L , L89V, I93L
PRdrv4 [§]	35.000	7000	L10F, I13V, D30N , K45I, I50L, L63P, A71I, V77I, N88D
PRdrv5 [§]	0.370	74	L10I, I13V, K14R, V32I , L33F, K43T, M46I, I47V , I54L , I62V, L63P, A71T, I72T, G73T, V77I, P79S, I84V , L90M
PRdrv6 [§]	1.800	360	L10I, I13V, G16E, L33F, M36L, N37T, P39S, K45R, M46L, I54V, K55R, I62V, L63P, A71V, G73D, V82T , I84V , L89V, L90M , I93L

Comparison of drug resistant HIV protease variants bearing multiple substitutions and the DRV binding affinity measured by isothermal titration calorimetry. Assays used different conditions, and may not be directly comparable to each other. Major mutations from Table 1 are shown in **bold** and underlined.

[†]Data taken from [42].

[‡]Selected *in vitro*. Other mutants are from clinical isolates.

[§]Data taken from [41].