HHS Public Access

Author manuscript

Trends Microbiol. Author manuscript; available in PMC 2017 July 01.

Published in final edited form as:

Trends Microbiol. 2016 July ; 24(7): 547–557. doi:10.1016/j.tim.2016.03.010.

Improving Viral Protease Inhibitors to Counter Drug Resistance

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Abstract

Drug resistance is a major problem in health care, undermining therapy outcomes and necessitating novel approaches to drug design. Extensive studies on resistance to viral protease inhibitors, particularly those of HIV-1 and hepatitis C virus (HCV) protease, revealed a plethora of information on the structural and molecular mechanisms underlying resistance. These insights led to several strategies to improve viral protease inhibitors to counter resistance, such as exploiting the essential biological function and leveraging evolutionary constraints. Incorporation of these strategies into structure-based drug design can minimize vulnerability to resistance, not only for viral proteases but for other quickly evolving drug targets as well, toward designing inhibitors one step ahead of evolution to counter resistance with more intelligent and rational design.

Keywords

drug resistance; protease inhibitors; HIV-1 protease; substrate envelope; structure based drug design; resistance mutations

Drug Resistance and Viral Proteases as Drug Targets

Drug resistance is a major health burden in a wide range of diseases from cancer to bacterial and viral infections, causing treatment failure as well as severe economic impact on the healthcare system. Drug resistance can be conferred via various mechanisms including decreased intracellular levels of drug such as due to efflux pumps, altered gene expression, and changes in drug target [1–4]. The most common mechanism of resistance to drugs against quickly evolving targets involves mutation of the targeted protein, including resistance to small molecule inhibitors of viral proteases.

Viral proteases are ideal drug targets as they are essential in the viral life cycle, and inhibiting the viral protease prevents the generation of new infectious viral particles. Most

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viruses that infect humans and cause disease encode at least one viral protease [5, 6]. These proteases are responsible for cleaving the viral polyprotein precursors at specific sites to release individual functional proteins, including *cis* cleavage of the protease itself. Certain viral proteases have also been reported to cleave host cell proteins such as translation initiation factors (eIF4 and eIF3d) in HIV to inhibit host protein translation [7, 8], and transcription factors in hepatitis C virus (HCV) to confound the innate immune response [9]. Although most viral proteases share general backbone folds and catalytic mechanisms with host cellular proteases, they are generally more compact likely due to evolutionary pressure to maintain a small genome [6]. Nevertheless, viral proteases are able to recognize and cleave diverse substrate sequences with distinct specificities.

HIV-1 and HCV Protease Inhibitors

Among medically relevant viruses, the viral protease most extensively investigated is inarguably the aspartyl protease encoded by HIV-1, with hundreds of sequences in the Stanford Database and crystal structures in the Protein Data Bank [10, 11]. HIV-1 protease is comprised of two identical chains of 99 amino acids each, with the active site located at the dimer interface and each monomer contributing a catalytic Asp to the active site (Figure 1a). In the unliganded state, HIV-1 protease is symmetric with highly flexible flaps that allow access to the active site. In the liganded state, these flaps close upon the bound substrate or inhibitor at the active site and become much more rigid. HIV-1 protease has been the target of extensive drug discovery and development efforts for decades and had a major role in launching the field of structure-based drug design. These efforts resulted in 9 FDA-approved HIV-1 protease inhibitors (PIs). All HIV-1 PIs are competitive inhibitors that bind at the protease active site. Although these PIs are very effective in inhibiting the wild-type protease and have significantly contributed to clinical treatment outcomes in combination therapy [12–14], resistance has emerged to all HIV-1 PIs.

HCV, which infects millions of people worldwide and causes chronic liver disease, liver failure and liver cancer [15, 16], encodes a chymotrypsin-like serine protease, NS3/4A (Figure 1b). HCV NS3/4A protease is a prime therapeutic target for direct-acting antivirals, with four FDA-approved inhibitors [17–21] and several in various stages of clinical development. However, even before the drugs were approved for the clinic, resistant viral variants have emerged [22–24]. Rapid emergence of resistance and low efficacy against genotypes other than HCV Genotype 1 has mandated combination therapies, which also decreased treatment duration and increased cure rates especially for Genotype 1 [25–27].

Mutations Confer Resistance by Selectively Weakening Inhibitor Binding but Retaining Specific Substrate Recognition and Cleavage

For a virus to become resistant to a PI, the viral genome acquires mutations that allow the protease to thwart inhibition by the drug but still retain the ability to cleave the viral polyprotein substrate at the required specific sites to allow viral maturation. HIV-1 evolves rapidly due to a high viral replication rate (10^7–10^9 newly infected cells/day in a patient [28]) and the error-prone mechanism of the viral reverse transcriptase, which generates a diverse pool of viral variants. This rapid evolution enables the targeted viral protease to

acquire mutations that abrogate the efficiency of inhibitor–protein binding. Many mutations already pre-exist at low levels even before the start of therapy in infected patients, and quickly become selected under drug pressure. Critically, these selected protease variants still retain their substrate recognition and cleavage activity and allow viral propagation. HIV-1 protease needs to process the Gag and Gag-Pro-Pol polyproteins at nine distinct sites, while HCV NS3/4A protease cleaves viral polyprotein precursors at four cleavage sites and cleaves two human immune proteins to confound the innate immune response. However, these cleavage sites are highly diverse in amino acid sequence, and unlike most known human proteases, the viral proteases do not have simple substrate sequence recognition motifs.

How is a viral protease able to recognize and cleave apparently dissimilar substrates with high specificity? And how can the targeted protease mutate to avoid inhibition but still process these substrates? The answer to these questions for HIV-1 protease led to major breakthroughs in elucidating molecular mechanisms underlying selection of primary mutations in drug resistance.

Substrate Envelope Explains Selection of Active Site Resistance Mutations

Although not similar in amino acid sequence, the substrates cleaved by HIV-1 protease all occupy a similar ‘shape’ or volume when bound at the active site, as revealed by high-resolution crystal structures of protease-bound peptides corresponding to cleavage sites in the viral polyprotein [29, 30]. Hence, the recognition motif for the viral protease is not a particular amino acid or sequence but a common shape assumed by the substrates, termed the substrate envelope (Figure 2). Similarly, the substrate envelope for HCV NS3/4A protease has also been determined as the overlapping volume shared by bound peptides in complex crystal structures [31] (Figure 3).

In addition to explaining how the viral proteases recognize their substrates, the substrate envelope effectively explains the mechanism of resistance due to active site mutations. Active site residues that make essential and conserved interactions with the substrate, including the catalytic side chains, cannot mutate to confer resistance without compromising substrate binding. However, whenever an inhibitor protrudes beyond the substrate envelope to contact a protease residue, that residue can mutate to differentially weaken contact with the inhibitor relative to substrate binding resulting in drug resistance. Accordingly, specific resistance mutations near the viral protease active site that confer resistance to a given inhibitor occur at positions where that inhibitor protrudes beyond the substrate envelope to make contact with the enzyme (Figure 2) that are not necessary for substrate recognition but compromise inhibitor binding.

Correlations between active site resistance mutations and protrusion of a specific inhibitor out of the substrate envelope have been demonstrated for a variety of HIV-1 and HCV PIs [29, 31–34]. HIV-1 protease signature resistance mutations (such as D30N to nelfinavir, I50V/L to amprenavir/atazanavir, G48V to saqunavir, and V82A to saquanavir/ritonavir) all occur where that particular inhibitor contacts the protease active site beyond the substrate envelope (Figure 2). Similarly, the major HCV NS3/4A protease resistance mutations occur

in the S2 pocket residues (R155, A156, and D168) that are contacted by large P2 extension moieties protruding out the substrate envelope [24, 31]. Hence, a structural analysis of the fit of a given inhibitor within the viral protease substrate envelope is a good predictor of active site residues that may mutate to confer resistance to that inhibitor.

Strategies in Designing Against Resistance

Drug design typically focuses on improving affinity against a single target of interest, typically the wild-type variant of the most common genotype in the case of viral proteases. Indeed, all the HIV-1 PIs have been designed on wild-type HIV-1 subtype B, the dominant viral strain in Europe and the Americas. Inadvertently, this current drug design paradigm may actually facilitate the occurrence of drug resistance, since the standard process is to develop potent compounds that very specifically target the wild-type structure of a single subtype. Polymorphisms in other subtypes can severely impact inhibitor potency and lead to different pathways to resistance, as seen in HCV genotype 3 [35] and HIV-1 [36]. Disrupting the activity of a single wild-type target is necessary, but not sufficient, for developing a robust drug that avoids resistance. Instead of trying to combat resistance only after resistant viral variants arise in clinic, we need a paradigm shift to incorporate elucidation of drug resistance mechanisms to the design strategy.

Detailed analysis of molecular resistance mechanisms for HIV-1 and HCV viral PIs has led to several strategies that can be incorporated into the design strategy toward more robust inhibitors, which should extend to other rapidly evolving drug targets (Box 1).

The first strategy is to design inhibitors that fit within the substrate envelope. This strategy originates from the realization that substrate envelope efficiently explains susceptibility to resistance due to active site mutations. As protrusions out the substrate envelope render an inhibitor vulnerable to resistance, avoiding such protrusions will minimize the chances of active site mutations that can confer resistance. Analogous pairs of inhibitors designed to stay within versus extend beyond the substrate envelope supported this strategy [37]. Darunavir (DRV) is the proof-of-concept for the viability and validity of this strategy and is the most potent and robust HIV-1 PI in the clinic [38–40]. Although not designed according to the substrate envelope, DRV fits very well within HIV-1 protease substrate envelope, avoiding unessential contacts that would render the inhibitor susceptible to resistance mutations. Accordingly, no single or even double mutation confers significant DRV resistance; protease needs to extensively evolve to accumulate at least ~7 mutations to escape DRV inhibition in vitro at therapeutic concentrations [38, 41]. In clinical trials, 11 mutations in protease were associated with resistance to DRV and the presence of at least 3 of these mutations was associated with a diminished virological response to this drug [42]. DRV also proves that achieving high potency (single digit picomolar) while staying within the substrate envelope is possible.

How widely applicable is the design strategy of using the substrate envelope to counter resistance due to active site mutations? Both HIV-1 and HCV proteases have diverse substrates that are recognized by shape, which defines the envelope. Similarly, most viral proteases also lack a sequence-based recognition motif and process diverse substrates that

also likely bind to protease in a conserved shape. However, multiple or diverse substrates are not a prerequisite to define a substrate envelope for a given target. The key to this strategy is to define target-substrate contacts (even for a single natural substrate) that are essential for biological function, and avoid any contacts beyond those in inhibitor design.

A complementary strategy in identifying residues that are essential to the target's biological function is exploiting knowledge on sequence conservation. Whether with or without selection pressure, the viral protease can evolve only if the enzyme maintains residues essential for function, including the catalytic residues. Designing inhibitors that pack on these strictly conserved residues minimizes chances of mutations that the enzyme can tolerate to confer resistance. The HCV NS3/4A PI MK-5172 is a good example where the extended P2 moiety protrudes out the substrate envelope but packs on the catalytic residues (rather than the mutation-prone S2 subsite) (Figure 3). In addition to the catalytic residues, other regions that cannot tolerate mutations can be identified by sequence alignment or by systematic probing of the mutational fitness landscape through saturating mutagenesis [43, 44]. In such an analysis, all possible point mutations can be introduced into the protease and the effect on enzymatic fitness measured. Residues that cannot tolerate mutations without compromising fitness may have less chance of mutating to confer resistance.

One strategy in designing against resistance is maximizing hydrogen bonds of the inhibitor with main chain atoms of active site residues. The idea here is that the protease backbone atoms are invariant regardless of the side chain, which can change due to a mutation [45, 46]. While less likely than a side chain hydrogen bond, main chain hydrogen bonds can still be readily lost due to steric effects or shifts in the inhibitor-binding mode due to side chain changes. Therefore, this strategy may be the most effective when the binding mode of natural substrates is taken into consideration, by either mimicking the hydrogen bonds of natural substrates or making sure the inhibitors do not violate the substrate envelope constraints.

Another strategy against resistance, proposed for both HIV-1 protease and reverse transcriptase and may be more generally applicable, is designing flexibility into the inhibitors so that they can slightly shift or change conformation in response to active site mutations [47–50]. One key caveat in applying this strategy may be having an 'anchor' moiety with extensive interactions that are invariant, which can help ensure sustained potency even when part of the inhibitor adapts in response to the mutation.

Increasing Potency to Avoid Resistance

Although common practice is to report fold-changes in affinity (K_i or IC50 values) with respect to wild-type protease, the absolute value of affinity against a given variant is what determines resistance at a given drug concentration. For instance, DRV has a K_i in the low picomolar range against wild-type HIV-1 protease. Even with a 10 or 100-fold change due to a mutation, DRV may still be more potent against a 'resistant' variant compared to other inhibitors with much lower affinity. Hence, increasing potency of a viral PI contributes to the barrier to resistance.

Potency of PIs can benefit from additional design strategies based on recent findings, in addition to standard structure based drug design. First, inhibitors can be modified to more optimally fill the substrate envelope, without compromising low susceptibility to resistance. For example, the P1' moiety in DRV can be extended without violating the substrate envelope, a strategy that resulted in inhibitors even more potent and more robust than DRV [51–53]. Similarly, a comparison of HCV NS3/4A inhibitors with the substrates reveals a large portion of the substrate envelope in the P4-P5 region not utilized by any current inhibitor [54] (Figure 3). Extending inhibitors to this untapped region of the protease active site can enhance inhibitor potency. Another strategy that significantly enhanced HCV PI potency is adding a macrocycle to link either the P2–P4 or P1–P3 moieties [55–57]. The macrocycle is thought to stabilize the inhibitor in a conformation competent for protease binding, thus increasing the entropic contribution to the free energy of binding. However, macrocycle design may need to be optimized between the rigidity required for enhancing entropy versus the flexibility required for adapting to a mutated active site [55, 58].

Can Resistance-Proof PIs Be Designed?

PIs of HIV-1 have two unique advantages toward becoming resistance-proof: (i) PIs have highly cooperative dose-response curves. This implies that at clinical levels of drug concentration, which is typically much higher than IC50 values, very high levels of (or complete) enzyme inhibition can be achieved [59–61]. (ii) PIs are the only transition-state analogs among direct-acting antivirals (DAAs). As transition-state analogs, HIV-1 PIs have unprecedented potential to achieve exceptional potencies, as enzymes have evolved to bind tightest to the transition state.

In clinical practice, emergence of drug resistance by HIV-1 is successfully thwarted by multidrug therapy where the rapid addition of high levels of a combination of drugs presents a high enough genetic barrier so that resistance does not develop. This happens even though resistance would evolve to each of the drugs if they were given sequentially and additively. This clearly shows that there is a limited sequence space the virus can occupy during the time of therapy initiation, and that there is not enough time (i.e. rounds of replication) to explore greater space during the period when viral load is falling in the presence of multiple drugs. Even though there is no imminent effort to completely replace multidrug therapy with a single inhibitor, the possibility of a 'resistance-proof protease inhibitor' is a compelling concept, and may find clinical use in maintenance therapy.

As increasingly potent transition state analogs are developed, the threshold may be crossed where the potency is so high that the addition of one, two, or three resistance mutations does not confer a useful level of resistance and thus does not allow sustained replication. This approach could lead to a path where single drug therapy could be a viable option in a maintenance strategy for treatment-naïve patients, greatly reducing the cost of lifelong therapy. In fact, therapy failure with DRV in such individuals is rarely associated with drug resistance mutations [62], suggesting that failure is primarily due to compliance or extreme metabolism of the drug. In few patients where DRV-containing regimens as first line of therapy fail, in addition to no mutations in the protease, the reverse transcriptase resistance mutations were only rarely observed [63]. Hence, DRV may be close to an inhibitor where

the appearance of the first resistance mutation is insufficient to confer enough of a growth advantage to allow evolution of subsequent resistance mutations, and further improvements in inhibitor potency could lead to resistance-avoiding PIs. A recent clinical study actually found DRV monotherapy to be acceptable for long-term management of HIV infection, with regular viral load monitoring and prompt reintroduction of combination treatment for those who rebound [64]. However, considering the scarcity of data on inflammation and clinical progression, long-term DRV monotherapy needs to be administered with caution and only in selected patients, and we may need to even further improve pharmacokinetics, potency and clinical outcomes for long-term PI monotherapy.

Remaining Challenges in Overcoming Resistance

While we have considerable knowledge on the molecular mechanisms underlying resistance conferred by various protease mutations, still multiple challenges remain in understanding and overcoming PI resistance (see Outstanding Questions). In many drug resistant protease variants, multiple mutations co-evolve to both decrease the affinity of a particular inhibitor, and increase the viability and fitness of the enzyme. In HCV, a single mutation is often enough to confer resistance to PIs, but variants with multiple mutations can be found in clinical samples, as well as multiple changes in amino acid sequence (polymorphisms) across different genotypes. In HIV-1, multi-drug resistant patient isolates contain multiple mutations with up to 25% of the amino acids in the enzyme able to mutate and contribute to resistance [65, 66]. The impact of such combinations of mutations on conferring drug resistance is not simply additive, but instead these mutations can have complex interdependent effects [67, 68]. One remaining challenge is to unravel the interdependency of multiple mutations in conferring drug resistance.

Another challenge is revealing the molecular mechanisms of resistance conferred by mutations at residues outside of the active site (Figure 4). While the substrate envelope efficiently explains the selection of active site mutations, the mechanism of resistance associated with mutations outside the active site still remain elusive. Mutations distal to the active site may compensate for the losses in enzymatic fitness or protein conformational stability due to the active site mutations [69, 70]. In HIV-1 protease, many of these mutations are known to compensate for enzymatic fitness, but these and others may also directly confer resistance. Residues outside the active site that mutate to confer resistance are in the flaps (M46, F53, and I54) and core of the enzyme (L24, L33, L76, N88, and L90). In HCV protease, the distal mutation V36M is commonly associated with resistance [71]. Incorporating the effect of such distal mutations into rational drug design remains a complete challenge. The effect of mutations away from the active site may be propagated to the active site through a network of interactions within the protease, similar to a domino effect, as suggested by a detailed analysis of changes in the interaction network of HIV-1 protease structure due to such mutations (Figure 4A) [72].

While explaining the mechanism by which such mutations outside the active site confer resistance is challenging, one likely mechanism is through alterations in protein dynamics (Figure 4B). HIV-1 protease is a flexible enzyme that undergoes extensive conformational changes to allow opening of the flaps and access to the active site [73–76]. These changes at

the flaps are coupled to extensive side chain repacking at the hydrophobic core, or hydrophobic sliding [77, 78]. Mutations that cause changes in this dynamic process can confer resistance by altering the balance between substrate processing versus inhibitor binding at the active site. Altered dynamics differentially impact substrate processing versus inhibitor binding, as the inhibitor needs to stay bound at the active site for efficient inhibition, while the substrates need to be processed and released for efficient turnover. Such changes in dynamics have been revealed in molecular dynamics simulations as well as experimental NMR and electron paramagnetic resonance (EPR) dynamics of HIV-1 protease drug resistant variants [79–82]. This resistance mechanism through changes in conformational dynamics propagating to the active site [72] may be common to distal mutations in other proteases.

Mutations in other viral proteins beyond protease can contribute to drug resistance. Viral maturation is a complex interdependent process where additional levels of resistance can occur due to mutations in other viral proteins, and especially the protease cleavage sites in the Gag and Gag-Pro-Pol polyproteins. Mutations in the HIV-1 Gag polyprotein co-evolve with primary resistance mutations in the protease [83–87] and some mutations can even directly confer resistance in the absence of protease mutations [85, 88–90]. Thus drug resistance occurs within a complex system of interactions between the protease and the viral and host substrates; with the interdependent mechanisms by which changes outside the protease contribute to resistance remaining to be elucidated. Whether there are certain key signature mutations and/or common mechanisms through which resistance is conferred in highly mutated viral variants is still to be explored.

Concluding Remarks

To avoid resistance, the design of inhibitors against viral proteases needs to consider the essential biological function and evolutionary constraints on the protease. This entails design not just against the wild-type target but considering all potential mutant variants that can exist and still carry out the protease’s biological function. Combining strategies to minimize vulnerability to resistance while enhancing potency can lead toward designing inhibitors that are more robust against resistance. Regardless of whether ‘resistance-proof’ inhibitors are achievable or not, lessons from HIV-1 and HCV proteases can be incorporated into structure-based drug design. Reaching ‘resistance-avoiding’ inhibition is achievable given the constraints on viral evolution as the population size plummets with the initiation of potent therapy. Instead of lagging behind evolution and waiting for resistance to emerge, we can move one step ahead of evolution to counter resistance with more intelligent and rational design.

Acknowledgments

We thank Shurong Hou for help with preparing the figures. This research was supported by National Institute of Health (NIH) Grant P01 GM109767.

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Box 1. Strategies to Design Potent Viral PIs Robust Against Resistance

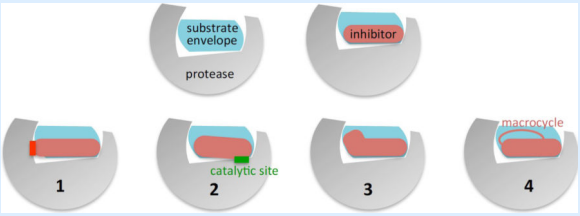

Figure I. Depiction of Strategies for Design of Inhibitors to Prevent Resistance

The substrate envelope of viral proteases can be incorporated into structure-based drug design to minimize the vulnerability of inhibitors to the emergence of resistance. The main strategies to design resistance-avoiding PIs include:

1. Stay within the substrate envelope: avoid contacts with protease residues (red bar) beyond those of the natural substrate(s).
2. Extend beyond the substrate envelope only if contacting invariant conserved residues such as the catalytic side chains (green bar), or toward the solvent.
3. Exploit untapped regions of the substrate envelope to further increase potency.
4. Add macrocycles to preposition the inhibitor in a binding-competent state, making sure the macrocycle itself obeys the rules above.

Outstanding Questions

- How do mutations outside the protease active site confer resistance? What is the molecular mechanism by which the impact of such mutations propagates to the active site?
- How is protein conformational dynamics related to drug resistance? Can we predict resistance from changes in dynamics?
- Are there key signature mutations that are pivotal in conferring resistance in highly mutated protease variants, and/or redundant mutations? Is there interdependency or synergy between coexisting mutations? Can we design inhibitors to avoid them?
- Can predictive and quantitative models of drug resistance be developed based on the kinetic balance between inhibitor binding versus substrate processing? Can the effect of multiple mutations in the protease, substrates sites, and other viral components be incorporated in the models?
- Is a ‘resistance-proof protease inhibitor possible? Is there a reachable potency threshold beyond which a protease inhibitor avoids clinical resistance?

Trends Box

- Viral proteases recognize substrates through a conserved shape, defining the substrate envelope.
- A variant that does not bind inhibitors efficiently but still processes substrates is resistant.
- Resistance mutations occur where inhibitors protrude outside of the substrate envelope.
- High potency inhibitors that fit within the substrate envelope can avoid resistance.
- Resistance-avoiding strategies need to be included in structure-based drug design.

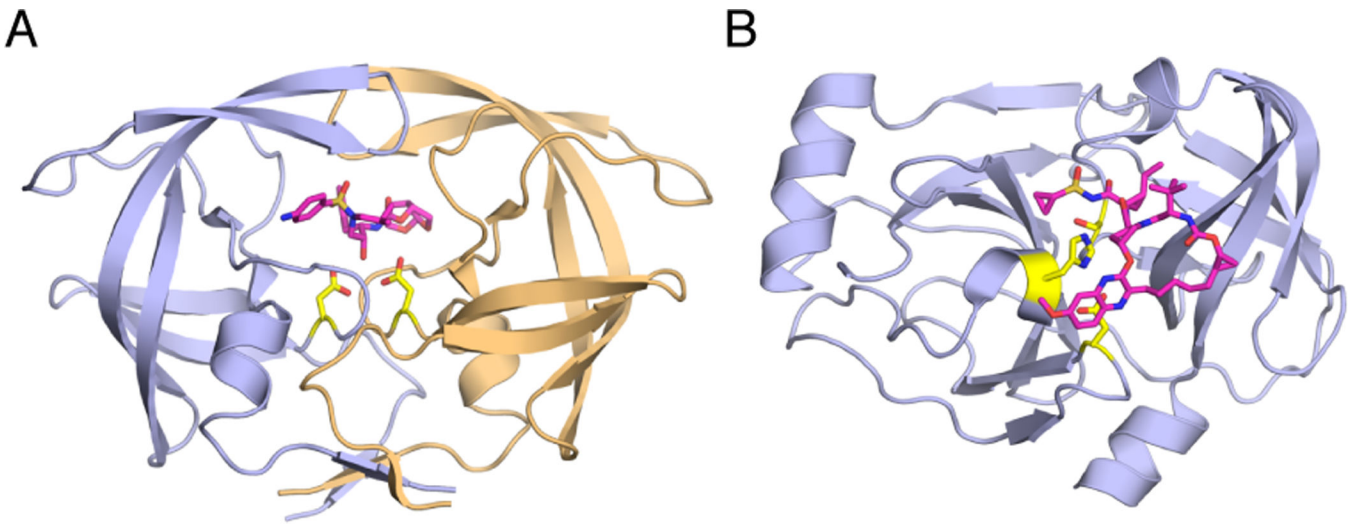

Figure 1.

HIV-1 and HCV Protease Structures. (A) HIV-1 protease bound to darunavir (PDB: 1T3R). The two monomers of HIV-1 protease are in light purple and gold. (B) HCV NS3/4A protease bound to MK-5172 (PDB: 3SUD). The inhibitors are in magenta and the catalytic residues in yellow sticks.

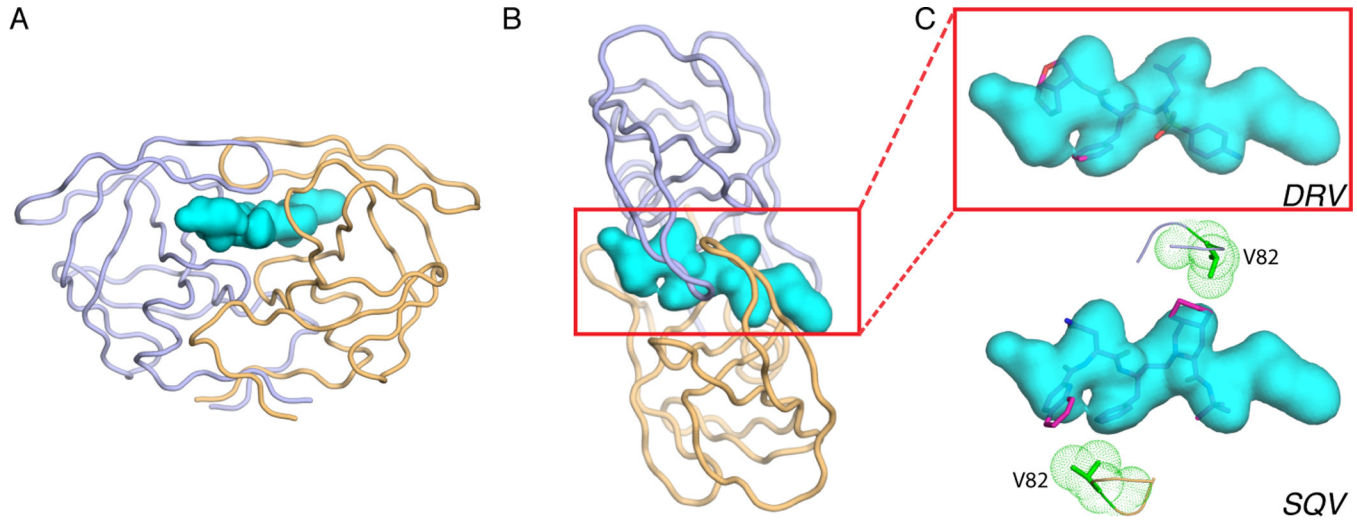

Figure 2.

Substrate Envelope of HIV-1 Protease and Fit of PIs in the Envelope. (A) The substrate envelope (teal volume) displayed at the active site of HIV-1 protease (light purple and gold ribbons), and (B) top view of this. (C) The fit of protease inhibitors darunavir (DRV, red sticks) and saquinavir (SQV, magenta sticks) in the substrate envelope. The side chain of protease residue V82, which mutates to A to confer resistance to SQV, is displayed as green sticks and mesh surface.

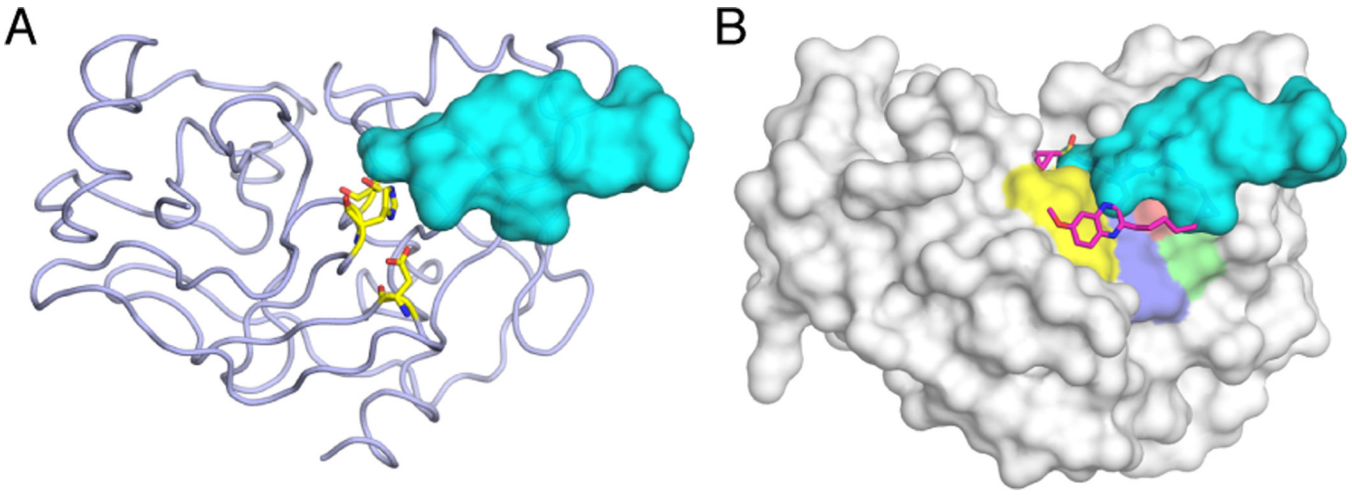

Figure 3.

Substrate Envelope of HCV NS3/4A Protease and the Fit of PI MK-5172 in the Envelope. (A) The substrate envelope (teal volume) displayed at the active site of HCV protease (light purple ribbon). The catalytic side chains are in yellow sticks. (B) The fit of protease inhibitor MK-5172 (magenta sticks) in the substrate envelope. The protease is displayed as a gray surface, with catalytic residues (yellow) and residues at the S2 subsite (purple, green, and red) that mutate to confer resistance colored on the surface. The large P2 extension moiety of MK-5172 protrudes beyond the envelope but avoids contacts with the S2 residues and instead packs against the catalytic residues.

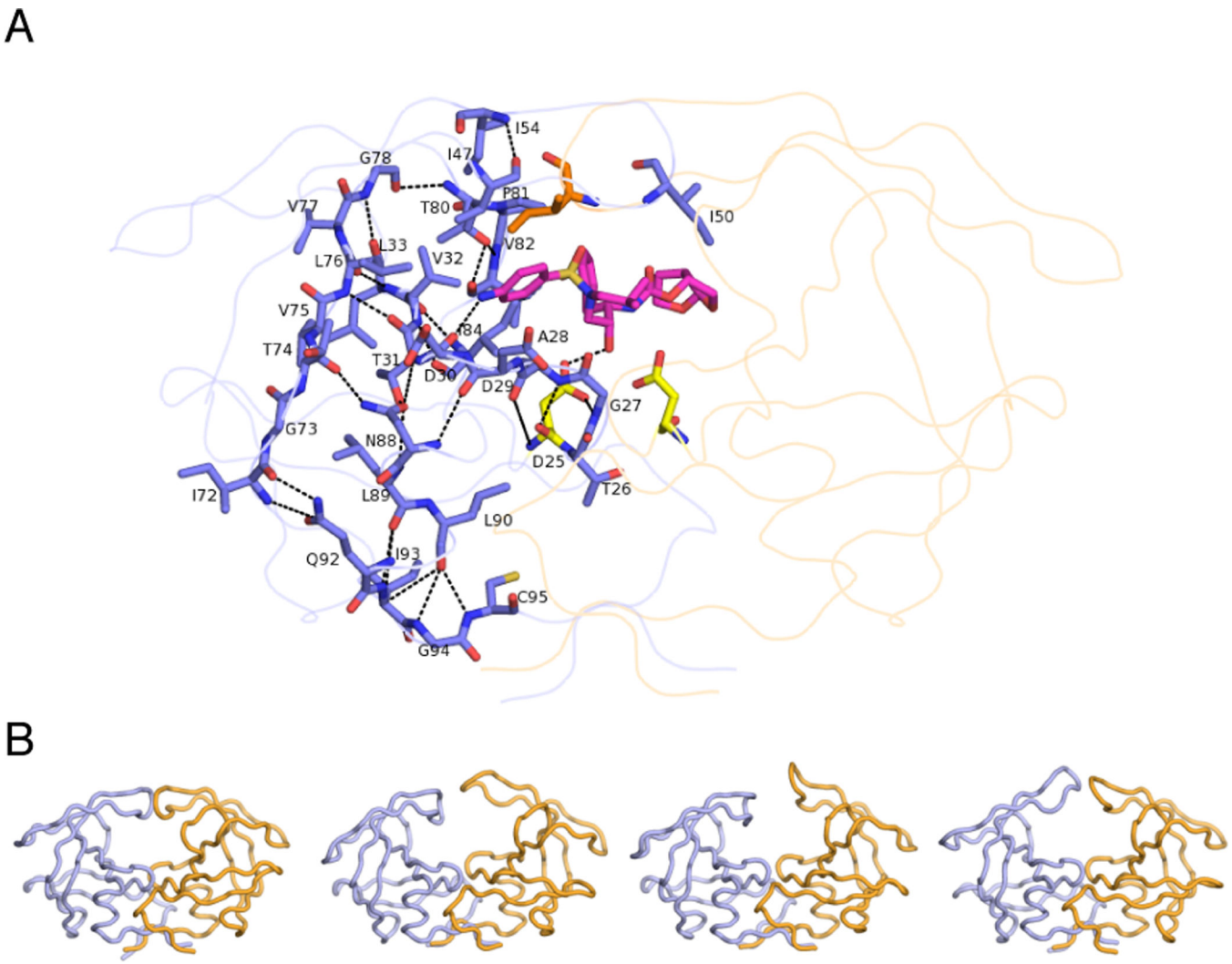

Figure 4.

Mutations Remote From the Active Site Can Contribute to Conferring Resistance. (A) The network hypothesis postulates that the impact of mutations away from the active site can be transmitted to the active site via the network of hydrogen-bonded interactions in the protein (dashed lines, displayed on one monomer of HIV-1 protease). (B) HIV-1 protease is highly dynamic, as revealed in snapshots from MD simulations, and mutations may modulate ligand binding to confer resistance by altering such conformational dynamics.