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HIV-1 protease inhibitors and mechanisms of HIV-1's resistance

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Abstract: Current anti-HIV drugs have significantly improved the prognosis of HIV infected patients so much so that it is now considered a chronic disease, and adherence to medications keeps non-detectable amounts of the virus in the body. However, HIV is still able to generate drug resistance substitutions. Protease inhibitors (PIs) in combination with other classes of anti-HIV drugs constitute an important part of the anti-HIV drug regimen. This article discusses some of the common resistance substitutions against PIs, mechanistic insight on resistance, and potential new inhibitors that can show efficacy against current resistant variants.

***Keywords*:** HIV-1, anti-HIV therapy, protease inhibitors, drug resistance, darunavir

Introduction

Since the identification of human immunodeficiency virus (HIV) as the causative agent for acquired immunodeficiency syndrome (AIDS) in the 1980s, significant progress has taken place not only in the diagnosis and treatment, but also in the education of vulnerable populations and in public health initiatives to combat the spread of the virus. The first set of anti-HIV drugs, AZT, ddI, and ddC targeted the reverse transcriptase of the virus (*1-3*). These drugs were crucial in the initial management of the disease and gave hope to millions of patients worldwide.

Subsequently, drugs targeting the viral protease and integrase were approved (*4-7*). More extensive understanding of the mechanism of HIV replication, cellular pathways involved, and involvement of viral reservoirs have increased the number of potential anti-HIV targets. More than twenty-five drugs have now been approved over the years, and many of them are combined for optimal effective doses (*4,8*). In spite of remarkable progress across many different areas to tackle the disease, several significant challenges in diagnosis and treatment remain. One of the continuing challenges is in HIV being able to generate drug resistance-associated amino acid substitutions that decrease the efficacy of treatment over a period of time (*9*). Research continues for the discovery and development of more potent, safe and long-acting inhibitors.

Protease inhibitors in clinical use

In December 1995, saquinavir (SQV) became the first protease inhibitor (PI) approved for the treatment of HIV-1-infected individuals by the USA Food and Drug Administration (FDA) (*10*). This was a watershed moment in the clinical care and disease management of HIV-1-infected patients. Till that time, AZT, ddI and ddC that targeted the HIV-1 reverse transcriptase, were in clinical use (*4*). However, treatment failed in many instances because of the resistance developed by HIV-1. Following SQV, other protease inhibitors such as ritonavir, lopinavir, atazanavir, fosamprenavir, and tipranavir secured FDA approvals between 1996 and 2005 (*4*). In 2006, darunavir (DRV) became the last PI approved for clinical use. DRV exerted its activity not only because it binds to the active site formed by the protease dimer, but also because it binds to protease monomers and prevents the formation of dimers (*11,12*). The high genetic barrier towards development of resistance of currently used anti-HIV medications, their much-improved safety profile and simplified dosing regimen have led to more rigorous standards for the initiation of clinical trials and the eventual approval of newer inhibitors.

Amino acid substitutions in protease that give rise to resistance

Drug-resistance-associated amino acid substitutions in HIV-1 protease result in a wide range of resistance and are somewhat arbitrarily categorized into major and minor substitutions (*9*). The major substitutions appear earlier during drug treatment and may result in a significant decrease in the efficacy of the drug. Minor substitutions often appear later, and may not

necessarily make the virus more resistant, but is thought to contribute to improving the replication fitness of the virus. Though the difference between major and minor substitutions is not clearly defined, mostly because of a lack of mechanistic understanding of how these substitutions contribute to resistance, such classification has helped in a simplistic understanding – hence we will use the same terminology with the caveat that some of the so called minor substitutions might be significantly contributing to HIV's resistance and the mechanism of their impact and contribution has not been fully deciphered so far. The following are some of the well-known resistance-generating major substitutions: D30N for nelfinavir; V32I for atazanavir and lopinavir; M46I/L for indinavir; I47V/A for darunavir, lopinavir, and tipranavir; G48V for saquinavir; I50L/V for atazanavir, darunavir, lopinavir and fosamprenavir; I54L/V/M for atazanavir, darunavir, and lopinavir; V82A for atazanavir, lopinavir, and indinavir; I84V for atazanavir, darunavir, lopinavir, fosamprenavir, and indinavir; and L90M for nelfinavir and saquinavir (Figure 1). Some residues commonly classified as minor resistant substitutions are L10F, K20M, L24I, L33F, M36I, and A71V (Figure 1). Some substitutions may be major for some PIs while such substitutions may be classified as minor for others. Clinically, substitutions may arise as a drug-resistance-conferring substitution(s) for one inhibitor, but can cause resistance against other inhibitors – a phenomenon known as cross-resistance.

New protease inhibitors to combat drug resistance

The quest to discover anti-HIV inhibitors that are more potent and long-acting with improved safety and pharmacological properties is an area of vigorous basic, pre-clinical, and clinical research. The design, synthesis, and virological assays of new chemical entities are required to improve the potency, resistance, and safety profile of PIs. In that regard, inhibitors that form polar interactions with backbone atoms of the protease have been examined (*13-15*). The rationale is that interactions of inhibitors with the protease backbone atoms are likely to be mostly maintained even if HIV-1's protease-encoding gene undergoes amino acid substitutions. Besides having moieties that have interactions with the backbone atoms, inhibitors with macrocyclic moieties that can more effectively occupy the active site cavity have also been explored (*15*). The crystal structure analysis of GRL-216, a protease inhibitor with a *bis*-tetrahydrofuran (*bis*-THF) and macrocyclic moiety showed that it not only formed several polar interactions with key protease residues, but also more effectively packed the S1′-S2′ binding pocket and formed better van der Waals (vdW) contacts with Val82 and Ile84 than did darunavir (*15,16*). Design and synthesis of inhibitors with more interactions with the protease flaps have also been explored. GRL-078 and GRL-079 have an alkylamine at the C-5 position of the P2-tetrahydropyrano-tetrahydrofuran (Tp-THF) and have a P2′ alkyl-aminobenzothiazole (Abt) moiety (*17*). These P2 and P2′ substituents form additional vdW interactions with the protease flaps, and are probably responsible for their impressive potencies against HIV_{NL4-3} and multi-drug-resistant HIV-1 variants (*17*). We reported GRL-044, a PI with a P2-Tp-THF, P1-methoxybenzene, and P2'-isopropyl-aminobenzothiazole (Ip-Abt) (*18*). The antiviral EC_{50} values of GRL-044 against wild-type $HIV\text{-}1_{NL4-3}$ and HIV-2 were 0.0028 and 0.0004 nM,

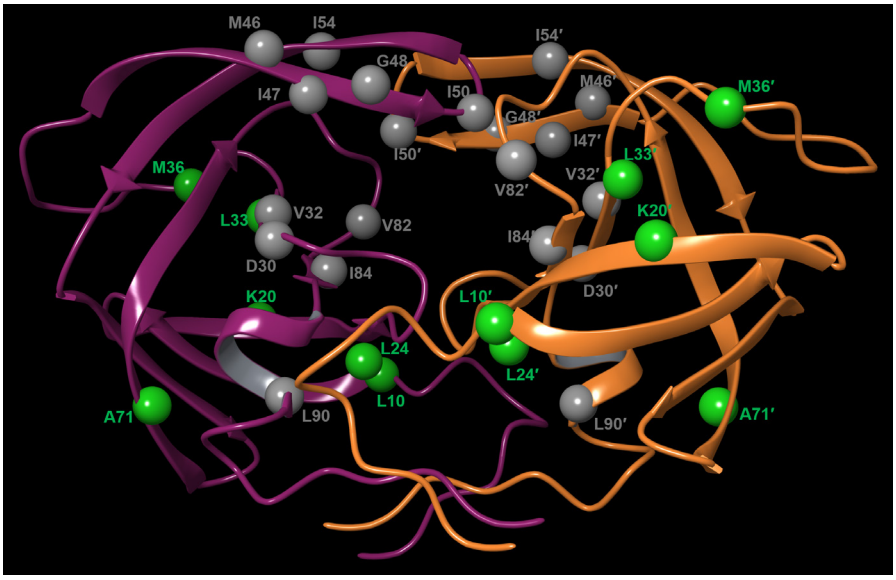

Figure 1. Drug resistance-associated mutations in HIV-1 protease. The drug-resistance-associated mutations are shown by small spheres corresponding to the locations of the alpha carbons. Mutations that are in general classified as major are shown in gray spheres and those generally classified as minor are shown in green spheres. The protease ribbons are shown in orange and maroon colors.

respectively. GRL-044 had antiviral EC_{50} values ranging from 0.065 to 19 nM against various PI-resistant HIV-1 variants (*18*). In selection assays, the emergence of HIV-1 variants resistant to GRL-044 was significantly delayed compared to that against DRV (18). Structural analyses showed that the larger size of GRL-044 over DRV, enabled GRL-044 to occupy a larger volume of the active-site hydrophobic cavity in the protease and contributed to the greater potency of GRL-044 against HIV-1 (*18*) (Figure 2 A-D). GRL-121 and GRL-142 were discovered by further optimizing the interactions of these inhibitors with HIV-protease (*19,20*). Both GRL-121 and GRL-142 have a P2 moiety consisting of 6-5-5-ring-fused crown-like tetrahydropyranofuran (Tp-THF) and a P2′ moiety consisting of a cyclopropyl aminobenzothiazole (Cp-Abt) moiety. The only difference between these inhibitors is in the P1 moiety: GRL-121 has a phenyl group whereas GRL-142 has a *bis*-fluorobenzyl group. While GRL-121 had impressive anti-HIV activity (*20*), GRL-142 had much better potency against wild-type and many drug-resistant variants with IC_{50} values in attomolar to picomolar ranges (*19*). X-ray crystal structure showed that GRL-142 had polar and non-polar interactions with many protease residues, and these interactions must be responsible for its strong binding to the active site in HIV-1 protease (Figure 3 A and B). GRL-142 was initially discovered as a protease inhibitor, but subsequent research demonstrated that it also had activity against HIV-1 integrase (*21*). The activity of GRL-142 against two viral proteins (protease and integrase) essential in the HIV-replication life-cycle is likely responsible for its attomolar to nanomolar activity against many HIV-1 variants. We further explored the effects of the number and location of fluorine substituents on the P1-phenyl moiety and replacement of Cp-Abt of GRL-142 with isopropyl-aminobenzothiazole (Ip-Abt) or isopropyl-aminobenzoxazole (Ip-Abo) as a P2′ ligand (*22*). While these molecules had very good activity against wild-type and many drug-resistant variants, they were not better than those of GRL-142 (*22*).

Darunavir and Val32Ile substitution in HIV-1 protease

DRV is the most widely used PI and has a high genetic barrier against the development of resistance by HIV-1. The presence of multiple mutations is often needed for HIV to develop resistance against DRV. It was reported that the combination of V32I, L33F, I54M and I84V substitutions (Figure 1) in the protease developed high resistance levels against DRV (*23*). HIV-1 having only V32I substitution is sensitive to DRV, in line with structural studies that show that Ile32 has a better vdW contact with DRV than does Val32 (*24*). Moreover, HIV-1 with V32I substitution had reduced viral fitness. We found that V32I substitution rarely occurs in HIV-1 protease but when it does occur, it triggers HIV-1 to accumulate additional substitutions such as L33F, I54M and I84V that allow HIV-1 to develop high-level resistance against DRV (*24*). Since DRV is the only PI once recommended as a first-line therapy, the study suggested that for patients already having V32I substitution, initiating or continuing DRV therapy might be potentially problematic.

Mechanisms of HIV-1's resistance to protease inhibitors

Crystal structures of HIV-1 protease and the PIs have been instrumental in deciphering the structural basis of drug resistance (*25-27*). These structures have illuminated the changes in interactions between the PIs

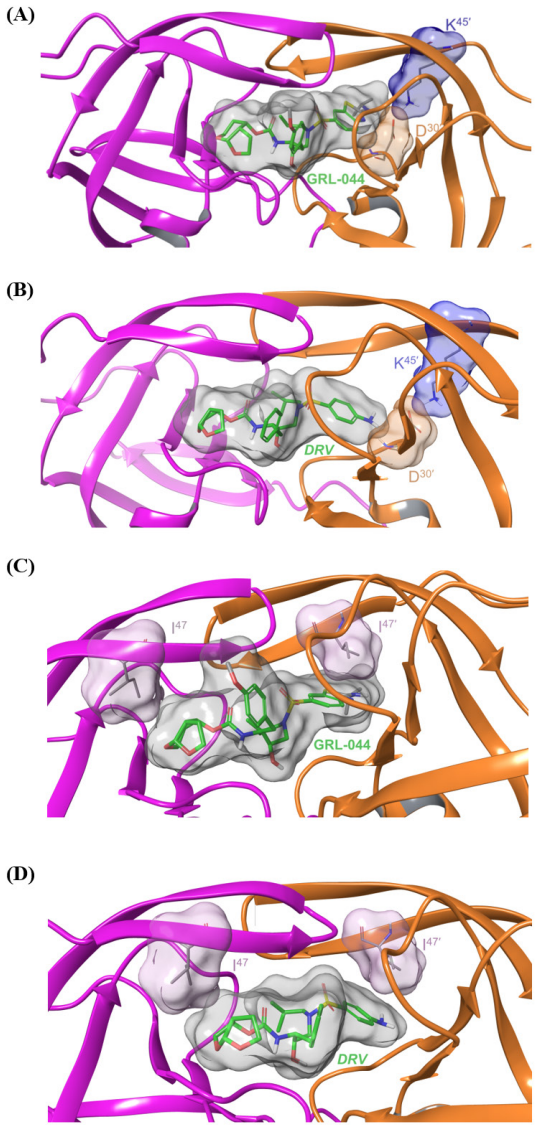

Figure 2. van der Waals surface interactions of GRL-044 and DRV. Comparison of the vdW surface interactions of GRL-044 and DRV with selected protease residues is shown in panels **A-D**. GRL-044 and DRV surfaces are in gray. D30', K45', and I47 surfaces are in orange, blue and plum, respectively. (Figure reproduced from Aoki *et al*. *Glob Health Med*. 2019; 1:36-48.)

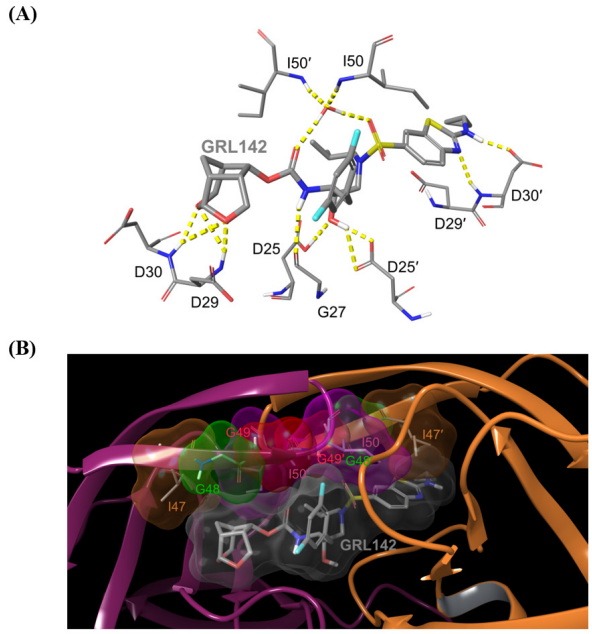

Figure 3. Polar and nonpolar interactions of GRL-142. Panel **A**: The hydrogen bond interactions (yellow dashed lines) of GRL-142, a highly potent PI, with active site residues are shown. Panel **B**: The van der Waals surface interactions of GRL-142 with HIV-1 protease flap residues are shown. The surface of GRL-142 is shown in gray; the surfaces of I47, G48, G49 and I50 are shown in aquamarine, green, red and magenta, respectively.

and HIV-1 protease with various substitutions. Pawar *et al*. determined the x-ray structure of GRL-1111 with HIV-1 protease with V32I, I47V and V82I substitutions (*26*). Though there is a significant loss of activity for the triple mutant (K_i: 31.4 ± 3.9 nm) compared to inhibition of PR_{wt} (K_i: 2 to10 pm), the x-ray-derived structure of GRL-1111 with the triple mutant protease showed only minor changes in interactions compared to interactions with PR_{wt} (*26*). Ghosh *et al*. overlayed the crystal structure of GRL-06579 with PR_{wt} against mutant proteases having two to eleven substitutions (*25*). These mutant proteases were crystalized with different PIs. Despite of the differences in inhibitors and protease amino acid sequences, the overlay of the alpha-carbon positions of the protease backbone produced root mean square deviation (RMSD) in the range of 0.5 -1.1 Å (*25*). This and other studies have shown that there is minimal structural fluctuation in the secondary structures of inhibitor-bound wild-type and drug-resistance-associated proteases. However, the side-chain configurations and contacts of these residues with the inhibitor are different between the wild-type and mutant proteases.

Recently, for an experimental PI and a keto-DRV-bound HIV-1 protease, a combination of x-ray and neutron diffraction was used to unambiguously assign the position of hydrogen atoms and the protonation/deprotonation states of the catalytic aspartates enabling differentiation of the tetrahedral intermediate as an oxyanion or a gem-diol (*28*). The study provided important insight on peptide hydrolysis catalyzed by HIV-1-protease and on the design of more potent inhibitors to overcome HIV-1 resistance (*28*).

Molecular dynamics (MD) simulations have shed light on the dynamical features of the protease-inhibitor complexes. They have helped understand the local variations and perturbations in the structural interactions. Foulkes-Murzycki *et al.* carried out MD simulations and proposed that the distal residues, many of which are in the hydrophobic core of the protease with limited solvent accessibility can easily exchange one hydrophobic vdW contact between PR_{wt} residues for another vdW contact between mutated residues with negligible energetic penalty (*29*). They postulated that changes in these hydrophobic contacts might be responsible for conformational changes in the protease (*29*). Rana *et al.* carried out MD and binding energy calculations to study the effect of M46I mutation on generating resistance against SQV (*30*). This resistance-associated mutation has clinically been observed in certain populations. They observed that M46I mutation induced wider opening of the protease flaps and significantly decreased the vdW interactions and free energy of binding of SQV to PR_{M46I} compared to PR_{wt} (*30*).

Wong-Sam *et al*. studied a protease with 10 mutations (L10F, V32I, M46I, I54L, L63P, A71V, L76V, I84V, L89V and L90M) that show resistance to LPV and DRV (*27*). They generated crystals of an apo form of this protease, and one bound to GRL-519, a potent experimental PI. The apo crystal structure of the mutant protease shows a highly curled flap conformation, and MD simulations showed extremely large fluctuations of the protease flaps (*27*). The total number of hydrogen bonds and non-polar interactions were identical for GRL-519 complexes with wild-type and the 10-mutant protease. However, the nature of interactions were different at the active site, flap-core interface, hydrophobic core, hinge region and the 80s loop (*27*). The study highlighted that mutations in highly resistant protease variants work cooperatively to reduce binding and generate resistance against PIs.

Discussion

As per the UN AIDS fact sheet (*https://www.unaids.org/en/resources/fact-sheet*), nearly forty million people globally were living with HIV-1 in 2023 out of which nearly thirty-one million had access to antiretroviral therapy. Protease inhibitors along with other classes such as nucleoside and non-nucleoside reverse transcriptase, fusion, attachment, and integrase strand transfer inhibitors have made significant impact in the quality of life and longevity of HIV-1-infected individuals. Still there is a long way to go. Darunavir, the last approved PI, has been in clinical use for more than fifteen years. Recently, it is used in combination with other nucleoside and/or integrase inhibitors (*4*). Though DRV has a high genetic

barrier against the emergence of resistance, mutations can still accumulate and decrease its potency (*9*). A lot of progress has been made in simplifying the dosage intervals and requirements. The recent FDA approval of lenacapavir, a long-acting HIV-1 capsid inhibitor which can be administered subcutaneously, has increased the available options (*31*). One of the continuing challenges with protease inhibitors is that most of them are readily metabolized by cytochrome P450-3A4. Ritonavir is a potent inhibitor of cytochrome P450-3A4, and is used as a pharmacokinetic booster when another P450-3A4-sensitive PI is administered. A side effect of ritonavir is that it causes a bad taste in the mouth – a factor that might also have negatively impacted wider adoption of nirmatrelvir, an inhibitor targeting the main protease of SARS-CoV-2 (*32*). A new anti-HIV-1 protease inhibitor not only needs to have significantly better potency against drug-resistant variants, but needs to have no toxicity and favorable pharmacokinetics and dosing requirements to be considered for clinical utility.

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