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Review

Comprehending the Structure, Dynamics, and Mechanism of Action of Drug-Resistant HIV Protease

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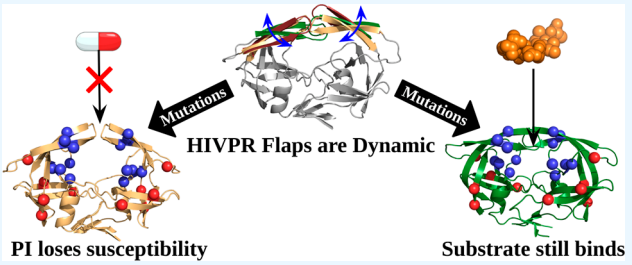
ABSTRACT: Since the emergence of the Human Immunodeficiency Virus (HIV) in the 1980s, strategies to combat HIV-AIDS are continuously evolving. Among the many tested targets to tackle this virus, its protease enzyme (PR) was proven to be an attractive option that brought about numerous research publications and ten FDA-approved drugs to inhibit the PR activity. However, the drug-induced mutations in the enzyme made these small molecule inhibitors ineffective with prolonged usage. The research on HIV PR, therefore, remains a thrust area even today. Through this review, we reiterate the importance of understanding the various structural and functional components of HIV PR in redesigning the structure-based small molecule inhibitors. We also discuss at length the currently available FDA-approved drugs and how these drug molecules induced mutations in the enzyme structure. We then recapitulate the reported mechanisms on how these drug-resistant variants remain sufficiently active to cleave the natural substrates. We end with the future scope covering the recently proposed strategies that show promise to deal with the mutations.



INTRODUCTION

The Human Immunodeficiency Virus (HIV) causes the disease called Acquired Immuno-Deficiency Syndrome or AIDS. HIV infection impairs the patient's immune system by affecting CD4 T lymphocyte counts, leading to immunodeficiency.[1] This makes the patient susceptible to opportunistic infections, like Salmonella, pneumonia, and herpes, and the compromised immune system makes these infections fatal. Consequently, AIDS has turned into a significant health issue affecting 38.4 million people worldwide and has led to a casualty of around 650,000 people in 2021 alone.[2] It was first detected in the United States in the 1980s and misnamed as Gay Related Immune Deficiency (GRID) or Gay Compromise Syndrome due to the occurrence of rare forms of pneumonia and other opportunistic infections among a high number of homosexual men.[3] Later, it was found to occur among women and intravenous drug users of both sexes.[4] In 1982, the Center for Disease Control (CDC) changed the name from GRID to AIDS. In 1983, Françoise Barré-Sinoussi and Luc Montagnier discovered a retrovirus in a patient with swollen lymph glands.[5] Later in 2008, they were awarded the Nobel Prize in Medicine for the discovery of HIV. In 1985, the first international AIDS conference in Atlanta was convened to discuss this new emerging disease. The FDA in 1987 approved the first drug for the treatment of HIV infection, azidothymidine (AZT),[6] which is an inhibitor of the reverse transcriptase enzyme of the virus. Since then, different classes of drugs, such as nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors, protease inhibitors (PIs), fusion inhibitors, and coreceptor antagonists[7] have been approved and used individually or in combination for the treatment of HIV-AIDS. The method of administering a cocktail of drugs referred to as Highly Active Antiretroviral Therapy (HAART) has prolonged and improved the quality of the patient's life but is still ineffective in eliminating the viral infection completely.

PIs are a class of drugs, often used in HAART. The HIV protease (HIV PR) is an integral enzyme in the life cycle of HIV as it is the only protease in the virus. It performs the proteolysis of gag and gag-pol, two long translated polypeptides, into all the structural proteins like matrix (MA), capsid (CA), and nucleocapsid (NC) and functional proteins like protease (PR), reverse transcriptase (RT), RNase H (RH), and integrase (IN).[8] There are currently 10 FDA-approved HIV PR drugs, namely, saquinavir (SQV), indinavir (IDV), ritonavir (RTV), amprenavir (APV), fosamprenavir (FPV), nelfinavir (NFV), lopinavir (LPV), tipranavir (TPV), atazanavir (ATV), and darunavir (DRV).[9,10] Nine of these ten inhibitors are transition state analogs—they competitively bind to the active site of the protease by mimicking the transition

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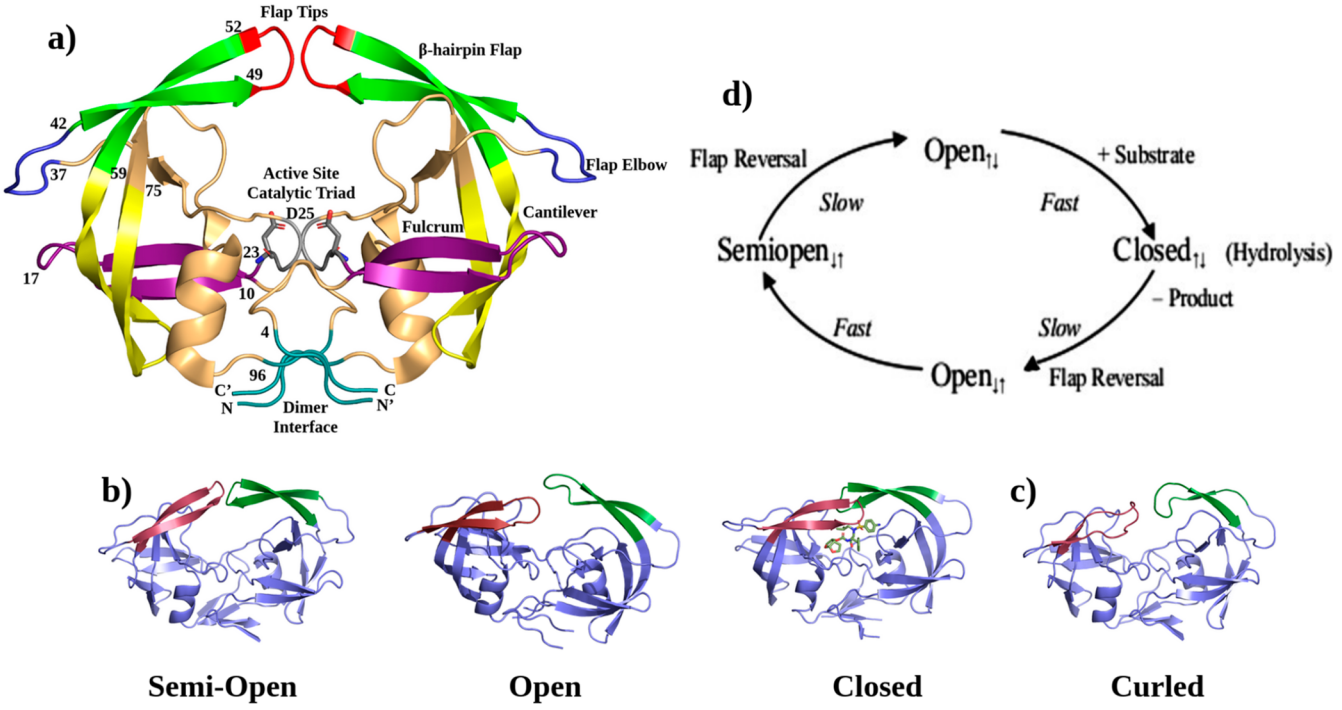

Figure 1. Structure of HIV protease. (a) Functionally relevant regions are shown in HIV PR structure with PDB ID 1HHP. Licorice gray: active site catalytic triad, residues 25−27; red: flap tips, residues 49−52; green: beta-hairpin flaps, residues 43−58; blue: flap elbow, residues 37−42; yellow: cantilever, residues 59−75; magenta: fulcrum, residues 10−23; and cyan: terminal dimer interface, residues 1−4 and 96−99. (b) Different conformational states of HIV PR: semiopen (PDB ID: 1HHP), open (PDB ID: 2PC0), and closed (PDB ID: 3SO9). (c) Curled flap conformation (PDB ID: 5B18), an alternative to the open conformation. (d) Flaps attain different conformations at different stages in the catalytic cycle. Figure adapted with permission from ref 28. Karthik et al. Copyright 2011 Wiley-Liss, Inc.

state of the substrates undergoing proteolysis, resulting in the inhibition of HIV PR activity.[11] TPV is the sole inhibitor that acts through a mechanism different from the nine others mentioned—it inhibits protease function by forming hydrogen bonds with the flaps, without mimicking the transition state.

The early studies on HIV PR were majorly focused on unraveling its nucleotide sequence and substrate specificity through chemical synthesis and gene expression in *E. coli*.[12,13] The first breakthrough in understanding the structure of HIV PR came in 1989 with the solving of its crystal structures.[14,15] Crystal structures of HIV PR with and without various inhibitor molecules, alongside computational studies like molecular dynamics (MD) simulations and protein−ligand docking, have unearthed a wealth of information about the protease and its inhibitors. This has paved the way for developing new and improved inhibitors against HIV PR. This method of designing inhibitors against HIV PR on the basis of the protease structure is considered to be one of the biggest success stories in Structure-Based Drug Designing (SBDD).[16] In this review, we aim to understand the structural and functional components of HIV PR, how inhibitors were designed through SBDD where the current pitfalls in drug designing lie in terms of drug-resistant mutants, and what mechanisms are being implemented to bypass the protease drug resistance.

STRUCTURAL AND FUNCTIONAL COMPONENTS OF HIV PROTEASE

The first crystal structure of HIV PR was determined in 1989 by Navia et al. at 3 Å resolution.[14] Since then, the number of structures of this enzyme has increased dramatically, as evident from the 666 plus structures deposited in the Protein Data Bank (PDB) of the wildtype and mutant HIV PR in free and ligand-bound forms. This abundance of information has helped identify various structural regions within the HIV PR with a shared analogy to microbial aspartyl proteases, including the active site region.[14] The functional form of HIV PR is a homodimer where each monomer is composed of 99 residues.[15] As seen in Figure 1a, the structural components of HIV PR consist of a beta-hairpin loop/flap, flap elbow, flap tip, fulcrum, cantilever in each monomer, and the active site catalytic triad at the dimer interface. The protease elbow region and terminal dimer interface are conceivably the two known allosteric sites. The beta-hairpin loops, commonly known as flaps, act as the gate and regulate the access of ligands to the active site pocket.[17] The flap elbow, fulcrum, and cantilever regions help in the opening of the flaps and regulate the flap dynamics.[18] Understanding the flap dynamics is crucial in modulating the function of the enzyme or in designing inhibitors against it.[17,19] Several attempts over the past decades have indicated that these flaps can exist in closed, semiopen, or fully open conformations,[18] as shown in Figure 1b. Through unrestrained MD simulations, Simmerling and co-workers have shown that an unliganded protease can exist in an ensemble of different conformations and interchange among the semiopen and closed states—predominantly populated by the semiopen state.[18] These authors also proposed an open flap confirmation to coexist for facilitating the ligand entry. This was later confirmed experimentally through the X-ray crystallographic structure (PDB ID: 2PC0) in 2007 by Heaslet et al.[20] An alternative to the open conformation, curled open, has also been detected via electron paramagnetic resonance (EPR)

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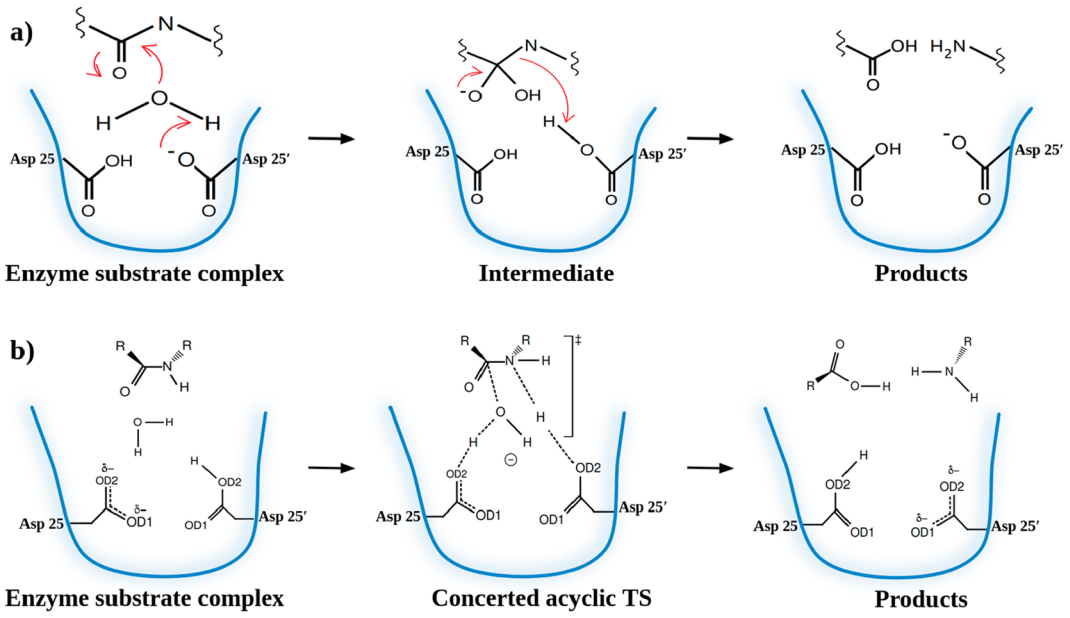

Figure 2. Mechanisms of substrate hydrolysis. (a) Step-by-step reaction mechanism that involves the formation of a tetrahedral intermediate, the rearrangement of which leads to the hydrolyzed products. (b) Concerted reaction mechanism that involves the formation of an acyclic transition state, where the water molecule and the protonated Asp attack the scissile peptide bond simultaneously.

studies.[21,22] Presented in Figure 1c, this curled conformation is hypothesized to be seen when salt bridges between the Glu-35−Arg-57 are lost due to a polymorphic mutation, E35D.[22] The ensemble of different conformational states of the flaps has also been evidenced by EPR studies, wherein the mutations and polymorphisms in HIV PR are shown to affect the population distribution of the flaps.[23,24]

NMR data have suggested that the ligand-free WT protease predominantly exists in the semiopen state.[17,25] However, the binding of ligand induces a change in flap conformation that leads to the formation of a closed state for the ligand−enzyme complex.[26,27] X-ray crystallographic studies have indicated that the enzyme attains an open flap conformation for the ligand entry prior to its binding.[20] In an MD simulation study, starting from the semiopen structure, we have elucidated the mechanism by which these different conformations of HIV PR transit from one to another.[28] We have found that the semiopen protease passes through a metastable open conformation in a slow transition and then quickly reaches to the closed state as the substrate enters the active site pocket (Figure 1d). It was also observed that the flap ordering or handedness undergoes a change from a bottom-to-top orientation in the semiopen state to a top-to-bottom orientation in the open state and remains the same in the closed state. After the substrate was hydrolyzed in the closed state, the protease transits to a different open conformation with bottom-to-top flap ordering before returning to the semiopen state rapidly in the same flap ordering. Another group used techniques such as temperature accelerated MD (TAMD) and string method in collective variables (SMCV) to show that disengagement of the flap tips at Ile50−Ile50′ is a major contributor in flap opening[29] and could be rate-limiting, which slows down the transition from the semiopen to open state. Many drug-resistant mutants of HIV PR are reported to show changes in flap conformations. For example, mutation of L76V leads to the loss of interaction of flaps with the protease core. This leads to increased flap mobility and rapid inhibitor dissociation.[30] Other examples include mutations such as M46I which affects the flap flexibility[31] and I50V which is attributed to the curling of flaps.[32]

UNDERSTANDING THE HIV PROTEASE ACTIVE SITE AND ITS ACTIVITY

HIV PR, an aspartic protease, has an active site consisting of a conserved catalytic triad of aspartate-threonine-glycine or D25-T26-G27, which hydrolyzes the scissile peptide bond. Preliminary studies showed that, like any other aspartic protease, HIV PR is also inhibited by pepstatin A, and when HIV PR undergoes point mutation at the active site from aspartate to asparagine, threonine, or alanine,[8,33,34] the inhibition is lost. Previous experimental studies provided evidence for the existence of various protonation states of the active site residues Asp25 and Asp25′, in double-protonated, single-protonated, and double-deprotonated states.[35] Notably, in the pH range of 2.5 to 7.0, HIV PR is reported to exist in a monoprotonated state; i.e., one catalytic aspartate is protonated, and the other is deprotonated according to NMR and neutron diffraction studies.[36,37] A recent study used constant pH MD simulation (CpHMD) to show that these varying protonation states of Asp25 and Asp25′ are crucial for the catalytic activity as it regulates the internal structural and flap dynamics, which in turn affects the binding affinity of the ligands to the protease.[38]

One of the currently accepted mechanisms of HIV PR activity is elucidated in Figure 2a. Here, one of the Asp is in a protonated state and the other in a deprotonated state, carrying a negative charge. The negatively charged Asp activates a nucleophilic water molecule, which in turn attacks the carbonyl group of the scissile bond, generating an oxyanion intermediate. This tetrahedral intermediate then undergoes rearrangement to produce hydrolyzed peptides.[39] This is a step-by-step mechanism. A different mechanism of concerted catalytic attack has also been proposed.[40] This has recently been tested by using quantum mechanical/molecular mechan-

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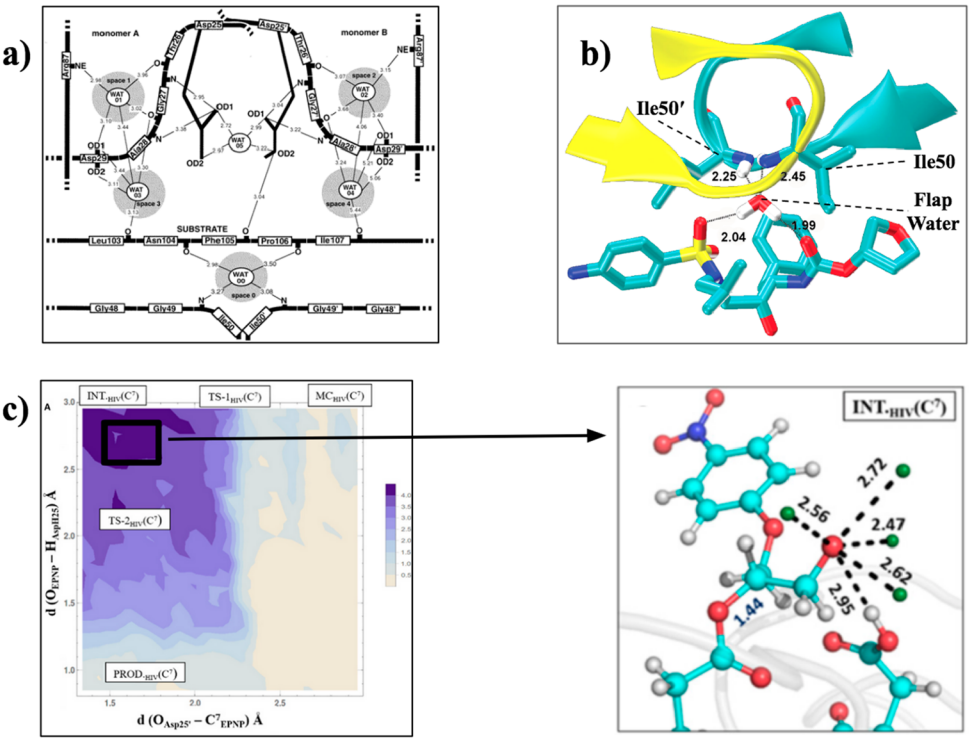

Figure 3. Structural water in HIV PR. (a) Catalytic water (WAT05) forms a bridge between the active site residues Asp25, Asp25′, and an incoming ligand, which is necessary for hydrolysis. Figure adapted with permission from ref 49. Okimoto et al. Copyright 2000 American Chemical Society. (b) Flap water bridges between the flaps and incoming ligand to assist flap closing. Yellow and cyan ribbons: flap A and B; licorice: the ligand; amprenavir; flap water and flap tip residues Ile50 and Ile50′ are labeled. Figure adapted with permission from ref 52. Singh et al. Copyright 2008 American Chemical Society. (c) Number of water molecules that appear at different states during the epoxide ring opening reaction. Water plays a cocatalytic role here. Inset shows the presence of multiple H-bonds between water (green spheres) and the epoxide molecule (ball-n-stick) in the intermediate state. Figure adapted with permission from ref 50. Ahsan et al. Copyright 2019 American Chemical Society.

ics hybrid (QM/MM) simulation studies.[41] In this study, the authors compared possible mechanistic routes for the catalytic activity of HIV PR passing through either a concerted acyclic TS or a concerted 6-membered cyclic TS with one or two water molecules. The concerted acyclic TS route had the lowest activation barrier among the three proposed models, and that compares well with the experimentally derived values for activation free energy. The mechanism is shown in Figure 2b. Once the substrate is in the catalytic cavity, the nucleophilic catalytic water and the electrophilic proton from the protonated Asp25 attack the scissile bond simultaneously, forming an acyclic TS. The scissile bond breaks, and the catalysis products are released in a one-step reaction process. To support this mechanism, many recent QM/MM-based studies have been devoted.[41,42] However, this is still a theoretical proposition and lacks an experimental proof to support the one-step concerted acyclic mechanism. On the other hand, the first mechanism, a stepwise model, has recently been verified through X-ray and neutron crystallographic studies where the tetrahedral oxyanion intermediate could be traced.[43]

BOUND WATER IN THE PROTEASE ACTIVE SITE

The crystal structures of HIV PR have shown several structural water molecules bound to their active site. NMR and MD simulation studies have also shown the presence of multiple water molecules in the ligand-bound HIV PR that interface the enzyme−ligand interactions.[44,45] These water molecules modulate the ligand binding affinity by altering the enthalpy and entropy of the ligand−protease interaction.[46−48] The two important structural water molecules traced in the HIV PR active site that play a crucial role in protease activity are the catalytic water and the flap water.[48] The catalytic water is buried in the active site and forms a bridge between the catalytic aspartates, Asp25-Asp25′, and the incoming substrate (Figure 3a).[43,49] It then acts as a nucleophile for hydrolyzing the substrate, as shown from the QM/MM studies.[41,50] This water molecule could be traced mostly in the crystal structures of the unliganded protease but is absent in inhibitor-bound structures as the inhibitors are designed to mimic the tetrahedral transition state of the bound substrates and water.[51] The flap water forms a bridge between the two flap tips and the bound ligand, thereby interfacing the protease−ligand interaction.

In a MD simulation study, we have shown that the flap water forms a tetrahedral H-bonding network by acting simultaneously as an acceptor for two H-bonds from Ile50−Ile50′ and as a donor of two H-bonds to the carbonyl group of the ligand.[52] Our results also highlighted that this water plays a crucial role in flap closing dynamics after the ligand enters and binds to the active site. During the simulations, the starting open flap conformation of HIV PR transformed to the closed conformation immediately after the flap water spontaneously appeared between the ligand and protease flaps to mediate the ligand−protease interactions, as shown in Figure 3b.[52] However, as a consequence of multiple mutations, this conserved flap water molecule was found to be missing in the multidrug-resistant variants of HIV PR, such as in

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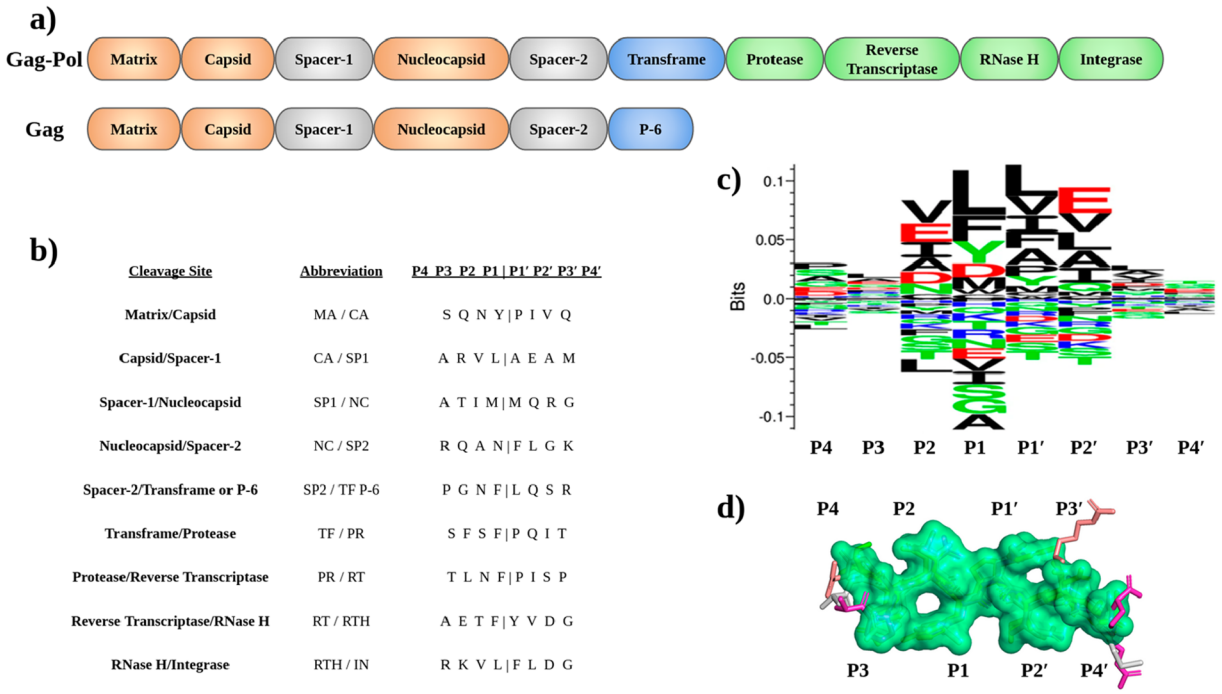

Cleavage Site	Abbreviation	P4 P3 P2 P1 | P1′ P2′ P3′ P4′
Matrix/Capsid	MA / CA	S Q N Y | P I V Q
Capsid/Spacer-1	CA / SP1	A R V L | A E A M
Spacer-1/Nucleocapsid	SP1 / NC	A T I M | M Q R G
Nucleocapsid/Spacer-2	NC / SP2	R Q A N | F L G K
Spacer-2/Transframe or P-6	SP2 / TF P-6	P G N F | L Q S R
Transframe/Protease	TF / PR	S F S F | P Q I T
Protease/Reverse Transcriptase	PR / RT	T L N F | P I S P
Reverse Transcriptase/RNase H	RT / RTH	A E T F | Y V D G
RNase H/Integrase	RTH / IN	R K V L | F L D G

Figure 4. HIV PR substrates. (a) Sequence of various structural and functional proteins in the gag-pol and gag polypeptides. (b) Sequence of amino acids and various cleavage sites in the substrates. (c) Sequence logo of cleavable octapeptides. Red: negatively charged residues, blue: positively charged residues, green: polar uncharged residues, black: hydrophobic residues. Figure adapted with permission from ref 63. Onah et al. CC BY license. Copyright 2022 The Authors. (d) Substrate envelope of HIV PR. Substrates are shown in licorice and in different colors, while the substrate envelope is shown in the surface representation and highlighted in green.

MDR769. These mutations cause the protease to have a "wide-open" conformation that is 8 Å wider than the "open" conformation of the wild-type protease.[53] As a result, the active site cavity expands, losing the network of interactions of the ligand with the active site and flap water.[54] There are several other conserved structural water molecules reported to be present in the protease active site. These water molecules aid in substrate recognition and maintenance of the susceptible protease conformation for action, as shown by several MD simulation studies and XRD data.[49,54−56]

Bound water was also reported to play a cocatalytic role in the epoxide ring-opening reaction during the irreversible inhibition of HIV PR by epoxide-based molecules.[50,57] In a recent QM/MM study, we have shown the spontaneous appearance of a group of water molecules in the reaction field that stabilize the epoxide ring through the formation of an oxyanion intermediate (Figure 3c).[57] These water molecules, by virtue of their "low-barrier H-bonds" with the epoxide ring, reduce the intrinsic reaction barrier while remaining structurally unperturbed and thus play a catalytic role in the reaction.[50] By quantum mechanical calculations, we have also shown that strong ionizing power of water allows better charge delocalization to stabilize the transition state and facilitates the reaction.[57]

HIV PR SUBSTRATES

HIV PR proteolyzes gag and gag-pol, the two long polypeptides, into the structural proteins, matrix (MA), capsid (CA), and nucleocapsid (NC) and functional proteins protease (PR), reverse transcriptase (RT), RNase H (RH), and integrase (IN), as shown in Figure 4a. *In vitro* kinetics studies have shown that a minimum length of seven amino acid residues is required for processing the substrate by HIV PR.[58,59] The substrate amino acids are denoted as Pn···P4−P3−P2−P1−P1′−P2′−P3′−P4′···Pn′, where the scissile peptide bond is between P1 and P1′. The active site of HIV PR has subsites S4−S1 and S1′−S4′ that interact with the residues P4−P1 and P1′−P4′ on the substrate, respectively.[60] HIV PR exhibits unique specificity in its substrate cleaving sites, as listed in Figure 4b. In general, the PR substrates are asymmetric and do not have a consensus sequence. The traditional classification of HIV PR substrates falls into three broad categories based on the amino acid type at the P1−P1′ positions: (a) aromatic−proline (e.g., MA-CA, TF-PR, and PR-RT), (b) hydrophobic−hydrophobic (e.g., CA-SP1, SP1-NC), and (c) others.[61]

Predicting new sequences recognized and cleaved by HIV PR can help design potent inhibitors with enhanced binding ability. In this context, numerous experimental and computational studies involving multiple sequence alignments, machine learning, etc. have been employed to predict the cleavage site and sequence specificity. In a recent work, from the kinetic measurements of 150 distinct HIV PR substrate variants, two motifs for the cleavage site of the substrates are proposed, namely, NΩ/PI (N: Asn, Ω: aromatic Phe or Tyr, P: Pro, I: Ile) and βΦ/ΦE (β: β-branched aliphatic, Φ: hydrophobic, Φ: hydrophobic, E: Glu).[62] A comprehensive bioinformatics study evaluated the performance of different machine learning models for predicting HIV PR cleavage sites for octapeptide sequences using descriptors such as bond composition, amino acid binary profile (AABP), and physicochemical properties.[63] The study suggested that the predictive performances of the logistic regression model and multilayer perceptron classifiers model are on par with the state-of-the-art linear support vector machine model. The authors also plotted a sequence logo from

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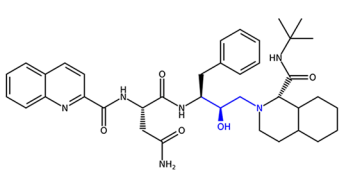
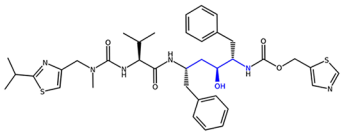
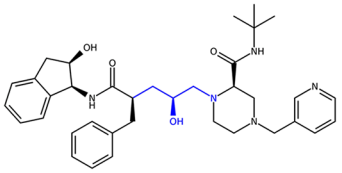
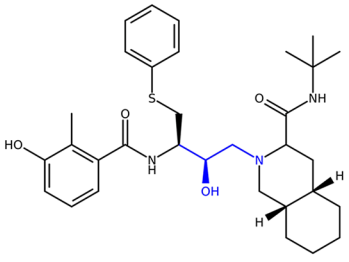
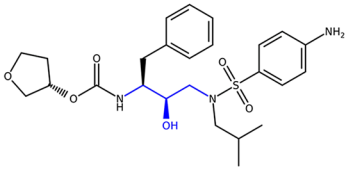
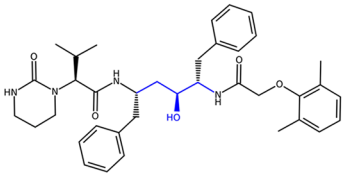
Table 1. FDA-Approved HIV PR Drugs, Their Structure, and Major Characteristics

FDA Approved Drugs against HIV PR		
Saquinavir (SQV) -1996		• Peptidomimetic drug designed to mimic Phe at P1, and Pro with decahydro-isoquinoline-3-carbon at P1′ of substrate • Poor bioavailability • EC50 ~ 37.7nM
Ritonavir (RTV) -1996		• Peptidomimetic drug designed with a long isopropyl thiazolyl P3 group • Also boosts the system concentrations of other protease inhibitors by acting as inhibitor of Cytochrome P450 3A4, which metabolizes other protease inhibitors • EC50 ~ 22nM
Indinavir (IDV) - 1996		• Peptidomimetic drug with a basic amine incorporated into its backbone to increase its potency • Increased the risk of renal toxicity and kidney stone formation • Low Solubility • EC50 ~ 5.5nM
Nelfinavir (NFV) -1997		• Peptidomimetic drug containing decahydro-isoquinoline-3-carbon and 2-methyl-3-hydroxybenzamide at its termini. • Also has an S-Phenyl group at P1 position which increases its potency. • EC50 ~ 30-60nM
Amprenavir (APV) - 1999		• Peptidomimetic drug derived from hydroxyethylene sulfonamide. • Benzyl group at P1 and Isobutyl at P1′ site, which incorporates well with pseudo symmetry of HIV PR active site • EC50 ~ 12-80nM
Lopinavir (LPV) - 2000		• Peptidomimetic drug designed with hydroxyethylene core, phenoxyacetyl at P2 and cyclic urea in P2′ position of substrate • higher potency against drug-resistant mutants • Approved as a co-formulation with low doses of ritonavir. • EC50 ~ 17 nM

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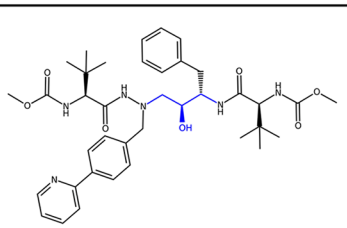
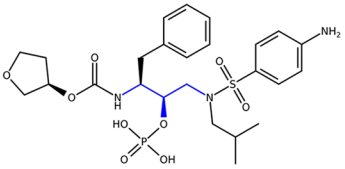
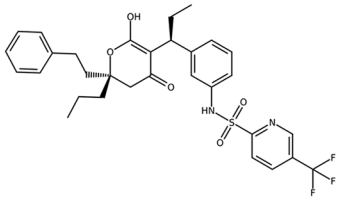
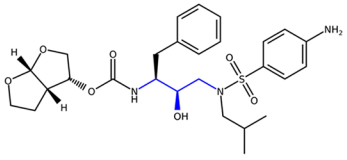
ACS Omega 2023, 8, 9748−9763

Table 1. continued

FDA Approved Drugs against HIV PR		
Atazanavir (ATV) - 2003		• Peptidomimetic drug designed with aza-hydroxyethylene core, phenyl pyridyl group at P1 and benzyl group at P1′ of substrate • good oral bioavailability and lesser side effects than other protease inhibitors • EC50 ~ 2.6-5.3nM
Fosamprenavir (FPV) - 2003		• Peptidomimetic drug which is the phosphate ester prodrug of APV • Replaced APV in HAART • Better bioavailability, lower pill burden and slower release • EC50 ~ 12-80nM
Tipranavir (TPV) - 2005		• Non-peptidomimetic drug with a dihydropyrone scaffold • Directly forms a H-bond with the flap residue Ile50 without the need for the bridge forming water molecule, as is required in the case of substrate or other inhibitors • Also inhibits the dimerization of HIV PR monomers[77] • EC50 ~ 30-70nM
Darunavir (DRV) - 2006		• Peptidomimetic drug derived from APV with a bis-tetrahydrofuran group in P2 site instead of a tetrahydrofuran • Has better hydrogen bond interaction with Asp29 • Also inhibits the dimerization of HIV PR monomers[77] • EC50 ~ 1-2nM

multiple sequence alignment of cleavable octapeptide sequences to predict the frequency of amino acids at each cleavable subsite (Figure 4c). Most of the predicted cleavable sites in the sequence logo match very well with the above-mentioned traditional classification categories.

Prabhu-Jeyabalan et al. noted that, after reaching the protease active site, the substrate binds to the active site in an extended conformation, burying approximately 900−1000 Å^2 area of the total binding pocket.[55] The substrate backbone forms conserved hydrogen bonds with protease residues Gly27, Asp29, Gly48, Arg8, and Asp30 and with water molecules that stabilize the extended conformation.[55] A large shape complementarity between the substrate and HIV PR binding pocket is also noted by these authors. An overlap of all the natural substrates that have been crystallized is shown in Figure 4d. It exhibits a common morphology with a toroid shape in the nonprimed residue side. The toroid shape primarily pertains to the overlap of packing of the different side chains and/or the change in backbone configurations of the different substrates. This consensus substrate envelope manifests to a conserved shape and volume occupied by all the natural substrates when bound to the protease active site.[64] This could explain how the HIV PR recognizes substrate cleavage sites despite their varied sequences.[55] This finding later brought the "substrate envelope hypothesis" that explains how mutations in the active site confer resistance only to the inhibitors and not to the substrates and also gave insight into designing mutation-resistant inhibitors.[64]

HIV PROTEASE DRUGS AND THE ASSOCIATED MUTATIONS

The success of structure-based drug discovery against HIV PR was primarily due to a detailed understanding of the structural and functional components of the protease. Inhibitors designed against HIV PR bind to the active site of the enzyme with high affinity by maintaining strong structural contacts, thus modulating the PR activity. As mentioned above, currently there are ten FDA-approved HIV PR inhibitors. All these drugs

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ACS Omega 2023, 8, 9748−9763

Table 2. List of Major and Minor Mutations in the HIV PR Driven by the Currently Used FDA-Approved Drugs in HAART[a]

PI	SQV/r	IDV/r	NFV	FPV/r	LPV/r	ATV/r	TPV/r	DRV/r
Major mutations	G48VM	V32I	D30N	V32I	V32I	V32I	V32I	V32I
	I54VTALM	L33F	L33F	L33F	L33F	L33F	L33F	L33F
	V82AT	M46IL	M46IL	M46IL	M46IL	M46IL	M46IL	I47VA
	I84V	I47V	I47V	I47VA	I47VA	I47V	I47VA	I50V
	N88S	I54VTALM	G48VM	I50V	G48VM	G48VM	I54VAM	I54LM
	L90M	L76V	I54VTALM	I54VTALM	I50V	I50L	V82LT	L76V
		V82ATFS	V82ATFS	L76V	I54VTALM	I54VTALM	I84V	V82F
		I84V	I84V	V82ATSF	L76V	V82ATFS		I84V
		N88S	N88DS	I84V	V82ATFS	I84V		
		L90M	L90M	L90M	I84V	N88S		
					L90M	L90M		
Minor mutations	L10IRV	L10IRV	L10FI	L10FIRV	L10FIRV	L10IFVC	L10V	V11I
	L24I	K20MR	M36I	G73S	K20MR	G16E	M36ILV	T74P
	I62V	L24I	A71VT		L24I	K20RMITV	K43T	L89V
	A71VT	M36I	V77I		F53L	L24I	Q58E	
	G73S	A71VT			L63P	E34Q	H69KR	
	V77I	G73SA			A71VT	M36ILV	T74P	
		V77I			G73S	F53LY	N83D	
						D60E	L89IVM	
						I62V		
						I64LMV		
						A71VITL		
						G73CSTA		
						I85V		
						I93LM		

[a]Except NFV, all other PIs are used in combination with Ritonavir, which is both an HIV PR and cytochrome P450 inhibitor (/r denotes that the PI was given in combination with RTV).

with the exception of TPV are peptidomimetic drugs designed to mimic the transition state of the protease−substrate complexes with a nonhydrolyzable hydroxyethylene core.[65−72] TPV is the only nonpeptidomimetic drug with a dihydropyrone ring scaffold which forms a direct bond with the flap (Ile 50, Ile 50′) residues.[73] Saquinavir, Ritonavir, Nelfinavir, and Indinavir belong to the first generation of HIV PR inhibitors designed to interact tightly with the protease binding pocket and have a high binding affinity. The remaining ones are second-generation HIV PR inhibitors, which have been designed to be effective against drug-resistant mutants and have greater bioavailability. Table 1 presents the structure and characteristics of FDA-approved HIV PR inhibitors. Even though the second-generation HIV PR drugs are performing better, the enzyme is still developing mutations resulting in reduced drug susceptibility. Consequently, in recent years, the third generation PIs came in the pipeline to overcome the drug resistance menace. These newer generations of drugs have a varied mode of binding and interactions with HIV PR and are designed to counteract the drug resistance. As an example, Ghosh et al. have used the "backbone binding" strategy to design inhibitors like TMC126 and GRL142 that tightly bind to the backbone atoms of the HIV PR.[74] Raines et al. have used the ability of boronic acid to form multiple hydrogen bonds with HIV PR to design boronic-acid-based inhibitors that show 20-fold greater binding affinity than DRV while maintaining their binding affinity even in the presence of mutations, such as D30N.[75] Bungard et al. have designed inhibitors with a novel bicyclic piperazine sulfonamide core, which can directly interact with flap residue Ile50, without the need of flap water molecules.[76] These inhibitor molecules are still under extensive research and/or in preclinical testing.

A major factor for the failure of the HIV PR inhibitors was a decrease in drug susceptibility due to mutations in the HIV PR. Mutation patterns depend on the inhibitors used for the treatment and their interaction with the protease. In some patients who received a combination of inhibitors (as in HAART), more complex mutations were seen in comparison to those who have received one PI treatment. The drug resistance is caused by the gradual accumulation of mutations in the HIV PR which do not hinder the protease function but block the competitive inhibition caused by the inhibitors. Since these mutants retain their substrate recognition competency but are no longer inhibited by the PIs, they cause a reduction in the binding affinity of the PI while maintaining the natural interactions with substrates.[78] The first observed drug-resistant mutation was V82A, which led to the loss of van der Waals contacts between the protease and inhibitor.[79,80] Several other mutations affecting van der Waals and hydrophobic interactions are also reported. Based on the site of the mutation and when it occurs during the treatment, mutations are classified as primary or secondary. Primary or major mutations are those that directly affect the PI binding; they occur in active site regions during the initial phase of PI therapy and can cause a decrease in drug susceptibility or virological failure. As these major mutations affect the active site, they increase the binding pocket volume or cause a reduction in the number of van der Waals or H-bonding interactions, effectively reducing the binding free energy of the PIs. They could also hinder the natural functioning of the protease and can thereby reduce the replicative capacity of the virus.[81,82] This sometimes leads to coevolution or coemergence of mutations in the substrates to compensate for this effect.[83,84] Another class of mutations that occur due to prolonged PI

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ACS Omega 2023, 8, 9748−9763

therapy are called secondary or minor mutations. Minor mutations work in tandem with the major mutations to decrease PI susceptibility more significantly and/or increase the replicative capacity of the virus. These secondary mutations are commonly seen as the natural polymorphic variants of the virus and are most often located at distal sites of the protease.[9,81,85] These minor mutations are away from the active site region and cause PI resistance indirectly.[86,87]

Table 2 presents a comprehensive list of the major and minor mutations seen in HIV PR after treatment with the currently used FDA-approved PIs in HAART.[88] These mutations are known to efflux the PIs through conformational changes in HIV PR. To understand these conformational changes, we performed a series of MD simulations of HIV PR−drug complexes where all the reported major and minor mutations are introduced in the protease sequence. All-atomic MD simulation was performed on each of the complexes for 100 ns each using AMBER20. After incorporating all the corresponding mutations in the open flap protease conformation of HIV PR (PDB ID: 2PC0), the drug molecule was docked in the enzyme active site, and subsequently the complexes were subjected to MD simulations. The simulation protocols were adapted from ref 52. The time-averaged structures of these complexes from MD simulation trajectories are shown in Figure 5. Interestingly, the flaps in all cases exhibited large-scale dynamics and remained in an open to wide-open conformation, despite the presence of the drug molecules in the active site.

In general, some mutations cause specific resistance to a particular PI, while some other mutations cause cross-resistance to multiple PIs.[9,89] For example, mutations like D30N and N88D are specific to NFV; I50L is specific to ATV; and V82L is specific to TPV. Meanwhile, cross-resistance mutations like V32I, G48V, I54VL, V82F, I84V, and L90M decrease susceptibility among most of the PIs, whereas mutations like I47V, G48M, I50V, L76V, V82A, and N88S decrease the susceptibility of four to five PIs.[90] On the other hand, certain mutations that lead to a drop in susceptibility for some PIs can increase the susceptibility for other PIs. For example, I50L lowers the susceptibility for ATV but increases the susceptibility for IDV, LPV, NFV, RTV, and SQV;[91] L76V lowers the susceptibility of LPV, DRV, APV, and IDV but increases the susceptibility for ATV and SQV;[92] and D30N lowers the susceptibility for NFV but increases the susceptibility for SQV and APV.[93] TPV is nonpeptidomimetic in nature and directly binds to the flap region (Ile50, Ile 50′) instead of interacting via a bridge water molecule as in the case of the other PIs. This leads to a nonconventional mutational profile for TPV, due to which it has been used in salvage therapy for patients with multi-drug-resistant HIV infection. However, over time, TPV has also failed due to accumulation of several unique mutations like K43T, Q58E, H69K, and V82L.[94]

An *in vitro* study on various HIV PR variants with combined active and nonactive site mutations (ANAMs) has shown that the inhibition constants (K_i) for several FDA-approved drugs increase significantly up to 78000-fold in the ANAM variant, compared to the wild-type protease (Table 3). On the other hand, the inhibition constant for the variants with active site mutations alone showed only up to a 33-fold increase. Thus, these results suggest that the nonactive site or secondary mutations play a very crucial role in reducing the PI susceptibility.[95] From the isothermal titration calorimetric (ITC) measurements, the same authors have shown that these mutations affect the binding enthalpy of the drug molecules to the protease, which eventually manifests in the reduced drug susceptibility (Figure 6a).[96]

Despite such interesting experimental observations, the mechanism of how these distal mutations affect PI susceptibility is not fully understood. An earlier MD simulation

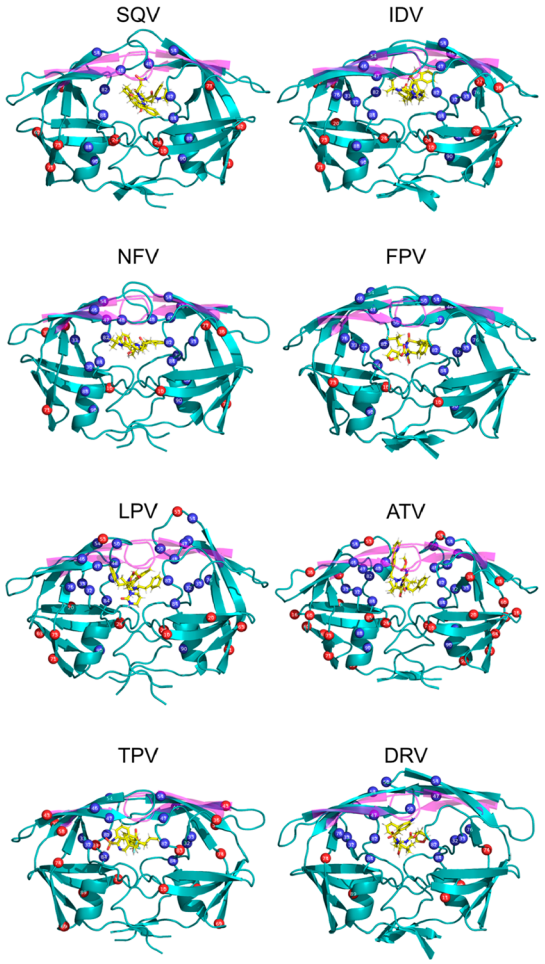

Figure 5. Mutations in HIV PR affect flap conformations. Time-averaged structures of the drug-bound HIV PR with reported major and minor mutations. All-atomic MD simulation was performed on each of the FDA-approved drug-bound variant HIV PRs for 100 ns each. The major and minor mutations are highlighted in blue and red spheres, respectively. The mutated PR structure is shown in cyan, while the flaps from the reference WT closed conformation (PDB ID 4LL3) are highlighted in magenta. Drug is in a yellow stick.

Table 3. K_i Values of Different FDA-Approved Drugs When Bound to the Wild Type (WT) and HIV-1 Protease Variants[a]

Inhibitor	WT (nM)	AM (nM)	NAM (nM)	ANAM (nM)
Indinavir	0.543 (1)	4.4 (8)	481 (890)	1102 (2030)
Nelfinavir	0.254 (1)	1.9 (7)	445 (1750)	732 (2840)
Saquinavir	0.463 (1)	2.0 (4)	755 (1630)	1948 (4200)
Ritonavir	0.027 (1)	0.91 (33)	340 (12600)	2107 (78000)

[a]AM: active site mutations, NAM: nonactive site mutations, ANAM: active and nonactive site mutations. The fold change in K_i values is shown in parentheses. Data adapted with permission from ref 95. Muzammil et al. Copyright 2003 American Chemical Society.

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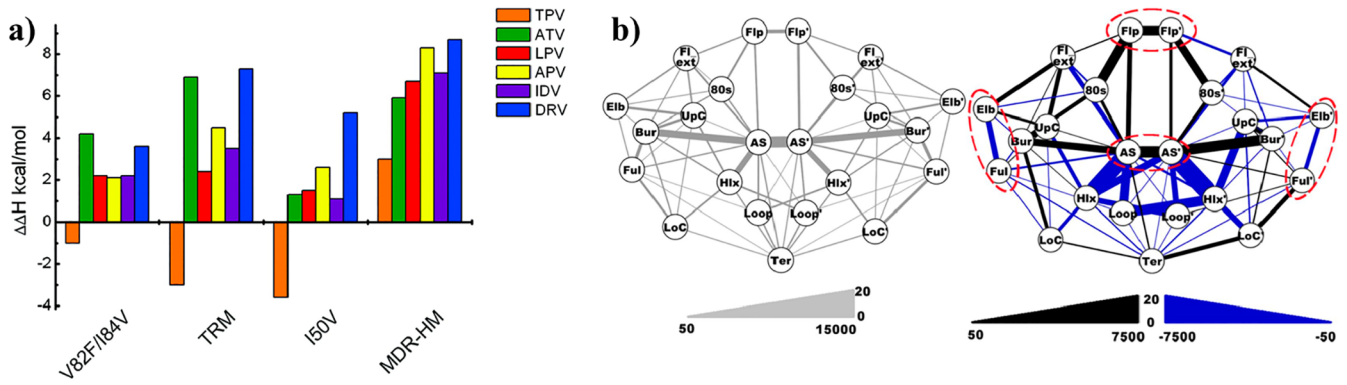

Figure 6. Mutations in HIV PR. (a) Loss in binding enthalpy of the drug molecules due to mutations in the protease. TRM (I13V/V32I/L33F/K45I/V82L/I84V) and MDR-HM (L10I/L33I/M46I/I54V/L63I/V82A/I84V/L90M) variants with both primary and secondary mutations exhibit greater loss in binding. Figure adapted with permission from ref 96. Muzammil et al. Copyright 2007 American Society for Microbiology. (b) Differential network of communications among the protease functional regions: WT (left) and NAM (right). Thicker edges represent greater information flow between the communities/functional regions. Blue edges represent stronger communications in the variant and black edges in the WT. Figure adapted with permission from ref 97. Appadurai et al. Copyright 2016 American Chemical Society.

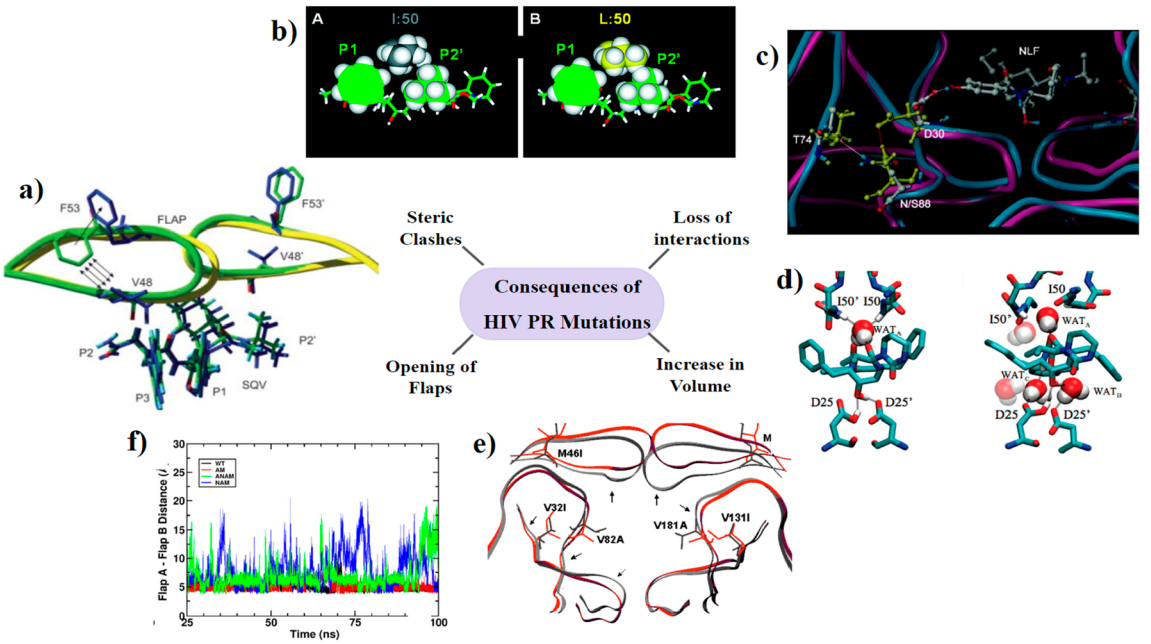

Figure 7. Some proposed mechanisms of drug resistance. (a) Flap sliding and exposure of Phe53 due to G48V mutation upon SQV binding. Figure adapted with permission from ref 98. Wittayanarakul et al. Copyright 2005 The Biophysical Society. (b) Induced I50L mutation due to ATV binding results in steric clashes with the tertiary butyl groups of ATV. Figure adapted with permission from ref 99. Yanchunas Jr. et al. Copyright 2005 American Society for Microbiology. (c) Loss of H-bonding interactions between the PR and NFV due to D30N mutation. Figure adapted with permission from ref 100. Wartha et al. Copyright 2005 American Chemical Society. (d) Decrease in vdW interactions and increase in electrostatic repulsion in the PR active site due to LPV binding caused increased water occupancy. Figure adapted with permission from ref 101. Sadiq et al. Copyright 2010 American Chemical Society. (e) Increased pocket volume due to primary mutations. Figure adapted with permission from ref 97. Appadurai et al. Copyright 2016 American Chemical Society. (f) Increased flap dynamics due to secondary mutations. Figure adapted with permission from ref 97. Appadurai et al. Copyright 2016 American Chemical Society.

study from our group has shown that certain distal, nonactive site mutations, e.g. K20R, L63P, and A71I, impart great stability to the enzyme and compensate for the loss in stability due to the primary mutations in the active site.[97] We have also shown that these distal mutations lead to an increase in active site volume and flap flexibility due to the decoupling of motion between the active site and flap regions. Network analyses further revealed that there exists a robust signaling pathway between the distal mutations and active site and flap residues in the WT, while it becomes weaker in the mutant protease. This brings about a negative interference of the distal regions on the flap residues in the variants with nonactive site mutations, leading to an unregulated flap opening and consequent drug efflux. A community-based network, built from the known functional regions of the enzyme, reconfirms these findings and in addition exhibits weaker intermonomer communications (AS-AS′, Flp-Flp′), implying easier dimer dissociation in the variant as depicted in Figure 6b.

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ACS Omega 2023, 8, 9748−9763

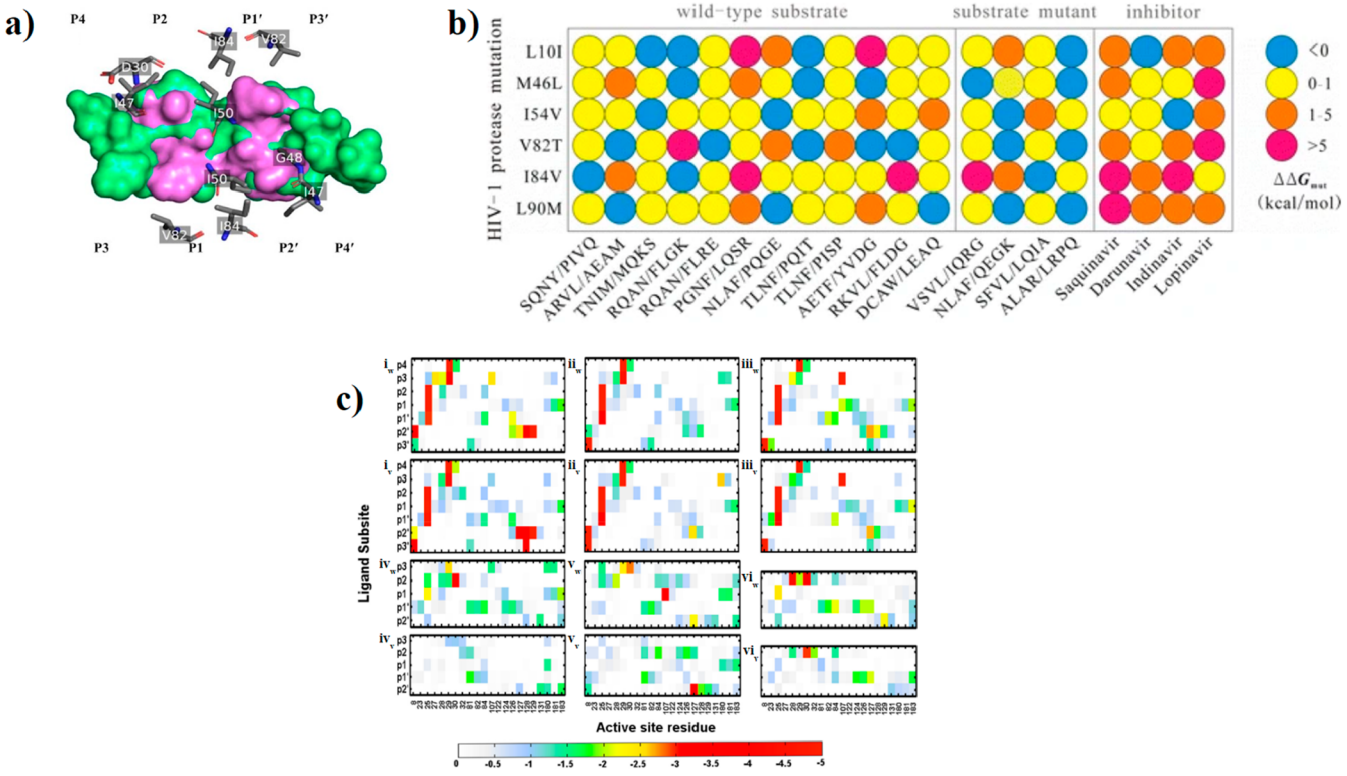

Figure 8. How PR variants remain functionally active. (a) Superposition of the inhibitors (pink) on the substrate envelope (green) shows that the former protrudes out of the envelope and is involved in multiple extra interactions with active site residues shown in gray licorice. (b) Coevolution of substrate mutations often compensates the binding, while the drugs suffer a loss in binding free energy due to PR mutations. Figure adapted with permission from ref 107. Ni et al. Copyright 2013 Springer Science Business Media New York. (c) Interactions of the substrates with WT and variant PR remain unaltered: (i_w) WT-p2-NC, (ii_w) WT-CA-p2, (iii_w) WT-RT-RH, (i_v) variant-p2-NC, (ii_v) variant-CA-p2, and (iii_v) variant-RT-RH, while those for the drug were reduced drastically: (iv_w) WT-saquinavir, (v_w) WT-indinavir, (vi_w) WT-nelfinavir, (iv_v) variant-saquinavir, (v_v) variant-indinavir, and (vi_v) variant-nelfinavir. The energy scale is in kcal/mol. Figure adapted with permission from ref 108. Appadurai et al. Copyright 2017 American Chemical Society.

MECHANISM OF DRUG RESISTANCE OF THE HIV PR MUTANTS

There is no unified mechanism that can explain how HIV PR resists the drugs. However, it has been generally accepted that HIV PR developed resistance through mutations. These mutations can cause steric clashes, alter hydrophobic and van der Waals interactions, or increase electrostatic repulsion with the drug. As a consequence, there is an increase in the active site size/volume, a decrease in binding energy, and/or a decrease in the H-bonding network when bound to the inhibitors. Figure 7 depicts some of the proposed mechanisms in the literature. In Figure 7a, the mutation G48V in HIV PR after SQV exposure causes steric clashes with the drug and the active site residues, which result in the flap sliding off and exposing the hydrophobic F53 to the solvent.[98] Mutation I50L results in major steric clashes with the tertiary butyl group at the P2 and P2′ sites of ATV (Figure 7b), which negatively affects the binding free energy and thus leads to flap opening.[99] Another mechanism is through an altered H-bonding network caused after the treatment with NFV. Here, the D30N mutation leads to a loss of the H-bond between the protease and NFV, resulting in a decrease of binding free energy. Another mutation N88S shifts the equilibrium to the unbound protease by altering the H-bonding pattern from D88-T74 to D88-D30. This prevents the crucial interaction of NFV with D30 by changing the orientation of D30 to face away from the binding pocket, making it less favorable to interact with NFV (Figure 7c).[100] Similarly, resistance to LPV was enthalpically driven due to a decrease in van der Waals interactions and an increase in electrostatic repulsion caused by expansion of the active site cavity and an increase of water occupancy, which alter the H-bond network between LPV and protease (Figure 7d).[101] An MD study, in combination with network analyses, from our laboratory has shown that active site mutations (AM) comprised of V32I, M46I, and V82A can increase the volume of the PR binding pocket by 21−31%, in comparison to the WT[97] (Figure 7e). We have also shown that secondary or nonactive site mutations (NAMs) increase the flap dynamics significantly, thus pushing the equilibrium toward the open-flap protease conformation, and thereby enhance the probability of drug efflux[97] (Figure 7f).

HOW MUTATIONS RESIST DRUG BINDING YET ALLOW SUBSTRATE CLEAVAGE

The mechanism by which the drug-resistant mutants are able to circumvent the PI binding but could function properly to cleave the natural substrates is not fully understood. One possible explanation is the substrate envelope hypothesis. Figure 8a shows an overlap of the substrate envelope and the bound inhibitors in the PR pocket. It can be seen that certain regions of the envelope occupied by the PIs protrude away from the conserved substrate envelope. According to the substrate envelope hypothesis, any PI with a shape that does not fit into the substrate envelope could form additional

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ACS Omega 2023, 8, 9748−9763

interactions with the protease that are not made by the natural substrates. These additional interactions around the active site can lead to primary mutations in the HIV PR with a resultant loss in binding affinity for the PI, while the network of interactions with the natural substrates remains unaffected.[64] Studies have shown that the deleterious effects of such mutations that lead to a reduction in catalytic activity are often rescued through other antagonistic mutations elsewhere in the HIV PR; e.g., the loss of catalytic efficiency brought about by the mutation D30N is restored with the mutation N88D,[102] and the loss of enzymatic efficiency due to L90M is partially resorted by L89V.[103] Recent *in vitro* studies have also shown another mechanism by which HIV PR still maintains its function while conferring drug resistance mutations, which is by mutating or changing the substrate (gag polyprotein) instead of the protease.[104] Several MD studies have shown how mutations negatively affect the drug binding free energy.[105,106] A combined QM/MM-PB/SA-based study to understand how HIV PR is still functional despite mutations has reported that the binding energy change due to the mutations, termed mutational energy, is not significant for substrates, whereas it is significantly large for inhibitors, leading to loss of inhibitory action of the inhibitors.[107] They also observed that the mutant forms of the substrates have favorable or negative mutational energy compared to the wildtype substrates, suggesting that the coevolution of mutations in the gag-pol could be one of the reasons for HIV PR still remaining functional while preventing the action of inhibitors (Figure 8b).

An MD-based study from our group using wildtype and mutant proteases with the substrate and PI reported that the substrate binding to the mutant protease shifted the equilibrium toward the closed flap state, whereas the inhibitor binding to the PR variants failed to do so. The loss of free energy of flap closure for the mutant−substrate complex with respect to the wildtype−substrate complex was less than that between mutant−PI and wildtype−PI complexes.[108] It was also observed that because the substrate was longer it was able to interact with more subsites of the protease, whereas the smaller PI forms only a limited number of interactions. Moreover, the substrate forms several H-bonds and nonpolar interactions with the conserved backbone atoms of Gly27, Gly126, Asp29, Asp128, Gly48, and Gly147, which remain intact in the mutant protease. On the contrary, the inhibitors interact predominantly via nonpolar hydrophobic interactions with protease side chains which are lost due to mutations (Figure 8c). This differential binding of substrates and drugs to the protease results in the mutant protease still functioning properly while developing drug resistance.

FUTURE PERSPECTIVE

From the above literature review, it is evident that drug resistance due to mutations in HIV PR is a major bottleneck in AIDS therapeutics. There are a few promising avenues that have been proposed to counteract the HIV PR drug resistance. One simple way is to enhance the levels of PI in the plasma. This is done by combining ritonavir (RTV) with the other HIV PR inhibitors. In these combination therapy strategies, RTV, which is also a cytochrome P450 inhibitor, increases the bioavailability of the other PIs by slowing down their metabolism and consequently enhancing the levels in plasma.[109] This enables the PI to show desired effects even when its susceptibility is lost due to drug-resistant mutations. All the FDA-approved PIs currently used in HAART are given as a combination with RTV for boosting, except NFV which is metabolized by a different enzyme CYP2C19 (Table 2). Another promising route to overcome or prevent drug resistance is to design a drug that falls within the substrate binding envelope as proposed by Schiffer and co-workers. In this formalism, it is proposed that a newly designed small molecule should not have any protrusions from the substrate envelope, such that it makes no extra interactions with the protease active site residues and makes them prone to mutate.[64] Also, drugs can be designed to extend in size or occupy more of the substrate envelope. APV and DRV are two such PIs that have a close fit with the substrate envelope and show good susceptibility against drug-resistant mutants. In recent years, significant focus has been devoted to designing a new set of PIs that present similar scaffolds as APV and DRV, and comply with the substrate envelope hypothesis.[110,111] Blocking the dimerization ability of HIV PR is another prospective strategy to combat drug resistance. Currently, only DRV and TPV are known to inhibit the protease dimerization, along with inhibiting the enzymatic activity.[77] An ESI-MS (electrospray ionization mass spectrometry) study on HIV PR mutants has suggested that DRV binds to the HIV PR monomer, presumably with a greater affinity than between the two monomers, and prevents the formation of the dimer.[112] To unravel the mechanism of dimerization inhibition by DRV, an MD study from our group has suggested that the drug realizes a different binding mode in the active site which sterically interferes with the formation of the flap−flap interface during dimerization.[113] Thus, despite the gloomy performance of the existing HIV PR drugs, the continuing research thrust on this important enzyme is showing promise to bring about new classes of drug molecules that can improvise the AIDS therapeutics.

We emphasize that almost all the published review articles on HIV PR focused either on HIV PR inhibitors or on the structural aspects of the enzyme. However, for such a protein which exhibits extremely high flexibility and allostery, the dynamics plays a crucial role in its function. While experimental techniques provided a wealth of detailed information about the HIV PR structure, it is the computer simulation techniques, particularly molecular dynamics (MD) simulations, which have unearthed the small-to-large-scale dynamics that this protein inherits. In this Review article, we present a comprehensive view of the structure and dynamics of HIV protease with an emphasis on how dynamics (apart from structure) can modulate the function; the available HIV PR drugs with an emphasis on how the mutations in the enzyme resist the drug binding yet maintain its hydrolytic activity; and an outlook on how to combat the drug resistance. In the process, we have covered a whole range of articles from structure to dynamics to drug-resistant mechanisms, combining both experimental and computational reports. To the best of our knowledge, this could be the first Review article that presents a comprehensive view of HIV PR research covering its structure, dynamics, mechanism of action, marketed drugs, and drug-resistant mechanisms, as unearthed both experimentally and computationally!

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Author Contributions

§A.D. and A.A. contributed equally to the work

Notes

The authors declare no competing financial interest.

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