Review

Subtype-Specific HIV-1 Protease and The Role of Hinge and Flap Dynamics in Drug Resistance: A Subtype C Narrative

Dean Sherry , Zaahida Sheik Ismail , Tshele Mokhantso , Yasien Sayed *

Posted Date: 10 June 2025

doi: 10.20944/preprints202506.0863.v1

Keywords: HIV-1 subtype C protease; hinge region dynamics; flap flexibility; genetic diversity; drug resistance mutations; protease inhibitors; RNA aptamers; allosteric inhibitors; dual-action inhibitors; peptide inhibitors

 doi:10.20944/preprints202506.0863.v1

Review

Subtype-Specific HIV-1 Protease and The Role of Hinge and Flap Dynamics in Drug Resistance: A Subtype C Narrative

Dean Sherry, Zaahida Sheik Ismail, Tshele Mokhantso and Yasien Sayed *

Protein Structure-Function Research Laboratory, University of the Witwatersrand, Johannesburg, South Africa

* Correspondence: yasien.sayed@wits.ac.za

Abstract: The HIV-1 aspartic protease is an effective target for the treatment of HIV/AIDS. Current therapy utilizes a selection of nine protease inhibitors (PIs) in combination with other classes of antiretroviral drugs. Although PIs were originally developed based on the knowledge of the HIV-1 subtype B protease, the existence of other HIV-1 subtypes and the effects of drug resistance on currently available PIs have become a major challenge in the treatment of HIV/AIDS. Specifically, the HIV-1 subtype C accounts for more than half of the global HIV infections. Considering the importance and relevance of the subtype C virus, in this timely chapter, we discuss the effect of polymorphisms in the HIV-1 subtype C protease on drug resistance, flap flexibility and hinge region dynamics. We discuss novel paradigms of protease inhibition that attempt to overcome the limitations of currently available inhibitors which fall short considering genetic diversity and resistance mutations.

Keywords: HIV-1 subtype C protease; hinge region dynamics; flap flexibility; genetic diversity; drug resistance mutations; protease inhibitors; RNA aptamers; allosteric inhibitors; dual-action inhibitors; peptide inhibitors

1. Introduction

The Human Immunodeficiency Virus (HIV), the causative agent of Acquired Immunodeficiency Syndrome (AIDS), continues to impose a significant global socio-economic impact, with developing countries generally being most greatly affected. The latest statistics on global infections estimate that ~39 million people were living with HIV as of 2023 [1]. The HIV/AIDS epidemic is particularly prominent in sub-Saharan Africa where roughly 70% of global infections occur; a fact that is reflected in these countries exhibiting the highest morbidity and mortality rates [1].

Since the discovery of HIV as the causative agent of AIDS, a significant amount of research has focused on understanding the biomolecular interactions that govern the lifecycle of the virus with the goal of identifying promising drug targets. The life cycle of the HI virus (Figure 1) begins with attachment of the viral spike glycoproteins (gp120/gp41) to the CD4 receptor present on the cell membrane of host CD4+ T-lymphocytes. With the aid of CCR5/CXCR4 co-receptors, the viral envelope fuses with the host cell membrane and a single-stranded positive sense RNA genome is released into the cytoplasm. Thereafter, the viral genome is reverse transcribed into a double-stranded DNA (dsDNA) molecule by the viral reverse transcriptase (RT) enzyme [2]. Reverse transcriptase synthesizes a single-stranded negative sense complementary DNA (cDNA) molecule using the viral RNA genome as a template. A complementary, positive sense DNA molecule is then synthesized using the cDNA as a template. dsDNA is formed by hybridization of the positive sense and negative sense DNA strands, which is then integrated into the host cell genome by the viral integrase (IN) enzyme [2]. The integrated viral genome is translated into viral polyprotein precursors, namely, Gag and Gag-Pol. These polyproteins, along with several copies of the ssRNA viral genome are transported to the cell membrane. Once the necessary viral components are assembled, membrane

 doi:10.20944/preprints202506.0863.v1

envelopment is initiated, followed by budding, resulting in the production of an immature virion [3]. Finally, maturation of the virion occurs through the action of the viral protease (PR), which cleaves the Gag and Gag-Pol polyproteins into enzymes and structural proteins. The resultant virion is mature and capable of infecting subsequent host cells.

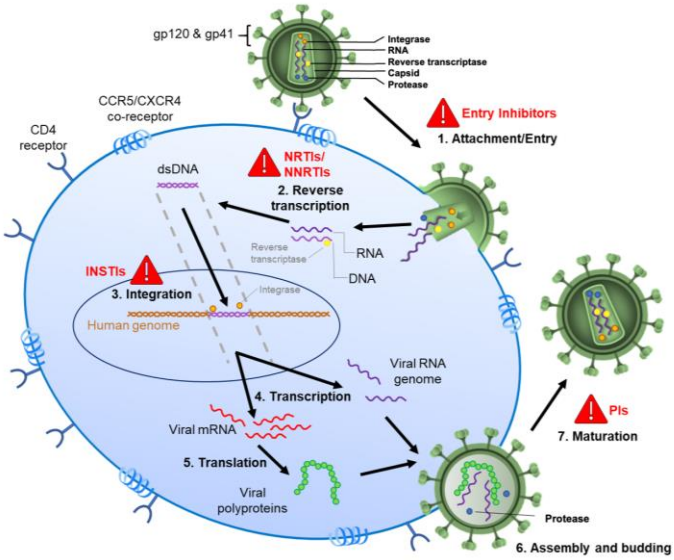

Figure 1. The life cycle of HIV-1. Stages of the life cycle are numbered. 1: Attachment/entry of the virus to CD4/CCR5/CXCR4 receptors on the host cell membrane to allow for subsequent fusion with the host cell. 2: Reverse transcription of the viral genome into double-stranded DNA. 3: Viral double-stranded DNA is transported into the nucleus and integrated into the host genome. 4: Transcription of the viral double-stranded DNA genome into single-stranded RNA genetic material and mRNA. 5: Translation of mRNA into viral Gag/Gag-Pol polyproteins. 6: Assembly and budding of immature viral progeny. 7: Maturation of the HIV virion via the action of the protease. Red triangles depict the stages of the life cycle that are major targets for antiretroviral therapy.

The identification of key steps in the virus life cycle has enabled the development of several classes of antiretroviral drugs targeting crucial steps in the production of infectious virions (Figure 1). Highly active antiretroviral therapy (HAART) make use of up to twenty different drug molecules including nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs) and fusion/entry inhibitors (Figure 1).

1.1. Genetic Diversity of HIV-1

The HIV-1 genome has three major genes typical of the *Retroviridae* family, namely, Group specific antigen (Gag), Polyprotein (Pol) and Envelope (Env). Gag encodes the viral structural proteins such as the Matrix (MA), Capsid (CA) and Nucleocapsid (NC), which account for approximately half of the mass of the HIV-1 virion. Pol encodes the enzymatic proteins responsible for the viral replication process, namely the RT, IN and PR enzymes. Env encodes the glycoproteins gp160 and gp120 which are found on the virion surface and help with viral attachment and infection of host cells. The HIV-1 genome additionally has regulatory genes Tat and Ref and accessory genes; Vif, Vpr, Vpu and Nef [4]. One of the key characteristics of HIV-1 is its high genetic diversity [5]. Three principal mechanisms contribute significantly to the genetic diversity observed within the virus. Firstly, the rapid replication rate of HIV, estimated to produce approximately 1010 virions daily, serves as a substantial driver of genetic variation [6]. Secondly, viral recombination plays a crucial role by facilitating the reassortment of genetic mutations across different viral genomes. Lastly, the inherent error-prone replication process of the reverse transcriptase (RT) enzyme,

 doi:10.20944/preprints202506.0863.v1

characterized by its low fidelity, further contributes to the accumulation of genetic diversity within HIV [5]. In fact, during replication, the error-prone nature of RT introduces up to 1 error for every 2000 base incorporations [7].

To date, two types of HIV have been identified: type 1 (HIV-1) and type 2 (HIV-2). HIV-1 has been shown to be more virulent than HIV-2 and accounts for the majority of global infections [8]. As such, global research efforts have focused on HIV-1. HIV-1 is further classified into the following groups: Group M (main), N (non-M or non-O) and O (outlier) and P (a group given its own name due to how different it is from M, N and O groups) [9]. The group which is predominantly responsible for the global HIV/AIDS pandemic is group M. This group is further classified in terms of subtypes, namely, subtype A, B, C, D, F, G, H, J, K and circular recombinant forms (CRFs) as illustrated in Figure 12.2. CRFs are epidemic strains that arise from the recombination of various strains of HIV [9]. Among all the subtypes within group M, subtype B is the most extensively studied and is predominant in the infections found in developed regions such as North America and Western Europe. These regions, often referred to as first-world countries, possess the necessary infrastructure, financial resources, and technological capacity to conduct advanced HIV research. Consequently, the majority of scientific studies in these countries have concentrated on HIV-1 subtype B. This focus has led to the development of drug treatments primarily targeted at subtype B, despite its accounting for only 12% of global HIV infections. In contrast, the HIV-1 subtype C which accounts for more than 50% of global infections but has remained largely understudied [10–12]. A greater genetic variation exists within HIV-1 subtype C (Figure 2; red) compared to all other HIV-1 subtypes including subtype B (Figure 2; blue). Although the subtype B-derived therapeutic interventions have successfully decreased fatalities and the spread of HIV-1, there has been an increase in the number of non-B subtype infections globally. These variants may evade current therapies, leading to increased drug resistance and treatment failures. Of particular concern is HIV-1 subtype C, which poses significant challenges in treatment efficacy and control.

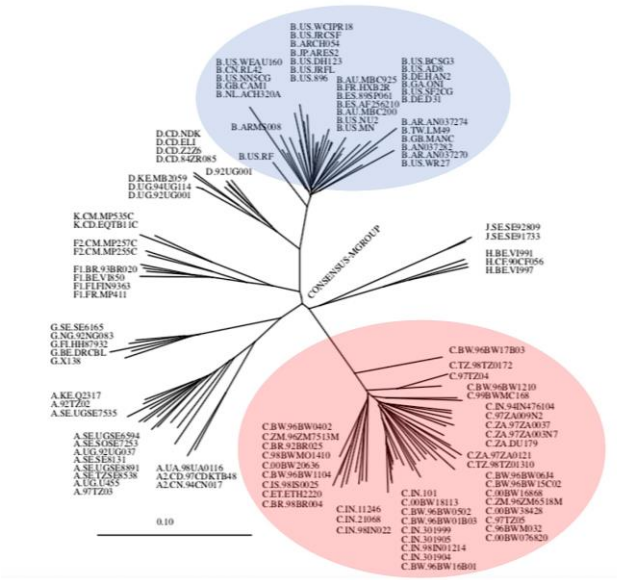

Figure 2. Radial phylogeny tree of HIV-1 subtypes under the consensus M group of HIV-1. The identity code for each subtype is as follows; subtype indicated by a letter, country and then identity code. The tree was made using the NJ method and a F84 distance model with equal site rate, with 43 sequences and 7963 characters including insertions, and gaps stripped. The HIV-1 subtype B and subtype C are indicated in blue and red, respectively. This figure was generated using the Los Alamos HIV Sequence Database: www.hiv.lanl.gov/.

 doi:10.20944/preprints202506.0863.v1

1.2. The Global Predominating Subtype: HIV-1 Subtype C

As mentioned previously, the HIV-1 subtype C is the predominant subtype in developing countries including Brazil, China, India, and South Africa. The South African HIV-1 subtype C protease (C-SA PR) is, in essence, the consensus subtype C PR (Los Alamos HIV Sequence Database, (www.hiv.lanl.gov/). The subtype C accounts for approximately 70% of infections in sub-Saharan Africa. There is evidence to suggest that the efficacy of inhibitors against subtype C is less than that of subtype B [10,11,13,14]. It has been postulated that the discrepancy in the efficacy of antiretroviral therapy across subtypes is due to quantifiable differences in the structural and functional properties of the viral protein targets. All currently available FDA-approved antiretrovirals were designed using the subtype B viral proteins as templates for rational drug design. However, as the scope of HIV research has increased to include HIV-1 non-B subtypes, it has become increasingly clear that certain crucial differences exist between antiretroviral drug targets across subtypes. In particular, the PR exhibits significantly decreased drug susceptibility in HIV-1 subtype C [10,11,13,14], perhaps suggesting that current antiretroviral drugs lack broad spectrum efficacy across different HIV-1 subtypes. Notably, the subtype C PR has been shown to be structurally and dynamically different from the subtype B PR which may explain the observed differences in drug efficacy (this is explored further in the subsection Naturally Occurring Polymorphisms in the HIV Protease).

Gag is the main structural polyprotein in HIV and its cleavage, by PR, results in the production of mature HIV virions [15–17]. During HIV maturation, the scissile bonds on the Gag polyprotein are targeted and cleaved by the PR, a process that is crucial for viral development. Therefore, these cleavage sites have been simulated in efforts to design competitive protease inhibitors which are capable of prohibiting viral maturation [18]. However, Gag mutations have previously been shown to alter drug susceptibility, particularly when they have coevolved with mutations in the PR [19–21]. The cleavage sites on HIV-1 subtype C Gag polyprotein; p17/p24, p2/NC, NC/p1, were shown to have the highest genetic variation in comparison to other Group M subtypes. Such variability in the Gag cleavage sites between subtypes may immensely affect the pathogenesis, viral fitness and drug-response of the HI virus [18,22,23].

The Gag polyprotein plays a significant role in the replicative capacity of HIV. Notably, a comparison of the Gag-PR-mediated replication capacity of HIV-1 subtype B and C revealed a reduced replicative capacity in subtype C [24]. Similar studies have also suggested that subtype C has a reduced viral fitness compared to other subtypes within group M [25,26] A decrease in replication capacity has been attributed to the presence of residues L483 and Y484 in the subtype B Gag sequence which are absent in the subtype C Gag. Deletion of these residues in the subtype B Gag consistently resulted in a decreased viral replication capacity in comparison to when these residues are present [24]. This natural difference in the subtype C may facilitate a slower progression of subtype C infections and, thus, increase the opportunity for new transmission events. Some studies suggest that the reduced replication kinetics of HIV-1 subtype C may increase the half-life of productively infected cells and these infected cells, if transmitted in genital fluid, may increase transmission risk compared to free virions [24].

1.3. Structure of the HIV-1 Subtype C Protease

The PR enzyme is an obligate homodimer composed of two identical 99 amino acid monomers (Figure 3). The amino and carboxyl termini residues found at the dimer interface (residues 1-4 and 94-99) are conserved across all HIV subtypes (Naicker et al., 2013). Notably, the terminal F99 residue has been shown to play a crucial role in the formation of the functional homodimeric PR. Indeed, the F99A substitution leads to the formation of structured monomeric molecules, inhibiting dimerization and completely abolishing enzymatic activity [27]. The active site contains a conserved catalytic triad, Asp25/Thr26/Gly27, which is characteristic of aspartyl proteases [28]. Each monomeric subunit contributes an Asp residue to the catalytic triad which is located at the bottom of the protease active site cavity (Figure 3). Entry of substrates or inhibitors into the active site is controlled by two β-hairpin loops termed the flaps (residues 46-54). The flap tips contain a conserved glycine-rich sequence, 46-

 doi:10.20944/preprints202506.0863.v1

MIGGIGGFI-54, which plays an important role in substrate recognition and binding [29,30]. The movement of these flexible flaps is largely aided by the hinge regions (residues 35-42 and 57-61) and regulate flap flexibility and stability. Opening of the flaps relies on the synergistic downwards motion of the cantilever (residues 62-78), fulcrum (residues 10-23) and hinge regions which also results in the rotation of each monomer about the β-sheeted dimer interface [13,31]. This combined movement is referred to as the hydrophobic sliding mechanism [32]. A dynamic image of the PR, revealed through NMR spectroscopy and molecular dynamics (MD) simulations, has revealed that the flaps exist in a dynamic equilibrium of heterogeneous flap conformations, namely, open, semi-open and closed (Figure 3) [33–35]. The mobility of the flaps is critical for the function of the PR as the open conformation allows for substrates or PIs to enter the active site cavity, while the closed conformation is responsible for the formation of intermolecular interactions with the substrate [29]. Traditionally, flap conformations are defined by measuring the distance between the alpha carbon (Cα) of the flap tip Gly51/Gly51' residues; where interatomic distances < 7 Å, 7-12 Å and > 12 Å are defined as closed, semi-open and open conformations, respectively. However, this definition is limited and can be affected by asymmetric movement of the flaps, as well as the occurrence of flap tip curling. As such, a more accurate description of these conformations are defined using the interatomic distance between the Cα of the catalytic Asp25/Asp25' residues and the flap tip Ile50/Ile50' residues [12,36]. Based on the new definitions, the conformations are consequently defined as interatomic distances of < 17 Å, 17-22 Å and > 22 Å for the closed, semi-open and open conformations, respectively (Figure 3).

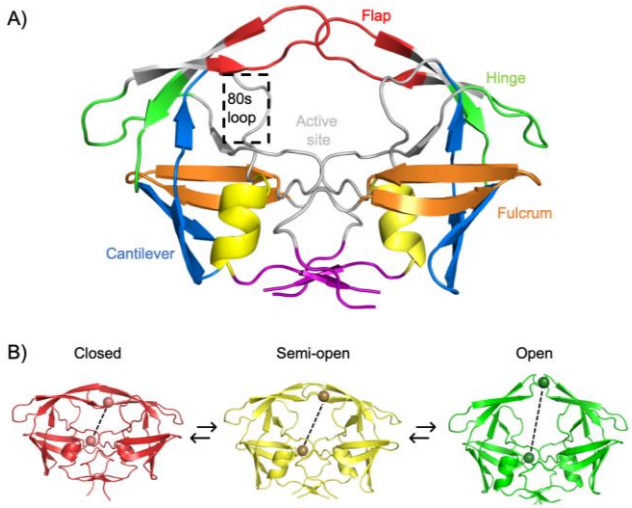

Figure 3. Structural representation of HIV-1 C-SA PR based on crystal structure. (**a**) The regions of the PR are represented by purple: an inter-leaved beta strand motif forming the dimer interface (residues 1-4 and 94-99), orange: fulcrum (residues 10-23), red: flap (residues 46-54), green: hinge (residues 35-42 and 57-61), blue: cantilever (residues 62-78), and yellow: α-helix (residues 86-93). The 80s loop (residues 79-84) is indicated in the black rectangular box. (**b**) Heterogenous flap conformers displayed by the HIV-1 PR. The closed (red), semi-open (yellow) and open (green) flap conformations exist in dynamic equilibrium. These conformations are defined by the distance between the Cα of Asp25 and Ile50 where closed, semi-open and open conformations are < 17 Å, 17-22 Å and > 22 Å, respectively. This figure was generated using PyMOL Molecular Graphics Software, Schrödinger LLC. PDB ID: 3U71.

1.4. Function of the HIV-1 Subtype C Protease

Understanding the structure of the PR active site and its mechanism of action has allowed for the design of protease inhibitors (PIs) which target the active site and impede the function of the PR.

 doi:10.20944/preprints202506.0863.v1

Regardless of the viral PR subtype, HIV-1 proteases use a general acid-base reaction mechanism for the hydrolysis of the carbonyl carbon scissile bond on a substrate protein. Briefly, two Asp25 residues of the PR (one from each monomer) exist in opposing states of protonation and coordinate a water molecule that acts as a nucleophile during catalysis. When a substrate (e.g., Gag) is bound to the active site of the PR, the coordinated water molecule performs a nucleophilic attack on the carbonyl carbon of the scissile peptide bond, resulting in the formation of a tetrahedral intermediate enzyme-substrate complex. Protonation of the scissile amide nitrogen atom results in the breakdown of the intermediate complex and the subsequent release of the cleaved peptide products.

Structurally, the active site cavity of the PR is divided into eight subsites, each accommodating specific side chains of the substrate/inhibitor (Figure 4) [37]. The scissile peptide bond is positioned between the P1 and P1′ site of the PR (Figure 5). The amino acids positioned toward the N-terminus on the substrate are designated P1, P2 and P3 while those toward the C-terminus are termed P1′, P2′ and P3′ (Figure 4). Each side chain of the peptide (P3 - P3′) binds a subsite formed by the PR residues, designated as S4 - S3′. A maximum of seven residues expanding from S4 - S3′ is needed for efficient catalysis [37].

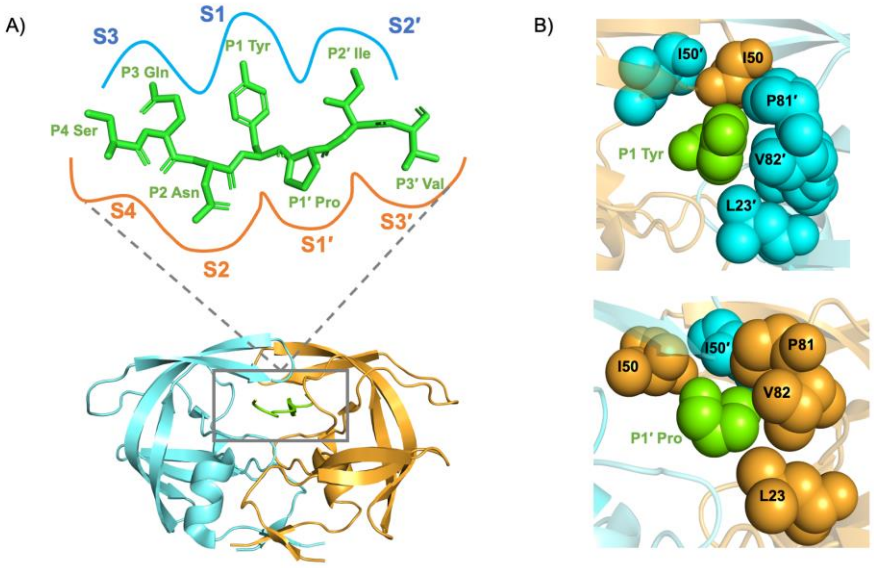

Figure 4. (**a**) Diagrammatic representation of the subsites of the HIV-1 protease active site and a Gag substrate. The subsites of the active site (gray box) are represented by S4 - S1 and S1' - S4' for those subsites toward the N- or C-terminus of the scissile bond, respectively. The PR monomers are colored blue and orange to illustrate each monomers contribution to the subsites. The substrate sites are denoted similarly: P4 - P1 and P1' - P4'. (**b**) The P1 and P1′ sites of the substrate fit into the S1 and S1′ subsites of the PR active site following a lock-and-key paradigm.

 doi:10.20944/preprints202506.0863.v1

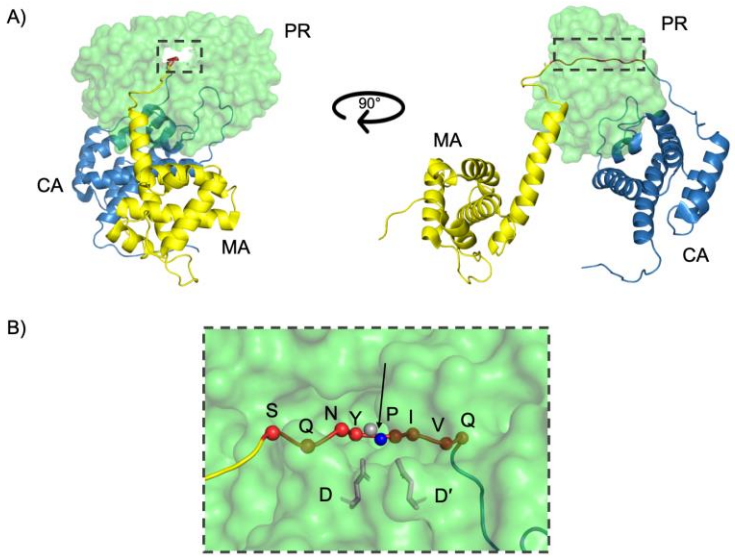

Figure 5. The function of the HIV protease. (**a**) PR (green) bound to the MA/CA cleavage site of the unprocessed Gag polyprotein (MA yellow, CA blue). The black box highlights the cleavage site being thread through the active site cavity. (**b**) The MA/CA cleavage site heptapeptide (SQNYPIVQ; red spheres) is coordinated in the active site cavity of the PR. The catalytic aspartic acid residues (D/D′) are indicated as blue sticks. The scissile peptide bond is indicated by the black arrow and occurs between the P1 carboxyl carbon (gray sphere) of the and the P1′ amide nitrogen (blue sphere). This figure was generated using PyMOL Molecular Graphics Software, Schrödinger, LLC. PDB ID: 6I45 (PR); 1L6N (MA-CA polyprotein).

1.5. Dynamics of the HIV-1 Subtype C Protease

In comparison to the amino acid sequence of the well-characterized HIV-1 subtype B protease, the C-SA PR contains eight naturally occurring amino acid polymorphisms (NOPs) [10,11,30,31]. These polymorphisms are located outside of the PR active site region as follows: T12S, I15V and L19I, which are found in the fulcrum region, M36I and R41K, in the hinge region, and H69K and L89M which are found in the cantilever and 80s loop, respectively. Lastly, I93L is found in the α-helix of the PR monomer. The hydrophobic core of the protease contains 20 amino acids, namely, L5, V11, I13, V15, I19, A22, L24, I26, L33, L38, I62, I64, I66, V75, V77, I85, M89, L90, L93 and L97 [11,32]. Notably, four of the NOPs that occur in the C-SA PR sequence are located within the hydrophobic core of the PR, namely, V15, I19, M89 and L93. The remaining four NOPs (T12S, M36I, R41K, and H69K) are closely associated with core residues. The presence of NOPs in the C-SA PR has a profound effect on the hydrophobic sliding mechanism by altering the dynamics of the PR and, more specifically, the way the flaps change conformation over time [31]. In the subtype B PR, the cantilever and hinge regions move over I15 in the hydrophobic core, and this allows anchoring of the flaps. In the C-SA PR, however, the hydrophobic interactions within the core are altered due to the presence of the I15V, L19I, M36I, H69K, L89M and I93L polymorphisms. Interestingly, the C-SA PR exhibits a higher propensity to sample open flap conformations when compared to the subtype B PR [11,38]. In addition, the C-SA PR has been shown to sample a wider variety of conformations exhibiting greater flap region conformational heterogeneity [39].

The hinge mutation, M36I, introduces an interesting effect on the flap dynamics of the C-SA protease. The E35-R57 salt bridge, which is adjacent to the M36I mutation, is the only salt bridge linking the flap and hinge regions of the subtype B PR and subtype C PR. Noticeably, the E35-R57 salt bridge of the C-SA PR is absent when observed via molecular dynamics simulations [31,40]. The E35-R57 salt bridge is thought to help lock the flaps into semi-open/closed conformations and its absence, therefore, contributes to the increased flap flexibility of the C-SA PR due to the M36I mutation altering the local geometry of the hinge region [31,40]. Furthermore, the L89M

 doi:10.20944/preprints202506.0863.v1

polymorphism is thought to increase the stability of the hinge, fulcrum and cantilever regions of the C-SA PR due to the extensive contacts M89 forms with neighboring residues [41]. It is evident, therefore, that results from studies such as these indicate that there are significant dynamic differences between B and C viral proteases which cannot be ignored. Considering this, using a B protease as a representative protease of all subtypes, and designing drugs against this subtype, is not prudent. NOPs in the C-SA PR work synergistically to alter both the hydrophobic sliding mechanism and the flexibility of the flaps, resulting in the dynamic behavior of the C viral PR being markedly different in comparison to the B viral PR.

2. HIV-1 Protease Inhibition

There are currently nine PIs approved for ART by the FDA, namely, amprenavir (APV, PubChem CID: 65016), atazanavir (ATV, PubChem CID: 148192), darunavir (DRV, PubChem CID: 213039), indinavir (IDV, PubChem CID: 5462355), lopinavir (LPV, PubChem CID: 92727), nelfinavir (NFV, PubChem CID: 64142), ritonavir (RTV, PubChem CID: 392622), saquinavir (SQV, PubChem CID: 60787) and tipranavir (TPV, PubChem CID: 54682461). All PIs directed at the subtype B PR exhibit relatively uniform intermolecular contacts with the active site, and function as competitive inhibitors by competing with the substrate for the active site of the PR [42]. PIs are specifically designed to mimic the tetrahedral transition-state observed during the proteolytic reaction [43]. The process of inhibitor binding shifts the PR flaps into the closed conformation where the flaps exhibit significantly reduced mobility.

Due to the highly specific configuration of the transition-state, transition-state analogs exhibit binding affinities for the PR active site that are in the picomolar range (pM) [44–46].Together with the presence of a non-hydrolysable hydroxyethylene core, PIs exhibit higher specificity for the PR than their corresponding substrates and inhibit the reaction mechanism of the PR [44–46]. However, due to the selective pressure imposed by PIs in conjunction with the mutation-prone nature of the viral RT, the virus rapidly accumulates sequence polymorphisms which favor inhibitor resistance. Indeed, amino acid polymorphisms associated with reduced drug susceptibility are observed at over half of the positions of the PR sequence [47].

2.1. NOPs, Drug-Induced Protease Mutations, and Inhibitor Resistance

The paradigm describing the accumulation of drug resistance mutations in the PR is centered around the presence of two distinct, but equally relevant, types of mutations. Generally, it is believed that resistance begins with the onset of primary mutations, which refer to amino acid substitutions that directly impact drug susceptibility. Most often, primary mutations occur directly within the active site cavity and affect drug binding through altering the geometry of the drug binding interface [48,49]. Since PIs are smaller and more rigid molecules relative to their substrate counterparts, inhibitors are unable to account for changes in local geometry of the interface. Therefore, primary mutations reduce drug binding affinity through altering the geometric complementarity between the active site and inhibitor. However, primary mutations still possess the potential to decrease the binding affinity of the natural substrates and, therefore, the PR sequence accumulates compensatory mutations in the hydrophobic core of the PR as well as in the Gag/Gag-Pol cleavage sites [50]. These non-active site mutations, or secondary mutations, are thought to compensate for the geometric perturbations caused by primary mutations in a manner that maintains the catalytic efficiency of the PR [51]. Together, the accumulation of complex combinations of primary and secondary mutations results in resistance through compromising inhibitor binding while maintaining selectivity for the naturally occurring substrate molecules. In some cases, NOPs that occur in non-B subtypes are resistance-associated mutations with respect to the subtype B PR. The key role that NOPs can play in reducing the PI susceptibility of non-B subtypes is highlighted from thermodynamic analyses of the binding of FDA-approved PIs between the subtype B and subtype C PR. These analyses have revealed that PIs bind to the subtype C PR with 3- to 24-fold weaker binding affinity in comparison to the subtype B [52]. Interestingly, when considering the subtype C PR, it has been shown that the

 doi:10.20944/preprints202506.0863.v1

NOPs present in the subtype C increase the propensity of the apo PR to adopt the open flap conformation [31,39]. Moreover, analysis of subtype C PR flap tip curling indicates that in some cases the inhibitor-bound complexes, such as ATV, DRV and TPV, remain in the semi-closed conformation and do not achieve the fully closed conformation [11,12,31,38]. Non-active site mutations have been shown to increase the mobility of the flap regions when in complex with inhibitors, suggesting that such mutations prevent PIs from successfully "locking down" the PR active site into the closed conformation [13,38].

As mentioned previously, substrate and inhibitor recognition rely on the motion of the PR flap regions. Moreover, the hydrophobic sliding mechanism illustrates that the flap regions exist in a delicate equilibrium that is maintained, at least partially, by the rearrangement of hydrophobic residues in the core of the PR. As such, it is postulated that mutations in the core of the PR, whether drug-induced or naturally occurring between subtypes, may alter the packing of the core due to changes in the size and shape of the mutated residues. The mechanism of action of resistance-conferring mutations that occur outside of that active site is not well-understood. However, it has been suggested that their presence in the PR sequence alters the dynamic nature of the enzyme; particularly the flap regions [13,32,53,54]. Since mutations of this nature are not located inside the active site, steric irregularities are not likely to be responsible for their mechanism of action in reduced inhibitor binding affinity. Rather, it is more likely that their presence alters the conformational equilibrium of the PR flaps, resulting in novel conformational heterogeneity in PI-resistant PR variants [12,32,54]. Therefore, when considering the inhibition of non-B subtypes it is likely that observable decreases in drug susceptibility to PIs can be attributed to the altered dynamics and flexibility of the flap regions of the PR. This fact is mirrored when considering the requirement for constellations of non-active site mutations to accumulate in MDR PR variants. It is clear that the occurrence of several non-active site mutations alter the conformational equilibrium of the PR flaps and induce novel conformational heterogeneity in PI-resistant PR variants [12,13,53–55]. It is evident, therefore, that drug resistance arises, at least partially, from alterations in the conformational equilibrium of the flap regions [13,56]. Therefore, differences in drug binding affinity are not only explicable due to the presence of NOPs in non-B subtypes but, rather, these inherent differences are explicitly expected to directly impact drug binding. It is prudent to consider the mechanistic details when designing the next generation of protease inhibitors. In conjunction with the mutations that accumulate in the PR sequence, the Gag and Gag-Pol polyproteins have been seen to accumulate sequence mutations that have been implicated in reduced drug susceptibility. This observation suggests a cooperativity between the PR and Gag/Gag-Pol mutations in order to obtain high levels of resistance [20]. It is noteworthy that the emergence of novel mutations in the PR sequence have been decreasingly reported, an observation that suggests that a limited number of mutations are tolerable within the PR sequence itself. However, the Gag polyprotein is approximately 500 amino acids in length, suggesting that the polyprotein would be more tolerant of a higher number of compensatory mutations in its sequence. Although the mechanism through which mutations in the Gag sequence contribute to PI resistance has yet to be fully understood, it is thought that these mutations work synergistically to simultaneously reduce intermolecular contacts with PIs while maximizing substrate binding.

2.2. Effect Of Insertion Mutations on The Dynamics Of HIV-1 Subtype C PR

Insertion mutations are a rather rare, but not insignificant, type of mutation that occurs in the PR sequence. Amino acid insertions rarely occur in non-B HIV-1 subtype strains and do not play a major role in drug susceptibility, only contributing to drug susceptibility in the presence of additional mutations [57,58]. Amino acid insertions in C-SA PR gene have been observed in treatment-naïve patients with a prevalence of 2.5% [58,59]. These insertions commonly occur in the hinge region resulting in changes to the active site pocket and flap mobility and could be involved in reduced drug susceptibility by altering binding affinities of PIs [30,60,61]. A hinge region insertion C-SA PR mutant, N37T↑V, was isolated from a South African PI-naïve infant (Ledwaba et al., 2019). In order to denote

 doi:10.20944/preprints202506.0863.v1

a mutation that involves the insertion of an amino acid, the "↑" notation was devised by Sayed and Achilonu. In addition to a mutation and insertion at codon 37 in the hinge region, N37T↑V contains ten background mutations, namely, I13V, G16E, I36T, P39S, D60E, Q61E, I62V, L63P, V77I and M89L. Often, changes occurring in the hinge region of the PR directly affect flap dynamics since the hinge is associated with the movement and flexibility of the flaps. Consequently, N37T↑V displays a modification in flap and hinge region dynamics with the flap region achieving a wider opening of the flap regions (Figure 6)[40]. A different hinge region mutant, containing a mutation and insertion at codon 36, was discovered in a HIV-positive mother who was treated with RT inhibitors but was PI-naïve. This mutant, termed I36T↑T, contained three additional background mutations, namely, P39S, D60E and Q61E. The I36T↑T hinge region mutant exhibited increased flap flexibility and movement in the presence of nelfinavir resulting in more semi-open conformations of the complex [62]. It is highly likely that the prevalence of insertion mutations will increase and, therefore, the effects of these insertions on the PR need to be explored.

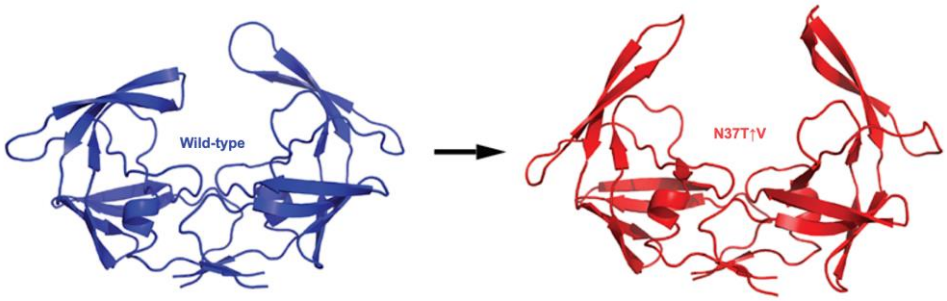

Figure 6. Comparison of WT (blue) and N37T↑V (red) hinge and flap dynamics. The fully open conformation of the N37T↑V flap region opens to a greater extent than the WT PR due to the increased flexibility of the hinge region due to the N37T↑V mutations.

3. Novel Paradigms for Drug Development and PR Inhibition

Despite the propensity for HIV-1 to acquire resistance to antiretroviral agents, PIs have generally been known to exhibit a high genetic barrier to resistance in the context of subtype B and subtype C infections [63]. The accumulation of resistance-conferring mutations in the PR due to prolonged exposure to clinically available PIs has been well documented [64,65]. Indeed, the accumulation of such mutations in the virus is the leading cause for therapy failure. These issues are compounded by the adverse side effects of antiretroviral regimens which result in the discontinuation of treatment. Thus, there remains a need for the development of novel antiretroviral drugs with better features such as greater potency, a higher genetic barrier to resistance, minimal or no side effects. Moreover, the role of NOPs in the resistance mechanism of non-B subtypes of HIV illustrate the need for effective drugs across subtypes.

3.1. Darunavir Scaffold-Based Next Generation Inhibitors

Darunavir, the latest PI to be approved for clinical use, maintains a high genetic barrier to resistance development. This means that DRV maintains inhibitory activity against wild type subtype B PR and resistant PR variants that are no longer susceptible to some older PIs. In the context of the subtype C PR, DRV has been shown to exhibit decreased affinity due to the presence of NOPs [52].

 doi:10.20944/preprints202506.0863.v1

However, the potency of DRV has still been shown to be greater when binding to the wild type subtype C PR in comparison to when in the presence of several resistance conferring mutations [31,66,67].

It is thought that the basis for the high genetic barrier to resistance that is exhibited by DRV is largely due to the formation of hydrogen bonds between the PI and the backbone atoms of the PR active site [68]. Specifically, the oxygens of the bis-THF moiety of DRV at the P2 ligand site (Figure 7, inhibitor 1) form strong hydrogen bonds with the amide group of the PR S2 subsite [68]. Designing inhibitors that interact with the backbone of the PR has been the basis of development for novel PIs. This is because bonds formed with the PR backbone are less likely to be affected by side chain mutations and, therefore, would be less susceptible to resistance-causing active site mutations. The X-ray structure of the subtype B PR in complex with DRV reveals that the P2′ ligand (4-aminosulfonamide) is involved in water-mediated hydrogen bonding with the backbone amide NH of Gly48′ through two crystallographic water molecules [69]. Based upon this observation, it has been hypothesized that modifying the P2′ ligand site to replace one of these two crystallographic water molecules may assist in maintaining strong hydrogen bonds with backbone Asp30, and potentially form a water-mediated hydrogen bond with the backbone of Gly48′ which is located in the flap region of the PR [70,71]. The basis of developing next-generation PIs that bind optimally with the backbone of the PR active site has, therefore, been largely driven by developing derivatives PIs based on the scaffold of DRV and its derivative, TMC-126 (Figure 7, inhibitor 2). New inhibitors have been designed by incorporating more complex chemical structures derived from modifications on three moieties of the DRV scaffold: namely, subsites P1, P2 and P2′. Several novel PIs with high potency have been reported that incorporate a variety of heterocyclic P2 ligands to enhance interactions with the S2 subsite [72–75], with the intention of increasing hydrogen bonding potential with Asp29 and Asp30.

 doi:10.20944/preprints202506.0863.v1

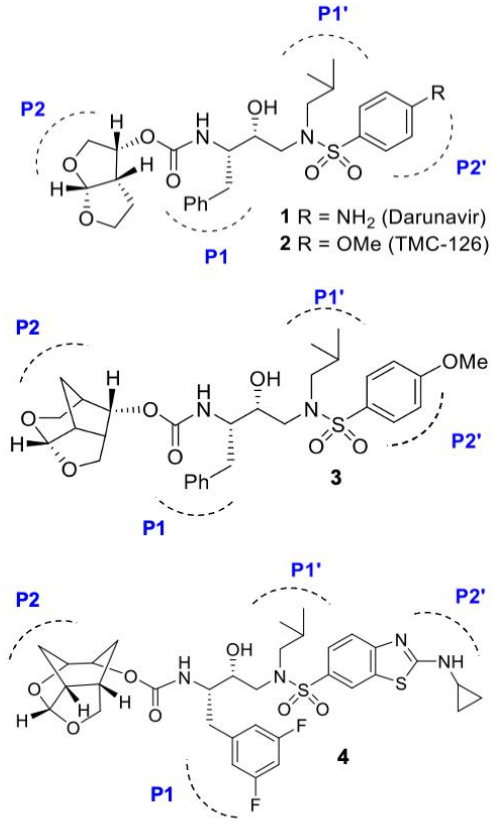

Figure 7. Structures of HIV PIs 1-4. Potent PIs have been developed based on the structural scaffold of darunavir (1), the most recent inhibitor to be approved for clinical use. Novel inhibitors (2, 3, 4) [76–78] are generated through modifying the moieties present at the P1, P2 and P2′ subsites of darunavir.

Additionally, optimization of both P1 and P2′ ligand moieties has proven to improve potency through improving the van der Waals interactions formed with the S1 and S2′ subsites, respectively [74–76,79]. As such, simultaneous optimization of both the P1, P2, and P2′ structural templates of DRV have been proposed for highly potent PIs [76]. The incorporation of a novel crown-like tetrahydropyrano-tetrahydrofuran (crown-THF) as the P2 ligand (Figure 7, inhibitor 3) has been shown to promote additional van der Waals interactions in the active site and these PI derivatives were seen to maintain potent antiviral activity against MDR PR variants [77,78]. Such crown-THF inhibitors have been shown to maintain potent inhibitory activity against MDR subtype B variants; through improved ligand-binding site interactions [77,80]. More recently, a new class of PIs containing cyclohexane fused-bistetrahydrofuran (Chf-THF) as the P2 ligands in combination with (R)- hydroxyethylaminesulfonamide isosteres as the P2′ ligands has shown promise for next generation inhibitors of the PR (Figure 7, inhibitor 4). This inhibitor has been shown to maintain exceptional antiviral potency against a panel of highly MDR subtype B variants. This new design shows promise for hydrogen bonding interactions with the backbone amide groups of Asp29 and Asp30 while the additional methylene groups around the bis-THF exhibit better van der Waals interactions with the S2 subsite [76]. The potency of such novel inhibitors has not been tested in the subtype C; however, the panel of highly MDR subtype B variants tested contain the resistance-

 doi:10.20944/preprints202506.0863.v1

associated mutations M36I/I93L which are resistance-associated NOPs in the subtype C. As such, it may be suggested that novel inhibitors containing crown-THF and Chf-THF moieties could possess the potential to maintain potency across subtypes.

3.2. Dual Action Inhibitors of HIV Reverse Transcriptase and Protease

The design of inhibitors that are capable of simultaneously inhibiting the action of the RT and PR represent a promising inhibitory paradigm. Potent dual inhibitors could radically change drug treatment regimens through minimizing the need for several drug compounds targeting various enzymes in the life cycle of HIV. Dual action inhibitors could decrease the pill burden on HIV positive individuals and decrease the extent of adverse physiological side effects. Together, these factors may increase the therapeutic compliance of those undergoing ART, thereby reducing the discontinuation rates of treatment. The difficulty with designing dual action RT/PR inhibitors lies in the differences in the structure and function of these two enzymes. For instance, the HIV-1 RT is an asymmetric heterodimer composed of a 560 amino acid subunit (p66) and a 440 amino acid subunit (p51) [81]. The p66/p51 RT heterodimer possesses two active sites (Figure 8); the DNA polymerization active site and the RNase H active site, both of which reside in the p66 subunit (Figure 8). The p66 contains several subdomains, namely, the fingers (residues 1-85 & 118-155), palm (residues 86-117 and 156-237), and thumb (residues 238-318) [81]. Moreover, the structure of NNRTIs, NRTIs and PIs differ structurally and chemically (Figure 8). However, there is evidence to suggest that the synthesis of inhibitors with dual action is feasible. Recently, coumarin derivatives were shown to exhibit moderate binding affinities (μM) to both the PR and RT enzymes of the subtype B [82] and subtype C [83]. In an effort to develop more potent dual inhibitors, several chemical changes were introduced, (i) To better fit the spindly binding pocket of RT, cinnamic acids or phenyl-propionic acids (Figure 9) with long linear linkers were used instead of coumarins. (ii) Aromatic rings were maintained in the P2 ligand site in order to optimize π–π interactions with both the PR and RT. (iii) Finally, aliphatic chains or rings were introduced in order to better adapt to both active site cavities [84].

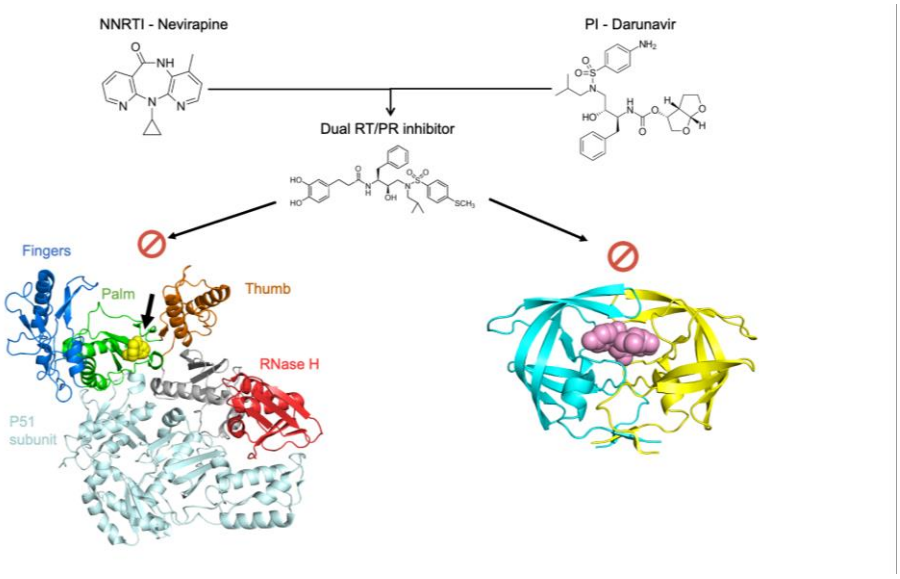

Figure 8. Dual inhibition of the HIV RT and PR enzymes. The structures of an NNRTI and PI are shown as well as a novel dual RT/PR inhibitor (compound 38c,[84]) . The viral RT is divided into subdomains, namely, fingers (blue), palm (green), thumb (orange) and an RNAse H domain (red). The black arrow indicates the location of the NEV ligand (yellow) in the DNA polymerization active site region of RT. The PR is shown in complex with DRV (pink). This figure was generated using PyMOL Molecular Graphics Software, Schrödinger, LLC. PDB ID: 1JLF, 4DQB.

 doi:10.20944/preprints202506.0863.v1

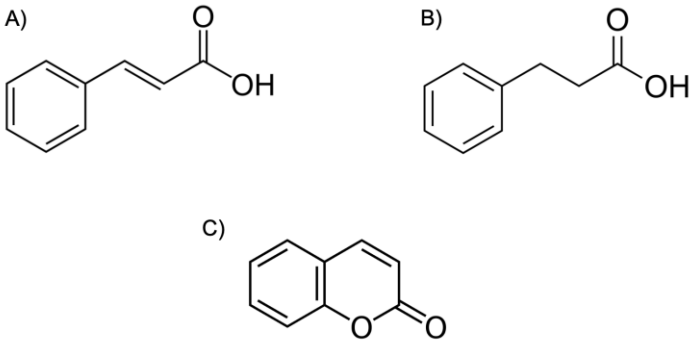

Figure 9. Structure of A) cinnamic acid (PubChem CID: 444539), B) phenyl-propionic acid (PubChem CID: 107) and C) coumarin (Pubchem CID: 323).

Notably, these newly synthesized inhibitors showed an increase in anti-RT activity by one or two orders of magnitude while maintaining potency against the PR. For example, compound 38c (Figure 8) exhibited IC_{50} values of 2.02 nM and 0.43 mM for the PR and RT, respectively, which are comparable to positive control inhibitors; DRV and EFV which exhibit IC_{50} values 1.52 nM and 0.091 mM for the PR and RT, respectively. This novel class of inhibitors illustrates that dual RT/PR inhibitors can be designed to simultaneously target the active site of both enzymes. The basis for targeting both ligand binding sites of RT and PR is thought to be due to the shared nature of interactions and uniformity of key residues shared by the active sites [84]. Molecular docking studies revealed that the dual inhibitory potency was largely due the presence of an aromatic ring as well as hydroxy groups at the P2 ligand site were crucial for the formation of hydrogen bonds and van der Waals interactions with both PR and RT.

3.3. Novel Fullerene Derivatives as Dual RT/PR Inhibitors

Fullerene is a carbon allotrope with pharmaceutically useful properties based on its three-dimensional structure. However, evaluating the bioactivity of fullerene is limited due to poor water solubility. As such, fullerene derivatives with hydrophilic moieties have been developed to study its potential bioactivity and usefulness. Interestingly, the pharmacological properties of fullerene are broad and include antibacterial activity [85], and inhibition of various enzymes including the HIV RT [86] and HIV PR [86,87]. With regard to the usefulness of fullerene derivatives as anti-HIV agents, several fullerene-based compounds have been reported to exhibit dual inhibitory potential for RT and PR [86]. In particular, fullerene derivatives bearing a hydroxy-methyl-carbonyl (HMC) moiety have been shown to bind favorably with the active site aspartic acid residues of the PR during docking simulations [88]. This is due to the HMC moiety mimicking the transition-state isostere of substrate processing, in a manner that is similar to the inhibitory mechanism of clinically available PIs. A study of fullerene derivatives containing a pyrrolidine ring and an alkyl chain linker to the HMC moiety exhibited potent PR inhibition properties in the low micromolar (μM) range [86]. Although the inhibition activities of these fullerene derivatives were weaker than the clinically available PI, ritonavir, cell-based assays revealed that these compounds exhibited membrane permeability comparable to that of ritonavir and no indications of serious cytotoxicity [86]. Thus, the suitability of fullerene-based derivatives as drug candidates for dual inhibition of RT/PR show promise for the development of novel drug candidates which could be used to improve anti-HIV therapy.

 doi:10.20944/preprints202506.0863.v1

3.4. Allosteric Inhibitors of the HIV Protease

The PR exists as an ensemble of conformations in equilibrium, namely, open, semi-open and closed. Allosteric inhibitors are small molecules that bind to the enzyme at an allosteric site, which is not at the same location as the active site, in a manner that affects the activity of the enzyme through modulating its shape, or possibly, its dynamics. The design of allosteric inhibitors of the PR is based on the assumption that the global dynamic nature of proteins is conserved, and so interfering with the dynamic equilibrium may interfere with the function of the enzyme. Indeed, it has been postulated that disturbances to this dynamic equilibrium are the basis of many non-active site resistance mutations [13]. Indeed, molecular dynamics simulations have illustrated that the conformational dynamics of drug-resistant PR variants are different from that of the WT PR [89,90]. Notably, a recent study combining machine learning and molecular dynamics simulations illustrated that drug-resistant PR variants converge to a similar feature phenotype, suggesting that drug resistance is mediated through specific mechanisms that are independent of the site or type of mutations [91]. As such, allosteric inhibitors that interfere with the global dynamics of the protein represent an encouraging prospect for future therapies. Allosteric inhibitors that are able to shift the conformational equilibrium of MDR PR towards behavior that resembles a more wild-type-like state may prove to be an effective course of action. The potency of such allosteric inhibitors has yet to be fully realized; however, the co-administration of allosteric inhibitors alongside traditional PIs may prove to be a promising model of inhibition. To date, four allosteric binding sites have been identified for the dimeric PR, namely, the Exo site, flap-top pocket, Eye site and Cantilever site (Table 1). Several small molecules have been shown to bind to these sites (Table 1, Figure 10) and, interestingly, crystal structures have revealed that existing PIs, APV and DRV, may bind to the flap-top pocket.

Table 1. Allosteric binding sites of the HIV PR and the associated molecules that bind to these sites.

Binding site	Associated residues	Allosteric binder	PDB ID	Reference
Exo site	K14 G16 G17 N18 L63	4D9	3KFN	36
	E65 L70	Br7	N/A	92
Flap-top pocket	Y42 P44 M46 K55 R57	1F1-N	4EJK	93
	P79	AK-2097	4TVG	93
		Br6	2AZC	94
		DRV	3UCB	95
		ATV	3OXV	96
Eye Site		DHQB	N/A	97
		HIVE 9	5VJ3	N/A
	V32 I47 G48 G49 I50 I54	NIT	N/A	98
	V56 G78 P79 T80 P81 I84			
		5NI	N/A	36
Cantilever site	P1 Q2 I3 K7 T12 C67	HIVE 9	5W5W	N/A

 doi:10.20944/preprints202506.0863.v1

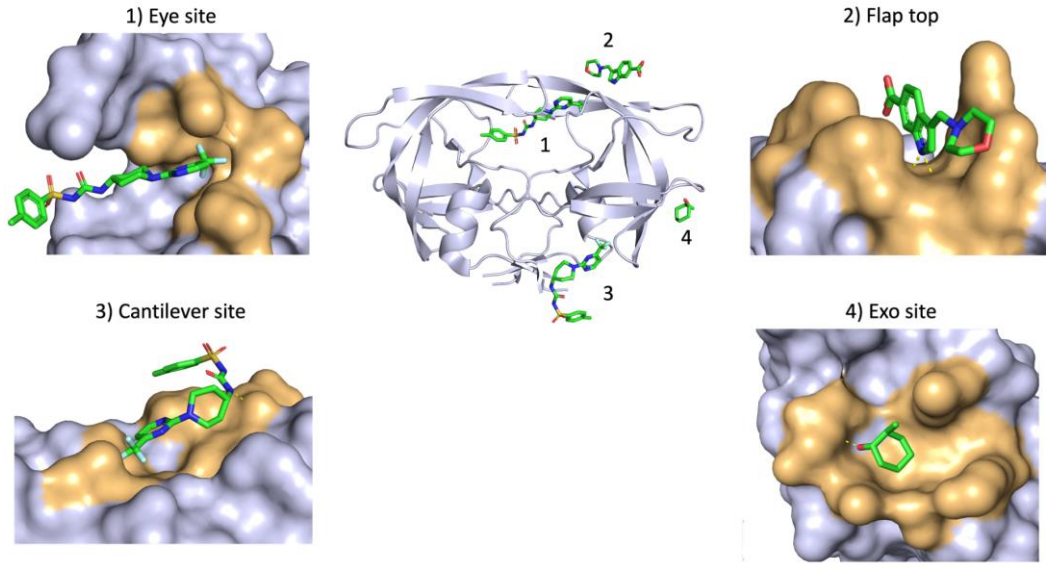

Figure 10. Allosteric binding sites identified for the HIV PR. Four allosteric binding sites for small molecules have been identified on the surface of the PR, namely, the Exo site, the flap-top pocket, the Eye site and the Cantilever site.

The exact mechanism of action for allosteric inhibition of the PR is not fully elucidated; however, some experimental evidence suggests that the modulation of these sites alters the mobility of the flaps. For instance, the Glu35-Arg57 salt bridge forms part of the flap-top pocket and has been shown to play an important role in the conformational stability of the flap regions [31,40]. As such, allosteric inhibitors that bind to the flap-top pocket may modulate the stability of the flaps via the Glu35-Arg57 salt bridge. Curling of the flap tips into the Eye site is thought to drive opening of the flap regions [97] and, as such, blocking the Eye site is hypothesized to prevent curling of the flap tips in a manner that prevents opening of the flaps to accommodate substrates. Alternatively, due to the location of the Eye site, allosteric inhibitors may prevent the flaps from closing fully which could negatively impact the catalytic efficacy of the PR. Either of these modes of binding provide a possible mechanism of action for inhibitors designed to target the Eye site of the PR through altering the dynamic equilibrium of the flaps in a manner that diminishes substrate binding and catalysis. More recently, the curling of the tip regions of the cantilever have been hypothesized to play a significant role in the functional dynamics of the PR [12] and, therefore, allosteric inhibitors that bind to this site and modulate the mobility of the cantilever tips could represent a novel mode of inhibition of the PR. There is evidence to suggest that co-administration of allosteric regulators, such as 1F1, 4D9 and Br6, restore the potency of clinically available PIs against MDR PR variants by restoring a more wild-type like behavior [94,97,98]. As such, when considering the importance of the global dynamic nature of the PR in its functionality, the use of allosteric inhibitors that complement the effect of active site inhibitors could restore drug potency against MDR HIV variants.

3.5. Biologics Targeting HIV Protease

3.5.1. RNA Aptamers

Aptamers are a class of short, single-stranded RNA or DNA oligonucleotides that can form higher order three-dimensional structures and possess high binding affinity (nM-μM) and selectivity for target molecules [99]. These target molecules include: proteins, peptides, toxins, small molecules, carbohydrates and whole cells. Such aptamers possess several characteristics that are advantageous for therapeutic purposes. Aptamers are smaller in size than other biologics, such as antibodies, but maintain selectivity and binding affinity that is comparable to their antibody counterparts. Unlike

 doi:10.20944/preprints202506.0863.v1

antibodies, aptamers can be synthesized in bulk with relative ease and they can be chemically modified to improve their pharmacokinetic and pharmacodynamic properties for specific tasks.

Anti-HIV PR RNA aptamers represent a unique class of potent inhibitors in anti-HIV therapy. RNA aptamers are able to bind to the PR and inhibit its proteolytic activity through non-competitive inhibition. The binding locations of RNA aptamers can be varied, however, since allosteric inhibition sites have been described for other small molecules it is thought that aptamers will successfully inhibit the PR through binding the hinge region, flap-top pocket, flap tips, and dimerization interface. Since aptamers are non-peptidic inhibitors, the goal of their design is interference with the dynamic equilibrium of conformations that the PR is known to sample. As such, aptamers are likely to bind to non-active site regions of the PR. However, aptamers may also prove to be effective dimerization inhibitors which represent a different inhibition mechanism which renders the enzyme inactive due to its obligatory dimeric nature. Since active site inhibitors target the dimer and are susceptible to resistance mutations in the active site it is suggested that dimerization inhibitors may overcome this limitation through interacting with the dimer interface of the PR. Examples of oligonucleotide aptamers are shown in Figure 11 which illustrates the complex structures of three aptamers bound to different regions of the PR monomer. MD simulations of these complexes revealed that aptamer complex A exhibited decreased flexibility in the hinge region of the PR [100]. In this complex, the aptamer forms interactions with residues of the hinge region and stabilizes their movement. The flexibility of the hinge region is known to play a crucial role in the conformational freedom exhibited by the flap regions of the PR [13,32,56]. Therefore, locking the hinge into a more stable conformation has been hypothesized as a method of preventing the function of the PR by restricting the conformational changes required for catalysis. The aptamer complex B (Figure 11) illustrated an increase in the global dynamics and flexibility of the PR backbone [100]. This indicated that the binding of the aptamer to this location that forms part of the “flap-top” pocket of the PR, serves to destabilize the PR which suggests that the dynamic equilibrium of the PR has been altered by this interaction. Complex C (Figure 11) revealed another form of inhibition through interacting with the dimer interface of the monomeric PR [100]. The ability of the aptamer to form stable interactions with the dimer interface would likely inhibit dimer formation and, therefore, the function of the PR. The ability of RNA aptamers to bind to various allosteric locations on the PR illustrate the diverse potential for the inhibitory function of these molecules in anti-HIV therapy.

 doi:10.20944/preprints202506.0863.v1

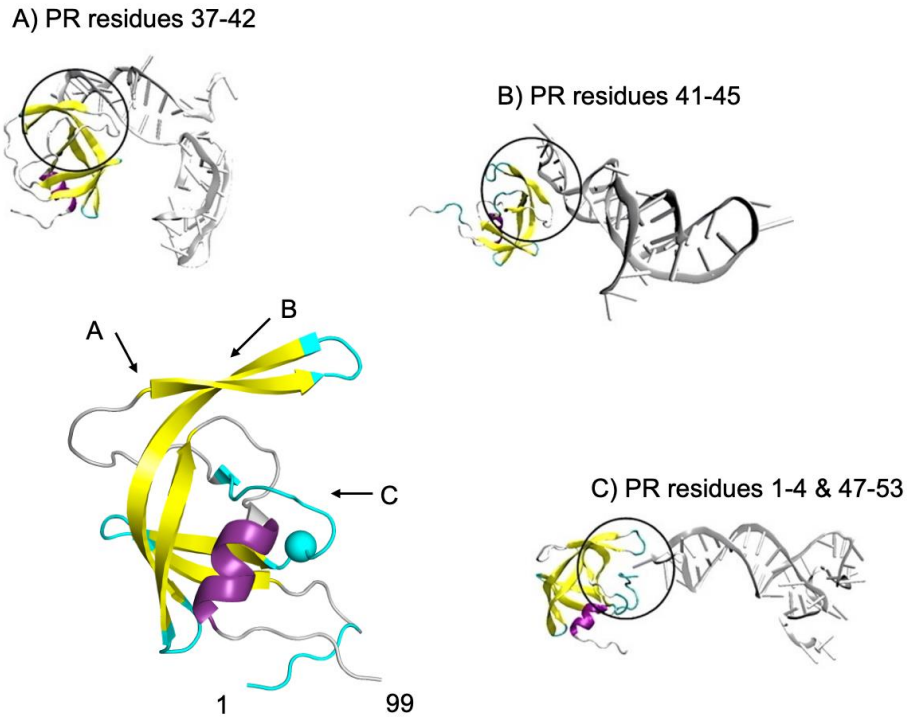

Figure 11. Sites of interaction between the PR monomer and three different oligonucleotide aptamers. Complex A is formed when the aptamer makes contact with the PR hinge and flap region residues (37-42), complex B forms with PR residues 41-45 of the flap region, while complex C makes contact with the N-terminal residues (1-4) and residues of the flap tip (47-53). The active site Asp25 residue is indicated by a cyan sphere.

3.5.2. Peptide-Based Inhibitors

Several peptide inhibitors of the HIV PR have been identified experimentally [101]. While active site mutations can alter the shape of the active site of the PR, peptides have shown great promise as novel protease inhibitors because of the versatile range of locations that are targetable by peptides, other than the catalytic domain. For example, peptides synthesized to be complementary to the N- or C-terminus of the PR (Figure 12) can successfully inhibit dimerization and, therefore, the activity of the PR through binding to the dimerization interface [102,103]. Perhaps most interestingly, peptide inhibitors have been suggested as potential inhibitors of the PR through shifting the thermodynamic equilibrium of the protein towards a denatured state [104]. Such inhibitors are designed to shift the thermodynamic equilibrium of the protein towards a denatured state and, in general, escape the straightforward paradigm of competitive inhibition and resistance mutations. These peptide inhibitors affect the conformational properties of the PR monomer and shift its thermodynamic equilibrium towards a state in which the active site is not formed, resulting in an inactive enzyme [104]. Peptides with the same sequence as segments of the hydrophobic core of the PR (Figure 12) have been suggested to successfully disrupt the PR monomer. Such folding inhibitors represent a novel and potentially powerful method of inhibiting the HIV PR enzyme [105,106]. In order for peptide-based inhibitors to be effective, their sequence must not resemble that of the natural Gag/Gag-Pol cleavage sites. As such, peptide-based biologics exhibit a form of non-competitive/allosteric regulation of the activity of the PR.

 doi:10.20944/preprints202506.0863.v1

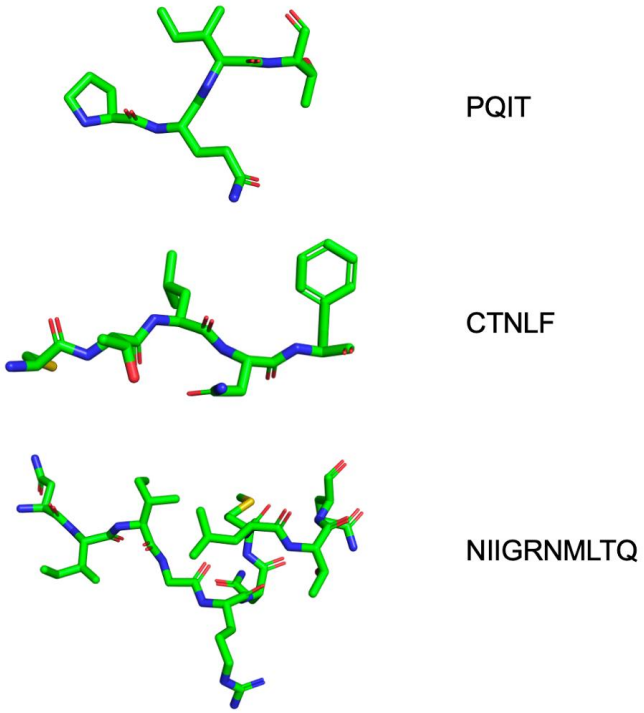

Figure 12. Peptide biologics designed to inhibit HIV-1 PR. Novel peptide biologics are designed using segments of the subtype B PR sequence as a template. Thermodynamic inhibitors resemble sections of the hydrophobic core of the subtype B PR (NIIGRNMLTQ) and disrupt its native structure by penetrating into and interacting with the hydrophobic residues in the core. Dimerization inhibitors are able to bind to and inhibit dimerization by mimicking the peptide sequence that comprises either side of the dimerization interface (PQIT/CTNLF).

4. Concluding Remarks

HIV/AIDS continues to pose a significant socio-economic burden on developing countries, particularly in sub-Saharan Africa. Approximately 39 million individuals are HIV positive globally, with 70% of infections occurring in sub-Saharan Africa. Currently available FDA-approved PIs used for antiretroviral therapy were designed using the HIV-1 subtype B PR as a template. The HIV-1 subtype B occurs mainly in North America and Western Europe and, as a result, the majority of global research has focused on this subtype. Rather, rational drug design approaches have not focused on the HIV-1 subtype C which, shockingly, accounts for more than 50% of global infections. There is evidence to suggest that FDA-approved PIs show diminished efficacy in the context of non-B PR subtypes. The significant genetic diversity of the HI virus means that substantial structural and behavioral differences are present at a fundamental level when comparing different HIV-1 subtypes. Such differences are likely to affect the efficacy of current drug regimens in non-B subtypes. Future research and drug development efforts must focus on the design of inhibitors that are effective across subtypes. Detailed structural, biochemical, and biophysical studies are required to produce novel drug molecules that possess high potency towards HIV-1 subtype specific protein targets.

Author Contributions: Conceptualization, Y.S. and D.S.; writing—original draft preparation, D.S, Z.I, and T.M.; writing—review and editing, Y.S., D.S., T.M., Z.I.; supervision, Y.S.; funding acquisition, Y.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Research Foundation Competitive Support for Rated Researchers, grant number CSRP170428229183, to Yasien Sayed. The views and opinions expressed are those of the authors and do not necessarily represent the official views of the NRF.

 doi:10.20944/preprints202506.0863.v1

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

MDPI	Multidisciplinary Digital Publishing Institute
DOAJ	Directory of open access journals
TLA	Three letter acronym
LD	Linear dichroism

References

1. UNAIDS, "Global HIV & AIDS statistics — Fact sheet," 2023. Accessed: Dec. 30, 2023. [Online]. Available: https://www.unaids.org/en/resources/documents/2023/UNAIDS_FactSheet
2. A. Jacobo-Molina and E. Arnold, "HIV reverse transcriptase structure-function relationships," Biochemistry, vol. 30, no. 26, pp. 6351–6356, Jul. 1991, doi: 10.1021/bi00240a001.
3. J. A. G. Briggs and H.-G. Kräusslich, "The molecular architecture of HIV," J. Mol. Biol., vol. 410, no. 4, pp. 491–500, Jul. 2011, doi: 10.1016/j.jmb.2011.04.021.
4. T. B. Faust, J. M. Binning, J. D. Gross, and A. D. Frankel, "Making Sense of Multifunctional Proteins: Human Immunodeficiency Virus Type 1 Accessory and Regulatory Proteins and Connections to Transcription," Annu. Rev. Virol., vol. 4, no. 1, pp. 241–260, Sep. 2017, doi: 10.1146/annurev-virology-101416-041654.
5. M. Peeters and P. M. Sharp, "Genetic diversity of HIV-1: the moving target," AIDS Lond. Engl., vol. 14 Suppl 3, pp. S129-140, 2000.
6. A. S. Perelson and P. W. Nelson, "Mathematical Analysis of HIV-I: Dynamics in Vivo," SIAM Rev., vol. 41, no. 1, pp. 3–44, 1999.
7. N. V. Bhagavan and C.-E. Ha, "Chapter 22 - DNA Replication, Repair, and Mutagenesis," in Essentials of Medical Biochemistry (Second Edition), N. V. Bhagavan and C.-E. Ha, Eds., San Diego: Academic Press, 2015, pp. 401–417. doi: 10.1016/B978-0-12-416687-5.00022-1.
8. S. Nyamweya, A. Hegedus, A. Jaye, S. Rowland-Jones, K. L. Flanagan, and D. C. Macallan, "Comparing HIV-1 and HIV-2 infection: Lessons for viral immunopathogenesis," Rev. Med. Virol., vol. 23, no. 4, pp. 221–240, Jul. 2013, doi: 10.1002/rmv.1739.
9. D. L. Robertson et al., "HIV-1 nomenclature proposal," Science, vol. 288, no. 5463, pp. 55–56, Apr. 2000, doi: 10.1126/science.288.5463.55d.
10. S. Mosebi, L. Morris, H. W. Dirr, and Y. Sayed, "Active-site mutations in the South african human immunodeficiency virus type 1 subtype C protease have a significant impact on clinical inhibitor binding: kinetic and thermodynamic study," J. Virol., vol. 82, no. 22, pp. 11476–11479, Nov. 2008, doi: 10.1128/JVI.00726-08.
11. P. Naicker and Y. Sayed, "Non-B HIV-1 subtypes in sub-Saharan Africa: impact of subtype on protease inhibitor efficacy," Biol. Chem., vol. 395, no. 10, pp. 1151–1161, Oct. 2014, doi: 10.1515/hsz-2014-0162.
12. D. Sherry, R. Worth, Z. S. Ismail, and Y. Sayed, "Cantilever-centric mechanism of cooperative non-active site mutations in HIV protease: Implications for flap dynamics," J. Mol. Graph. Model., vol. 106, p. 107931, Jul. 2021, doi: 10.1016/j.jmgm.2021.107931.
13. D. Sherry, R. Worth, and Y. Sayed, "Elasticity-Associated Functionality and Inhibition of the HIV Protease," Adv. Exp. Med. Biol., Aug. 2021, doi: 10.1007/5584_2021_655.
14. A. Velazquez-Campoy, S. Vega, and E. Freire, "Amplification of the effects of drug resistance mutations by background polymorphisms in HIV-1 protease from African subtypes," Biochemistry, vol. 41, no. 27, pp. 8613–8619, Jul. 2002, doi: 10.1021/bi020160i.
15. V. Marie and M. L. Gordon, "The HIV-1 Gag Protein Displays Extensive Functional and Structural Roles in Virus Replication and Infectivity," Int. J. Mol. Sci., vol. 23, no. 14, p. 7569, Jul. 2022, doi: 10.3390/ijms23147569.

 doi:10.20944/preprints202506.0863.v1

16. S. Mattei, M. Anders, J. Konvalinka, H.-G. Kräusslich, J. A. G. Briggs, and B. Müller, "Induced Maturation of Human Immunodeficiency Virus," J. Virol., vol. 88, no. 23, pp. 13722–13731, Dec. 2014, doi: 10.1128/JVI.02271-14.
17. J. Ning et al., "In vitro protease cleavage and computer simulations reveal the HIV-1 capsid maturation pathway," Nat. Commun., vol. 7, no. 1, p. 13689, Dec. 2016, doi: 10.1038/ncomms13689.
18. C. M. Parry, A. Kohli, C. J. Boinett, G. J. Towers, A. L. McCormick, and D. Pillay, "Gag Determinants of Fitness and Drug Susceptibility in Protease Inhibitor-Resistant Human Immunodeficiency Virus Type 1," J. Virol., vol. 83, no. 18, pp. 9094–9101, Sep. 2009, doi: 10.1128/JVI.02356-08.
19. F. M. Codoñer et al., "Gag-protease coevolution analyses define novel structural surfaces in the HIV-1 matrix and capsid involved in resistance to Protease Inhibitors," Sci. Rep., vol. 7, no. 1, p. 3717, Jun. 2017, doi: 10.1038/s41598-017-03260-4.
20. V. Marie and M. Gordon, "Gag-protease coevolution shapes the outcome of lopinavir-inclusive treatment regimens in chronically infected HIV-1 subtype C patients," Bioinforma. Oxf. Engl., vol. 35, no. 18, pp. 3219–3223, Sep. 2019, doi: 10.1093/bioinformatics/btz076.
21. C. T.-T. Su, D. W.-S. Koh, and S. K.-E. Gan, "Reviewing HIV-1 Gag Mutations in Protease Inhibitors Resistance: Insights for Possible Novel Gag Inhibitor Designs," Molecules, vol. 24, no. 18, p. 3243, Sep. 2019, doi: 10.3390/molecules24183243.
22. E. Dam et al., "Gag Mutations Strongly Contribute to HIV-1 Resistance to Protease Inhibitors in Highly Drug-Experienced Patients besides Compensating for Fitness Loss," PLOS Pathog., vol. 5, no. 3, p. e1000345, Mar. 2009, doi: 10.1371/journal.ppat.1000345.
23. M. Kožíšek et al., "Mutations in HIV-1 gag and pol Compensate for the Loss of Viral Fitness Caused by a Highly Mutated Protease," Antimicrob. Agents Chemother., vol. 56, no. 8, pp. 4320–4330, Aug. 2012, doi: 10.1128/AAC.00465-12.
24. M. W. Kiguoya et al., "Subtype-Specific Differences in Gag-Protease-Driven Replication Capacity Are Consistent with Intersubtype Differences in HIV-1 Disease Progression," J. Virol., vol. 91, no. 13, pp. e00253-17, Jun. 2017, doi: 10.1128/JVI.00253-17.
25. A. J. Marozsan et al., "Differences in the fitness of two diverse wild-type human immunodeficiency virus type 1 isolates are related to the efficiency of cell binding and entry," J. Virol., vol. 79, no. 11, pp. 7121–7134, Jun. 2005, doi: 10.1128/JVI.79.11.7121-7134.2005.
26. C. M. Venner et al., "Infecting HIV-1 Subtype Predicts Disease Progression in Women of Sub-Saharan Africa," EBioMedicine, vol. 13, pp. 305–314, Oct. 2016, doi: 10.1016/j.ebiom.2016.10.014.
27. P. Naicker, P. Seele, H. W. Dirr, and Y. Sayed, "F99 is critical for dimerization and activation of South African HIV-1 subtype C protease," Protein J., vol. 32, no. 7, pp. 560–567, Oct. 2013, doi: 10.1007/s10930-013-9517-y.
28. P. P. Mager, "The active site of HIV-1 protease," Med. Res. Rev., vol. 21, no. 4, pp. 348–353, Jul. 2001, doi: 10.1002/med.1012.
29. A. Gustchina and I. T. Weber, "Comparison of inhibitor binding in HIV-1 protease and in non-viral aspartic proteases: the role of the flap," FEBS Lett., vol. 269, no. 1, pp. 269–272, Aug. 1990, doi: 10.1016/0014-5793(90)81171-j.
30. J. Ledwaba, Y. Sayed, V. Pillay, L. Morris, and G. Hunt, "Low Frequency of Protease Inhibitor Resistance Mutations and Insertions in HIV-1 Subtype C Protease Inhibitor-Naïve Sequences," AIDS Res. Hum. Retroviruses, vol. 35, no. 7, pp. 673–678, Jul. 2019, doi: 10.1089/AID.2019.0012.
31. P. Naicker et al., "Structural insights into the South African HIV-1 subtype C protease: impact of hinge region dynamics and flap flexibility in drug resistance," J. Biomol. Struct. Dyn., vol. 31, no. 12, pp. 1370–1380, Dec. 2013, doi: 10.1080/07391102.2012.736774.
32. J. E. Foulkes-Murzycki, W. R. P. Scott, and C. A. Schiffer, "Hydrophobic Sliding: A Possible Mechanism for Drug Resistance in Human Immunodeficiency Virus Type 1 Protease," Structure, vol. 15, no. 2, pp. 225–233, Feb. 2007, doi: 10.1016/j.str.2007.01.006.
33. D. I. Freedberg et al., "Rapid structural fluctuations of the free HIV protease flaps in solution: Relationship to crystal structures and comparison with predictions of dynamics calculations," Protein Sci. Publ. Protein Soc., vol. 11, no. 2, pp. 221–232, Feb. 2002.

 doi:10.20944/preprints202506.0863.v1

34. V. Hornak, A. Okur, R. C. Rizzo, and C. Simmerling, "HIV-1 protease flaps spontaneously close to the correct structure in simulations following manual placement of an inhibitor into the open state," J. Am. Chem. Soc., vol. 128, no. 9, pp. 2812–2813, Mar. 2006, doi: 10.1021/ja058211x.
35. L. K. Nicholson et al., "Flexibility and function in HIV-1 protease," Nat. Struct. Biol., vol. 2, no. 4, pp. 274–280, Apr. 1995, doi: 10.1038/nsb0495-274.
36. A. L. Perryman, J.-H. Lin, and J. A. McCammon, "HIV-1 protease molecular dynamics of a wild-type and of the V82F/I84V mutant: Possible contributions to drug resistance and a potential new target site for drugs," Protein Sci., vol. 13, no. 4, pp. 1108–1123, 2004, doi: 10.1110/ps.03468904.
37. I. Schechter and A. Berger, "On the size of the active site in proteases. I. Papain," Biochem. Biophys. Res. Commun., vol. 27, no. 2, pp. 157–162, Apr. 1967, doi: 10.1016/s0006-291x(67)80055-x.
38. S. I. Maphumulo et al., "Exploring the flap dynamics of the South African HIV subtype C protease in presence of FDA-approved inhibitors: MD study," Chem. Biol. Drug Des., vol. 92, no. 5, pp. 1899–1913, 2018, doi: 10.1111/cbdd.13364.
39. J. L. Kear, M. E. Blackburn, A. M. Veloro, B. M. Dunn, and G. E. Fanucci, "Subtype Polymorphisms Among HIV-1 Protease Variants Confer Altered Flap Conformations and Flexibility," J. Am. Chem. Soc., vol. 131, no. 41, pp. 14650–14651, Oct. 2009, doi: 10.1021/ja907088a.
40. J. Zondagh, V. Balakrishnan, I. Achilonu, H. W. Dirr, and Y. Sayed, "Molecular dynamics and ligand docking of a hinge region variant of South African HIV-1 subtype C protease," J. Mol. Graph. Model., vol. 82, pp. 1–11, Jun. 2018, doi: 10.1016/j.jmgm.2018.03.006.
41. R. M. Coman et al., "The Contribution of Naturally Occurring Polymorphisms in Altering the Biochemical and Structural Characteristics of HIV-1 Subtype C Protease," Biochemistry, vol. 47, no. 2, pp. 731–743, Jan. 2008, doi: 10.1021/bi7018332.
42. A. Wlodawer and J. Vondrasek, "INHIBITORS OF HIV-1 PROTEASE: A Major Success of Structure-Assisted Drug Design1," Annu. Rev. Biophys., vol. 27, no. Volume 27, 1998, pp. 249–284, Jun. 1998, doi: 10.1146/annurev.biophys.27.1.249.
43. J. A. Partaledis et al., "In vitro selection and characterization of human immunodeficiency virus type 1 (HIV-1) isolates with reduced sensitivity to hydroxyethylamino sulfonamide inhibitors of HIV-1 aspartyl protease," J. Virol., vol. 69, no. 9, pp. 5228–5235, Sep. 1995, doi: 10.1128/jvi.69.9.5228-5235.1995.
44. E. Lefebvre and C. A. Schiffer, "Resilience to Resistance of HIV-1 Protease Inhibitors: Profile of Darunavir," AIDS Rev., vol. 10, no. 3, pp. 131–142, 2008.
45. M. A. Thompson et al., "Antiretroviral Treatment of Adult HIV Infection: 2010 Recommendations of the International AIDS Society–USA Panel," JAMA, vol. 304, no. 3, pp. 321–333, Jul. 2010, doi: 10.1001/jama.2010.1004.
46. P. A. Volberding and S. G. Deeks, "Antiretroviral therapy and management of HIV infection," The Lancet, vol. 376, no. 9734, pp. 49–62, Jul. 2010, doi: 10.1016/S0140-6736(10)60676-9.
47. T. D. Wu et al., "Mutation Patterns and Structural Correlates in Human Immunodeficiency Virus Type 1 Protease following Different Protease Inhibitor Treatments," J. Virol., vol. 77, no. 8, pp. 4836–4847, Apr. 2003, doi: 10.1128/jvi.77.8.4836-4847.2003.
48. Z. Chen, Y. Li, H. B. Schock, D. Hall, E. Chen, and L. C. Kuo, "Three-dimensional structure of a mutant HIV-1 protease displaying cross-resistance to all protease inhibitors in clinical trials," J. Biol. Chem., vol. 270, no. 37, pp. 21433–21436, Sep. 1995, doi: 10.1074/jbc.270.37.21433.
49. M. Markowitz et al., "Selection and analysis of human immunodeficiency virus type 1 variants with increased resistance to ABT-538, a novel protease inhibitor," J. Virol., vol. 69, no. 2, pp. 701–706, Feb. 1995, doi: 10.1128/jvi.69.2.701-706.1995.
50. F. Clavel and F. Mammano, "Role of Gag in HIV Resistance to Protease Inhibitors," Viruses, vol. 2, no. 7, Art. no. 7, Jul. 2010, doi: 10.3390/v2071411.
51. M. Nijhuis et al., "Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy," AIDS Lond. Engl., vol. 13, no. 17, pp. 2349–2359, Dec. 1999, doi: 10.1097/00002030-199912030-00006.
52. [52] A. Velázquez-Campoy et al., "Protease inhibition in African subtypes of HIV-1," AIDS Rev., vol. 5, no. 3, pp. 165–171, 2003.

 doi:10.20944/preprints202506.0863.v1

53. N. E. Goldfarb et al., "Defective Hydrophobic Sliding Mechanism and Active Site Expansion in HIV-1 Protease Drug Resistant Variant Gly48Thr/Leu89Met: Mechanisms for the Loss of Saquinavir Binding Potency," Biochemistry, vol. 54, no. 2, pp. 422–433, Jan. 2015, doi: 10.1021/bi501088e.
54. S. Mittal, Y. Cai, M. N. L. Nalam, D. N. A. Bolon, and C. A. Schiffer, "Hydrophobic Core Flexibility Modulates Enzyme Activity in HIV-1 Protease," J. Am. Chem. Soc., vol. 134, no. 9, pp. 4163–4168, Mar. 2012, doi: 10.1021/ja2095766.
55. S. V. Sankaran, S. R. Krishnan, Y. Sayed, and M. M. Gromiha, "Mechanism of drug resistance in HIV-1 protease subtype C in the presence of Atazanavir," Curr. Res. Struct. Biol., vol. 7, p. 100132, Feb. 2024, doi: 10.1016/j.crstbi.2024.100132.
56. V. Hornak, A. Okur, R. C. Rizzo, and C. Simmerling, "HIV-1 protease flaps spontaneously open and reclose in molecular dynamics simulations," Proc. Natl. Acad. Sci., vol. 103, no. 4, pp. 915–920, Jan. 2006, doi: 10.1073/pnas.0508452103.
57. M. Kozísek et al., "Ninety-nine is not enough: molecular characterization of inhibitor-resistant human immunodeficiency virus type 1 protease mutants with insertions in the flap region," J. Virol., vol. 82, no. 12, pp. 5869–5878, Jun. 2008, doi: 10.1128/JVI.02325-07.
58. J. Pereira-Vaz, V. Duque, L. Trindade, J. Saraiva-da-Cunha, and A. Meliço-Silvestre, "Detection of the protease codon 35 amino acid insertion in sequences from treatment-naïve HIV-1 subtype C infected individuals in the Central Region of Portugal," J. Clin. Virol., vol. 46, no. 2, pp. 169–172, Oct. 2009, doi: 10.1016/j.jcv.2009.06.019.
59. P. O. Bessong et al., "Resistance Mutational Analysis of HIV Type 1 Subtype C among Rural South African Drug-Naive Patients Prior to Large-Scale Availability of Antiretrovirals," AIDS Res. Hum. Retroviruses, vol. 22, no. 12, pp. 1306–1312, Dec. 2006, doi: 10.1089/aid.2006.22.1306.
60. S. B. Maseko et al., "I36T↑T mutation in South African subtype C (C-SA) HIV-1 protease significantly alters protease-drug interactions," Biol. Chem., vol. 398, no. 10, pp. 1109–1117, Oct. 2017, doi: 10.1515/hsz-2017-0107.
61. Z. K. Sanusi et al., "An insight to the molecular interactions of the FDA approved HIV PR drugs against L38L↑N↑L PR mutant," J. Comput. Aided Mol. Des., vol. 32, no. 3, pp. 459–471, Mar. 2018, doi: 10.1007/s10822-018-0099-9.
62. H. A. Lockhat et al., "Binding Free Energy Calculations of Nine FDA-approved Protease Inhibitors Against HIV-1 Subtype C I36T↑T Containing 100 Amino Acids Per Monomer," Chem. Biol. Drug Des., vol. 87, no. 4, pp. 487–498, 2016, doi: 10.1111/cbdd.12690.
63. M. Zazzi, "High genetic barrier antiretroviral drugs in human immunodeficiency virus-positive pregnancy," Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am., vol. 50, no. 6, pp. 895–897, Mar. 2010, doi: 10.1086/650748.
64. I. T. Weber, D. W. Kneller, and A. Wong-Sam, "Highly resistant HIV-1 proteases and strategies for their inhibition," Future Med. Chem., vol. 7, no. 8, pp. 1023–1038, Jun. 2015, doi: 10.4155/fmc.15.44.
65. I. T. Weber and J. Agniswamy, "HIV-1 Protease: Structural Perspectives on Drug Resistance," Viruses, vol. 1, no. 3, pp. 1110–1136, Dec. 2009, doi: 10.3390/v1031110.
66. D. Sherry, R. Pandian, and Y. Sayed, "Non-active site mutations in the HIV protease: Diminished drug binding affinity is achieved through modulating the hydrophobic sliding mechanism," Int. J. Biol. Macromol., vol. 217, pp. 27–41, Sep. 2022, doi: 10.1016/j.ijbiomac.2022.07.033.
67. J. Zondagh, A. E. Basson, I. Achilonu, L. Morris, H. W. Dirr, and Y. Sayed, "Drug susceptibility and replication capacity of a rare HIV-1 subtype C protease hinge region variant," Antivir. Ther., vol. 24, no. 5, pp. 333–342, 2019, doi: 10.3851/IMP3308.
68. A. K. Ghosh, Z. L. Dawson, and H. Mitsuya, "Darunavir, a conceptually new HIV-1 protease inhibitor for the treatment of drug-resistant HIV," Bioorg. Med. Chem., vol. 15, no. 24, pp. 7576–7580, Dec. 2007, doi: 10.1016/j.bmc.2007.09.010.
69. Z. Liu et al., "Conserved hydrogen bonds and water molecules in MDR HIV-1 protease substrate complexes," Biochem. Biophys. Res. Commun., vol. 430, no. 3, pp. 1022–1027, Jan. 2013, doi: 10.1016/j.bbrc.2012.12.045.

 doi:10.20944/preprints202506.0863.v1

70. J. Agniswamy, D. W. Kneller, A. K. Ghosh, and I. T. Weber, "Novel HIV PR inhibitors with C4-substituted bis-THF and bis-fluoro-benzyl target the two active site mutations of highly drug resistant mutant PRS17," Biochem. Biophys. Res. Commun., vol. 566, pp. 30–35, Aug. 2021, doi: 10.1016/j.bbrc.2021.05.094.
71. A. K. Ghosh et al., "Potent HIV-1 Protease Inhibitors Containing Carboxylic and Boronic Acids: Effect on Enzyme Inhibition and Antiviral Activity and Protein-Ligand X-ray Structural Studies," ChemMedChem, vol. 14, no. 21, pp. 1863–1872, Nov. 2019, doi: 10.1002/cmdc.201900508.
72. X. Bai et al., "Design and synthesis of potent HIV-1 protease inhibitors with (S)-tetrahydrofuran-tertiary amine-acetamide as P2–ligand: Structure–activity studies and biological evaluation," Eur. J. Med. Chem., vol. 137, pp. 30–44, Sep. 2017, doi: 10.1016/j.ejmech.2017.05.024.
73. [73] Y. Dou et al., "Design, synthesis and biological evaluation of HIV-1 protease inhibitors with morpholine derivatives as P2 ligands in combination with cyclopropyl as P1' ligand," Bioorg. Med. Chem. Lett., vol. 30, no. 7, p. 127019, Apr. 2020, doi: 10.1016/j.bmcl.2020.127019.
74. K. Hohlfeld, J. K. Wegner, B. Kesteleyn, B. Linclau, and J. Unge, "Disubstituted Bis-THF Moieties as New P2 Ligands in Nonpeptidal HIV-1 Protease Inhibitors (II)," J. Med. Chem., vol. 58, no. 9, pp. 4029–4038, May 2015, doi: 10.1021/acs.jmedchem.5b00358.
75. K. Hohlfeld, C. Tomassi, J. K. Wegner, B. Kesteleyn, and B. Linclau, "Disubstituted Bis-THF Moieties as New P2 Ligands in Nonpeptidal HIV-1 Protease Inhibitors," ACS Med. Chem. Lett., vol. 2, no. 6, pp. 461–465, Jun. 2011, doi: 10.1021/ml2000356.
76. A. K. Ghosh et al., "Structure-Based Design of Highly Potent HIV-1 Protease Inhibitors Containing New Tricyclic Ring P2-Ligands: Design, Synthesis, Biological, and X-ray Structural Studies," J. Med. Chem., vol. 63, no. 9, pp. 4867–4879, May 2020, doi: 10.1021/acs.jmedchem.0c00202.
77. A. K. Ghosh et al., "Design of Highly Potent, Dual-Acting and Central-Nervous-System-Penetrating HIV-1 Protease Inhibitors with Excellent Potency against Multidrug-Resistant HIV-1 Variants," ChemMedChem, vol. 13, no. 8, pp. 803–815, 2018, doi: 10.1002/cmdc.201700824.
78. A. K. Ghosh et al., "Design and Development of Highly Potent HIV-1 Protease Inhibitors with a Crown-Like Oxotricyclic Core as the P2-Ligand To Combat Multidrug-Resistant HIV Variants," J. Med. Chem., vol. 60, no. 10, pp. 4267–4278, May 2017, doi: 10.1021/acs.jmedchem.7b00172.
79. A. K. Ghosh et al., "Evaluation of darunavir-derived HIV-1 protease inhibitors incorporating P2' amide-derivatives: Synthesis, biological evaluation and structural studies," Bioorg. Med. Chem. Lett., vol. 83, p. 129168, 2023.
80. M. Aoki et al., "A novel central nervous system-penetrating protease inhibitor overcomes human immunodeficiency virus 1 resistance with unprecedented aM to pM potency," eLife, vol. 6, p. e28020, Oct. 2017, doi: 10.7554/eLife.28020.
81. L. A. Kohlstaedt, J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz, "Crystal Structure at 3.5 Å Resolution of HIV-1 Reverse Transcriptase Complexed with an Inhibitor," Science, vol. 256, no. 5065, pp. 1783–1790, Jun. 1992, doi: 10.1126/science.1377403.
82. S. Mishra, A. Pandey, and S. Manvati, "Coumarin: An emerging antiviral agent," Heliyon, vol. 6, no. 1, Jan. 2020, doi: 10.1016/j.heliyon.2020.e03217.
83. T. O. Olomola, R. Klein, N. Mautsa, Y. Sayed, and P. T. Kaye, "Synthesis and evaluation of coumarin derivatives as potential dual-action HIV-1 protease and reverse transcriptase inhibitors," Bioorg. Med. Chem., vol. 21, no. 7, pp. 1964–1971, Apr. 2013, doi: 10.1016/j.bmc.2013.01.025.
84. M. Zhu et al., "Design and biological evaluation of cinnamic and phenylpropionic amide derivatives as novel dual inhibitors of HIV-1 protease and reverse transcriptase," Eur. J. Med. Chem., vol. 220, p. 113498, Aug. 2021, doi: 10.1016/j.ejmech.2021.113498.
85. T. Mashino et al., "Antibacterial and antiproliferative activity of cationic fullerene derivatives," Bioorg. Med. Chem. Lett., vol. 13, no. 24, pp. 4395–4397, Dec. 2003, doi: 10.1016/j.bmcl.2003.09.040.
86. T. Yasuno et al., "Fullerene derivatives as dual inhibitors of HIV-1 reverse transcriptase and protease," Bioorg. Med. Chem. Lett., vol. 31, p. 127675, Jan. 2021, doi: 10.1016/j.bmcl.2020.127675.
87. S. H. Friedman, P. S. Ganapathi, Y. Rubin, and G. L. Kenyon, "Optimizing the Binding of Fullerene Inhibitors of the HIV-1 Protease through Predicted Increases in Hydrophobic Desolvation," J. Med. Chem., vol. 41, no. 13, pp. 2424–2429, Jun. 1998, doi: 10.1021/jm970689r.

 doi:10.20944/preprints202506.0863.v1

88. M. Ibrahim, N. A. Saleh, A. J. Hameed, W. M. Elshemey, and A. A. Elsayed, "Structural and electronic properties of new fullerene derivatives and their possible application as HIV-1 protease inhibitors," Spectrochim. Acta. A. Mol. Biomol. Spectrosc., vol. 75, no. 2, pp. 702–709, Feb. 2010, doi: 10.1016/j.saa.2009.11.042.
89. M. Souffrant, X.-Q. Yao, and D. Hamelberg, "Evolving Mutational Buildup in HIV-1 Protease Shifts Conformational Dynamics to Gain Drug Resistance," J. Chem. Inf. Model., vol. 63, no. 12, pp. 3892–3902, Jun. 2023, doi: 10.1021/acs.jcim.3c00535.
90. A. Wong-Sam et al., "HIV-1 protease with 10 lopinavir and darunavir resistance mutations exhibits altered inhibition, structural rearrangements and extreme dynamics," J. Mol. Graph. Model., vol. 117, p. 108315, Dec. 2022, doi: 10.1016/j.jmgm.2022.108315.
91. F. Leidner, N. Kurt Yilmaz, and C. A. Schiffer, "Deciphering Complex Mechanisms of Resistance and Loss of Potency through Coupled Molecular Dynamics and Machine Learning," J. Chem. Theory Comput., vol. 17, no. 4, pp. 2054–2064, Apr. 2021, doi: 10.1021/acs.jctc.0c01244.
92. T. Tiefenbrunn et al., "Small Molecule Regulation of Protein Conformation by Binding in the Flap of HIV Protease," ACS Chem. Biol., vol. 8, no. 6, pp. 1223–1231, Jun. 2013, doi: 10.1021/cb300611p.
93. T. Tiefenbrunn et al., "Crystallographic Fragment-Based Drug Discovery: Use of a Brominated Fragment Library Targeting HIV Protease," Chem. Biol. Drug Des., vol. 83, no. 2, pp. 141–148, 2014, doi: 10.1111/cbdd.12227.
94. A. Luchi, E. Angelina, L. Bogado, S. Forli, A. Olson, and N. Peruchena, "Flap-site Fragment Restores Back Wild-type Behaviour in Resistant Form of HIV Protease," Mol. Inform., vol. 37, no. 12, p. 1800053, 2018, doi: 10.1002/minf.201800053.
95. J. Agniswamy, C.-H. Shen, A. Aniana, J. M. Sayer, J. M. Louis, and I. T. Weber, "HIV-1 Protease with 20 Mutations Exhibits Extreme Resistance to Clinical Inhibitors through Coordinated Structural Rearrangements," Biochemistry, vol. 51, no. 13, pp. 2819–2828, Apr. 2012, doi: 10.1021/bi2018317.
96. S. Mittal et al., "Structural and Thermodynamic Basis of Amprenavir/Darunavir and Atazanavir Resistance in HIV-1 Protease with Mutations at Residue 50," J. Virol., vol. 87, no. 8, pp. 4176–4184, Apr. 2013, doi: 10.1128/JVI.03486-12.
97. K. L. Damm, P. M. U. Ung, J. J. Quintero, J. E. Gestwicki, and H. A. Carlson, "A poke in the eye: Inhibiting HIV-1 protease through its flap-recognition pocket," Biopolymers, vol. 89, no. 8, pp. 643–652, 2008, doi: 10.1002/bip.20993.
98. P. M.-U. Ung, J. B. Dunbar, J. E. Gestwicki, and H. A. Carlson, "An Allosteric Modulator of HIV-1 Protease Shows Equipotent Inhibition of Wild-Type and Drug-Resistant Proteases," J. Med. Chem., vol. 57, no. 15, pp. 6468–6478, Aug. 2014, doi: 10.1021/jm5008352.
99. A. D. Ellington and J. W. Szostak, "In vitro selection of RNA molecules that bind specific ligands," Nature, vol. 346, no. 6287, pp. 818–822, Aug. 1990, doi: 10.1038/346818a0.
100. M. Ajamgard, J. J. Sardroodi, and A. R. Ebrahimzadeh, "A Molecular Dynamics Study of the Inhibition of Monomeric HIV-1 Protease as An Alternative to Overcome Drug Resistance by RNA Aptamers as A Therapeutic Tool," ChemistrySelect, vol. 5, no. 29, pp. 9086–9096, 2020, doi: 10.1002/slct.202000990.
101. A. Qureshi, N. Thakur, and M. Kumar, "HIPdb: A Database of Experimentally Validated HIV Inhibiting Peptides," PLOS ONE, vol. 8, no. 1, p. e54908, Jan. 2013, doi: 10.1371/journal.pone.0054908.
102. L. M. Babé, J. Rosé, and C. S. Craik, "Synthetic 'interface' peptides alter dimeric assembly of the HIV 1 and 2 proteases," Protein Sci., vol. 1, no. 10, pp. 1244–1253, 1992, doi: 10.1002/pro.5560011003.
103. H. J. Schramm et al., "The inhibition of human immunodeficiency virus proteases by 'interface peptides,'" Antiviral Res., vol. 30, no. 2, pp. 155–170, May 1996, doi: 10.1016/0166-3542(96)00940-0.
104. S. Kimura, R. A. Broglia, and G. Tiana, "Thermodynamics of strongly allosteric inhibition: a model study of HIV-1 protease," Eur. Biophys. J. EBJ, vol. 41, no. 11, pp. 991–1001, Nov. 2012, doi: 10.1007/s00249-012-0862-0.
105. R. Broglia, Y. Levy, and G. Tiana, "HIV-1 protease folding and the design of drugs which do not create resistance," Curr. Opin. Struct. Biol., vol. 18, no. 1, pp. 60–66, Feb. 2008, doi: 10.1016/j.sbi.2007.10.004.

 doi:10.20944/preprints202506.0863.v1

106. R. A. Broglia, G. Tiana, L. Sutto, D. Provasi, and F. Simona, "Design of HIV-1-PR inhibitors that do not create resistance: Blocking the folding of single monomers," Protein Sci., vol. 14, no. 10, pp. 2668–2681, 2005, doi: 10.1110/ps.051670905.