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Molecular Analysis of Human Immuno-deficiency Virus-1 (South African subtype C) Protease Drug Resistance mutations emerging on Darunavir therapy

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Molecular Analysis of Human Immuno-deficiency Virus-1 (South African subtype C) Protease Drug Resistance mutations emerging on Darunavir therapy

A thesis submitted to the School of Pharmacy and Pharmacology, Faculty of Health Science, University of KwaZulu-Natal, Westville, for the degree of Master of Pharmaceutical Chemistry.

This is a thesis in which the chapters are written as a set of discrete research paper, with an overall introduction and conclusion. Typically, these chapters will have been published in internationally recognized, peer-reviewed journals.

This is to certify that the research reported in this thesis is the original work of Eniola Lilian Folarin.

As the candidate's supervisors, we have approved this thesis for submission.

Supervisor:

Signed: ---------- Name: **Prof G Kruger** Date: ----------

Co-Supervisor:

Signed: ---------- Name: **Prof J Lin** Date: ----------

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DEDICATION

I dedicate this thesis to my family. I am thankful for the love, encouragement and prayers.

Abstract

Human immunodeficiency virus as the causative agent of acquired immune deficiency syndrome remains a serious infectious disease and the leading cause of deaths worldwide. According to UNAIDS, approximately 37 million individuals are living with HIV/AIDS and 770,000 AIDS related deaths. HIV-1 subtype C strain is responsible for approximately 70 % of individuals living with HIV. Even with this staggering statistic, not many studies have been conducted on this subtype.

Currently, there exist no treatment that completely eradicates the virus from an infected individual. Although, three enzymes required by the virus to undergo intracellular replication have been targeted to delay the progression of the disease, these enzymes include; reverse transcriptase crucial for completion of the initial stages of HIV replication, integrase essential for the integration of pro-viral DNA into the host chromosomal DNA and finally the enzyme for which this study will focus only is protease which is vital for the development and assembly of infectious viral progeny. The HIV aspartyl protease plays a major role in the life cycle of the virus and has long been a target in antiviral therapy. This advancements in the knowledge of HIV biology, pathogenesis and pharmacology has led to unprecedented efforts to interpret basic findings in the development of novel antiviral drug therapies.

Nonetheless, the emergence of drug resistant mutations has hampered the efficacy of HIV-1 protease inhibition therapy. These mutations reduce the binding affinity of inhibitors while maintaining viable catalytic activity and affinity for the natural substrate. In HIV-1 protease, mutations at the following positions V32I, I50V,154M, and I84V are associated with subtle structural changes that confer resistance to protease inhibitors especially darunavir. These mutations located at or adjacent to the active site cavity, compromise drug susceptibility due to weak Van der Waals interaction and binding site distortion resulting in treatment failure. In this study we analysed the functional effects of these mutations on the HIV-1 South African subtype C protease. To understand how these mutations influence drug susceptibility in HIV-1 CSA protease, the mutations were introduced by site directed mutagenesis and confirmed by DNA sequencing. Over-expression and purification of wild-type and mutant protease. Followed by enzyme kinetics, inhibition (K_i) and thermodynamics studies carried out against six clinically approved drugs. Significant difference was not observed in the substrate affinity of the variant protease compared to the wildtype C-SA protease with a Michaelis constant (K_m)

values of 104 and 124 µM and turnover number (K_{cat}) of approximately 2.2 and 0.2 s^{-1} for variant and wildtype protease respectively. The six clinically approved drugs used in this study demonstrated reduced binding affinities and weaker inhibition towards the variant protease in comparison to the wild-type HIV-1 protease. Atazanavir, amprenavir, darunavir and saquinavir exhibited the weakest inhibition towards the variant protease with K_i ratio values of 163, 232, 465 and 247 respectively. Thermodynamic data showed less favourable Gibbs free binding energy in selected protease inhibitors towards the variant protease, largely due to decreased binding entropy. Vitality values for the variant protease against the selected protease inhibitors, confirm the impact of these mutations on the HIV-1 CSA protease. In the presence of these drug resistant mutations V32I, I50V,154M, and I84V the efficacy of the selected protease inhibitors used in this study is significantly reduced. Future studies would involve crystallization and structure determination. This will give an in-depth understanding on the structural interaction of the variant protease towards the protease inhibitors.

DECLARATION 1-PLAGIARISM

I, Eniola Lilian Folarin declare that

1. The research reported in this thesis, except where otherwise indicated, and is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
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I, Professor G. Kruger as supervisor of the Masters study hereby consent to the submission of this Masters thesis. The 'Turnitin' report generated a value of 13 % similarity.

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List of Articles for Publication

1. Targeting the major HIV-1 enzymes: Are we winning the battle in fighting the epidemic?
   Authors: **Sphelele Jeza,** Sibusiso Maseko and Eniola Folarin and Johnson Lin
   Review article for submission.
2. Key Darunavir Resistant Mutations in the HIV-1 C-SA Protease Alters Binding Affinity Across Protease Inhibitors: Kinetic and Thermodynamic Study
   Authors: Eniola L Folarin, Sibusiso B Maseko, Johnson Lin, Thavendran Govender, Tricia Naicker, Sooraj Baijnath, Glenn E.M Maguire, Gert Kruger.
   Eniola Folarin: performed the experiments and wrote the paper.
   Sibusiso B Maseko, Johnson Lin, Thavendran Govender, Tricia Naicker, Sooraj Baijnath, Glenn E.M Maguire, Gert Kruger: supervised the project.

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LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
APV	Amprenavir
ATV	Atazanavir
DRM	drug resistant mutation
DRV	Darunavir
CA	capsid protein
CDC	centers for disease control
CPRU	catalysis and peptide research unit
CRF	circulating recombinant forms
C-SA	South African subtype C
DNA	deoxy ribonucleic acid
Gag	group-specific antigen
gp120	surface glycoprotein
gp41	trans-membrane glycoprotein
HAART	highly active antiretroviral therapy
HIV	Human immune virus
HIV-1 PR rDRV	Mutant protease V32I, 150V, I54M, and I84V
IDV	Indinavir
IN	integrase
ITC	isothermal titration calorimeter
KZN	KwaZulu-Natal
LEDGE	Lens epithelium-derived growth
LPV	Lopinavir
MA	matrix protein
MD	molecular dynamics
MM	molecular machanical
Nef	negative regulatory factor
NFV	Nelfinavir
ONIOM	own n-layered integrated expression molecular orbital and mechanics

PI	protease inhibitor
QM	quantum mechanical
Rev	regulator of virion protein
RNA	ribonucleic acid
RT	reverse transcriptase
RTV	Ritonavir
SDM	site-directed mutagenesis
SQV	Saquinavir
TNF	tumor necrosis factor
TPV	Tipranavir
UNAIDS	The Joint United Nations Programme on HIV and AIDS
USA	United states of America
Vpr	viral protein R
Vpu	viral protein U

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CHAPTER ONE

General Introduction and Overview

1. Human Immuno-Deficiency Virus and Acquired Immune Deficiency Syndrome.

Human immunodeficiency virus (HIV) is known as the causal agent of acquired immune deficiency syndrome (AIDS). AIDS is the most advanced stage of HIV infection, characterised by a compromised immune system. HIV/AIDS is one of the world's most serious public health challenges till date. However, medical advances in treatments mean that people living with HIV very rarely develop AIDS when receiving effective treatment [1].

1.1. Origin of HIV and AIDS

AIDS was first recognized in 1981 among homosexual men falling sick and dying from rare opportunistic infections due to compromised immune system [2]. This would in fact be the beginning of the spread of the most devastating infectious disease till date.

In 1982, acquired immune deficiency syndrome was the name suggested by the center for disease control (CDC) in the United States. It was initially assumed that the infectious disease was mostly spread between gay men and people who self-injected drugs into their system. This lead scientists to believe the cause of infection revolved around lifestyle issues, reaction to semen, drugs, and multiple other infections [3]. It was subsequently evident through epidemiological reports that infectious disease AIDS was spread by means of bodily fluids and exposure to infected blood *via* sexual, prenatal, and percutaneous paths [4].

In 1983, it was documented by the CDC that AIDS could also be transmitted heterosexually, this finding potentially improved awareness that the HIV/AIDS wasn't merely restricted to gay men. In fact, it subsequently became clear that up to 80% of new infections globally were transmitted heterosexually [3]. Later in 1983, a hint of the exact root of AIDS was revealed to the world; Luc Montagnier and colleagues isolated tissue from a patient with acute lymphadenopathy prior to the development of AIDS. They then discovered a new human retrovirus, much different from the human T-lymphotropic virus (HTLV) known to cause T-cell leukemia and a demyelinating disease called HTLV-1 *via* electron microscopy [5]. In 1984,

Robert Gallo *et al*., were able to isolate the new retrovirus [6] independently. In 1986 Harold Varmus recommended that the new retrovirus be called human immunodeficiency virus [3].

In late 1986, the first blood test that enabled the screening of HIV was carried out due to the independent isolation of the retrovirus [7, 8]. The blood test did much to enhance hope in the scientific community such that Margaret M. Heckler was convinced within two years a vaccine against HIV would be obtainable [3]. Regrettably, understanding the many defenses of HIV has since proven difficult.

HIV/AIDS remains a crucial health issue all over the world. According to UNAIDS 2018 facts sheet, approximately 36.9 million people globally are living with HIV, and 770,000 people died of AIDS-related infections [9].

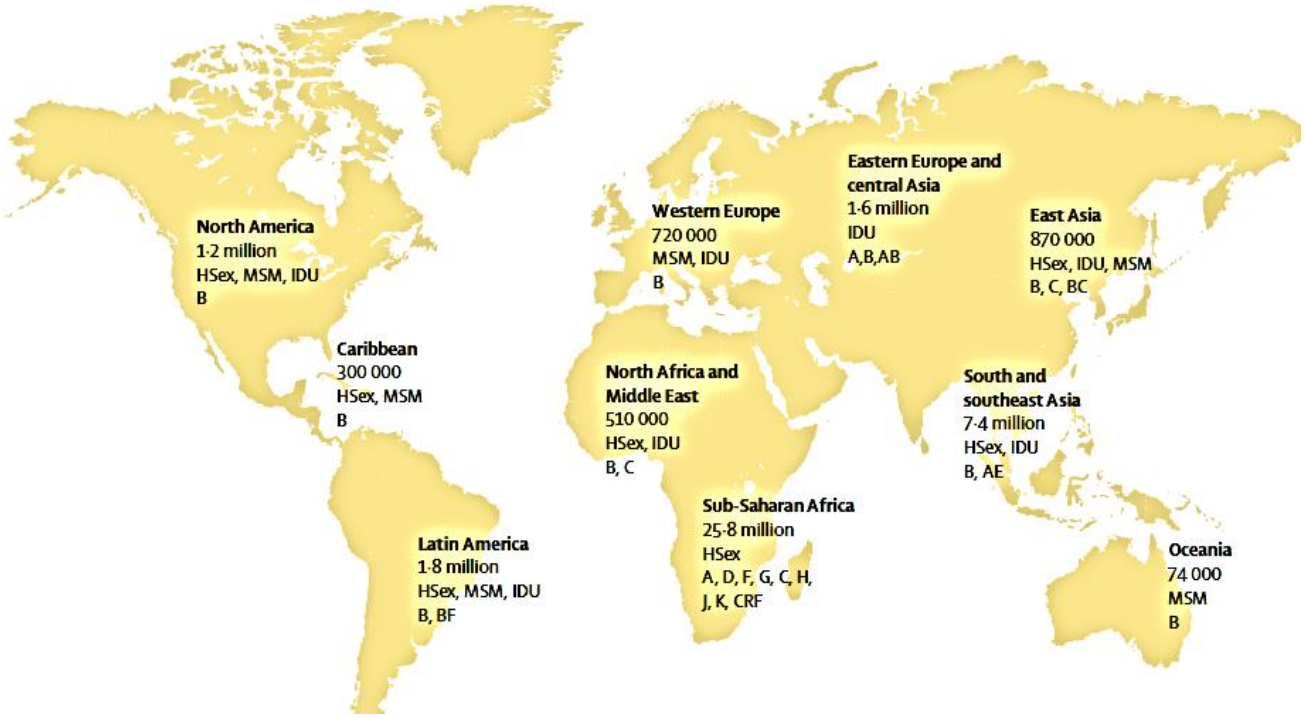


Figure 1.1. Global epidemiology and classification of HIV [10]. Heterosexual (HSex), Injection Drug Users (IDU), Homosexual Men (MSM).

Majority of individuals living with HIV are situated in middle to low income countries. Globally, an estimated 67 % are residing in Sub-Saharan Africa [9]. Amongst this group, an estimated 7.7 million live in South Africa, which saw 240,000 HIV infections in 2018 [9]. In South Africa, epidemiological data by mapping revealed KwaZulu-Natal (KZN) has the highest HIV prevalence (12.2 %) [11]. HIV infection disproportionally affects women. In 2016 sexually active women aged 15 - 24 made up 37 % of new infections [12].

1.2. Human Immunodeficiency Virus.

Since HIV was discovered as the causal agent of AIDS, it has been a subject of intense study considering the reasons for its sudden emergence, how it has spread epidemically, and its unique pathogenicity [1, 13]. HIV is known as a lentivirus or 'slow' virus whose genome is made up of ribonucleic acid (RNA) [14].

The HIV virus is described by a continuous deterioration in the immune response. Its infection is characterised by a drastic, irreversible depletion of mature T-helper cells with CXCR4, CCR5 chemokine receptors, and CD4+ T-cells surface proteins, which plays a significant role in the immune system [15].

The virus targets mainly lymphoid CD4+ T-cells, reduced CD4+ cell count results in failure to control T-helper cell homeostasis [16]. The blood of a healthy individual has CD4+T-cells levels that range between 700 to 1200 CD4+ T-cells/mm^3. The depletion of CD4+ T-cells to about 500 CD4+ T-cells/mm^3 in an HIV infected individual results in minor opportunistic infections such as herpes simplex (cold sores), warts, and some fungal infections like thrush, could arise [17]. The patient subsequently becomes more susceptible to major life-threatening infections and risk the development of AIDS related cancers when CD4+ count has fallen below 200 CD4+ T-cells/ mm^3 in the blood [15].

The depletion of CD4+ T-cells is also attributed to various mechanisms such as the upregulation of tumor necrosis factor-α (TNF), Fas ligand, and TRAIL [18, 19]. Death of unaffected CD4+ T cells occurs due to increased sensitivity towards death ligand-mediated apoptosis [10].

Genomic diversity of HIV differs in infected individuals, HIV-1 and HIV-2 are the two strains identified globally [20].

1.3. Classification and Global Epidemiology of HIV

Multiple factors contribute to the extensive genetic variation of HIV. These factors include numerous zoonotic transference from simian immunodeficiency virus (SIV) into the human populations, and recombination due to high mutation rates during retroviral replication, resulting in the generation of multi-resistant viral strains [21].

HIV has two major strains, HIV-1 and HIV-2. These strains are then subclassified into groups, subtype, and circulating recombinant form (CRF) [20]. The HIV-1 and HIV-2 strains are biologically and morphologically similar but differs by their mode of infection, genome (envelope glycoprotein and antigenic epitopes), and rate of disease progression [22, 23]. HIV-1 is over three times more infective and has a more rapid disease progression than HIV-2. As a result HIV-1 is the model strain used to study the virus [10]. HIV-1 is predominantly spread within the United States of America (USA), Africa, Europe, and most of the world, while HIV-2 infection is found to be more prevalent and localized within western Africa [15].

Genetic variation of the HIV-1 virus has three groups namely: Main group (M), Outlier group (O), and Non-M/O group (N). HIV-1 group M accounts for over 90% of global infections. This group is further classified into nine phylogenetic subtypes (A-D, F-H, J, and K) and >48 CRFs [24] (Fig 1.1). On a global scale, subtypes, A, B, and C are the most highly prevalent viral strains responsible for most cases of HIV infections.

Infections by Subtype A predominates in central and eastern Africa. These include countries such as Tanzania, Rwanda, Uganda, and Kenya, as well as countries in eastern Europe. Subtype A accounts for about 12.3% of global infections [24]. Subtype B is predominant in western Europe, Australia, and North America which only accounts for approximately 12% of infections globally, even with this statistical information, Antiretroviral therapies currently available are based on the study of subtype B (group M) [25].

HIV-1 subtype C (group M) infections dominate southern Africa from where it spread to India and other Asian countries [26]. More than 50% of global infection is attributed to the HIV-1 subtype C viral strain (Fig 1.1). There is an increasing need to focus Antiretroviral therapy studies on this subtype as infections by subtype C is increasing rapidly in Sub-Saharan African regions [27].

HIV-1 group O (outlier) is rare and is referred to as less imperative as it is responsible for < 2.2% of global infections. However, the strain has been isolated in France, including among patients of French origin such as Cameroon and Gabon [28]. Group N was identified in 1998 by Simon and colleague, and the strain is even less rampant than group O as less than 15 cases of infection by group N has been reported, all in individuals with French origin (Cameroon) [29, 30].

Central Africa is the most likely home for CRF01 created by recombination events, it has also been identified in Thailand to cause a heterosexual widespread in the late 1980s. CRF01 is also predominant in Southeast Asia [31]. The spread of these different CRFs affects the development vaccines against the virus, the rates of disease progression, and response to antiretroviral therapy in terms of drug resistance.

1.4. Structure and Life Cycle of HIV

HIV is a member of a retrovirus subgroup called lentiviruses or popularly known as “slow” viruses. Like all viruses, HIV cannot survive outside a living cell as it is dependent on the host cell’s replication machinery to reproduce itself. However, before entry into the human cell, the virion is described by a spherical outermost region, studded morphology with spicules, and a cone shaped core [22].

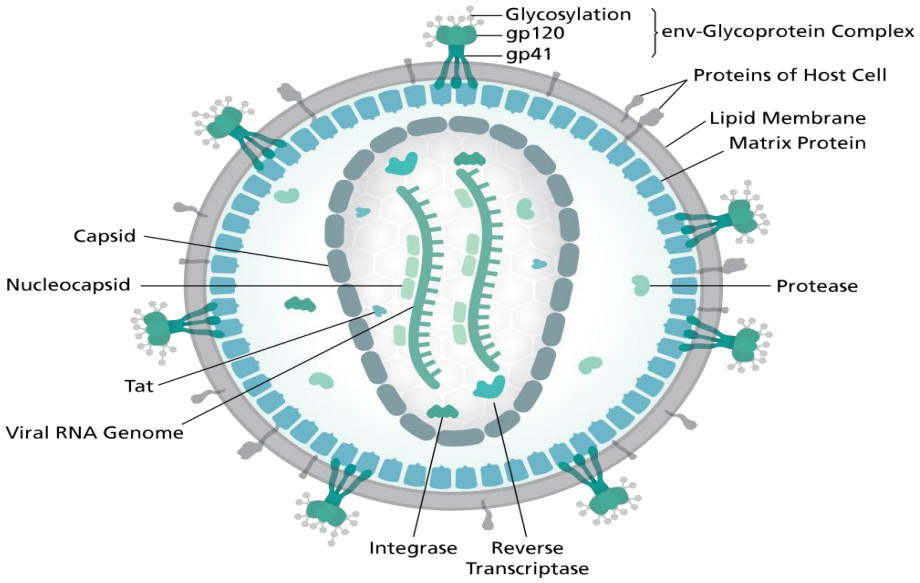


Figure 1.2. Structure of HIV virion [32] (open access)

The spherical virion is approximately 100nm in width, enveloped by a bi-layer lipid membrane formed by matrix protein (MA, p17), and seventy-two (72) knobs of Env trimer proteins surround the envelope. Trimers of the gp41 transmembrane (TM) protein anchor trimers of gp120 surface proteins (SU) where conformation-dependent neutralizing epitopes are found (Fig 1.2). Inner capsid protein p24 (CA) assemble to form a conical-shaped capsid in the innermost region of the virion [33]. Located within the inner capsid are two identical single strand genomic ribonucleic acids (RNA), some viral enzymes essential for the development of the virion (RT/RNase H and IN). Also enclosed in the virus particle are accessory proteins (Vif, Vpr, Nef), regulatory protein (Tat, Rev), and viral protease [34].

The lifecycle of the virion begins by transmission of infected blood and/or body fluids into the host. In a process called fusion, glycoprotein (gp120) present on the virion surface, attaches to the CD4+ receptors at the cell membrane. Subsequent interaction between CCR5, CXCR4 chemokine co-receptors present on the CD4 cell surface, and gp120 results in an irreversible

conformational change permitting fusion. The viral core is then release into the host cytoplasm [35, 36].

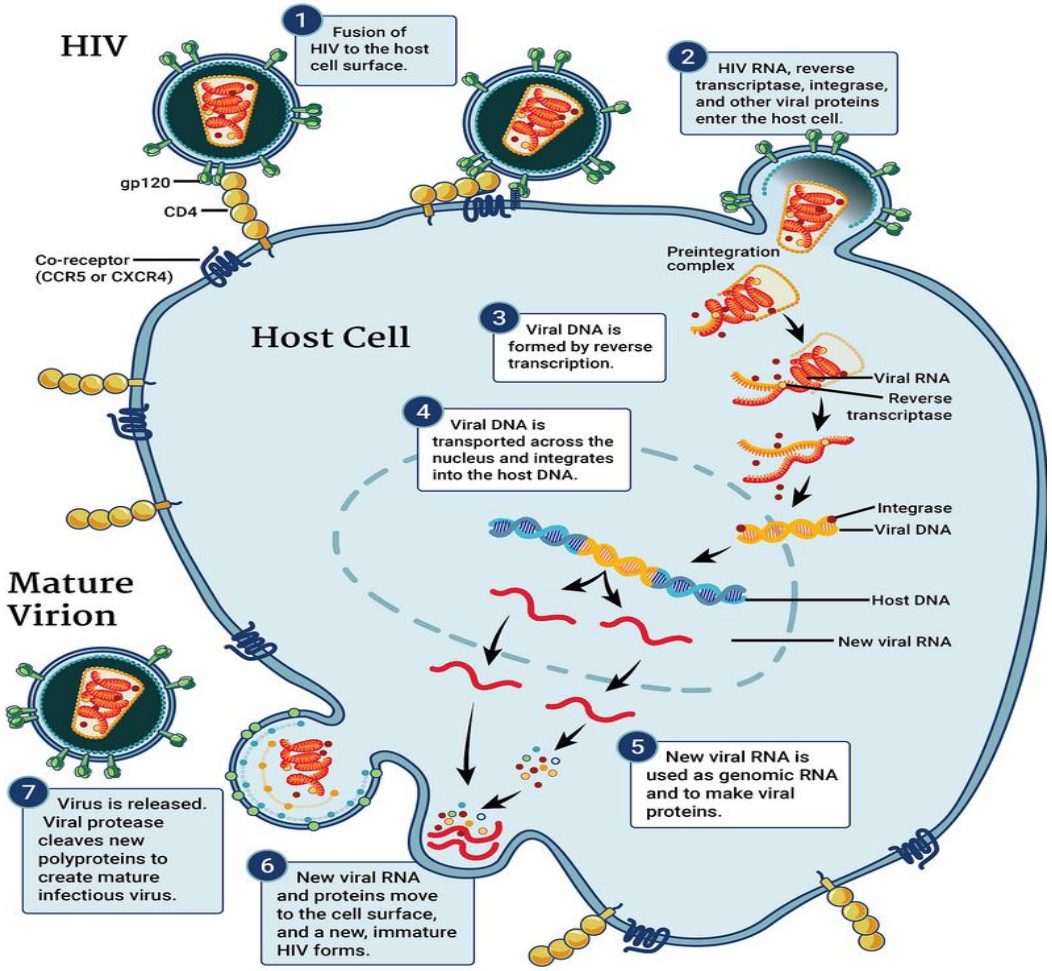


Figure 1.3. Diagrammatic representation of the Human Immunodeficiency Virus life cycle [37]. (open access)

The viral genome released post viral core disruption is reverse transcribed from ribonucleic acid (RNA) into deoxyribonucleic acid (DNA) by the virion's reverse transcriptase (RT) enzyme [38]. The transcription process is highly error prone as the RT enzyme has no proof-reading activity, meaning closely related viral variants are generated. Following transcription, the viral genome is inserted into the host genome by the virions integrase (IN) enzyme into the host cell genome, this process is facilitated by an integrase host binding factor called lens epithelium-derived growth factor (LEDGF/p75), present in the chromosomal DNA of the host. At this stage of infection, the host cell is irreversibly converted to a virus producing cell [10, 39].

The integrated viral and host's DNA is transcribed into multiple copies of new HIV RNA by means of the host's transcription machinery, then the mRNA is translated into proteins outside the nucleus [40, 41]. New copies of viral RNA and long chains of proteins assemble at the cell membrane forming immature HIV virion (non-infectious), which are then released out of the cell in a process known as budding [40, 42]. Once released out of the CD4 cell, the viral protease (PR) enzyme is responsible for breaking up long chains of proteins and re-assembling those proteins to form mature (infectious) virions [43, 44]. These newly mature virions are ready to infect more CD4 cells and commence the replication process again. Very quickly, the virus spreads throughout the body and can be transferred to others by contact with bodily fluids [45].

The design of anti-viral drugs to target HIV is focused on the various stages of the virion's life cycle summarized above. These drugs inhibit binding of the virus to a cellular receptor, Env-mediated cell membrane fusion, reverse transcription, integration of viral genome, and maturation by viral protease [46]. The viral protease enzyme is regarded as one of the most important proteins, as it is responsible for the maturation of the virion and will be discussed in detail.

1.5. HIV-1 Protease (PR)

HIV-1 PR enzyme is required for the processing of viral Gag and Gag-Pol polyproteins into structural and functional mature proteins. The PR enzyme is an invaluable therapeutic target, as the inhibition of PR activity results in noninfectious, immature virions [47].

1.5.1. Structure and Function of HIV-1 Protease

HIV-1 PR is characterised as an aspartic protease on the bases of the highly conserved sequence (two times Asp-Thr-Gly) triad at the active site of the enzyme. The aspartyl protease exists as a homodimer interrelated by a 2-fold crystallographic rotation with a single C_2-symmetric active-site [48]. A single monomer has 99 amino acids sequence. The tertiary structure of HIV-1 PR monomers consists typically of β-sheets. Four interdigitated strands make up the viral protease structure. The active site triad sequence (Asp25-Thr26-Gly27) is present at the dimer

interface within two corresponding β strand loops from each monomer connected by hydrogen bonds. A β-hairpin loop from each monomer composed of residues 44-57, protrudes over the active site and constitutes part of the substrate-binding cleft [49]. (Fig 1.4).

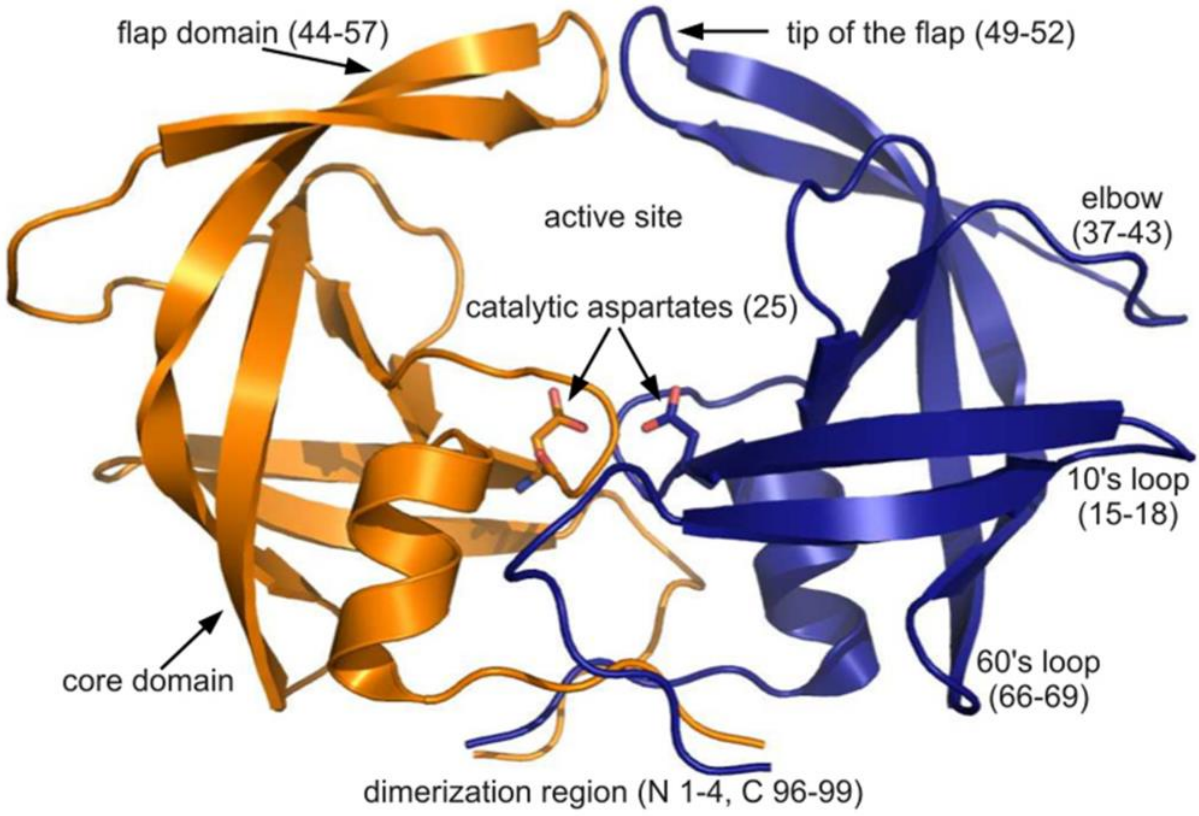


Figure 1.4. Dimeric structure of Human Immunodeficiency Virus protease [50] open access. Residue positions in bracket.

These β-hairpin loops, also called 'flaps' are crucial in the binding of substrate to the enzyme. The flexible flaps which can take an open, semi-open, and closed conformation also regulates substrate entry into the enzyme's active site [51]. Intermolecular interactions between charged residues at the ends of the binding cleft within opposing monomers stabilizes the dimeric form of the PR enzyme [52]. The catalytic mechanism of the PR must be understood to consider the goal of its effective inhibition.

1.5.2. The Catalytic Mechanism of HIV-1 Protease

In many biological processes, protease is essential for catalyzing the hydrolysis of peptide bonds with increased catalytic proficiency and high sequence specificity [53]. The PR enzymes can be divided into two classes according to their catalytic mechanism. The first class uses a water molecule as a nucleophile to hydrolyze the amide bond carbonyl of the substrate's scissile bond. Activation of the water molecule can be accomplished by the two aspartyl β-carboxy groups at the active site (aspartate proteases) or a zinc cation (zinc metalloproteinases) [54]. For the second class of enzymes, an amino acid side chains act as a nucleophile, which initiates the hydrolysis of amide bonds. Firstly, the nucleophilic atom present on the amino acid side chain (hydroxy group or thiol) is activated. This activated nucleophile attacks the carbonyl of the scissile amide bond to form an acyl intermediate (ester or thiolester). The acyl intermediate is then hydrolyzed by a water molecule to form a product [55].

In a mechanism proposed for aspartic protease; the negatively charged catalytic Asp will activate the closest nucleophilic water molecule in between the two catalyzing aspartate side chains. The activated water molecule then attacks the substrate's carbonyl group at the scissile bond to produce an oxyanion tetrahedral intermediate [53]. The intermediate is broken down by the protonated scissile amide N atom into the hydrolyzed product.

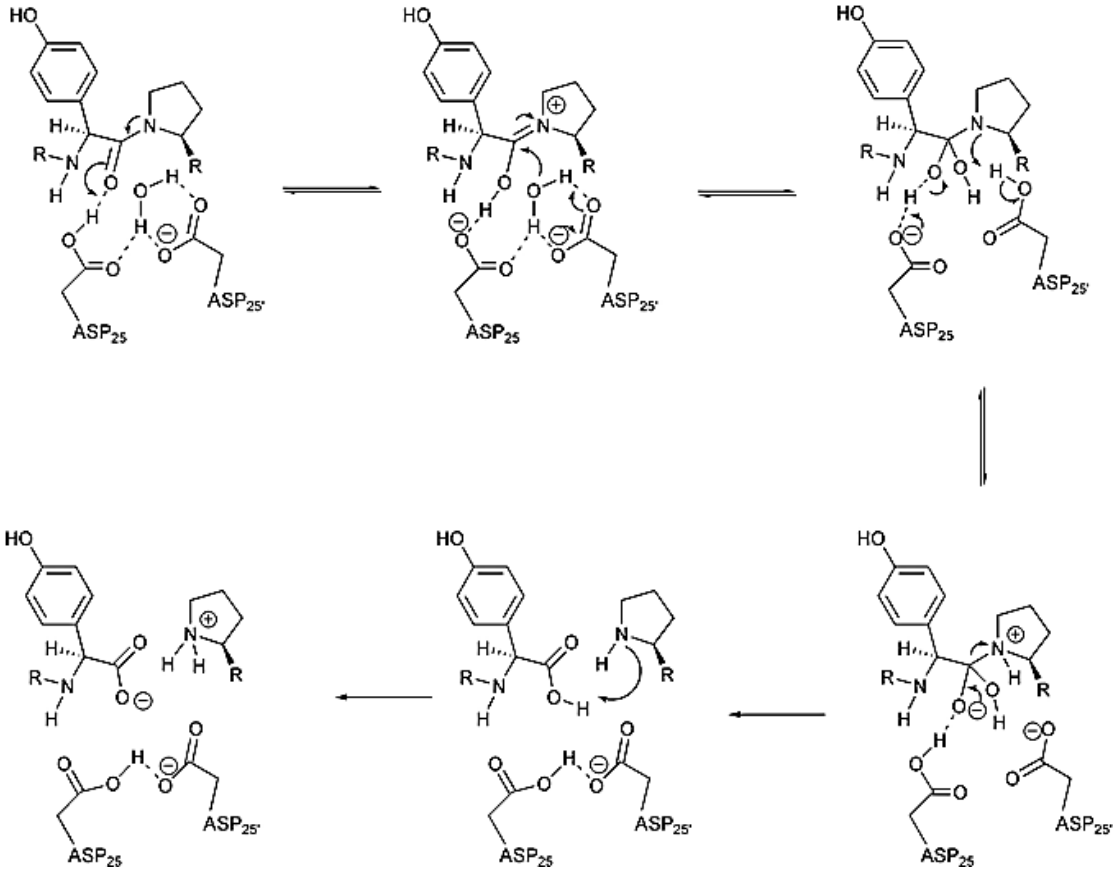


Figure 1.5. Proposed concerted mechanism of HIV protease action. The two catalytic Asp represented as 25 and 25′ to identify respective locations in individual subunits. Activation of conserved water molecule by the carboxylates acts as a nucleophile for peptide bond cleavage in the E-S complex [56].

Although HIV-PR belongs to the aspartic protease family and shares many features in their mechanism, the fully detailed mechanism is not completely understood. According to numerous studies, HIV-PR generally belongs to the second class of protease enzymes, where the scissile bond or cleavage site is positioned between substrate amino acid residues P1 and P1`. The scissile bond is the term given to the peptide bond at which hydrolysis or cleavage occurs [57]. According to standard nomenclature, amino acids neighboring the N-terminus and C-terminus are presented as P1, P2, P3, and P1`, P2`, P3`, respectively [58] (fig. 1.6).

These amino acids interact with water molecules involved in the recognition of the substrate or the release of the product. C2-symmetric subsites presented as S1, S2, S3, and S1`, S2`, S3` starting from the Asp-25 (catalytic aspartic residue), interacts with substrate or inhibitor non-covalently. These N and C-terminus subsites are located at the same positions.

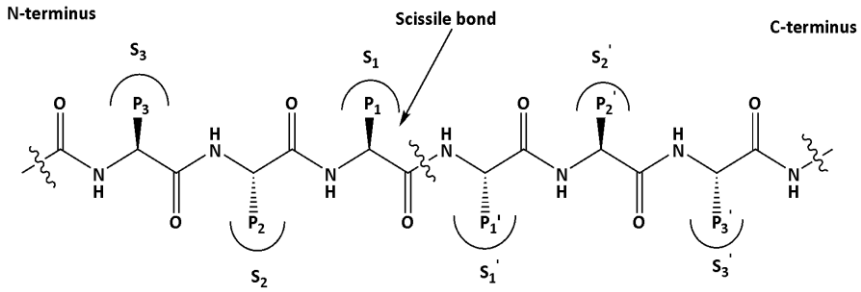


Figure 1.6. Standard nomenclature used to designate amino acid residues of peptide substrates (P). The corresponding binding sites on the protease are referred (S) to as subsites. [59]

The S1 and S1` subsite like the catalytic aspartate residue, is highly hydrophobic [59]. The following residues; Arg-8, Asp-25, Gly-27, Ile-50, Leu-23, Gly-49, Gly-48, Thr-80, Thr-81, and Val-82 are in contact with the P1/P1` side chains of the inhibitors or substrates. Except for Asp29/Asp29` and Asp30/Asp30`, the following residues; Ala-28/28`, Ile-84/84`, Ile-47/47`, Ile-50/50`, Leu-76/76`, Gly-49/49`, and Val-23/23` interacting with S2/S2` subsites are hydrophobic [60]. In comparison to the S1/S1` and S3/S3` binding subsites, S2/S2` has smaller interior pockets and are more specific in controlling the type and size of residues at P2/P2` in substrates or inhibitors [61]. These features have been implicated in the reduced tolerance of multi-drug resistant mutants of these binding site residues. Substrate specificity of HIV-PR is reportedly determined by S2/S2` binding subsites [62].

The S2/S2` subsite interacts primarily with protease inhibitors containing hydrophobic moieties, these inhibitors are usually shorter than the natural substrate and bind to the protease at the same binding pocket, even though their chemical structures may differ [63]. This same pocket binding of protease inhibitors provides insights into protease cross-resistance observed from individual mutation. The S3/S3` subsites adjacent to the S1/S1` contain mostly hydrophobic residues and has a broad residue specificity. The following residues; Asp-29/29`, Gly-48/48`, Gly-49/49`, Pro-81/81`, and Val-82/82` form the S3/S3` pocket and part of the S1/S1` binding pocket [64].

Limited information about protease-ligand complexes extending beyond P3/P3` pocket has deprived the understanding of subsites S4/S4` and S5/S5` binding pockets [65]. The HIV-PR proficiently cleaves octapeptide substrates using S4 and S3` as the main processing binding subsites, as such Jaskolski and co-workers were able to propose a new mechanism based on protease and octapeptide protease inhibitor (U-85548e) complex crystal structure [66].

Our group recently used MD simulation and a double layer ONIOM B3LYP/6-31++G method to explore the mechanistic pathway of the natural HIV-1 C-SA PR substrate. They reported that the recognition of the substrate depends heavily on its length and the sequence of amino acid [67].

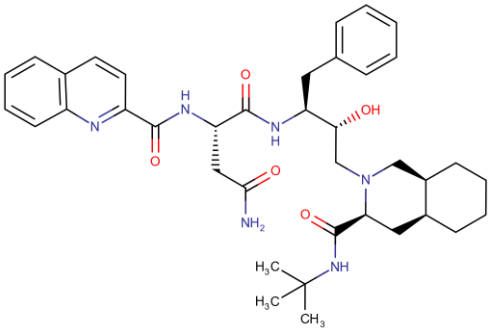
Various protease inhibition drugs have been designed and used today as antiretroviral therapeutic treatment. These anti-protease drugs will be discussed in the next section.

1.5.3. HIV Protease Inhibition

HIV protease inhibitors (PIs) contribute to the clinical, virologic, survival, and immunological benefits of an infected patient. Effective HIV protease inhibitor design and development hinges on a vast abundance of information regarding the structure and mechanism of the enzyme [53]. Other aspartic PIs designed to inhibit the actions of angiotensinogenase contributed ample information for HIV-PR inhibitor design [54]. Most of these aspartyl PR inhibitors were peptidomimetic substrate-based compounds replaced by non-cleavable inserts rather than the scissile-bond amino acid pattern [58]. Mandatory alterations were considered to improve oral bioavailability by reducing the peptidic character. This *de novo* approach has been priceless in facilitating the discovery of effective drugs against HIV protease.

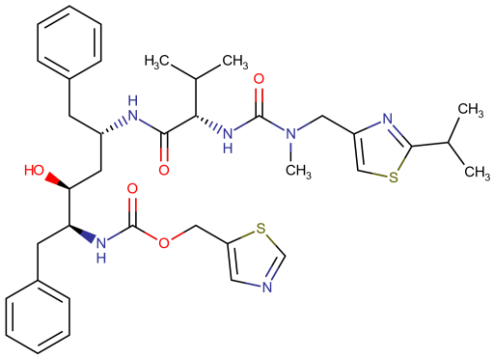
Till date, there are nine FDA approved drugs functioning as HIV-PR PIs. Based on their structural features, the drugs were divided into first- and second-generation PIs. First-generation PIs include the following; saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and the following PIs are second-generation PIs; lopinavir, atazanavir, tipranavir, and darunavir. The PIs are briefly described in the section to follow.

a) Saquinavir (SQV)



In 1995, the first ever PI was approved by the FDA. Saquinavir was developed by Hoffmann-La Roche. SQV has a hydroxyethlamine transition state analogue consisting of a bulky (S, S, S)-decahydro-isoquinoline-3-carbonyl (DIQ) moiety in P1` replacing proline in the natural substrate and quinoline P3 [68, 69]. The 90% and 50% inhibitory concentration (IC_{90} and IC_{50}) values at 6 nM and 0.4 nM respectively, makes SQV considerably a potent protease inhibitor [69]. Despite SQV being a very potent inhibitor, some of its shortcomings include poor oral bioavailability expeditious degradation by cytochrome P450 (CYP-450) *in vivo*. Structurally distinct inhibitors Ritonavir and Indinavir soon followed the release of SQV [70].

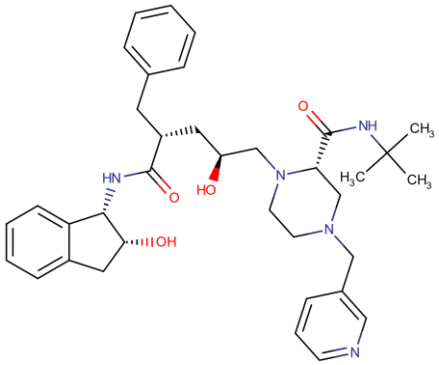
b) Ritonavir (RTV)



Ritonavir became the second HIV PI approved in the United States by the FDA. It was developed in 1996 by Abbott's laboratory, who attempted to modify the C2 symmetry by incorporating a C2 symmetric dihydroxy Phe-Phe isostere core [70]. Interestingly, lead optimization, revealed that detachment of a single hydroxyl group would not alter the potency of RTV. Which is why RTV has an all carbon pseudo-symmetric Phe-Phe hydroxyethylene

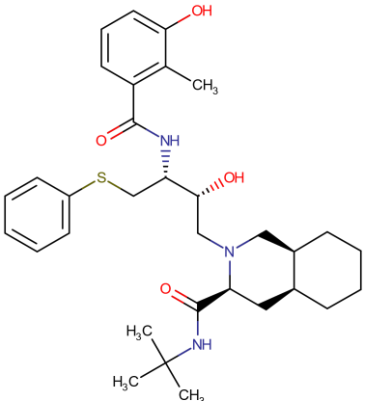
isostere core. RTV is a potent PI with IC_{90} and IC_{50} values of 10 nM and 70 nM respectively. Considering RTV's many adverse effects, it is no longer used as a PI on its own [71]. However, the potency of RTV against PI's metabolic enzyme CYP-450 3A encouraged it's use in low doses as a pharmacokinetic booster in highly active antiretroviral therapy (HAART) with other PIs [72, 73].

c) Indinavir (IDV)



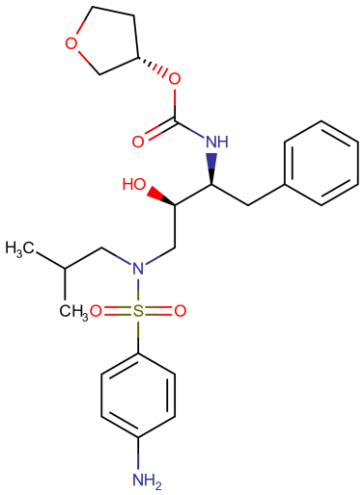
Indinavir was the third FDA approved drug released only two weeks after RTV's approval in 1996. It was developed by Merck & Co as a peptidomimetic transition state analog, which belongs to the hydroxyaminopentane amide (HAPA) compound class of PIs [74]. The synthesis of IDV required a Phe-Gly hydroxyaminopentane dipeptide isotere in place of P1-P1` portion. IDV's structural features consist of an aminohydroxyindane moiety in P2`, pridylmethylpiperazine moiety in PI-P2, and bulky hydrophobic groups that interact with the S2/S2` hydrophobic pockets [75]. It has a protease inhibitory potency (K_i) value of 0.5nM and good oral bioavailability. Unfortunately, IDV's short (1.8 h) half-life would require a multiple dose schedule to sustain viral load suppression.

d) Nelfinavir (NFV)



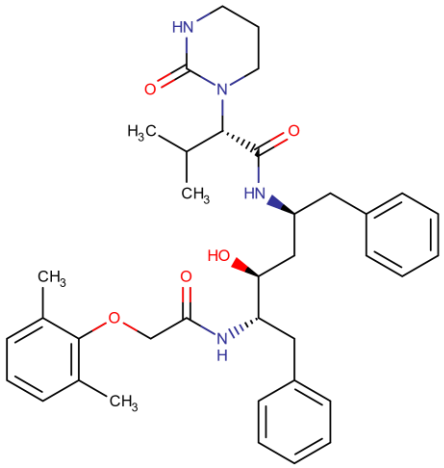
In 1997, FDA another PI. NFV was developed by Agouron Pharmaceuticals and Eli Lily. They replaced the P2 asparagine with a 2-methyl-3-hydroxybenzamide group at the C-terminal and trimmed the z(S,S,S)-decahydro-isoquinoline-3-carbonyl (DIQ) moiety at the N-terminal of SQV [76]. The hydroxy-methylamine isotere at the P1 pocket together with the aforementioned modifications was designed to enhance NFV's potency (K_i = 2 nM), improve bioavailability, and ultimately reduce the molecular weight. [77]

e) Amprenavir (APV)



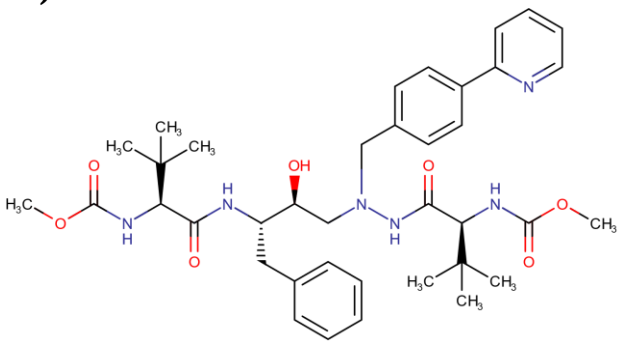
Amprenavir was developed by Vertex and Glaxo Wellcome, and approved by the FDA in 1999 [78]. APV belongs to a sulfonamide class of PIs, with the incorporation of a hydroxyethylammine-sulfonamide dipeptide isostere and a 3-hydroxyltetrehydrofuran moiety at the P2` site. The phenyl amide P2` group was designed to imitate the asparagine side chain and Asp29 residue interactions in SQV. APV, in comparison to previous PI's, contains fewer chiral centers. These improvements simplify the chemical synthesis and improve oral bioavailability. APV is moderately potent (K_i = 0.6 nM) [70].

f) Lopinavir (LPV)



Lopinavir was developed by Abbott Laboratories by improving on ritonavir's structural features, and approved in 2000 by the FDA [79]. It is used in combination therapy with ritonavir as a pharmacokinetic boosting agent. Resembling first-generation PI Ritonavir, LPV has a hydroxyethylene dipeptide isotere in the core region. A phenoxyacetyl group replaces the 5-thiazolyl group in the P2 pocket in RTV [73]. Also replaced is the 2-isopropylthiazolyl group in P2` site by a six-member cyclic urea. The replacement of this bulky P2 and P2` moieties in RTV significantly improves the potency (K_i = 1.3 pM) of LPV [53].

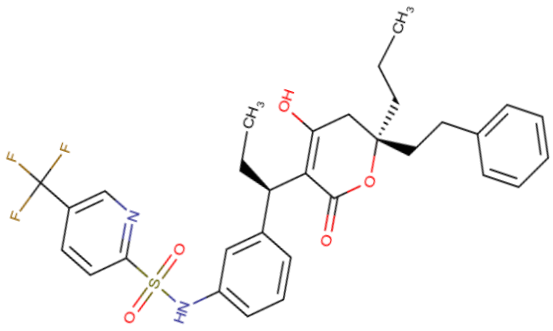
g) Atazanavir (ATV)



Atazanavir was developed by Bristol-Myers Squibb. The second-generation PI was approved for use in 2003. ATV structure consists of a 2-hydroxy-1,3-di-aminopropane transition state isotere with an aza-di-peptide core, an extended 4-(2-pyridinyl) phenylmethyl moiety at the P1` pocket and methyl-carbamate capped tert-leucine moiety at P2/P3 and P2`/P3` pockets [80]. The aza-dipeptide link between the P1-P2 pockets removes one of the three chiral centers found

in LPV, thus, allowing large-scale and straightforward synthesis. ATV is highly potent (K_i = 10 pM) with good oral bioavailability [81].

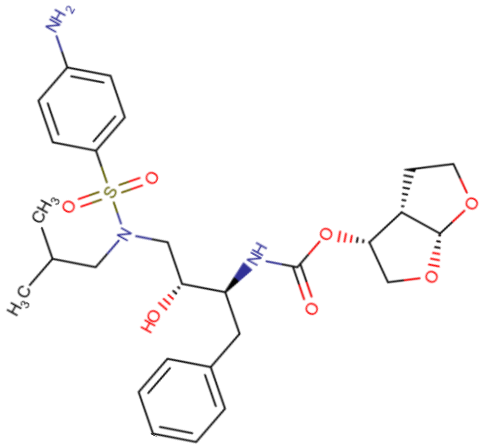
h) Tipranavir (TPV)



TPV approval followed closely after ATV in 2005. Boehringer-Ingelheim developed TPV from a class of lead compounds structurally similar to 4-hydroxycoumadin and 4-hydroxy-2-pyranone known to inhibit protease activity. TPV is a 4-hydroxy-5,6-di-hydro-2-pyrone sulfonamide based, non-peptidic inhibitor. Despite TPV being a non-peptidic inhibitor, its crystal structure displayed comparable interactions with protease like other peptidic inhibitors [82].

Other peptidomimetic inhibitors generally make hydrogen bond interactions directly with ile50/50` residues at the protease flap/hinge region. The case is different for TPV whose hydrogen bond interaction occurs indirectly with Asp25/25` using the central 4-hydroxy-2-pyranone sulfonamide moiety [83]. Nonetheless, TPV maintains its activity against numerous PI-resistant HIV strains, has a much higher barrier against resistance requiring as many as 16-20 mutations to significantly confer resistance. TPV is also a very potent inhibitor (K_i = 5 pM and an IC_{90} = 100 nM) [84].

i) Darunavir (DRV)



Darunavir was developed by Tibotec Inc. and approved by the FDA in 2006. It is the latest PI used in HAART. DRV is a non-peptidic inhibitor and a structural homolog of APV. Both compounds share the same hydroxyethyl amino-sulfonamide isostere. The difference is the incorporation of a condensed bis-tetrahydrofuranylurethane (bis-THF) moiety in DRV [85]. Bis-THF in DRV replaces the single tetrahydrofuran (THF) moiety of APV at the P2 site. This replacement improves hydrogen bond interactions with residues Asp29 and Asp30 at the protease active site. Thus, allowing DRV mimic the conserved hydrogen bonds of the natural substrate [86].

DRV generally has a broad specificity against highly mutated resistant protease strains, which explains its ability to fit within the suggested substrate-binding site. Ultra-high-resolution crystal structures revealed DRV's dual binding to the PR enzyme. DRV sometimes occupies a subsite pocket (S2 site) created by one flap casing the active site, this critical interaction is responsible for DRV's exceptionally high inhibitory potency with a K_i value of 15pM [87]. DRV binds up to 100 times more tightly than the structural homolog APV and nearly a 1000 times more than SQV, RTV, NFV, and IDV to the PR [88]. Clinical studies revealed that for both treatment naïve and experienced patients to develop resistance to DRV, a high number of cross resistance mutations across PIs must be observed. Thus, DRV is characterised by a high genetic barrier against PI resistant strains and new resistance development. DRV remains the most potent PI approved to date.

PI's generally share similar structures and binding patterns, which is the reason for the shared side effects amongst all protease inhibitors. Some common side effects of all PIs are summarized below (Table 1).

Table 1.1. Some of the common adverse effects associated with the 9 FDA approved PI

No.	HIV-1 Protease Inhibitors	Common Adverse Effects
1	Saquinavir	Fatigue, vomiting, nausea, abdominal pain or diarrhea. [89]
2	Ritonavir	hyperlipidemia, gastrointestinal symptoms (nausea, diarrhea and abdominal discomfort). [90]
3	Indinavir	Bloody urine, intense lower back pain, lipodystrophy syndrome. [91]
4	Nelfinavir	Bloating, diarrhea, abnormal pain, maldistribution and accumulation of body fat. [92]
5	Lopinavir	Increased liver enzymes and lipids, nausea, stomach upset, fatigue, heartburn, migraines, diabetes and lipodystrophy, Achilles tendinopathy. [93]
6	Amprenavir	Distressing burning sensation in legs and arm, increased appetite, thirst and cholesterol levels, skin rash, frequent urination.[94]
7	Atazanavir	Hyperbilirubinemia, skin rash, gastrointestinal symptoms, insomnia, heartburn, diabetes, liver toxicity. [95]
8	Tipranavir	Lipodystrophy, flatulence, chronic liver disease, gastrointestinal symptoms, decompensated hepatitis, fatigue, headaches, fever, diabetes, Intra-cranial hemorrhage. [96]
9	Darunavir	Gastrointestinal disease, liver toxicity, skin rash, diabetes, general body pain. [97]

1.6. HIV-1 Protease Drug Resistance Mutation

The effectiveness of protease inhibitors mentioned above has been severely hampered by the development of resistant mutations. As a result of the high error rates and lack of proof-reading ability during cDNA synthesis by the reverse transcriptase (RT) enzyme, HIV-1 PR is highly prone to the development of polymorphic mutations. The high error rate of the RT enzyme contributes a selective advantage for the virus survival in the host system. The accumulation of mutations within the PR allows viral evolution that prevents the binding of PIs to the protease enzyme [98, 99]. Different combinations of polymorphic mutations within the protease leads to PI resistance. Drug resistance is a slight rearrangement of the structure of protease to allow the recognition of the natural substrate and processing of the Gag and Gag-Pro-Pol polyprotein, while significantly altering effective inhibition by competitive inhibitor molecules.

HIV-1 protease polymorphic mutations are categorized into primary and secondary groups (Table 1.2) [100]. Mutated residues involved directly with the active site region of the enzyme are referred to as primary mutations. The following residues; 25-32, 47-53, and 80-84 form the active site region. Mutations occurring outside the active site region are called secondary mutations [101]. Secondary mutations are generally compensatory mutations located at the dimerization interface and in the flap region. The flap region, which is responsible for substrate regulation and ligand binding, is composed of the following residue; 39-57 [101]. The mutations in this region affect the flexibility of the flaps and alter the shape and access to the binding pocket reducing inhibitor binding at the active site.

Table 1.2. Summary of primary and secondary mutations. [56]

Primary Mutations	D30/N, L33/F, M46/I, I47/V, I50/V, V82/A/F/T, I84/V, L90/M
Secondary Mutations	L10/I/V, K20/M/R, L24/I, V32/I, L33/F, E25/D, M36/I, M46I/L, I47/V, I54/L/V, L63/P, A71/T/V, G73/S, V77/I, N88/D, L90/M

Both primary and secondary mutations are reported to significantly decrease the interaction of Van der Waals between the protease inhibitor and enzyme, resulting in reduced binding enthalpy [102, 103].

Mutations at 45 residues out of the 99 amino residues of a single protease enzyme monomer are reported to confer resistance to the drug. Of the 45 residues only 11 are marked as primary mutations directly affecting the enzyme active site region, making most mutations classified as secondary mutations [104]. All protease inhibitors have their own unique signature mutations [105, 106]. (Table 1.3)

Table 1.3. The most common clinically significant resistant mutations across all protease inhibitors [100, 107, 108].

No	HIV-1 Protease Inhibitor	Common Drug Resistant Mutation
1	Saquinavir	G48/V, L90/M
2	Ritonavir	K20/R, L33/F, M36/I/L, M46/L, I54/V, A71/V/T, V82/A/F/T/S, I84/V and L90/M
3	Nelfinavir	D30/N, L90/M
4	Indinavir	M46/I/L V82/A/F/T and I84/V
5	Lopinavir	V32/I, I47/V/A, L76/V and V82/A/F/T/S
6	Amprenavir	I50/V and I84/V
7	Atazanavir	I50/V, I84/V and N88/S
8	Tipranavir	I47/V, Q58/E, I74/P, V82L/T, N83/D and I84/V
9	Darunavir	I47/V, I50/V, 154/M/L, L76/V and I84/V

1.7. Polymorphic resistant mutations in HIV-1 subtype C (Southern Africa) protease

The available PIs currently used in antiretroviral therapy are optimized with increased binding affinity to wild-type HIV-1 subtype B protease rather than the most predominant polymorphic subtype C protease in sub-Saharan Africa. The high prevalence of some resistance mutations in non-B subtypes indicates that drug resistance may manifest more rapidly, and some non-B subtypes may be intrinsically resistant to some if not all antiretroviral drugs. HIV-1 subtype C contains naturally occurring polymorphic mutations within the hinge and cantilever regions, altering the hydrophobic core interactions, which allows anchoring of the flaps in HIV-1 subtype B [109]. Compared with subtype B, the conformational stability of the HIV-1 subtype C protease is slightly reduced, which indicates that the structural difference may comparatively affect the binding affinity of PIs to subtype C protease. Consequently, an important question concerns the efficacy of current protease inhibitors against non-B subtype protease [110].

1.8. Studies previously conducted on HIV-1 C-SA protease at CPRU

Extensive studies focused on the binding energies, and drug resistance mutations associated with PIs has been carried out for HIV-1 subtype B protease. Although HIV-1 subtype C is liable for over half of global infection, this subtype has not been addressed in enough studies. Our research unit has reported on the large-scale production and recovery of HIV-1 PR, which has been a problem due to its low yield, cytotoxicity, insolubility, and low activity. This study found that pET32a (Trx tag) expressed in BL21 (DE3) pLysS provided the quickest way to produce active HIV-1 protease [111].

A variant protease which harbours a mutation and insertion (I36T↑T) at position 36 of the C-SA HIV-1 protease mutation was reported by our research unit to significantly alter the inhibition and binding affinity of clinically approved protease inhibitors to the variant protease [112]. Another variant HIV-1 C-SA protease harbouring five mutations, ED35, I36G, two insertions at positions 38 S and L, and D60E was also reported by our research unit displayed a significant decrease in the binding of seven clinically approved PIs against the variant protease[113].

Computational analysis using molecular dynamic trajectories explored the flap dynamics of HIV-1 C-SA protease in the presence of second-generation clinically approved PIs. This study observed that other contributing factors, such as large Van der Waals interactions and low

solvation free energies, were contributing factors for the binding affinity of the PIs to the protease [109]. Computational analysis, together with experimental data, provided insights on the molecular interactions of clinically approved PIs against an L38L↑N↑L PR protease variant. This study reported reduced inhibitory activity compared to the HIV-1 C-SA wild-type [114]. Other computational studies using molecular dynamics (MD) and/or ONIOM approach for the calculation of free energies for mutant C-SA HIV-1 PR against FDA approved PIs [115-118] have been reported by our group.

Our group also reported various ways in which nanotechnology can play a vital role in the treatment of HIV infection by addressing toxicity, drug resistance, and suboptimal adherence problems [119-123]. In our laboratory we carried out several studies involving different families of pentacyloundecane (PCU) lactam peptides/peptoids and PCU diol peptide compounds synthesized as possible inhibitors for HIV-1 C-SA PR [124-130]. Studies on the effect of potential inhibitors to combat HIV-associated neurocognitive disorders [131, 132] and protease inhibitors [133] has been reported by our group. QM/MM and ONIOM studies have provided insight into HIV-1 PR catalyzing mechanisms [67, 134].

1.9. Molecular Gene Cloning

Advancements in understanding the molecular biology of cell's chemical structure and activity has relied heavily upon gene cloning studies [135]. Gene cloning is a recombinant DNA technology that allows for the study of specific genes, creating multiple copies of a gene for downstream applications, such as heterologous expression of a protein, mutagenesis, sequencing, and genotyping [136]. This method generally involves moving the desired fragment of DNA from one organism to a self-replicating medium, such as a bacterial plasmid. In a suitable host cell, the vector proliferates, generating numerous identic copies of not only itself but also of the inserted gene of interest to produce a new DNA fragment called recombinant DNA [137, 138]. Further replication of recombinant DNA occurs in progenies during host cell division. A colony or clone is formed following many cell divisions [139].

1.10. *In Vitro* Site Directed Mutagenesis

In vitro site directed mutagenesis (SDM) [140] is an invaluable molecular biology tool used in gene modification and for the study of protein structural and functional properties, based on the enzyme's structure, function, catalytic residues and catalytic mechanism [141]. Conventional SDM technique involves the use of custom designed oligonucleotide primers to deliberately change, insert, and delete single or multiple amino acid residues in a double-stranded DNA plasmid. The following are required to perform SDM; DNA/protein sequence and a suitable expression vector. The design of synthetic oligonucleotide primer to alter the target region involves a primer complementary to the target region on the template plasmid except for a clearly defined mismatch to introduce the mutation. Primer hybridization to the plasmid template (expression clone or amplified target gene by PCR) occurs at low stringency. The DNA synthesis is performed using a Taq polymerase, which has no proof-reading ability, followed by PCR amplification of the mutated product, ligation to a suitable vector and transformation into a suitable host. Currently, there are several mutagenesis kits commercially available with protocol variations and unique *Escherichia coli.* Strain (such as Phusion Site-Directed Mutagenesis from Thermo and the GeneArt system from life, Q5 Site-Directed Mutagenesis Kit). SDM is usually analysed by bioinformatic methods [142, 143].

1.11. Overexpression, Purification and Characterization of Protein

Recombinant protein production in microbial systems has indeed revolutionized molecular biology [136]. The ability to harvest and purify a great amount of a desired recombinant protein, permits a broad range of possibilities, including its use for study purposes to treat and diagnose the disease. A gene of interest cloned downstream of a promoter of an expression vector ensues the synthesis of recombinant proteins. Protein blueprints are stored in DNA and decrypted by highly regulated transcriptional processes to produce messenger RNA (mRNA), which is translated into polypeptide chains and then subjected to a post-translational modification to produce proteins [144, 145].

Following successful recombinant protein expression, it is generally desirable to purify the protein for different applications. Purification of proteins requires several processes to successfully isolate a specific protein from a complex mixture of proteins [146]. Protein

purification processes/protocol commonly involves a few chromatography steps. There are various chromatographic techniques to choose from, depending on the nature and size of the protein [147]. Some of these techniques include affinity chromatography [148], which separates proteins by an interaction between a peptide "tag" (Histidine and GST tags) appended to the recombinant DNA during expression and a specific immobilized substrate [149].

Ion exchange chromatography (IEC) [150] separates by net surface charge of protein through electrostatic interactions that occur between a charged stationary phase (anion exchange and cation exchange for positively and negatively charge stationary phase respectively) and the protein [150]. Hydrophobic interaction chromatography (HIC) [151] separates proteins based on their hydrophobicity and size exclusion chromatography (SEC) [152], which separates proteins based on the hydrodynamic radii using porous gels. The most used techniques for recombinant HIV-PR protein include affinity and ion-exchange chromatography [153].

Identification and characterization of purified protein of interest are one of the most important aspects of recombinant protein expression. It involves the downstream profiling of a protein's purity, functional, structural, and biochemical properties [154]. Several techniques are used to achieve protein characterization, the most commonly used is sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [155], which involves the separation of proteins by charge based on their mass and isoelectric points. Enzyme kinetics and inhibition studies are some of the major aspects required in the discovery and synthesis of new drugs.

1.12. Enzyme Kinetics

Enzyme kinetics is one of the core features of enzymology, it is the study of a chemical reaction catalysed by enzymes. Enzyme kinetics provides a greater understating of the catalytic mechanism of an enzyme, its role in metabolism processes, and the mechanism of enzyme inhibition [156]. Enzymes are characterised by fast reaction rates and substrate specificity. The general catalysis principle involves the catalyst (enzyme), providing a means of reducing the Gibbs energy barrier and increasing the reaction rates [157]. In enzyme kinetics experiments, an artificial substrate that could yield a brightly colored product (chromogen) is often used and easily monitored using a spectrophotometer or a colorimeter [158]. Spectrophotometric analysis is the most convenient assay used because it allows continuous measurement of light absorbance changes between substrate and product. The substrate forms an intermediate

complex with the enzyme (ES) and is immediately followed by the release of a product (P). This process can be modeled using Michaelis-Menten kinetics equation.

$$\mathbf{E + S \rightleftharpoons ES \rightarrow E + P} \qquad \text{(Equation 1) [159]}$$

The Gibbs energies and enthalpies for products and reactants remain unchanged by the enzyme regardless of the catalytic mechanism and kinetic energetics [160].

1.13. Enzyme Inhibition and Thermodynamics Studies

Enzymes can be controlled in ways that either decrease or favour their activity. In the case of enzyme inhibition, many drugs are designed to inhibit or reduce the activity of enzymes to halt the progression of diseases [157]. The general principle of enzyme inhibitors is to decrease the catalytic efficiency of the enzyme. Inhibitors are structurally comparable with the natural substrate that it can bind the active site of the enzyme (competitive inhibition) directly or bind outside the active site to a separate allosteric binding pocket (non-competitive inhibition) [161]. Inhibitors can be categorized based on their mode of inhibition as either an irreversible or reversible inhibitor. The reversible inhibitors bind noncovalently to the active site of the enzyme resulting in a temporary reduction of the enzyme's catalytic efficiency. Therefore, an equilibrium is occurring between the enzyme and inhibitor [162]. The irreversible inhibitor forms covalent bonds with the active site resulting in a complete loss of catalytic efficiency and an irreparable enzyme. The reaction continues progressively overtime [161]. The majority of the FDA PIs employ the reversible inhibition mechanism of action, which is the focus of this study [162].

The reversible inhibition is again categorized into competitive, non-competitive, and uncompetitive mode of enzyme inhibitor binding.

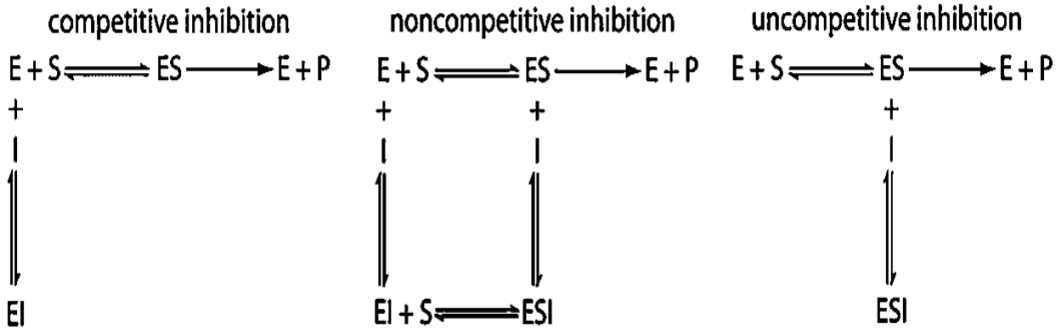


Figure 1.7. Illustration of enzyme catalysis reversible inhibition mechanisms. Enzyme - E, Product - P, Substrate-S, Enzyme-Substrate complex - ES, Inhibitor - I, Enzyme-Inhibitor complex - EI, Enzyme-Substrate-Inhibitor complex - ESI.

In competitive inhibition, there is competition between the inhibitor and substrate to bind at the enzyme's active site. This would result in the reduction of the enzyme's catalytic efficiency since the substrate is unable to bind to the enzyme-inhibitor (EI) complex. In the case of non-competitive inhibition, the substrate and inhibitor do not compete to bind at the enzyme's active site. The inhibitor binds at different binding pockets forming an ESI complex resulting in the loss of catalytic efficiency. Finally, with uncompetitive inhibition, the inhibitor binds only to the enzyme-substrate (ES) complex resulting in an inactive ESI complex that would ultimately lower the catalytic efficiency of the enzyme [163].

The type of reversible inhibition can easily be identified by observing how the presence of inhibitor affects the relationship between reaction rate and concentration of the substrate using a Lineweaver-Burk plot from Michaelis-Menten equations [164] (fig. 1.6).

Competitive Inhibition [165]

Competitive inhibitors will only bind to the enzyme (E) and not the enzyme-substrate (ES) complex. The equation is given by:

$$\mathbf{V_0} = \frac{\mathbf{Vmax}[\boldsymbol{S}]}{\mathbf{km}\left(\mathbf{1}+\frac{\mathbf{I}}{\mathbf{ki}}\right)+[\boldsymbol{S}]} \quad \text{(only } K_m \text{ changes)} \qquad \textbf{(Equation 2) [162]}$$

The K_m increases by substrate binding interference, but V_{max} is not affected because the inhibitor and substrate cannot bind to the enzyme at the same time.

Non-competitive Inhibition [165]

Non-competitive inhibitors are characterised by identical affinities for E and ES, V_{max} deceases, but K_m remains constant. The equation is given by:

$$\mathbf{V_0} = \frac{\mathbf{Vmax}}{\frac{\left(1+\frac{\mathbf{I}}{\mathbf{ki}}\right)}{\mathbf{Km}+[\mathbf{S}]}}[\mathbf{S}] \qquad \text{(only } V_{max} \text{ changes)} \qquad \textbf{(Equation 3) [162]}$$

The non-competitive inhibitor binds at a different site from the substrate-binding site, causing a reduction in catalytic rate of enzyme activity. This is usually a case of mixed inhibition.

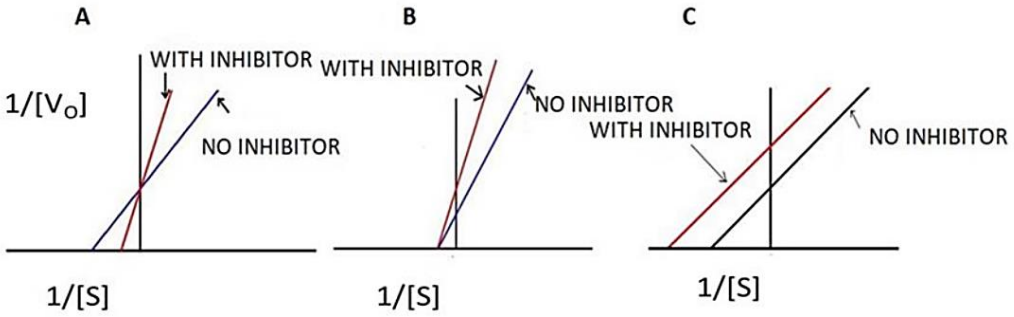


Figure 1.8. Illustration of reversible Enzyme inhibition showing the effects of inhibitor on kinetic parameters K_m and V_{max}. Competitive(A), Non-competitive(B) and Un-competitive inhibition(C) [111].

Uncompetitive Inhibition [165]

Uncompetitive inhibitors bind only to the ES complex. Thus, there is a decrease in K_m and V_{max}. The equation is given by:

$$\mathbf{V_0} = \frac{\frac{\mathbf{Vmax}\,[\mathbf{S}]}{\left(1+\frac{\mathbf{I}}{\mathbf{Ki}}\right)}}{\frac{\mathbf{Km}}{1+\frac{\mathbf{1}}{\mathbf{Ki}}}} + [\mathbf{S}] \quad \textbf{(Both } V_{max} \text{ \& } K_m \text{ changes)} \qquad \textbf{(Equation 4) [159]}$$

The release of product cannot be achieved when the inhibitor is bound to the enzyme-substrate (E-S) complex. This explains why V_{max} is reduced. The K_m is reduced due to increased binding efficiency.

Some techniques used in enzymology will be discussed in the section to follow. The section will provide a broad understanding of some molecular and quantitative techniques used to study protein-protein/drug interactions.

1.14. Techniques used in enzyme kinetics and inhibition studies

Several enzyme kinetics and inhibition techniques have been developed over the years. These kinds of experiments require techniques that are quick, simple, sensitive, and reliable. Two of the most common techniques employ the use of light, i.e., spectrophotometry and spectro-fluorometry. A modern technique employs the absorption and release of heat, i.e., isothermal titration talorimetry (ITC).

1.14.1. Spectrophotometry

Spectrophotometry [166] is a tool used to quantitatively measure the amount of light absorbed by a chemical substance as a function of wavelength. Spectrophotometric assays are the most common detection method in enzyme kinetics and inhibition. The release of the product from enzyme-substrate/inhibitor interaction generates a change in the absorbance signal. Formation of product is decreased when an inhibitor is present, resulting in a decrease in absorbance.

The decrease in absorbance is easily monitored with increasing drug concentration to determine the inhibition constant (K_i). The K_i is related to the Gibbs free energy by equation [159].

$$\Delta G = -RT \ln Ki \qquad \text{(Equation 5) [159]}$$

Where ΔG is the change in Gibb's energy, R is the ideal gas constant, T is temperature in kelvin.

1.14.2. Fluorescence Spectroscopy

Fluorescence spectroscopy [167] is an effective, and sensitive widely used quantitative analysis technique used to monitor the structure, dynamics, and interactions of protein in solution. A beam of light (ultra-violent light) excites electrons in molecules (fluorophores) [168] of some compounds, which causes them to emit light. Tyrosine, tryptophan, and phenylalanine amino acids are inherently responsible fluorescence of proteins. Fluorescence quenching, defined as a physiochemical procedure that reduces light intensity emitted by fluorescent molecules. This tool is used for measuring binding affinity between enzyme and inhibitor. A range of molecular interactions may lead to fluorophore quenching. These include excited state reactions, energy

transfer, molecular rearrangements, collisional quenching, and ground-state complex formation [169].

The Stern-Volmer equation allows calculation of product K_qT_0 from the graphical slope equation of F_0/F -1 plot against [Q] quencher concentration [170].

$$\frac{\mathbf{F_0}}{\mathbf{F}} = \mathbf{1} + \mathbf{K_Q T_0}[\mathbf{Q}] = \mathbf{1} + \mathbf{Ksv}[\mathbf{Q}] \qquad \textbf{(}\text{Equation 6) [170]}$$

Where F_0 & F are the fluorescence signals with and without a quencher (Q) respectively. K_Q is the quenching rate constant of the enzyme, K_{sv} represents the Stern-Volmer quenching constant and T_0 (10^{-8} s) is the average life-time of the fluorescent substance without the quencher.

Equation 7 is used to determine the equilibrium between molecules that are bound and/or free to equivalent sites on the enzyme [171].

$$\mathbf{log}\left[\frac{(\mathbf{F_0}-\mathbf{F})}{\mathbf{F}}\right] = \mathbf{log\,Kb} + \mathbf{n\,log}[\mathbf{Q}] \qquad \text{(Equation 7) [170]}$$

Where Kb and n represent the binding constant, and number of binding sites respectively.

Thermodynamic parameters from the reaction system are calculate using the Van't Hoff's equation [172]:

$$\mathbf{ln\,Kb} + \left(\frac{\mathbf{\Delta H}}{\mathbf{RT}}\right) + \left(\frac{\mathbf{\Delta S}}{\mathbf{R}}\right) \qquad \text{(Equation 8) [171]}$$

Here, Kb is equivalent to the K_{sv} constant at the corresponding temperature. ΔH and ΔS is the change in enthalpy of the system minus the temperature (T in Kelvin) and changes in the entropy of the system, respectively. R represents the ideal gas constant (8.314 J/mol-k). The change in enthalpy (ΔH) and entropy (ΔS) can be graphically determined from the equation of slope and intercept respectively by plotting $\ln K_b$ against 1/T. The following equation relates the ΔH to the ΔS for the determination of Gibbs' free (ΔG) energy of the reaction.

$$\mathbf{\Delta G} = \mathbf{\Delta H} - \mathbf{T\Delta S} \qquad \text{(Equation 9) [170]}$$

As much as fluorescence spectroscopy is still widely used to carry out binding affinity experiments, a more sensitive and simple technique known as ITC is rapidly being adopted by scientists.

1.14.3. Isothermal Titration Calorimetry (ITC)

ITC [173] is a widely applicable procedure applied to any enzyme-catalysed reaction provided the reaction is associated with enthalpy change. ITC experiment is a label-free experiment that works by directly measuring the amount of heat being released or absorbed during a molecular binding process (Fig 1.8). ITC can be used to determine binding affinities (K_d), binding stoichiometry (n), and enthalpy changes between two or more molecules in solution [174]. Data obtained from a full experimental run is then used to calculate other thermodynamic parameters (ΔS, ΔG) according to Equations 1 and 9.

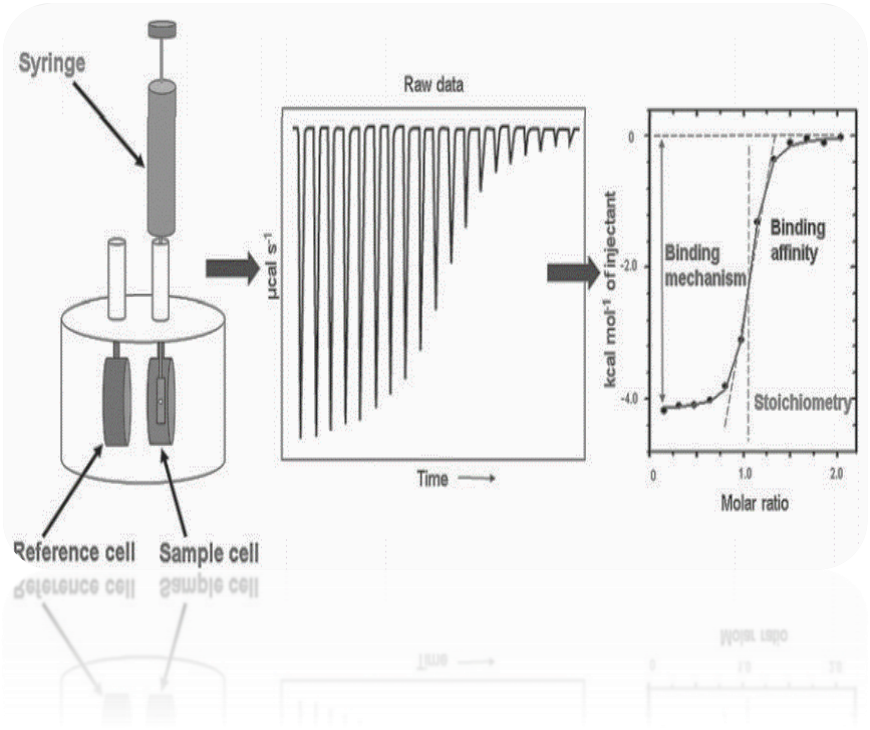


Figure 1.9. A Schematic representation of ITC. cells and injection syringe (left); Illustrative ITC data of titration experiment (middle); Illustrative binding isotherm of ITC experiment (right).

Other techniques used in enzyme kinetics and inhibition studies include surface plasmon resonance (SPR) [175] and microscale thermophoresis (MST) [176].

1.15. Study Rationale

This project focuses on the effects of key DRV resistance mutations in the C-SA HIV-1 protease framework. DRV is the newest FDA-approved PI and the only one prescribed for first-line therapy. Apparently, the dual mechanism of DRV's action is responsible for its high genetic barrier against resistance. However, eleven drug resistance mutations have been specifically associated with DRV resistance [177]. They can be classified into primary (I50/V, I54/M/L, I47/V and I84/V) and secondary (V11/I, V32/I, L33/F, I47/V, I54/L, G73/S, L76/V and L89/V) resistance mutations.

The I50/V, I54/M, V32/I, and I84/V mutations the protease at or adjacent to the active site cavity (residues 25 to 32, 47 to 54, and 80 to 84) [178]. The V32/I (valine/isoleucine) mutation is a major mutation across all PIs in the presence of secondary mutations. The mutation directly affects inhibitor interaction because the isoleucine residue is much larger in size than valine. The V32/I is reported to predispose HIV-1 to develop sensitive levels of DRV resistance [179]. The I50/V (isoleucine/valine) mutation affects inhibitor binding due to altered Van der Waals interactions close to the flap tips responsible for fastening the active site cleft upon ligand binding [180]. Amino acid residue substitution at positions V82 (valine to alanine) and I84 (isoleucine to valine) results in a compromised drug interaction, due to binding site distortion and weak Van der Waals interactions [181, 182].

Thus, V32/I, I50/V, I54/M, and I84/V major DRV resistant mutations were selected for this study. These mutations are observed within the framework of the subtype B virus, were reported to confer high levels of resistance, especially to DRV and cross-resistance across some clinically available PI's [164]. Therefore, this project aimed at investigating how the major darunavir resistant mutations within the C-SA protease frame affect darunavir and other protease inhibitor interaction.

1.15.1. Aims

The aim of this study is to analyse the functional effects of darunavir mutations in HIV-1 C-SA protease and to compare experimental binding energies of protease inhibitors between wild-type and mutant HIV-1 C-SA protease (Chapter 2).

In order to achieve this aim, the following specific objectives were determined:

1.15.2. Specific Objectives (Chapter 2)

- To clone, express, purify and characterise the wild type HIV-1 PR
- To perform site directed mutagenesis (L10I, I13V, L33I, S37N, R41K, L36I, C67A & C95A) on the wild type to avoid autocatalysis and cysteine oxidations
- To introduce DRV mutations (V32I, I50V, I54M, & I84V) into the newly synthesized wild type using site directed mutagenesis.
- To compare expression, activity, recovery and inhibitor binding of both wild type and mutated HIV PR
- To determine the binding energies for both the mutant and wild type using Fluorescence quenching
- To compare mutant interaction with other protease inhibitors using Fluorescence quenching

1.16. Thesis Outline

The thesis is presented in a paper format in which each chapter is dedicated to addressing one or two research question. In the first and last chapters, a general introduction and overall conclusion are provided, respectively, for the entire study.

The outline is therefore highlighted

Chapter 1: Introductory background of the study and study rationale.

Chapter 2: Key Darunavir Resistant Mutations in the HIV-1 C-SA Protease Alters Binding Affinity Across Protease Inhibitors: Kinetic and Thermodynamic Study. (paper 1)

Chapter 3: Overall Conclusion on the research outcome.

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CHAPTER TWO

Key Darunavir Resistant Mutations in the HIV-1 C-SA Protease Alters the Binding Affinity Across Protease Inhibitors: Kinetics and Thermodynamics Study

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Abstract

HIV-1 protease remain an important anti-retroviral target used in the treatment of HIV. However, drug resistant mutations seriously affect the efficacy of HIV-1 protease inhibition therapy. This problem is considered a major blockade in the treatment of HIV for the foreseeable future. Darunavir has quickly become one of the most commonly administered PIs in sub-Saharan Africa due its high barrier against resistance. In this study, enzyme kinetics, inhibition and thermodynamic studies were used to examine the functional effects of V32I, I50V,154M, and I84V mutations in recombinant HIV-1 C-SA protease. Mutations at these positions are associated with subtle structural changes that confer resistance across protease inhibitors especially darunavir resulting in treatment failure. A significant difference was observed in the catalytic efficiency (K_m/k_{cat}) in the variant (V32I, I50V,154M, and I84V) protease compared to the wildtype C-SA protease ($P < 0.0001$). The six clinically approved drugs used in this study demonstrated reduced binding affinities and weaker inhibition towards the variant protease in comparison to the wild-type HIV-1 C-SA protease. Darunavir and saquinavir exhibited the weakest inhibition towards the variant protease with 465- and 246-fold decrease respectively. Significant decrease in binding affinity was observed in all protease inhibitors towards the variant protease largely due to decreased binding entropy ($P < 0.0001$). Measurement of binding energy (ΔG) revealed that amprenavir and ritonavir exhibited the least decrease in binding affinity against mutant protease. Vitality values for the variant protease against the selected protease inhibitors, confirm the impact of these mutations on the HIV-1 CSA protease. We can therefore conclude that these mutations affect the efficacy of PIs used in this study.

1. Introduction

Human immunodeficiency virus (HIV) as the causative agent of acquired immune deficiency syndrome (AIDS), remains a serious threat to human life [1, 2]. In 2018, UNAIDS reported 36.9 million people living with HIV globally. According to global statistics approximately 26 million infected individuals reside in Africa and about 17 % in southern Africa. HIV is classified and grouped into subtypes based on their geographic distribution [3]. HIV-1 subtype C contributes to about 70 % of global infections and is the most prevalent strain within sub-Saharan Africa [4, 5]. Even with this staggering statistical data, very little experimental studies have been conducted on this strain. Therefore, it is crucial to focus therapeutic antiretroviral studies on HIV-1 C-SA infection.

In order to combat HIV infection, three enzymes required by the virus to undergo intracellular replication have been targeted to inhibit their action [6]. These enzymes include reverse transcriptase (RT) [7] crucial for completion of the initial stages of HIV replication, integrase (IN) [8] essential for the integration of pro-viral DNA into the host chromosomal DNA and finally the enzyme for which this study will focus, protease (PR) [9] which is vital for the maturation of infectious viral progeny.

HIV-1 protease (HIV-1 PR) is an aspartyl homodimeric enzyme responsible for cleavage of polyprotein products of viral Gag and Gag-Pol genes, producing functional and structural proteins that form a mature virion [10]. This process known as viral maturation, is the re-assembling of proteins to produce an infectious viral particle. Inhibition of HIV-1 PR action would mean that nascent (non-infectious) viral particles cannot attack other CD4+ cells and would therefore halt the progression of the disease. Thus, the vital role of HIV-1 PR in the life cycle of the virus has made the enzyme an important therapeutic target for HIV treatment. HIV protease inhibitors (PIs) were the first success of structure-based drug design [11]. Currently there are nine FDA-approved HIV-1 protease inhibitor, indinavir (IDV), amprenavir (APV), saquinavir (SQV), atazanavir (ATV), nelfinavir (NFV), ritonavir (RTV), tipranavir (TPV), lopinavir (LPV), and darunavir (DRV), all of which are competitive inhibitors binding at the active site of the protease enzyme [12]. Although these PIs treatment regimen are quite effective in improving quality of life and delaying the progression of the disease, mutations can arise in protease over time resulting in a resistant viral strain. Thus, making it difficult to

maintain strategies of effective treatment. Resistance to PIs occurs either directly (mature active PR with amino acid mutations) or indirectly (mutated polyprotein cleavage sites) [13].

Mutations that confer resistance to HIV-1 PR can be divided into two groups: primary resistance mutations which occurs within the active site and affects the binding of PIs and secondary resistance mutation which are present outside the active site of the enzyme. Secondary mutations affect the flexibility of the subdomain which indirectly influences PI binding [14]. More than 90 % of therapeutic failure in infected individuals is attributed to the emergence of resistant mutations to antiretroviral drugs [15]. Thus far, no effective treatment options exist to cease the emergence of drug resistant mutations (DRM) for such patients. Hence, structure-based design of drug molecules and knowledge on DRM is crucial in the search for the development of potential novel therapeutics for infected individuals [16]. Darunavir is one of the most widely used PIs, and the only PI recommended as a first-line therapeutic for treating HIV-1 infected patients. DRV has been reported to possess a high genetic barrier against resistant mutations [17]. Nevertheless, it is shown that the following PR mutations facilitates the development of DRV resistance: major (I47V, I50V, I54L/M, L76V, I84V) minor (V11L, V32I, L33F G73S, T74P, L89V). Our group recently reported the impact of I36T↑T and E35D↑G↑S mutation on HIV-1 C-SA protease. We found that the I36T↑T and E35D↑G↑S mutations located in the hinge region, decreased binding of seven clinical protease inhibitors except for amprenavir and ritonavir that showed the least decrease with IC_{50} values less than 10 nM [18, 19].

In this study, we assessed the effects of key darunavir drug resistant mutations (V32I, I50V, 154M, and I84V) on HIV-1 C-SA protease binding. These mutations are known to confer high level of resistance to DRV inhibitor in HIV-1 subtype B and as such were chosen for this study. Studies report that residue 50 of one monomer interacts with I54 of the other monomer upon ligand binding, this interaction stabilizes the bound conformation. Ile-Val at residue 50 and Ile-Met at 54 impact inhibitor susceptibility by altering the flap dynamic and ligand binding [20, 21]. The I84V mutation occurs in the binding pockets and greatly weakens the binding affinity of DRV and APV. The Val-Ile mutation increases the side chain of residue 32 which affects ligand binding in the active site cavity [22]. This study was aimed at investigating the functional effects of these key DRV mutations (V32I, I50V, 154M, and I84V) in HIV-1 C-SA protease and to compare experimental PI binding energies between HIV-1 C-SA wild-type (WT) and HIV-1 C-SA mutant protease.

2. Materials and Methods

2.1. Source of HIV-1 protease and expression plasmid

A pET 11b expression vector with a gene encoding the C subtype HIV-1 protease used in previous studies was used.

2.2. Oligonucleotide primer design

Oligonucleotide primers were designed for use with Geneart™ Site-Directed Mutagenesis PLUS System kit (Thermo scientific US) with the aid of the GeneArt® Primer and construct design tool. The mutagenic primers used to generate the L10I, I13V, L33I, S37N, R41K, L36I, C67A & C95A mutations in the Wild-type C-SA protease (Table S1). The V32I, I50V, I54M, I84V HIV-1 variant protease had the following primer sequence displayed in Table 1.

Table 1. Primer Sequence for the incorporation of V32I, I50V, I54M, I84V in HIV-1 C-SA protease.

Primer	Sequence
V32I Forward primer	5`- GAC ACT GGC GCT GAC GAC ACT **ATC** ATC GAA GAA ATC AAT CTG-3`
V32I Reverse primer	5`- CAG ATT GAT TTC TTC GAT GAT AGT **GTC** GTC AGC GCC AGT GTC-3`
I50V & I54M Forward primer	5`- AAA ATG ATC GGT GGC **GTC** GGC GGT TTT **ATG** AAA GTT CGT CAG-3`
I50V & I54M Reverse primer	5`- CTG ACG AAC TTT **CAT** AAA ACC GCC **GAC** GCC ACC GAT CAT TTT -3`
I84V Forward primer	5`-CCG ACT CCG GTT AAC **GTC** ATC GGC CGT AAC ATG-3`
I84V Reverse primer	5`-CAT GTT ACG GCC GAT **GAC** GTT AAC CGG AGT CGG-3`

Substituted residues are shown bold red.

2.2.1. Mutagenesis

The protease variant gene (V32I, I50V, I54M, I84V) was constructed using Geneart™ Site-Directed Mutagenesis PLUS System kit (Thermo scientific US) onto HIV-1 C-SA WT protease on a pET11b plasmid according to the kit protocol. L10I, I13V, L33I, S37N, R41K, L36I, C67A & C95A mutations were included to prevent autocatalysis and cysteine oxidation [23, 24] (Figure S1). Site-directed mutagenesis was performed according to the manufacturer's instructions. Briefly, plasmid DNA harbouring the wild-type HIV-PR was used as a template, amplified in a mutagenesis reaction with up to three overlapping primers containing the target mutations. The PCR products were generated through an incubation of 20 minutes at 37 °C and 2 minutes of 94 °C for methylation reaction followed by eighteen amplification cycles of 20 seconds at 90 °C to denature the DNA, 30 seconds at 57 °C to anneal the primers, 120 seconds at 68 °C for DNA extension and finally 5 minutes incubation at 68 °C. The reaction mix contained 10 X AccuPrime™ *Pfx* reaction buffer, 20 ng/µL of double stranded plasmid DNA template, 10 µM of each oligonucleotide primer mix. Accuprime™ *Pfx* (2.5 U/µL) and DNA methylase (4 U/µL) was then added. The plasmid was then recircularized at room temperature for 15 minutes in a recombination reaction. The recombination mixture was treated with 0.5 M EDTA to stop the reaction and used to transform into *Escherichia coli* DH5α™ -T1^{R} MAX Efficiency® competent cells supplied with the kit. Cells were plated onto LB agar plates supplemented with 100 µg/ml of ampicillin. Putative mutant plasmid DNA from screened colonies were sent for sequencing to ensure the presence of desired mutations incorporated into the cDNA.

2.3. Expression of HIV-1 C-SA Proteases

HIV-1- C -SA and the mutant protease were over-expressed as inclusion bodies in *Escherichia coli* (*E. coli*) strain BL21 (DE3), and BL21 (DE3) pLysS (Novagen, USA) [25]. Briefly, single positive colony of transformed cells was grown by shaking overnight at 37 °C in Luria-Bertani containing 100 µg/ml ampicillin and 35 µg/ml chloramphenicol (LBAC). The overnight culture (1 ml) was diluted in 100 ml of fresh LBAC medium at initial Optical Density (OD) measured at 600nm of 0.05. The culture was grown for 3hr at 37 °C in a shaker incubator. Over expression was induced by adding 1.0 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at early exponential phase of cell growth when OD measured at 600 nm was between 0.5 and 0.6. Cells

continued to grow in the shaker at 37 °C and harvested at 5000 *x* g at 4 °C for 10 min after 3 h induction. The cell pellet was resuspended in 10 ml of 50mM Tris HCL pH 7.9 buffer, the homogenized sample was disrupted by sonication (Omni International Sonic Ruptor 400 Ultrasonic homogenizer) at 50 kHz with 30 s on and off pulses for 10 min. Homogenized samples were placed in ice to reduce thermal effects of sonication. The sample was centrifuged at a high speed (13,000 *x* g) at 4 °C for 20 min. The insoluble fraction was resuspended in 50 mM Tris HCl pH 8 and 8 M Urea buffer and allowed to stand for a minimum of 1 h at room temperature. The mixture was clarified by centrifugation at 34,000 *x* g at 10 °C for 20 min and stored at 4 – 20 °C. Expression was then verified by SDS-PAGE.

2.4. Purification of HIV-1 C-SA Protease

HIV-1 PR purification was performed using an AKTA purifier 100-950 system (GE Health Care, USA). A 1:1 dilution of the solubilized protease in 50 mM Tris HCl pH 8 buffer was passed through a 5 ml HiTrap SPFF column (GE Healthcare). Previously equilibrated using 50 mM Tris pH 8, 4M urea. The column was then washed with 4 column volumes of the same buffer. Bound proteins were eluted with a linear NaCl gradient (0-1 M) in the same buffer at 2 ml/min flowrate. Fractions containing HIV protease were pulled and dialysed into a 10-fold dilution of 50 mM sodium acetate at pH 5, 10% glycerol, and 100 mM NaCl.

2.5. Enzyme Kinetics Studies

The measurement of enzymatic activity of wild-type and variant C-SA HIV-1 PR was monitored by hydrolysis of Abz-Arg-Val-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2 HIV-1 fluorogenic peptide substrate [25]. The fluorogenic substrate resembles the highly conserved KARVL/AEAM cleavage site in the Gag polyprotein precursor between the nucleocapsid (CA-p2) and capsid protein [26, 27]. The standard assay was performed in a microtiter plate at 20 °C containing 30-60 nM purified protease, 0-200 µM fluorogenic substrate, 50 mM sodium acetate, and 0.1 M NaCl (pH 5.0). A Jasco model V-550 spectrophotometer was used to measure HIV-1 fluorogenic hydrolysis by monitoring the decrease in absorbance measured at 300 nm. Enzymatic properties of protease (K_m, k_{cat} and k_{cat}/K_m) were calculated.

Michaelis-Menten reaction condition and Lineweaver-Burk plots constructed from the data, provided information on the enzyme kinetics parameters.

$$V = \frac{Vmax[S]}{(Km + [S])} \qquad \text{(Equation 1)}$$

The Michaelis-Menten equation. where [S] is substrate concentration, K_m is Michaelis constant, and V_{max} is maximum velocity of enzyme.

2.6. Enzyme Inhibition Studies

Inhibition constants (K_i), for the selected FDA approved inhibitors (atazanavir, amprenavir, darunavir, lopinavir, ritonavir and saquinavir) were obtained by measuring HIV-1 fluorogenic substrate rate of hydrolysis at 37 °C. The assay was performed using 100 nM protease in 50 mM sodium acetate, 0.1 M NaCl (pH 5.0) buffer, and 0-250 µM chromogenic substrate in the presence of increasing concentrations of inhibitor (0-10 nM). The competitive inhibition equation (Eqn 2) was used to calculate the K_i values.

$$v = \frac{Vmax\,[S]}{Km\left(1 + \frac{[I]}{Ki}\right) + [S]} \qquad \text{(Equation 2)}$$

Where [I] is inhibition concentration, K_m is Michaelis constant, K_i is inhibition constant, V is velocity and V_{max} is maximum velocity.

2.7. Vitality values calculations

To envisage the therapeutic benefits and/or effects of a given protease inhibitor over another, a vitality value (V) can be calculated using Equation 3. This value was calculated to determine the Comparison between the drug resistant mutant-PR and wild-type PR in the presence of the selected FDA approved inhibitors based on their catalytic efficiency values.

$$\text{Vitality (V)} = \frac{(\text{Ki} \times \text{kcat/Km})\text{m}}{(\text{Ki} \times \text{kcat/Km})\text{wt}} \qquad \text{(Equation 3)}$$

Where V is vitality, K_i is inhibition constant, k_{cat} is turnover number, m represents mutant and wt represents wild-type.

2.8. Fluorescence Quenching and Thermodynamics studies

Spectrofluorimetric analysis was carried out to determine tertiary structural anomalies induced in HIV-1 C-SA PR wildtype and mutant in the presence of protease inhibitors [28]. This experiment was conducted with a Jasco V-630 spectrofluorometer (Jasco International Co., LTD, Japan). Tryptophan residues served as a local probe in its microenvironment. The excitation wavelength for tryptophan residues was fixed at 295 nm and 482 nm the emission. This experiment is temperature dependent and was monitored at three different temperatures (298 K [25 °C], 303 K [30 °C] and 310 K [37 °C]). The fluorescence change was monitored over 10 min at 1 min incubation intervals as increasing concentrations of protease inhibitor (2 nM, 5 nM, 10 nM) were added to a reaction mixture of 250 nM HIV-PR wild-type or mutant in protease assay buffer (50 mM sodium acetate, 0.1 M NaCl pH 5) made to a final volume of 100 µl.

Calculations of thermodynamic parameters were performed according to Stern-Volmer (equation 4) and Van't Hoff graphical plots (equation 5).

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \qquad \text{(Equation 4)}$$

Where F_0 is florescence intensity in the absence of quenching, F is florescence intensity in the presence of quenching, K_{sv} is the Stern-Volmer constant and [Q] is quencher concentration in this case quencher is the drug/inhibitor.

$$\ln K_{sv} = -\left(\frac{\Delta H}{RT}\right) + \left(\frac{\Delta S}{R}\right) \qquad \text{(Equation 5)}$$

Where ΔH is the change in enthalpy, ΔS is the change in entropy, R is gas constant and T is the absolute temperature of the experiment.
Inhibition constant (K_i) of an inhibitor equals K_d in the case of competitive inhibition and as a result the Gibbs free binding energy (ΔG) were calculated from equation 6 [18].

$$\Delta G = RT \ln K_i \qquad \text{(Equation 6)}$$

Where ΔG is the Gibbs free binding energy, R is the gas constant, T is the experimental absolute temperature and K_i is the inhibition constant.

2.9. Statistical Analysis

The results are presented as the mean ± standard deviation. The data were analysed using an unpaired t-test and significance value was set to 0.05. GraphPad Prism 7 software program was used in the data analysis [29].

3. Results

3.1. Site-Directed Mutagenesis

Site-directed mutagenesis was used to incorporate the V32I, I50V,154M, and I84V substitutions in the cDNA encoding the HIV-1 C-SA protease WT. The entire plasmid DNA encoding these substitutions were sequenced to confirm the presence of the desired mutations and ensure that no other mutations were incorporated in the sequence. The plasmid containing the desired mutations is designated pET11b HIV-1 PR rDRV. Wild-type C-SA protease also carried L10I, I13V, L33I, S37N, R41K, L36I, C67A & C95A mutations.

3.1. Expression of HIV-1 Protease

HIV-1 PR WT and HIV-1 PR rDRV were expressed as inclusion bodies in *Escherichia coli* BL21 (DE3) pLysS cells. The over-expression of HIV-1 PR was assessed using SDS-PAGE (Figure 1).

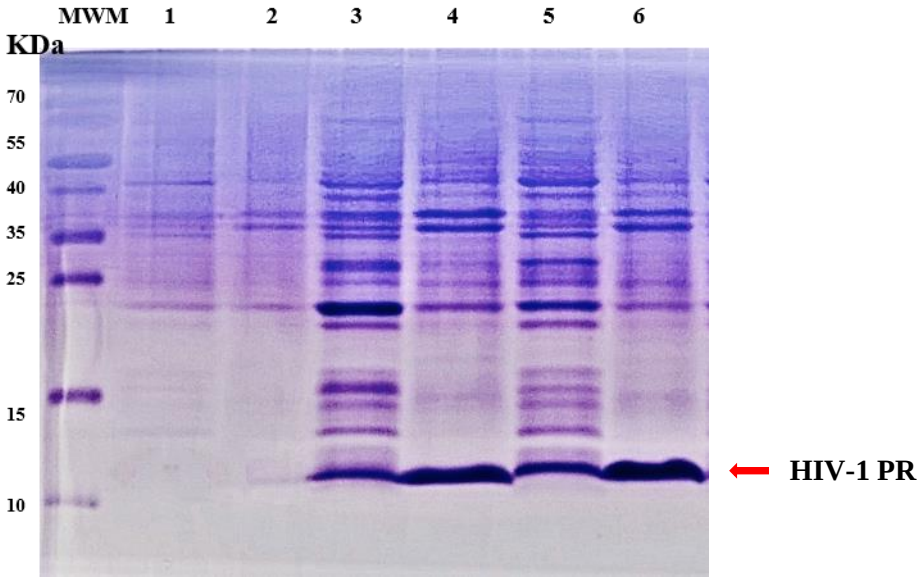

Figure 1: SDS-PAGE verification of over expressed HIV-1 protease induced by Isopropyl β-d-1-thioggalactopyranoside (IPTG).

MWM; molecular weight marker, 1 and 2 uninduced wild-type and mutant protease respectively, 3 and 4 soluble and insoluble fractions of induced wildtype protease respectively, 5 and 6 soluble and insoluble fractions of induced mutant protease respectively.

3.2. Purification of HIV-1 Protease

Over expressed protease recovered from inclusion bodies was purified using cation exchange chromatography.

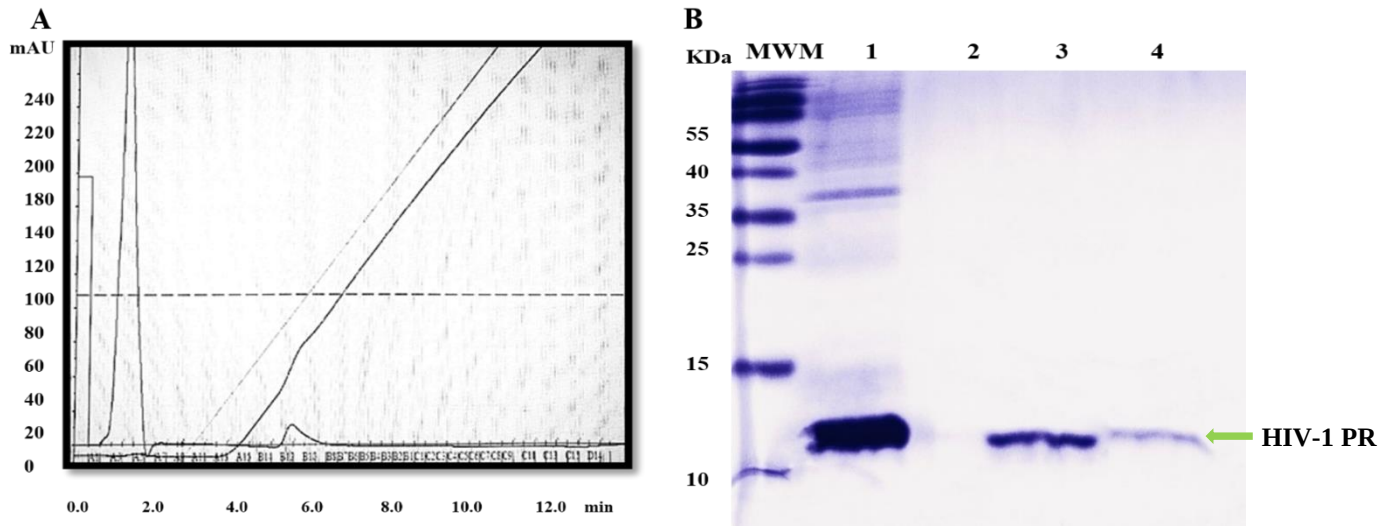

Figure 2: Purification of HIV-1 protease using ion exchange chromatography.

A; chromatogram showing bound (peak 2; B11, B12) and unbound (peak 1; A3) fractions. B; SDS-PAGE verification of purified HIV-1 protease from cation exchange AKTA purification, MWM; molecular weight marker, 1- crude protein, 2- unbound protein, 3- B11, 4-B12 bound proteins.

3.3. Enzyme kinetics

Displayed in Table 2 is a summary of the enzyme kinetics parameters of HIV-1 WT and HIV-1 PR rDRV. In the presence of the synthetic substrate (Abz-Arg-Val-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2), the mutant protease showed a decrease in (K_m) and catalytic efficiency (k_{cat}/K_m) compared to the wild-type protease. The wild-type protease displayed a higher (K_m) value, however when compared to the mutant protease it showed increased catalytic efficiency. This could be attributed to a high turnover (k_{cat}) number. A 90 % decrease in catalytic efficiency is observed for the mutant protease.

Table 2. Enzyme kinetic parameters of wild-type C-SA and mutant (I50V, I54M, V32I and I84V) protease using a synthetic substrate (Abz-Arg-Val-Nle-Phe(NO_2)-Glu-Ala-Nle-NH_2) (n = 3)

Parameters	Wildtype Protease	Mutant Protease
K_m (µM)	126.0±0.5	104.0±0.2
k_{cat} (s^{-1})	2.222±0.004	0.20±0.01
k_{cat}/K_m ($\mu M^{-1}/s^{-1}$)	0.018±0.000	0.0019±0.001

The decrease in K_m value for the mutant protease indicates high affinity for the substrate and continuous protease hydrolytic activity thus, increasing viral load which eventually lead to the progression of HIV to AIDS. The data for the C-SA protease WT are in agreement with values obtained previously [19, 30].

3.4. Enzyme Kinetics Inhibitions

The K_i values of PIs against the wild-type and mutant protease are shown in Table 3 below, calculated from Equation 2 (Figure S2). The K_i values were obtained by measuring in absorbance at 300 nm associated with the cleavage of the synthetic substrate and increasing concentrations of inhibitor.

The PIs can be been divided into three groups (more effective, effective, less effective). ATV, DRV and APV were the most effective against the wild-type protease as they had K_i values less than 200 pM. Lower K_i values is indicative of tighter binding to the wildtype protease.

Table 3. Summary of Inhibition constant (K_i) of PIs against wild-type and mutant protease.

Protease Inhibitors	K_i Wildtype Protease (pM)	K_i Mutant Protease(pM)	K_i ratio (Mutant/wild-type)
ATV	112.3±3.0	29510±30	163
APV	181.2±1.0	25530±41	232
LPV	204.4±5.0	16320±25	80
RTV	329.1±12.0	28510±10	87
DRV	108.6±3.0	50220±34	465
SQV	266.1±7.0	65670±20	247

LPV and SQV are classified as effective PIs against wild-type protease with K_i values of 204.4 pM and 266.1 pM respectively. RTV is considered less effective with a K_i value of above 300 pM. All six PIs were not effective against the mutant protease as they all had K_i values above 15000 pM. Overall the PIs showed decreased binding to HIV-1 PR rDRV compared to the wild-type protease. A K_i ratio (mutant/wild-type) is displayed in Table 3. The trend observed for mutant protease is much different than that of the wild-type. DRV, SQV and APV and displayed the weakest binding affinity of the PIs used in this experiment with approximately 465- 231- and 246-fold change respectively between the wildtype and mutant protease. RTV and LPV however displayed considerable binding with a fold change of 87 and 80 respectively.

3.5. Quenching and Thermodynamics

Thermodynamic parameters of inhibitor binding to the HIV-1 PR rDRV were compared with those of the wild-type using fluorescence quenching. Intrinsic tryptophan residue fluorescence can act as a probe to monitor conformational changes of proteins, due to high sensitivity to its local environment [31]. Using tryptophan residues as a probe to detect conformational changes is highly dependent on the number and location of tryptophan residue(s) in the amino acid sequence. HIV-1 protease in its dimeric form contains 4 tryptophan (Trp) located at Trp-6 and Trp-42 in each monomer. Trp-6 is the closest fluorophore to the active-site of the protease and is therefore expected to be the most affected by inhibitors than Trp-42. [31, 32]. These tryptophan residues in the protease frame behave as intrinsic quenchers by decreasing the quantum yield of fluorescence sensitive to the local environment. In this study, the presence of each inhibitor resulted in fluorescence quenching observed with Stern-Volmer plots. The linear regression plot allows the estimation of the Stern-Volmer quenching constant (K_{sv}) from the slope (Figure 3).

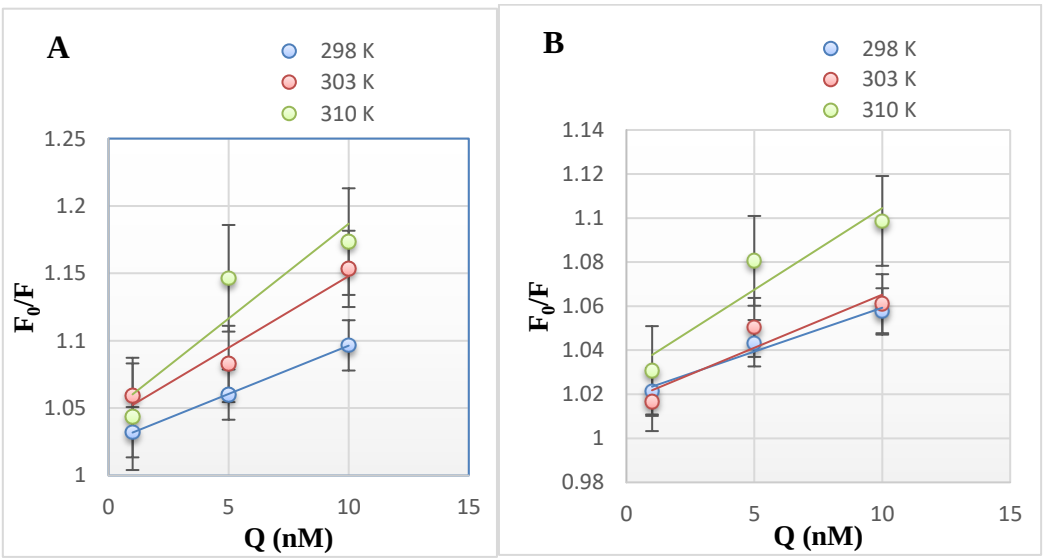

Figure 3: Examples of Stern-Volmer plots for fluorescence quenching of WT (A) and the mutant (B) protease treated with Amprenavir at different temperatures at 298 K; 303 K; 310 K respectively (n = 3).

High K_{sv} values indicates greater fluorescence quenching. The K_{sv} values of both wildtype and mutant protease displayed in Table 4 follows a similar trend as those of the inhibition constant (K_i) against the protease inhibitors. ATV displayed the best inhibition against wild-type and

mutant protease with K_{sv} values of 90.5 µM and 39.80 µM respectively. APV, DRV, LPV and RTV also displayed good inhibition against the wild-type protease. SQV showed the worst inhibition against wild-type and mutant protease with K_{sv} values of 42.70 µM and 14.00 µM respectively.

Table 4. Stern-Volmer quenching constants (K_{sv}) at 298 K for both wild type (WT) and mutant protease interacting with six PIs (n = 3).

	K_{SV} (μM^{-1}) at 298 k	**K_{SV} (μM^{-1}) at 298 k**
PROTEASE INHIBITOR	**WILD-TYPE PROTEASE**	**MUTANT PROTEASE**
APV	76.25±2.13	33.50±0.54
LPV	84.70±0.87	33.59±0.72
SQV	42.70±1.36	14.00±0.66
RTV	75.20±1.72	26.00±1.33
ATV	90.50±0.65	39.80±1.47
DRV	71.70±0.70	32.45±1.51

Van't Hoff's plot and calculation (Equation 5) assisted with understanding the interactive forces between the six selected protease inhibitors and the wild-type and mutant protease in a temperature dependent reaction (Figure S3).

The thermodynamic parameters (ΔG, ΔH and -TΔS) are summarized in T able 5 below. The Gibbs free energy (ΔG) of the PIs against the wild-type protease follows similar trend as the kinetic inhibition data. ATV and DRV are considered the best inhibitors against the WT protease as they displayed ΔG values of 14.13 and 14.15 kcal/mol respectively. All PIs displayed weak binding energies against the mutant protease with ΔG values of less than -11 kcal/mol. The high negative -TΔS values indicates that the reactions were mostly entropy driven. Although, RTV and LPV displayed negative ΔH values unlike the other PIs with positive ΔH values.

Table 5. A summary of experimental thermodynamic parameters for the six clinically approved HIV PR inhibitors (n = 3).

		PARAMETER		
DRUGS	**PROTEASE**	**ΔG (kcal/mol)**	**ΔH (kcal/mol)**	**-TΔS (kcal/mol)**
ATV	**WT**	-14.13	15.86	-29.99
	Mutant	-11.06	29.31	-40.37
LPV	**WT**	-13.76	-0.4	-13.36
	Mutant	-10.79	21.99	-32.78
SQV	**WT**	-13.60	31.29	-44.89
	Mutant	-10.72	33.79	-44.51
RTV	**WT**	-13.47	-4.93	-8.54
	Mutant	-10.70	14.57	-25.27
APV	**WT**	-13.83	7.37	-20.49
	Mutant	-10.37	12.41	-22.78
DRV	**WT**	-14.15	8.5	-21.23
	Mutant	-10.20	9.68	-19.88

Presented in Figure 4 is a comparison of the effects of the mutations in the binding of PIs to the wild-type. ΔΔG, ΔΔH and -TΔΔS values are the difference in free energy, enthalpy and entropy of PI binding.

To further investigate the changes in inhibitor binding thermodynamics due to drug resistance mutations, the differences in enthalpic and entropic contributions to the Gibbs free binding energy with respect to the HIV-1 WT protease were calculated. Amazingly, there is a substantial enthalpy-entropy compensation in the HIV-1 PR rDRV for most inhibitors.

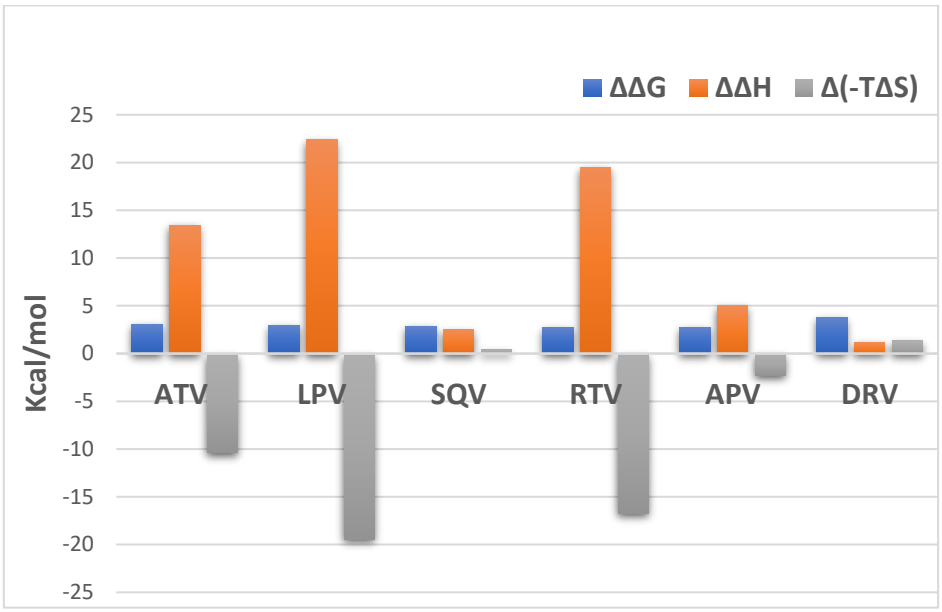

Figure 4: A comparison of changes in inhibitor binding thermodynamics of HIV-1 PR rDRV with respect to HIV-1 WT.

The entropic and enthalpic differences have similar magnitude but opposite signs, implying that they cancel out to yield fairly little changes in the overall Gibbs free binding energy change (ΔΔG). While SQV and DRV ΔΔG values are comparable to those for other PIs, the entropy-enthalpy compensation is non-existent. DRV and ATV showed the most decrease in binding energies with a ΔΔG value of 3.78 and 3.01 kcal/mol. compared to the other PIs. There is no observable trend found in the ΔΔH and -TΔΔS values of all PIs.

3.6. Vitality Value

Vitality is an empirical parameter used to compare the selective advantage of the V32I, I50V,154M, and I84V mutant protease in the presence of the clinically approved inhibitors. Vitality essentially provides a measurement of the biochemical or enzymatic of the mutant in the presence of selected inhibitor. Figure 6 displays the log vitality of the mutant protease.

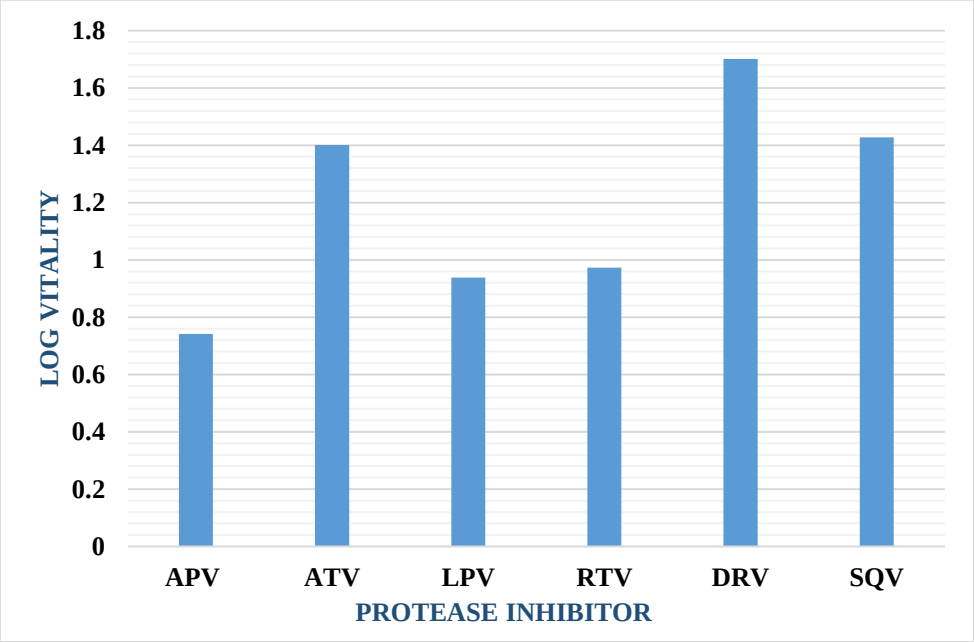

Figure 5: Log vitality values for the mutant protease with respect to the six selected protease inhibitors using wild type as a reference.

The mutant protease displayed the lowest vitality values of 0.74 for APV and is therefore the most effective against the mutant protease compared to other PIs. DRV, ATV and SQV had the highest vitality values of 1.70, 1.40 and 1.43 respectively. These values suggest that DRV, ATV, SQV, RTV and LPV may not be prescribed to infected individuals carrying these mutations. This data is consistent with the kinetic inhibition and thermodynamic data.

4. Discussion

HIV-1 protease is an attractive target for antiretroviral therapeutics. Darunavir's high genetic barrier against drug resistance has made it beneficial as a salvage therapeutic for patients failing other PI treatments. Nonetheless, a handful of mutations affect its potency. We choose four nonpolymorphic substrate-cleft signature mutations (V32I, 150V, I54M and I84V) within the inner hydrophobic cluster region reported to confer high level resistance to darunavir in HIV-1 subtype B [33, 34]. Thus, this study was set out to investigate the functional impacts of these resistant mutations in HIV-1 C-SA protease.

In other to characterise the enzymatic activity of WT and HIV-1 PR rDRV protease, these PR's were cloned into pET 11b vector in *E. coli* BL21 strain for their expression and purification. The recombinant proteases were successfully overexpressed, the pronounced ~11 KDa bands observed from the SDS-PAGE in (Figure 1) confirms expression of the PRs. Molecular mass of ~11 KDa for HIV-1 protease is consistent with the monomeric size previously reported [35]. Much of the protease is expressed in inclusion bodies and thus, present in the insoluble fraction. The recovery of the protease involved procedures adapted from Maseko and colleagues in 2018 [35]. The purity of the recombinant proteases was assessed using SDS-PAGE (Figure 2). Recovery from inclusion bodies produced an overall yield of 0.30 mg/L active protease, this value agrees with previously reported data [25].

Kinetic parameters for the WT and mutant protease are summarized in Table 2. HIV-1 PR rDRV had a lower K_m value relative to the WT, this data indicates higher affinity to the synthetic substrate. Studies reported that the changes induced by I84V mutation can maintain strong Van der Waals and hydrophobic interactions without losing significant affinity to the substrate [36]. The catalytic efficiency and turn over number were lowered by 11-, and ~10-fold respectively compared to the WT protease. Although the K_m value obtained suggests more affinity for substrate, it is not clear why the substrate is cleaved less efficiently by HIV-1 PR rDRV.

Based on the biochemical assay performed, K_i values were calculated for intrinsic measurement of selected inhibitor potency against WT and HIV-1 PR rDRV. It is important to note that the changes in inhibition constant are unique with respect to both inhibitor and mutant. Here, the affinity of clinical inhibitors used in this study (amprenavir, atazanavir, darunavir, lopinavir, ritonavir and saquinavir) for WT C-SA protease is in the nanomolar range and is

comparable with other findings [30]. It implies that the efficacy of the selected PIs against the naturally polymorphic WT C-SA protease is quite effective. DRV and ATV with K_i values of 108 pM and 112 pM were the most effective against WT. Furthermore, the binding affinity for the selected PIs was significantly ($P < 0.0001$) reduced against the HIV-1 PR rDRV relative to the WT (Table 3). The HIV-1 PR rDRV displayed large K_i values above 20000 pM, this large K_i values suggests limited interaction of PIs with HIV-1 PR rDRV.

Fluorescence quenching can be useful for inhibitor and protein interaction studies [37]. A variety of molecular interactions results in quenching [38], this effect was used to assess the binding affinity and thermodynamic parameters of amprenavir, atazanavir, darunavir, lopinavir, ritonavir and saquinavir, with WT and HIV-1 PR rDRV. Intensity of quenching is directly proportional to the Stern-Volmer quenching constant (K_{sv}) value. Results observed (Table 4) show that the K_{sv} values of both wildtype and mutant protease follow a similar trend as those of the inhibition constant (K_i) against the protease inhibitors. HIV-1 PR rDRV had lower K_{sv} values which is indicative of a higher fluorescence intensity.

Thermodynamic parameters (Table 5) such as ΔH (enthalpy) and ΔS (entropy) were determined for the selected PIs. PIs are competitive inhibitors, therefore their potency is directly related to the affinity with which they bind the protease molecule. The Gibbs free energy of binding (ΔG) can be used to determine the level of binding affinity [30]. The higher affinity of the inhibitor towards the WT C-SA PR is evident in the more negative ΔG value, thus confirming again that the HIV-1 PIs bind efficiently to the WT C-SA protease. As expected, ATV and DRV are considered the most effective against the WT C-SA protease (-14.13 and -14.15 kcal/mol respectively), interestingly these values are similar to values reported for HIV-1 subtype B and C-SA protease [18, 19, 39] . Autocatalysis and cysteine oxidation (Figure S1) mutations incorporated into the WT C-SA protease used in this study could account for the slight variations in ΔG values previously reported by our group for C-SA protease [19]. The comparison of binding energetics obtained for WT and HIV-1 PR rDRV, indicates that the main difference is in the magnitude of the entropy change. Entropy-enthalpy compensation could account for the reduced binding affinity of the PIs towards the HIV-1 PR rDRV. In agreement with previous reports, I50V/A71V variant protease resulted in compensatory changes for APV and ATV but not DRV [20, 40]. Based on the ΔΔG data Figure 4) APV and RTV showed the least change in binding energy between the WT and HIV-1 PR rDRV. An I36T↑T C-SA protease variant study also found APV and RTV to be the most effective PIs. [19].

In this study, the mutated residues contribute to the inner hydrophobic cluster around the substrate binding cavity, and hydrophobic interactions play an important role in the stability of proteins [41]. A structural analysis study reported that mutations to larger side chain as in V32I and I54M results in the formation of new hydrophobic interactions with flap residues 79 and 80 respectively restricting the flap movement. While, mutations to smaller side chains in I50V causes loss of internal hydrophobic interactions within the protease [34]. The formation of new interactions with the Asp25/25′ residue beside the dimer interface could be associated with reduced stability and altered catalytic parameters of this mutants as described in DRV and IDV studies [42].

5. Conclusion

In conclusion, examining the effects of V32I, I50V,154M, and I84V mutations in HIV-1 C-SA protease involved the use of several techniques. According to the enzyme kinetics data presented, it can be concluded that the mutant protease maintained substrate recognition and affinity. Therefore, the mutant protease can perform its proteolytic function. The selected PIs displayed less-favourable binding affinity to the mutant protease according to the thermodynamic data. This suggests that in the clinical environment, the efficacy of these PIs would be significantly reduced. These results prove that slight changes in protease molecule might have significant impact on conformationally constrained clinical inhibitor binding without a similar effect on the binding affinity of more relaxed molecules like the peptide substrates. Patients with these mutations may not be administered with ATV, SQV and DRV. Vitality data suggests that APV is still somewhat effective against the mutant protease and can be prescribed.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgements

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Appendix

Supplementary Information

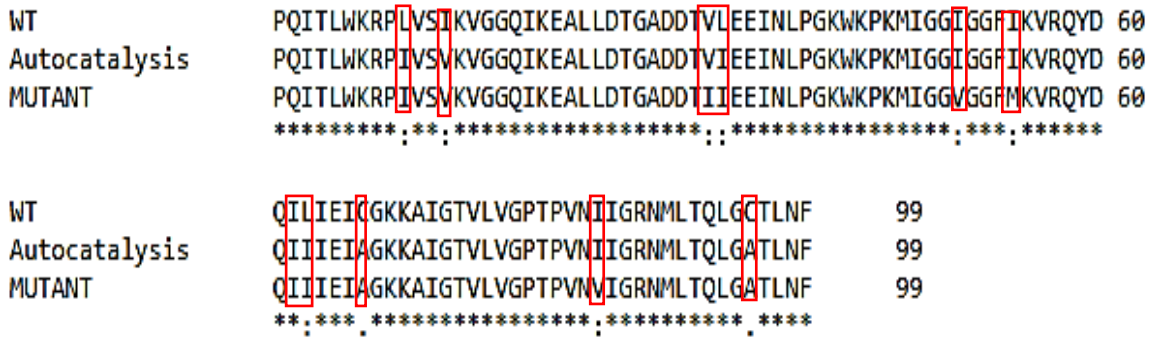

Figure S1. Multiple sequence alignment of HIV-1 C-SA protease (WT), autocatalysis and cysteine oxidation mutated WT(Autocatalysis) and HIV-1 PR rDRV protease (mutant).

Table S1. Primer Sequence for the incorporation for autocatalysis (L10I, I13V, L33I, L63I) and cysteine oxidation (C67A and C95A) prevention in WT HIV-1 C-SA protease.

Primer	**Sequence**
L10I & I13V forward primer	5′-TGG AAA CGT CCG ATC GTA TCC GTT AAA GTC GGT GGT CAG-3′
L10I & I13V reverse primer	5′-CTG ACC ACC GAC TTT AAC GGA TAC GAT CGG ACG TTT CCA-3′
L33I forward primer	5′-GGC GCT GAC GAC ACT GTT ATC GAA GAA ATC AAT CTG CCG-3′
L33I reverse primer	5′-CGG CAG ATT GAT TTC TTC GAT AAC AGT GTC GTC AGC GCC-3′
L63I & C67A forward primer	5′-CAG TAT GAT CAG ATC ATC ATC GAA ATC GCT GGT AAA AAA GCT ATC GGT-3′
L63I & C67A reverse primer	5′-ACC GAT AGC TTT TTT ACC AGC GAT TTC GAT GAT GAT CTG ATC ATA CTG-3′
C95A forward primer	5′-CGT AAC ATG CTG ACT CAG CTG GGT GCT ACT TTG AAC TTC-3′
C95A reverse primer	5′-GAA GTT CAA AGT AGC ACC CAG CTG AGT CAG CAT GTT ACG-3′

Substituted residues are shown red.

Inhibition Studies

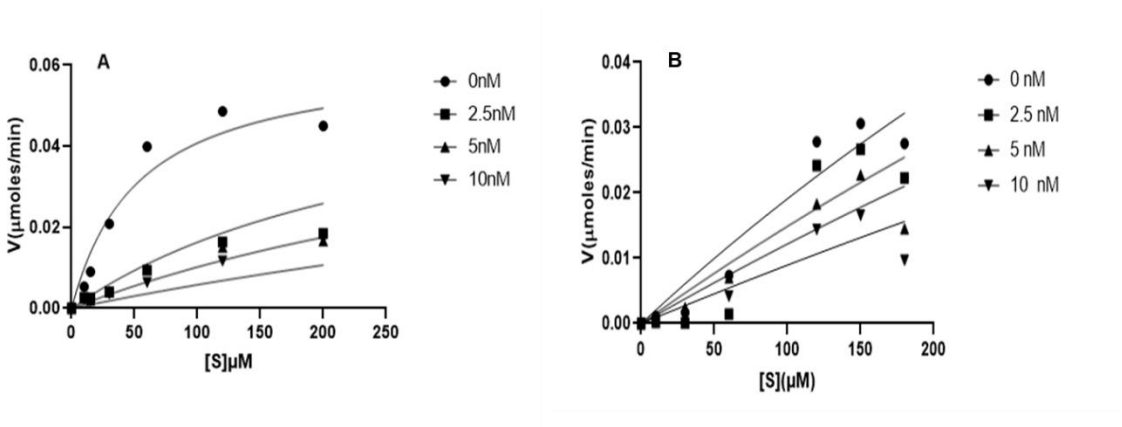

Figure S2. Inhibition (K_i) of the protease activities of wildtype (A) and HIV-1 PR rDRV (B) by APV.

Thermodynamic Studies

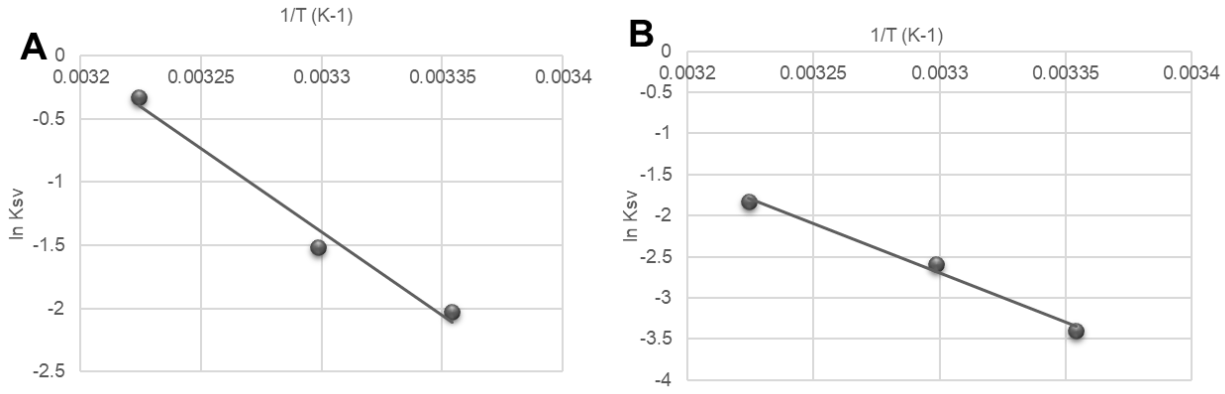

Figure S3. An example of Van't Hoff plots for the determination of thermodynamic data (ΔH and ΔS) for the interaction of Atazanavir, with HIV-1 protease (A) and mutant protease (B) at different temperatures. (n = 3).

CHAPTER THREE

Overall Conclusion of research outcome

1. Conclusion

Sub-Saharan Africa carries the burden of the highest HIV-1 infections globally [1]. Most HIV-1 infections in sub-Saharan Africa are caused by subtype-C rather than subtype-B for which the anti-retroviral drugs have been optimized. It is important to assess the effectiveness of anti-retroviral against other subtypes [2]. The development of anti-retroviral therapeutics has come a long way in managing the progression and transmission of the HIV disease. A major drawback in the treatment of HIV-1 infection has been the emergence of drug resistant viral strains [3].

HIV-PR is one the three significant enzymes that play a significant role in the HIV life cycle [4]. HIV-PR is responsible for viral maturation, therefore inhibiting the protease action will result in the production of immature non-infectious virions [5]. HIV-PIs especially darunavir are the backbone of salvage therapy [6]. DRV has a high genetic barrier against resistance and is the only PI recommended for line therapy [7]. Unfortunately, the efficacy of DRV is compromised by eleven PR residue mutations [8]. Till date there are no clinical anti-retroviral drugs available that can combat viral drug resistant strains. Therefore, more studies are focusing on drug discovery and development of potent inhibitors by understanding the mechanism of drug resistant viral strains.

The summation of the thesis is presented in this chapter. The first chapter covers the general introduction and overview of HIV-1 epidemiology, pathogenicity, and life cycle. The chapter covered much on the HIV-1 protease inhibition and resistance, the study rationale, and problem statement. The chapter also highlights an overview on enzyme molecular and quantitative techniques.

The second chapter reports on the functional effect of V32I, I50V,154M, and I84V mutations in recombinant HIV-1 C-SA protease on the efficacy of six clinically approved inhibitors (amprenavir, atazanavir, darunavir, lopinavir, ritonavir and saquinavir).

The successful expression, purification and yield of active of HIV-1 protease is reported in this chapter. The wild-type and mutant protease were characterised and kinetic parameters were

determined (K_m, K_{cat}, and K_{cat}/K_m). A higher affinity for the natural substrate demonstrated by a lower K_m value, was observed for the mutant protease. However, the mutant showed a reduced catalytic efficiency compared to the wild-type. In comparison with the C-SA wild-type, all six PIs ultimately showed weaker binding with the mutant protease.

The final phase of this study was to determine the thermodynamic parameters (ΔH, ΔS) for optimization of binding energy (ΔG). This data revealed that all inhibitor interaction with mutant protease was less favourable due to the reduced Gibb's energy values. However, APV and RTV seem to maintain some kind effectiveness against the mutant protease. Unlike DRV, which showed the least effectiveness against the mutant, and may not be prescribed to patients carrying these mutations. An interesting observation was the enthalpy-entropy compensation shown in APV, ATV, LPV and RTV, which accounts for the slight change in Gibb's energy. Vitality studies reveal that APV and RTV may be prescribed to patients with these mutations.

Future consideration for this study will involve X-ray crystallography [9] for diffraction and structure elucidation. This will provide a broad understanding on how the mutant interacts with the protease inhibitors. This could aid the development of protease inhibitors for resistant viral strains.

2. References

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