Characterizing Protease Inhibitor Failure in HIV-1 subtype C, using Ultra Deep Pyro-Sequencing and Homology Modeling.

By

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Preface

The experimental work described in this dissertation was carried out in the Hasso Plattner Research Laboratory at the HIV Pathogenesis Program at the Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, from April 2014 to October 2015 under the supervision of Dr Michelle Gordon.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it is duly acknowledged in the text.

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Dr Michelle Gordon (Supervisor)

Declaration

I, Avashna Singh, declare that:

i. The research reported in this dissertation, except where otherwise indicated, is my original work.

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Dr Michelle Gordon (Supervisor)

Ethical Approval

Ethical approval for this study was obtained from the Biomedical Research Ethics Committee of the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal (BR322/14).

Presentations

Part of this work was presented as a poster at the ”Second International Bioinformatics workshop on Molecular Biology and Evolution of Viruses, held in Salvador City, Bahia State, Brazil on the 26-30th of May 2014, the presentation was entitled “Characterizing Minority Variants in HIV-1 Subtype C infected patients failing a Darunavir based regimen using Ultra Deep Sequencing and Homology Modeling, in Kwa- Zulu Natal, South Africa.”

This work was also presented as a poster at the Keystone Symposia
“Mechanisms of HIV Persistence: Implications for a cure”, held in Boston, Massachusetts, United States of America, on the 26th of April – 1st of May 2015, the presentation was entitled “ Protease Inhibitor failure in Kwa-Zulu Natal, South Africa”.

This work was also presented as a poster presentation at the College of Health Sciences Symposia, 2015, held at the University of Kwa-Zulu Natal, Durban, South Africa. The poster was entitled “Drug resistance amongst patients failing Darunavir treatment”.

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To my mum, thank you for all you have instilled in me, my every achievement and success is yours.

This thesis is for my grandparents Mr. and Mrs. D Sivanath

The priceless gift of your love, care, and inspiration has seen me through each and every accomplishment
I am truly blessed to have you in my life.

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Abbreviations and Acronyms

3D	-	Three Dimensional
3'OH	-	3' Hydroxyl Radicals
3TC	-	Lamivudine
ABC	-	Abacavir
ABI	-	Application Binary Interface
AIDS	-	Acquired Immunodeficiency Syndrome
APS	-	Adenosine -5'-phosphosulfate
APV	-	Amprenavir
ART	-	Antiretroviral Therapy
ARTEMIS	-	Antiretroviral Therapy with TMC114 Examined In naïve Subjects
ARVs	-	Antiretrovirals
AS-PCR	-	Allele Specific PCR
ATP	-	Adenosine Triphosphate
ATV	-	Atazanavir
AVA	-	Amplicon Variant Analyzer
AZT	-	Azidothymine
BB2	-	Bead Buffer 2
BCC	-	Bond Charge Correction
BDD	-	Bead Deposition Device
cART	-	combination Antiretroviral Therapy
CCD	-	Charged Coupled Device
CCD	-	Catalytic Core Domain
CCR5	-	Chemokine Receptor type 5

CD4	-	Cluster of Differentiation 4
cDNA	-	complementary DNA
CHARMM	-	Chemistry of HARvard Macromolecular Mechanics
CRFs	-	Circulating Recombinant Forms
CSV	-	Comma Separated Values
CTD	-	C Terminal Domain
CXCR4	-	Chemokine Receptor type 4
d4T	-	Stavudine
ddi	-	Didanosine
DEPC	-	Diethlypyrocarbonate
DNA	-	Deoxyribonucleic Acid
dsDNA	-	double Stranded Deoxyribonucleic Acid
dNTPs	-	deoxynucleotide triphosphates
DRMS	-	Drug Resistance Mutations
DRV	-	Darunavir
DRV/r	-	Darunavir boosted with Ritonavir
DTG	-	Dolutegravir
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
EFV	-	Efavirenz
emPCR	-	emulsion PCR
ETR	-	Etravirine
EVG	-	Elvitegravir
FDA	-	Food and Drug Association
FTC	-	Emtricitabine
GAFF	-	Generalized Atomic Forcefield
gp120	-	glycoprotein 120
gp160	-	glycoprotein 160
gp41	-	glycoprotein 41
GROMACS	-	GROningen Machine for Chemical Simulations

HAART	-	Highly Active Antiretroviral therapy
HIV	-	Human Immunodeficiency Syndrome
HIV-1	-	Human Immunodeficiency Syndrome Type 1
HIV-2	-	Human Immunodeficiency Syndrome Type II
HR	-	Heptad Repeat
HTLV-III	-	Human-T-Lymphotrophic virus type III
IAS	-	International AIDS Society
IDV	-	Indinavir
IFEL	-	Internal Fixed Effect Likelihood
IN	-	Integrase
INSTI	-	Integrase Strand Transfer Inhibitor
IQR	-	Interquartile Range
KCL	-	Potassium Chloride
kDA	-	Kilo Dalton
KZN	-	Kwa-Zulu Natal
LAV	-	Lymphadenopathy Associated Virus
LPV	-	Lopinavir
LPV/r	-	Lopinavir boosted with Ritonavir
LTR	-	Long Terminal Repeat
MD	--	Molecular Dynamics
MIDS	-	Multiplex Identifiers
MPC	-	Magnetic Particle Concentrator
MRS	-	Magnetic Ring Stand
MVC	-	Maraviroc
MulV RT	-	Moloney Murine Leukemia Virus Reverse Transcriptase
NFV	-	Nelfinavir
NICD	-	National institute of Communicable diseases
NMR	-	Nuclear Magnetic Resonance
NNRTI	-	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	-	Nucleoside Reverse Transcriptase Inhibitor

NTD	-	N Terminal Domain
NVP	-	Nevirapine
ORF	-	Open Reading Frame
PCR	-	Polymerase Chain Reaction
PDB	-	Protein Data Bank
PI	-	Protease Inhibitor
PLANTS	-	Protein-Ligand ANT System
PR	-	Protease
PTP	-	PicoTiter plate
PTRAJ	-	Process Trajectory
qPCR	-	quantitative real time PCR
RAL	-	Raltegravir
RMSD	-	Root Mean Square Deviation
RNA	-	Ribonucleic Acid
RNaseH	-	Ribonuclease H
RPV	-	Rilpivirine
RT	-	Reverse Transcriptase
RT-PCR	-	Real Time PCR
SA	-	South Africa
SGA	-	Single Genome Analysis
SIV	-	Simian Immunodeficiency Virus
SQV	-	Saquinavir
RNA	-	Ribonucleic Acid
ssRNA	-	single stranded Ribonucleic Acid
TAM	-	Thymidine Analogue Mutation
TAR	-	Transactivation Response element
TBE	-	Tris Borate EDTA
TDF	-	Tenofovir
TE	-	Tris EDTA
TFP	-	Trans Frame Protein

TPV	-	Tiprinavir
UDPS	-	Ultra Deep Pyro-Sequencing
UNG	-	Uracil-N-Glycosylate
URFs	-	Unique Recombinant forms
X-Gal	-	X-Gal -5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abstract

The extensive roll-out of combination antiretroviral therapy (cART) has significantly improved the life expectancy for HIV-1 infected individuals in South Africa. Despite the inclusion of potent Protease Inhibitors (PIs) in second-line cART, many patients still fail treatment. The extent to which PI resistance contributes to treatment failure is not completely clear. In this study we report the prevalence of PI mutations amongst individuals failing a second-line Lopinavir (LPV/r) inclusive regimen. We also investigated if low frequency minority variants at LPV/r failure influence Darunavir (DRV/r) failure in a subset of patients using Ultra Deep Pyro-sequencing. Structural changes at DRV/r failure were investigated using Homology modeling. Models were constructed using the SWISS-MODEL webserver and visualized in Chimera v1.8.1. Darunavir was docked into each of the structures using the CLC Drug Discovery workbench ™ and Molecular Dynamics simulations was performed using the AMBER12 package. Our study reports a 24% prevalence of PI resistance mutations, slightly higher than other studies. A distinct pattern of PI resistance mutations was found: M46I+I54V+L76V+V82A, present in 13/37 (35%) of those with PI mutations. Darunavir resistance mutations detected following DRV/r failure included V11I, V32I, L33F and I54L. There were no minority variants detected at LPV/r failure that could have influenced DRV/r failure. Distinct conformational changes were evident in both the LPV/r-resistant and DRV/r-resistant model. Molecular docking showed that the inhibitory potency of DRV was lowered in the mutated DRV/r-resistant model and to a lesser extent in the LPV/r-resistant model. These results show that resistance mutations greatly contribute to DRV drug susceptibility. This work will contribute to the clinical management of patients failing treatment and will also assist in the design of new and improved ARVs.

CHAPTER 1

Literature review

1.1 Origins of HIV/AIDS

The Acquired Immunodeficiency Syndrome (AIDS) was first identified in 1981 where the Atlanta based Centre for Disease Control described the occurrence of rare opportunistic infections and severely compromised immunity amongst patients (CDC, 1981). The disease was prevalent amongst homosexual men, blood transfusion recipients, intravenous drug users, children and sexual partners, suggesting that the etiological agent was transmitted by body fluids (Freed, 2007). Thus far, AIDS has been described by many as the most "devastating infectious disease" yet to emerge (Gottlieb et al., 1981, Barre-Sinoussi et al., 1983, Popovic et al., 1984). In 1983, Dr Luc Montagnier and Dr Francois Barre-Sinoussi from the Pasteur Institute in France were able to isolate a retrovirus believed to be the cause of AIDS, which at the time was named the Lymphadenopathy-Associated virus (LAV) (Barre-Sinoussi et al., 1983). In 1984, Dr Robert Gallo isolated a virus called the Human–T–Lymphotrophic Virus Type III (HTLV-III) (Gallo et al., 1984). A few years later it was then apparent that both LAV and HTLV- III were in fact the same virus and was later named the Human Immunodeficiency Virus (HIV). HIV was then established as the causative agent of AIDS and was renamed HIV type 1 (HIV-1) to distinguish it from a related, but less prevalent AIDS-causing virus, HIV-2 in 1986 (Clavel et al., 1986).

HIV belongs to the Lentivirus genus of the Retroviridae family, and is believed to be a descendant of the Simian Immunodeficiency Virus (SIV) carried by certain non-human primates (Hemelaar, 2012). Like HIV, SIV belongs to the Retroviridae family comprising of more than 40 Lentiviruses, having high sequence homology to HIV (Sharp and Hahn, 2011). HIV-2 is distantly related to HIV-1, but is closely related to SIV, that causes immunodeficiency in macaques (Chakrabarti et al., 1987, Guyader et al., 1987). SIVs were found in different primates from Sub-

Saharan Africa and included African green monkeys, sooty mangabeys, mandrills, chimpanzees, and others. SIVcpz which infects chimpanzees is considered the origin of HIV-1 while SIVmm which infects sooty mangabeys is thought to cause HIV-2 (Silvestri et al., 2007). This provided the first evidence that AIDS had emerged in both humans and macaques as a result of a cross-species transmission event with Lentiviruses from different primate species (Sharp PM, 1994). This transmission was possibly the result of hunting, butchering and the consumption of infected raw meat from primates, a common practice at the time (Hemelaar et al., 2011). The phylogenetic relationship between SIV and HIV is illustrated in Figure 1.1.

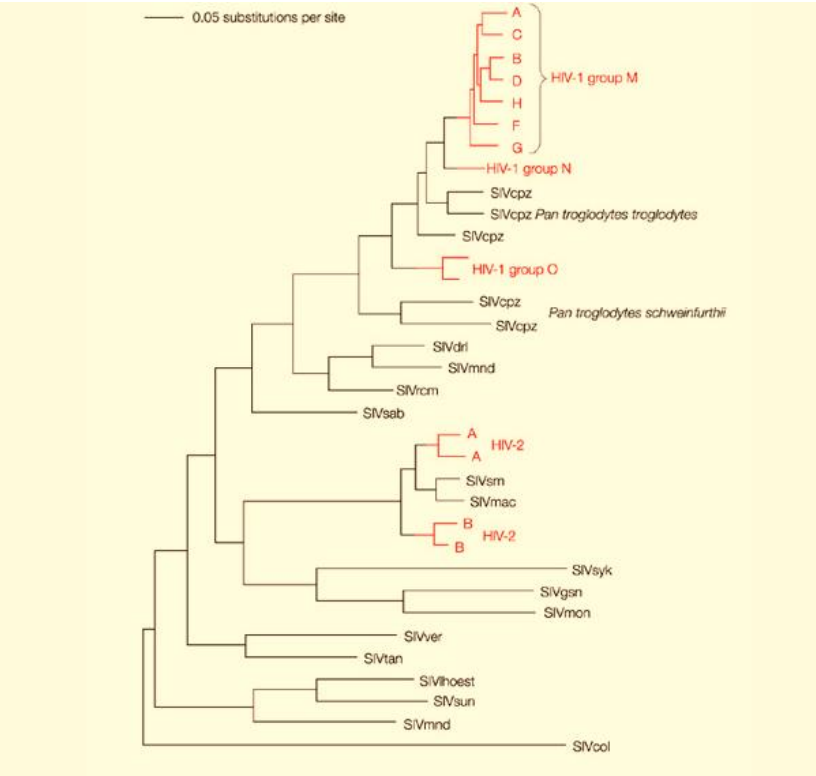


Figure 1.1 Maximum-likelihood tree of HIV-1 and HIV-2. The red branches represent independent cross-species transmission events from SIV isolated from other primates. Different groups of HIV-1 are shown: M (Main), O (Outlier) and N (non M/non O) representing independent introductions from chimpanzees (SIVcpz). Similarly HIV-2 was introduced from Sooty Mangebeys (SIVsm) on several occasions. SIVcol; black and white colobus, SIVdrl; drill, SIVgsn; greater spot nosed monkey, SIVlhoest; L'Hoest monkey, SIVmac; macaque. SIVmnd;

mandrill, SIVmon; Campbells mon monkey, SIVrcm; red-capped monkey, SIVsab; Sabaceus monkey, SIVsun; sun-tailed monkey, SIVsyk; Sykes monkey, SIVtan; tantalus monkey and SIVver; vervet monkey. The tree was constructed based on 34 published sequences of the viral polymerase gene accessed from Genbank. Taken from Rambaut et al (2001).

1.2 Classification of HIV

The diversity of HIV-1 stems from three independent introductions of the virus from chimpanzees to humans (Kandathil et al., 2005) as mentioned in section 1.1. As a result, there are four different phylogenetic lineages of HIV, comprising Group M, O, N and P (Simon et al., 1998, Ayouba et al., 2001, Plantier et al., 2009, Vallari et al., 2011). The HIV-1 epidemic is primarily due to Group M (Hahn et al., 2000, Buonaguro et al., 2007, Hemelaar, 2012) and comprises 11 phylogenetic subtypes (A-K) excluding the recombinant forms, while HIV-2 comprises subtypes (A-H) (Buonaguro et al., 2007). HIV is classified based on sequences that are derived from multiple sub-genomic regions of the same isolate, or from full-length genome sequence analysis. As a result phylogenetic relationships between different subtypes within the viral genome can be determined. These isolates are referred to as inter-subtype recombinant forms and are hypothesized to have originated in individuals that have had multiple infections with viruses belonging to two or more subtypes (Buonaguro et al., 2007). Once the virus is seen in at least three epidemiologically un-related individuals these recombinant forms are referred to as Circulating Recombinant Forms (CRFs) (Peeters, 2001).

1.3 Geographical distribution of HIV subtypes

Molecular epidemiological studies have shown that HIV-1 subtypes exist within distinct geographical regions (Bessong, 2008, Hemelaar, 2012). This distribution is largely driven by viral migration accompanied by a resulting founder effect or possibly the prevalence of a specific route of transmission. Therefore certain subtypes predominant within a specific population (Buonaguro et al., 2007). HIV-1 Group O appears to be endemic to Cameroon and neighboring countries of West

Central Africa and is representative of 1-5% of HIV-1 positive patients (Peeters et al., 1997). While group N viruses are generally found in parts of Central Africa (Plantier et al., 2009). Group M viruses drive the HIV-1 pandemic as they appear to have adapted well to new host species and have thus been able to spread around the world generating multiple genetic subtypes (Buonaguro et al., 2007). Subtype A is found in Central and Eastern Africa, including Kenya, Uganda, Tanzania, Rwanda as well as in the Eastern European countries. Subtype B is the most disseminated variant and is representative of the main genetic form in Western and Central Europe, including the Americas and Australia. It is also prevalent in South East Asia, Northern Africa, the Middle East and has been seen amongst South African and Russian homosexual men (Buonaguro et al., 2007). The most prevalent HIV-1 subtype is subtype C and is prominent in Sub-Saharan Africa, Eastern African countries, India and the Southern region of Brazil (Buonaguro et al., 2007). The presence of CRFs within the global pandemic is becoming more prevalent than before, accounting for 18% of infections in Southeast Asia (CRF01-AE) and in West Central Africa (CRF02-AG) (Buonaguro et al., 2007).

1.4 HIV-1 Structure

HIV-1 has a spherical morphology measuring between 100-120nm in diameter (Sierra et al., 2005, Marsden and Zack, 2013). HIV-1 comprises two copies of non-covalently linked, positive sense single stranded Ribonucleic Acid (ssRNA), tightly bound to the Nucleocapsid (p7) and enclosed within a viral protein capsid (p24). Also within the capsid is the late assembly protein (p6), viral enzymes: protease (PR), reverse transcriptase (RT), integrase (IN), as well as many viral proteins *Vpu, Vif, Vpr* and *Nef*. The cone shaped capsid is surrounded by the matrix (p17) which provides integrity to the virion. As the capsid buds from the host cell, part of the host cell membrane is retained by the virus forming an envelope that surrounds the capsid. Anchored within the envelope are proteins from the host cell as well as approximately 70 copies of glycoprotein 120 and 41, (gp120 and gp41). These glycoproteins allow the virus to fuse and attach to target cells thus initiating the

infectious replication cycle (Sierra et al., 2005, Marsden and Zack, 2013, Shum et al., 2013). The structural features of HIV-1 are shown in Figure 1.2.

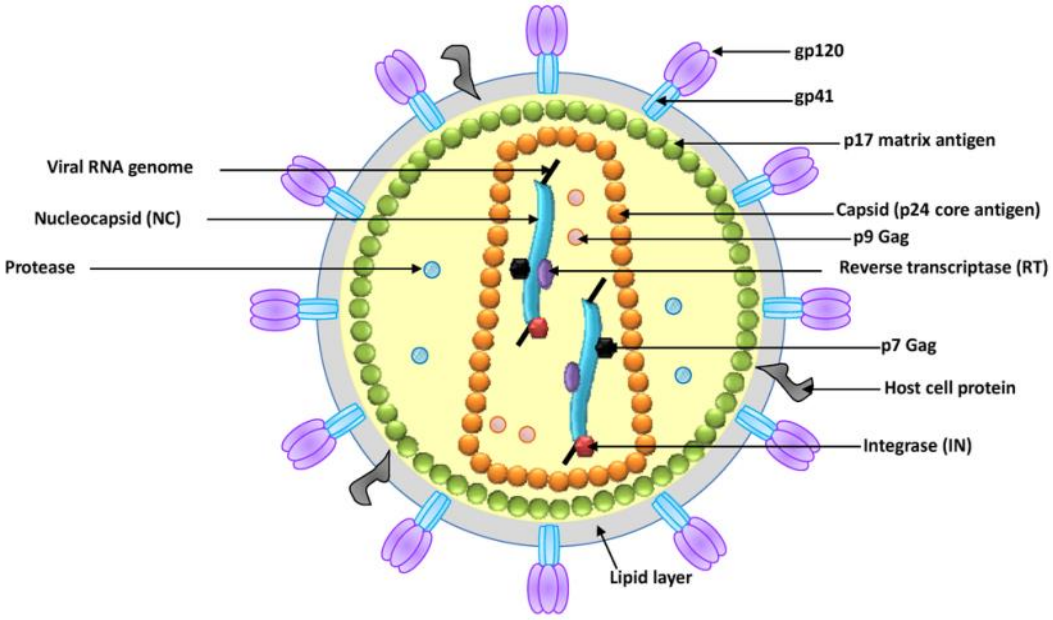


Figure 1.2 Structural features of HIV-1 required for the formation of mature infectious virions. Taken from Shum et al (2013).

1.5 HIV-1 genome arrangement

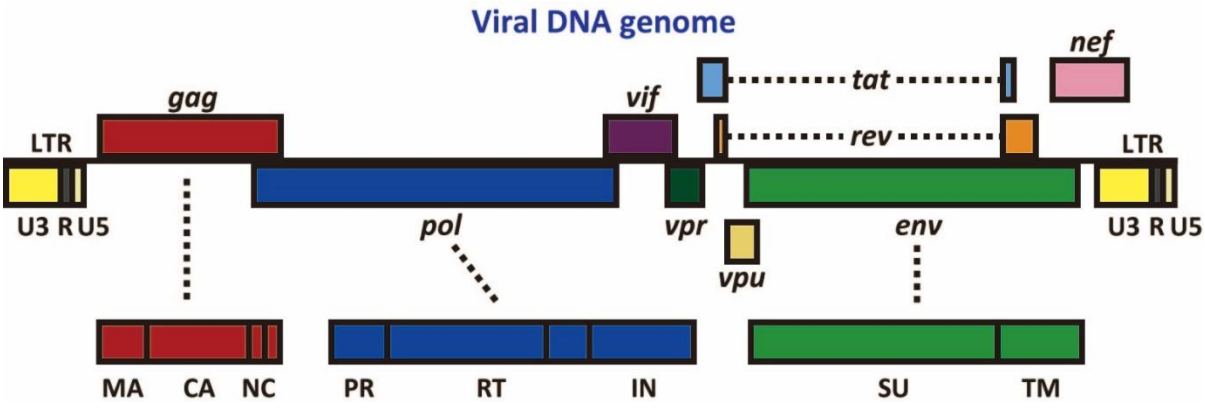


Figure 1.3 Genomic arrangement of HIV-1. Taken from Suzuki et al (2012).

The 9.2kb ssRNA molecules contain nine Open Reading Frames (ORFs). Both edges of the Ribonucleic Acid (RNA) molecule contain the LTR (Long Terminal Repeat) which are regulatory regions that flank the coding region. As indicated in Figure 1.3, each LTR contains a U3, R and a U5 coding region. U3 is a unique non-coding region of (200-1200nt) which forms the 5' end of the provirus following reverse transcription and contains binding sites for cellular transcription factors. The R region is much shorter (18-250nt) and forms a direct repeat at both sides of the genome, as a result this region is "terminally redundant". This region also contains the Transactivation response element (TAR) which is important for tat-mediated transactivation. The U5 is a unique, non-coding region of (75-250nt) and comprises the first part of the genome to be reverse transcribed, thus forming the 3' end of the provirus genome (Lodish, 2000). The *gag-pol* gene encodes for all structural viral proteins which are the matrix, capsid, nucleocapsid (p7), the p6 protein and the two spacer proteins (p2 and p1). The *gag-pol* poly-protein is made due to a ribosomal frame shift that generates structural proteins (Matrix, Capsid, p2, and p6), the Trans-Frame Protein (TFP) and the three viral enzymes. PR, RT and IN. The *env* gene encodes the glycoprotein 160 (gp160) containing the exterior gp120 and the transmembrane gp41, two regulatory (*Tat* and *Rev*) and four accessory (*Vif, Vpr, Vpu and Nef*) genes. These protein-coding regions are flanked by the 5' and the 3' LTR that are necessary for reverse transcription, integration and gene expression (Sierra et al., 2005).

1.6 HIV-1 replication cycle

During the HIV-1 replication cycle gp120 binds to a CD4 receptor on the surface of the target cell. Upon binding, the gp120 undergoes a conformational change resulting in binding to the co-receptors, either Chemokine receptor type 5 (CCR5) or Chemokine receptor type 4 (CXCR4) found on the membrane of the target cell. This attachment triggers gp41 transmembrane proteins; Heptad Repeat 1 and 2 (HR1 and HR2) to interact with each other resulting in the formation of a stable six helix bundle structure (Melikyan et al., 2000).This completes the fusion of the viral

and host cell membranes, permitting the release of the nucleocapsid containing viral RNA into the host cell cytoplasm. Following un-coating, the viral RNA is reverse transcribed by viral RT into double stranded Deoxyribonucleic acid (dsDNA) or proviral Deoxyribonucleic acid (DNA). Viral IN catalyzes the transport of the DNA into the nucleus as part of the pre-integration complex. Viral DNA is then inserted into the host cell's chromosomes and the integrated provirus is maintained for the entire lifespan of the host cell. Viral RNA is transcribed by host cell polymerases from the integrated provirus for translation into protein, following RNA splicing for certain viral proteins. New virions then assemble and bud from the plasma membrane releasing immature virions. Viral PR then cleaves the poly-proteins within the virion for the production of mature infectious virus particles (Marsden and Zack., 2013).

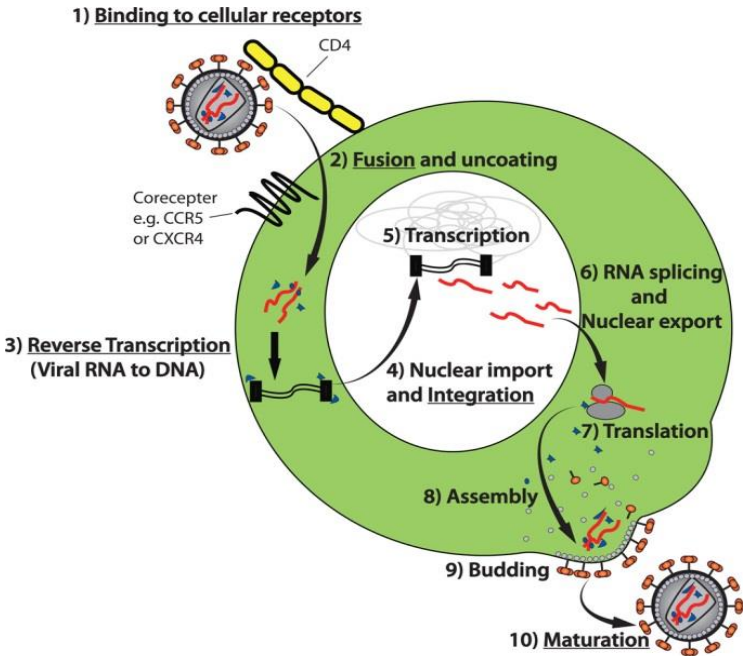


Figure 1.4 Essential steps of the HIV-1 replication cycle (1) HIV-1 virions bind to CD4 and a co-receptor on target cells. (2) The viral envelope proteins mediate fusion of the viral and host cell membranes, viral RNA is released into the host cell cytoplasm. (3) Viral RNA is reverse transcribed into dsDNA by HIV-1 RT. (4) dsDNA is translocated into the nucleus, IN catalyzes the integration of the dsDNA into the host cells chromosomes (5) Transcription of the HIV-1 genome is mediated by host cell polymerases (6) and (7) RNA is exported to the cytoplasm for

translation or in-corporation into new virions (8) and (9). New virions then assemble and bud from the plasma membrane. (10) Viral PR cleaves HIV poly-proteins into subunits, producing infectious, mature virions. Taken from Marsden and Zack et al (2013).

1.7 Antiretroviral treatment

The advent of Antiretroviral Therapy (ART) has transformed HIV-1 infection from a once “devastating infectious disease” to a now treatable and manageable chronic disease. Highly active antiretroviral therapy (HAART), also referred to as combination Antiretroviral Therapy (cART), is a customized combination of three drugs from at least two different classes of Antiretrovirals (ARVs). These drugs are prescribed for HIV-1 infection and are based on viral load, CD4 count, as well as patient dependent disease symptoms. HAART inhibits various stages in the HIV-1 replication cycle thereby controlling viral load and delaying the onset of disease progression (Marsden and Zack., 2013). Thus far, cART has played a vital role in improving the quality of life, and has been able to restore and improve immunological functions. Most importantly, cART has significantly reduced HIV-AIDS related morbidity and mortality within South Africa (SA) and globally (Mugavero and Hicks, 2004, Rong et al., 2007, Tanser et al., 2013, Kiepiela P, 2014). There are different classes of ARVs that constitute cART in SA, namely, the Non-Nucleoside Reverse Transcriptase Inhibitors (NRTIs), the Nucleoside-Reverse Transcriptase Inhibitors (NNRTIs), Protease Inhibitors (PIs), Integrase Strand Transfer Inhibitors (INSTIs), and Maturation inhibitors, each of these drugs target a specific step in the HIV-1 replication cycle.

1.8 Drug resistance and the role of Minority Variants

HIV-1 infection *in vivo* is characterized by rapid viral replication and the generation of 10^{10}-10^{11} virions daily, performed by the error prone HIV-1 RT that lacks proof reading functionality (Tang and Shafer, 2012). As a result, an average of one mutation per genome per replication cycle is introduced daily (Drake and Holland,

1999, Duffy et al., 2008). This combination of viral replication, recombination and subsequent evolution results in an extraordinary genetic diversity characterized by the co-existence of viral variants called “quasispecies”. The presence of these viral quasispecies imply that circulating viruses are not represented by a unique virus genotype, but rather by a “swarm” of different yet genetically related viral variants (Domingo et al., 2012). Thus, within any chronically infected un-treated individual there is continual evolution and co-existence of every possible mutation (Coffin, 1995, Ribeiro et al., 1998). Viral quasispecies are subject to varying selection pressures, enabling them to either dominate or decay within the population (Clavel, 2004). The wild-type variant has a better replicative ability in the absence of therapy, while drug resistant variants have a better replicative ability in the presence of therapy (Paredes and Clotet, 2010).

Drug resistance can either be acquired (primary drug resistance) or transmitted (secondary drug resistance). Acquired resistance occurs when drug resistant variants gain a selective advantage over the wild-type, and thus increases in frequency. Acquired resistance may also lead to the further accumulation of mutations and can either increase drug resistance or improve viral fitness (Devereux et al., 1999, Izopet et al., 2000, Miller et al., 2000, Birk et al., 2001). If treatment is interrupted these mutations decay due to a fitness cost, and are replaced by a fitter wild-type virus. These viruses remain incorporated within the viral quasispecies and are referred to as “minority drug resistant variants”. These low frequency variants can re-emerge if ART selective pressure is introduced again (Metzner et al., 2005).

Standard population based sequencing cannot detect variants that occur at <20% and therefore minority variants are not detected. This phenomenon is a fundamental concept and may account for the common trend of treatment failure accompanied by the absence of drug resistance mutations (DRMs). Nevertheless these variants are present and more sensitive assays are required for their detection. Some minority drug resistant variants have been shown to persist after treatment with certain ARVs (Tang and Shafer, 2012), especially the NNRTI drug

class. In particular, mutations that are associated with Efavirenz (EFV) and Nevirapine (NVP) have been shown to persist at low levels of <20% for as long as 12-24 months after treatment has stopped (Flys et al., 2005, Palmer et al., 2006, Loubser et al., 2006). These minority variants are important to study as they may have implications for subsequent therapy with second-generation NNRTIs. A pooled analysis from Nicot et al (2012) explained that there is a significant association between the presence of NNRTI minor variants and the risk of treatment failure (Nicot et al., 2012).

Transmitted resistance refers to resistant viruses that are transmitted from person to person through contact with blood, sexual intercourse or mother to child transmission (Wensing et al., 2005). It is well known that in the absence of therapy, the stability of these transmitted variants decreases, and they can easily revert back to wild-type or exist as minor variants (Brenner et al., 2004, Bezemer et al., 2006). However, if the transmitted variant has a high replication capacity, the transmitted variant is likely to persist for extended periods in blood and semen (Little et al., 2008). Patients harbouring these variants may have sub-optimal treatment responses in comparison to those patients without these variants (Little et al., 2008). Given that most ARVs are structurally similar and interact with similar target sites, resistance to one drug may inherently result in resistance to other drugs within the same drug class. This phenomenon limits the spectrum of ARVs that can be used in subsequent salvage regimens, and poses a major threat to the clinical efficacy of ART.

1.9 HIV-1 Reverse Transcriptase structure

HIV-1 RT is an asymmetric heterodimer comprising two subunits, a 66kDa (p66) and a 51kDa (p51) subunit. The p51 subunit is composed of the first 440 amino acids of the RT gene while the p66 subunit is made up of all 560 amino acids of the RT gene. Both the p66 and the p51 have an identical primary structure, however the p51 subunit lacks the 120 amino acid, Ribonucleic acid H (RNase H) domain found in the C-terminal region of p66 (Menendez-Arias, 2010). Both of these

subunits are derived by cleavage of the *gag-pol* poly-proteins by viral PR (Menendez-Arias, 2010). The polymerase domain contains four sub-domains, which are regarded as the “fingers” residues (1-85), (118-155), the “palm” (86-117), (156-236), the “thumb “(237-318) as well as connection sub-domain residues (319-426) (Kohlstaedt et al., 1992, Menendez-Arias, 2013). The p51 sub-domain also folds into the same four domains but in different positions relative to each sub-domain (Sarafianos et al., 1999). This sub-domain is non-enzymatic and has a structural role for the stabilization and folding of the p66 subunit. HIV-1 RT plays a significant role in the viral life cycle as a DNA polymerase to copy either a DNA or RNA template or as an RNaseH, to cleave the RNA portion of the RNA-DNA duplex. Both functions are performed together for the conversion of RNA into linear dsDNA (Menendez-Arias, 2010).

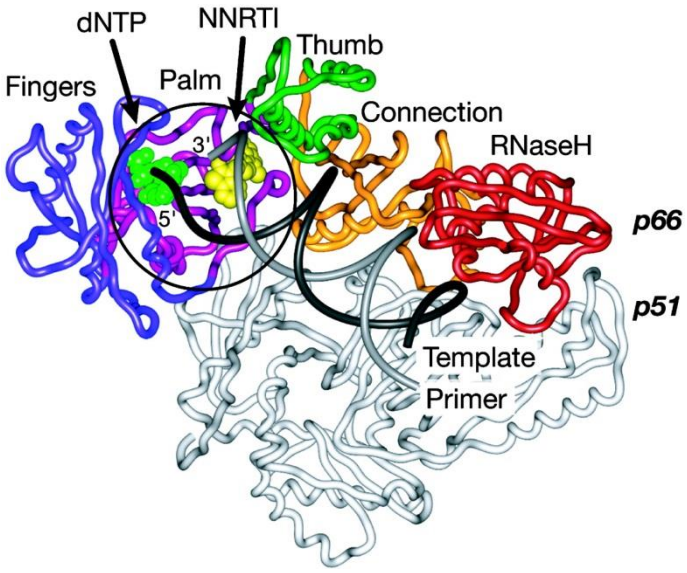


Figure 1.5 Structural components of HIV-1 Reverse Transcriptase, complexed with a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI). Taken from Pata et al (2004).

1.9.1 HIV-1 Reverse Transcriptase Inhibitors

Due to the integral role HIV-1 RT plays in the viral lifecycle it has become an attractive target in developing ARVs, making RT-inhibitors a major component of ARV regimens (Alcaro et al., 2011). Reverse Transcriptase Inhibitors that are utilized in clinical settings can either be classified as NRTIs or NNRTIs. NRTIs are essentially chain terminators and following phosphorylation by cellular kinases they are incorporated by RT into the nascent chain of viral DNA. Deoxynucleotides (dNTPs) that contain a terminal hydroxyl group are essential to ensure that the DNA helix structure continues to grow, however NRTIs lack this component and once incorporated the DNA chain is terminated, inhibiting viral DNA synthesis (Menendez-Arias et al., 2011, Tang and Shafer, 2012, Menendez-Arias, 2013). NNRTIs, prevent HIV-1 replication by binding to the hydrophobic pocket within the RT enzyme near the active site. As a result the flexibility of the enzyme is compromised preventing DNA synthesis (Tang and Shafer et al., 2012).

1.9.2 Resistance to Nucleoside Reverse Transcriptase Inhibitors

Once the drug enters the cell, it is subject to intracellular phosphorylation and conversion into triphosphate derivatives, which compete with the natural cellular dNTPs for incorporation into the growing DNA strand. There are two mechanisms of resistance that has been described (Menendez-Arias, 2013). The first involves a discriminatory exclusion pathway where RT retains the ability to recognize the natural dNTPs but discriminates and subsequently excludes the NRTI, preventing the drug from binding. Mutations that are involved in this pathway are referred to as “discriminatory mutations” and include K65R, K70EG, L74V, Y115F, Q151M and M184V (Tang and Shafer, 2012). Owing to the low genetic barrier of NRTIs, resistance emerges quite quickly; a common example includes M184V, which selectively impairs the ability of RT to incorporate the NRTIs: Lamivudine (3TC) and Emtricitabine (FTC), by steric hindrance (Sarafianos et al., 2009). M184V also hyper-sensitizes HIV-1 to Azidothymine (AZT) and Tenofovir (TDF) (Tang and Shafer, 2012) but more importantly this mutation reduces viral fitness and is

associated with lowering the plasma HIV-1 RNA 0.5 logs lower than that of the wild-type virus (Tang and Shafer, 2012). The second mechanism is one involving an excision pathway, that selectively removes the NRTI triphosphate that has been incorporated into the growing DNA chain (Sarafianos et al., 2009, Tang and Shafer, 2012). Chain termination is then bypassed allowing DNA replication to continue (Hughes et al., 2008). A common example of the excision mechanism is the Adenosine triphosphate (ATP) mediated excision of AZT by pyro-phosphorylation (Figure 1.6) (Sarafianos et al., 2009). Most of the amino acids that are involved in resistance via the excision mechanism are present within the fingers or the palm of RT and are in positions that could affect the binding of an incoming dNTP (Sarafianos et al., 2009).

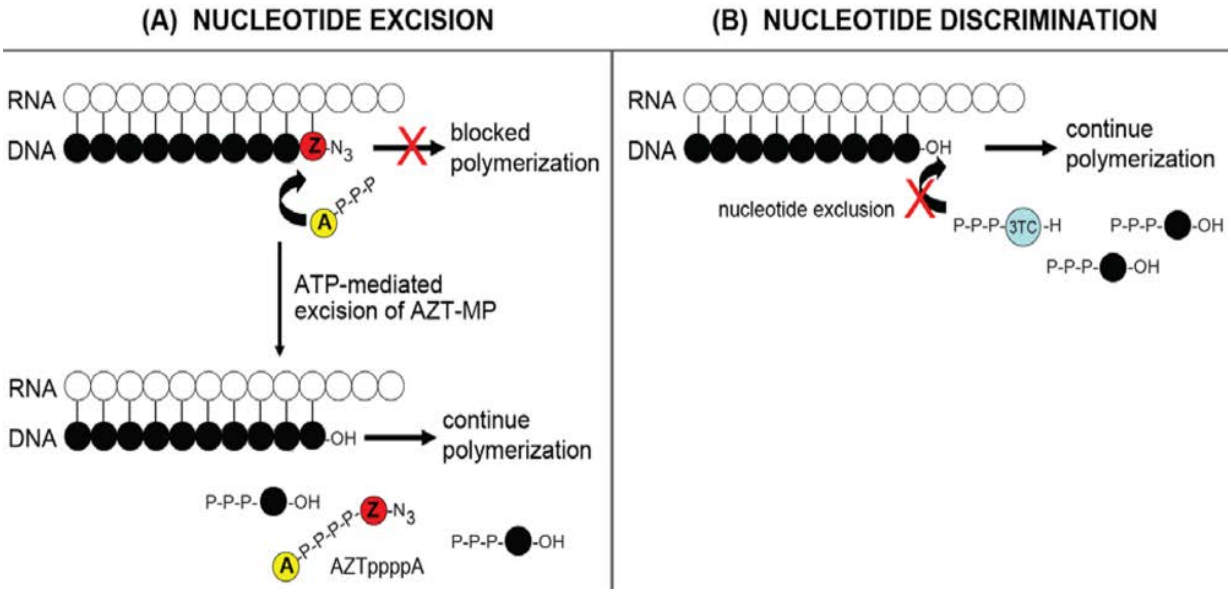


Figure 1.6 Drug resistance to Nucleoside Reverse Transcriptase Inhibitors via the excision and exclusion "discriminatory" pathways. Taken from Delviks-Frankenberry et al (2010).

Other mutations involved in this pathway include the Thymidine Analogue Mutations (TAMs) as illustrated in Figure 1.7. These mutations confer resistance to AZT, Stavudine (d4T) and partially contribute to Abacavir (ABC) and Didanosine

(ddi) resistance (Vandamme et al., 2004). Evolution within these TAM pathways results in increasing levels of resistance and cross-resistance to other NRTIs.

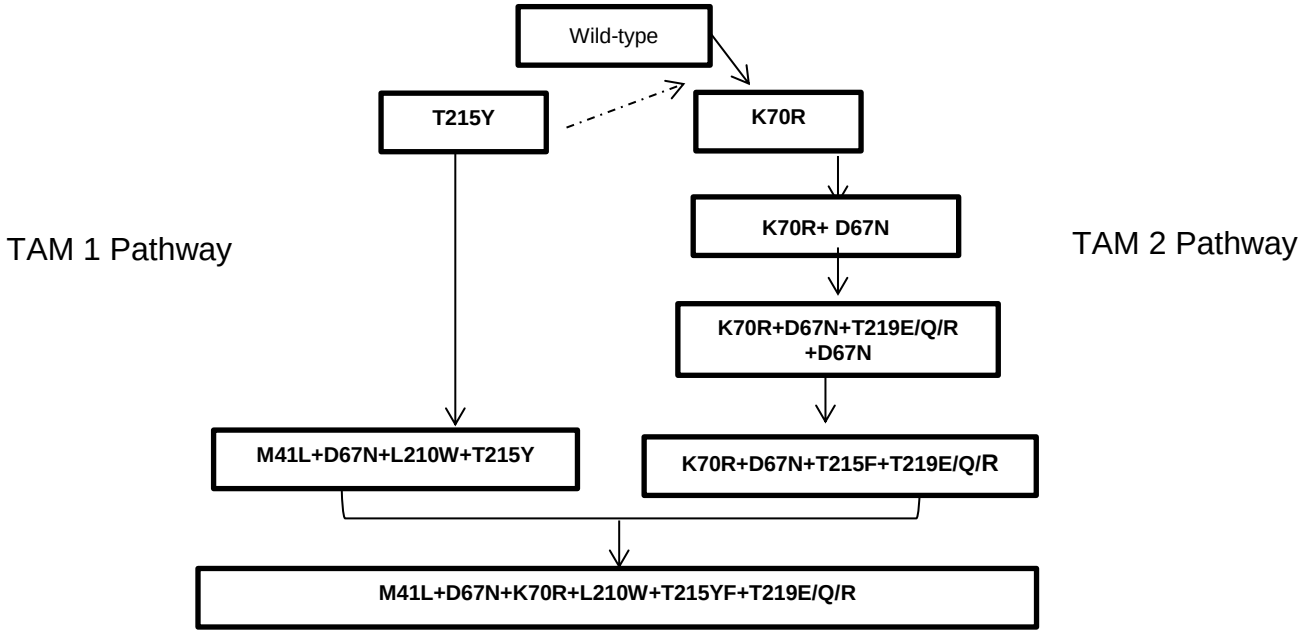


Figure 1.7 Schematic of two distinct pathways of Thymidine Analogue Mutations. Mutation K70R confers low-level resistance to AZT. Subsequently T215Y emerges later in different viral genomes and outgrows the K70R mutant. T215Y requires a double nucleotide mutation (ACC –TAC). Persistent replication of T215Y in the presence of non-suppressive therapy results in the appearance of the TAM 1 pathway. D67N mutation appears in viruses that already harbor K70R, with the eventual accumulation of K219E/Q/R and T215F (TAM 2 pathway). Persistent replication with TAMs can result in mixed patterns of TAM 1 and 2. Adapted from Hu et al (2006).

1.9.3 Resistance to Non-Nucleoside Reverse Transcriptase Inhibitors

A major limitation of NNRTIs is their relatively low genetic barrier, where only a single point mutation is required to cause resistance (Tang and Shafer, 2012). High level resistance to NVP and EFVs requires between one to two mutations while the second-generation NNRTI, Etravirine (ETR) requires two mutations for high-level resistance (Tang and Shafer, 2012). Nearly all of the NNRTI resistance mutations are present within or adjacent to the NNRTI binding pocket (Ren and Stammers, 2008). NNRTI mutations can be divided into three groups, Cluster 1: L100I,

K103N, V106A and V108I; Cluster 2: Y181C, Y188L/C/H, and G190S/A and Cluster 3: P225H, M230L, and P236L. The first two groups are mutations present on the opposite side of the NNRTI binding pocket of RT in the vicinity of p66, while the third cluster is present in the p51 subunit (Sarafianos et al., 2009). NNRTI resistance mutations have minimal effects on replication capacity due to the low fitness costs they incur (Wirden et al., 2003, Alcaro et al., 2011). However the long plasma half-life of these inhibitors may result in periods of monotherapy allowing the selection of drug resistant virus. This phenomenon can become problematic during periods of non-adherence to multi-drug regimens, where there may be intervals of NNRTI monotherapy, as other drugs have a shorter half-life. This, in addition to active replication results in resistance evolution (Tang and Shafer, 2012).

1.10 HIV-1 Protease

HIV-1 PR belongs to a family of aspartic proteases and is a symmetrically assembled homo-dimer containing two identical subunits of 99 amino acids. The dimers are held together by interdigitated β sheets formed at the base of the enzyme by the N and C termini of each dimer (Hughes et al., 2008). The substrate binding cleft or active site is located in between the two subunits and has the signature Aspartate-Threonine-Glycine sequence common to aspartic proteases (Brik and Wong, 2003). The active site is covered by two extended β sheet glycine-rich loops known as “molecular flaps” which are highly flexible and are part of the substrate-binding cavity. The flaps open slightly to enable substrates into the active site and subsequently close keeping the substrate in place by hydrogen bonds and van de Waals interactions (Pietrucci et al., 2009, Perez et al., 2010).

1.10.1 HIV-1 Protease Inhibitors

Extensive characterization of the structural components of HIV-1 PR and its substrates have led to the advent of PIs (Wensing et al., 2010). PIs bind to the active site of viral PR and have an integral function in the inhibition of cleavage of post-translational proteins thereby preventing the production of mature infectious virus (Wensing et al., 2010, Fun et al., 2012). Thus far, nine PIs have been clinically approved for use by the Food and Drug Association (FDA) and include Saquinavir (SQV), Indinavir (IDV), Ritonavir, Nelfinavir (NFV), Amprenavir (APV), Lopinavir (LPV), Atazanavir (ATV), Tiprinavir (TPV) and Darunavir (DRV). All PIs with the exception of TPV are competitive peptidometric inhibitors containing a hydroxyl-ethylene core that prohibits cleavage by PR (Turner et al., 1998, Wensing et al., 2010). Since the advent of PIs several strategies have been implemented to improve the clinical outcome of these drugs, such as the co-administration of PIs with a sub-optimal dose of Ritonavir. Ritonavir is an inhibitor of the cytochrome P450 3A4 enzyme that considerably improves the bioavailability and half-life of PIs, resulting in a higher PI plasma concentrations (Wensing et al., 2010, Fun et al., 2012).

1.10.2 Resistance to Protease Inhibitors

Resistance to all PIs has been noted and the genetic basis of resistance well documented (Wensing et al., 2010). Resistance to PIs occurs in a sequential process resulting in the accumulation of primary and secondary mutations. Primary or “major mutations” arise first due to an amino acid substitution within the substrate-binding cleft of viral PR resulting in an overall enlargement of the catalytic site. As a result the binding efficiency of the PI is diminished leading to a reduction in drug susceptibility and thus drug resistance. In addition binding of the natural substrate to PR is also compromised resulting in decreased viral replication capacity (Nijhuis et al., 1999). Examples of primary mutations include D30N, V32I, M46IL, G48VM, I50VL, I54VTAML, L76V, V82ATFS, I84V, N88S and L90M. Secondary “minor mutations” emerge later and can only cause resistance in the

presence of major mutations; however they play a critical role in improving viral replication and are also referred to as “compensatory” mutations (Wensing et al., 2010). PI mutations have also been observed in *gag* where they are commonly found in or close to the cleavage sites. These mutations are thought to adapt the virus to the altered substrate binding cleft of the mutant drug resistant viral PR (Fun et al., 2012).

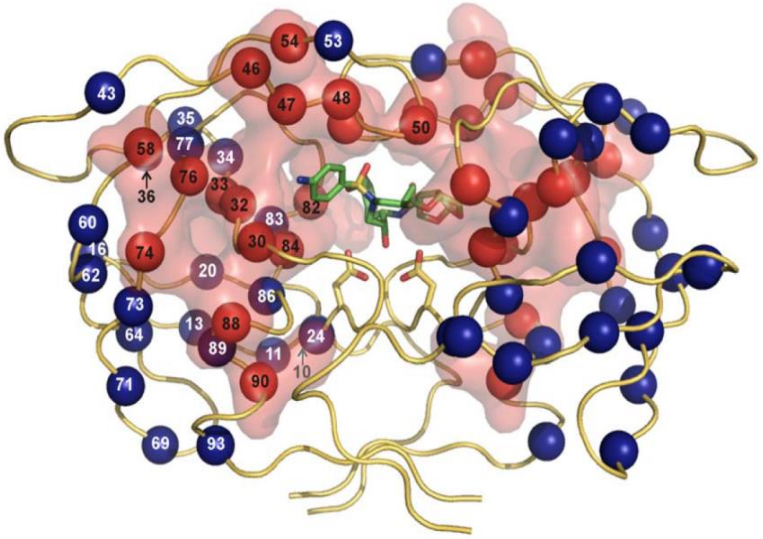


Figure 1. 8. Three-dimensional structure of HIV-1 viral Protease. Mutated residues are shown with their C-α atoms with spheres. Major mutations (red) and minor mutations (blue), semi- transperant solvent accessible surfaces are shown in red. Within the active site, a stick model of DRV is bound. Taken from Pokorna et al (2009).

1.11 HIV-1 Integrase structure

HIV-1 IN is a 288 amino acid protein of 32kDa encoded by the end of the *pol* gene and forms part of the *gag-pol* polypeptide precursor, from which it is released by viral PR during cleavage (Suzuki et al, 2012). HIV-1 IN consists of three independent structural and functional domains that are connected by flexible linkers as indicated in Figure 1.9: the N terminal Domain (NTD), the catalytic core domain (CCD), and the C terminal domain (CTD). Each of these domains either directly or indirectly contribute to the IN-DNA interaction. The NTD is well conserved and encompasses residues (1-49) and is linked to the CCD (50-212)

through a linker segment of amino acids (47-55), that has the HH-CC (2 Histidine's and 2 Cysteine's) motif. This motif makes up the Zinc binding site and possibly favors protein multimerization, a fundamental process in integration (Delelis et al., 2008, Suzuki et al., 2012). The CCD is composed of a mixture of α helices and β sheets and comprises a triad of aspartic (D) and glutamic acid (E) residues termed the DDE motif, which is important for the catalytic activity (Suzuki et al., 2012). This domain is well conserved and is important for binding of the viral DNA extremities via residues Q148, K156 and K159 (Delelis et al., 2008, Suzuki et al., 2012). The CTD amino acids (212-288) bind non-specifically to DNA and are involved in the stability of the complex with DNA. The CTD displays the greatest degree of genetic variation across the retrovirus family (Delelis et al., 2008, Suzuki et al., 2012).

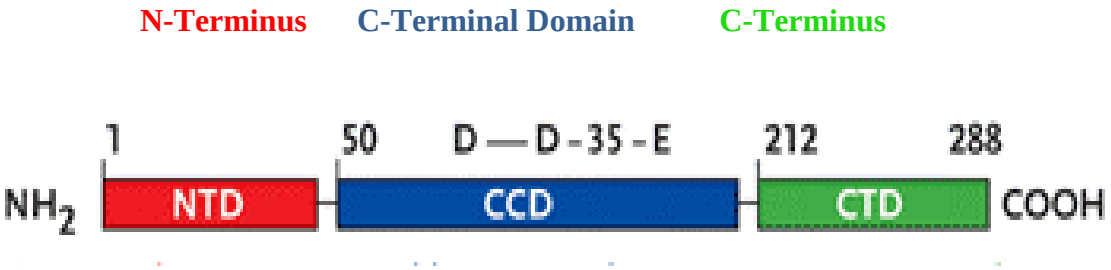
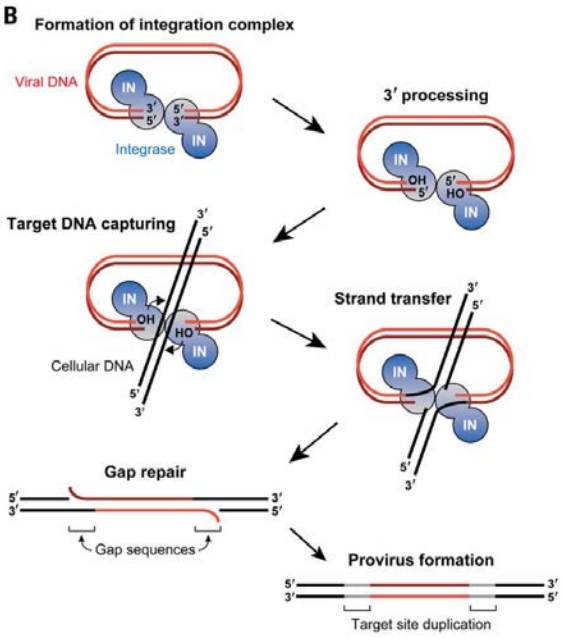


Figure 1.9 Three independent domains of HIV-1 Integrase, the N-Terminal Domain (NTD), the Catalytic Core Domain (CCD) and the C-Terminal Domain (CTD) (Taken from Low & Muesing et al (2006).

1.11.1 HIV-1 Integrase function

Retroviral mediated integration occurs via three biochemically distinct steps (Craige et al., 2001). Firstly, IN specifically recognizes the viral attachment sites in the 5' and the 3' LTRs of newly synthesized viral DNA ends. This allows for the 3' processing of viral DNA, HIV-1 IN catalyzes the removal of two nucleotides adjacent to the highly conserved CA dinucleotide from the 3' end of the LTR region. As a result the DNA is chemically activated through the formation of 3' hydroxyl radicals (3 'OH) at the terminal ends of viral DNA, allowing its participation in the subsequent reaction. The second step involves the insertion of the 3' processed, activated viral DNA strand into the target DNA via a single trans-

esterification reaction referred to as the “strand transfer step”. The viral DNA ends are brought into close proximity with the target DNA by IN, allowing a nucleophilic attack by the 3’OH radical on the target DNA (Suzuki et al., 2012). Following which, IN ligates both the 3’ OH radical terminal of the viral DNA to the 5‘ phosphoryl ends of the target DNA and a new phosphor-diester bond is established. Intermediate DNA products with un-repaired gaps between the non-ligated 5’ends of viral DNA as well as the 3’ ends of target DNA are formed. These un-repaired gaps result in the short duplication of target DNA sequences flanking both ends; this is followed by the formation of imperfect inverted repeats upon sealing of the nick. Following the ligation reaction the gaps in the intermediate DNA products are repaired to yield fully functional integrated provirus. This step creates an intermediate product where the DNA is joined to the target DNA by the 3’ ends of both strands. DNA repair is therefore required to seal the nick between the 5’ end of the viral DNA and the 3’ end of the target DNA, in order to complete the provirus formation (Suzuki et al., 2012). This reaction is shown in Figure 1. 10.



Figures 1.10 The role of HIV-1 integrase (IN) during retroviral integration. The process is initiated by IN recognition of both ends of viral DNA and the subsequent

removal of 2/3 nucleotides from each of the 3' ends, a process referred to as "3' processing". Integrase captures the target DNA (chromosomal DNA) which is then cleaved in a "staggered manner" via the exposed hydroxyl group on the viral DNA ends. The 3' end of the viral DNA and the 5' end of the target DNA is simultaneously linked during the "strand transfer" step. In infected cells, the excision of the mispaired 5' viral DNA ends and the incorporation of the single strand gaps are carried out by unknown cellular enzymes. Taken from Suzuki et al (2012).

1.11.2 HIV-1 Integrase Inhibitors

Viral integration is a key process for retroviral replication hence the use of HIV-1 INSTIs has become popular for antiviral activity. Raltegravir (RAL) was the first INSTI approved for use by the FDA in 2007 for treatment naïve and experienced patients. However it was only made available

in SA in 2011 and is recommended as a component of a patient specific third line treatment (Gary Maartens, 2011). Dolutegravir (DTG) and Elvitegravir (EVG) are new investigational INSTIs still in clinical trials (Abram et al., 2013). Both RAL and EVG have shown good efficacy *in vitro* and in clinical studies (Ceccherini-Silberstein et al., 2010). (Gary Maartens, 2011). Raltegravir has a low genetic barrier where only a single point mutation is required to cause resistance. Therefore it is not surprising that resistance to this drug has also emerged. Resistance associated with RAL is characterized by the presence of three major signature IN mutations Y143RC, Q148HRK and N155H (Malet et al., 2008, Abram et al., 2013). Secondary accessory mutations further increase the level of resistance and often restores replication capacity. These mutations occur at the CTD residue S230, suggesting a direct role for the other domains in IN enzymatic function (Mbisa et al., 2011, Parczewski et al., 2012). Natural polymorphisms within IN; T97A, E138K, V151I, G163R, V165I, V201I, I203M and T206S, can influence the rate at which resistance develops and can either increase resistance, restore viral fitness or contribute to both (Rhee et al., 2008, Mbisa et al., 2011, Parczewski et al., 2012). However more work is needed to confirm the roles of these polymorphisms.

Primary resistance to EVG is characterized by mutations T66I, E92Q, T97A, S147G, Q148R, N155H, and less commonly T66AK, E92G, and Q148HK (Quashie et al., 2012). Dolutegravir is a relatively novel INSTI and no major DTG associated drug resistance mutations have been identified as yet. Although it is known that the accumulation of multiple mutations is required for resistance due to the drugs higher genetic barrier. Nevertheless substitutions associated with this drug include E92, L101, T124, S153, and G193 (Quashie et al., 2012).

1.12 Highly Active Antiretroviral Therapy in South Africa

The National Treatment Program was implemented in SA in 2004 (Kiepiela P, 2014). South Africa currently has the largest ART program in the world with an estimated 1.79 million people on ART; approximately 85% have access through the public sector, 4% through non-governmental community treatment programs and 11% from the private sector (Johnson, 2012). Kwa-Zulu Natal (KZN) has the highest prevalence of HIV-1 infection globally, but fortunately has been able to provide the highest number of HIV-1 testing per province as of 2014, as well as the highest number of sites that are accredited to provide ART (Massyn et al, 2014). South Africa utilizes standardized first and second-line regimens based on drug tolerability and safety efficacy for each patient. Current guidelines in SA are illustrated in Figure 1.11 (Department of Health, 2013). Despite the intervention of ART programs and their role in reducing HIV-1 burden, various obstacles limit its use including, drug toxicity, adverse effects, treatment adherence issues, co-infection with other pathogens, individual pharmacokinetics and drug resistance

Eligibility to begin lifelong Antiretroviral Therapy

Patients presenting with	Regardless of CD4 count, ART is given to	Regardless of CD4 count, ART is given to patients
• CD 4 < 500µl/ml irrespective of clinical stage of disease • Priority given to patients with CD4 < 350cells/µl	• Patients presenting with all types of Tuberculosis (TB) • Pregnant or breastfeeding women • Known co-infection with Hepatitis B Virus (HBV)	• With advanced HIV-1 disease (WHO Stage 3 & 4) • Patients with CD 4 <200 cells/µl are fast tracked

First Line Therapy- 2NRTIs +1 NNRTI	Regimens
New Adult Patients including pregnant women	TDF+3TC/FTC + EFV/FDC FDC Preferred
Adolescents	ABC+ 3TC+ EFV
Contraindication to EFV – psychiatric co-morbidity	TDF + FTC/3TC+ NVP
Contraindication to TDF- Renal disease & Anemia or use of Nephrotoxic drugs	AZT+3TC+EFV or NVP
Contraindication to TDF & AZT Renal disease & Anemia/ use of nephrotoxic drugs/aminoglycosides	d4T + 3TC+EFV or NVP
Contraindication to TDF, AZT, d4T Renal disease/Anemia/ peripheral neuropathy use of nephrotoxic drugs	ABC+3TC+EFV or NVP
Currently on d4T regimen	TDF + 3TC/FTC+ EFV FDC preferred
Infants and children <3 years < 3kg	ABC+3TC+LPV/r
Infants and children 3 years and older or above 10kg	ABC+3TC +EFV
If patients present with a viral load >1000 copies per ml then switch to second line LPV treatment	

Second Line Therapy 2 NRTIs + 1 PI	
Adults and Adolescents	
Failing on a TDF based 1st line regimen	AZT+3TC+ LPV/r
Failing on a d4T based 1st line regimen	TDF+3TC/FTC and LPV/r
Dyslipedia or diarrhea associated with LPV	Switch LPV to ATV/r
Infants and Children	
Failing first line ABC+3TC+LPV/r or d4T+3TC+LPV/r	Consult with expert for advice
Failed 1ST line NNRTI regimen	
ABC+3TC +EFV/NVP	AZT+ 3TC+LPV/r
d4T+3TC+EFV for NVP	AZT+ABC+LPV/r
If HIV RNA >1000 copies/ml on 2nd line for >1 year, repeat viral load after 6 months and monitor adherence, drug interaction, tolerability and psychological problems If patients present with VL <1000cpm - Continue with second-line LPV/r treatment, or if VL >1000cpm - Specialist referral and genotypic testing requested. Patient placed on salvage regimens with DRV/RAL/ETR.	

Figure I.11 Schematic of the current South African Antiretroviral guidelines for HIV-1 infection (Adapted from the Department of Health (2013).

1.13 Second-line Antiretroviral Therapy

The PI Lopinavir is a first-generation PI that was approved in 2000, and was one of the first PIs to be co-formulated with Ritonavir as Kaletra™ (Chandwani and Shuter, 2008). Lopinavir is currently marketed as Aluvia™ and is administered in combination with two NRTIs, constituting the second-line regimen in SA, as previously mentioned (Department of Health, 2013). This drug has demonstrated high efficacy in both treated and naïve patients (Chandwani and Shuter, 2008). Despite the relatively high genetic barrier, resistance to LPV/r is characterized by mutations L10IRV, K20M, L24I, V32I, L33F, M46IL, I47AV, I50V, F53L, I54VLAMTS, L63P, A71VY, G73S, L76V, V82AFTS, I84V, and L90M (Kempf et al., 2002, Tang and Shafer et al., 2012). Nonetheless, LPV/r has still shown good efficacy in the presence of five or less primary resistance mutations; however the presence of additional mutations compromises its efficacy (Kempf et al., 2002).

Genotypic analysis of first-line failures in SA suggests that the majority of patients remained susceptible to a second-line treatment of AZT, ddi and LPV/r (Marconi et al., 2008, Orrell et al., 2009, Wallis et al., 2010). Some studies have shown good virological outcomes with a LPV/r inclusive second-line therapy, when used in conjunction with adherence counseling

(Hosseinipour et al., 2010, Johnston et al., 2012, Garone et al., 2013, Schoffelen et al., 2013). However, some patients experience mortality due to the high toxicity of LPV/r, threatening the drugs long-term use (Osinusi-Adekanmbi et al., 2014). A few studies have reported on second-line failure in Africa (Pujades-Rodriguez et al., 2011, Levison et al., 2011, Wallis et al., 2011) . Amongst these studies, the first major PI mutations were seen at least six months after treatment (Osinusi-Adekanmbi et al., 2014). Treatment failure in most studies were attributed to adherence issues, delayed start in second-line treatment and mortality associated with LPV/r (Wallis et al., 2011, Ajose et al., 2012, Garone et al., 2013, Osinusi-Adekanmbi et al., 2014, Van Zyl et al, 2013).

With the numerous challenges that resource limited settings experience, it is imperative to preserve and maximize second-line treatment options (Garone et al.,

2013). Despite patients failing second-line treatment, little has been reported on the PI resistance mutations seen in HIV-1 subtype C (Wallis et al., 2011). Thus far some studies have shown infrequent or a low prevalence of major PI drug resistance mutations (Riddler et al., 2008, El-Khatib et al., 2010, Wallis et al., 2011, Ajose et al., 2012, Levison et al., 2012, Osinusi-Adekanmbi et al., 2014,). A similar trend has been reported for HIV-1 infection with B viruses (Riddler et al., 2008).

1.14 Third-line Antiretroviral Therapy

South Africa is one of the only countries in Sub-Saharan Africa that has access to third-line ART for patients that have failed both first and second-line treatment (Department of Health, 2013). Antiretroviral drugs, DRV/r, ETR, RAL and Maraviroc (MVC) are registered in SA for use in third-line treatment. There are no stipulations regarding the choice of third-line drugs however, decisions are made by an expert panel on the basis of a genotypic resistance test (Meintjes et al., 2014). Nonetheless there are some guidelines to assist in the design of these regimens such as: adherence counselling prior to initiating third-line treatment; first generation NNRTIs should not be included as they do not impair viral fitness; 3TC is recommended as this mutation selects for M184V which impairs viral replication; other drugs that can be considered include RAL, ETR, and or Rilpivirine (RPV). However, RAL is favoured as it is an entirely new drug class with no risk of cross-resistance; MVC is extremely expensive and can only be used following a tropism test which shows that the patient has tropism for the CCR5 co-receptor; the use of double boosted PIs and the use of a PI with the broadest resistance profile (currently DRV) is preferred (Meintjes et al., 2014).

Darunavir, previously known as TMC114, is commercially known as PREZISTA™ and gained FDA “accelerated approval” in 2006, following results from various clinical trials (Wolfe and Hicks, 2009). Darunavir is a specifically engineered HIV-1 PI that has a greater binding affinity within the active site of viral PR than other known PIs (Wolfe and Hicks, 2009, Tang and Shafer, 2012). Like other PIs, DRV is also co-administered with a low dosage of Ritonavir. Darunavir is structurally

similar to APV but shows a greater binding avidity and binds almost 1000 times tighter than Ritonavir, SQV or NFV (King et al., 2004). This drug has become popular for use, owing to a number of favourable properties such as its high tolerability, dosing flexibility, high potency and high genetic barriers for resistance. Three major structural attributes are responsible for the high genetic barrier i) The Pico molar binding affinity to wild-type PR binding site ii) the ability to form numerous backbone to backbone hydrogen bonds with the PR substrate binding cleft and iii) the ability to adopt a conformation that fits within the substrate envelope of viral PR (Saskova et al., 2009).

POWER 1 and 2 studies (Performance of TMC114 when evaluated in Highly Treatment Experienced patients with PI resistance) evaluated the use of DRV/r in triple class (NRTI/NNRTI/PI) experienced adults with viral loads >1000cpm harbouring at least one primary PI DRM. Promising results with significant reductions in viral load and improvements in CD4 were shown (Katlama et al., 2007). Another trial, “The Antiretroviral Therapy with TMC114 Examined In naïve Subjects (ARTEMIS), evaluated DRV/r versus LPV/r efficacy in treatment naïve patients and confirmed a virological failure rate lower than 12% in the DRV/r patients compared to 17.1% for LPV/r patients during the 96 week analysis (Aratesh K, 2005).

1.14.1 Darunavir resistance profile

Data from a pooled 24 week POWER 1 and 2 trials identified 11 DRV/r resistance associated mutations as currently listed by the International Aids Society (IAS) this is illustrated in Table 1.1. (de Meyer et al., 2008).

Table 1.1 Darunavir resistance associated mutations. Those highlighted in blue indicate DRV/r mutations associated with virological failure. Adapted from de Meyer et al (2008).

Protease Codons	**11**	**32**	**33**	**47**	**50**	**54**	**73**	**76**	**84**	**89**
Consensus	V	V	L	I	I	I	G	L	I	I
DRV/r	I	I	F	V	V	L/M	S	V	V	M

Owing to the high genetic barrier of DRV/r, more than three DRMs in addition to a number of background resistance associated mutations are required for DRV/r resistance (de Meyer et al., 2008). Studies on subtype B have shown that the prevalence of DRV/r mutations within routine clinical settings is low (Mitsuya et al., 2007, Wolfe and Hicks, 2009).

1. 15 Genetic variability in HIV-1

HIV-1 naturally varies in genetic content by 35% between subtypes (Wainberg and Brenner, 2012). This variability is strongly associated with differences in genes coding for viral enzymes. Inter-subtype differences in PR and RT are between 10-12% at the nucleotide level and 5-6% at the amino acid level (Gonzalez et al., 2003). This variability strongly drives the spectrum of mutations that emerge under drug selection pressure (Gonzalez et al., 2003). Three factors that influence which drug resistance mutations develop in different subtypes are: (i) Inter-subtype differences in codon usage - associated with differences in nucleotide and mutational motifs and refers to the number of transitions and trans-versions required for ARV resistance. Viral isolates may encode different amino acid substitutions, thus influencing the rate and type of resistance mutations that may emerge. ii) Inter-subtype amino acid differences - associated with minor structural changes in drug targets; as a result different mutations may emerge under the same drug pressure, (iii) Inter-subtype differences at the nucleotide level at regions surrounding nucleotides involved in drug resistance (Tang and Shafer, 2012).

1.15.1 Variability in subtype C

Subtype C has several distinct genomic features that contribute to its high genetic variability, and include a prematurely truncated *rev* ORF, an extra NF Kappa β binding site in the LTR and an enlarged *Vpu* protein (Ndung'u et al., 2001, Gordon et al., 2003). It has also been shown that subtype C PR has a higher enzyme activity than PR from other subtypes (Velazquez-Campoy et al., 2001). Despite this level of genetic diversity in subtype C, these viruses are conserved at the amino acid level as a large proportion of amino acid substitutions are silent and result in no overall change in the amino acid sequence. However, the presence of these naturally occurring polymorphisms amongst the different HIV-1 subtypes is still debatable. Various studies have reported the possible contribution of these polymorphisms to drug resistance as indicated in Table 1.2 (Bessong et al., 2008, Bandaranayake et al., 2010, Wainberg and Brenner, 2012).

Table 1.2 Some genetic differences that predispose across the different subtypes of HIV-1 Protease (Adapted from Santos and Soares et al (2010).

Substitutions	Drug	% In Subtype B	Signatures	Polymorphisms
I13V	TPV	13%	90-98% Subtypes A, G and CRF02	4-78% in other Non B
K20I	ATV	2%	93-98% Subtype G, CRF02	1-3.5% in Subtype A, F, CRF01
M36I	ATV, IDV, NFV, TPV	13%	81-99% Non B Subtypes	-
H69K	TPV	2%	96-97% Subtype A, C, G, CRF01/02	2% in Subtype F
V82I	ATV	2%	87% in Subtype G	1-6% in Non B
I93L	ATV	33%	94% in Subtype C	5-40% in Non B

The presence of these genetic signatures and polymorphisms in PR are regarded as compensatory mutations in subtype B, and could influence the susceptibility to PIs. As a result this could create a major barrier for the clinical success of cART,

as patients with non-B subtypes may fail treatment quicker than those with B viruses (Santos and Soares, 2010).

The preferential selection of K65R by subtype C viruses is a common example of this variability (Brenner et al., 2006, Doualla-Bell et al., 2006, Invernizzi et al., 2013). This selection is based on a template-dependent mechanism as illustrated in Figure 1.12. The nucleotide sequence at codons 64-65-66 differs between subtype B and C. Viral isolates of subtype C contain a homo-polymeric stretch of adenine bases that cause RT to pause during the synthesis of dsDNA from ssDNA templates. This process is template specific but independent of the RT enzyme (Lessells et al., 2012). Subsequent mis-alignment of the template and the primer leads to the AAG to AGG change resulting in the K65R mutation (Coutsinos et al., 2011). As a result K65R is predominately found in subtype C viruses (Brenner et al., 2006, Doualla-Bell et al., 2006, Invernizzi et al., 2013). For subtype B, there is a distinct pausing at codon 67 facilitating the generation of D67N and TAMs as opposed to K65R (Coutsinos et al., 2011).

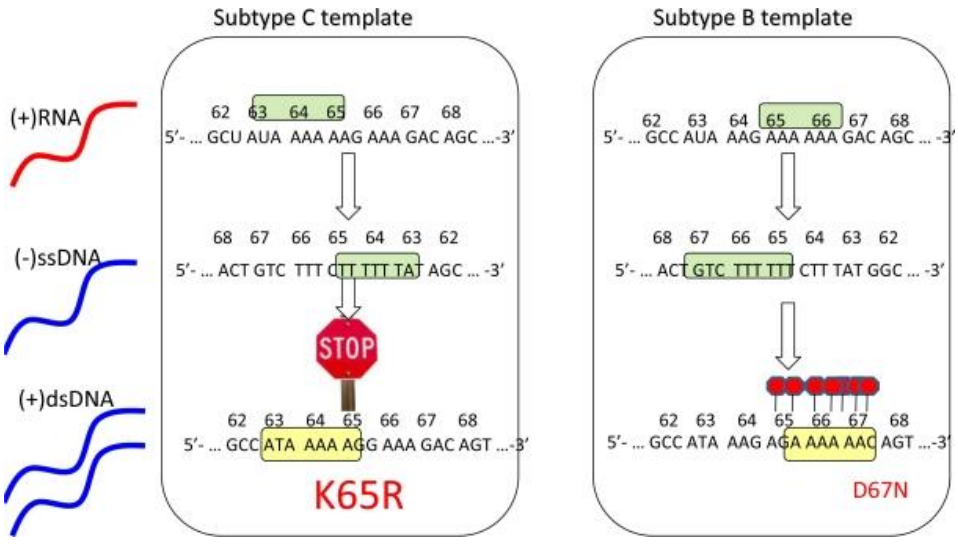


Figure 1.12 Schematic of the template dependent mechanism of subtype C versus subtype B viruses to select for the K65R mutation and the D67N Thymidine Analogue Mutation respectively Taken from (Wainberg and Brenner, 2010).

It is therefore imperative that patients are carefully monitored upon the initiation of HAART, as it is important to bear in mind that ARVs are designed to particularly target subtype B viruses, and differences in treatment responses should be expected (Doualla-Bell et al., 2006, Toor et al., 2011, Wainberg and Brenner, 2012, Sui et al., 2014).

1.16. Drug resistance testing

For clinicians to design efficient regimens, prior knowledge of mutations conferring resistance to a particular drug is required (Meintjies et al., 2014). This is achieved by resistance testing whose primary goal is to provide a list of detailed mutations and drug susceptibility profiles for patients receiving ARVs. There are two types of resistance testing, firstly phenotypic assays, which investigates drug susceptibility by directly measuring viral growth rate relative to a wild-type reference virus in the presence of varying concentrations of drug, while genotypic testing assesses the genetic composition of HIV-1 variants indicating the presence of resistance mutations. This involves sequencing of virus populations from plasma, the resultant nucleotide sequence encompasses the complete 99 amino acids of HIV-1 PR and the 5' polymerase-coding region of HIV-1 RT, both of which are viral enzymes targeted by ARVs. Additionally viral IN and gp41 testing can also be performed if required. Drug resistance algorithms such as the IAS and the Stanford Drug resistance database are used to interpret differences between patient derived sequences and a wild-type reference strain, usually subtype B. This results in the generation of a list of mutations that aid in predicting drug susceptibility (Harrigan and Cote, 2000). Genotypic resistance testing has proven to be useful in a number of clinical studies, in that it can improve both virological and immunological outcomes of ART (Harrigan and Cote, 2000, Medeiros et al., 2007, Paredes i Deiros et al 2009). Genotyping is generally preferred over phenotyping due to the relatively shorter turnaround time, its cost effectiveness and its ability to identify evolving resistance (Harrigan and Cote, 2000, Tang and Shafer, 2012).

Unfortunately with phenotypic testing the effects of antagonistic mutations can be obscured. A common example is the presence of K65R and M184V; the phenotypically the 3TC resistance mutation will be detected however seeing as K65R reduces TDF susceptibility two fold, the presence of the TDF resistance will be obscured due to the presence of M184V which increases TDF susceptibility two fold. Nevertheless phenotyping has proved useful in testing the susceptibility of recently approved drugs (Tang and Shafer, 2012).

1.16.1 Drug resistance testing in South Africa

In many parts of the world, resistance testing is recommended for patients that are newly diagnosed with HIV-1 or for patients failing first-line treatment (Vandamme et al., 2004). However, for resource-constrained settings like SA, routine testing includes only a six monthly viral load test, while resistance testing is only performed for those patients failing second-line therapy (Department of Health., 2013). Unfortunately this has numerous implications for patients, in that they are given first-line treatment without prior knowledge of baseline mutations and possible transmitted drug resistance. Both of these may increase in frequency in the presence of drug selection pressure and hence compromise the efficacy of subsequent treatment. Some retrospective and prospective studies have demonstrated that the presence of drug resistance mutations prior to starting a regimen is an independent predictor of the success of that regimen (DeGruttola et al., 2000, Demeter and Haubrich, 2001, Hanna and D'Aquila, 2001). A German study showed that almost 10% of the drug naïve patients harbored pre-existing mutations within RT, suggesting that prior knowledge of these mutations before treatment are useful for designing effective regimens (Harrigan and Cote, 2000). Additionally, patients failing their first-line regimen are placed onto second-line therapy comprising a PI and recycled NRTIs. Given that the PI component may work relatively well in suppressing viral replication, patients may have already mounted NRTI resistance, causing them to fail treatment quicker. Despite the inability to frequently conduct resistance testing, these settings face several other

challenges. Many patients are left on failing first-line regimens for extended periods of time, resulting in the accumulation of mutations. Levison et al (2011) showed that the accumulation of RT mutations is detrimental to the success of subsequent second-line therapy (Levison et al., 2011). Thus, resistance testing is an important tool for making informed treatment decisions and guiding patient management within a clinical setting.

1.17 DNA sequencing

The advent of DNA sequencing has revolutionized considerably over the past 50 years encompassing a variety of new platforms (Figure 1.13). Many were developed in first world countries where resources and funds are more readily available. Despite this, many of these applications are slowly filtering their way through to developing countries like SA.

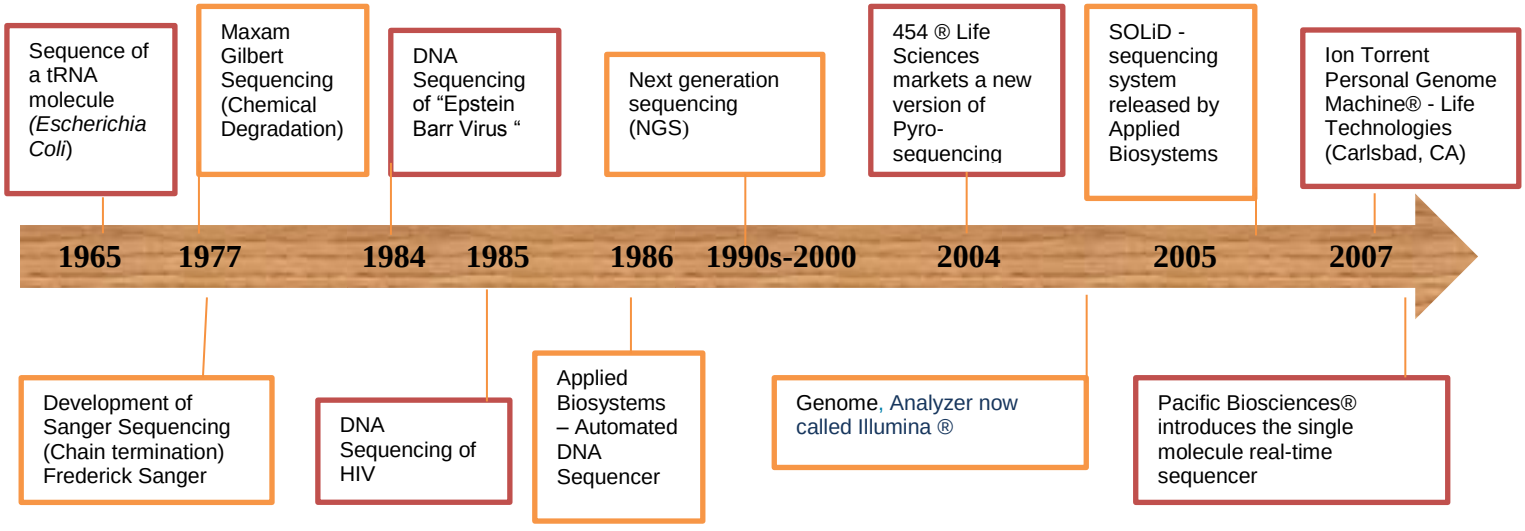


Figure 1.13 Timeline depicting the evolution of DNA sequencing since 1965 (Adapted from Quinones- Mateu et al (2014).

1.18 Sanger sequencing versus Ultra Deep Pyro-Sequencing

Standard resistance genotyping employs the Sanger based method for sequencing. However it is well known that this method can only detect >20% of the virus population and viruses occurring at < 20% are missed (Halvas et al., 2010, Stelzl et al., 2011). Figure 1.14 reiterates the ability of deep sequencing to detect more variants than Sanger sequencing. Fortunately, evolution of other sequencing methods such as, Next generation sequencing (NGS) can detect these low frequency variants and have been adopted in many studies. Quiñones-Mateu et al (2014) reported that the past 13 years has seen the highest number of scientific publications for HIV-1 studies employing deep sequencing, while in 2013 alone 57 HIV-1 studies using Ultra Deep Pyro-Sequencing (UDPS) were published (Quinones-Mateu et al., 2014).

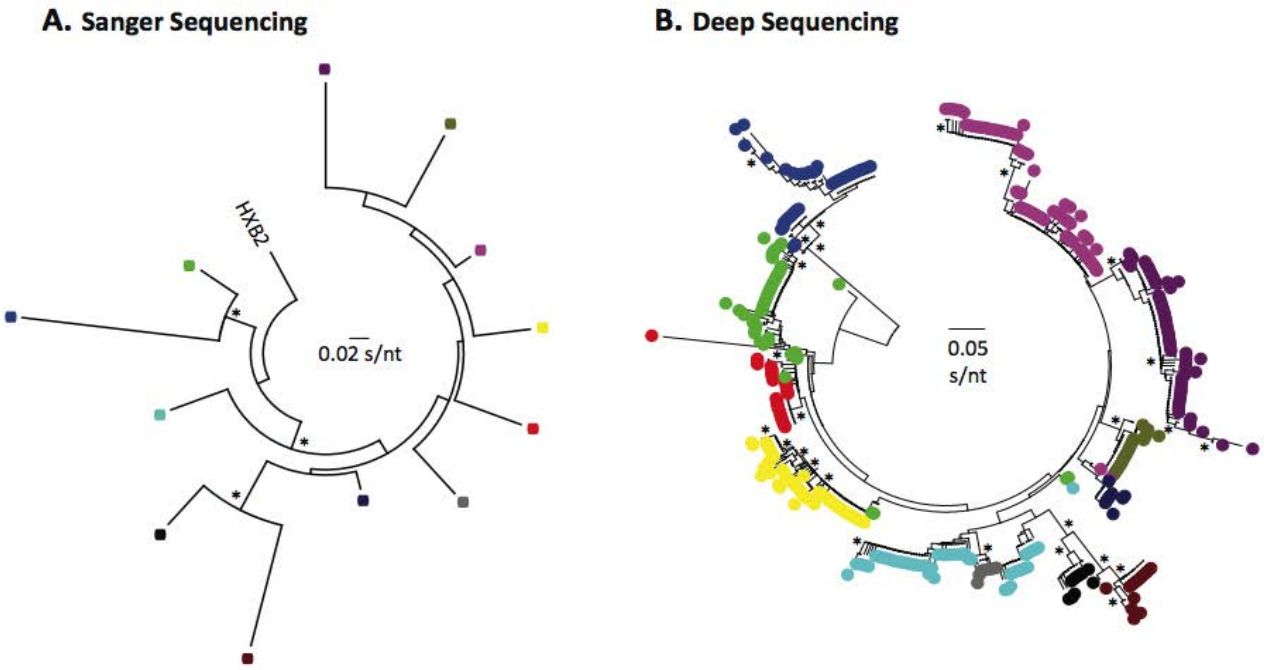


Figure 1.14 Neighbour-joining phylogenetic trees. Differences between Sanger and Ultra Deep Pyro-sequencing (UDPS). A) Trees for 12 HIV-1 infected individuals, comprising 105bp fragments corresponding to the HIV-1 V3 region of the gp120 envelope. B) Phylogenetic tree for deep sequencing reads, (frequency <1%) for the same 105bp fragments. Coloured dots indicate each unique variant. Bootstrap resampling (1000 data sets) of the multiple alignments tested the statistical robustness of the trees, an asterisk indicates percentage values >75%

and (s/nt) - represents the substitutions per nucleotide (Quinones-Mateu et al., 2014).

1.19 Detection of minority variants

There are numerous methods to detect minor variants: point mutation assays, phenotypic assays and sequencing assays.

1.19.1. Point mutation assays

Point mutation assays are highly sensitive and can detect minor variants present within the viral quasispecies (Gianella and Richman, 2010). An inherent disadvantage of most of these assays is that only a single point mutation at a time can be detected, and other polymorphisms in nearby codons are missed (Gianella and Richman, 2010). Examples of these assays include Allele Specific PCR (AS-PCR) and the LigAmp assay (Table 1.3).

1.19.2 Sequencing assays

Sequencing assays offer the ability to analyse the entire sequence context identifying genetic linkage of each mutation and has become one of the most widely used techniques in a research setting. There are different methods which can be used and include; clonal sequence analysis, single genome analysis (SGA) and UDPS.

1.19.2.1 Clonal sequence analysis

The desired Polymerase Chain Reaction (PCR) amplified product is inserted into a vector comprising an antibiotic resistance gene, and a colour selection marker such as the *LacZ* gene which is used to provide blue white screening using X-gal is employed. Source DNA is generally obtained by pooling products of parallel PCRs. The final recombinant construct is then transformed into bacterial cells and plated onto solid media with an antibiotic that restricts growth of bacteria without the plasmid. After selection of recombinant clones using blue-white screening, recombinant DNA is extracted and subsequently sequenced. The number of

colonies examined predicts the sensitivity of the assay (Paredes i Deiros et al 2009, Gianella and Richman, 2010).

1.19.2.2 Single Genome Analysis

For SGA, many single viral genomes from a plasma sample are obtained. After an RNA extraction and complementary DNA (cDNA) synthesis using specific or random primers, the cDNA is serially diluted 1:3 to a maximum dilution of 1:187. Thereafter ten individual real-time PCR (RT-PCR) amplifications are performed for each of the cDNA dilutions. In accordance with Poisson's distribution, the cDNA dilution that yields PCR products in three of the ten reverse transcription-PCRs contains one copy of cDNA per positive PCR about 80% of the time. As a result 70 PCRs are performed using the cDNA dilution that yields approximately 30% positive reactions. These reactions are then screened by Agarose gel electrophoresis and sequenced using direct dideoxyterminator sequencing. DNA sequences that are derived from 20-40 single genomes are analysed per sample (Gianella and Richman, 2010).

1.19.2.3 Ultra Deep Pyro-Sequencing

Ultra Deep Pyro-sequencing is an emulsion-based massively parallel sequencing technology, particularly suited for an in-depth analysis of a population of heterogeneous genomes such as retroviruses. DNA library fragments are immobilized onto micron sized capture beads, respective enzymes and reagents are placed in a water mixture, which is injected into small cylindrical plastic containers containing synthetic oil. A combination of these materials together with vigorous mixing creates droplets around the beads resulting in micro-reactors. Each of these micro-reactors will ideally contain only one bead with a unique fragment of DNA. Any competing and contaminating sequences are excluded, the enzyme component in the reaction causes the isolated DNA fragments to be amplified into millions of copies during the emulsion PCR (emPCR). This parallel

amplification of fragments results in a copy number of several million per bead. The emulsion is then broken and the amplified fragments remain bound to the beads. The clonally amplified beads are enriched and loaded layer by layer onto a PicoTiter plate (PTP). After the addition of sequencing enzymes individual nucleotides are flown across the plate in a fixed order. The addition of one or more nucleotides that are complementary to the template strand results in a chemi-luminescent signal that is recorded by a Charge Coupled Device (CCD) camera, these light signals are processed and subsequently transmitted to a computer generating a report with the identified DNA sequences for each clone (Paredes i Deiros et al 2009, Gianella and Richman, 2010, Avidor et al., 2013, Quinones-Mateu et al., 2014). With recent technological improvements, the use of UDPS for the detection of minor variants is becoming more widespread and has led to many different platforms.

Table 1.3 Comparison between different methods for detecting minority variants (Adapted from Paredes I Deiros et al (2009), Gianella and Richman et al (2010).

Method	**Point Mutation Assays**		**Sequencing Assays**		
Assays	AS-PCR	LigAmp	Clonal Sequence Analysis	SGA	UDPS
Advantages	-Inexpensive -Quick turnaround time -Less labor intensive	-Increased specificity -Inexpensive -Quick turnaround time Less labor intensive	-Simple approach -Relatively cheap	-Detection of linked mutations	-Quick turnaround time -Moderately to highly sensitive
Disadvantages	-One allele per experiment can be studied- No linked mutation analysis -Underestimates mutant proportions	-One allele per experiment can be studied -No linked mutation analysis -Not quantitative	-Labor intensive -Capacity to detect minor variants is limited -Slow turnaround time	-Slow turnaround time -Labor intensive -relatively expensive	-Expert bioinformatics support required, -Expensive Labor intensive
Number of mutations	1	1	Multiple	Multiple	300-400bp
Linked mutations	No	No	Yes	Yes	Yes
Sensitivity (%)	0.01-<1	<1-12	<1-10	<1-10	<1
Cost	*	**	***	****	*
Labor intensity	**	**	*	*****	*******

1.19.2.4 Deep sequencing platforms

There are four deep sequencing platforms currently available, 454 ™ (454 Life Sciences/Roche, Branford, CT), Illumina ™ (Illumina, Inc, San Diego, CA), Ion Torrent™ (Ion Torrent/Life Technologies, South San Francisco, CA) and PacBio™ (Pacific Biosciences, Menlo Park, CA) (Rothberg JM et al, 2011, Quinones-Mateu et al, 2014). Comparisons of these platforms are shown in Table 1.4. Each of these

platforms can generate a vast amount of valuable sequencing data, but differ from each other in terms of the quantity and quality of data produced. Many factors must be considered when selecting a deep sequencing platform most importantly, the desired applications that are required, resource availability, costs incurred for reagents and consumables to perform experiments, existing infrastructure as well as personnel to perform the assays (Quinones-Mateu et al., 2014).

Table 1. 4 Comparisons between the four most popular sequencing platforms as of 2014. The 454™ (GS Junior and GS FLX+ systems), Illumina™ (MiSeq v2 and HiSeq 2500 systems), Ion Torrent™ (Ion Personal Genome Machine, 318 v2 chip, Ion Proton™ chip systems, and Pacific Biosciences™ (PacBio RS II SMRT). Adapted from Quinones-Mateu et al (2014).

Platform	**454™**		**Illumina™**		**Ion torrent™**		**PacBio™**
	454 GS Junior	GS FLX+	MiSeq	Hi Seq	PGM	Proton	RS II
Amplification method	Emulsion PCR on beads		Bridge PCR *in situ*		Emulsion PCR on beads		No PCR required
Chemistry	Synthesis Pyro-sequencing		Synthesis (Reversible Termination)		Synthesis (H+Detection)		Single Molecule, real time sequencing
Advantages	Long reads, Maturity		Easy work flow, Maturity		Low cost, fast run		Longest reads
Disadvantages	Homo-polymer misreads, expensive, labour intensive		Short reads, Long run		Homo-polymer, mis-reads		High error rate, expensive
Primary Error	Indels		Substitution		Indels		Indels
Error rate	~1%		~0.1%		~1%		~13%
Read length (bp)	400	700	250	125	400	200	8500
Run time (hours)	10	20	39	276	7	4	2

Reads that are generated by UDPS provides an efficient tool for studying viral diversity, HIV transmission and disease progression (Quinones-Mateu et al, 2014). Interestingly, a number of studies have reported the use of UDPS for the detection of minority HIV-1 drug resistant variants at levels as low as 0.1-1% (Wang et al., 2007, Johnson et al., 2008, Archer et al., 2012, Dudley et al., 2012, Xiaobai et al.,

2014). Majority of these studies have employed the 454™ sequencing platform while others have used platforms like Illumina™ and Ion Torrent™.

1.19.2.5 Applications of Ultra Deep Pyro-Sequencing

As previously mentioned, studies have shown that UDPS can detect minority drug resistance variants occurring at < 20% of the circulating virus population which are often missed by bulk sequencing (Dudley et al., 2012, Fisher et al., 2012, Nicot et al., 2012, Avidor et al., 2013, Garcia-Diaz et al., 2013, Mohamed et al., 2014). The detection of drug resistant minority variants has been employed in various clinical settings (Fisher et al., 2014). A study by Avidor et al (2013) found PI DRM V32I (12.8%) in a single patient that had failed DRV/r treatment. This mutation was unfortunately missed by bulk sequencing at the time of testing and as a result the patient continued DRV/r treatment for 19 months, until this mutation was detected much later by bulk sequencing (Avidor et al., 2013). A study by Le et al (2009) described a correlation between minority variants and historical ARV use in treatment experienced patients (Le et al., 2009).

Others studies have shown that minor variants can predict ARV failure to NNRTI-inclusive regimens (Simen et al., 2009), as well as identify possible ETR resistance at low frequencies. (Varghese et al., 2009). This data is useful for resource limited settings, where patient treatment histories are not always readily available. Patients harboring these variants are exposed to almost twice the risk of virological failure upon initiating treatment. These variants may increase in the presence of drug therapy, and can also result in cross-resistance to other drugs within the same drug class (Kapoor et al., 2004, Simen et al., 2009, Varghese et al., 2009, Nicot et al., 2012). This suggests that these variants may act as predictors of treatment failure despite adequate adherence levels. However, more work will be required to fully elucidate the role of minority variants in HIV-1 subtype C infected patients failing PI-inclusive treatment.

1.20 Computational methods to study HIV-1 Protease

The structure of HIV-1 PR is fundamental for the design of PIs. As a result, various computational methods such as (i) homology modeling (ii) molecular docking and (iii) molecular dynamics simulations have been employed (Schwede et al., 2003, Hao et al., 2012). This information has been used to understand HIV-1 PR drug resistance and the implications it may have for new and improved ARVs. Most PIs are substrate-based inhibitors that bind with great specificity to the active site of viral PR. Slight structural changes within the active site, the flaps or other regions not in direct contact with the inhibitor can compromise the drug-binding interaction (Schwede et al., 2003, Hao et al., 2012).

1.20.1 Homology modeling

Homology modeling is an effective method to reliably generate three-dimensional (3D) models of a protein from the amino acid sequence at the atomic level. It relies on the identification of one or more known protein structures (template) that is likely to resemble the structure of the target sequence. This is followed by an alignment that maps residues in the target sequence to residues in the template sequence (Marti-Renom et al., 2000). There are essentially five steps that are performed (i) template selection, (ii) alignment (iii) model building (iv) energy minimization (v) model evaluation and (vi) model validation. Various software packages are available for model construction, two popular packages include Modellar (Sali and Blundell, 1993), and SWISS-MODEL (Nayeem et al., 2006).

SWISS-MODEL was used in this study and is a fully automated protein modeling server, that comprises three integrated components; (i) the pipeline- which is a suite of software tools and databases for automated protein structure modeling, (ii) the workspace, which is a web based graphical user workbench, and lastly (iii) the repository which is a continuously updated database of homology models for a set of organism proteomes (Schwede et al., 2003). In order to visualize the resultant 3D structure, programs such as UCSF Chimera (Pettersen et al., 2004), SWISS-

pdb-Viewer (Guex and Peitsch, 1997), or The Pymol Molecular Graphics System 2002 (DeLano Scientific, San Carlos, CA, USA) are amongst the many that can be used.

1.20.2 Molecular docking

Molecular docking is used to predict the preferred orientation of one molecule (receptor) to a second molecule (ligand) when bound to each other in order to form a stable complex (Lengauer and Rarey, 1996). There are three approaches describing the ligand-receptor interaction. First: the "lock and key" theory as proposed by Fisher et al (1890) where both the ligand and the receptor are treated as rigid bodies. Second the induced fit theory created by Koshland et al (1958) which states that the active site of a protein is continuously reshaped due to the ligand-protein interactions implying that the ligand and the receptor are treated as flexible during docking (Lengauer and Rarey, 1996). It has also been documented that proteins can undergo much larger conformational changes, and is shown by a recent model referred to as a "conformation ensemble", proposed by Boyong et al (2003). This model describes proteins as a pre-existing ensemble of conformational states; the plasticity of the protein permits a switch from one state to another (Lengauer and Rarey, 1996). Each of these models focuses on a particular aspect of the recognition process; the lock and key theory introduces the principle of 3D complementarity, the induced fit model explains how the complementarity is achieved and the ensemble model depicts the conformational complexity of proteins. When structures are treated as flexible, binding events are determined more accurately as opposed to rigid models (Jenwitheesuk and Samudrala, 2003). In addition, the number of possible conformations increases exponentially requiring a large amount of computing power.

Various algorithms have been designed to deal with such conformations and are usually incorporated into molecular docking software packages. Examples of some of these software packages include AutoDock, Glide, FlexX, CDocker, LigandFit,

MCDock, Dock 6.0, GOLD, and a relatively new package known as the CLC drug discovery Workbench ^{TM} (CLC, Bio, Qiagen, Valencia, CA). This software employs algorithms from a previously well-known package " Molegro Virtual Docker". The CLC Workbench enables the use of a variety of components to study the molecular mechanisms of proteins while the drug discovery component can be used for molecular docking,

The docking simulation involves two important aspects (i) the search algorithm and (ii) the docking scoring function –the docking function used by the CLC workbench is the PLANTS (Protein-Ligand ANT System) (Korb et al., 2009) score which has a good balance between accuracy and computational evaluation time. This score mimics the potential energy change, when the protein and the ligand bind together. As a result the ligands can be ranked in terms of binding stability; generally ligands with a low energy score have a better stability and are used for further *in vitro* testing. This is represented by the simple equation (Score = S_{target} -S_{ligand} + S_{ligand}). Previous studies have employed molecular docking studies to investigate the level of drug susceptibility for a range of PIs (Toor et al., 2011, Saxena, 2013). A study by Toor et al (2011), demonstrated that in the presence of certain DRMs, binding scores differed and correlated with drug susceptibility scores in accordance with the Stanford drug resistance algorithms (Toor et al., 2011). Docking is extensively used in the pharmaceutical industry for the design and discovery of novel inhibitors (Sousa et al., 2006).

1.20.3 Molecular Dynamics

A Molecular Dynamics (MD) simulation is a computational method that calculates the time dependent behavior of a molecular system, predicting the physical motion of atoms and molecules

(Lipkowitz, 1990). These simulations provide an insight of the fluctuations and conformational changes that occur amongst proteins and nucleic acids. The resultant trajectory of the molecule is determined using Newton's laws of motion

(Lipkowitz, 1990). During the simulation, the molecule is set into motion by heating to a specific temperature to overcome potential energy barriers and achieve more stable conformations at high temperatures. The force on each particle (F_i) is determined, first as a function of time, which is equal to the negative gradient of the potential energy ($F_i = -\frac{\partial U}{\partial ri}$) where U is the potential energy function and r is the position of the particle. By Newton's second law of motion, the acceleration of a particle is determined by dividing the force acting on it by the mass (M) of a particle ($a_i = -\frac{Fi}{Mi}$). The change in velocities of the particle is equal to the integral of acceleration (a) over time and the change in position is equal to the integral of velocity over time. ($dv = \int adt$), ($dv = \int vdt$). The kinetic energy can be described in terms of both the velocities and momenta of the particles, where (N) is the number of particles. $K(v) = \frac{1}{2}\Sigma\frac{N}{i=1} m_i V_i$ and $K(p) = \frac{1}{2}\Sigma\frac{N}{i=1}\frac{pi^2}{mi}$. As a result, the total energy in the system is referred to as Hamiltonian (H), which is the sum of the kinetic and potential energies; H (q.p)=K (p)+ U (q), where q is the set of Cartesian coordinates, p is the momenta of the particles and U (q) refers to the potential energy function. The velocities (v_i) is the first derivative of the positions with respect to time. $v_i(t) = \frac{d}{dt} qi\ (t)$ where $qi(t)$, refers to the atomic positions at a time (t). Using the initial coordinates of the system, atoms move to new positions and new velocities at time (t) can be determined, thereby generating new conformations (Jensen, 1999, Parul Sharma, 2012).

Software packages that can be used to perform these MD simulations include packages like, Assisted Model Building with Energy Refinement (AMBER), (Chemistry of HARvard Macromolecular Mechanics (CHARMM), and GROningen Machine for Chemical Simulations (GROMACS). For this study, AMBER (Case et al, 2005) was used for the analysis. AMBER incorporates a set of programs that uses force fields to perform MD simulations of chemical structures. The AMBER package comprises four programs; Antechamber, tleap, Sander and Process

Trajectory (PTRAJ). Antechamber automates the process of parameterization and is used to build topology files required for the simulation, tleap is used to prepare the system for the simulation programs. Sander is a central simulation program that is used to perform energy minimization and MD. PTRAJ is used to perform the analysis of the simulation results and provides outputs that can be used for other downstream analysis such as the calculation of the Root Mean Square Deviation (RMSD) values and the determination of binding affinities for complexes (Marcos Vinícius R. Garcia et al., 2012).

MD simulations have been used to study the dynamics and flexibility of HIV-1 PR flaps that may be influenced by the presence of mutations (Liu et al., 2008, Agniswamy et al., 2012, Naicker et al., 2013). A previous study was able to use MD simulations to show a unique binding mode within an open conformation of the PR flaps, here the inhibitor was found laying roughly perpendicular to the usual active site binding pocket. As a result they were able to identify a new binding site for DRV that may have implications for improved drug design (Zhang et al., 2014). In another study MD simulations was used to show that that the flap region of the subtype C PR has more fluctuations over time in comparison to a subtype B PR. Other structural modifications were also identified that could result in the reduced susceptibility to PIs (Naicker et al., 2013). It is therefore an important tool for investigating the implications of resistance mutations to drug binding interactions (Liu et al., 2008, Agniswamy et al., 2012, Naicker et al., 2013).

1.21 Project rationale and aims

Aims:

1. To investigate the prevalence of protease, reverse transcriptase and integrase resistance mutations present in patients failing a Protease Inhibitor inclusive regimen.
2. To use homology modeling to construct models for LPV/r-resistant and DRV/r-resistant isolates, to compare the effects of Darunavir treatment on HIV-1 Protease.
3. To utilize Molecular Dynamics and molecular docking simulations to investigate the stability of mutated and un-mutated complexes of HIV-1 PR.

Objectives:

1. To genotype PR and RT using an in house assay as well as the HIV-1 Viroseq Genotyping system, for all patients failing LPV/r.
2. To genotype PR, RT and IN for patients that failed LPV/r, and were treated with DRV/r.
3. To perform UDPS using the Roche 454™ Platform, for the detection of minor variants for patients that failed LPV/r and thereafter DRV/r.
4. To generate homology models for both a LPV/r-resistant and a DRV/r-resistant isolate, using a wild-type subtype C sequence as a template,
5. To compare overall conformational changes in the both models, in order to identify the implications of DRV/r resistance on HIV-1 PR structure
6. To perform molecular docking, of the PI DRV/r into the LPV/r-resistant, DRV/r-resistant and the wild-type model.
7. To perform MD simulations in AMBER to determine the stability of generated structures.

CHAPTER 2

Resistance genotyping of the HIV-1 *pol* gene following second-line LPV/r failure.

2.1 Introduction

The advent of cART is a milestone in HIV-1 treatment policies; unfortunately the emergence of drug resistance mutations undermines its efficacy leading to treatment failure (Mugavero and Hicks, 2004, Toledo et al., 2010). Data on the mutations seen after first-line failure in SA show a high frequency of NNRTI mutations (Marconi et al., 2008, Singh et al., 2011, Manasa et al., 2013, Wallis et al., 2014) as opposed to second-line failure studies, which report infrequent LPV/r resistance, but a significant accumulation of NRTI/NNRTI mutations (Riddler et al, 2008, Wallis et al, 2011, Levison et al, 2012, Schoffelen et al 2013). These studies attributed non-adherence to be the major cause of treatment failure rather than drug resistance. However for those patients that present with resistance, there is growing concern around the emergence of additional mutations that may limit the efficacy of newer generation ARVs.

Most studies describe LPV/r failure in patients infected with subtype B viruses, however C infections dominate the epidemic in Sub-Saharan Africa. The few studies describing second-line failure in SA were conducted in the Western Cape, Gauteng, Limpopo provinces and other regions in Africa. However, there is no data from KZN, the province with the highest prevalence of HIV-1 infection, and the highest number of patients receiving ARVs (Kiepela et al, 2014). Lastly, most of these studies were based on small cohort sizes, with the exception of one study by Van Zyl et al (2013). Here we investigate the contribution of DRMs to PI-inclusive ART failure within a large cohort of subtype C infected patients from KZN.

2.2 Materials and Methods

2.2.1 Study population

Patients failing their second-line LPV/r inclusive regimen that were referred to the Infectious Diseases Clinic at King Edward VIII Hospital were recruited, having met the study requirements. In addition, patients were also recruited from the Sinikithemba ARV Clinic at McCord Hospital in Durban, KZN. Patients were enrolled if they had a viral load of >1000cpm and were on a PI-inclusive regimen for more than six months. Ethical approval for the study was obtained from the Biomedical Research Ethics Committee (BF068/08). Subtype C sequences from treatment naïve patients were downloaded from the Los Alamos sequence database (http://www.hiv.lanl.gov/).

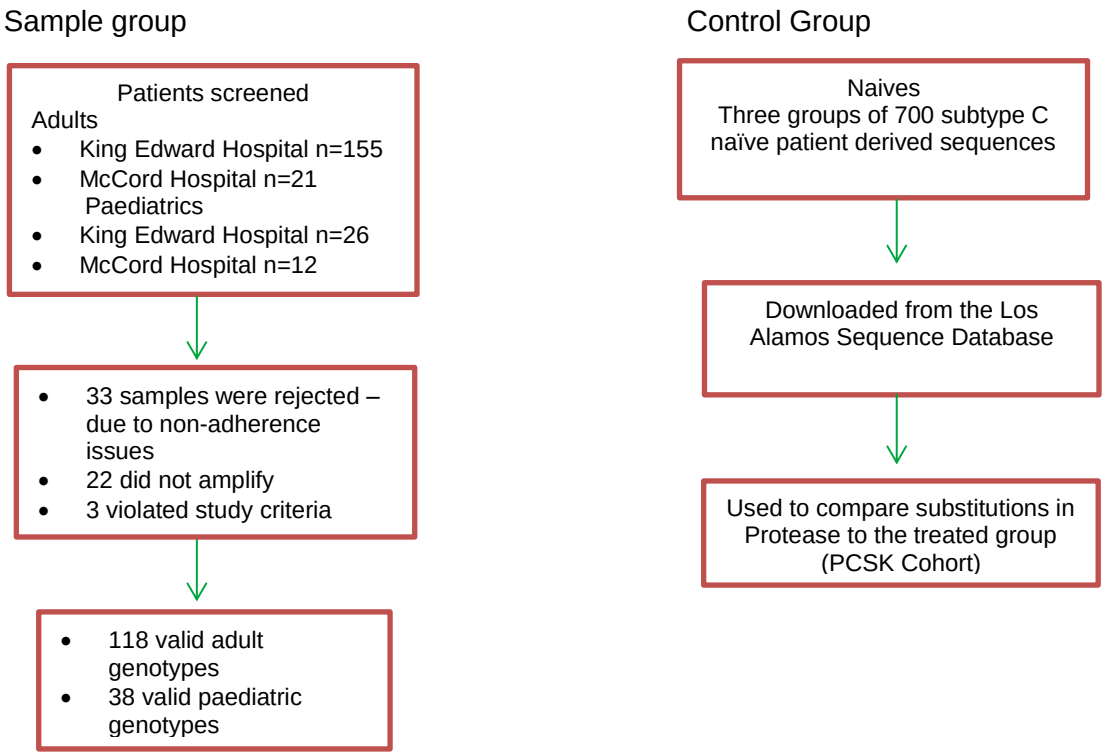


Figure 2.1 Sample and control groups used in the study. Accession numbers are provided in
Appendix A

2.2.2. Sample collection and processing

Two tubes of blood (four mls each) were collected in Ethylenediaminetetraacetic acid (EDTA) tubes and processed within 24 hours of reception. Tubes were centrifuged at 1000 X g for ten minutes at room temperature to allow for the separation of the plasma and buffy coat, each of which was then stored in 1.5ml cryovials at -80°C until further use.

2.2.3 In-house HIV-1 drug resistance genotyping assay

The protocol for the in-house drug resistance assay was obtained from the National Institute of Communicable Diseases (NICD) and was further optimized and validated for use in our laboratory (Pillay et al., 2008). The assay is used to detect mutations in the PR and RT regions of the HIV-1 *pol* gene.

2.2.3.1 RNA extraction

An RNA extraction involves the isolation of RNA particles from plasma followed by purification of the viral RNA. Prior to extraction, 500µl of plasma was added to a screw capped 1.5ml tube and labeled with the sample details as well as an orientation marking to indicate the position of the pellet after centrifugation. Plasma was then centrifuged at 25000 X g at 4°C for one hour in an Eppendorf ultracentrifuge. After removal of the supernatant, the RNA was extracted using the QiAmp Viral RNA Kit (Qiagen, Valencia, CA) according to the manufacturers instructions. Isolated RNA was then stored at -80°C until required.

2.2.3.2 cDNA Synthesis

The HIV-1 genome was reverse transcribed using a gene specific primer (IN3-10pmol) and the Superscript III RT enzyme (Invitrogen, Life Technologies, Carlsbad, CA). Master mix reactions were prepared in the pre-amplification area. All reagents, with the exception of enzymes, were thawed at room temperature. Enzymes were kept in the freezer until use. Once thawed, reagents were gently vortexed and spun in a minifuge to collect droplets. A mix of one µl of dNTPs

(10mM/µl) and one µl of the forward primer IN3 (10pmol/ µl) was prepared in a 0.2ml tube and kept at room temperature until use. A cDNA master mix was prepared in a 1.5ml tube as indicated in Table 2.1.

Table 2.1 Reagents used to prepare a master mix reaction for the cDNA synthesis reaction.

Reagent	Volume for 1 reaction (µl)	Final concentration
5X First Strand Buffer	4	1X
0.1M DTT	1	5mM
RNase out (40U/µl)	1	40U
Superscript III (200U/µl)	1	200U
Final Volume	7	

The cDNA master mix was used within 30 minutes of preparation. For the reverse transcription reaction, ten µl of thawed RNA was added to the mix of primer and dNTPs, and was placed in the thermocycler under the reaction conditions indicated in Table 2.2

Table 2.2 Thermocycling conditions used for the cDNA synthesis reaction.

Temperature	Time	Process
65°C	5 minutes	Relax RNA secondary structure
4°C	1 minute	Cool to optimal enzyme activity
Pause manually *		
50°C	60 minutes	Reverse transcription
70°C	15 minutes	Inactivates superscript III
4°C	>10 minutes	Hold

*After the 65°C step the thermocycler was paused and eight µl of freshly prepared cDNA master mix was added into each tube and mixed well. Tubes were placed back onto the thermocycler for the remaining conditions.

Once complete, one µl of RNase H (1U/µl) (Invitrogen, Life Technologies, Carlsbad, CA) was added for the degradation of ssRNA at 37°C for 20 minutes, the reaction was held at 4°C until further use.

2.2.3.3 PCR amplification

A nested PCR amplification of PR and RT genes from cDNA was done using the Expand Long Template PCR system (Roche Applied Science, Branford, CT, USA) making use of four primers as indicated in Table 2.3.

Table 2.3 Primer sequences used for the nested PCR. The locations relative to HXB2 are also shown.

Primer name	HXB2 positioning	Primer sequence (5'-3')
G25REV(forward)	1867-1892	GCAAGAGTTTTGGCTGAAGCAATGAG
AV150(forward)	2036-2062	GTGGAAAGGAAGGACACCAAATGAAAG
IN3(reverse)	4246-4212	TCTATVCCATCTAAAAATAGTACTTTCCTGATTCC
POL M4 (reverse)	3892-3870	CTATTAGCTGCCCCATCTACATA

A first round and a second round master mix was prepared in two separate 1.5ml tubes and stored in the fridge until required. An extra reaction containing no sample was used as the negative control in all instances.

Table 2.4 Reagents and volumes used for the first round PCR reaction.

Reagent	Volume for 1 reaction (µl)	Final Concentration
DEPC Treated Water	16.3	-
10 X Buffer + $MgCl_2$ (17.5mM)	2.5	1X
dNTPs (10mmol/µl)	0.88	0.35Mm
AV150 (10pmol/µl)	0.25	0.125µm
IN3 (10pmol/µl)	0.25	0.125µm
Expand long template (5U/µl)	0.38	1.9U
Final	20.5	

Table 2.5 Reagents and volumes used for a second round PCR master mix reaction

Reagent	Volume for 1 reaction (µl)	Final Concentration
DEPC Treated Water	39	-
10 X Buffer + $MgCl_2$ (17.5mM)	5	1X
dNTPs (10mmol/µl)	1.75	0.35Mm
G25REV (10pmol/µl)	0.5	0.125µm
POL M4 (10pmol/µl)	0.5	0.125µm
Expand long template (5U/µl)	0.75	1.9U
Final	47.50	

Once the cDNA program was complete, the tubes were removed from the thermocycler and centrifuged in a minifuge to collect the droplets. Thereafter 4.5µl of the cDNA was added into each of the respective first round master mix tubes. The reaction was then run under the conditions shown in Table 2.6, with the ramp speed set to maximum and the reaction volume to 25 µl.

Table 2.6 Thermal cycling conditions used for the first and second round PCR reaction.

No of cycles	Temperature	Time	Process
1	94°C	2 minutes	Initial Denaturation
10	94°C	10 seconds	DNA Denaturation
	52°C	30 seconds	Primer annealing
	68°C	2 minutes	Primer extension
25	94°C	15 seconds	DNA Denaturation
	52°C	30 seconds	Primer annealing
	68°C	2 minutes +20 sec/cycle	Primer extension
1	68°C	7 minutes	Final Extension
	4°C	∞	Hold until ready

Once the reaction was complete the tube was spun down again and 2.5 µl of the first round product was added into the respective second round PCR master mix. The tube was placed in the thermal cycler and the reaction volume set to 50µl. The reaction was run under the same cycling conditions as the first round reaction.

2.2.3.4 Gel electrophoresis

To confirm the size of the product that was amplified, the PCR products were electrophoresed on a 1% Agarose gel. Gel loading dye was prepared by adding one µl of Gel Red (Biotium, USA) to 50µl of Gel loading buffer (Sigma-Aldrich, USA) and vortexed well before use. Two µl of a one kb ladder (Invitrogen, Life Technologies, Carlsbad, CA) was mixed well with two µl of the Gel loading dye and was loaded into the first well of the gel, two µl of each PCR product was mixed with two µl of the Gel loading dye and loaded into the remaining wells. The gel was placed in an electrophoresis tank containing 1 X TBE (Tris Borate EDTA Buffer) (Sigma-Aldrich, USA) and electrophoresed at 100V/cm for 45 minutes on an Electrophoresis Power Supply- EPS 301 (Amersham Biosciences, Sweden). The gel was then examined under Ultraviolet light using the GelVue UV Trans-illuminator (SynGene, London). The negative control well was examined for any visible bands apart from primer dimer <100bp, as bands >100bp indicate possible contamination and the PCR would have to be repeated. Sizes of the amplified products were checked against the ladder and were 1.7kb in size. Products were stored at -20°C until purification.

2.2.3.5 Amplicon Purification

PCR products were purified using the QIAmp PCR Purification kit (Qiagen, Valencia, CA), according to the manufacturers instructions. Purified products were run on an Agarose gel once again using a one kb DNA ladder (Invitrogen, Life Technologies, Carlsbad, CA) in order to quantify the products for sequencing.

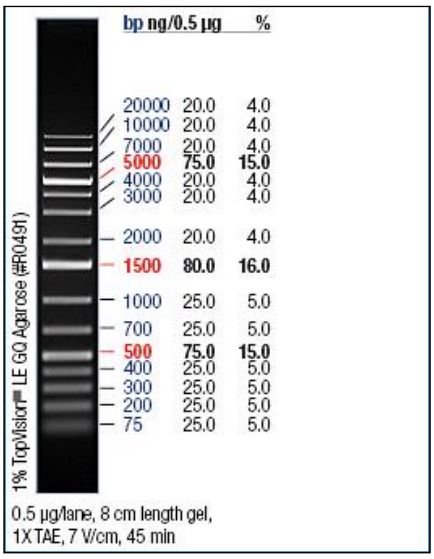


Figure 2.2 One kb DNA ladder showing the different size products on a 1% Agarose gel. Taken from the GeneRuler 1kb plus ladder package insert (Invitrogen, Life Technologies, Carlsbad, CA).

2.2.3.6 Sequencing reaction

Once the products were quantified, they were diluted to 20ng/µl using PCR grade water. Direct sequencing of the PCR amplification products was performed using the BigDye Terminator v3.1 cycle sequencing chemistry with six sequencing primers as listed in Table 2.7.

Table 2.7 Primer sequences and their relative HXB2 positioning, used during the sequencing reaction.

Primer Name	HXB2 positions	Primer sequence
PolM0(3.2pmol) (forward)	2251-2272	TCCCTCAGATCACTATTTGGCA
PolM1(3.2pmol) (forward)	2610-2632	GTTAAACAATGGCCATTGACAGA
PolM4(3.2pmol) (reverse)	3892-3870	CTATTAGCTGCCCCATCTACATA
PolM8(3.2pmol) (reverse)	3323-3302	CTGTATATCATTGACAGTCCAG
PolMG(3.2pmol) (reverse)	2823-2798	A TTGAACTTCCCAGAAGTCTTGAGTT
AV150(3.2pmol) (forward)	2036-2062	GTGGAAAGGAAGGACACCAAATGAAAG

A master mix was made in separate 0.2ml tubes for each primer and comprised the reagents listed in Table 2.8.

Table 2.8 Reagents and volume used to make the sequencing reaction master mix

Reagent	Volume for 1 reaction (µl)	Final Concentration
DEPC treated water	1.6	-
5X Sequencing buffer	2	1X
BigDye Terminator ready reaction mix	0.4	-
Primer 3.2pmol/µl	1	0.256 µM
Total	5	

Thereafter five µl of each master mix was aliquoted into a 96 well optical plate (Applied Biosystems, CA. USA) and five µl of the diluted sample was added into each well according to the designated plate layout. The plate was sealed with adhesive foil, gently vortexed and centrifuged to collect droplets. The adhesive foil was then removed and replaced with a rubber mat which was secured using an applicator to ensure all wells were properly sealed. The plate was then placed in the thermocycler and run under the conditions listed in Table 2.9.

Table 2.9 Thermal cycling conditions used for the sequencing reaction.

No of cycles	Temperature	Cycles	Process
1	96°C	1minute	Initial denaturation
35	96°C	10 seconds	DNA Denaturation
	50°C	5 seconds	Primer annealing
	60°C	4 minutes	Primer extension
	4°C	∞	Hold until ready

2.2.3.7 Purification of sequencing products

Once the reaction was complete, the sequencing products were purified immediately. Prior to beginning the purification, a Sodium Acetate-ethanol mixture was prepared by adding one µl of a 3M Sodium Acetate (pH 5.2) which was required per well, and 25µl of 100 % Absolute ethanol. The plate was removed from the thermocycler and one µl of 125mM EDTA (Sigma-Aldrich, USA) was added into each well using a multichannel pipette followed by 26µl of the freshly prepared Sodium Acetate-ethanol solution. The plate was sealed with adhesive foil and gently vortexed for five seconds and centrifuged at 3000 X g for 20 minutes. During this time two sheets were made by folding paper towel into a thick mat, matching the size of the 96 well plate. After the 20 minutes the plate was inverted onto the paper towel and placed in the centrifuge holder at 150 X g for five minutes. During this time a 70% ethanol solution was prepared, and was stored in the fridge until use. Thereafter 35µl of freshly prepared ethanol was added into each well using a multichannel pipette. It was important to work quickly at this step to prevent drying out of the fragments. The plate was placed in the centrifuge at 3000 X g for five minutes. After this time the paper towel was placed back on the plate for drying and centrifuged at 150 X g for one minute. Thereafter the late was dried in a thermal cycler at 50°C for five minutes and was then stored at -20°C until sequenced.

2.2.3.8 Sequencing on the 3130xl Genetic Analyzer

Prior to sequencing, the dried pellets in each well were reconstituted by the addition of ten µl of Hi-Di Formamide (Applied Biosystems, CA, USA), and denatured on a thermal cycler at 95 °C for five minutes, 10 °C for two minutes and 4°C on hold. The ABI 3130xl Genetic Analyzer (Applied Biosystems, CA, USA) was then prepared for sequencing by the addition of Polymer (POP7) (Applied Biosystems, CA, USA) and 10 X EDTA Buffer (Applied Biosystems, CA, USA). The plate was then placed into the instrument and sequenced overnight.

2.2.3.9 DNA sequencing analysis

Sequences were obtained from the sequencer in Application Binary Interface (ABI) format and was imported into Sequencher v5.0 software (Gene codes, Ann Arbor, MI) for analysis. Sequences were trimmed and assembled to form contigs; chromatograms were then analyzed for the presence of mixtures and ambiguities. The consensus sequence was then exported in FASTA format into ClustalX v2.1 (Thomson et al, 1994) where sequences were automatically aligned against each other and an HXB2 subtype B reference sequence. Alignments were manually checked in Bioedit v.5.0.9 (Ibis Biosciences, An Abbott Company, CA. USA). To ensure no cross-contamination occurred between samples, PAUP v4.0 (Swofford et al, 2002) was used for the construction of neighbor-joining phylogenetic trees. Trees were viewed in Figtree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) and were checked for unusual clustering and short branch lengths. Sequences were submitted to the Stanford drug resistance database (http:/hivdb.stanford.edu) for genotypic interpretation. Reports were generated containing mutations and drug susceptibility profiles. The software interprets the sum of predicted resistance scores as being susceptible, potential low-level resistance, low-level resistance, intermediate resistance or high-level resistance.

The Viroseq HIV-1 Genotyping system was used for those samples that did not amplify by the in-house genotyping assay.

2.2.4 Viroseq HIV-1 Genotyping system

The Viroseq genotyping system (Celera Diagnostics, CA, USA). is a commercial kit used for the identification of mutations within the entire PR gene and approximately two thirds of the RT gene in the *pol* ORF, amplifying a 1.8kb product.

2.2.4.1 RNA extraction as described in section 2.2.3.1.

2.2.4.2 Reverse Transcription PCR

All reagents were thawed at room temperature, except for the enzymes, these were kept in the freezer until use. After thawing, reagents were vortexed gently and spun to collect droplets. Enzymes were not vortexed, only spun briefly. The PCR master mix was prepared first in a 1.5ml micro-centrifuge tube according to the Table 2.10. AmpliTaq Gold ™ was used due to its high specificity and efficiency while the Amp Erase Uracil-N-Glycosylate (UNG) has an anti contamination chemistry and aids in eliminating false positives.

Table 2.10 Reagents and volumes used to make up the PCR reaction master mix.

Reagent	Volume (µl/sample)	Final concentration
HIV PCR mix	29.5	-
AmpliTaq Gold polymerase (5U/µl)	0.5	2U
AmpErase UNG (1U/µl)	1.0	1U
Final volume	31.0	

The mix was vortexed briefly and spun for five seconds to collect droplets and was kept in the fridge until required. Thereafter the reverse transcription master mix was prepared as listed in Table 2.11.

Table 2.11 Reagents and volumes used to make up the reverse transcription master mix

Reagent	Volume (µl/sample)	Final concentration
HIV RT Mix	8	-
RNAse Inhibitor (20U/µl)	1	20U
Murine Leukemia Virus (MuLV) Reverse Transcriptase (50U/µl)	1	50U
DTT (100mM)	0.4	2mM
Final Volume	10.4	

The master mix was vortexed gently and centrifuged for ten seconds to collect droplets, the mix was used within 30 minutes of being prepared. Thereafter ten µl of thawed RNA was added into labeled 0.2ml PCR tubes, the RNA was then placed in the thermal cycler and run at 65°C for 30 seconds to relax the RNA structure, followed by 42°C for five minutes to cool to optimal enzyme activity, the reaction was then paused and tubes were removed from the machine. Ten µl of the freshly prepared RT master mix was added into each of the RNA tubes and mixed well. The tubes were then placed back onto thermal cycler and held at 42°C for 60 minutes for the reverse transcription reaction and then 99°C for five minutes to inactivate the Moloney Murine Leukemia Virus Reverse Transcriptase (MuLV RT) enzyme. The reaction was held at 4°C for at least ten minutes until use. Tubes were then spun for ten seconds and 30µl of the freshly prepared PCR master mix was added into each tube. Tissue was used to open each tube to prevent aerosol contamination. The samples were then loaded onto the thermal cycler and run under the conditions listed in Table 2.12.

Table 2.12 Thermal cycling conditions used for the reverse transcription PCR.

No. of Cycles	Temperature	Time	Process
1	50^{o}C	10 min	UNG activation
1	93^{o}C	12 min	AmpliTaq Gold activation and UNG inactivation
40	93^{o}C 64^{o}C 66^{o}C	20 sec 45 sec 3 min	DNA denaturation Primer annealing Primer extension
1	72^{o}C	10 min	Final extension
-	4^{o}C	HOLD (max 24 hours)	-

Following amplification the products were stored at -20 ^{o}C until further use.

2.2.4.3 PCR purification

PCR products were purified using the reagents provided in the Viroseq kit (Celera Diagnostics, CA, USA). Briefly a micon-100 spin column is inserted into one of the micron 1.5ml collection tubes, 300µl of Potassium Chloride (KCl) (200mM) (Celera Diagnostics, CA, USA), was added to the top of each column together with the entire 50µl of the PCR product. The micro-tube caps were secured to the top of each of the column and centrifuged for 15 minutes at 1400-1769 X g. Following which, 300µl of deionized water was added to the top of each column, caps were replaced and centrifuged again at the same speed for 15 minutes. For the elution, 35µl of sterile filtered water was added to each of the columns, which were then inverted into an empty micron tube and spun for five minutes at 1400-1769 X g. The columns were then removed and discarded, leaving behind 40-50µl of purified PCR product which can be stored for up to two weeks at -20 ^{o}C before use. The purified products were then run on a 1% Agarose gel. Briefly, six µl of product was mixed with five µl of gel loading dye and promptly loaded into the respective wells. Six µl of the DNA Mass Ladder provided with the kit was added to one of the wells. The gel was then electrophoresed at 10V/cm for 30 minutes, and was viewed as previously described. The concentration of the product was then determined using the scale indicated in Table 2.13.

Table 2.13 Band sizes and corresponding concentrations of DNA relative to a DNA mass ladder.

Band	DNA amount per six µl
2.0 kb	100 ng
1.2 kb	60 ng
0.8 kb	40 ng
0.4 kb	20 ng
0.2 kb	10 ng

Table 2.14 Concentrations and corresponding dilutions for purified samples.

If the intensity of the 1.5 kb PCR product band is:	Dilutions
> 100 ng	Make a 1:10 dilution with sterile filtered H_2O (e.g. 5 µl sample + 45 µl ddH_20)
60 – 100 ng	Make a 1: 4 dilution with sterile filtered H_2O (e.g. 13 µl sample + 39 µl ddH_20)
40 – 60 ng	Make a 1: 2 dilution with sterile filtered H_2O (e.g. 25 µl sample + 25 µl ddH_20)
20 – 40 ng	Adjust the volume of the sample to 50 µl (minimum volume for sequencing)
<20 ng	Sample is not suitable for sequencing , however the number of cycles can be increased to 35

Once the samples were diluted they were vortexed for five seconds and spun to collect droplets, the samples were stored at -20 ^oC until required.

2.2.4.4 Cycle sequencing reaction

Twelve µl of each of the custom primer mixes namely A, B, C, F, G and H and eight µl of the diluted sample were added directly to a 96 well optical plate into each of the respective wells and mixed well. The plate was vortexed for five seconds and centrifuged to collect droplets. The plate was then placed in the thermocycler and run under the thermal cycling conditions listed in Table 2.15, the number of cycles were increased if very feint bands were amplified.

Table 2.15 Thermal cycling conditions for the sequencing reaction

Temperature	Time	No of cycles	Process
96 ^{o}C	10seconds	25	Denaturation
50 ^{o}C	5seconds		Primer annealing
60 ^{o}C	4 minutes		Primer extension
4 ^{o}C	∞		Hold

Purification of sequencing products and running of the 3130XL Genetic Analyser (Applied Biosystems, was performed as previously described in section 2.2.3.8. However the filter set E and BigDye v3 was selected on the sequencer.

2.2.4.5 Analysis of results

Sequences were analyzed using the Viroseq HIV-1 genotyping system software v2.5 (Celera Diagnostics, CA, USA), which assembles the sequences, generating a consensus using primers from both PR and RT which are then aligned to an HIV-1 HXB2 reference sequence. Within the editing window, the codon positions, amino acid translation, reference sequence, sample sequence and the electropherograms are viewed. Sequences were edited and mixtures called, based on the following guidelines i) broad peaks near the beginning of a sequence are possibly dye blobs, ii) secondary peaks should be at least 30% of the primary peak iii) a sequence is considered a mixture if both the opposite sense sequences contain a secondary peak two times above the local noise iv) One sequence contains a secondary peak 30% of the primary peak and the sequence in the opposite direction contains a secondary peak <30%. In rare situations, a mixture will be seen in only one of the two sequences. In these cases, the secondary peak must be at least 30% of the primary peak and three times the local noise. Subtyping was performed using the REGA HIV-1 Subtyping tool version 3.0 (www.bioafrica.net/rega-genotype/html/). Thereafter the FASTA sequences were submitted to the Stanford drug resistance database for genotypic interpretation. Phylogenetic trees were drawn using PAUP v 4.0 and viewed in Figtree v1.4.1, to

ensure no cross contamination between samples had occurred as previously described in section 2.2.3.9.

2.2.4.6 Positive selection

Codon aligned sequences were uploaded to the HyPhy package on the Datamonkey webserver (http://datamonkey.org), which determines the rate of synonymous (D_s) to non synonymous (D_N) amino acid substitutions occurring at each site in the sequence. Ds<Dn is indicative of conservation of codon sites while Dn>Ds indicates more variability and diversification. The HyPhy package employs four approaches to determine sites under a positive selection pressure; here the test was performed using the Internal Branch Fixed Effect Likelihood (IFEL) as well as the Single Likelihood Ancestral Reconstruction (SLAC) algorithms using the HKY84 nucleotide substitution model (0.05 level of significance). SLAC is the most conventional counting method that involves reconstruction of the ancestral sequences using a single most likely ancestral reconstruction that considers all possible ancestral reconstructions or sampling from ancestral reconstructions (Kosakovsky Pond & Frost, 2005). The IFEL method determines the selection pressure that occurs on the internal branches of a tree.

2.2.4.7 Statistical analysis

Graph Pad Prism v5.0 (GraphPad Software, California) was used for all statistical testing. Descriptive statistical analysis was performed to determine the median and interquartile range (IQR) for age and viral load. Fischer's Exact test was used to compare the frequency of PR substitutions in treatment naïve and exposed patients. A p-value of <0.01 was considered significant (***). The test was performed by comparing the treated group to three separate groups of 700 sequences each. Substitutions with a 0.01 (**) and 0.001 (***) significant difference in at least two of the three groups were reported.

2.3 Results

2.3.1. Patient characteristics

A total of 156 patients failing their LPV/r inclusive regimen were studied, comprising 118 adults and 38 paediatric patients. Amongst the adults, 84 were female and 34 were male; the median age was 38 (IQR 32-43). For the paediatric patients, 25 were female and 13 were male; the median age was 12 (IQR 5-14). The median viral load for adults and paediatrics following LPV/r therapy was 53862 IQR (14192-185431) and 64298 (IQR 9753-213494) respectively. The median CD4 count (cells/µl) in adults (n=102) prior to LPV/r treatment was 135 IQR (72-209) and for paediatrics (n= 25) 280 IQR (114-473.5). CD4 data following LPV/r failure was not available for these patients. All patients were infected with HIV-1 subtype C. The most common treatment regimen amongst the adults was, AZT+ddi+LPV (n=66), TDF+3TC+LPV (n=27), while for the paediatric patients AZT+ddi+LPV (n=11), d4T+3TC+LPV (n=9) and ABC+3TC+LPV (n=7). In order to assess patient adherence, personal interviews were performed by clinicians, as well as records of pharmacy collection dates four months prior to genotyping. Consistent pharmacy collection dates were only available for 107 adult patients, of which 65 (61%) of patients had regular pharmacy collection dates. Most patients were on LPV/r treatment for less than one year.

2.3.2 Prevalence of Drug Resistance Mutations

Table 2.16 Prevalence of Drug Resistance Mutations (DRMs) within the cohort.

Resistance Category	Adults (n=118)	Paediatrics (n=38)	Adults and Paediatrics (n=156)	
*Any DRM	68(58%)	25(66%)	93(60%)	
^PI only	2(2%)	1(3%)	3(2%)	37 (24%)
-PI+other	28(25%)	6(18%)	34(22%)	
§NRTI+other	50 (42%)	20(53%)	70(45%)	
=NRTI DRMs only	2(2%)	5(13%)	7(4%)	
~NNRTI DRMs only	17(14%)	4(11%)	21(13%)	
//NNRTI+other	61(52%)	16(42%)	77(49%)	
--NRTI+NNRTI only	44(37%)	12(32%)	56(36%)	
#NRTI+NNRTI+PI	25(21%)	3(8%)	28(18%)	

*Any DRM - refers to patients that had mutations across any of the three drug classes
^PI only- refers to patients that had only PI mutations and no other mutations in the NRTI/NNRTI drug classes
-PI+other – refers to the number of patients that had PI mutations in combination with mutations from other drug classes (NRTI+NNRTI).
§NRTI+other - refers to the number of patients that had NRTI mutations in combination with mutations from other drug classes (PI+NNRTI).
=NRTI DRMs only – refers to the number of patients that had NRTI DRMs only and no other DRMs
~NNRTI DRMs only - refers to the number of patients that had NNRTI DRMS only and no other DRMs
//NNRTI+other – refers to the number of patients that had NNRTI mutations in combination with mutations from other drug classes (PI+NRTI).
--NRTI+NNRTI only - refers to patients that had only NRTI and NNRTI mutations and no PI mutations
#NRTI+NNRTI+PI - refers to patients that had mutations across all three drug classes

Within this cohort, 93 (60%) patients presented with any DRM, mostly prevalent in the NRTI n=70 (45%) and NNRTI n=77 (49%) drug classes. Amongst those patients harbouring resistance mutations, PI mutations were found in 37 (24%) patients; 30 were adults and seven were paediatric patients. All except three

patients had PI mutations in combination with other DRMs. Interestingly, 36% of patients presented with NRTI+NNRTI mutations, suggesting some adherence to at least this component of the regimen.

2.3.3 Protease Inhibitor mutations

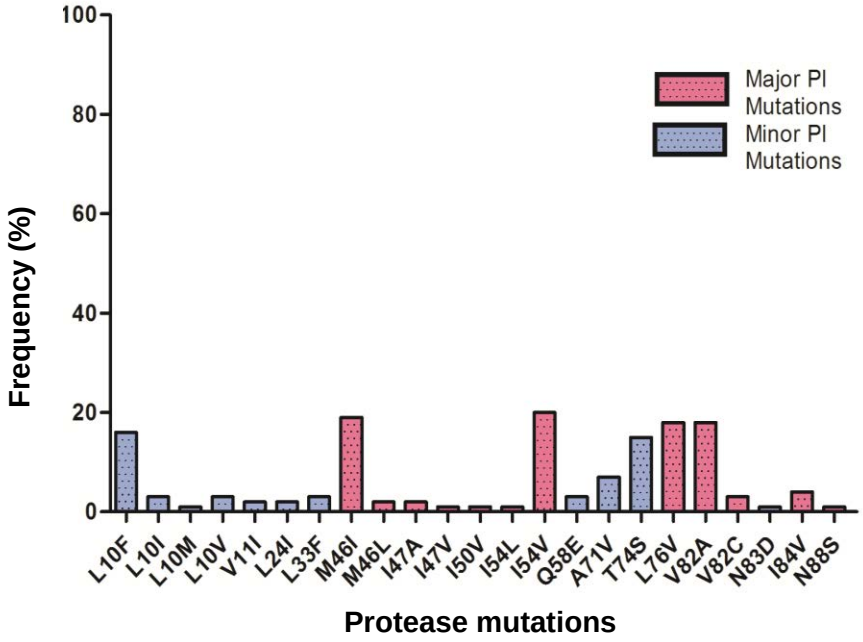


Figure 2.3 Frequency of Protease Inhibitor mutations in adult patients (n=118). The major mutations are shown in pink and the minor mutations are shown in blue.

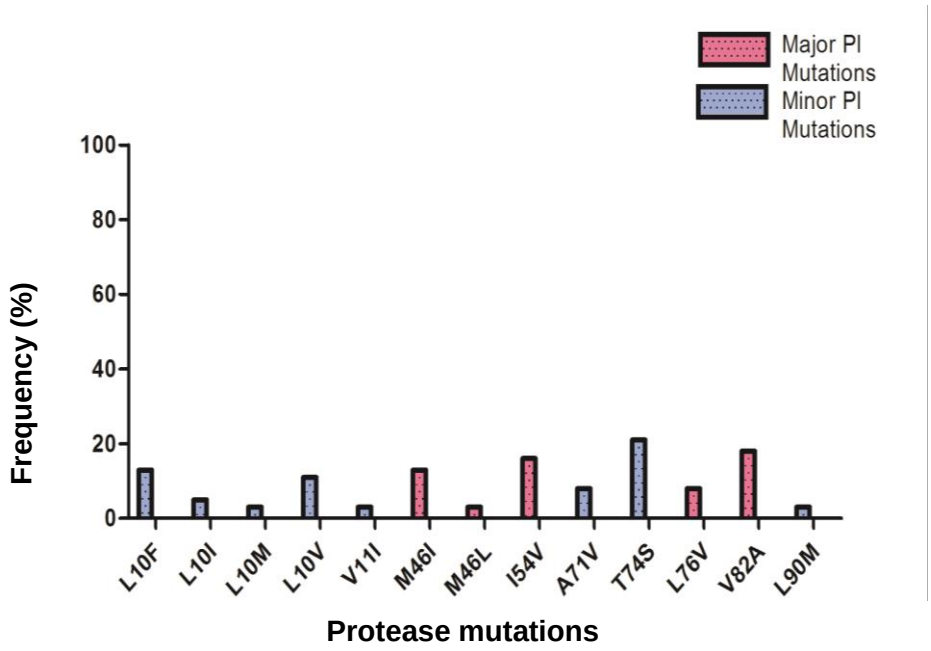


Figure 2.4 Frequency of Protease Inhibitor mutations in paediatric patients (n=38). The major mutations are shown in pink and the minor mutations are shown in blue.

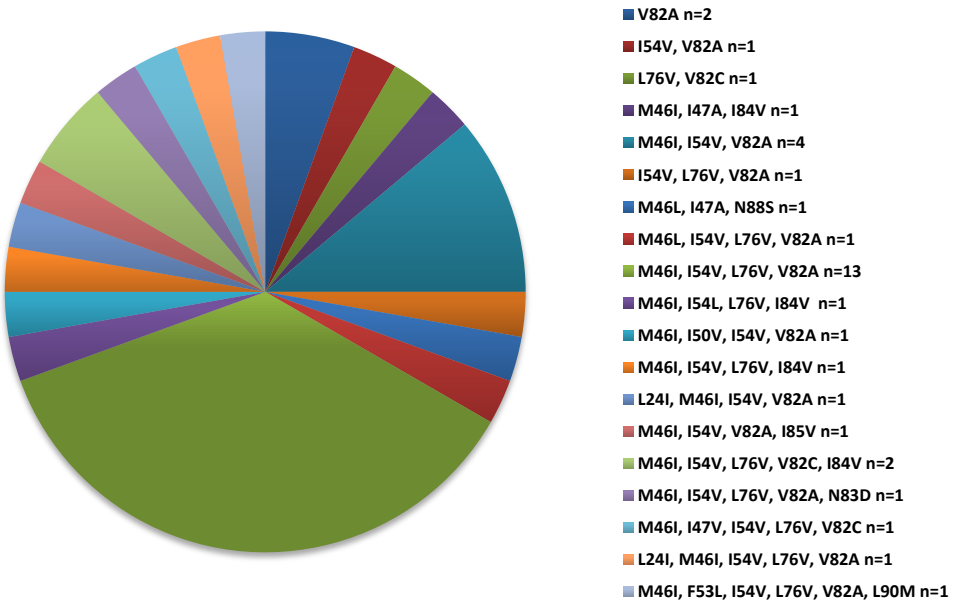


Figure 2.5 Pattern of Major Protease Inhibitor mutations amongst adult and paediatric patients with PI resistance [n=36, 24%].
*One paediatric patient presented with V11I only, a minor PI DRM, not indicated in this graph

Amongst the adult patients the most frequent major PI mutations were I54V [n=24, 20%]. M46I [n=23, 19%], L76V [n=21, 18%], V82A [n=21, 18%]. The most frequent minor PI DRMs found were L10F [n=19, 16%] and T74S [n=18, 15%]. Amongst the paediatrics studied, the most frequent major PI mutations were V82A [n=7, 18%], I54V [n=6, 16%], M46I [n=5, 13%], while the most frequent minor PI mutations were T74S [n=8, 21%], L10F [n=5, 13%] and L10V [n=4, 11%]. V82A was the only major PI DRM occurring alone, while some patients had up to six PI mutations. Interestingly, four of the patients with six PI mutations were on LPV/r for less than a year. The most common pattern of mutations was M46I, I54V, L76V and V82A (n=13) as shown in Figure 2.3. V11I was the only minor PI DRM occuring alone. In order to identify novel mutations selected as a result of PI drug pressure, a comparison between treated and naïve sequences was performed. A significant increase in frequency in the treated group were only found at positions known to cause drug resistance: L10F, K20R, M46I, I54V, A71V, L76V, and V82A (Fischer's exact Test, $p<0.001$). No novel mutations in PR were identified.

2.3.4 Reverse Transcriptase Inhibitor mutations

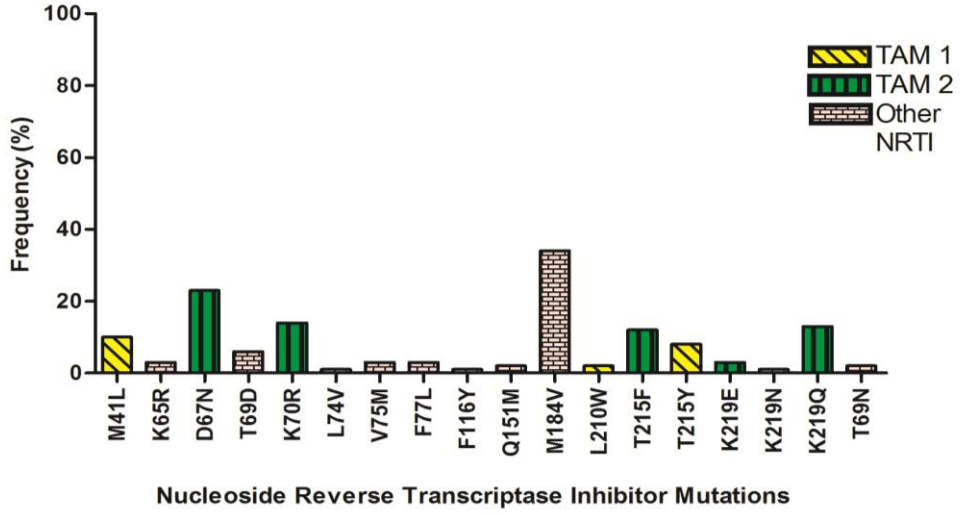


Figure 2.6 Prevalence of Nucleoside Reverse Transcriptase Inhibitor mutations amongst adult patients n=118.

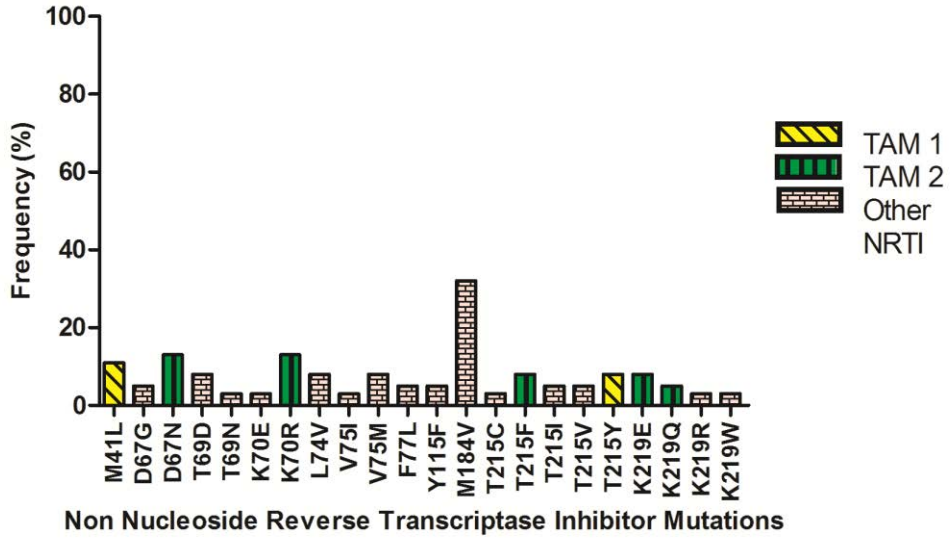


Figure 2.7 Prevalence of Non-Nucleoside Reverse Transcriptase Inhibitor mutations amongst paediatric patients n=38

Amongst adult and paediatric patients, M184V was the most prevalent NRTI DRM: [n=40 (34%)] and [n=12 (32%)], respectively. There were 39 adult patients that presented with TAMs: 22 had TAM 2 only, two had TAM 1 only and 15 had both TAM1+2. For the paediatric patients, 12 patients harboured TAMs: seven had TAM 2 only, and three had TAM 1 only, while two had both TAM 1 + 2. K65R was found in only three adult patients despite 27 patients receiving TDF-inclusive second-line treatment. Interestingly, paediatric patients harboured many substitutions at codon 215, (T215C/F/I/V/Y). However T215F/Y were the only substitutions that occurred at this codon amongst the adult patients.

Figure 2.8 Non–Nucleoside Reverse Transcriptase Inhibitor mutations amongst adult patients n=118. The most prevalent NNRTI mutation was K103N.

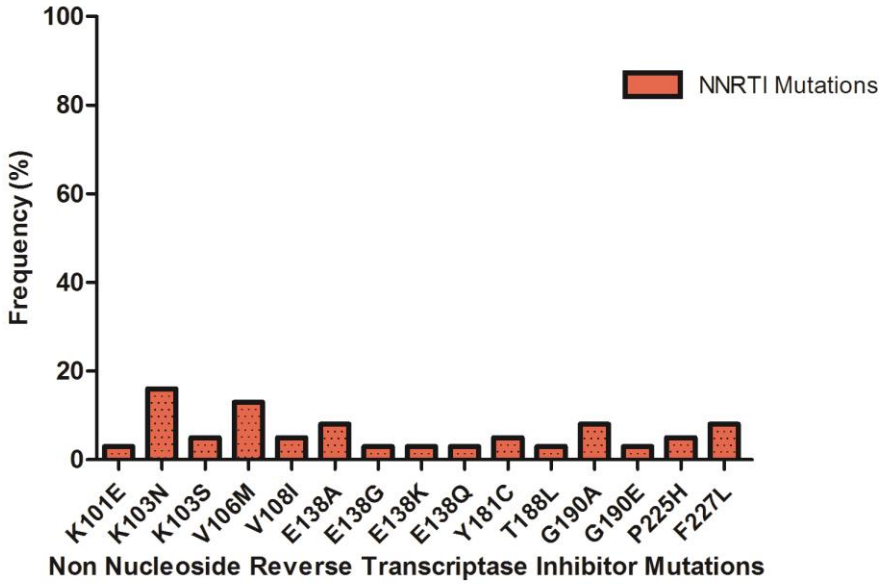


Figure 2.9 Non–Nucleoside Reverse Transcriptase Inhibitor mutations amongst paediatric patients n=38. The most prevalent NNRTI mutations were K103N and V106M.

For the NNRTI drug class, 61 (52%) adult patients had NNRTI mutations; K103N was the most prevalent occurring in [n=58 (49%)]. While [n=16 (42%)] paediatric patients had NNRTI mutations, the most prevalent NNRTI mutations were K103N [n=6 (16%)] and V106M [n=5 (13%)]. In addition, F227L was present only in the paediatric group.

2.3.5 Positive selection

Table 2.17 A test for positive selection amongst the sample and control groups (n=156 and n=423) respectively.

	Subjects			
Algorithm	Treated (n=156)		Naïve (n=423)	
	PR	RT	PR	RT
Both SLAC and IFEL	12, 19, 63	36, 48, 123, 184.	12, 15, 19, 63, 74	36, 123, 165, 173, 174
IFEL only	20, 82	219	None	None
SLAC only	15, 37,74.	162, 165, 166, 173, 174, 200.	37, 60	48, 162, 166, 200

Positive selection using the IFEL algorithm identified 11 positively and 138 negatively selected sites amongst the treated patients, while SLAC identified 16 positively and 173 negatively selected sites. Amongst the naïve patients, IFEL detected 22 positively and 381 negatively selected sites, while SLAC found 25 positively and 394 negatively selected sites. Sites that were selected in the treated group only were 20 and 82 in PR and sites 184 and 219 in RT. Most of the other positively selected sites were common in both the treated and naïve groups.

2.4 Discussion

In this large cohort of second-line failure patients from KZN SA, 60% of the patients harboured any DRM, with only 37 patients (24%) harbouring viruses with major LPV/r mutations. While still low, this is higher than reports from other provinces in SA (Wallis et al, 2011, Van Zyl et al., 2013). The prevalence of PI DRMs in other studies have been largely attributed to non-adherence (Wallis et al, 2011, Ajose et al, 2012, Levison et al, 2012, Osinusi-Adekanmbi et al., 2014).

In addition, it has been shown that for PIs, the time spent in the mutant selection window during which drug resistant viruses are rapidly selected is very short, and with non-adherence, the wild-type virus rapidly emerges and gains a selective advantage (Rosenbloom et al., 2012, Siliciano and Siliciano, 2013). However the characteristic higher genetic barrier of LPV/r may also account for the lower frequency of PI DRMs. The genetic barrier for the NNRTI and NRTIs are lower and would consequently result in a higher frequency of NNRTI/NRTI DRMs (Tang and Shafer et al, 2012). A third possibility is the development of PI resistance mutations in other parts of the viral genome, such as gag and envelope.

Amongst those patients with PI resistance the most prevalent PI DRMS were M46I, I54V, L76V and V82A, all of which are major PI DRMS (Tang and Shafer et al, 2012). The most frequent minor PI DRMs were L10F, T74S, and A71V. Major mutations are often selected first and alter the drug binding site of PR for both the drug and the natural substrate, resulting in a less fit virus with reduced infectivity, while the accessory mutations play a compensatory role in restoring viral fitness (Race, 2001). Two mutational pathways describe LPV/r failure, firstly mutations (M46I, I54V and V82A) which retain sensitivity to DRV/r, versus. a second pathway described by patients harbouring the L76V mutation, and less commonly (V32I, I47A and I50V), this describes a pathway conferring DRV/r cross-resistance (Tang and Shafer et al, 2012). The L76V mutation is a distinctive LPV/r DRM in both

subtype B and C and has a strong impact on DRV/r resistance (Lambert-Niclot et al., 2008). Interestingly, the major PI DRMs appeared to be accumulated in distinct patterns, where V82A was the only mutation that occurred alone, followed by V82A+ I54V, then V82A+I54V+M46I. This combination of mutations was seen in four patients, and has been previously reported for subtype B and C. The addition of L76V to this combination was seen in 13 patients and was accompanied by the presence of an accessory PI DRM, L10F in all 13 patients. While this has also been observed by Van Zyl et al (2013) in subtype C patients, this is distinctly different to subtype B. The L10F mutation could improve replication capacity, as shown by Race et al (2001) where variants containing a pattern of M48I/L63P/V82T/I84V and L10R/M46I/L63P/V82T/I84V were able to replicate at the same at the rate as wild-type virus. (Race et al, 2001). However, more work will be required to investigate the fitness costs associated with the most frequent pattern of mutations (M46I/V82A/I54V/L76V/L10F) seen in our study. Interestingly there were four patients that were able to accumulate six drug resistance mutations within less than one year of receiving LPV/r treatment, while seven patients were able to accumulate four DRMs between the first and second year of LPV/r treatment, largely suggesting that mutations in PR can develop quickly despite the drug's high genetic barrier (Tang and Shafer et al, 2012).

To further identify mutations that arose as a result of exposure to LPV/r, a comparison of our LPV/r treated patients with downloaded treatment naïve sequences was performed. A significant increase in frequency in the treated group was found for DRMs: L10F, K20R, M46I, I54V, A71V, L76V, and V82A and no novel PR mutations were detected. While, the K20R mutation is regarded as an accessory mutation that plays a compensatory role in patients that have other PI resistance mutations, other studies have found a high prevalence of K20R in non-B treatment naïve patients (Bessong et al, 2008, Descamps et al., 2009). A study by Descamps et al (2009) showed that the K20R mutation results in a better virological response to a DRV/r containing regimen, as opposed to the K20I

mutation (Descamps et al. 2009). A71V is considered a minor PI mutation and is present in (2-3%) of the untreated population and increases in frequency in patients receiving PIs. The co-existence of V82A and A71V results in a significant increase in viral fitness (Race et al, 2001).

Mutation M184V was the most frequent NRTI mutation amongst both adults and paediatric patients, attributed to the use of 3TC/FTC during first or second-line treatment. Despite 27 patients receiving TDF-inclusive regimens, only three patients harboured the K65R mutation, the presence of the TAMs prevents the selection of K65R, and thus may account for the low frequency of this mutation (Tang and Shafer et al, 2012). The high percentage of the NNRTI mutation, K103N, is a result of an NNRTI-containing first-line regimen. Many studies have shown that K103N can persist between one to three years after stopping NNRTI treatment, due to the fitness advantage conferred by this mutation (Cong et al., 2007). Fortunately, the second-generation NNRTI, ETR would still be a good option for the majority of patients, as the presence of K103N does not confer cross-resistance to ETR (Tang and Shafer et al, 2012).

Positive selection results indicated that the majority of codons in PR were under negative selection pressure, suggesting that very little sequence variation can be tolerated in order to preserve PR functional activity. (Gordon et al, 2003, Banke et al., 2009). Positively selected sites that were common in both the treated and naïve groups are most likely the result of immune selection pressure and not the influence of drug pressure. The codons under positive selection in PR (in both naïve and treated isolates included: 12, 15, 19, 37, 63 and 74 and in RT: 36, 48, 123, 162, 165, 166, 173, 174 and 200. Most of these codons are part of the genetic signature of C viruses (Banke et al, 2009). The only codons that were positively selected in PR in the treated isolates only, were at codons 20 and 82; codon 82 is associated with the major PI DRM, V82A, while codon 20 is an accessory PI DRM and may also play a compensatory role in improving fitness but also results in a

>20 fold decrease in LPV/r susceptibility (Barber et al., 2012). For RT, codons 184 and 219 were positively selected in the treated group only, and are associated with the DRMs M184V and K219E/Q/R/W/N respectively, and are an indication of RT-inhibitor treatment. It is evident that selection pressure due to ARV therapy is the main force driving the development of DRMs in the *pol* gene (Banke et al, 2009).
In summary, PI drug resistance does not seem to be the major cause of LPV/r failure given that only 24% of patient's harboured resistance to LPV/r. Intermittent drug-adherence may account for the emergence of PI resistance amongst these patients.

Overall non-adherence seems to be the major cause of treatment failure in the remaining patients. Measuring the LPV/r concentration using serum levels or hair sampling may also help to out rule adherence as the primary cause of failure. However sampling at random as opposed to a longitudinal analysis may not be completely accurate and is thus subject to variability in these patients. Additionally, the high genetic barrier associated with PIs, as well as the presence of DRMs in other parts of the viral genome such as *gag* or *env* may also be likely contributors to treatment failure. For those patients without resistance, LPV/r and recycled NNRTIs/NRTIs can still be used in the presence of added adherence counselling. Despite the distinct pattern of four PI DRMS (M46I, I54V, V82A, and L76V) identified amongst those with PI resistance, most patients still retained susceptibility to DRV/r and will thus still be a good third-line option. However, for those harboring the L76V mutation cross-resistance to DRV/r is likely, nevertheless this drug can still be used, as additional DRV/r resistance associated mutations are required for high-level DRV/r resistance.

CHAPTER 3

Drug resistance amongst patients failing both Lopinavir and Darunavir using Ultra Deep Pyro-Sequencing.

3.1 Introduction

Access to third-line ART in SA is limited to patients who have documented resistance to LPV/r and must be authorized by an expert committee. For these patients, DRV/r and other ARVs such as ETR and RAL, depending on the patient's resistance profile and ART history, are usually prescribed (Department of Health and Human Services, 2015). Darunavir is a second-generation PI that was developed for use in treatment-experienced patients. Despite this, studies have shown that distinct DRV/r resistance associated mutations can develop and contribute to treatment failure (Delaugerre et al., 2008, de Meyer et al., 2008, Lambert-Niclot et al., 2008, Descamps et al., 2009). This aspect of the study describes DRV/r resistance mutations in patients from KZN, and investigates the role of low frequency mutations (or minority variants) present at LPV/r failure and DRV/r failure. Minority populations are generally missed by Sanger sequencing, however the clinical value of detecting these variants is well described in the literature (Le et al, 2009, Avidor et al, 2013). Therefore, minority variants that are present at LPV/r failure may inherently increase in frequency and contribute to DRV/r resistance. Here, the Roche 454 platform^{TM} (Branford, CT, USA) was used to perform UDPS for the detection minority variants in PR, RT and IN. The IN sequences serve as baseline data prior to RAL treatment. Given the limited availability of treatment options beyond third-line, these drugs must be preserved and used sparingly.

3.2 Materials and Methods

3.2.1 Study population

Twenty patients from the PCSK cohort were switched to a third-line regimen comprising a combination of either DRV/SQV/ATV or 3TC/TDF/AZT, ETR and RAL. Of these patients, seven presented with viral loads >1000cpm and were genotyped; six of these patients showed distinct DRV/r resistance associated mutations while the remaining patients did not show any additional PI mutations. Five of the six patients were sequenced using UDPS at LPV/r and DRV/r failure. The sixth sample was not available for analysis.

3.2.2 Ultra Deep Pyro-Sequencing

The Roche 454^{TM} platform was used for UDPS according to the manufacturer's instructions. The procedure incudes an RNA extraction, RNA purification, cDNA synthesis, PCR amplification, generation of the HIV-1 amplicon library, amplicon purification, quantification, qualitation, amplicon pooling, emulsion PCR (emPCR), and sequencing.

3.2.2.1 RNA extraction

The assay recommends using HIV-1 infected plasma with a viral load of >2000 copies per ml. Briefly, one ml of each plasma sample was aliquoted into a 1.5ml screw capped tube and labeled with an orientation marking indicative of the pellet. Tubes were centrifuged in an ultracentrifuge at 25000 X g for two hours at 4°C. After removal of the supernatant, RNA was extracted using the QiAmp Viral RNA Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The final elution was performed using 50µl of Buffer AVE supplied with the extraction kit. Bacteriophage MS2 carrier RNA (10ng/µl) (Roche Life Science, Branford, CT, USA) was added to the freshly extracted RNA to improve the stability of the RNA.

3.2.2.2 RNA purification

RNA was purified using the Agencourt RNA Clean XP beads (Beckman, Coulter, Brea CA). These beads are paramagnetic once placed in a magnetic field, and allow the collection and re-suspension of the beads once the magnetic field is removed. The purification was carried out in a 96 well optical plate where 45µl of freshly extracted RNA was added into columns A1-10. Before use, the AMPure RNA clean XP beads were thoroughly vortexed for three minutes until a homogenous suspension was formed, following which 81µl was added into each of the sample wells and mixed five times. The plate was incubated at room temperature for ten minutes and then placed on a 96 well Magnetic Ring Stand (MRS) (Life Technologies, Carlsbad, CA) for five minutes. The supernatant was carefully removed and discarded without disturbing the beads. Thereafter 200µl of freshly prepared 70% ethanol was added into each of the sample wells and the plate was gently swirled five times around the MRS to dislodge the beads. The plate was then placed on the MRS for 30 seconds. The supernatant was removed and discarded, a total of three 70 % ethanol washes were performed. Following the final wash, all ethanol was completely removed and beads were left to air dry for no longer than five minutes. For the final elution, 15µl of nuclease free water was added into each of the sample wells and mixed ten times. The plate was placed back on the MRS for one minute, during this time the V Type HIV-1 cDNA 1-1 primer plates (Roche, Applied Science, Mannheim, Germany) containing lyophilized primers were centrifuged at 900 X g for 30 seconds to collect droplets and was placed on a plate cooler for at least five minutes before use. The plates contained primers spanning PR, RT as well as IN. Thereafter, 13.5µl of the supernatant from the RNA elution was transferred into the corresponding wells of the cDNA primer plate. The positive and negative control was included in well 11 and 12 respectively. The plate was sealed with an adhesive foil and centrifuged at 900 X g for 30 seconds.

3.2.2.3 cDNA Synthesis and PCR amplification

The cDNA synthesis was performed using the Transcriptor First Strand cDNA synthesis Kit (Roche Applied Science, Mannheim, Germany). All reagents except enzymes were vortexed for five seconds and centrifuged for ten seconds before use. The cDNA master mix was prepared in a 1.7ml micro-centrifuge tube as indicated in Table 3.1.

Table 3.1 Reverse Transcriptase Master Mix

Reagent	**(µl)/well**	**Final Concentration**
5X Transcriptor RT Reaction Buffer	4	1X (8mM)
dNTPs (10mM)	2	1mM each
Protector RNASE Inhibitor (40U/µl)	0.5	20U
Transcriptor Reverse Transcriptase (20U/µl)	0.5	10U
Total Volume	7	

The master mix was gently vortexed, spun in a bench top minifuge and placed on ice until use. The plate was then obtained and placed on the thermocycler at 65°C for ten minutes, and promptly cooled on ice for two minutes. Seven µl of the freshly prepared cDNA master mix was added into each of the wells and mixed well. The plate was sealed with adhesive foil and centrifuged at 900 X g for 30 seconds. The reaction was run on the thermocycler at 50°C for 60 minutes, 85°C for five minutes and left on hold at 4°C for no longer than two hours. Once the reaction was complete the plate was centrifuged again and one µl of RNase H (Life Technologies, Invitrogen, Carlsbad, CA) was added to each well. The plate was centrifuged again and placed on the thermocycler at 37°C for 20 minutes and then 4°C on hold.

Multiplex Identifiers (MIDs) were added onto each primer sequence as indicated in Figure 3.1. This allows a system of barcoding and an automated software identification of samples after they have been pooled together.

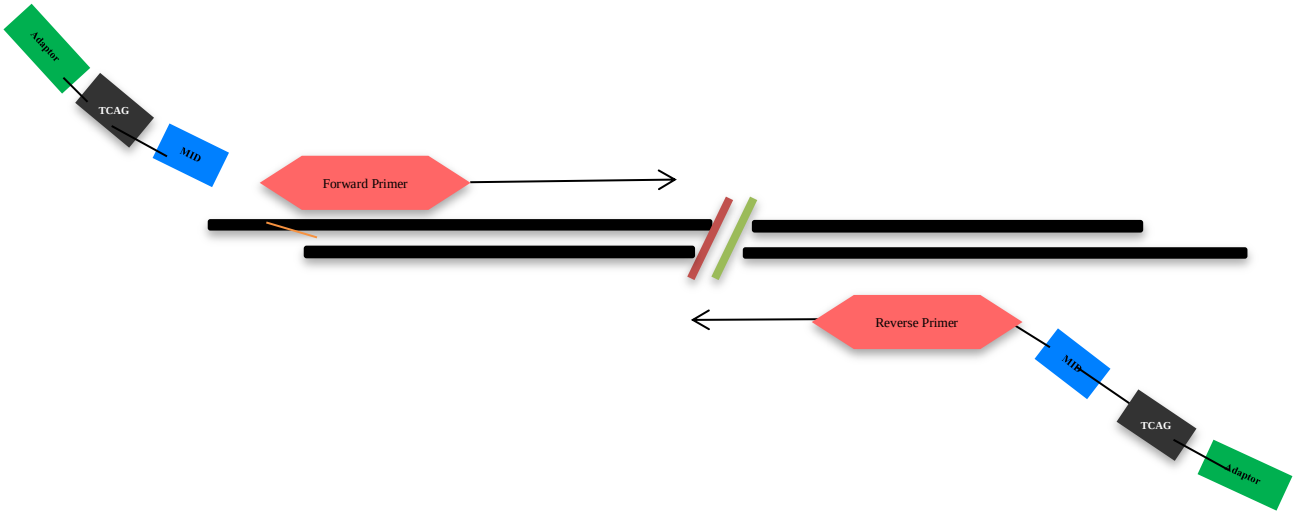


Figure 3.1 Illustration of the Multiplex Identifiers. Each well in the primer plate contains amplicon specific primers comprising adaptors that are 5' extensions with binding sites for the forward (A) (Primer A- Key) 5- CGTATCGCCTCCCTCGCGCCA and reverse (B) (Primer B – Key) 5' CCTATCCCCTGTGTGCCTTGGCAGTC, to which the key sequence (TCAG) is attached. The key sequence refers to a known sequence of four nucleotides which is followed by the MID. (Adapted from the Amplicon Library Preparation Method Manual, Roche 454^{TM} Life Sciences, Branford, CA, USA).

A PCR master mix was made using the Fast Start Hi Fidelity PCR System (Roche Applied Science, Mannheim Germany) which encompasses a unique blend of Fast Start Taq DNA polymerase and a thermostable chemically modified proofreading protein without polymerase activity. The PCR master mix was made as indicated in Table 3.2 (Roche 454 ^{TM}, Life Sciences, Branford, CA, USA).

Table 3.2 Composition of the PCR master mix using the Fast Start Hi-Fi Kit.

Reagent	(µl)/well	Final concentration (mM)
Nuclease Free Water	16.5	-
Fast Start Hi Fidelity Reaction Buffer w/o $MgCl_2$ (10X)	2.5	1 X
$MgCl_2$ (25mM)	2.25	2.56
dNTPs (10mM)	0.5	0.23
Fast Start High Fidelity Enzyme Blend (5U/µl)	0.25	1.25U
Total volume	22	

The PCR mix was gently vortexed, centrifuged and placed on ice until use. PCR primer plates were prepared as previously described for the cDNA plates in section 3.2.2.3. Using a multichannel pipette 22µl of PCR master mix was added into each well of rows A-E of the PCR primer plate using a new tip for each well. The cDNA plate was removed from the thermocycler and centrifuged at 900 X g for 30 seconds to collect droplets and was placed on a plate cooler. Using a multichannel pipette three µl of cDNA was then transferred from row A into each of the rows (A-E) of the PCR plate containing the master mix. The PCR plate was sealed, centrifuged and run under the following thermal conditions. Once the reaction was complete the plate was stored at -20°C until required.

Table 3.3 Thermal cycling conditions for the PCR amplification.

No of cycles	Temperature	Time
1	95°C	3 minutes
43	95°C	30 seconds
	55°C	20 seconds
	72°C	50 seconds
1	72°C	8 minutes
1	4°C	Hold

3.2.2.4 Amplicon purification

Purification of amplicons was performed using the Agencourt Ampure XP Kit (Beckman Coulter, Brea, CA, USA). Firstly, amplicons were immobilized onto a new 96 well optical plate-containing 22.5µl of molecular biology water; 22.5µl of the amplicons were transferred into corresponding wells. The bottle of Ampure beads was thoroughly vortexed for three minutes to ensure a homogenous suspension and 45µl was added into each well and mixed at least five times. The plate was incubated at room temperature for ten minutes to allow the paramagnetic beads containing amplicons to bind. The plate was placed on the MRS for five minutes to allow the separation of the beads from the supernatant, which was then removed and discarded using a multichannel pipette. The next step involved contaminant removal, where 100µl of freshly prepared 70% ethanol was added into each of the wells. The plate was then swirled five times across the MRS to dislodge the beads into the liquid and was placed back on the MRS for one minute. The supernatant was removed and discarded; this process was repeated. After the last wash it was important to remove as much of the ethanol as possible. The plate was then left to air dry for five to ten minutes. Thereafter, using a multichannel pipette, 20µl of freshly prepared 1 X Tris EDTA (TE) buffer (Sigma, Aldrich, USA) was added into each well. The plate was swirled across the MRS and thereafter incubated for one minute at room temperature. Lastly, the clear supernatant was removed and transferred onto a new 96 well optical plate keeping the same plate layout.

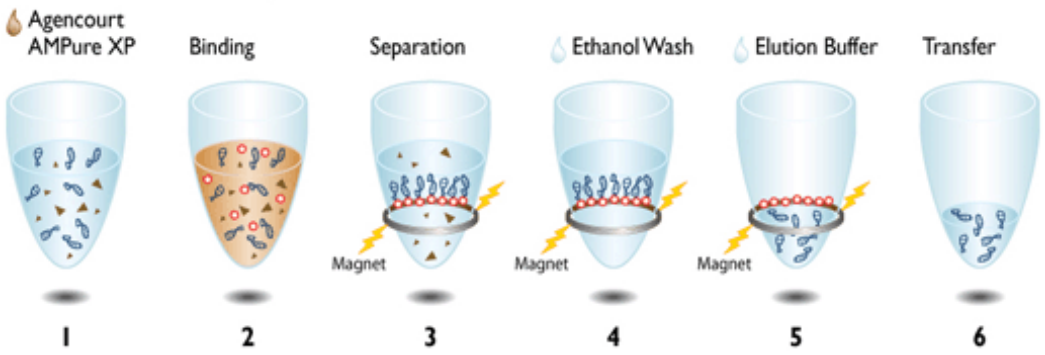
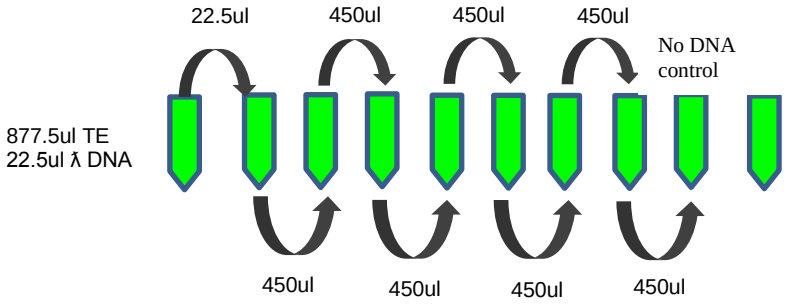


Figure 3.2 Amplicon purification using Agencourt Ampure XP beads (Beckman Coulter, Brea, CA, USA).

3.2.2.5 Quantification of amplicons

The Quant-iT Picogreen dsDNA Assay kit (Invitrogen, Life Technologies, Carlsbad, CA) employs Picogreen, an ultra-sensitive fluorescent nucleic acid stain for quantitating dsDNA. Both the Lambda DNA standard and the Picogreen reagent were thawed according to the manufacturer's instructions. Lambda DNA was serially diluted across ten 1.5ml micro-centrifuge tubes, as indicated in the Figure 3.3.



A

Tube	Concentration (ng/µl)	Tube	Concentration (ng/µl)
1	250	6	7.81
2	125	7	3.91
3	62.5	8	1.95
4	31.2	9	0.98
5	15.63	10	0

B

Figure 3.3 A) Serial dilution to quantify the amplicons. This was performed using the Lambda DNA standard, tube one contained 877.5µl of I X TE buffer and tubes 2-10 had 450µl of 1 X TE buffer, at each transfer tubes were vortexed and spun for five seconds before transferring into the next tube. B) Final concentrations for each of the serial dilutions.

From each of the serially diluted tubes 100µl was transferred into the first row of a black 96 well Micro plate (Bio-Rad, Hercules, CA. USA) and 97µl of 1 X TE Buffer was added into all the sample wells using a multichannel pipette followed by the addition of three µl of the purified sample. The Picogreen reagent was prepared by making a 1:200 dilution (three µl Picogreen + 600µl 1X TE Buffer) this was vortexed well and 100µl was added into all wells and mixed at least five times. The plate was read on the Glomax Modulus Micro plate Flourometer (Promega, State of Wisconsin, USA) as described by the Quant-It Picogreen dsDNA Assay Kit insert. The Comma Separated Values (CSV) file was exported from the Glomax and used to construct a standard curve using Relative Fluorescent Units (RFU) versus concentration. An R-value of >0.98 was considered acceptable.

Table 3.4 The DNA standard and the sample values were used to determine the concentration of each amplicon in molecules/µl

Molecules/µl	Sample concentration (ng/µl) x 6.022 x10^{23}
	656.6 X 10^{9} x Amplicon length (bp)

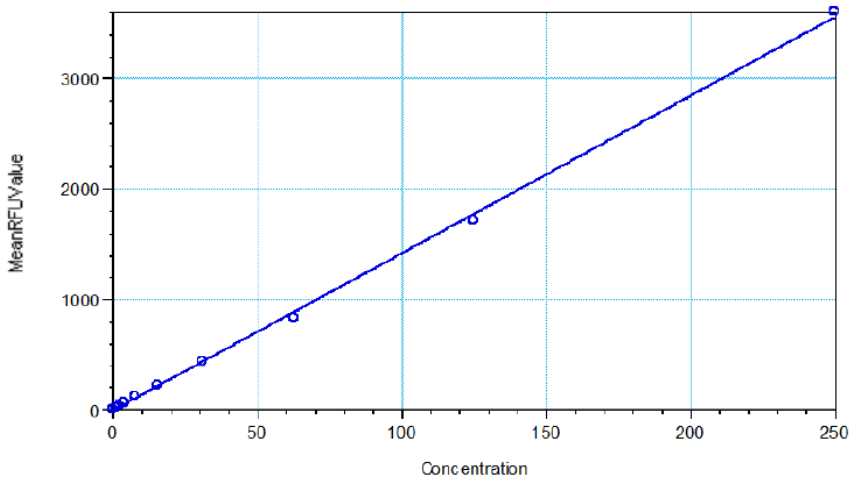


Figure 3.4 Standard curve used to quantitate amplicons by the Quant -it Picogreen dsDNA assay kit (Invitrogen, Life Technologies, Carlsbad, CA).

3.2.2.6 Amplicon qualitation

Amplicons that were quantified at <5ng/µl were further evaluated on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Procedures describing the use of the bioanalyzer is described in Appendix B. The bioanalyzer is used to ensure that all amplicons are between 400-500bp. Amplicons with bands that are less than 300bp are indicative of primer dimer. If these primer dimer bands were less than a 3:1 molar ratio relative to the correct sized products then these amplicons were further purified. This was done to prevent the preferential amplification of primer dimer during the sequencing reaction. Primer dimers that were greater than a 3:1 molar ratio relative to the product were not included. The bioanalyzer also provides an electropherogram which indicates an upper marker and a lower marker, the middle peak is indicative of the product at 400-500bp while other peaks indicate primer dimer. This is shown in Figure 3.5.

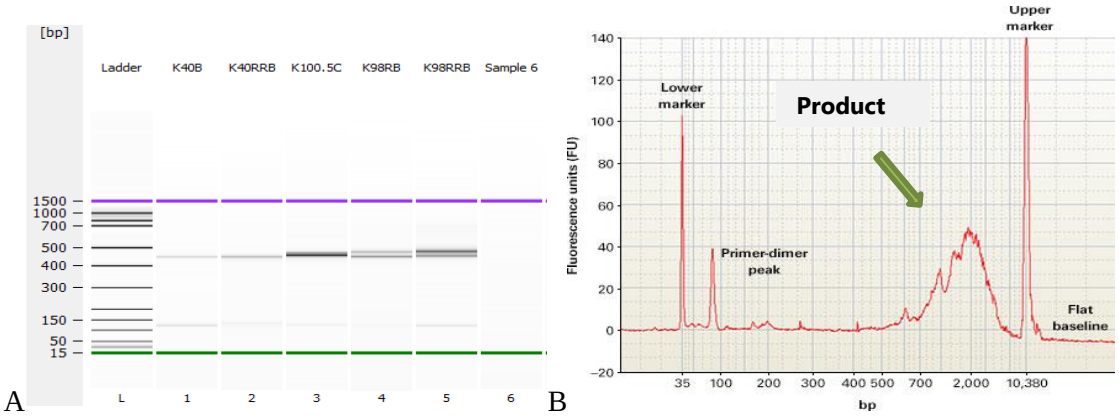


Figure 3.5 A) Gel picture generated by the bioanalyzer. All samples showed products between 400-500bp, primer dimer was present at 100bp in a few samples, however these were <3:1 ratio relative to the products and were thus ignored. B) Electropherogram shows slight primer dimer <3 times the height of the products can be seen.

3.2.2.7 Normalization of amplicons

All amplicons had to be present at equimolar ratios for the emPCR. Therefore amplicons were normalized to $5x10^9$ molecules/µl based on the concentration obtained from the dilution calculator on the my454.com website (my454.com). Dilutions were performed using 1 X TE buffer. In a new 96 well plate the required volumes of 1 X TE buffer and sample was added. The sample pool was then prepared by transferring all the samples from each column into the respective last row of each column. The final master pool was prepared by removing the entire volume of sample from the last row of the plate and transferring into a single 1.7ml eppendorf. The final master pool was diluted from $5x10^9$ to $1x10^9$ molecules/µl (ten µl of the 5 $x10^9$ master pool + 40µl of 1 X TE buffer) and was vortexed gently. This master pool was then purified to ensure all short fragments were removed before the emPCR.

3.2.2.8 Purification of the master pool.

In a single well of a new 96 well plate, 22.5µl of molecular water + 22.5µl of the $1x10^9$ molecules/µl master pool + 45µl of Ampure XP beads and was mixed at least five times to ensure a homogenous suspension, the plate was incubated for ten minutes at room temperature and five minutes on the MRS. With the plate on the MRS the supernatant was removed and discarded without disturbing the beads followed by the addition of 100µl of freshly prepared 70% ethanol. The plate was swirled across the MRS to wash the bead pellet and thereafter placed back on the MRS for one minute. The supernatant was removed and discarded, the process was repeated to obtain a total of two washes after the last wash, as much of the ethanol as possible was removed and the beads were left to air dry for five minutes. To re-suspend the beads, 22.5µl of 1 X TE buffer was added and the plate was swirled across the MRS followed by an incubation of one minute on the MRS. The supernatant was removed and transferred to a new well on the same plate, 22.5µl of molecular water and 45µl of Ampure beads was added and mixed

well. The procedure was repeated as before, once the 1 X TE buffer was added the supernatant was removed and transferred to a new 1.7ml tube for quantification.

3.2.2.9 Quantification of the twice-purified master pool

The twice purified master pool was quantified using the Quant it dsDNA Picogreen assay as previously described in section 3.2.2.5. Ten eppendorf tubes were labeled 1-10, and 441µl of 1 X TE buffer was added to the first tube and 225µl of 1 X TE was added into the remaining tubes. Nine µl of DNA standard was added into the first tube and vortexed for five seconds making a 1:50 dilution of the DNA standard, 225µl from tube one was transferred into tube two and vortexed, the serial dilution continued to tube nine where 225µl was removed and discarded leaving tube ten as the no DNA control. One hundred µl of each standard was transferred onto the black 96 well Micro plate, into a single column and 90µl of 1 X TE buffer was added into one of the wells. This was followed by the addition of ten µl of the twice-purified master pool. A 1: 200 dilution of Picogreen was prepared and 100µl was added into the respective wells containing the standard and the sample. The plate was then read on the Glomax Modulus Micro Plate Flourometer as described in section 3.2.2.5. The master pool was then diluted to $2x10^6$ molecules/µl using 1 X TE buffer, constituting the final purified master pool.

3.2.2.10 Emulsion PCR

Emulsion PCR refers to a process of emulsion based clonal amplification whereby DNA library fragments are attached to micron sized beads. The emPCR was performed on sepharose beads carrying immobilized primers that are complementary to the B adaptors of the library. For clonal amplification of a single molecule per bead a 1:1 ratio of library fragments to beads is required (Avidor et al, 2009). Therefore correct library quantitation is crucial, as no readable sequence can be obtained from those beads that have no DNA fragments or less than one fragment bound. During the initial process of the amplification reaction, the DNA

library fragments capture beads, and enzyme reagents are subject to vigorous shaking in small cylindrical plastic containers. This creates water droplets around the beads, referred to as an emulsion. The water droplets generated by the emulsion act as PCR micro-reactors containing biotinlyated primers, buffers, nucleotides, salts and DNA polymerases that causes a single and isolated DNA fragment in each droplet to be amplified into millions of copies of DNA, which are clones of a single library molecule (Roche 454 ^{TM} Life Sciences, Branford, CA, USA). The emPCR was performed according to the methods provided in the Roche 454 ™ emulsion PCR Manual (my454.com) and is described in Appendix C.

3.2.2.11 Sequencing

The GS Junior uses sequencing by synthesis approach whereby ssDNA is synthesized by synthesis of the complementary strand one base pair at a time. The reaction utilizes ssDNA as a template, sequencing primers, the enzymes DNA polymerase, ATP sulfurylase, Luciferase and Apyrase, as well as two substrates, Adenosine 5' phosphosulfate (APS) and Luciferin. The nucleotide bases TCAG are flowed sequentially in the same order across the PTP. As one of the four dNTPs is added to the sequencing reaction, while the DNA polymerase catalyzes its incorporation onto the DNA strand. During this time a phosphodiester bond between the dNTPs is formed, resulting in the release of pyrophosphate (PPi). ATP sulfyrase converts the PPi to ATP in the presence of APS. ATP is utilized for the conversion of Luciferin to Oxyluciferin catalyzed by Luciferase resulting in the production of light intensity that is proportional to the amount of ATP used in the reaction. This light is then detected by a CCD camera as a peak. The height of the peak is proportionate to the number of nucleotides that were incorporated, while the intensity of the light signal is proportional to the amount of ATP synthesized, which is also proportional to the number of nucleotides added to the growing chain. The system is regenerated with Apyrase, which degrades ATP and the un-incorporated dNTPs (to form dinucleotide monophosphate [dNMP]), thereby

eliminating any background noise during the sequencing run. As the process continues the complementary DNA strand grows and the nucleotide sequence is determined based on the signal peaks seen in the pyrogram.

3.2.2.12 Sequencing method

Reagents were supplied as three individual kits from Roche; the GS Junior Sequencing Kit Reagents and Enzymes, Sequencing Kit Buffers and the Sequencing Kit Packing Beads with Supplement CB (Roche 454 ™ Life Science, Branford CA, USA). Kit components were all thawed according to the manufacturer's instructions. The sequencing reaction was performed according to the Roche 454™ Sequencing method manual and is described in Appendix C.

3.2.2.13 Post Analysis – using the Amplicon Variant Analyzer (AVA)

The Amplicon Variant Analyzer (AVA) is a component of the standard GS junior software suite used for identifying sequence variants derived from moderate to high-depth sequencing. Before using AVA, the HIV-1 assay extra processing script (runAnalysisCollabHIV_v3.0.gz), was installed which acts as an interface for downstream analysis software. Its purpose is to automate the generation of an appropriately configured AVA project, trigger AVA processing, de-multiplex samples, align reads, as well as extract and export the read alignments in FASTA format for third party analysis software. Following installation of the script, the standard FASTA format (SFF) files were imported into AVA, for the generation of an AVA project. The frequency of variants were determined by the proportion of reads with a pattern of variation, all of the aligned reads that span the pattern provides an estimate of the variant frequency. The frequency of resistance mutations were reported in a summary table according to the IAS and the Stanford Drug resistance Database algorithms (Avidor et al, 2013).

3.2.2.14 Quality control

Despite the Roche 454™ platform being a powerful and efficient deep sequencing tool, with widespread applications, this technology is error prone in several aspects. Well documented bias includes errors introduced by stretches of homo-polymers and GC content; primer related selective amplification and *in vitro* recombination, as well as sampling error due to low template availability, all of which can result in substantial uncertainty for rare variants (Knapp et al, 2014). Nonetheless many studies have been able to show that 454™ deep sequencing is fairly reproducible with an error rate of ~1% (Huse et al, 2007, Mitsuya et al 2007, Kohlmann et al, 2011, Minoche et al, 2011, Knapp et al, 2014).

3.3 Results

3.3.1 *Longitudinal analysis for patients on a DRV/r-inclusive regimens*.

Results below show a longitudinal analysis of patient data, performed using the ART-AiDE (Antiretroviral Therapy –Acquisition and Display engine, Version 1.1 beta), accessed online from the Stanford Drug Resistance Database (http://dbpartners.stanford.edu/DDCRP/pages/art_aide.html).

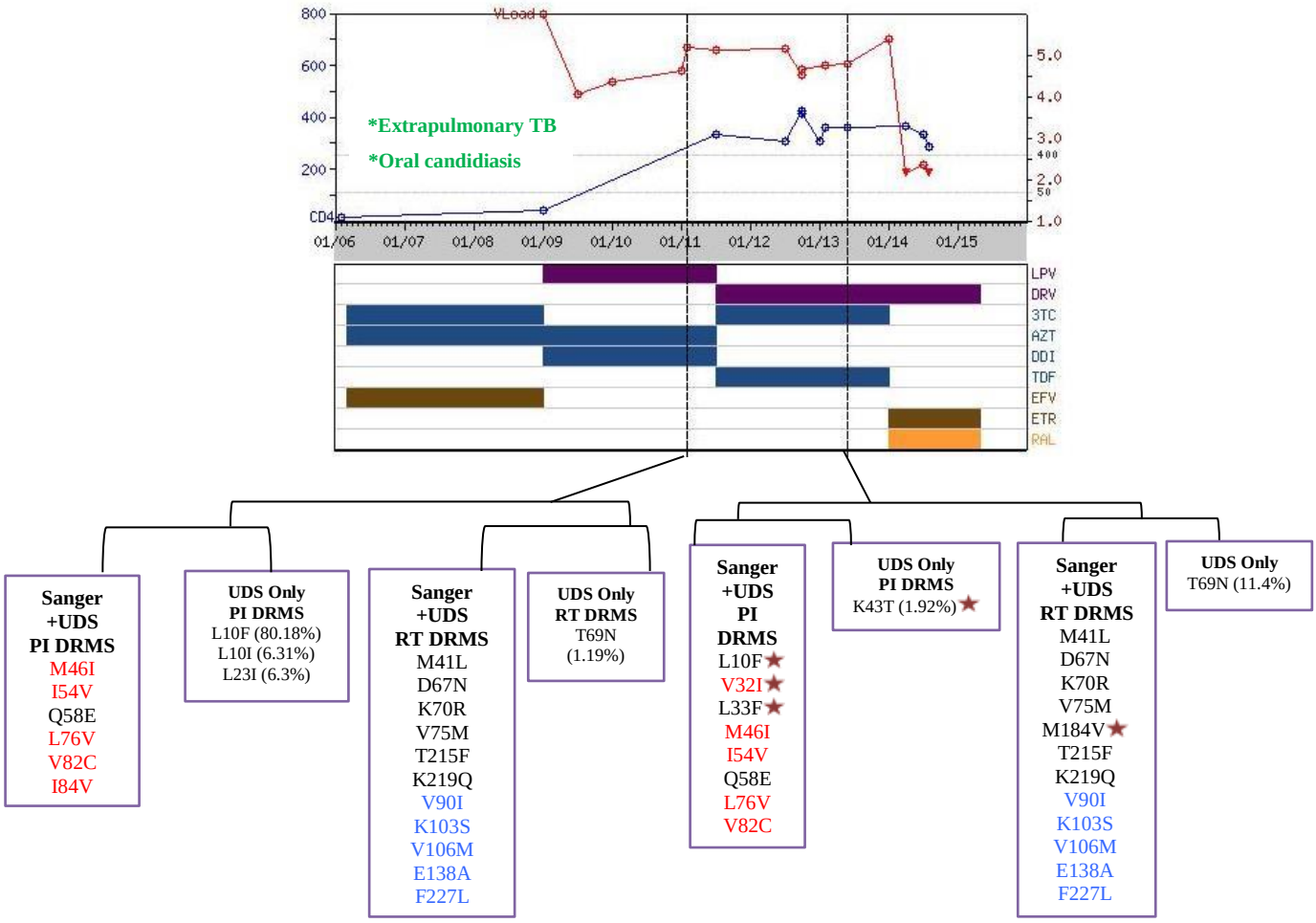


Figure 3.6 Longitudinal Analysis for PID 040 showing mutations and minor variants identified. Major PI DRMS are indicated in red, while minor PI DRMs are shown in black. NRTI DRMS are shown in black and NNRTI DRMs are shown in blue. Opportunistic infections during treatment are indicated in green. Red (★) indicates the emergence of additional mutations between time points.

Patient 040 was initiated on first-line treatment in 2006, presenting with a CD4 count of 20cells/μL extra-pulmonary TB, and oral candidiasis. In 2009, a high viral load of 150 000cpm prompted a switch to second-line LPV/r treatment. On the 9th of February 2011, the viral load still did not suppress and the patient was sent for genotyping. Sanger sequencing and UDPS identified major PI DRMs: M46I, I54V, L76V, V82C, I84V and one minor PI DRM (Q58E). Other PI mutations found included T12S, I15V, L19I, K20R, M36I, R41K, L63P, H69K, and L89M. UDPS alone found L10F that was missed by Sanger sequencing; this is an accessory mutation that causes reduced susceptibility to certain PIs. In addition to the PI DRMs detected, UDPS also detected minor variants (those variants occurring at < 20% of the circulating virus population), L10I and L23I; these mutations reduce the susceptibility or increase the replication of viruses having other resistance mutations. L23I is an uncommon non-polymorphic mutation associated with NFV/r resistance. Patient 040 had intermediate resistance to DRV/r and high-level resistance to all other PIs. Sanger and UDPS found NRTI mutations (M41L, D67N, K70R, V75M, T215F, and K219Q). UDPS alone found minor variant T69N (1.19%). A combination of these mutations resulted in low-level resistance to 3TC/FTC and high-level resistance to all other NRTIs. NNRTI mutations found by both assays were (V90I, K103S, V106M, E138A, F227L) thus conferring high-level resistance to NVP and EFV, low-level resistance to RPV and potential low-level resistance to ETR. After initiating DRV/r treatment in 2011, patient 040 presented with a detectable viral load two years later and was thus genotyped again. In addition to the PI DRMs found at the first time point, Sanger and UDPS found minor mutations L10F, V32I and L33F, conferring high-level resistance to all PIs. UDPS alone detected K43T (1.92%); this mutation is associated with TPV/r resistance. Sanger and UDPS identified the same NRTI mutations at the previous time point, in addition to M184V, which now resulted in high-level resistance to 3TC. Initially patient 040 presented with high-level TDF resistance, however at the second genotype this changed to intermediate TDF resistance, possibly due to the presence of M184V, which increases susceptibility to TDF. Minor variant T69N

(11.4%) was detected again by UDPS only, and increased in frequency since the first genotype. T69N is a non-polymorphic NRTI resistance mutation that is weakly selected in those patients receiving NRTIs; however their effects on NRTI susceptibility are not well studied (Tang and Shafer et al, 2012). The NNRTI resistance profile did not change from the first genotype. Patient 040 did not achieve virological suppression thus a new salvage regimen with DRV+RAL+ETR was initiated in January 2014. This regimen worked well and was able to reduce the viral load, resulting in an increase in the CD4 count. Patient 040 continues to receive this treatment.

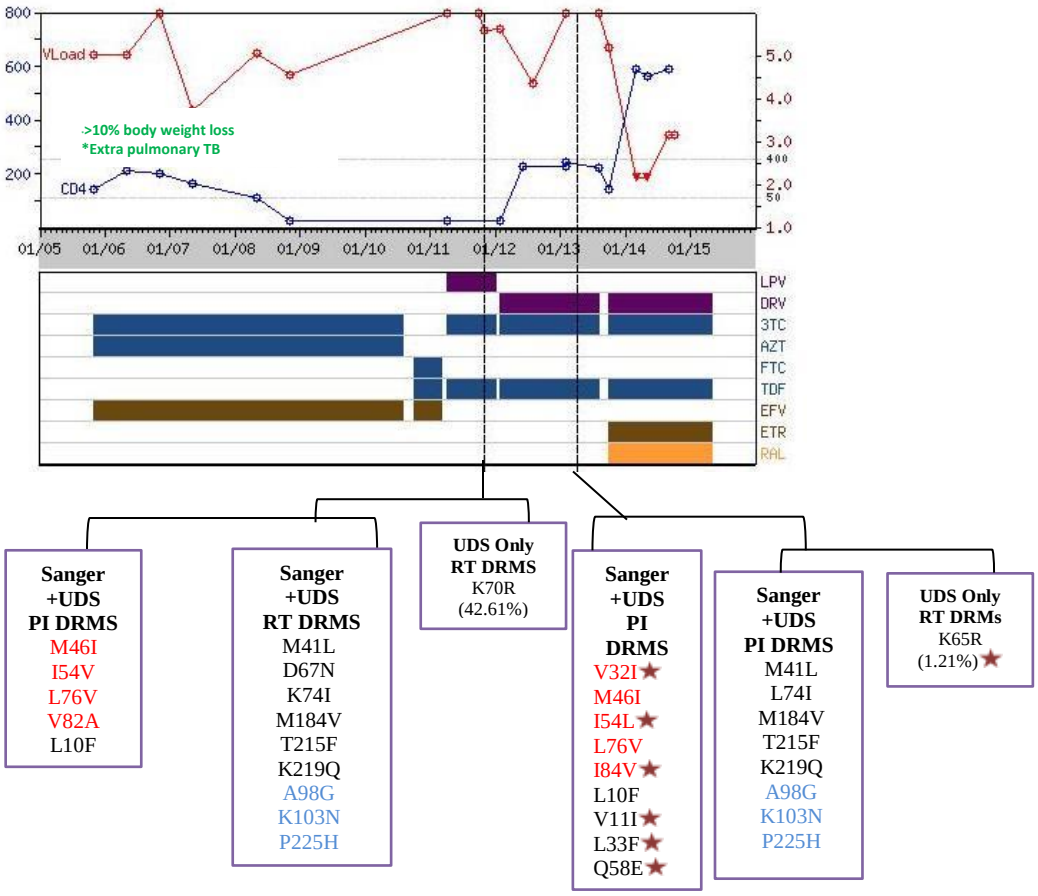


Figure 3.7 Longitudinal Analysis for PID 071 showing mutations and minor variants identified. Major PI DRMS are indicated in red, while minor PI DRMs are shown in black. NRTI DRMS are shown in black and NNRTI DRMs are shown in blue. Opportunistic infections during treatment are indicated in green. Red (★) indicates the emergence of additional mutations between time points.

In November 2005 patient 071 initiated first-line treatment with, AZT+3TC+EFV; however the viral load never fully suppressed. In October 2010 AZT was stopped and TDF was started in addition to the remaining drugs in the regimen. After approximately one year, the viral load was still high, reaching 270 0000cpm, with a low CD4 count of 28cells/µl. Patient 071 was switched to second-line LPV/r treatment, and the viral load still did not fully suppress. A few months later genotyping was performed and both Sanger sequencing and UDPS identified major PI DRMs (M46I, I54V, L76V, V82A) and one minor PI DRM (L10F). Other PI mutations found were I13V, G16E, L10I, K20R, M36I, R41K, L63H, H69K, L89I and I93L. There were no PI minority variants that were detected by UDPS. Patient 071 had low-level resistance to DRV/r and TPV/r, intermediate-level resistance to ATV/r and SQV/r and high-level resistance to the remaining PIs. Both Sanger and UDPS detected NRTI mutations (M41L, D67N, K74I, M184V, T215F and K219Q) as a result patient 071 now had intermediate resistance to TDF and high-level resistance to the remaining NRTIs. In addition to the NRTI mutations detected by Sanger, UDPS also detected K70R; this mutation causes intermediate resistance to AZT and possible low-level resistance to d4T and TDF. Both Sanger and UDPS identified NNRTI DRMs (A98G, K103N and P225H). Fortunately patient 071 was still susceptible to ETR and RPV, but had high-level resistance to the remaining NNRTIs.

Virological suppression was not achieved and thus treatment with DRV+TDF+3TC was initiated in February 2012, which decreased the viral load only slightly. A few months later the viral load began increasing and patient 071 was genotyped again. In addition to the PI DRMs found at the first genotype, both Sanger and UDPS identified three new major PI DRMS, V32I, I54L and I84V, as well as minor PI DRMs V11I, L33F and Q58E. Other PI mutations found were I13V, G16E, L19I, K20R, M36I, R41K, K55R, L63H, H69K, L89I and I93L. No minority variants were identified at this time point. Patient 071 now had high-level resistance to all PIs with the exception of TPV/r (intermediate resistance). The same NRTI and NNRTI mutations were present from the first genotype as detected by both Sanger and

UDPS, except D67N, which was no longer present, as patient 071 was no longer receiving AZT. UDPS alone found, NRTI mutation K65R (1.21%). Despite the switch to a DRV/r-inclusive regimen, the viral load did not fully suppress and in October 2013 a new salvage regimen with DRV+TDF+3TC+RAL+ETR was started. This treatment was effective in reducing the viral load and in March 2014, the viral load was <150cpm. The CD4 count also increased to 594cells/µl, patient 071 continues to receive this treatment.

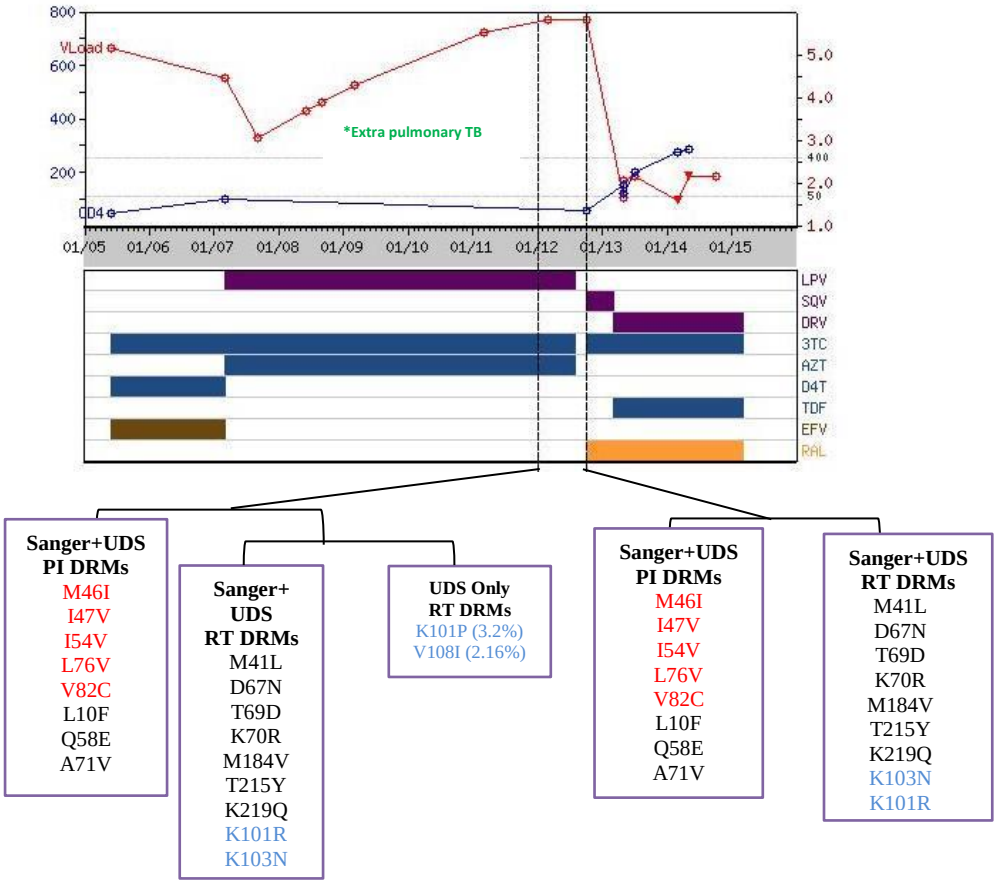


Figure 3.8 Longitudinal Analysis for PID 086 showing mutations and minor variants identified. Major PI DRMS are indicated in red, while minor PI DRMs are shown in black. NRTI DRMS are shown in black and NNRTI DRMs are shown in blue. Opportunistic infections during treatment are indicated in green. Red (★) indicates the emergence of additional mutations between time points.

In 2005 patient 086 presented with a very high viral load of 150 000cpm and a low CD4 count of 52 cells/µl. As a result, a second-line LPV/r inclusive regimen was

initiated, however the viral load still did not suppress and thus genotyping was performed again. Both Sanger and UDPS identified major PI DRMs (M46I, I47V, I54V, L76V, V82C) and minor PI DRMS (L10F, Q58E, A71V) resulting in high-level resistance to LPV/r, IDV/r, NFV/r, FPV/r, intermediate resistance to DRV/r, ATV/r, TPV/r and low-level resistance to SQV/r. Other PI mutations identified were T12S, I15V, G16E, L19E, K20T, E35D, M36I, R41K, L63T, H69R, L89M and I93L. No minority variants were found at this time point.

Both Sanger and UDPS identified NRTI DRMs, M41L, D67N, T69D, K70R, M184V, T215Y, K219Q) which resulted in high-level resistance to all NRTIs with the exception of TDF for which patient 086 had intermediate resistance. Only two NNRTI DRMs were found by both Sanger and UDPS (K101R and K103N). This resulted in high-level resistance to EFV and NVP. UDPS alone found minor variants K101P and V108I. This regimen was continued for seven months while awaiting a new salvage regimen. Prior to the new treatment a resistance test was performed and the same mutations were found by both Sanger and UDPS, however no minor variants were detected. A regimen comprising SQV+3TC+RAL was initiated in 2013, which reduced the viral load slightly however the viral load was still not fully suppressed. In March 2013, SQV/r was stopped, DRV/r and TDF was initiated in addition to the remaining drugs in the salvage regimen. The treatment worked well and in March 2014 the viral load decreased to <40 copies/ml. Patient 086 continues to receive this treatment.

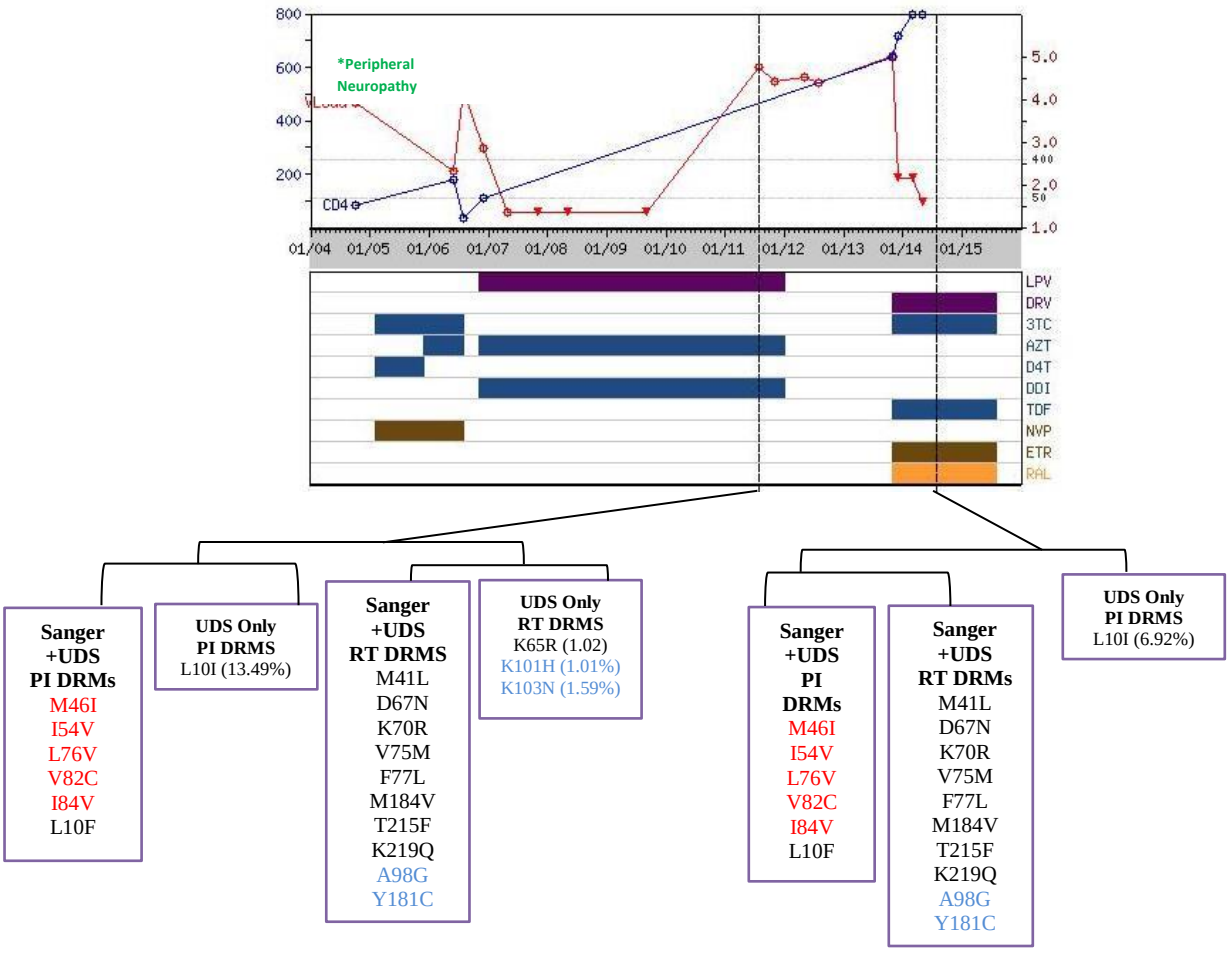


Figure 3.9 Longitudinal Analysis for PID 098 showing mutations and minor variants identified. Major PI DRMS are indicated in red, while minor PI DRMs are shown in black. NRTI DRMS are shown in black and NNRTI DRMs are shown in blue. Opportunistic infections during treatment are indicated in green.

Patient 098 initiated first-line treatment in 2005 with d4T+3TC+NVP, less than a year later, d4T was switched to AZT, due to peripheral neuropathy. In August 2006 a high viral load of 16000cpm and a low CD4 of 42cells/µl prompted a switch to a second-line LPV/r–inclusive regimen that reduced the viral load to <25cpm. This new regimen did not fully supress the viral load and in June 2012 a genotypic test was performed. Both Sanger and UDPS identified major PI DRMS (M46I, I54V, L76V, V82C, I84V) and one minor PI DRM L10F, resulting in intermediate resistance to DRV/r and high-level resistance to all other PIs. Other PI mutations

found were T12S, I15V, L19V, K20R, M36I, R41K, D60E, Q61H, L63P, H69K, and L89M.

UDPS alone identified minor variant L10I, which can cause reduced susceptibility to or increased replication of viruses with other PI resistance mutations. NRTI mutations M41L, D67N, K70R, V75M, F77L, M184V, T215F, and K219Q were identified by both Sanger and UDPS resulting in high-level resistance to all NRTIs. NRTI DRM, K65R was found by UDPS only. NNRTI mutations A98G and Y181C were detected by both UDPS and Sanger, resulting in intermediate resistance to EFV, ETR, RPV and high-level resistance to NVP. UDPS alone found K101H and K103N, reflecting prior NNRTI use.

After a nine month delay due to costs associated with salvage drugs within the public health care sector, patient 098 was eventually initiated on a third-line regimen comprising DRV+ETR+TDF+3TC+RAL. However in August 2014 the viral load was still detectable and a genotypic test was performed again. Both Sanger and UDPS identified the same PI DRMs from the first genotype and as a result patient 098 still maintained intermediate resistance to DRV/r. Other PI mutations found were T12S, I15V, L19V, K20R, M36I, R41K, D60E, Q61H, L63P, H69K, and L89M. UDPS alone found L10I again however the frequency of this mutation decreased since the first genotype. Both Sanger and UDPS found the same NRTI and NNRTI mutations since the first genotype. Patient 098 continues to receive this treatment.

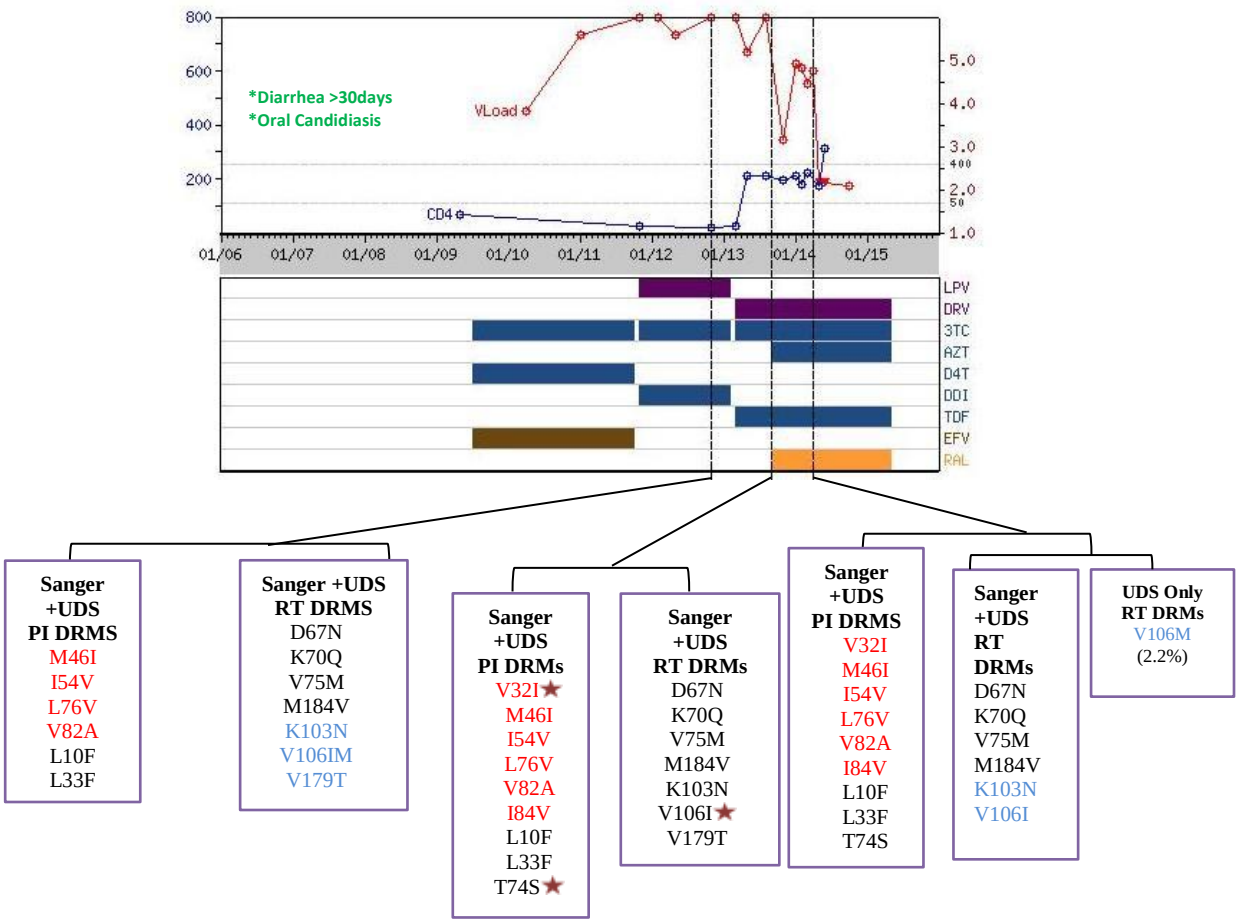


Figure 3.10 Longitudinal Analysis for PID 100 showing mutations and minor variants identified. Major PI DRMS are indicated in red, while minor PI DRMs are shown in black. NRTI DRMS are shown in black and NNRTI DRMs are shown in blue. Opportunistic infections during treatment are indicated in green. Red (★) indicates the emergence of additional mutations between time points.

Patient 100 presented with stage IV disease and initiated first-line treatment in 2009. In November 2011 a high viral load of 8418642cpm and a low CD4 count of 27cells/µl prompted a switch to a second-line LPV/r inclusive treatment, however this did not reduce the viral load. In November 2012, patient 100 was still not doing well presenting with a viral load of 1 million cpm and a very low CD4 count of 24cells/µl. A genotypic resistance test was performed, both Sanger and UDPS detected major PI DRMs (M46I, I54V, L76V, and V82A) and minor PI DRMs (L10F, L33F), resulting in low-level resistance to DRV/r and TPV/r, intermediate resistance

to SQV/r and high-level resistance to all other PIs. Other PI mutations found were I13V, K14R, I15V, L19I, K20R, E35D, M36I, N37D, R41K, H69K, L89I and I93L. No PI minor variants were detected at this time point. Both Sanger and UDPS detected NRTI DRMs (D67N, K70Q, V75M, M184V) resulting in low-level resistance to AZT and TDF, intermediate resistance to ABC, ddi, and high-level resistance to 3TC/FTC and d4T. NNRTI DRMs detected by both Sanger and UDPS included K103N, V106IM, and V179T. These DRMs resulted in resistance to all NNRTI drugs, with the exception of ETR and RPV. V179T is a rare non-polymorphic mutation rarely selected in those patients receiving NNRTIs, and is associated with minimal reductions in ETR and RPV susceptibility (Tang and Shafer et al, 2012).

As the viral load did not suppress, patient 100 was switched to a third-line regimen DRV/r+TDF+3TC in March 2013. This treatment resulted in only a slight decrease in viral load and the CD4 count did not change, as a result a resistance test was performed again. Three additional PI DRMs were detected by both Sanger and UDPS: V32I, 184IV and T74S and resulted in intermediate resistance to DRV/r and TPV/r. Other PI mutations found were I13V, K14R, I15V, L19I, K20R, E35D, M36I, N37D, R41K, H69K, L89I and I93L. No minority variants were detected at this time point. The NRTI and NNRTI DRMs remained the same from the first genotype as detected by both Sanger and UDPS. In August 2013, a new salvage regimen with DRV+RAL+TDF+3TC+AZT was administered. The viral load was still relatively high and in April 2014, a genotypic resistance tests was performed again. Both Sanger and UDPS detected the same PI DRMs. L63P was now present, however the I15V and I93L mutations were no longer present. The same NRTI and NNRTI DRMs were present from the previous genotype however the NNRTI DRM, V179T was no longer present. UDPS found one minor variant V106M reflecting prior NNRTI use. Patient 100 continued with this treatment and in May 2014, the viral load was <150cpm and the CD4 count was 318cells/µl. Patient 100 is currently receiving this treatment.

3.3.2 Baseline Integrase substitutions prior to Raltegravir treatment

Table 3.5 Baseline integrase associated polymorphisms detected by Sanger sequencing as well as minority variants detected by UDS.

PID	Mutations detected by Sanger sequencing and UDS	Minor Variants detected by UDS only
PID 40R	D25E, P30A, M50T, I84L, T93N, L101I, T112V, S119P, T122I, T125A, K136Q, V201I, L234I, M275V, D278A, S283G	V72N (7.33%), R127K (4.48%),
PID 71R	S17N, D25E, V31I, I84IM, E96D, L101I, T112V, T124A, T125A, K136Q, V201I, L234I	None
PID 86R	D3K, E11K, S17N, A23G, D25E, E35K, M50I, I72V, I84M, F100Y, L101I, T112V, I113L, T124A, T125A, K136Q, V201I, L234I, D278A, S283G	R127K (15.39%)
PID 98R	S17N, R20K, V31I, I72V, I89L, F100Q, L101I, T112V, T124G, T125A, K136Q, I182V, V201I, T206S, L234I, R269K, D278A	None
PID 100R	E11D, S24N, D25E, V31I, L101I, T112V, K136Q, V201I, K215N, L234I, R269K, D278A, S283G, D286N, V151I	V72I (5.64%), I84M (6.75%), T125A (6.75%), I135V (6.75%)

3.4 Discussion

All patients harbored major and minor PI DRMS both at LPV/r and DRV/r failure. Other PI mutations that were found are subtype C genetic polymorphisms some of which are implicated in drug resistance, particularly K20R, L63P, and L89M (Tang and Shafer, 2012). The K55R mutation was seen only after DRV/r failure in two patients and plays a compensatory role in restoring viral fitness in the presence of major PI DRMS, in particular M46L (Margerison et al., 2008).

Following DRV/r treatment patients quickly developed PI mutations, which included (V11I, V32I, I54L and T74S) that were not present at LPV/r failure. These mutations changed the resistance profile for two patients from low-level DRV/r

resistance to intermediate DRV/r resistance. Two patients presented with intermediate DRV/r resistance before and after DRV/r treatment; patient 098 harbored L10F, M46I, I54V, L76V, V82C and I84V interestingly no additional mutations were found following DRV/r treatment. Patient 086 harbored the I47V mutation in addition to other PI DRMs, which is included in the Tibotec DRV GSS (Tang and Shafer et al, 2012) and may account for the intermediate DRV/r resistance seen in this patient before treatment. One patient harbored I54L, which changed the resistance profile from low-level to high-level resistance, suggesting that this mutation is required for complete DRV/r resistance. The presence of these mutations in addition to other PI mutations (V11I, V32I, L33F, I47V, I54L, L76V, I84V) are important for conferring DRV/r resistance, and have been reported in other studies as DRV/r-associated resistance mutations (Clotet et al., 2007, Madruga et al., 2007, Lambert-Niclot et al., 2008). All patients harbored L76V, a characteristic LPV/r-resistance associated mutation, which is involved in the pathway to DRV/r cross resistance, often accompanied by V32I, I47A and I50V (Tang and Shafer et al, 2012). A study by Lambert-Niclot et al (2008) suggested that the presence of L76V at baseline reduces the risk of accumulating additional DRV/r resistance mutations (Lambert-Niclot et al., 2008). This may account for the four patients that presented with only intermediate DRV/r resistance despite accumulating DRV/r resistance mutations.

Two patients (PID 098 & PID040) presented with minority variants at LPV/r failure, which were L10I and L23I. One of these patients (PID 040) presented with L10F detected by UDPS only but missed by Sanger sequencing. This mutation was detected by Sanger sequencing only after DRV/r failure. Nonetheless patient 040 already had PI DRMs conferring high-level resistance to all PIs with the exception of DRV/r, thus L10F would not have altered the drug susceptibility profile. The remaining three patients did not present with any PI minority drug resistant variants. These results suggest that Sanger sequencing is sufficient for the detection of PI mutations and the added sensitivity provided by UDPS does not

impact on the interpretation of resistance profiles. All patients presented with resistance to the NRTI component of the regimen, limiting the use of these drugs in subsequent regimens. Only three patients presented with NRTI minor variants, which were T69N and K65R. Mutation, T69N occurred in a single patient at a frequency of (1.19%) and thereafter increased to (11.4%) at the second time point. This mutation may have implications for subsequent treatment with ddi. In addition, two patients presented with the K65R mutation, given that this mutation occurs within a homo-polymeric stretch and is subject to sequencing errors, their presence as true variants is debatable (Lessells et al., 2012).

The NNRTI mutation, K103N, persisted from first-line failure in 3/5 patients studied. This mutation is well known for its persistence due to the low fitness cost it confers (Flys et al., 2005, Loubser et al., 2006, Palmer et al., 2006, Cong et al., 2007). Other NNRTI mutations included V90I, A98G, E138A and V106I, which may have implications for subsequent treatment with second-generation NNRTIs in salvage regimens. The presence of NNRTI minority variants (K103N, K101H, K101P, V108I, V106M), reflect the prior use of NNRTIs during first-line treatment.
Overall, minority variants did not play a significant role in treatment failure. However the presence of certain accessory mutations that were found may have a compensatory role in viral fitness (Race, 2001). The clinical benefit of maintaining PIs in failing regimens is controversial, as it is well-known that resistant viruses are associated with diminished viral fitness, reduced infectivity, and incomplete processing of viral proteins (Wensing et al., 2010). Despite the superiority of DRV/r versus LPV/r, none of the patients were able to achieve complete virological suppression. Poor adherence seems to be a major barrier for these patients.

For IN, several baseline substitutions were identified in all patients. Accessory mutation, V151I was present in-patient 100 and is a polymorphic mutation selected in patients receiving RAL and *in vitro* by EVG. This mutation appears to have little or no effect on INSTI susceptibility in the absence of major INSTI mutations.

Patient 086 harbored the accessory mutation M50I, a subtype B polymorphism that is seen in 10-25% of INSTI naïve patients (Wares et al., 2014). Other studies have found this polymorphism in combination with the R263K mutation in patients failing RAL treatment (Wares et al., 2014). However this mutation does not restore the loss in HIV-1 infectivity associated with R263K. M50I alone will not negatively impact IN strand transfer activity or HIV replication capacity. However some studies have shown that it may slightly decrease HIV-1 susceptibility to EVG. The clinical relevance of this mutation will require further studies. Other IN polymorphisms that were found may have a compensatory role when present with other primary mutations; further work in this regard is required (Quashie et al., 2012).

Patients within this setting urgently require adherence counseling in order to preserve current ARVs and prevent the emergence of drug resistance mutations. More data, from larger cohorts are required in order to fully understand the virological outcomes associated with third-line ART in KZN, SA.

CHAPTER 4

Comparison of HIV-1 Protease at LPV/r versus DRV/r failure using computational structural analysis

4.1 Introduction

Within HIV-1 PR, 45 of the 99 residues have been associated with drug resistance (Volberding and Deeks, 2010). Eleven of the 45 have been associated with direct changes in the active site and are regarded as primary mutations. The remaining 34 of the 45 residues occur outside the active site and are often regarded as secondary mutations that are thought to indirectly interfere with the drug binding interaction, while also improving the overall fitness and stability of HIV-1 PR (Ragland et al., 2014). Some structural studies have described how these secondary mutations affect the drug binding interactions, however data on their specific functions and mechanisms of resistance are not extensive (Ragland et al., 2014). Thus far it has been shown that in the presence of drug resistance mutations there is an expansion of the active site; the volume of the amino acid side-chains within the active site decreases; there is a loss of van de Waals interactions between the inhibitor and HIV-1 PR; there is a reduction in the active site specificity; the mobile flaps are found to be further apart; there are structural differences between the side-chains due to changes in the amino acid sequence and the distance between certain residues occurring in combination differ (Logsdon et al., 2004, Kuiper et al., 2015). Many of these studies were based on experimental crystal structures of HIV -1 PR derived from X-ray crystallography or Nuclear Magnetic Resonance (NMR) spectroscopy. However, it is difficult and time consuming to obtain these experimental structures, thus in this study we employed homology modeling, a reliable and accurate method for the generation of modeled enzyme systems (Xiang, 2006).

We use these structures to describe conformational changes in LPV/r-resistant and DRV/r-resistant forms of HIV-1 PR. The homology models were generated from a single patient presenting with high-level DRV/r resistance following DRV/r failure. The drug binding interaction was investigated using molecular docking, and a MD simulation was performed to assess the stability of the final structure. This component of the study will provide an insight on the impact of LPV/r and DRV/r DRMs on PR in the context of subtype C.

4.2 Materials and Methods

4.2.1 Homology modeling

Sequences from a single patient (PID-071) from the PCSK cohort was used to construct homology models at LPV/r and DRV/r failure. Amino acid sequences were uploaded onto the SWISS-MODEL server (http://swissmodel.expasy.org/). A template search was performed for models that best matched the query sequence with the highest percentage identity. The template was selected and the model was constructed using built-in algorithms on the SWISS-MODEL server. Structures were viewed and analyzed in Chimera v1.8.1 (Petterson et al., 2004).

4.2.2 Structural analysis

Modeled structures were superimposed onto each other to perform the structural comparisons. Firstly, the atomic structures of mutated residues from the LPV/r-resistant model were visualized and compared to those of the DRV/r-resistant model. Secondly, to further understand conformational changes that occur between LPV/r and DRV/r failure, the distance between residues of each protein chain was measured. Lastly, the LPV/r resistant and DRV/r resistant models were compared to a crystal structure of PR bound to DRV (PDB ID: 4DQB); this was done to investigate how structural changes between the two time points may alter drug binding. The distance between residues in the direct vicinity of DRV were

measured for both models. We also investigated the influence of mutated residues within the active site on neighboring residues in both models.

4.2.3 Molecular docking

To assess changes in the drug-binding interactions between PR and DRV at LPV/r failure versus DRV/r failure, DRV was docked into each of the models using the CLC Drug discovery Workbench™ v2.4.1 software package (CLC Bio-Qiagen, Aarhus, Denmark). All ligands were downloaded from the ZINC[12] database (Irwin et al., 2012) in mol2 format. Briefly, all structures were imported one at a time onto the CLC Workbench; waters and hydrogen's were removed and each structure was saved as a molecule project. In order to identify the target region within the protein, suitable binding pockets were identified for DRV. This was performed by selecting the "find binding pockets" option within the toolbox and default parameter settings were used (binding pockets with volumes less than 20 $Å^3$ were ignored). The desired binding site was shown using green spheres within the center of the protein. Once the binding site was found, the "set up binding site" option was selected. Before commencing with docking, the ligand was inspected and validated, to ensure that there were proper connections between atoms, bond orders, hydrogen atom positions, and atom hybridizations. The selected binding sites and ligand were saved as a "Molecule project". Docking was then performed, using the "dock ligands" option in the drug-design toolbox, and the molecule project as an input. The default docking parameters were selected, which included the number of iterations for each ligand in the search for an optimal binding mode (n=100). This is considered a good balance between search completeness and cost. Increasing this number would have lead to more extensive binding mode searches, requiring more computational time. The number of docking results returned was set to one for each ligand, allowing only the best binding mode to be returned.

4.2.4. Molecular Dynamics simulations using Implicit solvation

The AMBER12 Molecular Dynamics package was accessed remotely via the Centre for High Performance Computing (http://www.chpc.ac.za/) and was used to perform the MD simulations. The LPV/r-resistant and DRV/r-resistant models were used for the simulation as described in section 4.2.1.

4.2.4.1 Preparation of the ligands

The atomic force field parameters of the ligands were obtained using the ANTECHAMBER module, which employs the Generalized Atomic Force Field (GAFF) and Bond Charge Correction (BCC) charges. The AMBER force field ff99SB was used to perform the MD simulation. The Parmchk program contains a database for parameterization calculations and was used to create the physico-chemical parameters of the solvation box. The resultant parameter file is employed by tleap in AMBER12 to add hydrogen atoms to the ligand. The receptor was then loaded in PDB format for the formation of the complex (ligand+receptor). The default radii angles were set and the ligand, receptor and complex topology files were saved. Additional Chlorine ions were added wherever necessary, in order to neutralize the system and balance the charge of HIV-1 PR. The protein was then placed in the center of the cubic box with TIP3PBOX8.0, which was used as the water model for this solvation. This water model specifies that the molecule should have a buffer of at least 8.0Å between the structure and the periodic box wall. Lastly the final solvated topology and coordinate files were saved (Simmerling et al., 2002).

4.2.4.2 Energy minimization and equilibration

Energy minimization was performed to obtain the most favorable configuration of the model. Each of the parameters that was used to perform the minimization is provided in detail in Appendix D. Briefly, the solvated complex was equilibrated by performing an energy minimization using the SANDER module in a constant volume by 2500 cycles of steepest descent minimization; this was followed by 750

cycles of conjugated gradient minimization. The systems were then gradually heated from 0K to 300K over 50ps of heating, 50ps of density equilibration with weak restraints on the complex followed by 0.25ns of constant pressure equilibration at 300K.All simulations were run using the Shake algorithm to constrain the bonds that contained hydrogen atoms, and the temperature was controlled using the Langevin thermostat. The backbone Root-Mean-Square-Deviation (RMSD), (which is a measurement of how the internal atomic co-ordinates change relative to the minimized molecular structure co-ordinates) (Coutsias et al., 2004), was determined using the PTRAJ module in AMBER. PTRAJ is a comprehensive program used to analyze MD trajectories. The program requires a topology file as well as a perl script specifying the input and output requirements. These are shown in Appendix D. Average summaries for the density, temperature and the total energy were obtained using the process_mdout.perl script provided in the Amber Tools package. This data was used to generate separate plots for the RMSD, density, and equilibration versus time. This was performed to ensure that all structures were stable. (Simmerling et al., 2002).

4.3 Results

4.3.1 Homology modeling

The LPV/r resistant model harbored polymorphisms: I13V, G16E, L19I, K20R, M36I, R41K, K55R, L63H, H69K, L89I, I93L as well as major (M46I, I54V, L76V and V82A) and minor (L10F) PI resistance mutations. The DRV/r-resistant model harbored the same polymorphisms. In addition, major (V32I, M46I, I54L, L76V) and minor (L10F, V11I, L33F and Q58E) PI DRMs, were also present. However, the patient no longer had the V82A mutation. Both these sequences were modeled using a South African wild-type HIV-1 subtype C virus (PDB ID: 2R5Q) resolved by X-ray crystallography (2.3Å) as a template. The final modeled structures are indicated in Figure 4.1.

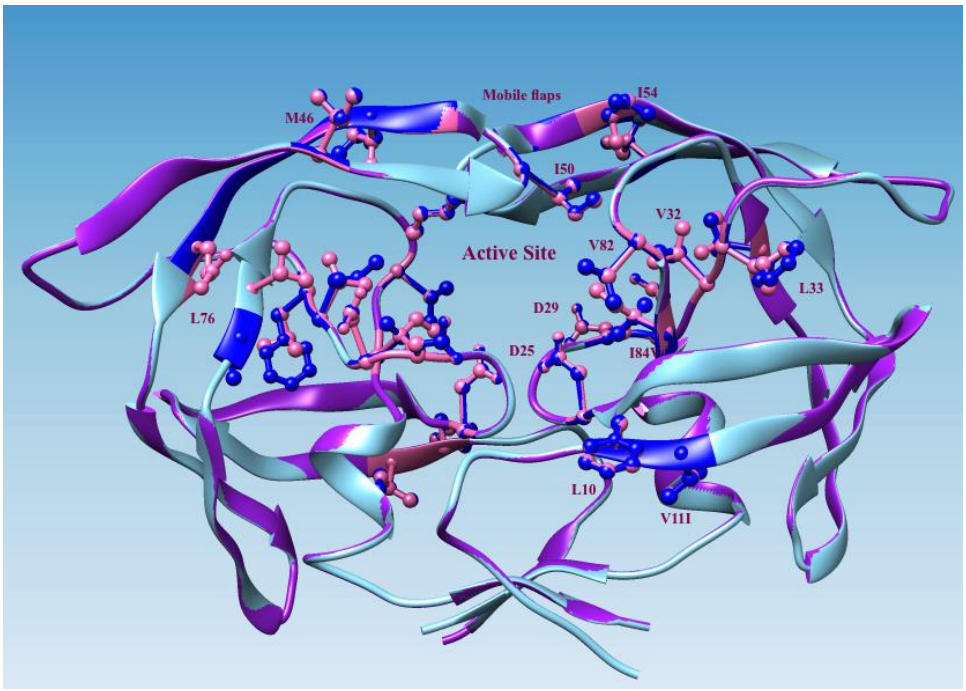
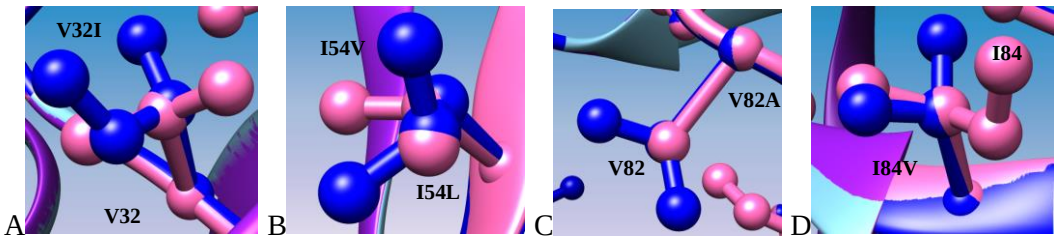


Figure 4.1 Three-dimensional model of HIV-1 Protease. The LPV/r-resistant model is shown by purple traces with pink side-chains and the DRV/r-resistant model is shown by light blue traces with blue side-chains. The model was generated in SWISS MODEL and visualized in Chimera v1.8.1.

4.3.2 Structural analysis

Residues that differed between the LPV/r-resistant and the DRV/r-resistant models are indicated in Figure 4.2 and Figure 4.3.



A B C D

Figure 4.2 Major PI mutations that differed between the LPV/r-resistant (pink side-chains) and DRV/r-resistant (blue side-chains) models. A) Mutant V32I is a substrate cleft mutation that makes direct contact with the inhibitor; the mutant form appears slightly larger than the un-mutated form. B) The I54L/V residue is a flap-tip mutation, the 154L mutant form seems slightly larger than the I54V mutant C) V82A is regarded as a substrate cleft mutation, and appears shorter and smaller

than the un-mutated form. D) The I84V mutant is also a substrate cleft mutation and has shorter amino acid side-chains in comparison to the un-mutated form.

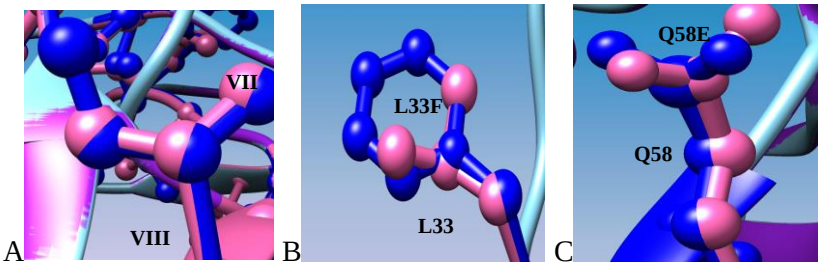


Figure 4.3 Minor PI mutations that differed between the LPV/r-resistant (pink side-chains) and DRV/r-resistant (blue side-chains) models. A) Residue V11, occurs within the distal regions of the enzyme and thus does not seem to have direct contact with the inhibitor/substrates within the active site, the mutant side chains appear to be longer than the un-mutated chains. B) The L33F mutant shows a large aromatic side chain that protrudes into the hydrophobic pocket of the enzyme, occupying more space than the un-mutated form. C) The Q58E mutant is located distally and distinct structural changes can be seen in comparison to the un-mutated form.

4.3.2.1.Distances between side-chains in the absence of the inhibitor

The distance between the side-chains of certain residues in the LPV/r-resistant model and the DRV/r-resistant model was measured. This comprised all mutated residues present in both models as well as those residues that were not mutated but present within the active site and flap regions. These results are indicated in Table 4.1.

Table 4.1 Distance between the protein chains of selected mutated and un-mutated residues of both models studied.

LPV/r-resistant model		DRV/r-resistant model		Difference in distance between chain A and B (Å)
Residue	Distance between chain A and B (Å)	Residue	Distance between chain A and B (Å)	
L10F	30.505	L10F	30.504	0.001□
V11	24.500	V11I	24.553	0.053□
D25	4.720	D25	4.701	0.019□
D29	18.641	D29	18.646	0.005□
V32	19.778	V32I	20.108	0.33□
L33	32.124	L33F	31.191	0.9333□
M46I	25.417	M46I	25.413	0.004□
L50	11.168	L50	11.040	0.128□
F53	18.376	F53	18.378	0.002□
I54V	14.315	I54L	13.986	0.329□
L76V	26.476	L76V	26.211	0.265□
V82A	18.296	V82	18.172	0.124□
I84	11.315	I84V	14.165	2.85□
Q58	36.544	Q58E	39.593	3.049□

*We considered a 0.1Å difference as a notable change in conformation between the two structures. Those with a 0.1Å difference that were also mutated are shaded in green, while the residues that were not mutated but showed a (>0.1Å) difference are shaded in grey. Those that were mutated/un-mutated that showed only slight differences (<0.1Å) are not shaded. Arrows (□/□) indicate if the distance increased or decreased from LPV/r to DRV/r failure.

Amongst those residues that were mutated and showed a noted change in distance, V32I, resulted in an increase in distance from LPV/r to DRV/r failure. Other residues that increased in distance (>0.1) from LPV/r to DRV/r failure included residues 58 and 84, I84V appeared shorter than the un-mutated form, thus would occupy less space making the structure wider. The remaining residues (33, 54, 76, and 82) decreased in distance between the two models. Each of these residues in their mutated states seemed to occupy more space than the un-mutated forms, thereby reducing the distance between the two protein chains. Residue 50 was not mutated, but the distance between the protein chains with this

residue showed a reduction from LPV/r to DRV/r failure. The remaining residues showed very slight differences in distance between the protein chains.

4.3.2.2 Distance between side-chains in the presence of the inhibitor.

The distance between some of the residues likely to make direct contact with the inhibitor was measured, as shown in Figure 4.4.

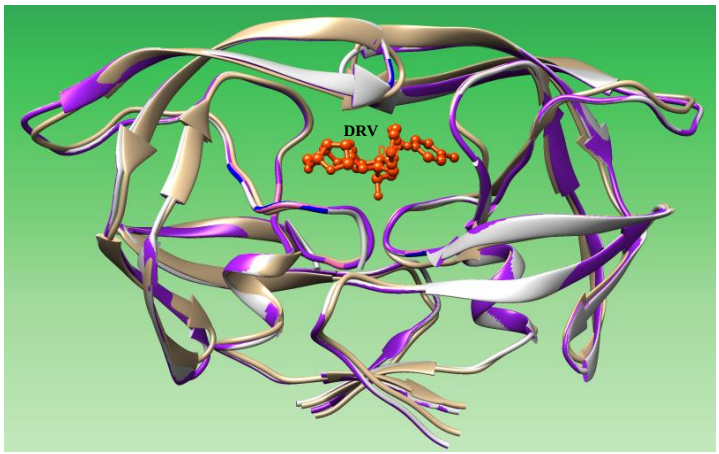
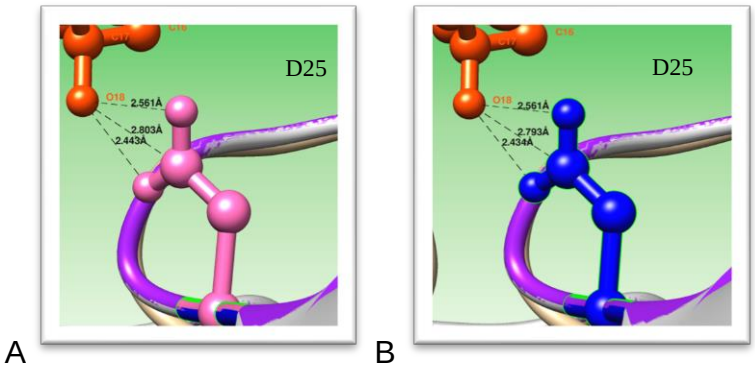
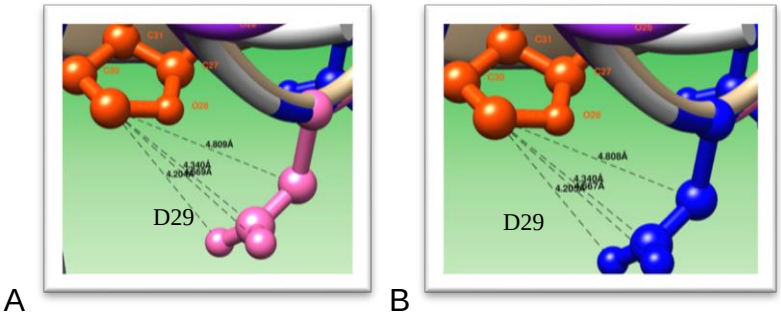


Figure 4.4 Three-dimensional model of HIV-1 PR with Darunavir bound. The LPV/r-resistant model is shown by purple traces the DRV/r-resistant model is shown with grey traces, the inhibitor Darunavir is shown within the active site in orange. The model was generated in SWISS MODEL and visualized in Chimera v1.8.1.



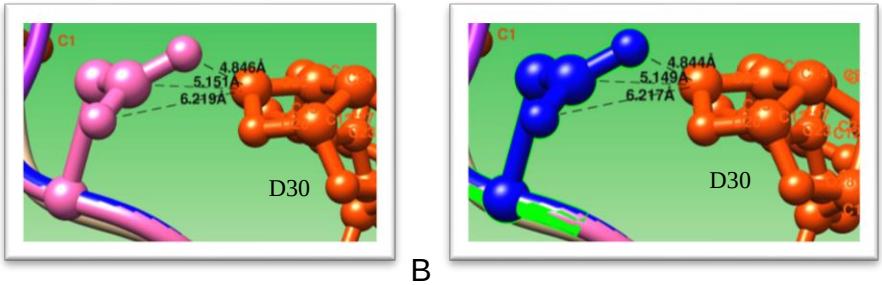
A B

Figure 4.5 Residue D25 side-chain interactions with the PI-DRV shown in orange. (A) LPV/r-resistant model, side-chains are indicated in pink (B) DRV/r-resistant model, side-chains are shown in blue. The distances between each of the side-chain interactions with the inhibitor differed only slightly.



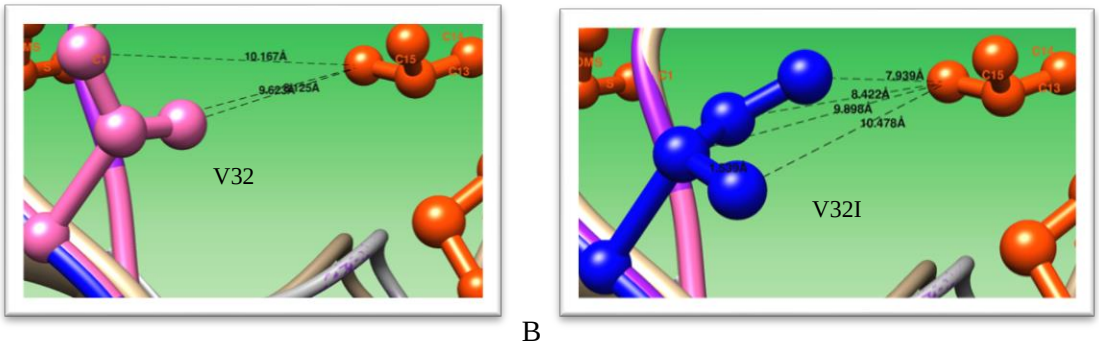
A B

Figure 4.6 Residue D29 side-chain interactions with the PI-DRV shown in orange (A) LPV/r-resistant model, side-chains are indicated in pink (B) DRV/r-resistant model, side-chains are shown in blue. The distances between each of the side-chain interactions with the inhibitor differed only slightly.



A B

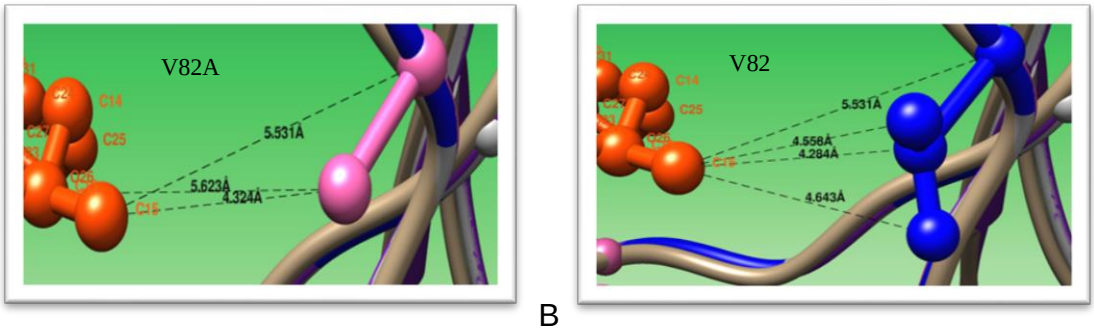
Figure 4.7 Residue D30 side-chain interactions with the PI-DRV shown in orange (A) LPV/r-resistant model, side-chains are indicated in pink (B) DRV/r-resistant model, side-chains are shown in blue. The distances between each of the side-chain interactions with the inhibitor differed only slightly.



A B

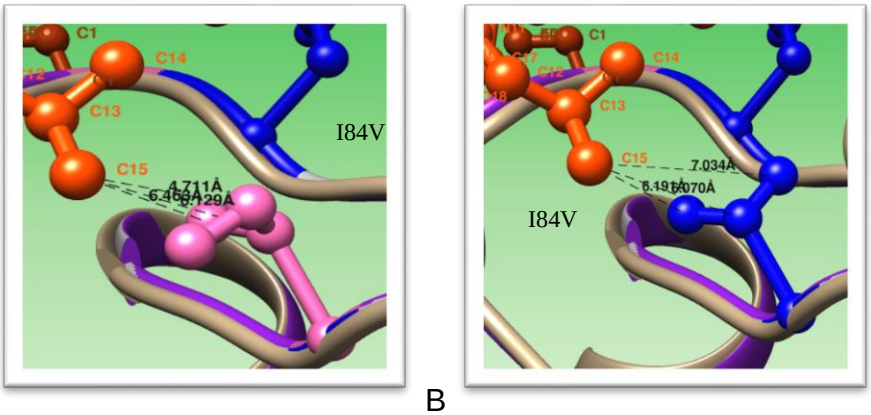
Figure 4.8 Residue V32 side-chain interactions with the PI-DRV shown in orange (A) LPV/r-resistant model, side-chains are indicated in pink (B) DRV/r-

resistant model, side-chains are shown in blue. The distances between each of the side-chain interactions with the inhibitor differed.



A B

Figure 4.9 Residue V82 side-chain interactions with the PI-DRV shown in orange (A) LPV/r-resistant model, side-chains are indicated in pink (B) DRV/r-resistant model, side-chains are shown in blue. The distances between each of the side-chain interactions with the inhibitor differed.

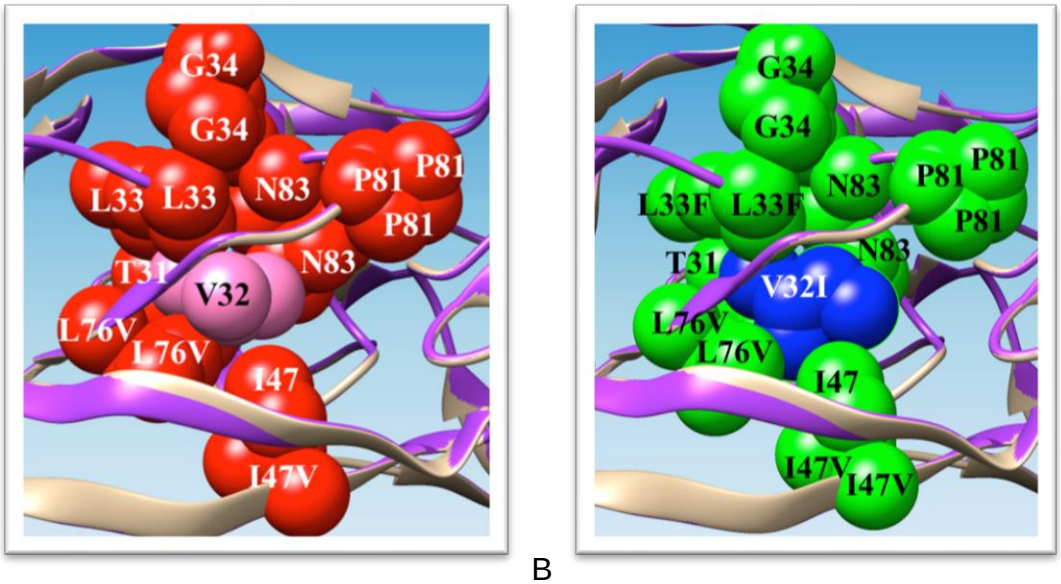


A B

Figure 4.10 Residue I84 side-chain interactions with the PI-DRV shown in orange (A) LPV/r-resistant model, side-chains are indicated in pink (B) DRV/r-resistant model, side-chains are shown in blue. The distances between each of the side chain interactions with the inhibitor differed.

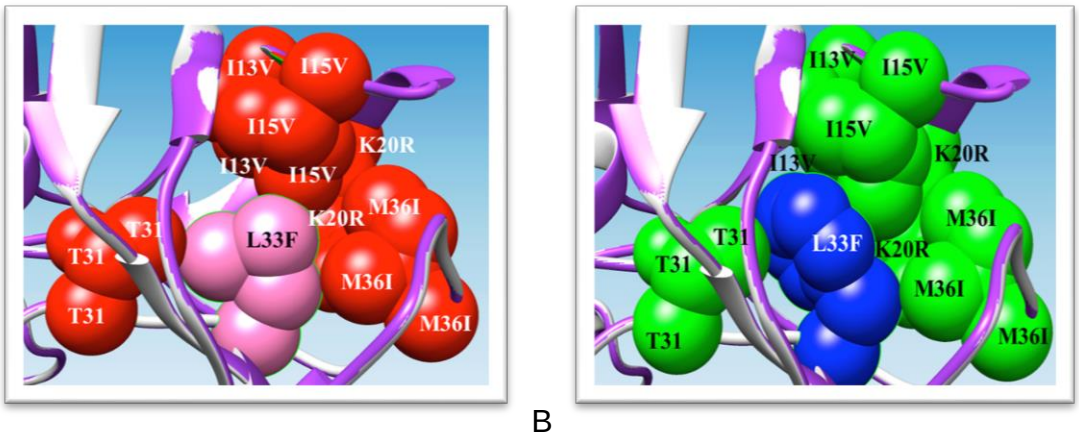
4.3.2.3 Interactions with surrounding residues

The interaction of certain active site residues that were mutated were visualized in sphere form, to gauge the interactions with neighboring residues. Those having more interactions with surrounding residues may affect their locations and thus will have implications for overall drug binding due to the altered structure.



A B

Figure 4.11 Mutant V32I, interactions with surrounding residues. A) Side chain of V32 (pink spheres) in the LPV/r-resistant model, shows interactions with surrounding residues (red spheres) are slightly less in comparison to B) V32I (blue spheres), in the DRV/r-resistant model, the mutant moves closer into the active site compared to the un-mutated form. This indicates that the mutant form will have more interactions with the surrounding residues in comparison to the un-mutated form. These further interactions may influence the positions of these surrounding residues.



A B

Figure 4.12 Mutant L33F, interactions with surrounding residues. A) Side chain of L33 (pink spheres) in the LPV/r-resistant model show that there are fewer interactions with surrounding residues in comparison to the B) DRV/r-resistant model with blue side chains, this mutant form tends to have more interactions with

the surrounding residues, thus occupying more space. There seems to be more interactions with the I15V residue in particular

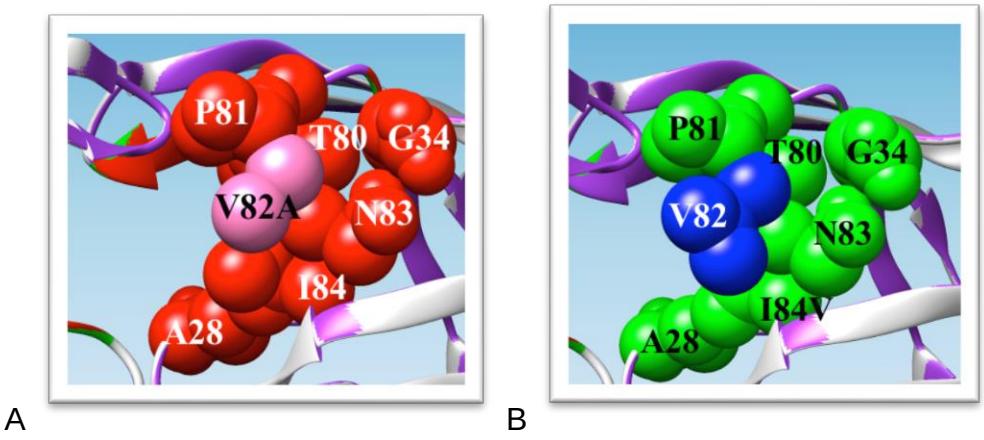


Figure 4.13 Mutant V82A, interactions with surrounding residues. A) Side chain of V82A (pink spheres) in the LPV/r-resistant model, shows that this mutant form looses interactions with surrounding residues and occupies less space in the structure, while in B) the un-mutated form seems to retain these interactions.

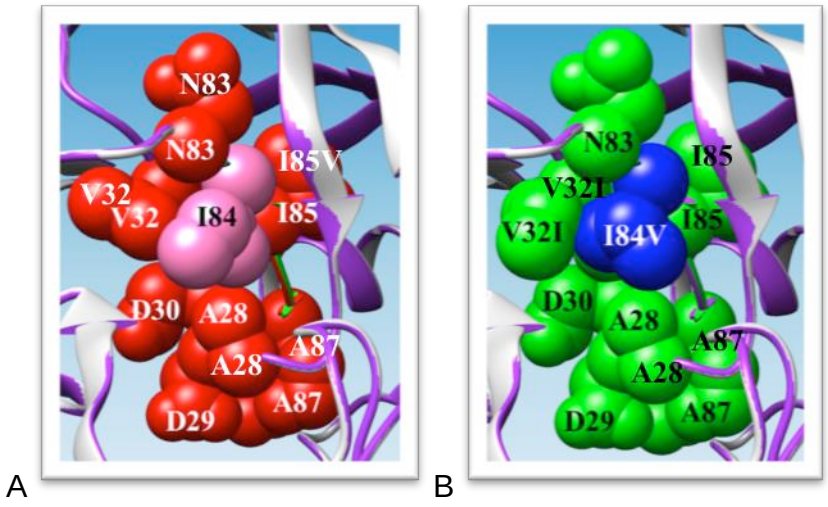


Figure 4.14 Mutant I84V, interactions with surrounding residues. A) Side-chain of I84 (pink spheres) in the LPV/r-resistant model appears larger thus occupying more space and has more interactions with neighboring residues in comparison to B) the DRV/r-resistant model shown by (blue spheres), here the 184V mutant form appears smaller and has fewer interactions with neighboring residues.

4.4 Molecular docking

Table 4.2 Binding scores derived from molecular docking for the LPV/r-resistant and DRV/r-resistant models.

Structures	Ligands	Binding scores (kcal/mol)
Wild-type model (2R5Q)	DRV	-20.04
LPV/r failure model	DRV	-13.36
DRV/r failure model	DRV	-11.56

These results show that the wild-type model had the lowest binding score indicative of the most stable complex. For the LPV/r-resistant model and the DRV/r-resistant model the binding scores increased, suggesting that the inhibitory potency was lowered.

4.5.Molecular Dynamics simulations

Following energy minimization, the RMSD versus time was plotted. This was done for each of the models studied as shown in Figure 4.15.

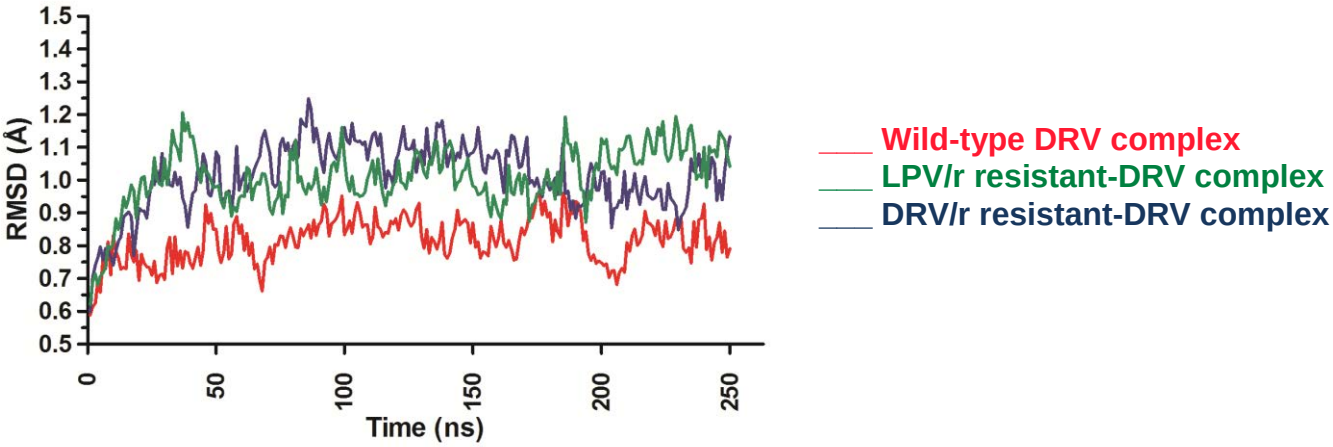


Figure 4.15 RMSD values versus time for the wild-type-DRV complex (red), the LPV/r-resistant-DRV complex (green) and the DRV/r-resistant –DRV bound complex (blue).

Equilibrium was reached in each of the structures after a 0.25ns simulation at an RMSD of 0.79A. 1.04A, 1.15A for the wild-type, LPV/r-resistant and DRV/r-resistant complexes respectively. The equilibrium seemed to converge well for the wild-type-DRV complex, as opposed to the other two models. Larger fluctuations in the latter models may be attributed to the presence of DRMs that seem to propagate throughout the enzyme system.

4.6 Discussion

The emergence of drug resistance mutations in PR enables the enzyme to evade inhibition and maintain biological function (Ragland et al., 2014). Structural studies have revealed resistance mechanisms amongst mutated residues in HIV-1 PR. For example, the role of the M36I mutation in regulating the size of the active site, by shifting the L33 and V77 residue inward (Ode et al, 2007), or the asymmetrical changes caused by the L76V mutant, where one side of the active site constricts and the other side widens (Ragland et al., 2014). Our study investigated how the HIV-1 PR structure is altered following LPV/r-inclusive second-line treatment and DRV/r-inclusive third-line treatment. Distinct differences were found in each of the mutated residues in the DRV/r-resistant model; where some side-chains (V82, I84V) appeared smaller and shorter, while others (V11I, V32I L33F and I54L) were larger and longer occupying more volume. Some of these side-chain changes also resulted in distinct differences in the width between dimers of the LPV/r-resistant and DRV/r-resistant structures. The presence of V32I, Q58E and I84V in the DRV/r-resistant model caused the dimers to be further apart in comparison to the LPV/r-resistant model. The short side-chains of I84V occupy less space and results in a slight expansion of the active site cavity. Other studies report that in the presence of both I84V and V82A, there is a profound expansion of the active site

(Logsdon et al, 2004), however given that our DRV/r model lacks the V82A mutation, active site expansion in this model is minimal. Residues with larger side-chains (L33F, I54L, and V82) resulted in the dimers being closer together. These results clearly show that following DRV/r failure there are significant structural alterations in PR that contribute to DRV/r resistance.

Distances between the active site residues (D25, D29, D30) and DRV differed only slightly. This was probably because DRV was designed to form more hydrogen bonds with the main chain atoms of Asp29 and Asp 30, which are not easily disrupted and can withstand minor structural alterations caused by resistance mutations (Lui et al., 2008). In our study the V32I mutant showed increased interactions with the inhibitor compared to the un-mutated form. This mutant appeared to move inward toward the active site and had more interactions with surrounding residues.

In the DRV/r-resistant model the I84V mutant had shorter side-chains, thus losing its interactions with DRV, and also appeared to have fewer interactions with surrounding residues. This could be attributed to the possible rearrangements with the 80s loop caused by V32I, that is known to push DRV away from the I84 residue and toward the 50s region (Ragland et al., 2014). Similarly the mutant form of V82A in the LPV/r-resistant model also appeared shorter in comparison to its un-mutated form, thus loosing interactions with DRV and surrounding residues. Other residues that do not make direct contact with DRV can also result in overall structural rearrangements within PR. This is especially true for the L33F mutant that presented with bulky aromatic side chains thus having increased interactions with residues within the hydrophobic pocket, defined by residues (I13, I15, K20, A22, T31, M/V36, L38, I64, I66, V75, V77, N83, and I85). These increased interactions causes the structure to become rigid and loose flexibility and as a result, certain regions can no longer move to accommodate the inhibitor (Kuiper et al., 2014).

Docking results showed that the potential inhibitory potency of DRV was lowered in both the LPV/r and DRV/r resistant models. Despite the absence of DRV/r-resistance associated mutations in the LPV/r-resistant model, DRV did not bind as efficiently as it was bound to the wild-type model. This is largely attributed to the presence of certain LPV/r mutations that contribute to DRV resistance, specifically the L76V mutant that is known to confer DRV/r cross-resistance (Barber et al, 2012). The worst drug binding score was returned for the DRV/r-resistant model, suggesting that the drugs inhibitory potency is greatly lowered in the presence of multiple resistance mutations. However, the results obtained from molecular docking need to be further confirmed by performing an end state energy analysis on generated MD trajectories using the MMPBSA.py script. This approach has been shown to be more accurate than other scoring methods (Rastelli et al., 2010). Molecular Dynamics simulations showed that the wild-type complex was more stable than the LPV/r-resistant and DRV/r-resistant models. The fluctuations seen on the RMSD plots can be attributed to the presence of DRMs that alter the enzyme dynamics.

Despite the superiority of DRV for highly treatment-experienced individuals, we find that its efficacy is considerably affected by the presence of DRMs. A combination of these structural alterations amongst certain localized residues, as well as those occurring in a variety of positions collectively contribute to the shape and flexibility of the overall structure and are thus likely to contribute to drug resistance (Ragland et al., 2014)

CHAPTER 5

General discussions and conclusions

HIV-1 PR is an ideal drug target owing to its vital role in proteolytic processing (Tomasselli et al., 1990). The inclusion of PIs in cART, is the standard of care for HIV-1 treatment globally. Here in South Africa, LPV/r is a key component of the second-line regimen, and DRV/r is recommended for third-line treatment (Department of Health, 2013). Unfortunately, the selection of PI-resistant viral variants leads to drug resistance and poor treatment responses (Tang and Shafer et al., 2012). However, to date second-line failure studies report a low frequency of LPV/r DRMs with a significant accumulation of RT-inhibitor DRMs (Riddler et al., 2008, Wallis et al., 2011, Levision et al, 2012). These studies ascribed treatment failure to non-adherence as opposed to drug resistance.

From our cohort of 156 second-line failures from KZN, only 24% harboured LPV/r DRMs, thus we also attribute non-adherence as the primary reason for treatment failure. Despite this low occurrence of PI DRMs, our findings are higher in comparison to other regions (Wallis et al, 2011, Van Zyl et al, 2013). Interestingly, we also observed a distinct pattern of PI DRMs, where V82A occurred alone, followed by V82A+I54V then V82A+I54V+M46I, and lastly V82A+I54V+M46I+L76V+L10F, representing the most prevalent PI DRM pattern. For the RT-inhibitor drug class, the NRTI mutation M184V was the most prevalent attributed to 3TC/FTC treatment. In the NNRTI drug class the K103N mutation was most frequent, and is known to persist from first-line failure (Tang and Shafer et al., 2012).

For those patients presenting with high-level LPV/r resistance, the third-line regimen usually consists of DRV/r and a combination of ETR, RT-inhibitors and or RAL (Department of Health, 2013). Amongst those patients in our cohort that were

switched to DRV/r, five patients failed their third-line treatment within less than a year of receiving DRV/r. We longitudinally assessed these five patients using UDPS to investigate the possibility of drug resistant minority variants at LPV/r failure that may predict DRV/r failure. Other studies have reported that the presence of resistant minority variants can influence treatment outcomes in patients (Fisher et al., 2012, Nicot et al., 2012, Avidor et al., 2013, Gianella et al., 2013). In our study we found, that 2/5 patients presented with PI minority variants at LPV/r failure (L10I and L23I). These were not seen at DRV/r failure. One patient presented with minor variant K43T at DRV/r failure only. These variants are compensatory mutations and are not known to cause DRV/r failure. For the RT-inhibitor class the K65R variant occurred in 2/5 patients this mutant occurs within a homo-polymeric region and is possibly a sequencing artifact. Several NNRTI varaints were identified that have persisted from first-line failure, the persistence of these mutations has also been reported elsewhere (Fisher et al.,2012).

Our results established that minority variants at LPV/r failure did not influence DRV/r failure. However distinct DRV/r resistance mutations were selected relatively quickly in majority virus populations, despite the drugs high genetic barrier. Exceptionally high viral loads were seen in each of these patients despite DRV/r treatment, inferring that DRV/r was unable to suppress viral replication. However this could also be attributed to non-adherence. Nonetheless, most (80%) of patients developed additional DRV/r resistance mutations, implying that these patients did take their medication intermittently. Furthermore the presence of LPV/r resistance mutations conferring cross-resistance to DRV/r could have influenced DRV/r failure.

In the single patient that presented with high-level DRV/r resistance, we investigated the structural implications on viral PR before and after DRV/r treatment. Structural differences between the LPV/r-resistant and the DRV/r-resistant model were evident. We showed that resistance mutations caused

alterations to the side-chains at certain residues that influenced the width between the dimers in each model.

However, the distance between the active site residues (D25, D29, D30) and DRV/r differed only slightly, despite various other conformational changes in these structures. In contrast the I84V mutant had reduced interactions with the inhibitor, further contributing to resistance. Mutated residues such as V32I had increased interactions with DRV/r, however these interactions tend to have detrimental effects for other residues (Ragland et al., 2014). Additionally the L33F mutant protrudes deeply into the hydrophobic regions, and has increased interactions with surrounding residues, consistent with the findings of Kuiper et al (2015). In summary, mutations located within the active site as well as in other regions of the enzyme contribute to the overall conformation and drug binding interaction of viral PR. Each of these conformational changes account for the high-level of drug resistance experienced by this patient at DRV/r failure.

Molecular docking studies confirmed these findings, as the inhibitory potency of DRV/r was lowered in the DRV/r-resistant model and to a lesser extent in the LPV/r-resistant model. The binding scores of the LPV/r-resistant model was similar to the DRV/r-resistant model. This is attributed to the presence of certain LPV/r resistance mutations that are involved in the pathway to DRV/r cross-resistance, that inherently confer baseline DRV/r resistance prior to treatment. Molecular Dynamics simulations showed that LPV/r and DRV/r-resistant models seemed to fluctuate more than the wild-type model, indicating that PI DRMs contributed to the overall stability of the structure. Our results confirm that drug resistance considerably compromises the drug binding interaction within viral PR. Despite the potency and ability of DRV/r to withstand a certain degree of mutations, multiple mutations of this kind can severely compromise its efficacy. Studying these physical impacts of mutations on drug binding affinity provides a detailed

perspective on how the enzyme can accommodate such changes but also provides a basic platform for the design of new and improved PIs.

Patients within our setting urgently require adherence counseling to preserve current ARVs and prevent the emergence of drug resistance mutations. One of the major limitations of our study was that we could not accurately measure drug levels for either LPV/r or DRV/r at the relevant time points in order to fully assess patient adherence. Furthermore, CD4 data for the majority of these patients were not available; thus we do not know the full clinical profile of these patients. Additionally, while longitudinal analysis of patient's revealed valuable information, a larger sample size may provide more information on treatment outcomes.

CHAPTER 6

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APPENDIX A

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AY878068	AY900909	DQ011175	DQ093586	DQ164127	DQ222278	DQ351229
AY878070	AY900912	DQ011176	DQ093587	DQ164129	DQ222279	DQ351230
AY878071	AY900914	DQ011177	DQ093588	DQ205288	DQ222280	DQ351231
AY878072	AY900915	DQ011178	DQ093589	DQ207941	DQ222281	DQ351232
AY900740	AY900917	DQ011179	DQ093590	DQ222243	DQ222282	DQ351233
AY900750	AY900920	DQ011180	DQ093591	DQ222244	DQ275642	DQ351234
AY900752	AY900936	DQ013271	DQ093592	DQ222245	DQ275643	DQ351235
AY900759	AY900953	DQ013277	DQ093593	DQ222246	DQ275644	DQ351237
AY900760	AY900954	DQ056302	DQ093594	DQ222247	DQ275645	DQ369976
AY900774	AY900955	DQ056404	DQ093595	DQ222248	DQ275646	DQ369977
AY900775	AY900956	DQ056405	DQ093596	DQ222249	DQ275647	DQ369978
AY900777	AY900957	DQ056406	DQ093597	DQ222250	DQ275648	DQ369979
AY900780	AY901965	DQ056408	DQ093598	DQ222251	DQ275649	DQ369980
AY900782	AY901966	DQ056409	DQ093599	DQ222252	DQ275650	DQ369981
AY900785	AY901967	DQ056410	DQ093600	DQ222253	DQ275651	DQ369982
AY900787	AY901968	DQ056411	DQ093601	DQ222254	DQ275652	DQ369983
AY900797	AY901969	DQ056412	DQ093602	DQ222255	DQ275653	DQ369984
AY900806	AY901970	DQ056413	DQ093604	DQ222256	DQ275654	DQ369985
AY900832	AY901971	DQ056414	DQ093605	DQ222257	DQ275655	DQ369986
AY900834	AY901972	DQ056415	DQ093607	DQ222258	DQ275656	DQ369987
AY900835	AY901973	DQ056416	DQ164104	DQ222259	DQ275657	DQ369988
AY900836	AY901974	DQ056417	DQ164105	DQ222260	DQ275658	DQ369989
AY900837	AY901975	DQ056418	DQ164106	DQ222261	DQ275659	DQ369990
AY900839	AY901976	DQ060209	DQ164107	DQ222262	DQ275660	DQ369991
AY900843	AY901977	DQ060212	DQ164108	DQ222263	DQ275661	DQ369992

DQ369993	DQ396399	DQ660000	DQ660048	DQ826607	EF186941	DQ660066
DQ369994	DQ445631	DQ660001	DQ660050	DQ826608	EF186942	DQ660067
DQ369995	DQ445632	DQ660003	DQ660051	DQ826609	EF186943	DQ660068
DQ369996	DQ445633	DQ660004	DQ660052	DQ826610	EF186944	DQ660069
DQ369997	DQ445634	DQ660005	DQ660053	DQ826611	EF186945	DQ660070
DQ396364	DQ445635	DQ660006	DQ660054	DQ826612	EF186946	DQ660071
DQ396365	DQ445637	DQ660007	DQ660055	DQ826613	EF186947	DQ660073
DQ396366	DQ659965	DQ660008	DQ660056	DQ826614	EF186948	DQ660074
DQ396367	DQ659966	DQ660009	DQ660057	DQ826615	EF186949	DQ660075
DQ396368	DQ659967	DQ660013	DQ660058	DQ826616	EF186950	DQ666390
DQ396369	DQ659968	DQ660014	DQ660059	DQ826617	EF186951	DQ826595
DQ396370	DQ659969	DQ660015	DQ660060	DQ826618	EF186952	DQ826596
DQ396371	DQ659970	DQ660016	DQ660061	DQ826619	EF186953	DQ826597
DQ396372	DQ659972	DQ660017	DQ660062	EF064480	EF186954	DQ826598
DQ396373	DQ659973	DQ660019	DQ660063	EF120244	EF195277	DQ826599
DQ396374	DQ659974	DQ660020	DQ660064	EF120254	EF368549	DQ826600
DQ396375	DQ659975	DQ660021	DQ660065	EF120336	EF368642	DQ826601
DQ396376	DQ659976	DQ660022	DQ660066	EF120337	EF368697	DQ826602
DQ396377	DQ659977	DQ660023	DQ660067	EF122539	EF368731	DQ826603
DQ396378	DQ659978	DQ660024	DQ660068	EF143496	EF368743	DQ826604
DQ396379	DQ659979	DQ660025	DQ660069	EF143499	EF368805	DQ826605
DQ396380	DQ659980	DQ660026	DQ660070	EF143501	EF368807	DQ826606
DQ396381	DQ659982	DQ660027	DQ660071	EF143502	EF368846	DQ826607
DQ396382	DQ659983	DQ660028	DQ660073	EF143518	EF368925	DQ826608
DQ396383	DQ659984	DQ660029	DQ660074	EF143521	DQ660051	DQ826609
DQ396384	DQ659985	DQ660031	DQ660075	EF143527	DQ660052	DQ826610
DQ396385	DQ659986	DQ660032	DQ666390	EF143528	DQ660053	DQ826611
DQ396386	DQ659987	DQ660033	DQ826595	EF143529	DQ660054	DQ826612
DQ396387	DQ659988	DQ660035	DQ826596	EF143532	DQ660055	DQ826613
DQ396388	DQ659989	DQ660036	DQ826597	EF143533	DQ660056	DQ826614
DQ396389	DQ659990	DQ660037	DQ826598	EF157888	DQ660057	DQ826615
DQ396390	DQ659991	DQ660038	DQ826599	EF186933	DQ660058	DQ826616
DQ396391	DQ659992	DQ660039	DQ826600	EF186934	DQ660059	DQ826617
DQ396392	DQ659993	DQ660041	DQ826601	EF186935	DQ660060	DQ826618
DQ396393	DQ659994	DQ660042	DQ826602	EF186936	DQ660061	DQ826619
DQ396394	DQ659995	DQ660043	DQ826603	EF186937	DQ660062	EF064480
DQ396395	DQ659996	DQ660045	DQ826604	EF186938	DQ660063	EF120244
DQ396396	DQ659998	DQ660046	DQ826605	EF186939	DQ660064	EF120254
DQ396397	DQ659999	DQ660047	DQ826606	EF186940	DQ660065	EF120336

EF120337
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APPENDIX B

Use of the Agilent 2100 Bioanalyzer

The DNA 1000 Kit was used to carry out the assay. Reagents were thawed and prepared according to the manufacturer's instructions. Briefly, the chip priming station together with the syringe kit, base plate and syringe clip were set up according to the manufacturer's instructions. A new DNA 1000 chip was removed from its seal and placed on the chip priming station; nine µl of the gel dye mix was pipetted to the bottom of the well-marked with a G. The plunger was positioned at the one ml marking, the chip priming station was closed the plunger was pressed down and held by the syringe clip for 60 seconds. This allows the distribution of the gel throughout each of the wells through a pressurization action. Thereafter the clip was released allowing the plunger to move back to its one ml position. The chip priming station was opened and nine µl of the gel-dye mix was added into the remaining two wells marked with a G. Five µl of the DNA marker was added into the well-marked with the ladder symbol as well as into each of the 12 sample wells. One µl of the DNA ladder was added to the well with the ladder symbol, and one µl of each amplicon was added into the respective sample wells, one µl of deionized water was added to any remaining empty wells. The chip was then removed from the chip priming station and placed on the IKA vortex (Roche, Life Sciences, Branford, CT, USA) at 2400rpm for 60 seconds. The chip was inserted into the Agilent Bioanalyzer and run within five minutes on the DNA 1000 series assay, after 30 minutes the gel and an electropherogram were viewed on the Agilent Software program (Agilent Technologies, Santa Clara, CA, USA).

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APPENDIX C

Emulsion PCR

1. Preparation of Mock Mix and Pre-Emulsion

Reagents were obtained from the GS Junior Titanium emPCR Kit (Lib-A) (Roche Applied Sciences, Mannheim, Germany) and were thawed according to the manufacturer's instructions. A 1 X mock mix was prepared using molecular grade water. The provided emulsion oil was vortexed well and poured into the IKA Turrax Stirring Tube (Roche Applied Science, Mannheim, Germany) two mls of the freshly prepared 1 X mock mix was added. The Ultra Turrax Tube Drive (UTTD) (Roche Applied Science, Mannheim, Germany) was set at 4000rpm for five minutes. During this time two Live Amplification mixes were prepared in 1.7ml micro-centrifuge tube as indicated in Table 1 the tubes were stored on ice until required.

Table 1. Preparation of Live Amp Mix A and B for the emulsion PCR.

Reagent (Amp A)	Volume (µl)	Reagent (Amp B)	Volume (µl)
Molecular Biology Grade Water	205	Molecular Biology Grade Water	205
Additive	260	Additive	260
Amp Mix	135	Amp Mix	135
Amp Primer A	40	Amp Primer A	40
Enzyme mix	35	Enzyme mix	35
Ppiase	1	Ppiase	1
Total	676	Total	676

2. DNA library capture

Capture beads have oligonucleotides that are complementary to adaptor A and B of the amplicons bound on their surface. Each of the capture beads A and B were washed twice with a freshly prepared 1 X Wash buffer by a spin rotation (quick spin the tube in one orientation, rotate the tube and quick spin again), the supernatant

was then removed. An input library of one copy per bead corresponding to the addition of 2.5µl of sample was added to capture bead A and 2.5µl to capture bead B. Six hundred µl of Live Amp Mix B was added to the tube of captured library B, this was vortexed and transferred to the Turrax stirring tube set at 2000rpm for five minutes. Thereafter 600µl of the Live Amp Mix A was added to the tube of captured library A and vortexed at the same conditions. Thereafter, 100µl of the emulsion was dispensed using a combitip into a new 96 well optical plate. The plate was sealed with strip capped sealers and placed on the thermocycler under the following reaction conditions, one cycle of 94°C for four minutes, 50 cycles of 94°C for 30 seconds, 58°C for 4.5minutes, 68°C for 30 seconds; the reaction was held at 10 °C until use. The plate was left in the thermocycler for no more than 16 hours.

3. Bead Recovery

The beads were recovered by breaking the water in oil emulsion using isopropanol and ethanol solutions. The recovered beads represent a heterogeneous population comprising several null beads which are those beads that have no library fragments captured, mixed beads that have captured more than one fragment as well as the DNA beads that have correctly captured a single library fragment. Null beads and mixed beads will not produce any readable sequence data and are therefore removed. The emulsion was aspirated into a 50 ml tube using a vacuum assisted transpette provided in the GS Junior Oil and Breaking Kit (Roche. 454^{TM} Science, Branford, USA). The wells were washed twice with 100µl of isopropanol per well and the amplified beads (approximately 15ml) were collected in a 50 ml falcon tube.

4. Bead Washes

In order to recover the collected emulsion, beads were washed as described below and all centrifugation steps were performed at 930X g for five minutes. The 50ml tube of collected emulsions was vortexed well and washed by adding isopropanol to a final volume of 35ml.The pellet was vortexed again, then centrifuged and the

supernatant was carefully poured out. Ten mls of enhancing buffer was added to the tube and vortexed well. It was important to completely re-suspend the bead pellet and ensure all aggregates were completely broken. The beads were washed again by re-suspending in a final volume of 40ml of isopropanol, vortexed and centrifuged. An additional wash using isopropanol followed by ethanol, both to a final volume of 35ml was performed, the supernatant was removed and enhancing buffer to a final volume of 35ml was added. Following centrifugation the supernatant was removed, leaving behind at least two mls. The pellet was re-suspended and the final DNA bead suspension was transferred to a 1.7ml siliconized micro-centrifuge tube provided in the kit. Following a spin rotation the supernatant was removed and discarded and the remaining bead suspension was added to the tube and the spin rotation repeated. The 50ml tube was rinsed with an additional one ml of enhancing buffer and added to the 1.7ml tube. The bead pellet was thoroughly rinsed twice with one ml of enhancing buffer by spin rotation and discarding of the supernatant.

5. DNA library bead enrichment

A 10N Sodium Hydroxide (NaOH) (Sigma Aldrich,St Louis, Missouri, USA) solution was prepared by dissolving 2g of NaOH in five mls of Molecular biology water. This was used to prepare a Melt solution, with 125µl of 10N NaOH and 9.875ml of molecular biology grade water. Beads were washed twice with one ml of Melt solution followed by a two-minute incubation at room temperature. After a spin rotation the supernatant was removed, discarded and 45µl of annealing buffer, 15µl of enrich primer A and 15µl of enrich primer B was added to the beads and vortexed well. The tube was placed on a heated block at 65°C for five minutes and then promptly cooled on ice for two minutes. The beads were washed with one ml of enhancing buffer for a total of three washes; each wash was followed by a spin rotation and removal of the supernatant

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6. Preparation of enrichment beads

Enrichment beads were vortexed thoroughly for one minute to re-suspend the beads. Beads were washed by placing the tube on the Magnetic Particle Concentrator (MPC) (Invitrogen, Life Technologies, Carlsbad, CA) for three minutes, removing the supernatant and adding 500µl of Enhancing buffer. The enrichment tube was then placed on the MPC to pellet the beads and the wash was repeated. Following the final wash, 80µl of enhancing buffer was added to the beads and vortexed. All of the washed enrichment beads were added to the 1.7ml tube of DNA beads and vortexed. The tube was placed on the lab rotator at room temperature for five minutes and thereafter pelleted on the MPC for five minutes, followed by removal of the supernatant. DNA beads were washed between six to ten times with one ml of Enhancing buffer to remove the white beads that do not contain DNA until the aspirated supernatant was clear i.e. no white beads were present. The enriched DNA beads were washed twice with 700µl of Melt solution, vortexed for five seconds and then pelleted on the MPC. The supernatant containing enriched DNA beads was transferred to a new 1.7ml tube. After a spin rotation the supernatant was removed and discarded and one ml of annealing buffer was added for a total of three washes. Finally, 100µl of annealing buffer was used to re-suspend the beads.

7. Bead counting

After the enrichment, beads were counted on the GS Junior emPCR Bead Counter v2 (Roche, 454 ^{TM} Life Science). The recommended input for a sequencing run is 500 000 enriched DNA beads. The bead counter was held at eye level, and the position of the pellet was noted. Ideally the pellet should be located within the window and above the bottom edge, indicative of 500 000 beads. If the number of positive beads was too high and the enrichment exceeded the threshold parameter of 20%, this indicated that the bead population contained too many beads and was discarded.

8. Sequence primer annealing

To anneal the sequencing primers, 15µl of sequencing primer A and 15µl of sequencing primer B was added to the 1.7ml tube and vortexed. The tube was placed on a heating block at 65°C for five minutes and promptly cooled on ice for two minutes. The beads were washed twice with one ml of enhancing buffer and stored at 2-8°C for up to two weeks before sequencing.

Sequencing reaction

1. Pre-Wash

Before beginning the sequencing run, the instrument fluidics was thoroughly washed by performing a Pre-wash, according to the manufacturer's instructions (GS Junior Titanium Series, Sequencing Method Manual).

2. Preparation of the Bead Deposition Device (BDD)

The BDD was washed twice using Nano Pure water and left to air dry. The PTP plate was then installed onto the BDD according to the manufacturer's instructions (GS Junior Titanium Series, Sequencing Method Manual).

3. Preparation of Bead Buffer 2 (BB2)

From the sequencing kit buffers, 6.6ml of Supplement CB was added to the Buffer CB bottle and thoroughly mixed by inverting the bottle ten times. Forty mls of this mix was transferred to a 50ml falcon tube and labeled Bead Buffer 2 (BB2); this was placed on ice until required. The tube of Apyrase was thawed and centrifuged at 9300 X g for five seconds and 6.5µl was added to BB2 and was gently inverted ten times to mix the contents and thereafter placed on ice. Using a single motion, 380µl of BB2 was pipetted into the loading port of the BDD and centrifuged for five minutes at 1620 X g. It was important to ensure that the centrifuge was correctly balanced with the provided adaptors during the centrifugation process.

4. Preparation of the bead layers

Four kinds of beads were prepared and loaded onto the PTP

- Preparation of DNA beads

Control beads XLTF were centrifuged in a micro-centrifuge for five seconds at 9300 X g and was resuspended by pipetting up and down at least five times. Six µl of the control beads and 500µl of BB2 were added to the previously prepared enriched DNA beads. After gentle vortexing the tube was sealed with parafilm and placed on the Lab Rotator for 20 minutes at room temperature.

- Preparation of the packing beads

Packing beads were washed three times by adding one ml of BB2 and vortexing at high speed until a homogenous suspension was formed. The tube was centrifuged at 9300 X g for five seconds following which the supernatant was carefully removed and discarded. After the final wash, 200µl of BB2 was added, vortexed and kept on ice until use.

- Preparation of the enzyme and PPiase Beads

Enzyme beads and PPiase beads were washed three times in parallel by the addition of one ml of BB2, care was taken not to mix tips. The tubes were vortexed at medium speed and placed on the MPC for 30 seconds thereafter the supernatant was carefully removed and discarded. After the third wash, 400µl of BB2 was added to the Enzyme beads and 410µl of BB2 was added to the PPiase beads and vortexed at medium speed. PPiase beads were then placed on ice until use. Enzyme Pre-Layer and Enzyme Post-layer were prepared as indicated in Table 2 and the tubes were stored on ice until required.

Table 2 Preparation of enzyme beads Pre-Layer and Post-Layer for the sequencing reaction

Reagent	Enzyme Beads Pre-Layer (µ/l)	Enzyme Beads Post- Layer (µ/l)	Total volume (µ/l)
BB2	300	180	410
Enzyme Beads	110	230	410

- ***Preparation of the DNA and Packing Beads***

The DNA bead tube was removed from the Lab Rotator and centrifuged at 9300 X g for five seconds to pellet the beads. BB2 was removed leaving behind approximately 50µl. Polymerase and Polymerase Cofactor tubes were centrifuged at 9300 X g for five seconds. Forty µl of Polymerase, 20µl of Polymerase Cofactor and 65µl of BB2 was added to the tube of DNA beads. The mixture was vortexed at medium speed for five seconds and incubated on the Lab Rotator for ten minutes at room temperature. The tube of Packing Beads was vortexed at high speed and 175µl of packing beads was added to the DNA mixture, vortexed at low speed and incubated on the Lab Rotator for five minutes at room temperature.

5. Preparation of Buffer CB

The tube of 0.1M DTT was thawed, vortexed at high speed for five seconds and one ml was added to the bottle of Buffer CB and was inverted ten times. Using a serological pipette, 44ml of Substrate TW was transferred to the bottle of Buffer CB. The bottle was inverted ten times to mix and thereafter placed in the dark.

6. Priming the GS Junior

The GS Junior was primed following the manufacturer's instructions in the user handout (Roche, 454 Life Science, Branford, CA, USA). The bottle of Buffer CB and the cassette containing sequencing reagents were placed in the instrument.

7. Deposition of bead layers

Beads were deposited onto the PTP by pipetting 350µl of each bead suspension through the loading port of the BDD. Using centrifugal sedimentation, beads were packed to the bottom of the wells of the plate. After each layer was centrifuged, the supernatant was removed before the next layer was added.

8. Initiating the sequencing run

The GS Junior Titanium, PTP Kit Cartridge was prepared according to the manufacturer's instructions, full processing for amplicons was selected at 200 cycles, the plate was loaded onto the instrument and the sequencing run was started. The instrument fluidics were calibrated for 30 minutes followed by the sequencing run for nine hours

APPENDIX D

Input files for the Molecular Dynamics simulation

Energy minimization	Heating Stages
minimize ras-raf &cntrl imin=1,maxcyc=2500,ncyc=750, cut=8.0,ntb=1, ntc=2,ntf=2, ntpr=100, ntr=1, restraintmask=':1-242', restraint_wt=2.0 /	heat ras-raf &cntrl imin=0,irest=0,ntx=1, nstlim=25000,dt=0.002, ntc=2,ntf=2, cut=8.0, ntb=1, ntpr=500, ntwx=500, ntt=3, gamma_ln=2.0, tempi=0.0, temp0=300.0, ntr=1, restraintmask=':1-242', restraint_wt=2.0, nmropt=1 ig= -1 / &wt TYPE='TEMP0', istep1=0, istep2=25000, value1=0.1, value2=300.0, / &wt TYPE='END' /
Density	Equilibration
heat ras-raf &cntrl imin=0,irest=1,ntx=5, nstlim=25000,dt=0.002, ntc=2,ntf=2, cut=8.0, ntb=2, ntp=1, taup=1.0, ntpr=500, ntwx=500, ntt=3, gamma_ln=2.0, temp0=300.0, ntr=1, restraintmask=':1-242', restraint_wt=2.0, ig=-1	heat ras-raf &cntrl imin=0,irest=1,ntx=5, nstlim=1000000,dt=0.002, ntc=2,ntf=2, cut=8.0, ntb=2, ntp=1, taup=2.0, ntpr=1000, ntwx=1000, ntt=3, gamma_ln=2.0, temp0=300.0, ig= -1 /

Script: Calculation of the RMSD using PTRAJ

Trajin equil.mdcrd.gz 1:250 1
Reference sample_solvated.inpcrd

Rms reference out equil.rmsd @CA,C,N

APPENDIX E

Distances between residues and the inhibitor

Model at LPV failure			Model at LPV failure			Difference in distances
Residue	Location	Distance	Residue	Location	Distance	
D25	O18-OD1	2.443	D25	O18-OD1	2.434	0.009
	O18-CG	2.803		O18-CG	2.793	0.01
	OD2-O05	2.561		OD2-O05	2.561	0
D29	C29-OD2	4.204	D29	C29-OD2	4.205	0.001
	C29-CG	4.34		C29-CG	4.34	0
	C29-OD1	4.669		C29-OD1	4.667	0.002
	C29-CB	4.809		C29-CB	4.808	0.001
D30	C25-OD2	4.846	D30	C25-OD2	4.844	0.002
	C25-CG	5.151		C25-CG	5.149	0.002
	C25-OD1	6.219		C25-OD1	6.217	0.002
V32I	C15-CG1	8.125	V32I	C15-CG1	8.422	0.297
	C15-CB	9.623		C15-CB	9.898	0.275
	CG2-C15	10.167		CG2-C15	10.478	0.311
I50	CG1-C3	6.444	I50	CG1-C3	6.447	0.003
	CD1-C3	7.537		CD1-C3	7.557	0.02
	CB-C3	6.326		CB-C3	6.325	0.001
V82	CB-C15	4.324	V82A	CB-C15	4..284	0.04
	CB-C14	5.695		CG2-C15	4.588	1.107
	CA-C15	5.531		CG1-C15	4.643	0.888
I84	CG2-C15	6.191	I84V	CG2-C15	6.463	0.272
	CB-C15	6.07		CB-C15	6.129	0.059
	C15-CG1	7.034		C15-CG1	4.711	2.323