UNIVERSITY OF KWAZULU-NATAL

THE DIFFERENTIAL INFLUENCE OF HIV-1 SUBTYPE C,

NUCLEOSIDE ANALOG RESISTANCE MUTATIONS: K65R, A62V, S68N AND Y115F

SUSCEPTIBILITY TO TENOFOVIR.

By

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Student Number: 217079876

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Virology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences,

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Supervisor: Michelle L. Gordon (PhD)

Preface

The experimental work described in this dissertation was carried out in the Hasso Plattner Research Laboratory of the HIV Pathogenesis Programme at the Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, from February 2018 to February 2019 under the supervision of Michelle Lucille Gordon (PhD).

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.

Signed: _____ Date: <u>13 August 2019</u>

Onyisi Christiana Didamson (Candidate)

Signed:_____Date:_____<u>14 August 2019</u>_____

Michelle Lucille Gordon (PhD) (Supervisor)

Declaration

I, Onyisi Christiana Didamson, declare that:

1. The research reported in this dissertation, except where otherwise indicated is my original work.
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Michelle Lucille Gordon (PhD) (Supervisor)

Ethical Approval

Full ethical approval was obtained for this study from the Biomedical Research Ethics Committee of the Nelson R. Mandela School of Medicine, University of KwaZulu-Natal (BREC Ref No: BE600/17).

Presentation

Didamson OC and Gordon ML. Impacts of Tenofovir Selected Mutation of HIV-1 Subtype C on Replication Capacity and Tenofovir Susceptibility at the University of KwaZulu Natal, Annual Laboratory Medicine and Medical Sciences Research Symposium, 6th September 2019. (Oral poster presentation).

Dedication

This thesis is dedicated to God for His faithfulness, grace, preservation, provision, and strength upon my life through this research. Indeed, He is my Ebenezer.

Also, is dedicated to my loving husband, children (Shams and Shim), Mum (Roseline Uzoka), and my late Dad (John Uzoka). You are all an inspiration to me

Abstract

The use of Tenofovir Disoproxil Fumerate (TDF) for the treatment of HIV-1 infection has been recommended for the first-line as well as a second-line antiretroviral regimen in South Africa, due to its high antiretroviral activity and low toxicity level. However, the efficacy of the drug could be threatened by the emergence of drug resistance mutations. The development of TDF resistance poses a public health threat. TDF resistance can be acquired through a selection of the K65R mutation or the K70E mutation (though less frequently) under TDF selection pressure. Besides, K65R and K70E mutations, recent studies have identified other mutations associated with TDF resistance such as A62V, K65N, S68G/N/D, K70E/Q/T, L74I, V75L, and Y115F. These mutations were particularly observed to be in association with the K65R mutation and were reported to be more common in HIV-1 subtype C viruses. Also, these mutations could cause high-level resistance to TDF, especially when in combination with K65R. However, in-vitro studies are required to demonstrate their influence on viral fitness and TDF susceptibility. In this study, we investigated the impact of K65R, A62V, S68D, Y115F, and K65R+S68N on replication capacity and TDF susceptibility. The reverse transcriptase (RT) region was amplified from a drug-naive HIV-1 subtype C isolate obtained from a patient enrolled in the Tropism study (BREC: BF088/07) and cloned into a TOPO vector using a TOPO TA cloning kit. The HIV-1 RT mutations (K65R, A62V, S68D, Y115F, K65R+A62V, K65R+S68D, K65R+S68G,

K65R+S68N, and K65R+Y115F) were introduced into the TOPO+RTsubC recombinant using the Quikchange lightning Multi site-directed mutagenesis kit. Next, recombinant viruses were created by co-transfection of the mutant RT amplicons and a pNL4-3-deleted-reverse transcriptase (RT) (pNL43ΔRT) backbone into GXR cells by electroporation. The replication capacity of the mutant viruses was assessed using a replication method that utilized a green fluorescent protein (GFP) reporter cell line and flow cytometry. We evaluated the replication capacity using the exponential growth curve function in Excel to determine the percentage GFP-expressing cells between days 2 and 6. The impact of the mutant viruses on susceptibility to TDF was performed in a luciferase-based assay. The 50% inhibitory concentration (IC50) was calculated using Graph Pad Prism. Drug susceptibility was expressed as the fold change in IC50 of mutant virus compared with the wild type virus.

Of the 5 TDF- selected mutants analysed: A62V, K65R, and Y115F mutants display a reduction in replicative fitness whereas, S68D and K65R+S68N showed high viral fitness. Interestingly, the TDF-selected resistance mutations we analysed, showed high susceptibility (A62V, S68D, and Y115F) and reduced susceptibility (K65R and K65R+S68N) to TDF. Our findings support the hypothesis that TDF-selected mutations only confer reduced susceptibility to TDF. Hence, further study is needed on various combinations of TDF-selected resistance mutations to further solidify this claim.

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Abbreviations and Acronyms

3TC	Lamivudine
ABC	Abacavir
AID	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ART	Antiretroviral treatment
ARV	Antiretroviral
ATP	Adenosine Triphosphate
ATV	Atazanavir
AZT	Zidovudine
bp	base pair
cART	Combined antiretroviral therapy
CCO	Clinical cut-off
cDNA	Complementary DNA
cpx	Complex
CRFs	Circulating recombinant forms
d4T	Stavudine
ddC	Zalcitabine
ddI	Didanosine
DHHS	Department of Health and Human Services
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside Triphosphate
DRMs	Drug resistance mutations
dsDNA	Double Stranded Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
Env	Envelope Protein

ETR	Etravirine
FBS	Foetal Bovine serum
FC	Fold change
FDA	Food and Drug Administration
FTC	Emtricitabine
Gag	group-specific antigen
GFP	Green fluorescent protein
Gp120	Glycoprotein 120
Gp41	Glycoprotein 41
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HIVDB	HIV database
IC50	50% inhibitory concentration
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
LB	Luria Bertani
LMICs	Low and medium-income countries
LPV/r	Lopinavir boosted with Ritonavir
LTR	long terminal repeat
MDR	Multidrug resistance mutation
ml	Millilitre
mM	Millimolar
NDOH	National department of health
Nef	Negative regulatory factor
ng	Nanogram
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor

℃	Degrees Celsius
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pNL4-3ΔRT	pNL4-3 deleted reverse transcriptase
Pol	Polymerase
PR	Protease
RC	Replication capacity
Rev	Regulator of expression of virion particles
RLU	Relative light unit
RNA	Ribonucleic acid
RT	Reverse transcriptase
RTIs	Reverse transcriptase inhibitors
RT-PCR	Reverse transcription polymerase chain reaction
SANAC	Southern Africa national AIDS Council
SDM	Site-directed mutagenesis
SIV	Simian immunodeficiency virus
TAM	thymidine analogue mutation
Tat	trans-activator of transcription
TCID50	50% tissue culture infective dose
TDF	Tenofovir
VF	Virologic failure
Vif	Viral infectivity factor
Vpu	Viral protein unique
Vpx	Virus protein x
WHO	World health organization
WT	wildtype
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
β	Beta

γ	Gamma
μg	Microgram
μl	Microliter
μM	Micromolar

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Chapter One

Literature Review

1.1   Introduction

Over 77.3 million individuals have been HIV positive following the HIV-1 outbreak, with about 35.4 million deaths related to AIDS globally. In 2017, 36.9 million people were reported to be having HIV while approximately 21.7 million individuals were on antiretroviral treatment worldwide. Approximately 1.8 million individuals were diagnosed with new HIV infections and 0.94 million died from AIDS-influenced-illness in the same year (UNAIDS, 2018). Sub-Saharan Africa bears the highest HIV burden globally, with about 4.2% of individuals having the virus, representing approximately two-thirds of the HIV global burden (UNAIDS, 2018). Within this region, South Africa (where HIV-1 subtype C dominates) has the highest HIV-1 burden which is also the highest globally (WHO, 2018). In South Africa, the prevalence rate of HIV infection, new diagnosis, and AIDS-linked mortality are 19%, 15%, and 11% respectively. However, the KwaZulu-Natal (KZN) province, which is the location of this study has the highest infection with about 40.8% infected individuals (UNAIDS, 2018). Interestingly, there has also been a huge decrease in new HIV infections and AIDS-linked mortality since 2010 by 49% and 29% respectively (UNAIDS, 2018).

In 2017, South Africa had a total of 7.2 million individuals having HIV, 270000 of newly HIV infected individuals and 110000 mortality of AIDS-related illness (UNAIDS, 2018). To combat this epidemic, the most effective interventions towards this goal was the initiation of "highly active antiretroviral therapy (HAART)" for the prevention and treatment of HIV (UNAIDS, 2016, WHO, 2012). Large-scale interventions have been placed among the main global health goals, to eliminate the epidemic spread by 2030 (UNAIDS, 2016) as well as achieving universal ART access and the UNAIDS 90-90-90 target by 2020. In 2017, 4.4 million (61%) infected individuals had access to antiretroviral treatment, representing 20% of individuals on antiretroviral therapy in the world and 47% had their viral load suppressed (SANAC, 2017, UNAIDS, 2018).

In the last few decades, great achievements have been made through ART intervention among which include: great improvement in the life of HIV-1 individuals; drastic reduction in the mortality and morbidity rate of AIDS-associated illness; and huge decrease in mother-to-child HIV-1 spread (Granich et al., 2009, UNAIDS, 2016, Weng et al., 2016, WHO, 2012, Williams et al., 2011). However, in spite of these profound benefits and the large ART scale-up programme in South Africa, the prolong usage of these ARVs has led to the development of both acquired and transmitted drug-resistant mutations that confer cross-resistant and limit the benefits of subsequent treatment option (Hamers et al., 2013, Rhee et al., 2015, Sigaloff et al., 2012, Steegen et al., 2016). The evolution of HIV-1 drug resistance arises due to the following factors: lack

of proofreading in the HIV-1 reverse transcriptase (RT) enzyme in the course of replication, high turnover HIV-1 of multiplication, increased reservoir of the provirus strains during infection, recombination of different DNA sequences affecting the same target cell resulting in a large number of quasi-species and rapid evolution under drug selection pressure (Coffin, 1995, Katzenstein, 2006, Li and Kuritzkes, 2013, Shafer, 2002). Also, other contributing factors include poor compliance, inadequate drug absorption, a low genetic barrier to resistance, and the presence of pre-existing drug resistance mutations. The ultimate effect of drug resistance mutations is virologic failure leading to treatment failure (Coffin, 1995, Skhosana et al., 2015). Studies have reported that during treatment on first-line NRTI/NNRTI based treatment regimens, about 10% - 30% of individuals experienced virologic failure (VF) and more than half of these individuals acquired drug-resistant viruses (Aghokeng et al., 2014, McMahon et al., 2013, Rhee et al., 2015).

In 2010, South Africa implemented the use of TDF for both first-line and second-line antiretroviral treatment, as well as for pre-exposure prophylaxis in conjunction with the World Health Organization (WHO) ART treatment recommendations for adult HIV-1 infection (Bennett et al., 2008, NDOH, 2014, WHO, 2013). The effective use of TDF can be limited by the development of drug resistance mutations. Studies have identified the K65R mutation as the main TDF resistance mutation (TenoRes-Study, 2016, Rhee et al., 2017, HIVBD, 2019). Also, the K70E mutation is an alternative pathway of TDF resistance; however, its occurrence is not common (TenoRes-Study, 2016, Rhee et al., 2017, HIVBD, 2019).

 A previous study observed a greater than 50% incidence of K65R in viruses isolated from Sub-Saharan Africa (TenoRes-Study, 2016). Similarly, an increase in the incidence of the K65R resistance mutation has been observed in South African TDF treated patients (Skhosana et al., 2015, Sunpath et al., 2012), although Hoffmann and colleagues reported a low prevalence of the K65R mutation (Hoffmann et al., 2013). Another study showed that K65R was the most commonly transmitted or pre-treatment drug resistance mutation in ART-naive individuals in South Africa (Steegen et al., 2016). Furthermore, recent studies have observed other mutations "A62V, K65N, S68G/N/D, K70E/Q/T, L74I, V75L and Y115F" commonly found with TDF resistance in patients on TDF treatment and are seen to be co-selected particularly with K65R. These mutations were discovered to be higher in subtype C compared to subtype B (Maphumulo, 2016, Margot et al., 2016, Rhee et al., 2017). Therefore, it is paramount to determine the phenotypic implication of these mutations. In this study, we analysed the impact of the following TDF selected mutations: A62V, K65R, S68D, Y115F and K65R+S68N of HIV-1 Subtype C on viral replication capacity and susceptibility to TDF.

1.2   Human Immunodeficiency Virus

The human immunodeficiency virus (HIV) is "the aetiological agent of acquired immunodeficiency syndrome (AIDS)" and dates back in 1983. Since then, the epidemic is yet to be completely eradicated, but

rather under manageable control (Sampathkumar et al., 2012). HIV belongs to the class of virus known as Lentivirus of the family of Retroviridae and subfamily Orthoretrovirinae (ArbeitskreisBlut, 2016, Klimas, 2008). HIV is categorised into two, known as HIV-1 and HIV-2 based on genetic features and viral antigen. HIV-1 is the most prevalent and account for the global AIDS epidemic. It is also more infective, virulent and has high transmissibility rate. While, HIV-2 has lower pathogenic potentials and is mostly confined to West Africa due to its lower transmissibility rate (Kanki and Travers, 1994, O'Donovan et al., 2000). However, HIV-1 and HIV-2 likewise affect the immune system and develop an illness that compromises the immune system (Klimas, 2008, Sampathkumar et al., 2012, Santoro and Perno, 2013). While, in HIV-2 AID develop at a slower rate, with a higher CD4 count compared to HIV-1 (Esbjornsson et al., 2018, Martinez-Steele et al., 2007).

HIV has its origin from non-human primate harbouring the retrovirus Simian immunodeficiency virus (SIV). The SIV infecting humans represent two different zoonotic transmission sources namely, chimpanzee (SIVcpz), and sooty mangabeys (SIVsm) and are closely related to HIV-1 and HIV-2 respectively (Chavan, 2011, Katzenstein, 2006, Peeters and Sharp, 2000, Sampathkumar et al., 2012). Nevertheless, irrespective of not having the same origins, HIV-1 and HIV-2 share in total about 55% similarity on the nucleotide level (Li et al., 2015, Motomura et al., 2008). While, on the amino acid level about 55% similarity in Gag and Pol, and 35 % similarity Env (Motomura et al., 2008). However, a study has reported high genetic divergence between HIV-1 and HIV-2 with more than 50% sequence differences in the genes encoding the envelope proteins (Mourez et al., 2013). Also, the replication fitness and cytopathicity between the virus types have shown varying outcomes, despite having similar transmission routes and target cells (Arien et al., 2005).

Furthermore, both HIV-1 and HIV-2 have been characterized into groups, subtypes, and recombinant forms, with HIV-1 being well described into various groups, subtypes, sub-subtypes and circulating recombinant forms. Presently, nine groups denoted as A-I have been described for HIV-2, initially known as subtypes (Sauter and Kirchhoff, 2019, Visseaux et al., 2016), with A, B and D as the most predominant group (Santiago et al., 2005, Visseaux et al., 2016). Also, some recombination have been seen to be present between intergroup, while information on HIV-2 subtype is not available (Santiago et al., 2005, Ibe et al., 2010, Visseaux et al., 2016),

1.3   HIV-1

1.3.1   Groups and Subtypes
Phylogenetic analysis from different geographical areas have established 4 distinct clades of HIV-1 namely: "group M (main), N (new, or non-M, non- O), O (outlier) and P (putative group)", nevertheless, all manifest

the same clinical symptoms (Klimas, 2008, Peeters and Sharp, 2000, Santoro and Perno, 2013, Sharp and Hahn, 2011). The M group which was the first to be isolated in 1983 (Barré-Sinoussi et al., 1983), is the major strains found globally, and its account for the worldwide AIDS pandemic (Sharp and Hahn, 2011). The second to be isolated was Group O in 1990 and is not as widespread as the group M (De Leys et al., 1990) with about 1% of the worldwide HIV-1 infection. It is highly confined to Cameroon and surrounding countries (Peeters et al., 1997).

The third to be discovered was group N in 1998 from an individual originating from Cameroon (Simon et al., 1998), and it is not as disseminated as group O; with just 14 cases of infection reported (Vallari et al., 2010). While, the fourth to be identified was group P in 2009 from a 62-year-old Cameroonian woman residing in France (Plantier et al., 2009). Presently, only two strains of group P have been described, and are closely related phylogenetically; however, proof of any connection between the two strains is lacking (Vallari et al., 2011). The use of molecular clock analysis has postulated that a common ancestor of HIV-1 major groups moved into the human population in the mid-20th century and it is considered to have originated from chimpanzees and/or gorillas (Katzenstein, 2006, Korber et al., 2000). While group N, O and P are restricted to Central Africa (Sharp and Hahn, 2011).

HIV-1M group is characterized by vast divergent subtypes and circulating recombinant forms (CRFs). Phylogenetically, the group M viruses are classified into pure subtypes and CRFs. There are currently nine subtypes namely: "A, B, C, D, F, G, H, J, and K", with A and F subtypes further sub-classified into A1-A6 and F1and F2 respectively (Foley et al., 2016). The while circulating recombinant forms (CRFs) result when an individual is infected by multiple HIV-1 subtype variants either by coinfection or superinfection, giving rise to recombinant viral genome made up of regions from each of the diverse subtypes. The recombination formed is a consequence of template switching and the reverse transcriptase switches between the two templates in the course of the reverse transcription process. Therefore, sequences relating to a CRF have similar recombination split points in the genome. If there are huge epidemic transmission and dissemination of these new recombinants within a population, they are considered being circulating variants and are categorized as CRF (Peteers, 2000, Neher and Leitner, 2010). Also, the variant must be identified from two unrelated persons and completely sequenced (Carr et al., 1998). When more than three subtypes are present the designation "cpx" (complex) is employed (McCutchan, 2006).

Presently, over 98 different CRFs have been registered in the Los Alamos National Laboratory (www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html), and they account for 16.7% of all HIV infections in the world (Hemelaar et al., 2019). The characterization of HIV-1 into subtypes and CRFs confirm HIV diversity and also describes the several cross-species distributions seen in Africa, where the

initial HIV-1 isolate was derived (Katzenstein, 2006). HIV-1 subtypes vary in nucleotide sequence by 30% -40% in the envelope gene, 8–10% variation in the pol/gag genes, 20% variation within subtypes, and differ by 25 and 35% across subtypes (Peeters and Sharp, 2000, Santoro and Perno, 2013, Hemelaar et al., 2006).

1.4   Global distribution of HIV-1

HIV-1 subtype B is the dominating subtype in America, Europe and Australia, CRF-01_AE dominates in Asia, and CRF02_AG in Western Africa (Figure 1.1). While the largest HIV-1 subtypes and CRFs variation is found in Central Africa, the origin of HIV. HIV-1 subtype C is prevalent in southern Africa, the horn of Africa, India and China. (Figure 1.1) (Maartens et al., 2014, Tebit and Arts, 2011, Bbosa et al., 2019).
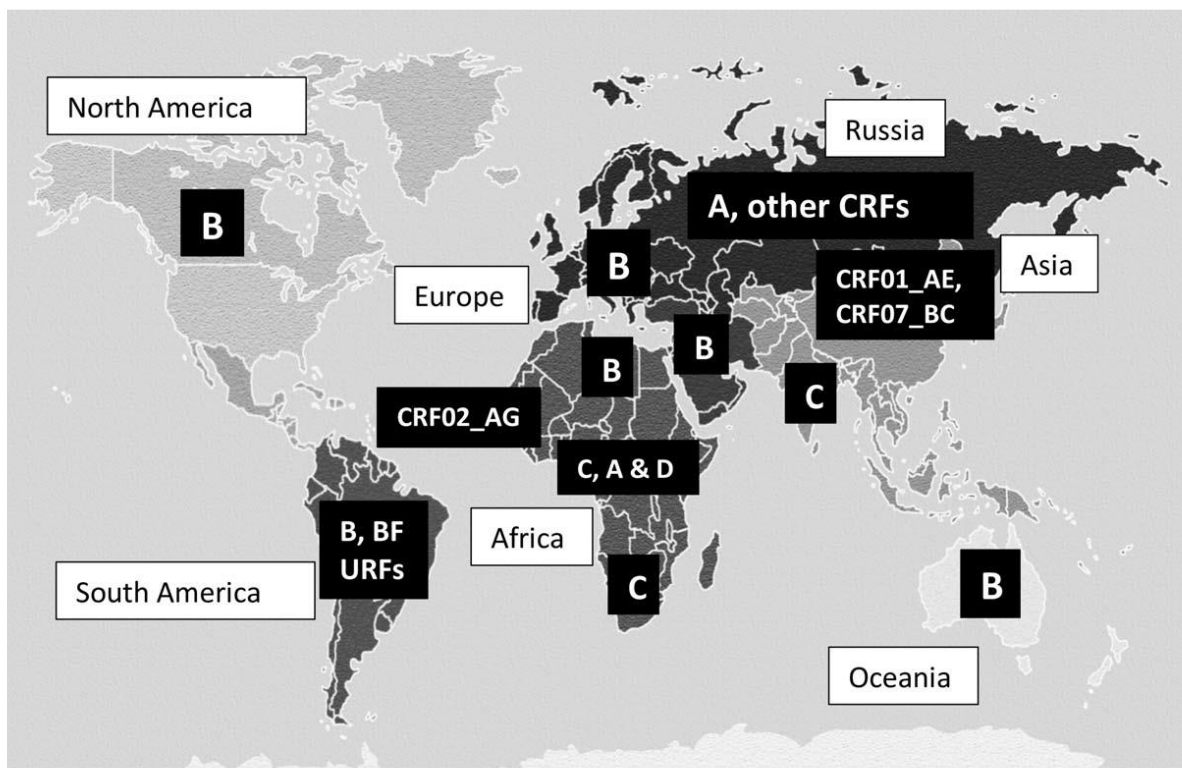
Figure 1.1: Global distribution of HIV-1 subtypes, CRF and URF. Taken from Bbosa et al., (2019). Abbreviations: CRF – circulating recombinant form; URF – unique recombinant form.

A recent update in the global distribution of HIV-1 subtypes, CRF and URF by Hemelaar et al showed that subtype C is the more dominant type (46.6%), then by subtype B (12.1%), subtype A (10.3%), subtype G (4.6%), subtype D (2.7%) and any of subtypes F, H, J or K (0.9%). The CRF02_AG was prevalent in 7.7% of the global population with CRF01_AE in 5.3% of the population, with other recombinants accounting for 3.7% (Hemelaar et al., 2019). Abbreviations: CRF – circulating recombinant form; URF – unique recombinant form.

1.5   HIV-1 Structure

HIV-1 is a spherical enveloped retrovirus with a diameter of about 120nm that consists of the following components namely, the viral envelop, matrix, capsid, nucleocapsid, and the viral genome. The viral envelope is bi-lipid layers consisting of two envelop proteins, glycoprotein (gp) gp120 and gp41 which facilitate the attachment and fusion of the virus to the affected cell to initiate infection (figure 1.2). These envelope proteins are potential target against HIV-1 infection (Rajarapu, 2014). Within, the viral envelope is the matrix, which surrounds the capsid (p24), consists of the p17 viral protein that maintains the integrity of the virion particle (figure 1.2). The capsid (p24) encloses the nucleocapsid (p7/p6) that is tightly bound to the viral genetic material (RNA). The nucleocapsid also contains enzymes consisting of, reverse transcriptase, protease ribonuclease and integrase required for viral replication process (Rajarapu, 2014).

1.6   HIV-1 genome organization

HIV-1 genome is made up of about 9720 base pairs and nine genes, flanked by the long terminal repeat (LTR) at the 5' and 3' ends (figure 1.3) (Frankel and Young, 1998, Gallo et al., 1988, Muesing et al., 1985). The nine genes consist of three structural genes and six accessory genes. The structural genes consist of: "group-specific antigen (gag), polymerase (pol) and envelope (env)" that encode vital information crucial for the generation of new viral particles. The gag gene reading frame encodes four structural proteins that form the viral core: "the capsid (CA) p24, the matrix (MA) p17, the nucleocapsid (NC) p7 and p6 protein" (King, 1994, Mushahwar, 2007). The second reading frame, the pol genes codes for the viral enzymes, reverse transcriptase (RT, p55), ribonuclease H (RNase H, p15), or RT plus RNase H (p66), protease (PR, p12), and integrase (IN) (Frankel and Young, 1998, Votteler and Schubert, 2008) and are target for antiretroviral drugs (Levy, 2007). The Env viral glycoprotein the third reading frame codes for surface gp120 and transmembrane gp41 (King, 1994, Mushahwar, 2007, Frankel and Young, 1998). The six accessory genes consist of: negative regulatory factor (nef), regulator of expression of virion particles (rev), trans-activator of transcription (tat), viral protein R (vpr), viral infectivity factor (vif), and viral protein unique (vpu), that encode for proteins that regulate the potential of HIV-1 to enter a cell, generate of viral particles, or initiate pathogenesis (Frankel and Young, 1998). Vpx (virus protein x) in place of vpu, is seen in HIV-2, is proposed to be accountable for the low pathogenic nature of HIV-2 (Vicenzi and Poli, 2013). The long terminal repeat (LTR) at each end of the HIV-1 genome has a binding site which facilitates and boost viral expression (http://www.hiv.lanl.gov/).
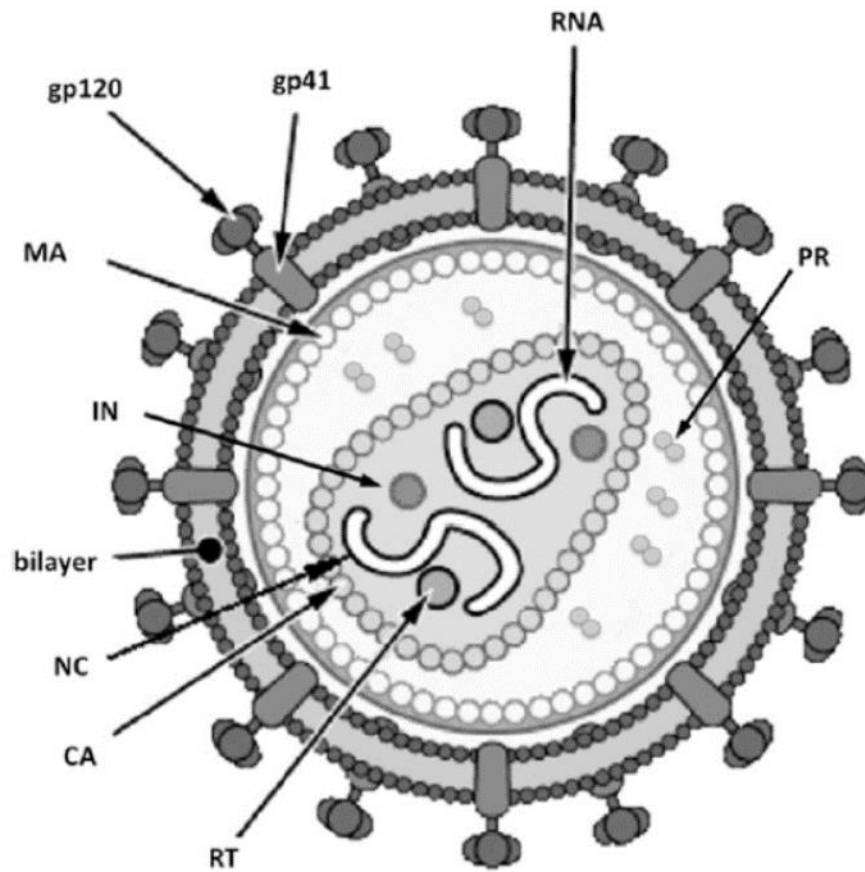
Figure 1.2: General features of HIV-1 virion. Taken from Rajarapu (2014).

Abbreviations: NC- Nucleocapsid; CA- Capsid; RT- Reverse transcriptase; IN- Integrase; PR- Protease and MA- Matrix protein.
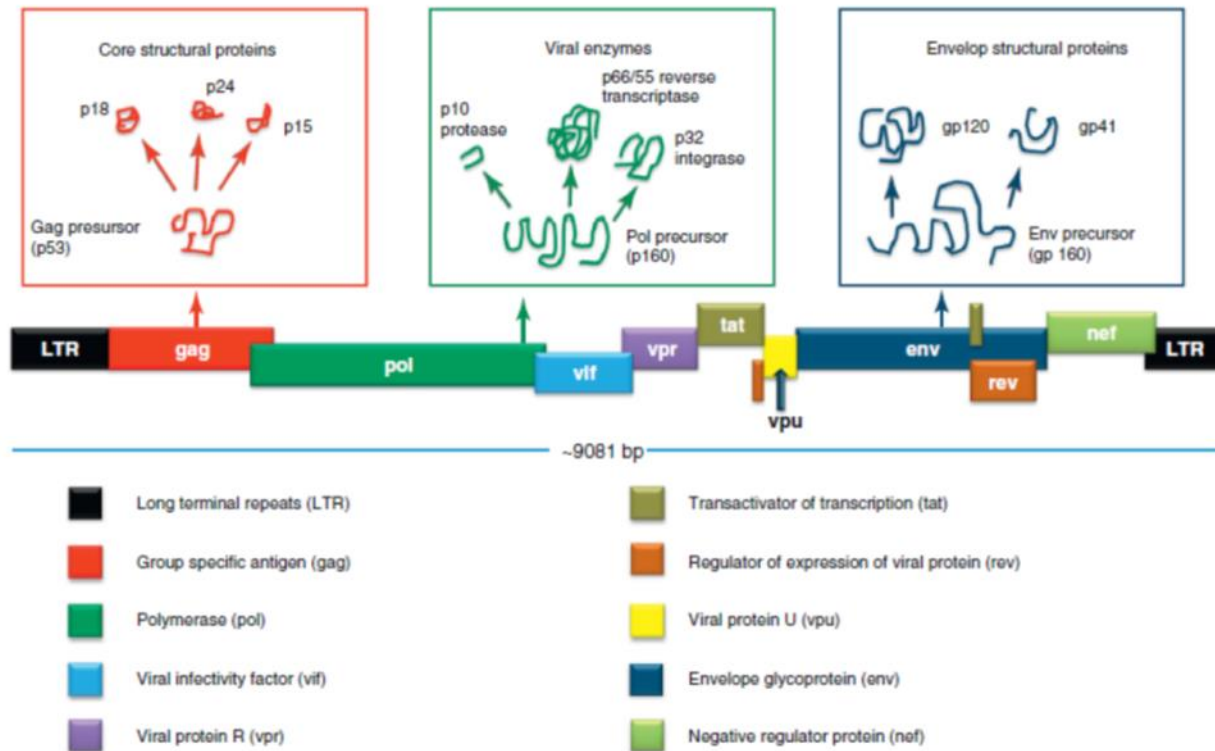
Figure 1.3: Genomic organization of HIV-1. Taken from Rajarapu (2014).

1.7   Infection of human cells and HIV-1 Replication cycle

The interaction of a protein-protein complex characterizes the first steps of HIV infection. The surface glycoprotein (gp) 120 of the well-formed HIV particles attach to the CD4 receptor of the host cell[1]. Upon fusion one of the two CD4 receptor: "C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4)" is activated which accelerates the release of the gp41 protein for efficient membrane binding, resulting in the merging of the viral envelope and the CD4 receptor into the host cell (ArbeitskreisBlut, 2016). The viral core enters the cytoplasm and un-coats releasing the diploid viral RNA genome and "the viral enzymes (RT, IN and PR)" (Rajarapu, 2014). The RT enzyme transcribes the single-stranded viral DNA into two strands of proviral DNA.

The proviral DNA is transferred into the nucleus and is incorporated into the host genome by the IN enzyme (Rajarapu, 2014). This stage of integration concludes the HIV infection of the cell and the maintenance of continuous infection (ArbeitskreisBlut, 2016). The new provirus can reproduce, as the host cell replicates fusing existing cellular transcription mechanism which results in the synthesis of new RNA and RNA that codes for the viral proteins. The viral proteins and components merge at the surface of the host surface and

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[1] CD4 positive cells such as T helper cell, macrophages, dendritic cells and astrocytes are the primary target of HIV infection

are spliced by the protease enzyme into functional units. The new complete viruses are assembled and bud off from the surface of the host cell into extracellular space, acquiring an envelope and attachment spike (Rajarapu, 2014, Klimas, 2008). Following infection with HIV, about two days is needed to produce the first progeny viruses from the infected cell with a daily turnover rate of 10^8- 10^9 viral particles. This is due to the error in the RT proofreading activity. It is postulated that under one replication cycle under no antiretroviral drug, an evolutionary rate of 1 in 10^4 of nucleobase in a genome do occur, resulting in genetically distinct, but closely related viruses circulating in the body known as the quasispecies (Santoro and Perno, 2013). While under antiretroviral drug pressure, rapid virus turnover together with a high mutation rate is the main mechanism behind the development of HIV variants with antiretroviral drug resistance (Santoro and Perno, 2013).
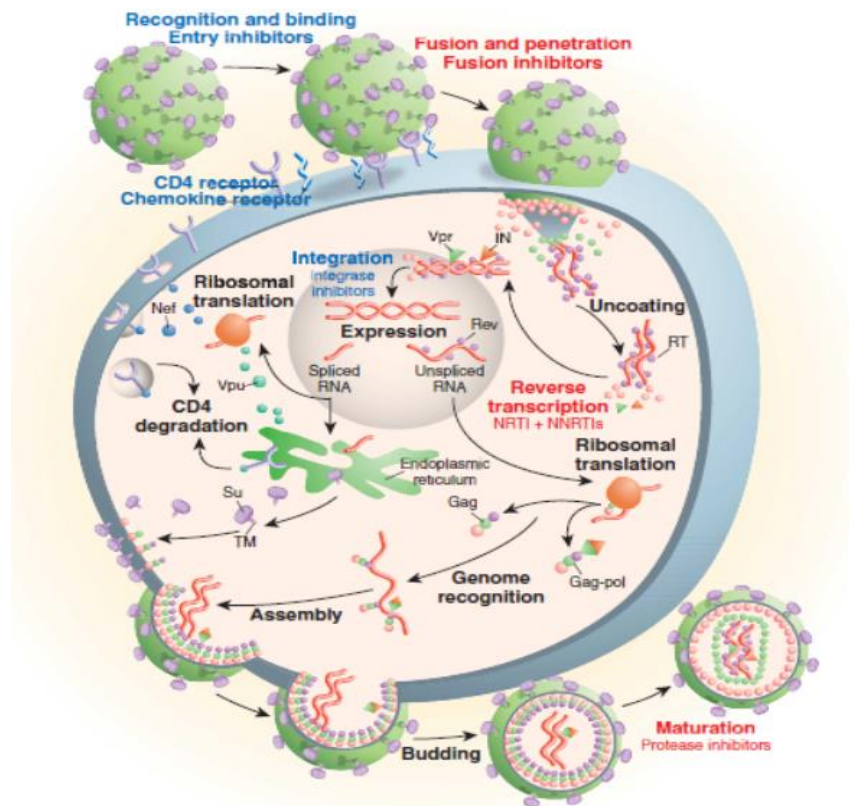
Figure 1.4: HIV-1 Replication Cycle. Taken from (Pomerantz and Horn, 2003).

1.8   HIV-1 Reverse Transcriptase

The HIV-1 RT enzyme converts the virus single-stranded RNA into two strands of DNA that are incorporated into the host genome (Sarafianos et al., 2009, Shafer, 2002). The HIV-1 RT has two catalytic sites, the N-terminal polymerase and the C-terminal RNase H. The N-terminal polymerase carries out two

enzymatic roles which are, RNA- and DNA-dependent DNA polymerization. While the C-terminal RNase H splits RNA from RNA/DNA hybrid to release the new DNA strand required as a template for DNA synthesis. These two catalytic sites of RT, work together to generate a double-stranded linear DNA from RNA (Sarafianos et al., 2009, Li et al., 2016, Huang et al., 1998). The HIV-1 RT is a heterodimer composing of a p66 and p51 subunit. These subunits are products of the Gag-Pol polyprotein precursor cleavage through the action of the viral protease (PR). The p66 subunit is comprised of 560 amino acids and is made up of three domains: the polymerase, RNase H and the connection domain that joins the polymerase and the RNase H domain (Sarafianos et al., 2009, Shafer, 2002, Kohlstaedt et al., 1992). The polymerase domain further consists of four subdomains: fingers (amino acid (aa) residues 1–85 and 118–155), palm (86–117 and 156–236 amino acid residues), thumb (237–318 amino acid residues), and connection (319–426 amino acid residues) (Sarafianos et al., 2009). While the p51 subunit comprised of 449 amino acids of the pol gene and is catalytically non-functional but provide the platform for the enzymatic function of the p66 subunit (Shafer, 2002). The p51 subunit also consists of four subdomains and has a similar sequence: "fingers, palm, thumb, and connection", although, the orientation is quite different from the p66 subunit (Kohlstaedt et al., 1992, Sarafianos et al., 2009).
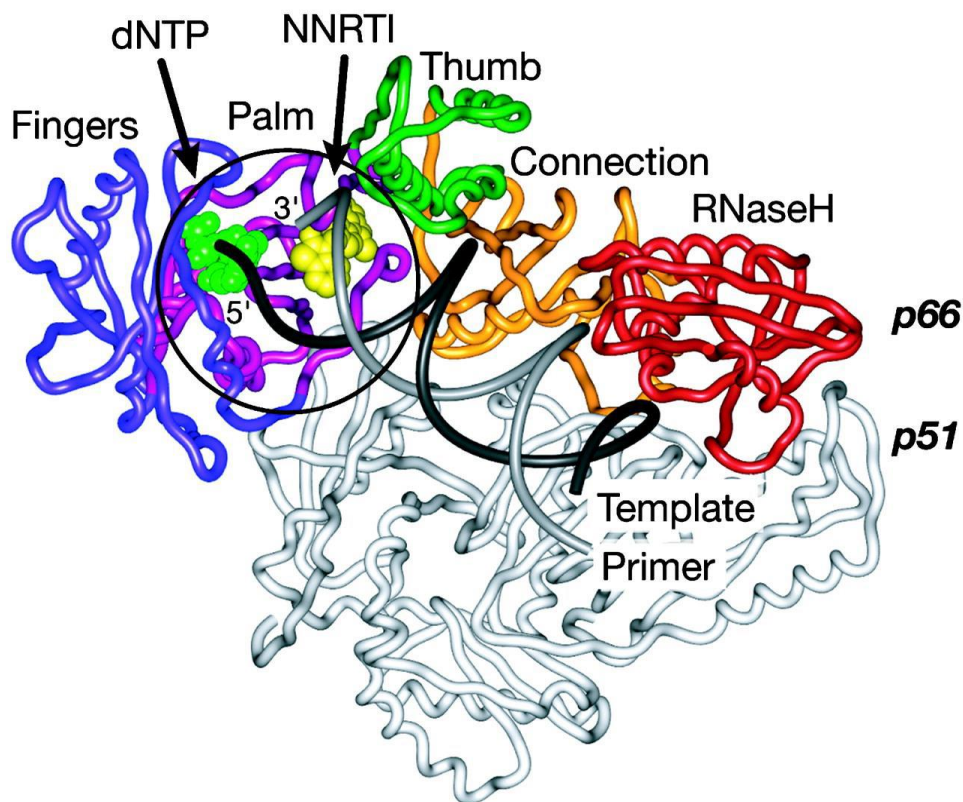
Figure 1.5 Reverse Transcriptase structure with two subunits p66 and p51. Taken from Huang et al., (1998).

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1.9 HIV-1 Antiretroviral (ARV) Drug

 The first ART to be authorized by the USA Food and Drug Administration (FDA) was zidovudine (AZT) in the 1980s (Brook, 1987, Ezzell, 1987). In the 1990s, the HIV-1 treatment was transformed by the development of inhibitors of two key HIV-1 enzymes (RT and PR) and the advent of combined antiretroviral therapy (cART) for efficient and long-lasting treatment (Arts and Hazuda, 2012, Gulick et al., 1997, Staszewski et al., 1996). The combined therapy which consists of three different antiretrovirals (ARVs) known as HAART (highly active antiretroviral therapy) has profound benefits. These benefits include the following: a) decline in the mortality and morbidity rate of HIV-1 infection and AIDS, changing the deadly disease into a controllable persistent illness, and consequently increasing the life span of an HIV infected individual; b) drastic curtailment of viral replication; c) reduction of plasma HIV-1 viral load below identification threshold; and restoration of the immune function (Arts and Hazuda, 2012, Staszewski et al., 1996). Following this development and advantage, HAART was immediately incorporated for clinical use and treatment of HIV individuals who are initiating treatment newly as well as treatment-experienced individuals.

Currently, the Food and Drug Administration (FDA) has authorized over 30 antiretroviral drugs (ARVs) for clinical use (DHHS, 2018). These antiretroviral drugs target several key viral processes and are categorised into seven classes. These include "nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)", "non-nucleoside reverse transcriptase inhibitors (NNRTIs)", "protease inhibitors (PIs)", "fusion inhibitors", "inhibitors of co-receptors", "integrase strand transfer inhibitors (INSTIs)" and "a CD4 post-attachment inhibitor" (Markham, 2018). Four of these classes of the ARVs target the function of the three key viral encoding enzymes, RT, PR and IN, while the fusion inhibitors and inhibitors of co-receptors block fusion and/or the entry of the virus to the host cells (Arts and Hazuda, 2012, De Clercq, 2009). See Table 1.1 for the classification of ARVs.

Table 1.1: Major classes of ARVs and their mode of actions

Drug class	Mode of action	Name of antiretroviral drug (abbreviation)
CD4 post-attachment inhibitor	Block the post-attachment process of the viral particle to their target cells	Ibalizumab (IBA)
Entry/fusion inhibitors	Prevent the fusion of the virus to their target cells	Enfuvirtide (T-20)
CRI	Interfere with the entry of HIV in the target cell	Aplaviroc (APL) and Maraviroc (MVC)
NRTIs	DNA)chain terminators and inhibit reverse transcription	Zidovudine (ZDV), Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T), Lamivudine (3TC), Abacavir (ABC), TDF (TDF) and Emtricitabine (FTC)
NNRTIs	Bind to the hydrophobic pocket inhibiting the RT enzyme	Nevirapine (NVP), Efavirenz (EFV), Etravirine (ETV), Rilpivirine (RPV) and Doravirine (DOR),
Integrase inhibitors (IN)	Inhibit the attachment of proviral DNA to host cell genome	Raltegravir (RAL), Elvitegravir (EVG), Dolutegravir (DTG and Bictegravir (BIC).
Protease inhibitors (PIs)	Target the viral enzyme required for cleavage of viral precursors and final assembly of viral particles	Saquinavir (SQV), Indinavir (IDV), Ritonavir (RTV), Nelfinavir (NFV), Amprenavir (APV), Lopinavir (LPV), Atazanavir (ATV), Fosamprenavir (FPV), Tipranavir (TPV) and Darunavir (DRV).

Abbreviations: NRTIs -Nucleoside reverse transcriptase inhibitors; NNRTIs- Non-Nucleoside reverse transcriptase inhibitors.

1.10   Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Non-nucleoside reverse transcriptase inhibitors bind to the amino acids inside the hydrophobic binding loop within reverse transcriptase leading to a conformational change around the active site, hence interfering with the chemical step that is crucial for DNA synthesis (Clavel and Hance, 2004, Sarafianos et al., 2009, Shafer, 2002). Conformational analyses of HIV-1 RT have revealed that the hydrophobic binding loop does not occur in the absence of the NNRTIs. In the event of the NNRTIs opening the pocket, it limits some of the movements of the enzyme that are crucial for DNA synthesis. Hence DNA synthesis cannot occur in the absence of the movements (Clavel and Hance, 2004, Kohlstaedt et al., 1992, Sarafianos et al., 2009). Examples of NNRTIs that have been approved by the FDA for use are NVP, EFV, ETR, RPV, and DOR (DHHS, 2018).

1.10   Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs)

Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) are deoxyribonucleic acid (DNA) chain terminators that inhibit the additional elongation of the proviral DNA during reverse transcription. There are eight NRTIs that have been approved by the FDA, seven nucleoside analogues: Abacavir (ABC), Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T), Zidovudine (AZT), Emtricitabine (FTC), Lamivudine (3TC) and one nucleotide analogue: TDF. These NRTIs form the backbone of the first-line regimen in most countries (Shafer, 2002, Cihlar and Ray, 2010). This study will focus more on TDF and the resistance mutations associated with this drug.

These nucleoside and nucleotide analogues are prodrugs that requires activation by the host cellular enzymes through a process called phosphorylation. The phosphorylated NRTIs that was converted to the active triphosphate form compete with the cells naturally produced substrate (deoxynucleoside triphosphates, dNTPs) for insertion into the newly formed complementary DNA (Shafer, 2002). This prevents additional elongation of the DNA chain, owing to the absence of the 3'OH group that is essential for the formation of the phosphodiester bond during complementary cDNA synthesis, resulting in DNA chain termination (Das and Arnold, 2013). Nevertheless, the effectiveness of these drugs is jeopardized by the emergence of HIV-1 drug resistance mutations (Shafer, 2002).

1.12   Mechanism of HIV-1 Drug Resistance

HIV-1 drug resistance is described as the presence of viral strains that can decrease drug effectiveness in relation to the wild-type viruses (Shafer, 2002). These evolve through the following factors: HIV-1 reverse transcriptase (RT) proofreading error in the course of replication, high turnover rate of the HIV-1 multiplication, increased reservoir of provirus strains during HIV-1 infection, genetic hybridization due to virus of difference sequence affecting the similar cell resulting in large number of quasi-species and rapid

evolution under drug selection pressure (Clutter et al., 2016, Coffin, 1995, Katzenstein, 2006, Li and Kuritzkes, 2013, Shafer, 2002). Other contributing factors are non-compliance, low genetic barrier resistance, treatment interruption due to drug stock out and drug interaction (Clutter et al., 2016, Hamers et al., 2013). Furthermore, the evolution of drug resistance relies on the rate at which the virus continues to replicate under sub-optimal therapy, the ability of the virus to gain some mutations and their impact on the drug activity and growth fitness (Clutter et al., 2016, Shafer, 2002). Conversely, drug-resistant viruses occur daily in treatment-naïve individuals although these strains are hardly detected due to reduced viral fitness compared to drug-susceptible strains when there is selective drug pressure (Perelson and Ribeiro, 2013). Most drug resistance mutations of clinical significance are non-polymorphic as they emerge only under selective drug pressure and are rarely detected in untreated individuals (Clutter et al., 2016, Tang and Shafer, 2012).

Drug resistance mutations (DRMs) can develop due to either a single DRM or by multiple DRMs and can, therefore, be grouped into primary and secondary mutations. Primary DRMs reduce drug susceptibility by themselves while secondary (accessory) DRM improves the fitness of primary DRMs or promote reduced susceptibility (Clutter et al., 2016, Shafer, 2002, Tang and Shafer, 2012). However, this classification is not strictly defined as a mutation that is major for one drug can be minor for another drug (Shafer, 2002). However, both primary and secondary mutations in the RT gene can limit the efficacy of reverse transcriptase inhibitors (RTIs) (namely, NRTIs and NNRTIs).

1.13   Mechanisms of NNRTI resistance

Mutations in RT nevertheless can develop resistance to the NNRTIs. The hydrophobic binding pocket harbours the majority of the NNRTI resistance mutations. The commonly observed resistance mutations in individuals on NNRTIs therapy are K103N and Y181C. Additional NNRTI resistance mutations seen in individuals include: "L100I, K101E, V106A, V179D, Y188L, G190A, and P236L". The occurrence of these mutations can be alone or in combination (Sarafianos et al., 2009). However, the main challenges with NNRTIs are the low genetic barrier to resistance (one mutation in the binding site can sufficiently decrease the drug activity) and secondly, its association with mutations at multiple codons (Sarafianos et al., 2009).

Table 1.2: Overview of NNRTIs resistance mutations. Taken from (HIVBD, 2019)**.**

Major Non-Nucleoside RT Inhibitor (NNRTI) Resistance Mutations								
	100	101	103	106	181	188	190	230
Cons	L	K	K	V	Y	Y	G	M
DOR	I	EP		AMI	CIV	LCH	SE	L
EFV	I	EP	NS	AM	CIV	LCH	ASEQ	L
ETR	I	EP			CIV	L	ASEQ	L
RPV	I	EP			CIV	L	ASEQ	L
NVP	I	EP	NS	AM	CIV	LCH	ASEQ	L

Abbreviations: Cons- Consensus**;** DOR**-**doravirine; EFV- efavirenz; ETR- etravirine; RPV- rilpivirine and NVP- nevirapine.

1.14   Mechanisms of NRTI resistance

The occurrence of NRTI resistance involves two main mechanisms, primer unblocking and a discriminatory mechanism (Clavel and Hance, 2004, Shafer, 2002, Shafer and Schapiro, 2008). The primer unblocking mechanism involves mutations that cause the resumption of reverse transcription and DNA synthesis by enhancing the excision of chain-terminating NRTIs, thereby facilitating primer unblocking through pyrophosphorolysis. The mutations that act through this mechanism include: "M41L, D67N, K70R, L210W, T215F/Y, K219Q/E (called the thymidine analogue mutations-TAMs)" and the T69 insertions seen with multi-nucleoside and most of the secondary mutations. These primer unblocking mutations have less effect on polymerase activity (Clavel and Hance, 2004, Shafer, 2002, Shafer and Schapiro, 2008). While in the discriminatory mechanism, the mutations favour the incorporation of the cells' natural dNTP substrate than the NRTIs during polymerization, preventing the addition of the NRTIs to the growing DNA chain (Sarafianos et al., 1999a, Shafer and Schapiro, 2008, Clavel and Hance, 2004, Shafer, 2002). These mutations occur at or close the NRTIs binding site on the RT gene, and often tend to reduce the catalytic polymerase activity in-vitro (Clavel and Hance, 2004, Shafer, 2002). Mutations that act through the discriminatory mechanism (blocking NRTIs incorporation) includes, M184V/I, K65R, L74V, Q151M (multi-nucleoside mutation) (Clavel and Hance, 2004, Shafer and Schapiro, 2008). The M184V mutation develops in viruses in patients receiving 3TC or FTC therapy (Arts and Hazuda, 2012), while K65R emerges with TDF, ddC, ddI, d4T, and ABC treatment (Margot et al., 2002). Usually, K65R hardly develops in individuals on AZT-containing regimens as a result of its antagonism to the TAMs (Parikh et al., 2006). In

the presence of K65R, TDF susceptibility is restored by the M184V mutation; hence individuals that fail 3TC or FTC with M184V on TDF rarely develop K65R (Deval et al., 2004).

The RT function and viral replicative fitness can decrease due to several primary and secondary NRTI mutations (or combination of both) (Quinones-Mateu and Arts, 2006). Though reduced replicative fitness due to NRTI resistance mutations have a possible clinical advantage, a high level of resistance can occur under drug selection pressure due to an accumulation of more mutations. While under no drug selection pressure, the loss of replicative fitness caused by drug resistance mutations is restored by the accumulation of secondary mutations (Arts and Hazuda, 2012).

Table 1.3: Overview of NRTIs resistance mutation. Taken from (HIVBD, 2019)

Major Nucleoside RT Inhibitor (NRTI) Resistance Mutations													
	Non-TAMs					TAMs						MDR	
	184	65	70	74	115	41	67	70	210	215	219	69	151
Cons	M	K	K	L	Y	M	D	K	L	T	K	T	Q
3TC	VI	R										Ins	M
FTC	VI	R										Ins	M
ABC	VI	R	E	VI	F	L			W	FY		Ins	M
TDF	***	R	E		F	L		R	W	FY		Ins	M
ZDV	***	***	*	*			L	N	R	W	FY	QE	Ins	M

Abbreviations: Cons- consensus; 3TC-lamivudine; FTC- emtricitabine; ABC- abacavir; TDF-tenofovir; AZT- Zidovudine; TAMs- Thymidine analogue mutations and MDR- multidrug resistance mutations.

1.15   Tenofovir Disoproxil Fumerate (TDF)

The TDF is a nucleotide analogue RT inhibitor and a very effective antiretroviral agent for treating HIV-1 infected individuals (both those starting treatment newly and already on treatment) (Margot et al., 2002, Squires et al., 2003). It has a durable intracellular half-life, less toxic and is formulated as a once-daily single tablet (Gallant et al., 2006). The TDF treatment in individuals having drug resistance can result in a decrease of about 0.6log10 HIV-1 RNA copies/ml of plasma by week 24 (Squires et al., 2003) and it is also active against most resistant strains associated with NRTI (Miller et al., 2001).

However, the effectiveness of TDF can be threatened when treatment failure emerges due to non-compliance and HIV drug resistance mutations. "Treatment failure is defined as the emergence of disease

following HAART introduction". Failure can be evaluated by the following indicators: a) clinical indicator (the presence of new opportunistic infections); b) immunological indicator (low CD4 count); or c) virologic indicator (viral RNA reappearance above the detection limit of 200copies/ml (Aldous and Haubrich, 2009). Viral load measurement has been recommended as the ideal measure for evaluating and detecting therapeutic failure since 2010 (WHO, 2010).

Therefore, in areas where viral load measurement is not accessible, response to antiretroviral therapy (ART) and treatment failure can be evaluated by clinical examination as well as immunologic examination (WHO, 2010). A study has observed that patients who failed first-line therapy especially for a prolonged period have a higher probability of experiencing suboptimal therapy, develop rapid drug resistance and fail treatment (Todd et al., 2006). Thus, timely identification of TDF resistance mutations is crucial as it can assist in predicting treatment efficiency and prevent resistance, which is cost-effective at the long run.

1.16   TDF resistance Profile

HIV-1 subtype C infection in patients, on which treatment is failing, develop TDF resistance 2.44 times more compared to individuals with other subtypes. In addition, individuals on nevirapine are 50% more likely to develop TDF resistance compared to individuals on efavirenz. Likewise, individuals on lamivudine have 50% likely chance to develop TDF resistance compared to individuals on emtricitabine (TenoRes-Study, 2016). Tenofovir resistance is often associated with the K65R signature mutation and is proposed to rapidly appear in in-vitro selection in subtype C compare to other subtypes (Brenner et al., 2006, Invernizzi et al., 2009).

Studies have reported that in low- and medium-income countries (LMICs), 20% - 60% of patients with incomplete viral suppression develop DRM with TDF based first-line treatment (Rhee et al., 2017, TenoRes-Study, 2016). There are two routes to TDF resistance: the K65R and the K70E/Q routes. The rate of emergence of K65R and K70E/Q mutations in patients with virologic failure is about 40% and 10% respectively (TenoRes-Study, 2016). The combination of K65R and K70E results in high replication capacity impairment and as such are hardly seen occurring together (Kagan et al., 2007).

However, when alone, K65R has high resistance to TDF as well as diminished replication capacity compared to K70E (Kagan et al., 2007, Melikian et al., 2012, Shafer, 2017, Sluis-Cremer et al., 2007). In patients failing TDF based first-line treatment, mutations that have been found include S68G/N/D, Y115F, A62V, L74I, K70N/T/G, K65N, and T69 deletions with prevalence a of 20%, 12%, 10%, 6%, 3.2% 0.7% and 0.3% respectively (Shafer, 2017, TenoRes-Study, 2016). These mutations are higher in subtype C compared to subtype B (Margot et al., 2016, Rhee et al., 2017). Some of these mutations are frequently

found with K65R (Rhee et al., 2017). However, the implication of these mutations on TDF in subtype C sequences needs further phenotypic investigation.

Viruses harbouring K65R is found to have a high fitness cost when in association with A62V and S68G (Svarovskaia et al., 2008), while Y115F is found to diminish TDF activity (Margot et al., 2006b, Melikian et al., 2012). Greater than 5- fold resistance to TDF are often found when in association with type 1 thymidine analogue mutations (TAM). On the other hand, TDF-selected DRMs when occurring together with cytosine-analogue DRMs M184V/I confer about 2-fold resistance to TDF (Margot et al., 2006b, Melikian et al., 2012, Shafer, 2017). Most TDF-associated DRMs have been shown not to cause resistance to AZT (Shafer, 2017). However, cross-resistance to other NRTIs have been commonly observed with TDF-associated DRMs. "K65R/N, Y115F, L74I, and K70E/Q/N/T/G" cause cross-resistance to Abacavir (ABC) (Margot et al., 2006b, Melikian et al., 2012). While K65R causes less resistance to 3TC and FTC when compared with M184V/I (Margot et al., 2006b, Melikian et al., 2012, Shafer, 2017).

1.17   Lysine (K) 65 Role in HIV-1 RT

The lysine (K) 65 is very crucial in polymerization fidelity, drug resistance and is located in the nucleotide-binding pocket, the location for drug resistance mutations, leading to reduced sensitivity of most nucleoside RT inhibitors (NRTIs). K65 constitutes part of the nucleobase binding site found between the β3 and β4 chain that make up the dynamic loop of the finger domain of the HIV-1 RT, which is essential for the polymerization process. K65 also supports the incorporation and proper alignment of the inbound dNTP into the growing DNA chain via the formation of a salt bridge between the epsilon-amino group of K65 and the γ phosphate of the inbound nucleotide. However, in the presence of K65R, the nucleotide-binding interaction greatly alters the γ-phosphate (Garforth et al., 2014, Huang et al., 1998).

1.18   Mechanism of K65R Mutation

K65R arise from a G-to-A change (AAA to AGA) and was initially found in individuals who were treated with the 2'-3'dideoxynucleoside inhibitor, ddC (Zhang et al., 1994) and have a broad resistance to other NRTIs with exception of AZT (Deval et al., 2004, Margot et al., 2002, Wensing et al., 2017). K65R distorts the conformation of the nucleotide incorporated in the polymerase functional site, while still maintaining the firmness of the same nucleotide necessary for polymerization (Zhang et al., 1994). K65R reduces the steric flexibility of the RT as a result of the arrangement of the guanidinium sheets of the R65 and R72 bases (Sarafianos et al., 1999a). In the K65R mutant RT, stacking of the guanidinium of the arginine with the incoming nucleotide renders the R65 and R72 residues inflexible. This steric inflexibility is proposed to be the mechanism behind the reduced polymerization rate seen in K65R mutant virus, by favouring the incorporation of dNTP over NRTIs, which alter the uptake of ATP (Das et al., 2009).

1.19   K65 Resistance Mutation and Subtype C

Studies have shown that non-subtype B viruses treated with d4T/ddI, d4T/3TC or TDF/3TC often develop the K65R mutation (Doualla-Bell et al., 2006, Hawkins et al., 2009, Wallis et al., 2010). Clinical studies have reported increase treatment failure and increase development of the K65R mutations in HIV subtype C individuals than in subtype B receiving TDF (Skhosana et al., 2015, Sunpath et al., 2012, TenoRes-Study, 2016, Theys et al., 2013). The above finding is consistent with the study by (Smit et al., 2017), which reported that patients experiencing treatment failure with HIV-1 subtype C viruses are highly susceptible to develop K65R mutations with double fold prevalence than other subtypes. Furthermore, in-vitro studies have shown that K65R developed more quickly in subtype C than in subtype B, in the presence of increasing concentrations of TDF (Brenner et al., 2006, Coutsinos et al., 2010, Garforth et al., 2014, Theys et al., 2013) (Theys et al., 2013).

This variation is likely due to the following mechanisms (figure 1.4): the nature of the subtype C RNA template (Coutsinos et al., 2011), distinctive polymorphisms at codons 64(AAA), 65(AAG) and 66 (AAG), pausing activities at codon 65 due to a poly-adenine region that permits wrong arrangement, insertions, deletions, strand chain and recombination, thereby promoting the development of K65R during reverse transcription (Brenner and Coutsinos, 2009, Coutsinos et al., 2011).
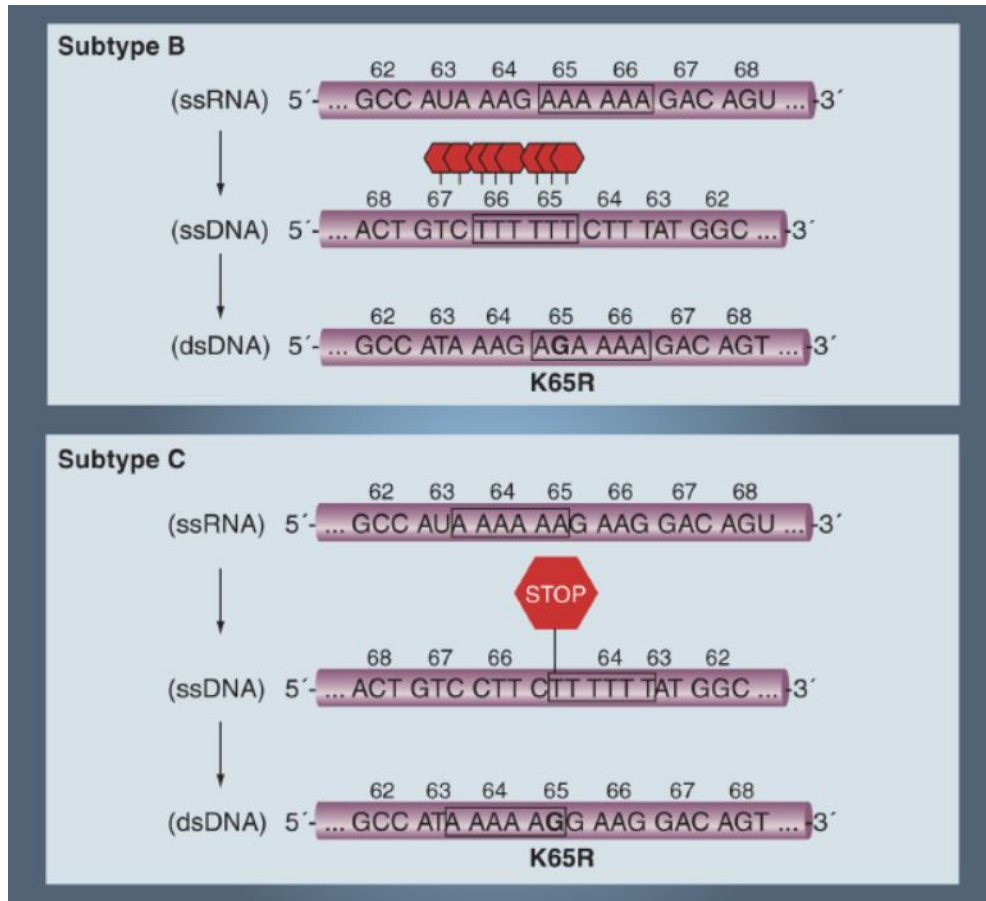
Figure 1.6: The increased selection of K65R in subtype C. The selection of K65R is initiated by the poly-adenine stretches at position 63 and 65. The stop occurs in poly stretches in position 65 (Brenner and Coutsinos, 2009).

However, other studies could not determine variations in response rates between subtype B and C due to the above mechanism (Xu et al., 2009, White et al., 2002, Günthard and Scherrer, 2016); rather they found that the variation was due to differences in demographics and clinical characteristics such as adherence, standard of care and delay in switching after treatment failure leading to the accumulation of drug resistance mutations (Günthard and Scherrer, 2016, White et al., 2016).

1.20   HIV-1 Drug-Resistant Testing

The occurrence of HIV-1 drug resistance mutations can be monitored and investigated through drug resistance testing (Granich et al., 2009). Resistance testing is an essential tool for proper care of HIV infected individuals particularly when changing their treatment plan in the event of incomplete virologic response, as this helps in the identification and measurement of drug resistance (Gunthard et al., 2019, Hirsch et al., 2008, Wensing et al., 2017). Resistance testing can also guide in deciding the right regimen

in the individuals newly initiating treatment, considering the increasing rate of transmitted drug-resistant viruses (Haubrich, 2004b). Presently, there are two types of HIV-1 drug resistance testing: genotypic resistance assay and phenotypic drug resistance testing (Schutten, 2006, Sen et al., 2006, Shafer, 2002).

1.21   Genotypic Assay

Genotypic resistance assays detect mutations that cause the loss of drug susceptibility by DNA sequence analysis of the gene of interest in the virus genome. The sequence generated is compared with the sequence of the reference control virus to identify variations (Schutten, 2006, Sen et al., 2006). Genotypic tests involve the following steps: viral RNA extraction from plasma, reverse transcription and amplification of the cDNA by PCR. The amplicons are then sequenced on an automated DNA sequencer. This generates complete data on the viral genotype (Schutten, 2006). There are two approaches to genotypic assay: commercial assay kits and the in-house protocol.

Commercially available genotypic resistance tests kits include: "HIV-1 GenotypR PLUS (Specialty Laboratories), TRUGENE HIV-1 genotyping test (Visible Genetics), VircoGEN II (Virco), ViroSeq HIV genotyping system (PE Applied Biosystems), GeneSeq (ViroLogic), HIV-1 Mutation Analysis (Focus Technologies), HIV ViroTYPE (Rheumatology Diagnostics Laboratory), GenoSure (LabCorp and Virco), and HIV-1 Genotype (Quest Diagnostics)" (Liu and Shafer, 2006, Sen et al., 2006). These kits are also packaged with interpretation tools; the two widely used commercial genotypic assays authorized by the FDA are: "TRUGENE HIV-1 genotyping test (Visible Genetics) and ViroSeq HIV genotyping system (PE Applied Biosystems)" (Schutten, 2006).

In-house methods are mostly used by laboratories due to its low cost. On the other hand, the in-house method is faced with inadequate assay validation and lack of general standardization. Yet, there is not much difference between the two approaches as shown by blinded quality assurance programmes (Hirsch et al., 2008). Both commercial assay kits and the in-house approaches use Sanger's sequencing approach for the identification of HIV-1 resistance mutations. Although Sanger-based genotypic assays are the most commonly used methods, they are limited in their sensitivity as they can only identify variants above 15%-20% of the viral population, omitting low minority variants (viral quasispecies) that may occur at low abundance of the viral population (Fox et al., 2014, Gibson et al., 2014, Halvas et al., 2006, Palmer et al., 2005).

Studies have demonstrated that these minority variants can impact significantly on patient treatment outcome (Cozzi-Lepri et al., 2015, Li et al., 2013a). There are technologies available that can detect these minority variants for as low as about 5% and above. These include: "next-generation sequencing" (Ram et al., 2015), "allele-specific-PCR (ASPCR, oligonucleotide ligation-based assays (OLA)", "a Ty1/HIV-1 RT

hybrid system (TyHRT)", "single-genome sequencing (SGS)", and "a line probe assay (LiPA)" (Halvas et al., 2006, Metzner, 2006). Another shortcoming of the genotypic assay is the inability to identify different unusual mutational patterns and interpreting multiple groups of mutations (Puertas et al., 2012, Shafer, 2002). However, genotypic testing is preferred than the phenotypic assay because of the following benefits: it is inexpensive, short analytical time, precise evaluation, easy to perform, readily available online interpretation algorithms (Hirsch et al., 2008, Puertas et al., 2012, Sen et al., 2006).

1.21.1 Interpretation of Genotypic Resistance Assay

Interpretation of genotypic resistance result is essential as there are diverse drug resistance mutations (DRMs) in complicated combinations (Gunthard et al., 2019). There are several tools available to guide in the proper interpretation of genotypic results. These tools consist of several collections of resistance algorithms and are constantly reviewed to give outlines and explanation of resistance mutations for each antiretroviral drug (HIVBD, 2019, Shafer, 2017, Wensing et al., 2017). Two types of genotypic test interpretation system exist; the rule-bases systems and the algorithm based or machine-learning system (Doring et al., 2018, Gunthard et al., 2019).

The rule-based system relies on set rules and expertise input. It is the widely used interpretation system as it takes into account other forms of information. This system is reproducible, transparent and informative; however, it is prone to bias (Gunthard et al., 2019). Examples of rule-based interpretation system include "the French National Agency for Research on AIDS and Viral Hepatitis", "Rega", "HIV Genotypic Resistance- Algorithm Deutschland", "AntiRetroScan", and "the Stanford HIV Drug Resistance Database" (Doring et al., 2018, Gunthard et al., 2019, Liu and Shafer, 2006, Puertas et al., 2012). In this system the virus is grouped into three; "susceptible", "intermediate resistant" and "resistant" (Liu and Shafer, 2006, Sen et al., 2006). While the Stanford HIV Drug Resistance Database and AntiRetroScan have five levels of resistance such as "susceptible", "potential low-level resistance", "low-level resistance", "intermediate resistance", and "high-level resistance" (Liu and Shafer, 2006, Sen et al., 2006).

The algorithm-based interpretation system utilizes statistical models and machine learning algorithms. A classic example of the algorithm-based interpretation system includes the geno2pheno (Liu and Shafer, 2006) and the SHIVA system (Doring et al., 2018). The difference between the geno2pheno and the SHIVA software algorithm-based interpretation system relies on the approach in which the data set, and the machining learning algorithm is applied. For instance, vector regression is used in geno2pheno, while SHIVA uses random forest (Doring et al., 2018).

1.22   Phenotypic resistance assay.

Phenotypic assays quantify the concentration of drugs required to prevent HIV growth in cell culture (Hirsch et al., 2008, DHHS, 2018). These assays assess the correlation of the replication of a HIV-1 test sample to the reference strains (wild-type virus) in drug concentration. Similar to the genotypic assay, the phenotypic assay uses PCR to amplify the HIV-1 gene of interest. These assays generate a recombinant virus by inserting the gene of interest derived from a patient into a reference molecular clone lacking the equivalent gene(s) of interest (Clutter et al., 2016, Hirsch et al., 2008, Sen et al., 2006). This recombinant virus contains an indicator (reporter) gene and is used in-vitro to infect host cells in culture. The growth of the recombinant virus in the presence/absence of ARVs can be determined by a single cycle phenotypic assay or a multiple cycle phenotypic assay (Dykes and Demeter, 2007). The activity or the expression of the reporter gene at varying concentration is a measure of the viral replication and is compared with a wildtype HIV strain's replication capacity (Hirsch et al., 2008, Shafer, 2002).

1.22.1 Types of Phenotypic Assay

There are two commercially available phenotypic assays: PhenoSense™ (Monogram Biosciences) (a single round phenotypic assay) and Antivirogram^{TM} (Virco) (a multiple-cycle phenotypic assay) (Hertogs et al., 1998, Petropoulos et al., 2000, Sen et al., 2006). Both assays are recombinant based and they both amplify the gene of interest from HIV-1 RNA derived patient plasma via polymerase chain reaction (PCR). However, the approached in which the amplified product is introduced into the deleted recombinant molecular HIV-1 clone backbone differs; the Phenosense assay employs ligation while Antivirogram employs homologous recombination (Zhang et al., 2005). For both assays, cell line are infected with a standardized amount virus, and virus replication competency is determined in the presence and absence of varying concentrations of ARVs.

In the PhenoSense™ (Monogram Biosciences) assay, the recombinant HIV-1 vector lacking the corresponding gene of interest has another deletion in the envelope gene (env) and insertion of luciferase gene insertion. The env deletion enables a single round of infection of the recombinant virus, while the luciferase acts as a reporter gene. Drug susceptibility and replication capacity are determined through luciferase production in the test sample relative to the reference strain (Sen et al., 2006, Zhang et al., 2005). In the Antivirogram^{TM} (Virco) assay, the recombinant virus is cultured in multiple rounds of infection. Drug susceptibility and replication capacity are determined through cell killing in the cell culture by measuring the expression of the tetrazolium dye production which relates to the number of live cells available following multiple rounds of infection (Sen et al., 2006, Zhang et al., 2005). The sensitivity and reproducibility of Phenosense assay are higher than Antivirogram (Zhang et al., 2005).

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1.22.2 Interpretation of Phenotypic Resistance assay

The results obtained from phenotypic assays are presented as fold change (FC) which is the drug concentration that suppress viral multiplication by 50% (IC50) of the test sample divided by the IC50 of the wild-type reference strain (Haubrich, 2004a, Clutter et al., 2016, Hirsch et al., 2008, Parkin et al., 2004a, Sen et al., 2006, Haubrich, 2004b) (Figure 1.5).
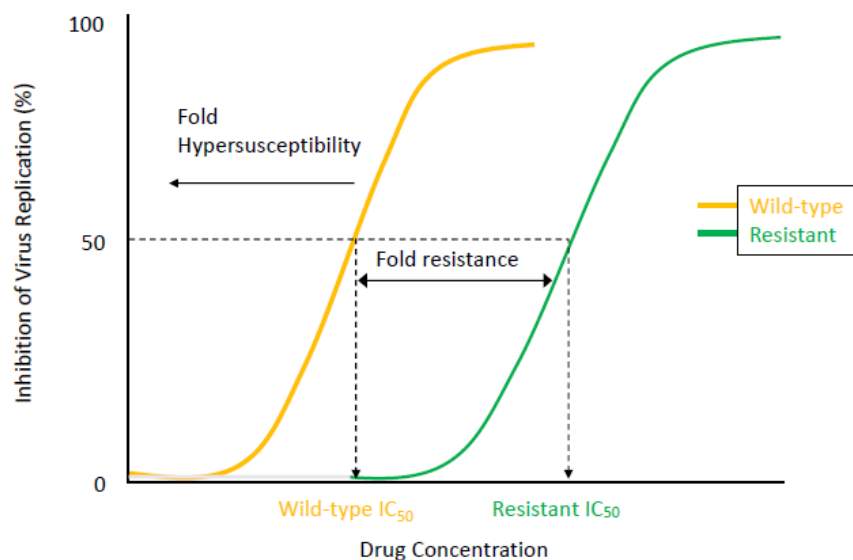
Figure 1.7: Interpretation of phenotypic test. Drug concentration requires to prevent virus growth in-vitro to 50.0% (50.0% inhibitory concentration, IC_{50}) compare to a wild-type (WT) reference virus. Taken from Haubrich, (2004).

Interpretation of Phenotypic assay is essential, due to variation in fold change susceptibility of each ARV that exist. Hence, to establish the relevance of FC in the susceptibility threshold or cut-off values are used and are categorized into three cut-offs (Parkin et al., 2004a, Gunthard et al., 2019).

1. Technical cut-offs- this measures the reproducibility of reference HIV-1 strain sensitivity to an ARV drug. However, this does not portray the true intrinsic difference observed in a patient without treatment (Hirsch et al., 2008, Parkin et al., 2004a, Sen et al., 2006).

2. Biological cut-offs: this measures the natural variation in IC50 of various wild-type strain obtained from HIV-1 infection individuals without treatment (Hirsch et al., 2008, Parkin et al., 2004a, Sen et al., 2006).

3. Clinical cut-offs: these are cut-offs values obtained from clinical trials in relation to patient responses to treatment. It consists of two cut-offs: the lower cut-off (intermediate resistance) which

is the lowest fold decrease at which slight resistance in the drug activity is observed; and the upper cut-offs (full resistance) at which there is complete drug inactivity (Hirsch et al., 2008, Parkin et al., 2004a, Sen et al., 2006).

Phenotypic assays are conducted on individuals presented with complicated forms of drug resistance mutations patterns and hence are more reliable than genotypic assays. However, discrepancies in the use of cut-off mark limits it uses (Hirsch et al., 2008).

1.23   HIV-1 Replication Capacity/ Fitness

HIV-1 replication capacity or fitness is the tendency of a virus to multiply in a given condition and this can be influenced by several interwoven factors such as host factors (e.g. genetic makeup, targeted cells and immune response), viral factors (e.g. HIV-1 replication cycle which involves several stages) and antiretroviral therapy factors (Daar, 2005, Nicastri et al., 2003, Nijhuis et al., 2001).   The measure of HIV-1 replication fitness in-vitro can be an efficient tool in determining prognosis or treatment outcome *in-vivo*. Therefore, replication capacity or fitness assays can be used to know when to begin therapy and change a failing regimen which is a prognostic tool for an antiviral outcome. A general feature of replication capacity assays is the relativity of the mutant virus to the control strain (Miao et al., 2008).

1.23.1 Measuring HIV-1 Replication Capacity

There are different *in-vivo* and in vitro methods available for the measurement of viral replication capacity. *In-vivo* methods are presumed to be more significant. On the other hand, they are hard to carry out and it depends on the amount of virus measured (Daar, 2005). Although an animal model can be used to measure viral fitness *in-vivo*, it still does not present the exact condition of the patient *in-vivo*. *In-vivo* fitness is determined by measuring viral kinetics in plasma**.** *In-vitro* methods measure the intrinsic potential of the virus to multiply relative to the reference control strains. This method can be achieved by evaluating the enzymatic activity of the virus (Back et al., 1996), *in-vitro* replication with a complete virus, growth competition assays or recombinant strains having a gene of interest of the virus (Kellam and Larder, 1994, Quinones-Mateu and Arts, 2006).

1.23.2 Measure of HIV-1 Replication Fitness In-Vitro

There are numerous methods available for measuring viral fitness *in-vitro*, these include "growth competition versus parallel infections, whole virus versus recombinant virus assays, multiple cycle versus single-cycle assays, reporter gene versus a viral gene or gene product to monitor virus growth and the use of cell lines versus primary human cells" (Dykes and Demeter, 2007, Nijhuis et al., 2001, Quinones-Mateu and Arts, 2006) (Table1.4).

Table 1.4: Description of replication capacity assay. taken from (Dykes and Demeter, 2007).

Methods	Brief description
Parallel infection assay	Sample and control are cultured in different media. Easy to perform but less sensitive, subject to experimental differences.
Growth competition assay	Sample and control are cultured together in the same medium. Can easily detect minute changes in fitness compared to parallel infections.
Single-cycle replication assay	Test is performed in an env-deleted molecular backbone in one round of infection
Multiple-cycle replication assay	Test is performed in several rounds of infection with the env gene intact
Whole virus assay	Test is conducted using complete viral genome derived from patient PBMCs
Recombinant virus assay	Test is conducted by inserting the gene of interest derived from a patient sample into a molecular clone.
Direct measure of virus replication	Viral growth is determined by measuring a viral protein e.g. p24
Use of reporter gene	Determine the viral growth by measuring the expression of a reporter gene produced by the recombinant virus
Use of cell line	Test is conducted in an engineered T-cell line
Primary human cell	Test is conducted in primary human cells

1.24 Project Rationale

Tenofovir is "a nucleotide analogue RT inhibitor introduced for the treatment of HIV infection"(Squires et al., 2003). However, the efficacy of the drug can be limited by the evolution of drug resistance mutations. TDF resistance can be acquired through the K65R mutation or the K70E mutation (though less frequently) under TDF selection pressure. The development of TDF resistance poses a public health threat, since TDF is currently used in the first-line regimen as well as in the second-line regimen in South Africa, where HIV-

1 subtype C predominates. Studies from our group and others have revealed additional mutations such as "A62V, K65N, S68G/N/D, K70E/Q/T, L74I, V75L and Y115F" frequently found in association with patients under TDF treatment and were higher in subtype C compared to subtype B (Maphumulo, 2016, Rhee et al., 2017). However, their influence on TDF susceptibility in HIV-1 subtype C warranted further investigation.

1.25   Project Aim and Objectives

Aim

1. To determine the impact of TDF-selected mutants (K65R, K65R/S68N, A62V, S68D/G/N and Y115F) of HIV-1 subtype C on replication capacity and TDF susceptibility.

Objectives

1. To generate TDF-selected mutants (K65R, K65R/S68N, A62V, S68D and Y115F) of HIV-1subtype C using site-directed mutagenesis.

2. To demonstrate the effects of TDF-selected mutants (K65R, K65R/S68N, A62V, S68D/G/N and Y115F) of HIV-1 subtype C on replication capacity and TDF susceptibility.

Hypothesis

- It is expected that under drug selection pressure, TDF-selected mutations will impact on replication capacity and TDF susceptibility.

CHAPTER 2

GENERATION TENOFOVIR-SELECTED RESISTANCE

MUTATIONS OF HIV-1 SUBTYPE C

Chapter Two

Generation Tenofovir-Selected Resistance Mutations of HIV-1 Subtype C

2.1 Introduction

Tenofovir disoproxil fumarate (TDF) is an approved constituent of first- and second-line antiretroviral regimens in the WHO HIV treatment guidelines (WHO, 2010), and is an effective antiretroviral agent for treating both newly and treatment-experienced HIV-1 infected individuals (Margot et al., 2002, Squires et al., 2003). It has durable half-life within the cell, is less toxic and is administered as a once-daily one tablet (Gallant et al., 2006).

TDF is unique among the other approved NRTIs by exhibiting an effect against a broad range of NRTI resistant strains (Miller et al., 2001). However, the use of TDF can be compromised when treatment failure emerges due to non-compliance and the development of drug resistance mutations which result from staying on the failing regimen for a prolonged period (Todd et al., 2006). Identification of TDF resistance mutations can help to predict treatment efficacy and thus allow efficient drug use, stop resistance and maintain cost-effectiveness of the National ARV treatment plan.

Besides the commonly selected K65R mutation associated with TDF resistance in HIV-1 subtype C, studies have also reported other mutations such as "A62V, K65N, S68G/N/D, K70E/Q/T, L74I, V75L and Y115F" (Maphumulo, 2016, Rhee et al., 2017, TenoRes-Study, 2016). However, their phenotypic impact needs further investigation. This study investigated the most frequently observed mutations associated with TDF resistance such as K65R, A62V, S68G/N/D, Y115F and combinations of these mutations with K65R. In this chapter, we concentrate on the generation of TDF-selected resistance mutations of HIV-1 subtype C virus using site-directed mutagenesis.

2.2 Materials and Methods

2.2.1 Study Sample

A plasmid containing the RT region of an HIV-1 subtype C treatment naïve sample (obtained from patient PKE4N from the tropism study (BREC Ref No: BF088/07) was used for the site-directed mutagenesis reactions. The UKZN Biomedical Research Ethics Committee authorized the current study (Ref No: BE600/17). The RT region was amplified from this plasmid to confirm the presence of the amplicon and prepare for the site-directed mutagenesis reaction.

2.2.2 Amplification of HIV-1 Reverse Transcriptase (RT)

PCR was performed utilizing a TaKaRa Ex Taq HS enzyme kit (Takara Biotechnology, Japan). For the PCR, chimeric forward and reverse primers that were specifically corresponding to NL4-3 backbone on

each side of RT were used, resulting in an overlap that was necessary for transfection. All reagents except the enzyme were thawed and vortexed for five seconds to mix. The master mix was prepared as shown in Table 2.1

Table 2.1: Reaction components and volumes for the PCR

Reaction component	Volume per reaction(µl)	Final Concentration
PCR Graded water	37	**-**
10X Ex Taq Buffer[a]	5	**1X**
dNTP mixture (2.5mM each)	4	**(200µM)**
Forward primer(10µM)	0.8	**(0.16µM)**
Reverse primer ((10µM)	0.8	**(0.16µM)**
TaKaRa Ex Taq (5 U/ul)	0.25	**1.25U**
plasmid DNA product	**2**	**-**
Total Volume	**59.85**	

    a. **Ex Taq buffer contained 20mM MgCl2, resulting in a 2 mM final concentration**

Table 2.2: Primer sequences used for the PCR

Primer Name	Sequence	HXb2 Position
P66_F	5'_ CTGCGGACATAAAGCTATAGGTACAGTATTAGTAGGACC TACACCTGTCAACATAATTGGAAGAAATCTGTTGACTCAGATT GGCTGCACTTTAAATTTT _3'	$2450 \rightarrow 2549$
P66_R	5'_TCTCCTTGCTACTACAGGTGGTAGGTTAAAATCACTAGCC ATTGCTCTCCAATTACTGTGATATTTCTCATGTTCTTCTTGGGCC TTATCTATTCCATCTAAAAA_3'	$4329 \leftarrow 4230$

Primers sequence was a gift from Dr Mark Brockman (Simon Fraser University, Canada).

Table 2.3: Thermocycler conditions for the PCR.

Segment	Cycle	Temperature (°C)	Time	Step
1	1	94	2 minutes	Initial activation step
2	40	94	30 seconds	Denaturation
		60	30 seconds	Annealing
		72	2 minutes	Extension
3	1	72	7minutes	Final extension
4	-	4	∞	Hold

2.2.3   Gel Electrophoresis

 Gel electrophoresis was performed to verify the PCR product. A 1% agarose gel was made by adding one agarose tablet (0.5g) (Bioline, USA) to 50 ml of 1X TBE buffer (Sigma-Aldrich, USA) (component: 89mM Tris base, 89mM Boric acid, 2 mM EDTA) in a 200 ml flask. The flask was kept for approximately 10 minutes at room temperature and then heated in a microwave till the agarose was completely melted. The solution was cooled to the touch and poured into a casting tray. The gel was allowed set for 20 to 30 minutes. It was then placed in an electrophoresis tank; 1X TBE buffer was poured into the tank until the gel was fully immersed. Two microlitres (µl) of each sample and two µl of gel loading dye (Sigma-Aldrich, USA) were mixed, then loaded into the wells in the gel. The gel loading dye was prepared by adding one µl of gel red (Biotium, USA) to 50µl of loading buffer. A one kb ladder (Invitrogen, Life Technologies, Carlsbad, CA) (Figure 2.1) was included on the gel. The gel was processed at 120 V, 500 mA for 40 minutes on an Electrophoresis Power Supply- EPS 301 (Amersham Biosciences, Sweden). The gel was viewed using the Chemi Doc MP Imaging System (BioRad) (Figure 2.2). The negative control was examined for any visible bands apart from primer dimer <100bp, as bands >100bp to check for any contamination. The size of the amplified product was checked against the ladder; the expected size was 1.879kb. The product was stored at -20°C until purification.

2.2.4   Amplicon Clean up

The PCR product was cleaned using the QIAmp PCR purification kit (Qiagen, Valencia, CA), as per the manufacturer's instructions. The purified product was measured using nanodrop to determine the concentration of the products for Cloning.

2.2.5   Cloning into pCR™II-TOPO® Vector

This PCR product was ligated into a TOPO PCR II vector from the TOPO TA Cloning kit (Invitrogen, USA) for use in site-directed mutagenesis. A ligation reaction was performed following the manufacturer's directions. Briefly, two µl of the purified product was added to a ligation mixture, along with one µl of the vector. The reaction was incubated at room temperature for 30 minutes.

2.2.5.1 Transformation

Top10 competent cells from the TOPO TA cloning KIT (Invitrogen, USA) were transformed with the ligation mixture, as per manufacturer's instructions. Briefly, two µl of the ligation reaction was added to one vial of TOP10 competent cells (50µl) and incubated on ice for 30 minutes. The mixture was exposed to 30 seconds of heat shock at 42° C and placed immediately on ice for two minutes. Two hundred and fifty µl of S.O.C media (supplied with the kit) was added to each reaction mixture. After a one-hour incubation at 37°C with shaking at 230 rpm, the ligation mixtures were plated out onto pre-warmed agar containing

ampicillin (100 µg/ml). The plates had been prepared 30 minutes prior with 40µl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (50 mg/ml solution) to allow for blue/white selection.

Blue/white selection was used to select clones containing the insert. The site of ligation of the PCR product is found in the coding sequence of the *lacZα* gene of the pCR™II-TOPO® Vector. This gene expressed the enzyme β-galactosidase. The activity of this enzyme on X-Gal generates blue colonies. Successful ligation of an insert into the vector interrupts this gene, preventing the production of the blue precipitate, producing white colonies. For this study, ten white clones were selected at random for screening by PCR.

2.2.6 Verification of the PKE4N clone by colony PCR and sequencing

2.2.6.1 Colony PCR

Colony PCR, followed by gel electrophoresis, was used to determine the presence of the RT-containing insert in the TOPO plasmid as follows: ten colonies were picked from the transformation plate, touched on another LB-ampicillin agar plate (master plate) to keep a record of the colonies, and added in 10 µl PCR graded water. This suspension was briefly vortexed and boiled in a thermocycler at 95°C for five minutes. Two µl of this solution was used as the template DNA for the screening PCR. *TaKaRa Ex Taq* HS version PCR kit (Takara Biotechnology, Japan) was used for the PCR as described in section 2.2.2.

A 1% agarose gel was used to confirm the presence of a PCR product as described in section 2.2.3. The gel was examined using the Chemi Doc MP imaging system (BioRad). PCR products were purified from colonies positive for a PCR product using the QIAmp PCR Purification kit (Qiagen, Valencia, CA), as per manufacturer's instructions.

2.2.6.2 Sequencing of the colony PCR product

After the products were quantified, they were diluted to 20ng/µl using PCR graded water. Direct sequencing of the PCR amplification products was conducted using the BigDye Terminator v3.1 cycle sequencing chemistry kit (Applied Biosystems, CA. USA) with six sequencing primers as listed in Table 2.4.

Table 2.4: Primer sequences and their relative HXB2 positioning, used during the sequencing reaction.

Primer Name	Sequence (5' – 3')	HXB2 Positions
PolM4	CTATTAGCTGCCCCATCTACATA	3870 ← 3892
PolM0	TCCCTCAGATCACTCTTTGGCA	2251→ 2272
PolM1	GTTAAACAATGGCCATTGACAGA	2610 → 2632
PolMG	ATTGAACTTCCCAGAAGTCTTGAGTT	2798 ← 2823
PolM8	CTGTATATCATTGACAGTCCAG	3302 ← 3323
IN5	GATTTGTATGTAGGATCTGA	3105→3124
P66_F	CTGCGGACATAAAGCTATAGGTACAGTATTAGTAGGACC TACACCTGTCAACATAATTGGAAGAAATCTGTTGACTCAGATT GGCTGCACTTTAAATTTT	2450 → 2549
P66_R	TCTCCTTGCTACTACAGGTGGTAGGTTAAAATCACTAGCC ATTGCTCTCCAATTACTGTGATATTTCTCATGTTCTTCTTGGGCC TTATCTATTCCATCTAAAAA	4329←4230
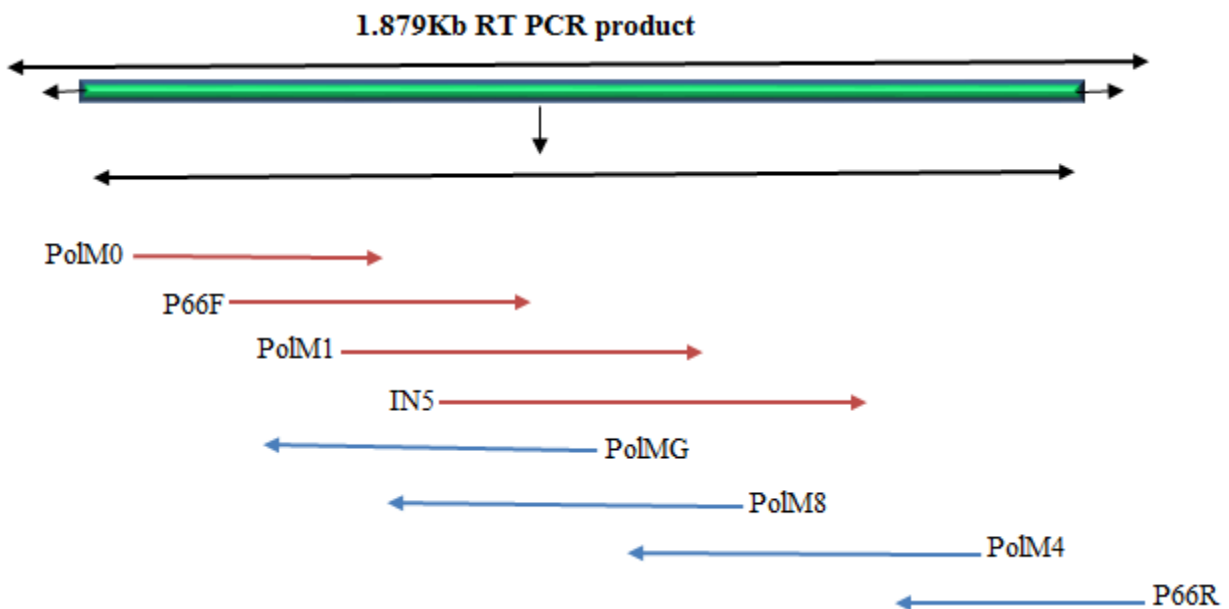
Figure 2.1: Layout of primers used to sequence the HIV-1 RT. Eight primers were used to sequence the reverse transcriptase region of HIV-1.

A master mix was made in separate 0.2ml tubes for each primer and comprised the reagents outlined in Table 2.5.

Table 2.5: Reagents and volume used to make the sequencing reaction master mix

Reagent	Volume per reaction (µl)	Final Concentration
PCR graded water	1.6	-
5X Sequencing buffer	2	1X
BigDye Terminator ready reaction mix	0.4	-
Primer 3.2pmol/µl)	1	0.32µM
Total	5	-

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 2.6.

Table 2.6: Thermal cycling conditions used for the sequencing reaction.

Segment	Cycle	Temperature(°C)	Time	Step
1	1	96	1 minutes	Initial denaturation
2	35	96	10 seconds	DNA denaturation
		50	5 seconds	Primer Annealing
		60	4 minutes	Primer Extension
-		4	∞	Hold

The sequencing products were purified immediately following temperature cycling. To each well, one µl of EDTA was added, followed by 26 µl of a 1 in 26 solutions of 3M sodium acetate in 100% ethanol. The plate was covered by foil, briefly vortexed and centrifuged at 3000 x g for 20 minutes. The plate was carefully taken out from the centrifuge, inverted onto a folded paper towel and centrifuged at 150 x g for

one minute to eliminate the liquid. Immediately following this step, 35 µl of ice-cold ethanol was added to each well, the plate was then centrifuged at 3000 x g for five minutes. Once again, the plate was carefully taken out from the centrifuge, inverted onto a folded paper towel and centrifuged at 150 x g for one minute. The plate was dried at 50°C for five minutes and kept at -20°C. Before sequencing, the dried pellets were re-suspended in 10µl formamide and denatured at 95°C for three minutes. Samples were put into the ABI 3130 Genetic Analyzer (Applied Biosystems, California). Resulting chromatograms were viewed in Bioedit v.7.0.5.3 (Ibis Biosciences, An Abbott Company, CA, USA).

The colony verified to contain the correct RT PKE4N TOPO clone sequence was then inoculated into three ml LB-ampicillin broth (100µg ampicillin per one ml broth) followed by incubation at 37 °C and 250 rpm for two hours. This culture was then used to inoculate 100 ml of fresh LB-ampicillin broth, at the same concentration, which was incubated overnight at 37 °C and 250 rpm. The RT PKE4N clone plasmid was then purified from these bacterial cells by a maxi-prep procedure (Qiagen) according to the manufacturer's instructions.

2.2.7   Site-Directed Mutagenesis

The principle of the Agilent QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent, Technologies) is shown in the figure 2.2 below.

2.2.7.1 Selection of Mutants

Mutations used for this study were selected based on the most prevalent mutations associated with TDF treatment in subtype C (A62V, K65R, S68G/N/D, and Y115F) as indicated by a previous master's student (Maphumulo, 2016) and the literature (Rhee et al., 2017).

2.2.7.2 Mutant Primer Design

Mutant primers were created employing the web-based Quikchange Primer Design Program accessible online at www.agilent.com/genomics/qcpd.

The mutant primer design was based on the following criteria:

1. Both the mutant primer sequences had the designated mutations and bind to the same sequence of the complementary strands of the plasmid.
2. Primers were between 25 and 45 bases long with a melting temperature of ≥78°C.
3. The desired mutation was in the centre of the primer sequence with 10-15 bases of the original sequence on either side.
4. A minimum GC content of 40%, ending with at least one C or G.
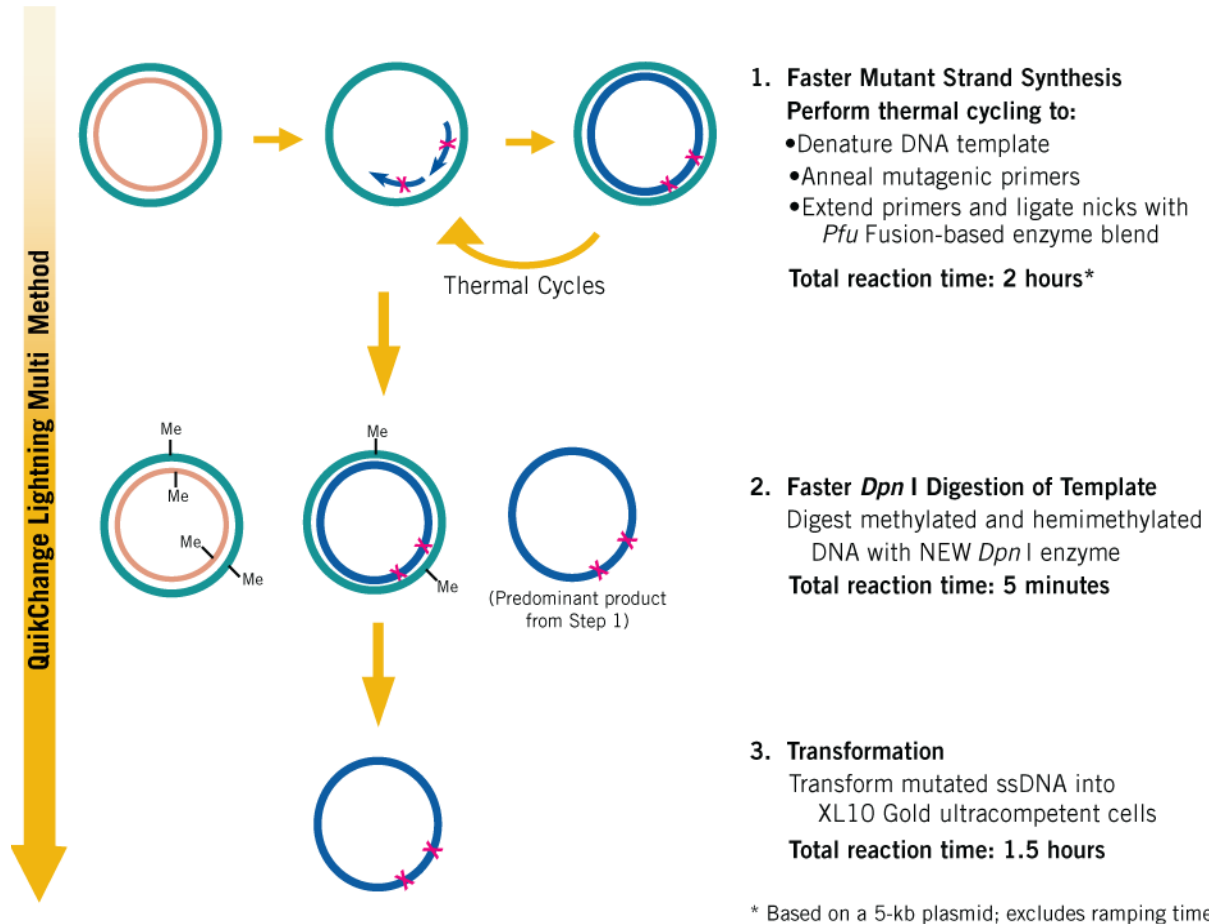
Figure 2.2: Overview of the QuikChange Lightning Multi Site-Directed Mutagenesis method. (Taken from the QuikChange Lightning Multi Site-Directed Mutagenesis Kit instruction manual).

The primers were designed specifically for the PKE04N TOPO clone containing a subtype C drug naïve sequence lacking any resistance-associated mutations in reverse transcriptase; the appropriate segment was selected and uploaded into the above website mentioned. The molecular features of the PKE04N TOPO clone can be seen in Figure 2.3. Table 2.7 shows the mutations needed to generate the designated amino acid sequences of the selected variants, standard desalting purified oligonucleotide primers were ordered from White Science, South Africa.
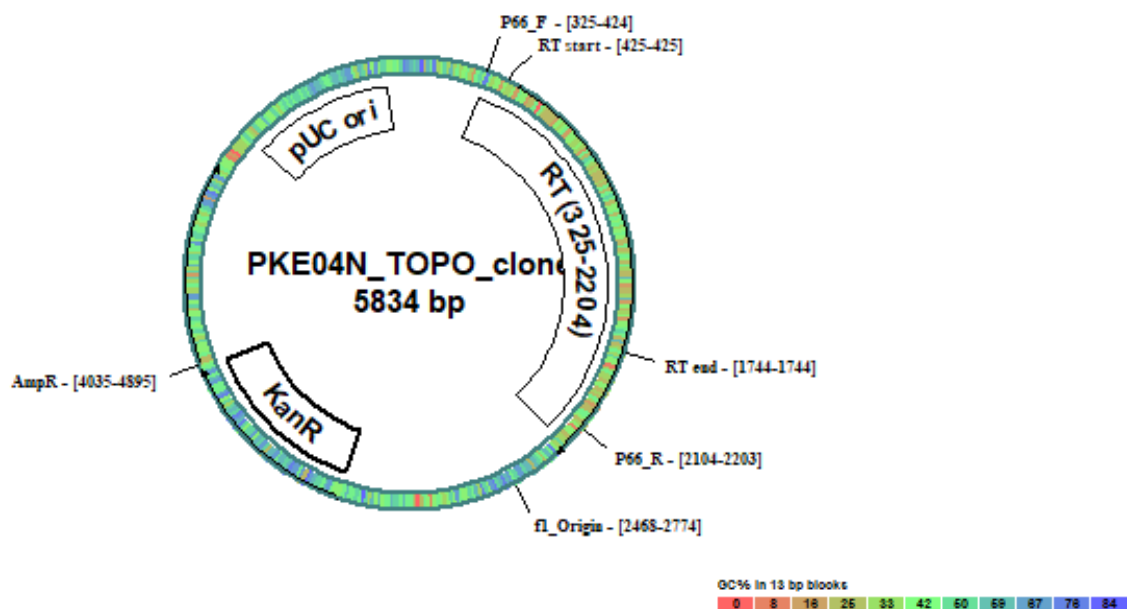
Figure 2.3: Molecular features of the PKE04N TOPO clone containing the RT region from patient PKE04N of the tropism study. The PKE04N clone was used as a template to generate all mutants. Labels P66_F and P66_R indicate the binding position of PCR primers. Image generated with pDRAW 32 (AcaClone_Software).

Table 2.7: Mutations required to generate the desired amino acid sequences of the selected variants

Mutant[a]	Primer Sequence[b] (5' to 3')	HXB2 Position
K65R	ATATAACACTCCAGTATTTGCCATAAAAAGGAAGGACAGTACTAAG	2714→2759
A62V	TCCATATAACACTCCAGTATTTGTCATAAAAAAGAAGGACAGTACTA	2711→2757
S68D	CAGTATTTGCCATAAAAAAGAAGGACGATACTAAGTGGAGGAAATT AGTAGATT	2725→2778
Y115F	ATTAGATGTGGGGGATGCATTTTTTTCGGTTCCTTTAGATG	2873→2913
S68G	TATTTGCCATAAAAAAGAAGGACGGTACTAAGTGGAGGAAATTAGTA	2728→2774
S68N	ATTTGCCATAAAAAAGAAGGACAATACTAAGTGGAGGAAATTAGTAG	2729→2775

    a. In order, to create the mutation combinations K65R_A62V, K65R_S68D, K65R_S68G, K65R_S68N, K65R_Y115F, independent primers were not utilized, instead, primers designed to create the single mutants were used in combination in the site-directed mutagenesis reaction.

    b. The table contains the forward primer sequences (5' – 3' direction) and the reverse primer sequences are the reverse complements of the forward primers. The mutated codon in each primer is underlined and the nucleotide change is highlighted.

2.2.7.3 Mutagenesis Reaction

The PKE04N TOPO plasmid was mutated using the Quikchange® Lightning Multi Site-Directed Mutagenesis kit (Agilent Technologies, California, USA) as per manufacturer's instructions with relevant primers containing mutations of interest. Briefly, a PCR reaction for both mutant viruses and positive control was prepared as per Table 2.8. Each PCR reaction was cycled in a thermocycler using the following parameters: 95°C for two minutes, 30 cycles of 95°C for 20 seconds, 55°C for 30 seconds and 65°C for two minutes and 54 seconds followed by a five-minute cycling at 65°C and a two-minute hold at 37°C. Following the completion of the PCR cycle, one µl of Dpn1 was added to each tube and incubated at 37ºC for five minutes to digest parental DNA.

Table 2.8: Summary of PCR reaction mix components for both sample and control mutagenic reactions, using the QuikChange lightning site-directed mutagenesis kit.

Component	Sample (µl)	Control (µl)	Final concentration
PCR water	16.75	18.5	-
10x QuikChange lightning reaction buffer	2.5	2.5	1x
Quiksolution	0.75	0	-
Forward Primer	1	1	100 ng
Reverse Primer	1	1	100 ng
Control primer mix	0	1	Proprietary, no information in kit[*]
dNTPs	1	1	Proprietary, no information in kit[*]
QuikChange enzyme blend	1	1	1 U/µl
ds-DNA template (PKE04N in TOPO)	1	0	100 ng/ µl
ds-control plasmid	0	1	Proprietary, no information in kit[*]
Total	25	25	

*: the stock concentration was not provided, hence the final concentration could not be determined. However, the required volume was used as specified in the protocol that came with the kit.

2.2.7.4 Transformation of the XL-Gold ultra-competent cells

XL10-Gold ultra-competent cells (Agilent) were utilized for transformation according to the manufacturer's instructions. Briefly, cells were removed from -80°C freezer and placed on ice to thaw for five minutes. A total of 45 µl of cells and two µl of beta-mercaptoethanol were incubated on ice for 10 minutes. Next DpnI treated DNA from the mutagenesis reaction one point two (1.2) µl was added and incubated on ice for 30 minutes. The reaction mixture was subjected to a 30-second heat pulse at 42°C and

immediately placed on ice for two minutes. Preheated S.O.C media (250µl) was added to each reaction and incubated at 37°C with shaking at 230 rpm for one hour. Afterwards, 100 µl of the transformation reaction was plated on LB-ampicillin agar plates which had 40 µl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 10 µl of 10 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), which as a whole served for easy identification of colonies that were efficiently transformed (i.e. white colonies showed efficient transformation while blue colonies were not efficiently transformed). Plates were incubated overnight at 37ºC for at least 16 hours.

2.2.7.5 Mutant screening

After incubation, five single white colonies were picked and touched to a master-plate before being boiled at 95ºC in 10 µl of PCR water. The master-plate was incubated overnight at 37ºC for at least 16 hours and stored at 4ºC. The DNA from the boiled colony was amplified by RT- PCR, as described in section 2.2.6.1, and sequenced (described in section 2.2.6.2), to confirm the presence of the correct mutation.

Following the confirmation of the inserted mutation the corresponding colony from the preserved master-plate was picked and cultured in LB-broth at 37ºC for 16 hours. Mutant plasmid DNA was then purified from the colonies confirmed to contain the correct mutant sequence employing the Qiagen maxi-prep procedure as per the manufacturer's instructions. DNA was quantified using the nanodrop and aliquots were kept at -80ºC.

2.2.8   Preparation of CEM-GXR25 cells

The "CEM-GXR25 green fluorescent protein (GFP) reporter T-cell line "(i.e. GXR cells was donated by Dr Mark Brockman) is replication-competent as they have the CD4 receptor and express the CXCR4 and CCR5 co-receptors. They also have a GFP expression cassette, which enhances the detection of infected cells using flow cytometry.

A frozen aliquot (i.e. 1 ml) of about one million GXR cells (stored in dimethylsulfoxide [DMSO], Sigma) was taken from a liquid nitrogen freezer (Custom Biogenics Systems, Romeo, USA) directly into a preheated 37ºC water bath. The tube of cells was gently agitated in the water bath until the contents were thawed. Afterwards, the tube of cells was transferred into a T25 flask (Corning-Costar, New York, USA) having four ml of pre-warmed R10 culture medium and incubated at 37ºC and 5% CO2 for 24 hours in a humidified Heraeus incubator (Thermo Scientific). R10 media comprised of RPMI-1640 (Sigma), supplemented with 50 U/ml penicillin-streptomycin (Gibco, New York, USA), 10 mM N-2-hydroxyethyl piperazine-N'-2ethanesulfonic acid (HEPES; Gibco), 2 mM L-glutamine (Sigma) and 10% foetal bovine serum (FBS; Gibco).

After 24 hours, the contents of the T25 flask was transferred into a 15 ml falcon tube and centrifuged at 1,500 rpm for 10 minutes to remove DMSO. Cells were then re-suspended in one ml of pre-warmed R10 and transferred into a T25 flask containing nine ml of pre-warmed R10. The flask was then incubated at 37ºC and 5% CO2 for a further 24 hours. Following incubation, cells were counted by adding 10 µl of thoroughly mixed cell culture to 10 µl of trypan blue (Bio-Rad, Hercules, USA). A total of 10 µl of this mixture was inserted into a TC20 cell counting slide (Bio-Rad) which was subsequently loaded into a TC20 automated cell counter (Bio-Rad). The output of the TC20 cell counter was the cell concentration (i.e. cells/ml). The volume of cell culture used to obtain a required number of cells was calculated as follows:

Volume of cell culture (ml) = number of cells required ÷ cell concentration (cells/ml)

Cells were kept at a concentration of 250 000 cells/ml in a final volume of 30 ml in a T75 flask (Corning). Cell growth was monitored every second day and if not used for experiments, 80% of the cell culture was removed and replenished with fresh pre-warmed R10. Cells were maintained for a maximum of two months, after which time a new aliquot of GXR cells was thawed and prepared for use.

2.2.9   Generation of Chimeric Viruses

2.2.9.1 RT amplification by PCR
The purified mutant PKE04 TOPO clone was amplified using the TaKaRa Ex Taq HS enzyme kit (Takara, Shiga, Japan) and the set of 100 nucleotides (which are chimeric forward and reverse primers that specifically correspond to NL4-3 backbone on each side of RT to generate an overlap necessary for transfection). Two PCR mixtures of 50 µl were prepared for each sample as described in section 2.2.2. Agarose gel (1%) electrophoresis was used as described in section *2.2.3 to* visualize the resulting PCR fragment of 1.879 kb.

2.2.9.2 pNL4-3Δ RT plasmid digestion
The pNL4-3ΔRT plasmid (donated by Dr Mark Brockman) was used to generate the mutant viruses. The pNL4-3ΔRT plasmid glycerol stock (in STBL3 cells) was taken from -80 ºC freezer, thawed on ice and 30µl was added to a mixture of Luria-Bertani (LB) (Sigma Aldrich, St Louis, USA) broth and ampicillin (100 ml of LB broth and 100 µl ampicillin). This was incubated in a shaking incubator at 37ºC and 230 rpm for 16 hours. The plasmid was extracted and purified using the Plasmid Maxi Kit (QIAGEN) as per the manufacturer's instructions and quantified using a nanodrop spectrophotometer (Thermo Scientific, Delaware, USA). The quantified plasmid was aliquoted and stored at -80ºC. The purified plasmid was then digested with BstEII (New England Biolabs, USA) to obtain a linear DNA fragment. Digestion was conducted on the morning of the co-transfection experiment to minimize the re-ligation of the restriction

site. The digestion reaction was prepared as shown in Table 2.9 and incubated in a water bath at 60°C for 2 hours.

Table 2.9: *BstEII* **digestion reaction.**

Reagent component	Volume
Plasmid (pNL43ΔRT)	10μg
10X Buffer	1/10 reaction volume
100X BSA	1/100 reaction volume
BstEII Enzyme 10U/ul	2 U/μg plasmid
Sterile Water	Makeup to the final desired volume

2.2.9.3 Co-transfection of the RT insert into CEM-GXR25 cell

The RT fragment was co-transfected with the linearized pNL4-3Δ RT plasmid into CEM-GXR25 cells. For the co-transfection steps, the CEM-GXR25 cells were counted using an automated TC20 cell counter (Bio-rad, South Africa) ensuring the viability of greater than 80%. For each mutant, four million cells were resuspended in 300μl of R10 medium, 90μl of RT PCR product and 10 μg digested plasmid was added to an electroporation cuvette and mixed. Samples were electroporated at 250 volts and 950μF. The contents were then rested in the electroporation cuvette at room temperature for five minutes. Using a Pasteur pipette, the contents were added to a T25cm flask containing nine ml of R10 medium, one million cells and 5μg/ml of polybrene (Sigma- Aldrich). The flasks were placed in an incubator at 37°C, 5% CO2. On day five, five ml of fresh R10 medium was added to the culture.

On day seven, the percentage of GFP positive cells was measured using flow cytometry on a FACS Calibur (BD Biosciences, San Jose, USA). Afterwards, on every second day, two ml of culture was removed of which one ml was prepared for flow cytometry and the remaining one ml discarded. The culture was then replenished with two ml of fresh R10 medium. When cells reached ~30% infection, the virus in the culture supernatant was harvested.

2.2.10 Monitoring viral growth by flow cytometry

To determine percentage infection, one ml of culture was placed in cluster tubes (Corning Costar) and centrifuged at 1500 x g for 10 minutes to pellet cells. The supernatant was discarded, and the pellet was vortexed for a few seconds. Cells were fixed in 200 μl of 2% paraformaldehyde (PFA) and vortex. The CEM-GXR25 cells were gated and data for GFP positive cells were acquired, using flow cytometry. Cultures above the threshold of 0.05% GFP+ cells were considered positive for infection.

2.2.11 Harvesting of Virus

The viruses were harvested when ~30% infection was attained. The whole culture contents were transferred into 50ml falcon tubes and centrifuged at 1500 rpm for 10 minutes at four degree Celsius (°C). The supernatant was removed and aliquoted into cryovials and stored at -80°C pending usage.

2.3   Result

2.3.1   Site-directed Mutagenesis (SDM) of Mutants

Eleven SDM reactions which consist of six single mutants SDM reactions and five combinations of double mutants and were introduced into a PKE04N TOPO clone. The following number of colonies were obtained from the SDM transformation reactions for each reaction (Table 2.10 and 2.11)

Table 2.10: Colonies from single mutants SDM transformation reactions

Mutants	The approximate number of colonies in a plate
A62V	250
K65R	100
S68G	500
S68N	4
S68D	400
Y115F	50

Table 2.11: Colonies from double mutants SDM transformation reactions

Mutants	The approximate number of colonies in a plate
K65R+A62V	>120
K65R+S68G	>300
K65R+S68N	>300
K65R+S68D	>200
K65R+Y115F	1

2.3.2   Colony screening of the Mutants

We picked four colonies at random for each of the transformation reaction, conducted colony PCR and checked for the presence of positive PCR products by gel electrophoresis (Table 2.12 and 2.13) and (Figure 2.4- 2.8).

Table 2.12: Colony screening of single mutants

Mutants	Number of colonies Screened	Number of positive PCR product present
A62V	4	3
K65R	4	2
S68G	4	4
S68N	4	3
S68D	4	4
Y115F	4	4

Table 2.13: Colony screening of double mutants

Mutants	Number of colonies Screened	Number of positive PCR Product present
K65R+A62V	4	4
K65R+S68G	4	4
K65R+S68N	4	4
K65R+S68D	4	4
K65R+Y115F	1	1
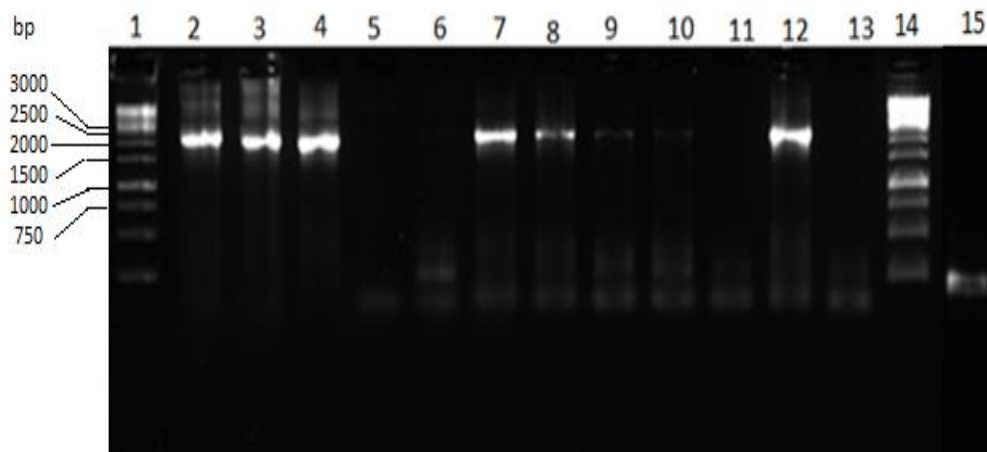
Figure 2.4: Gel Electrophoresis of single mutants. Lane 1, 14 (1kb ladder), lane 2-5 (S68N), Lane 6-9 (A62V), Lane 10- 13 (K65R) lane 15 (Negative control).
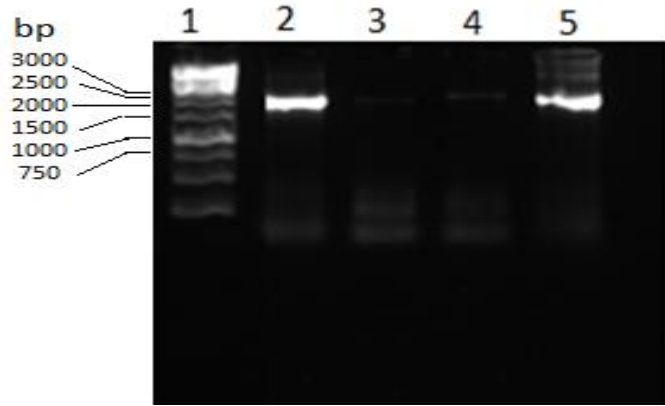
Figure 2.5: Gel Electrophoresis of S68D single mutants. Lane 1 (1kb ladder) and lane 2-5 (S68D).
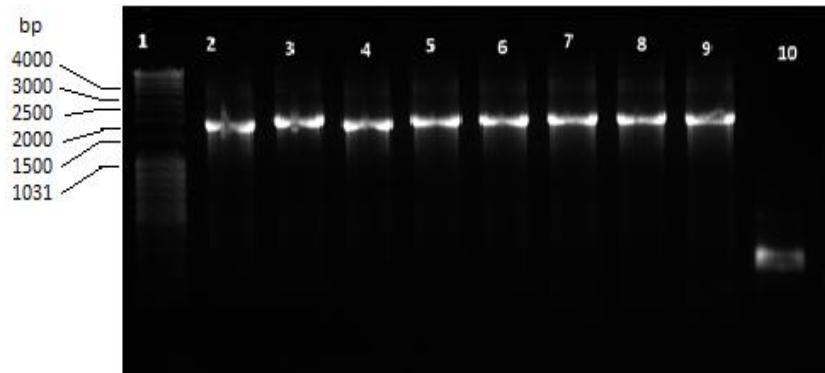
Figure 2.6: Gel Electrophoresis of single mutants and DNA ladder. Lane 1 1KB ladder, lane 2-5 (S68G), lane 6-9 (Y115F) and lane 10 (Negative control).
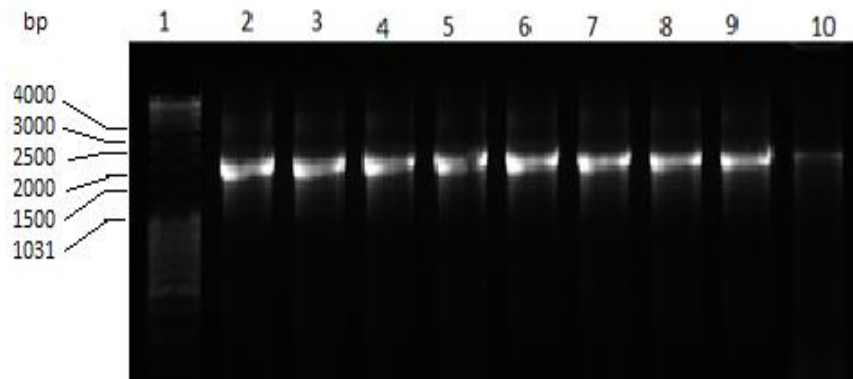
Figure 2.7: Gel Electrophoresis of double mutants. Lane 1 1KB ladder, lane 2-5 (K65R_S68G), lane 6-9 (K65_S68D) and lane 10 (K65R_Y115F).
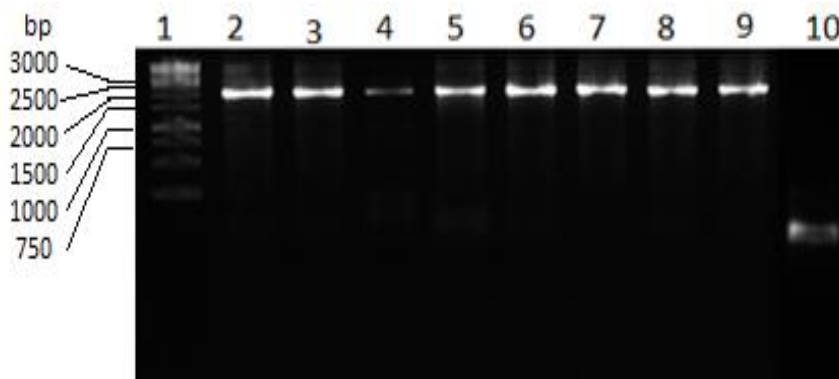
Figure 2.8: Gel Electrophoresis of double mutants. Lane 1 shows the 1KB ladder, lane 2-5 the K65R_A62V mutant, lane 6-9 the K65R_S68N mutant and lane 10 the Negative control.

2.3.3. Sequencing of Mutants

Sequencing was performed on all the colony positive for PCR products of the 11 SDM reactions performed. Seven of the mutant plasmids had the designated mutated sequence, while they remain four mutants that did not have the expected mutated sequence had the original sequence without the mutation. From the seven successful mutated sequences, all the six single mutants had the desired mutation. While only one of the double mutants had both the designated mutations present out of the five-double mutant SDM reactions carried out. See Table 2.15 and Figure 2.7(a-h) for the chromatogram peaks of the successful mutants generated.

Table 2.14: Mutants with and without the designated mutated sequence.

Mutants	Designated mutated sequence
A62V	Present
K65R	Present
S68G	Present
S68N	Present
S68D	Present
Y115F	Present
K65R+A62V	Absent
K65R+S68G	Absent

K65R+S68N	Present
K65R+S68D	Absent
K65R+Y115F	Absent

A. PKE04N Wildtype

PKE04N WT   CAATATTTGCCATAAAAAAGAAGGACAGTACTAAGTGGAGA
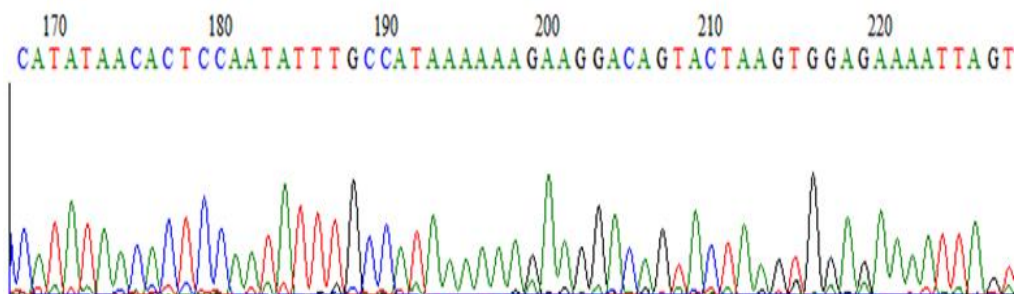
B. A62V

Pke04n WT   CAATATTTG<u>CC</u>ATAAAAAAGAAGGACAGTACTAAGTGGAGA

A62V   CAATATTTG<u>T</u>CATAAAAAAGAAGGACAGTACTAAGTGGAGA
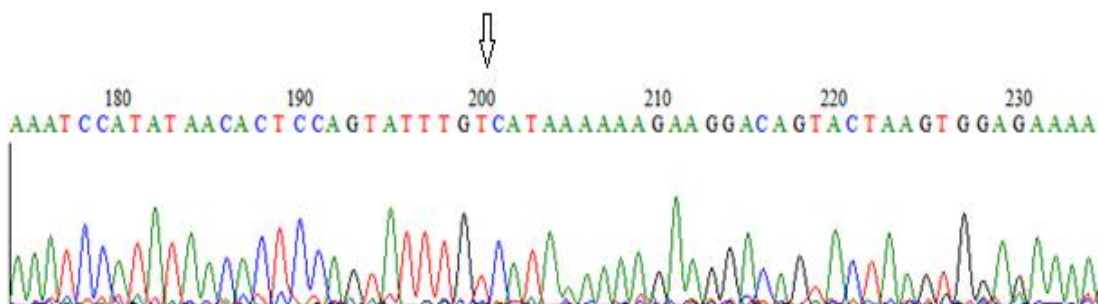
C. K65R

PKE04N WT   CAATATTTGCCATAAAAA<u>A</u>GAAGGACAGTACTAAGTGGAGA

K65R   CAATATTTGCCATAAAAA<u>G</u>GAAGGACAGTACTAAGTGGAGA
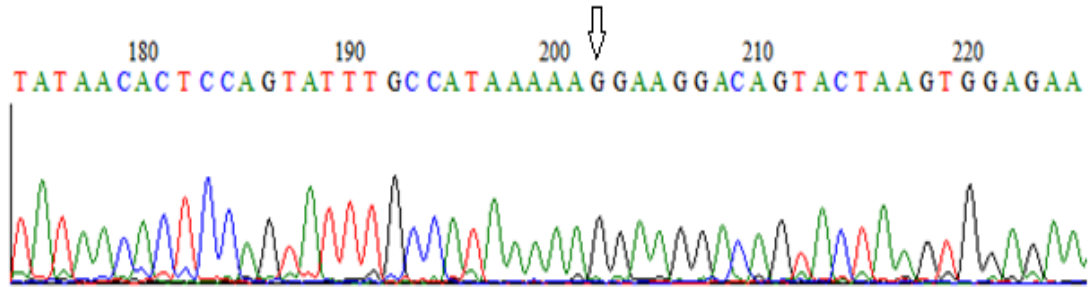
D. S68N

PKE04N WT   CAATATTTGCCATAAAAA<u>A</u>GAAGGACAGTACTAAGTGGAGA

S68N   CAATATTTGCCATAAAAAAGAAGGACA<u>A</u>TACTAAGTGGAGA
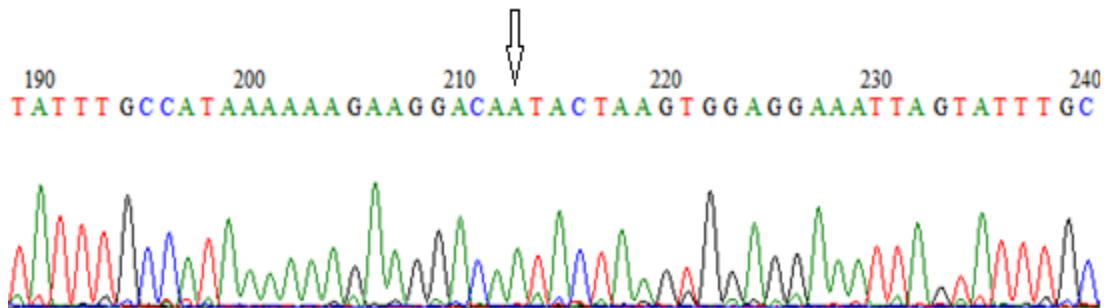
E. S68D

PKE04N WT   CAATATTTGCCATAAAAA<u>A</u>GAAGGACAGTACTAAGTGGAGA

S68D   CAATATTTGCCATAAAAAAGAAGGAC<u>GA</u>TACTAAGTGGAGA
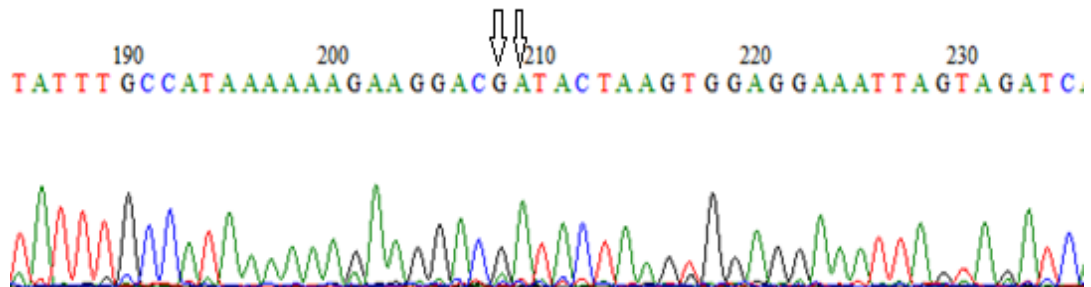
F. S68G

PKE04N WT   CAATATTTGCCATAAAAA<u>A</u>GAAGGAC<u>A</u>GTACTAAGTGGAGA

S68G   CAATATTTGCCATAAAAAAGAAGGAC<u>G</u>GTACTAAGTGGAGA
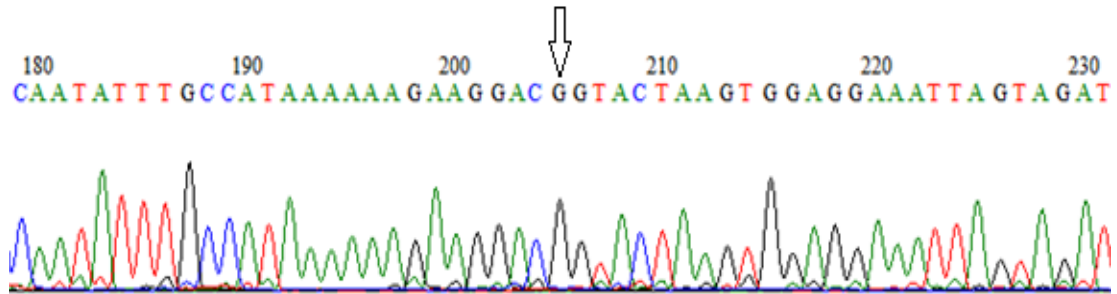
G. Y115F

PKE04 WT   TAGATGTGGGGGGATGCAT<u>A</u>TTTTTCAGTTCCTCTAGATGAA

Y115F   TAGATGTGGGGGGATGCAT<u>T</u>TTTTTCAGTTCCTCTAGATGAA
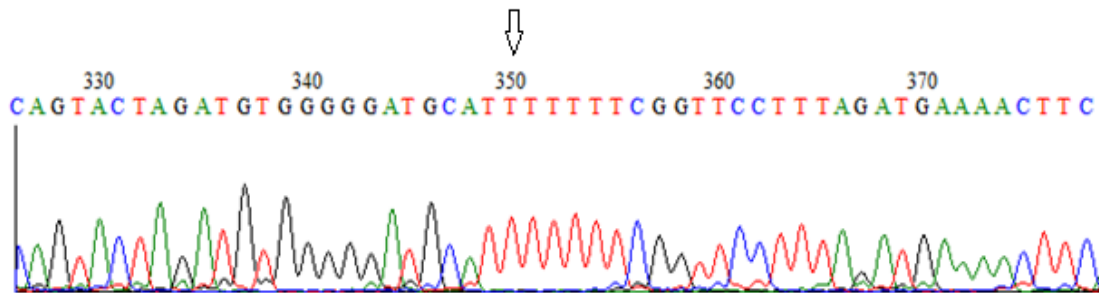
H. K65R_S68N

PKE04N WT   CAATATTTGCCATAAAAA<u>A</u>GAAGGACA<u>G</u>TACTAAGTGGAGA

K65R_S68N   CAATATTTGCCATAAAAA<u>G</u>GAAGGACA<u>A</u>TACTAAGTGGAGA
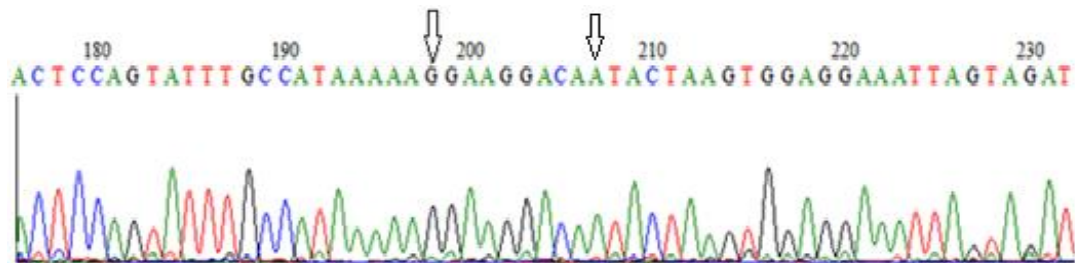
Figure 2.9: The chromatogram peaks of the sequence traces. The underlined and highlighted base indicate the base change in comparison with the PKE04N WT, while the arrow on the chromatogram peaks indicate the designated base (a)PKE04 Wildtype, (b) A62V at position 200, (c)K65R at position

202, (d) S68N at position 213, (e) S68D at position 208 and 209, (f) S68G at position 205, (g) Y115F at position 350 and (h) K65R_S68N at position 198 and 207.

2.3.4   Growth Kinetics of Mutants

The mutants, as well as the wildtype viruses, were harvested when the percentage infectivity was between 25 to 35% and kept at -80˚C pending when replication capacity assay (RCA) was carried out. As seen in Figure 2.10, the first virus to be harvested was the subtype B wildtype (pNL4-3) as it attained 56.59% infectivity on day seven, followed by the subtype C wildtype (PKE04N) and K65R on day eight with infectivity of 33.81% and 35.44 respectively. Viruses K65R+S68N, A62V and Y115F were harvested on day 11 with infectivity of 53.79%, 35.29%, and 45.23% respectively. Mutant S68D was harvested on day 13 (40.37% infectivity). S68G and S68N did not achieve the needed infectivity and could not be used for subsequent experiments.
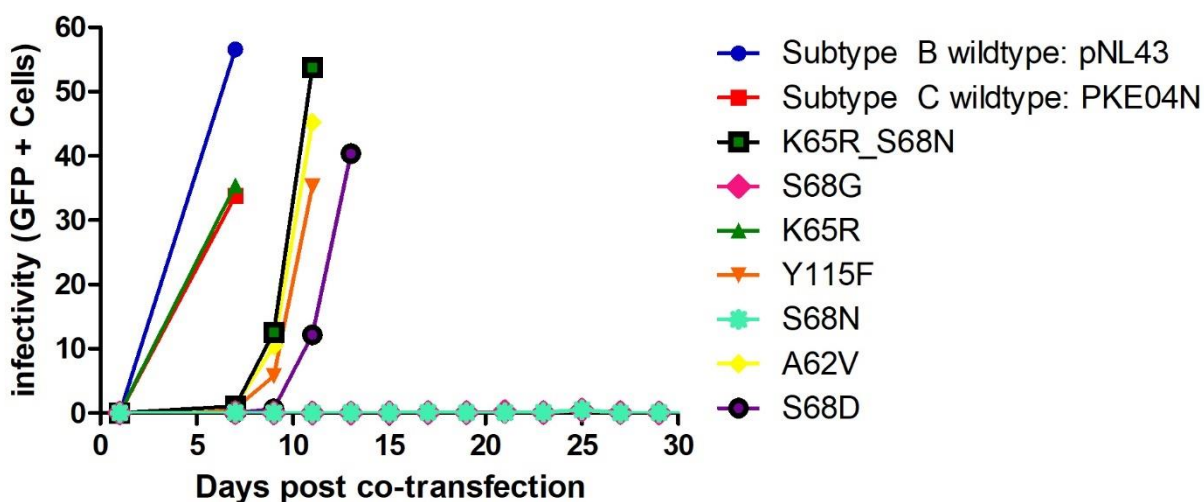
Figure 2.10: Infectivity of mutants during co-transfection. The NL43Δ RT, PCR product of the mutant were co-transfected into CEM-GXR25 by electroporation and viral infectivity was monitored regularly till ~30% infectivity was attained.

2.4   Discussion

Studies have reported mutations "A62V, K65N, S68G/N/D, K70E/Q/T, L74I, V75L and Y115F" to be accompanying TDF resistance in subtype C, in addition to the preferentially selected mutation K65R (Maphumulo, 2016, Rhee et al., 2017, TenoRes-Study, 2016). However, their phenotypic impact on subtype C needed further investigation. In this chapter, we generated the most frequently observed mutations associated with TDF resistance: K65R, A62V, S68G/N/D, Y115F and combinations with K65R using SDM. In the SDM experiment, we observed the successful and efficient generation of single mutants

compared to the generation of the double mutants. The difference may have been due to the primer pair self-annealing. This could be eliminated by designing primers with the desired mutation inserted at four bases away from the 5′-end and a minimum of six to eight bases from the 3′-end, rather than the usual 10–15 bases at each side (Zheng et al., 2004). However, due to time constraints, this was not done in the current study but will be used for future experiments.

For the single mutants generated (A62V, K65R, S68D, S68G, S68N, and Y115F), independent primers harbouring the designated mutation were used, whereas for the double mutants (K65R+A62V, K65R+S68D, K65R+S68G, K65R+S68N, and K65R+Y115F) primers designed to create the single mutants were used in combination in the site-directed mutagenesis reaction. However, we were not able to generate the desired combinations due to primer-dimer or as a result of the proximity of mutated positions (codon 62, 65 and 68). This is in line with earlier findings (Zheng et al., 2004, Weiner et al., 1994). Therefore, for the successful generation of double mutations, primers designed with both the mutations should have been utilized (Tian et al., 2010, Zheng et al., 2004). However, since K65R and Y115F are not close to each other, template switching during PCR may have been responsible (Abram et al., 2014, Nikolenko et al., 2004, Pathak and Temin, 1990). Also, it is possible that more colonies needed to be screened to detect the double mutants.

In this study, the PCR products of the seven successfully generated mutants (A62V, K65R, S68D, S68G, S68N, Y115F, and K65R+S68N) and the pNL4-3 deleted RT were co-transfected and viral infectivity was monitored until about 30% infectivity was attained and then harvested. Seven out of the nine recombinant mutants attained the expected infectivity and successfully yielded viral replicating stock within the limited time (31 days), while two of the mutants (S68G and S68N) did not attain the expected infectivity and could not be used for subsequent experiments. This failure may be an indication that these mutations come with a fitness cost, resulting in a non-infectious virus. This is in line with earlier findings by Garcia-Lerma et al (2000) and others who found that S68G came with a fitness cost and that S68G/N are secondary mutations that do not acquire resistance directly; rather, they are found in association with other mutations (García-Lerma et al., 2000, Mathiesen et al., 2003, Margot et al., 2006b, Svarovskaia et al., 2008).

CHAPTER 3

IMPACTS OF TENOFOVIR SELECTED RESISTANCE MUTATION OF HIV-1 SUBTYPE C ON REPLICATION CAPACITY AND DRUG SUSCEPTIBILITY

Chapter Three

Impacts of Tenofovir Selected Resistance Mutation of HIV-1 Subtype C on Replication Capacity and Drug Susceptibility

3.1   Introduction

South Africa, in line with World Health Organization (WHO) ART treatment guidelines for adult HIV-1 infection, introduced the use of tenofovir (TDF) for "first-line antiretroviral treatment" and for "pre-exposure prophylaxis" following its universal approval, due to its profound benefits over stavudine (d4T) which is toxic (Bennett et al., 2008, NDOH, 2014, WHO, 2013). Currently, the South African national treatment program first-line regimen consists of TDF/zidovudine (AZT) and lamivudine (3TC) or emtricitabine (FTC) if there are contraindications and efavirenz (EFV) or nevirapine (NVP) for pregnant women used as a fixed-dose combination. While the second-line regimen consists of AZT/TDF and 3TC or FTC and 1 protease inhibitor(PI) with ritonavir(r) as a booster (lopinavir (LPV/r) or atazanavir (ATV/r) (NDOH, 2014). The TDF can also be used for second-line treatment, as long as it antiretroviral activity is still maintained, preceding the emergence of the resistant virus after previous treatment exposure   (Rhee et al., 2017).

However, the efficacy of the drug can be jeopardized by the evolution of drug resistance mutations. The TDF resistance can be acquired through the K65R or K70E mutation pathway (though less frequently) under TDF selection pressure (TenoRes-Study, 2016, Rhee et al., 2017, HIVBD, 2019). The K65R mutation prevalence ranges from below 20% in Europe and North America to more than 50% in sub-Saharan Africa (TenoRes-Study, 2016). Tenofovir is currently used as a first-line regimen in many countries including South Africa, where the predominant subtype is subtype C, have documented a high rate of the K65R resistance mutation in TDF treated patients (Skhosana et al., 2015, Sunpath et al., 2012) although a low prevalence of the K65R mutation has also been reported (Hoffmann et al., 2013). Furthermore, increasing levels of transmitted drug resistance or pre-treatment drug resistance in ART-inexperienced individuals in South Africa with K65R have been documented (Steegen et al., 2016).

Furthermore, K65R causes broad resistance to nucleoside reverse transcriptase inhibitors (NRTIs) and is one of the major factors responsible for virologic failure in patients on a triple nucleoside regimen not containing zidovudine (Margot et al., 2002, Shafer, 2002). K65R also occurs in combination with other mutations that increase TDF resistance such as, "A62V, S68G/N/D, K70E/Q/T, L74I, V75L, and Y115F". These mutations were frequently observed in patients on a TDF regimen and were higher in subtype C compared to subtype B (Margot et al., 2016, Rhee et al., 2017). However, in the absence of these

accompanied mutations, it causes intermediate resistance to TDF (Margot et al., 2016, Miller et al., 2004, Skhosana et al., 2015, Stone et al., 2004, Svarovskaia et al., 2008).

In this chapter, we investigated the impact of selected TDF resistance mutations of HIV-1 Subtype C with a focus on K65R, A62V, S68D, Y115F and K65R+S68N on viral replication capacity and drug susceptibility.

3.2 Materials and methods

3.2.1 Mutant selection

The Quick-change Multi site-directed mutagenesis kit (Agilent) was employed to generate the following mutations K65R, A62V, S68D, Y115F and K65R+S68N as described in chapter two section 2.2.7.3. These mutants successfully yielded replicating virus stocks and were used for replication and drug susceptibility testing.

3.2.2 Replication Capacity Assays (RCA)

3.2.2.1 Virus titration

Virus titres were performed in triplicate for each of the chimeric viruses generated. Titres served to determine the amount of virus needed to obtain a multiplicity of infection (MOI) of 0.3% on day two of a replication capacity assay (Brockman et al., 2007). Briefly, a total of 1 million GXR cells in 100 μl of R10 was placed in each well of a 24 well culture plate (Corning Costar) followed by the addition of 400 μl of the harvested virus. The culture plate was incubated at 37ºC and 5% CO_2 for 24 hours. The following day, one ml of pre-warmed R10 was placed in each well and the plate was again incubated at 37ºC and 5% CO_2 for 24 hours. On day two, the contents of each well were thoroughly mixed and 500 μl of culture was removed and prepared for flow cytometry, to measure the percentage of cells infected. The percentage of cells infected was used to calculate the amount of virus required to achieve 0.3% on day two of the replication capacity assays. The formula used was as follows:

The volume of virus needed for the RCA (μl) = (0.3% ÷ % of cells infected on day 2 of the titre) *400 μl

3.2.2.2 Replication Capacity Assay

Replication capacity assays (RCA's) were conducted in GXR cells over a timeframe of seven days to determine the exponential growth of the virus as earlier described (Miura et al., 2009, Brockman et al., 2007). Each assay was performed in triplicate for each of the recombinant viruses generated and in duplicate in an independently repeated experiment. Each assay included a negative control (GXR cells only) and positive control (pNL43-WT virus).

Briefly, a total of one million GXR cells in 100 μl of R10 was added to each well of a 24 well culture plate (Figure 3.1). The required amount of virus (i.e. amount obtained from the viral titration step) was diluted in R10 to obtain a final volume of 400 μl. The diluted virus was added to the various wells of the 24 well culture plate and then incubated at 37ºC and 5% CO_2 for 24 hours. The following day, one ml of pre-warmed R10 was added to each well and incubated for 24 hours again. Thereafter from day two to six, 500μl of culture was removed daily to measure infectivity by flow cytometry and replenished with 500μl fresh pre-warm media.

To determine the viral replication capacity, the exponential increase in percentage infected cells from day two to six post-infection was calculated using the semi-log method in Microsoft Excel. Then the "logest" function in Excel was employed to estimate the log10 exponential curve, with the best fit to the data. The value obtained represents the slope of the curve, and then transformed to its' natural log. All replication values were normalized by dividing the slope of the exponential growth of each virus by the slope of the exponential growth of the pNL43-WT reference strain. Triplicate slope values were averaged.

Figure 3.1: Representation of a plate layout for the Replication Capacity assay. NEG: Negative

3.2.3   *Phenotypic Drug Susceptibility Testing*

Phenotypic drug susceptibility assays were conducted to determine the drug susceptibility of five selected mutants. The TDF drug was used (gotten from the AIDS Research and Reference Reagent Programme, Division of AIDS, NIAID, NIH). In this study, a TZMBL cell-based single-cycle phenotypic drug

susceptibility assay was employed and viral titres were also performed using the same assay as previously reported (Montefiori, 2009).

3.2.3.1 TZMBL cells preparation

TZMBL cells are a luciferase reporter-based HeLa cell line. They are adherent cells that express CD4 and CCR5 receptors and encode a luciferase gene which is regulated by the HIV-1 promoter. They are replication-competent and infected cells are detected by luminescence. TZMBL cells were gotten from the AIDS Research and Reference Reagent Programme, Division of AIDS, NIAID, NIH.

An aliquot of TZMBL cells (one ml) was taken from a liquid nitrogen freezer and quickly put in a water bath at 37ºC. The cells were transferred to a 15 ml falcon tube containing 10 ml of pre-warmed Dulbecco's Modified Eagle's Medium (DMEM; Sigma), supplemented with 10% FBS (Gibco), 50 U/ml penicillin-streptomycin and 10 mM HEPES. The 15 ml falcon tube was centrifuged at 1,200 rpm for 10 minutes. The supernatant was discarded, pellets were re-suspended in fresh DMEM (five ml) and transferred to a T25 flask containing an additional five ml of DMEM. The T25 flask was incubated at 37ºC and 5% CO_2 for 48 hours. After 48 hours, cells were visualized using a Zoe® fluorescent imager (Bio-Rad). If cells were not 80% confluent, media was discarded and replenished with fresh pre-warmed DMEM. If cells were 80% confluent, they were counted and used in experiments or passaged.

Cells were counted by first detaching them from the monolayer. This was done by decanting culture medium from the flask, quickly washing the monolayer with sterile PBS and gradually adding 2.5 ml of 0.25% trypsin-EDTA (Sigma) to the cell monolayer. The flask was incubated at room temperature for two minutes, followed by the removal of trypsin-EDTA and further incubation at 37ºC and 5% CO_2 for four minutes. Thereafter, 10 ml of pre-warmed DMEM was added and the wall of the T25 flask, containing the cell monolayer, was repeatedly rinsed to detach cells. The content of the flask was thoroughly mixed. Cells were counted by adding 10 μl of thoroughly mixed cell culture to 10 μl of trypan blue (Bio-Rad, Hercules, USA). A total of 10 μl of this mixture was inserted into a TC20 cell counting slide (Bio-Rad) which was then loaded into a TC20 automated cell counter (Bio-Rad). The output of the TC20 cell counter was the cell concentration (i.e. cells/ml). The volume of cell culture used to obtain a required number of cells was calculated as follows:

Volume of cell culture (ml) = number of cells required ÷ cell concentration (cells/ml)

The number of cells required was removed and used in experiments, whilst the remaining cells were maintained at 250,000 cells/ml in DMEM in a T25 culture flask, incubated at 37ºC and 5% CO_2. Cells were

monitored by microscopy, fed and passaged every 48 hours. Cells were maintained for a maximum of one month, after which time a new aliquot of cells was prepared for use in experiments.

3.2.3.2 Single-cycle virus titration

A single-cycle infection assay as previously reported (Montefiori, 2009) was conducted in the no drug medium, to measure the amount of virus required to achieve the average 50% tissue culture infective dose (TCID50) of each virus. Virus titrations were set up in a 96 well tissue culture plate (Corning Costar). A total of 100 μl of DMEM was placed in all wells of the plate. Then, 25 μl of virus (generated in 3.2.2) was transferred to the first 4 wells (column 1, rows A-D for one virus and rows E-H for a second virus). A five-fold serial dilution was then performed. All wells of column 12 were virus-free; this served as the cell control. A total of 10,000 TZMBL cells in 100 μl of DMEM and 0.05 g/μl of diethylaminoethyl-dextran hydrochloride (DEAE dextran; Sigma) was added to all wells. The plate was then placed in the incubator at 37ºC and 5% CO2 for 48 hours. Following incubation, 100 μl of culture medium was pipetted out from each well and 100 μl of Bright Glo luciferase reagent (Promega) was added. The plate was allowed to stay at room temperature for two minutes, the contents of wells were thoroughly mixed and 100 μl of culture from each well was transferred to corresponding wells of a 96 well black solid bottom microplate (Promega). Luminescence (i.e. indicator of infectivity) was measured using the Victor Nivo multimode microplate reader ((Perkin-Elmer).

Data are expressed as relative light units (RLUs) and positive infection was quantified using a cut-off of 2.5 times that of the control RLU. The TCID was calculated by selecting the dilution that yielded 150,000 RLU (+/- 15,000 RLU) (Montefiori, 2009).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
B	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
C	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
D	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
E	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
F	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
G	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC
H	Dil 1	Dil 2	Dil 3	Dil 4	Dil 5	Dil 6	Dil 7	Dil 8	Dil 9	Dil 10	Dil 11	CC

Figure 3.2: Virus titration plate setup. Virus 1 (row A-D) virus 2 (rows E-H). CC, Cell control wells (cells only).

3.2.3.3 Single-Cycle Phenotypic Drug Susceptibility Assay

A single-cycle phenotypic assay was performed. The drug concentrations for TDF used in this study ranged from 50 μM to 0.39 μM. Each assay comprised of a cell control (cells only, no virus or drug), a virus control (virus and cells only, no drug) and virus experiments (virus, cells, and drug).

Briefly, 10,000 TZMBL cells (in 100 μl of DMEM, 0.05 g/μl of DEAE dextran) and two-fold serial dilutions of TDF drug was infected with the relevant amount of virus that yielded 150,000 RLU equivalents (+/- 15,000 RLU), in a 96 well culture plate. The plate was placed in the incubator for 48 hours at 37ºC and 5% $CO2$. After which, 120 μl of supernatant was removed and 100 μl of Bright Glo reagent added. The plate was allowed to stay for two minutes at room temperature. Thereafter, the contents of each well were mixed and transferred to corresponding wells of a black solid bottom microplate. HIV-1 infection was determined by luciferase expression via the Victor Nivo multimode microplate reader ((Perkin-Elmer).

The extent to which the drug inhibited viral replication (i.e. percentage inhibition) was determined as the ratio of the difference in RLUs between the test wells and the cell control wells (i.e. negative/cells only) and the difference between the virus control wells (i.e. virus without exposure to drugs) and the cell control well and multiplying the result by 100 for each virus. The calculation is given below:

%inhibition = (test wells – cell control wells) ÷ (virus control wells – cell control wells) * 100

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The concentration of drug needed to prevent viral replication by 50% (i.e. IC50) was calculated by fitting the percentage inhibition results to a sigmoidal dose-response curve (with a variable slope) in GraphPad Prism.

Fold change in drug susceptibility (i.e. differences in the amount of drug needed to obtain the IC50 between the mutant virus and a reference virus) was calculated by dividing the IC50 of each virus by the IC50 of the NL43-WT reference strain.

3.2.3.4 Phenotypic Drug Susceptibility Interpretation

For this study, three classifications of drug susceptibility were used to group the mutant viruses these included: susceptible (S), reduced susceptibility (RS) and resistance (R). For viruses to be considered as susceptible, their FC was required to be below the lower FC cut-off established in literature. To be grouped as having reduced susceptibility the FC values were required to be greater than the lower FC cut-off level. In this study we use 1.4 lower FC cut-off level for TDF based on published lower clinical cut-off (CCO) as reported in the literatures (Lu et al., 2002, Miller et al., 2004, Parkin et al., 2004b, Whitcomb et al., 2003, Winters et al., 2008, Murray et al., 2008, Margot et al., 2015) as well as in the Monogram assay.

Table 3.1: TDF susceptibility clinical cut-offs

	Fold Change
Susceptibility (S)	<1.4
Reduced susceptibility (RS)	1.4-4.0
Resistance (R)	>4.0

The drug susceptibility of each mutant virus was measured in triplicate using TDF. Replicate assays were performed at least two days apart in independently repeated experiments.

3.2.4   Statistical Analyses

GraphPad Prism version 5.0 software (La Jolla, CA, USA) was employed for all the statistical analyses performed. To determine variations between the wild-type and the mutants a one-way Analysis of Variance (ANOVA) with Tukey post hoc testing was conducted. To evaluate agreement concordance between data either a Pearson's correlation test or a Spearman's Rank correlation test was applied to identify correlation coefficient (r) values. Student T-test was used to compare data between any two groups. A p-value of <0.05 was expressed as statistical significance

3.3 Results

3.3.1 *Viral Replication Kinetics*

CEM-GXR25 cells were infected with viruses harbouring drug resistance mutations and compared to those infected with the wild-type. The course of infection was followed daily over seven days. Viral replication kinetics as determined by daily FACS analysis (Figure 3.3). There were no significant differences (P=0.3360) in the replication kinetics between the wildtypes and the mutants as shown in Figure 3.3. We observed from day two to four of the assay, the mutants, as well as the wildtype viruses, grew relatively slowly, however, S68D and the wildtype viruses grew exponentially on day five, while K65R, K655+S68N, A62V, and Y115F grew exponentially on day six. The mutant S68D had the highest growth rate, both wildtype controls as well as, K65R had similar growth rates on day six, while Y115F had the lowest growth rate on day six (Figure 3.3).
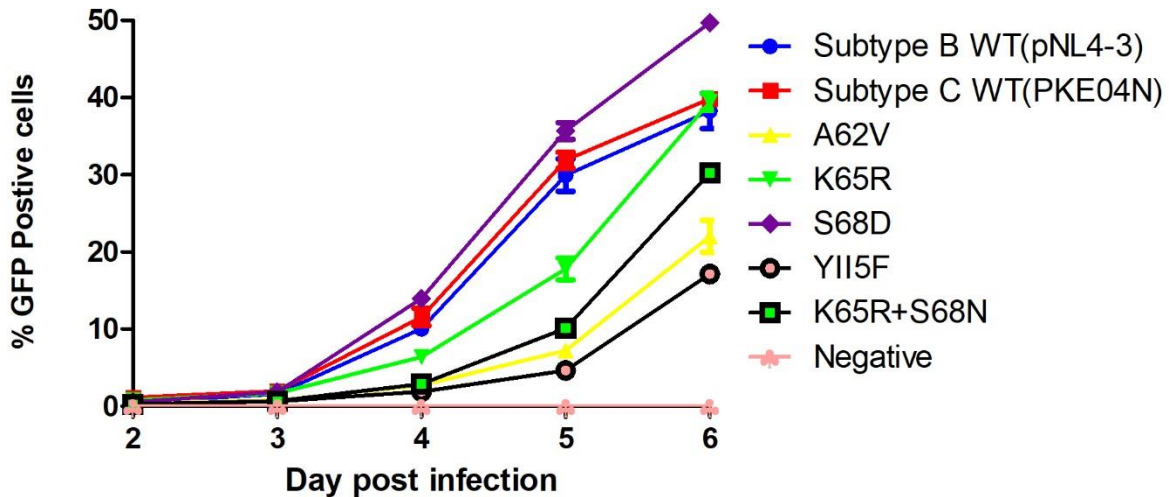
Figure 3.3: Replication kinetics of mutant viruses.

3.3.2 Validating reproducibility of the replication *assay*

Each replication assay was conducted in triplicate and repeated (duplicate) at least three days apart. All replication capacity values were normalized to the growth of pNL4-3 WT. Spearman's correlation of the duplicate assays showed concordance of replication capacity between replicates (Spearman's correlation r=0.7279, p=0.0002) (Figure 3.4). This highlights the reproducibility of the assay.
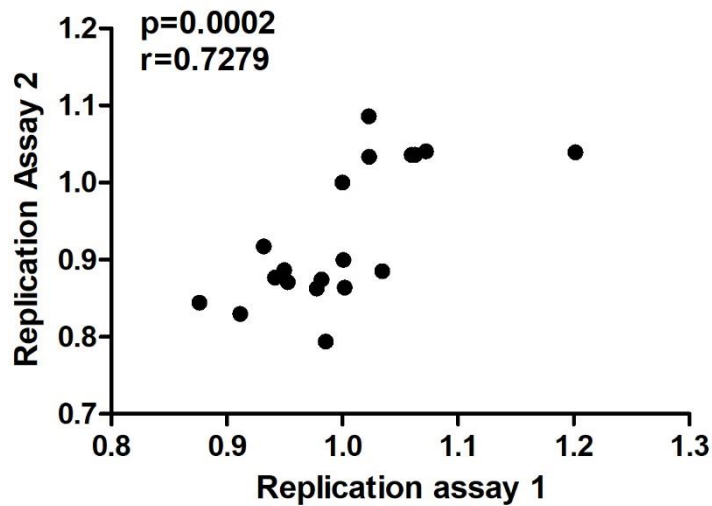
Figure 3.4: Comparison of duplicated replication assays. Pearson's correlation showed a high concordance between the two data sets indicating reproducibility of the assay.

3.3.3   *Replication capacity of chimeric HIV variants*

The replication capacity assay measures the competency of a virus to replicate, determined by the percentage GFP positive infected cells over seven days. In this study, we determine the relative replication capacity of the TDF selected variants (A62V, K65R, S68D, Y115F, and K65R+S68N) generated by site-directed mutagenesis in a multi-cycle parallel assay. Viral replication capacity was determined as the slope of the log GFP+ cells measured in the kinetics experiments. In this experiment, a replication capacity of one (1) represents the wild-type reference virus (Figure 3.5). There were significant differences (p<0.001) in the viral infectivity between the wildtypes and the mutants as shown in Table 3.2 and Figure 3.5. Viral infectivity was normalized to pNL4-3 for all variants and ranged from 0.86 to 1.05. Mutant K65R_S68N had the highest infectivity while subtype C wildtype (PKE04N) and A62V had the lowest (Figure 3.5). K65R mutation in combination with S68N displays a significantly higher replication capacity compared to K65R mutation alone (p<0.001) (Table3.2) (Figure 3.6).
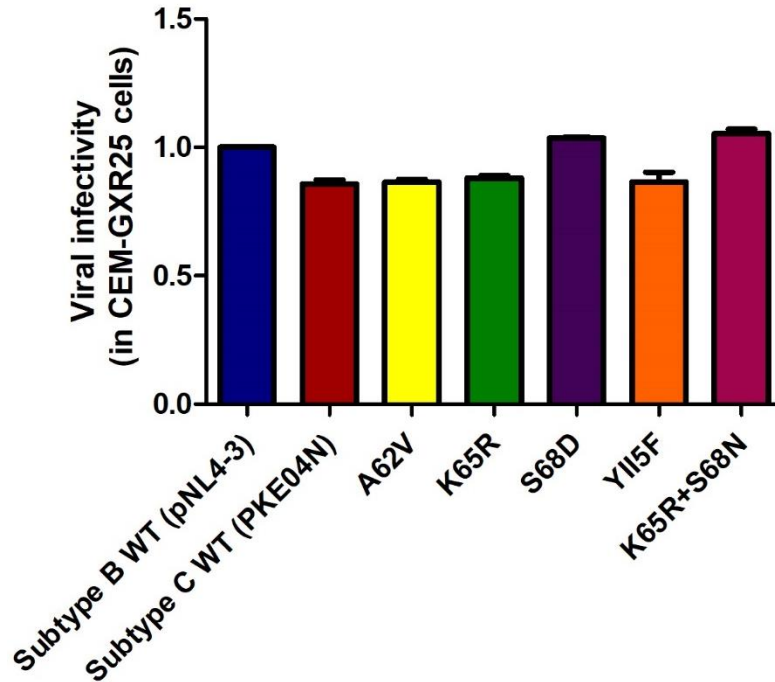
Figure 3.5: Replication capacity of mutant viruses. CEM-GXR25 cells were infected in triplicate with the virus variants and evaluated with the wild-type. The infectivity rate was measured as the slope of log GFP$_+$ cells analysed in the kinetics experiments.

Table 3.2: Replication capacity relative to the wildtypes

Virus variant	Replication capacity	Tukey's Multiple Comparison Test Significance p-value		
		Subtype-B wildtype (pNL4-3)	Subtype-C Wildtype (PKE04)	K65R
Subtype-B wildtype (pNL4-3)	-	-	-	-
Subtype-C wildtype (PKE04)	0.86 ± 0.025	***	-	-
A62V	0.86 ± 0.021	**	ns	-
K65R	0.88 ± 0.02	**	ns	-
S68D	1.04 ± 0.002	ns	***	-
YII5F	0.87 ± 0.06	**	ns	-
K65R+S68N	1.05 ± 0.03	ns	**	***

(-) not applicable; (ns) not significant. * P value < 0.05; ** P value < 0.01; *** P value < 0.001.
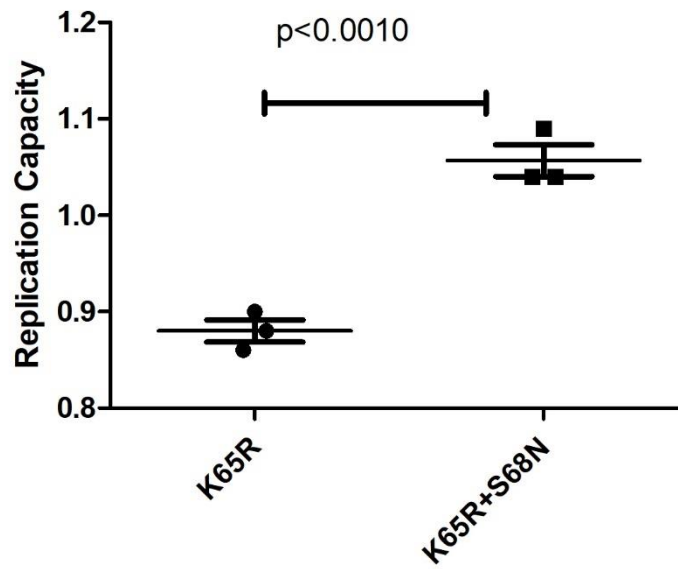
Figure 3.6: The effect of K65R and K65R+S68N on replication capacity. The student's t-test between the two-group showed a significant difference (p<0.0010).

3.3.3.1 Number of mutations in the sequence versus replication capacity

In this study, the mutants were grouped into two classes based on the number of nucleotides change in the sequence; single nucleotide change mutants (A62V, K65R, and Y115F, class 1) and double nucleotide change mutants (S68D and K65R+S68N, class 2). All class 1 mutant viruses displayed lower replication capacity (RC) ranging from 0.86-0.88 compared to the wildtype (WT), while class 2 mutants viruses had similar RC as the WT, ranging between 1.04 and 1.05 (Figure 3.7). This study showed significant differences (p<0.0016) between class 1 and class 2 viruses. Class 1 mutant viruses had similar RC as the subtype C WT, whereas class 2 was similar to that of the Subtype B WT (Figure 3.8). Also, class1 viruses are less fit than class 2 viruses. Furthermore, the correlation between the number of nucleotide mutation in the sequence and replication capacity was assessed. The number of nucleotide mutation in the sequence correlate well with Replication Capacity, however not statistically significant (Figure 3.9). Data showed that the number of nucleotide change may impact on replication capacity and viruses with an increase in nucleotide change in the sequence may likely be associated with high RC. Also, Variants at RT codon 68 (S68D and S68N in combination with K65R) had RC values of 1.04 and 1.05 respectively. This suggests that double nucleotide change probably restore the fitness cost of mutation at codon 68 of the RT.

Figure 3.7: Comparison of replication capacity based on the number of nucleotides change in the sequence. Class 1(single nucleotide mutation virus); Class 2 (double nucleotide mutation virus). Mann Whitney test between the two classes showed a significant difference (p<0.0016).

Figure 3.8: Comparison of the replication capacity of variants to the wildtype. Student's t-test between the two-group showed a significant difference (p<0.0001).

Figure 3.9: Correlation of the number of nucleotides change in the sequence with replication capacity. **Pearson correlation (r) and p-value were calculated as shown. Results displayed a significant correlation between the number of nucleotides change in the sequence and replication capacity.**

3.3.4 Phenotypic Drug Susceptibility

3.3.4.1 Correlation of Replication Capacity and IC50

No correlation between replication capacity and IC50 was observed for the mutant viruses phenotyped with TDF. This indicated that IC50 was independent of replication capacity (Figure 3.10).
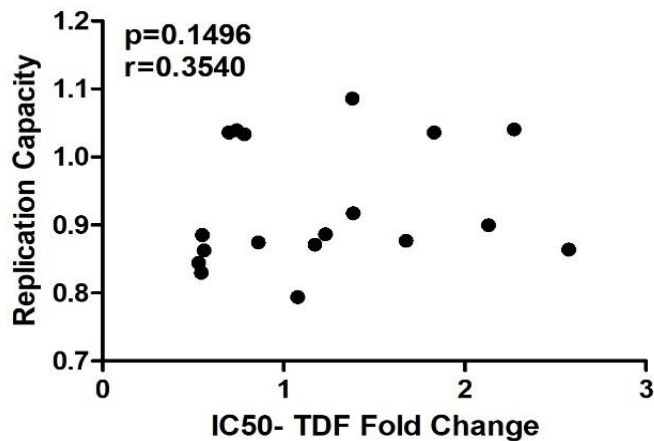
Figure 3.10: Correlation of IC50 with replication capacity for mutant viruses treated with TDF. **Spearman correlation (r) and p-value were calculated as shown. No correlation between IC50 and replication capacity was demonstrated.**

3.3.4.2 Number of nucleotide mutations in the sequence and IC50 for TDF.
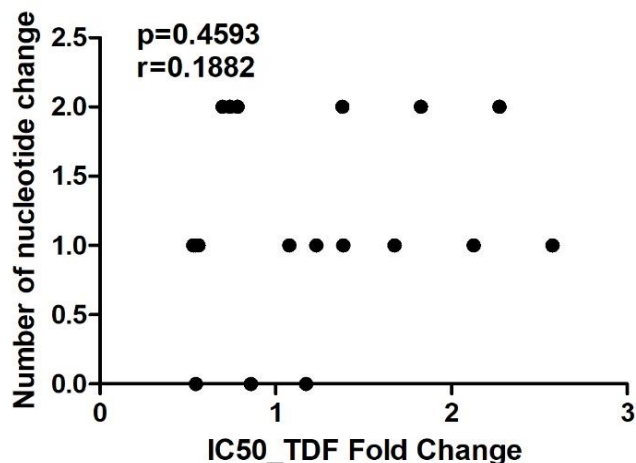
Figure 3.11: Comparison between the number of nucleotides introduced in the sequence and IC50 for TDF. Spearman's correlation showed no significant correlation between the number of nucleotides introduced in the sequence and IC50 for TDF.

3.3.4.3 Phenotypic drug susceptibility profiles of TDF selected recombinant Mutants

Five mutant viruses, as well as subtype B and C wildtypes, were used in the phenotypic drug susceptibility assay. The FC in IC50 and associated standard deviation, for TDF, is provided for each virus. Also, the susceptibility profile of each virus is provided, where S represents susceptible viruses and RS represents viruses with reduced susceptibility (Table3.3 and Figure 3.12). The cut-off value for TDF is highlighted in section 3.2.3.4 and Table 3.1. Of the five mutants phenotyped only, two displayed reduced susceptibility to TDF (K65R and K65R+S68N and three mutants (A62V, S68N, and Y115F) were susceptible to TDF (Table 3.3 and Figure 3.12). Also, the dose-response curves corresponding to the susceptible HIV-1 subtype B (pNL4-3) control virus strain and the sample virus are displayed for TDF (Figure 3.13).

Table 3.3: Overview of IC50 and FC of TDF for HIV-1 RT selected variants.

Variants		TDF		Susceptibility profile
		Mean IC50 (μM) ± SD[a]	Fold change (FC) ±SD[b]	
Subtype-B wildtype: pNL4-3		3.77 ± 0.05	1	S
Subtype-C wildtype: PKE04N		3.20 ± 0.47	0.86 ± 0.51	S
A62V		2.06 ± 0.01	0.55 ± 0.04	S
K65R		7.97 ± 0.25	2.13 ± 0.30	RS
S68D		2.80 ± 0.13	0.70 ± 0.08	S
Y115F		4.66 ± 0.22	1.23 ± 0.18	S
K65R+S68N		6.84 ± 0.3	1.83 ± 0.35	RS

[a] The IC50 (50% inhibitory concentration) values were estimated by calculating inhibition of luminescence in Tzm-bl cells. Values are averages ± standard deviations from at least two independent experiments of triplicates. [b] Fold change in IC50 compared with the wild type. Average fold change was calculated by the average fold of the fold change (mutant versus wildtype) calculated of each replicate assay. Abbreviations: FC – fold change; RS – reduced susceptibility; S – susceptible; SD – standard deviation.

D.

FC= 2.13

% Inhibition vs Log Concentration μM (TDF)

Subtype B wildtype: pNL4-3
K65R

E.

FC=2.6

% Inhibition vs Log Concentration μM (TDF)

Subtype C wildtype:PKE04N
K65R

F.

FC=0.74

% Inhibition vs Log Concentration μM (TDF)

Subtype B wildtype: pNL4-3
S68D

G.

FC=1.0

% Inhibition vs Log Concentration μM (TDF)

Subtype C wildtype:PKE04N
S68D

H.

FC=1.23

% Inhibition vs Log Concentration μM (TDF)

Subtype B wildtype: pNL4-3
Y115F

I.

FC=1.7

% Inhibition vs Log Concentration μM (TDF)

Subtype C wildtype:PKE04N
Y115F

Figure 3.12: TDF susceptibility profiles (A-K). Dose-response curves of the susceptible subtype B wildtype (pNL4-3) (blue), subtype C wildtype (PKE04N) (green) and to the mutant (red) are shown. (Doses in µM in the x-axes). IC50 values were determined using a sigmoid dose-response curve with variable slope (GraphPad Prism, version 5.0), and fold change (FC) values were determined by dividing the IC50 of the mutant by the wildtype IC50 for TDF.

3.4   Discussion

Various studies have investigated the impact of the TDF resistance mutations on replication capacity and drug susceptibility in HIV-1 subtype B as well as in Subtype C with more emphasis on K65R. In those studies, K65R showed a RC ranging from 0.20 to 0.82 relative to the WT virus (Miller, 2004b, Miller et al., 2004, Xu et al., 2015, Xu et al., 2009, Cong et al., 2007, Dapp et al., 2013) and FC of 1.7 to 5.0 in TDF susceptibility (Wainberg et al., 1999, Srinivas and Fridland, 1998, Rhee et al., 2006, Li et al., 2013b, Miller et al., 2003, Kagan et al., 2007, Lanier et al., 2004, Vermeiren et al., 2007). Recent studies have found more drug resistance mutations: "A62V, K65N, S68G/N/D, K70E/Q/T, L74I, V75L, and Y115F" in patients receiving TDF treatment. These mutations are often in association with K65R and are frequently seen in subtype C (Maphumulo, 2016, Margot et al., 2016, Rhee et al., 2017, TenoRes-Study, 2016). Therefore, it was necessary to investigate their phenotypic effects.

In this chapter, replication capacity was measured for five mutants (A62V, K65R, S68D, Y115F, and K65R+S68N) generated by site-directed mutagenesis. These mutants were selected as they were more frequently associated with TDF resistance in patients receiving TDF based regimen and also yielded replication-competent viruses. These mutations were evaluated for their impact on replication capacity and drug (TDF) susceptibility in an HIV-1 subtype C virus.

Replication capacity and drug susceptibility profile of HIV-RT selected resistance variants to TDF

The A62V mutant showed reduced replication capacity compared to the WT virus. This is in line with previous studies (Maldonado and Mansky, 2018, Dapp et al., 2013). Although in this study, the reduced RC was somewhat higher (0.86 fold) compared to previous studies reports (0.67 and 0.57-fold) (Dapp et al., 2013, Maldonado and Mansky, 2018). These differences may have been due to subtype variation. Also, A62V showed increased susceptibility (0.55-fold change) to TDF. This finding supports the argument that A62V does not confer resistance to TDF (Cases-Gonzalez et al., 2007, Maldonado and Mansky, 2018).

The K65R mutation has been well characterized in both subtype B and C viruses (Deval et al., 2004, Miller, 2004a, Miller et al., 2004, White et al., 2002, Xu et al., 2009, Xu et al., 2015). In this study, K65R mutation displayed reduced RC (0.88) compared to the WT virus. This outcome is similar to previous findings (Svarovskaia et al., 2008); however, other studies reported that the K65R mutation had the same replication capacity as the wild-type virus (García-Lerma et al., 2003, Gu et al., 1994b). In addition, lower RC values (range 0.24 to 0.60 relative to the WT virus) have also been reported (Xu et al., 2015, Xu et al., 2009, Cong et al., 2007, Dapp et al., 2013) than that obtained in this study.

Several approaches have been employed to determine HIV-1 replication capacity (Quinones-Mateu, 2001, Quinones-Mateu and Arts, 2002, Quinones-Mateu and Arts, 2006), oftentimes with varying outcomes as a result of the type of assay utilized to evaluate viral replication. A study using primary HIV-1 isolates have likewise demonstrated reduced replication in viruses harbouring the K65R mutation (Weber et al., 2005). In this study, however, we used *pol* recombinant viruses (based on site-directed mutagenesis) as previously described to determine the implication of this mutation on replication capacity (Brockman et al., 2007, Brockman et al., 2006). Furthermore, a clinical study has reported a reduction in the viral replication capacity in patients harbouring K65R mutation with the RC range of 0.20 to 0.82%, of the wild type (Miller, 2004b, Miller et al., 2004). Also, studies have likewise demonstrated reduced replication with the K65R mutation using either single-cycle or competition assays (Deval et al., 2004, White et al., 2002). Therefore, discrepancies between the assay method or the impact of other mutations not found in this recombinant mutant virus may be attributed to the variation in the result obtained. Instead, one study has revealed that reduction in replication is primarily connected to the decrease intake of the nascent substrate by the K65R mutant RT (Deval et al., 2004).

In addition, K65R mutation displays reduced susceptibility to TDF with a 2.1 FC relative to the WT virus. This is in line with previous studies who also found a 2.1 and 2.3 FC relative to the WT virus (Kagan et al., 2007, Lanier et al., 2004). However, other previous studies reported highly reduced susceptibility to TDF with a fold change ranging from 3.0 to 5.0 (Wainberg et al., 1999, Srinivas and Fridland, 1998, Rhee et al.,

2006, Li et al., 2013b). While Miller in 2003 reported a 1.7 fold change to TDF (Miller et al., 2003). These variations observed are possibly due to the reasons highlighted above.

The mutant K65R+S68N in this analysis showed RC similar to the WT virus, while it displayed an increase in RC compared to K65R alone in the absence of the drug. A 1.8-fold decrease in susceptibility to TDF was seen for the double mutant, whereas K65R alone resulted in a 2.1-fold reduction in TDF susceptibility. This result is supported by (Melikian et al., 2012) who showed that K65R+S68N displayed a 1.9-fold reduction to TDF. However, higher fold change ranging from 2.8 to 3 have been shown by other studies (Rhee et al., 2006, Margot et al., 2015, Margot et al., 2006b).

The S68D mutation exists as a polymorphism of RT at codon 68 and not much attention has been given to this mutation. In this study, S68D displayed an RC similar to the WT virus and a 0.74-fold change to TDF, as expected from a natural polymorphism. Our findings are in agreement with the previous findings on S68G (García-Lerma et al., 2000). Further studies will be needed to explain the effect of different amino acid variants at RT codon 68. To our knowledge, this is the first study to investigate S68D alone on replication capacity and susceptibility to TDF.

The Y115F mutation is not frequently accompany drug resistance; it is found in about 10% of patients on abacavir (ABC) treatment alone and in 1% of patients on combination treatments. It has been observed to be found frequently in combination with the K65R, L74V, and M184V mutations (Stone et al., 2004, Miller et al., 2000). In our investigation, we analysed the Y115F mutation alone, which showed a 0.87 replication capacity compare to the wild-type virus in the absence of TDF and a fold change of 1.23 in the presence of TDF. This outcome is similar to previous studies stating that Y115F has little impact on TDF susceptibility in the absence of K65R (Harrigan et al., 2000, Miller et al., 2000, Stone et al., 2004, Vermeiren et al., 2007, Lanier et al., 2004). In contrast, Ross and colleagues in 2006 reported that Y115F displayed a fold change of 1.8 to TDF (Ross et al., 2006).

Number of nucleotides introduced in the sequence, replication capacity and TDF susceptibility

In this present study, we assessed the implication of the number of nucleotides introduced in the sequence with replication capacity as well as with the FC in IC50 of TDF. The results displayed a significant correlation between the number of nucleotides introduced in the sequence and replication capacity. There was also no correlation between the number of nucleotides introduced in the sequence and susceptibility to TDF. Further, no correlation was observed between drug susceptibility and replication capacity implying that replication capacity cannot be used as a measure of treatment outcomes.

However, some of the effects of these mutations on RC might have been facilitated by the number of base (nucleotide) changes in the sequence as observed. In this study, mutants were classified into two groups (single nucleotide mutants and double nucleotide mutant) based on the number of nucleotide changes in the sequence. For example, the single nucleotide mutant viruses (A62V, K65R, and Y115F) had the lowest RC (0.86, 0.88 and 0.87) compared to that of the WT, whereas the double nucleotide change viruses (S68D and K65R_S68N) had RC (1.04 and 1.05) similar to the WT. This finding is in concordance with the previous report that resistant HIV-1 isolates with more nucleotide change may display high replication capacity under a condition where fitness is essential (Wain-Hobson and Morse, 1994); however, another study suggested that high nucleotide alteration was neither associated with reduced nor high fitness (Dapp et al., 2013).

3.5   Conclusion

In this chapter, we described the impact of selected TDF resistance mutations on viral replication capacity and TDF susceptibility. Mutant A62V, K65R, and Y115F viruses displayed reduced replicative fitness, suggesting that these less adapted viruses might be beneficial and better managed than wild-type viruses (Deeks, 2001, Quinones-Mateu, 2001). In addition, out of the five mutants analysed, three of the mutants (A62V, S68D, and Y115F) were susceptible to TDF and the mutants with K65R (K65R and K65R+S68N) had reduced susceptibility. Thus, additional investigations are needed to evaluate the effects of combinations of these single mutations with K65R on replication capacity and drug susceptibility.

CHAPTER 4

DISCUSSION AND CONCLUSION

Chapter Four

Discussion and Conclusion

4.1   Discussion

In this study, we investigated the impact of TDF selected resistance mutations on replication capacity and TDF susceptibility on the HIV-1 subtype C virus. To have a better understanding of the impact of TDF selected resistance mutations, we first employed site-directed mutagenesis to generate the desired mutations of interest. These mutations were grouped into two: single resistance mutants (A62V, K65R, S68D, S68G, S68N, and Y115F) and double resistance mutant in combination with K65R (K65R+A62V, K65R+S68D, K65R+S68G, K65R+S68N, and K65R+Y115F. Furthermore, these resistance mutations were chosen as they were observed to be found frequently in patients on TDF treatment (Maphumulo, 2016, Margot et al., 2016, Rhee et al., 2017, Shafer, 2017). All the single resistance mutants were successfully generated, while only one of the double resistance mutants (K65R+S68N) out of five was successfully generated. Failure to generate these other double resistance mutants may be due to the nature of the primer used in this study. Single independent primers were used in combinations as opposed to the use of single independent primers harbouring the desired double mutations due to the proximity of the positions (codon 62, 65 and 68) (Zheng et al., 2004, Weiner et al., 1994). The following mutants: A62V, K65R S68D, Y115F, and K65R+S68N were successfully generated. We then analyse the effect of these mutants alongside the subtype B and C wild-types viruses on the replication capacity and TDF susceptibility.

In our analysis of the replication capacity of TDF-selected mutant, we demonstrated that mutants with only one nucleotide base change in the sequence had significantly lower replication capacity relative to the WT virus than mutants with two/double nucleotide base change in the sequence as they are fitter. This observation is consistent with past findings, highlighting that resistant HIV-1 isolates with more nucleotide changes may be more adapted under a condition where fitness is favourable (Wain-Hobson and Morse, 1994). In contrast, another study hypothesized that an increase in nucleotide change was neither associated with reduced nor high fitness (Dapp et al., 2013).

The A62V mutation is situated in the finger region of HIV-1 RT, specifically in the flexible β3-β4 strand region and is usually observed in various mutational combinations such as the T69SSS insertion complex (Cases-Gonzalez et al., 2007) and the multi-dideoxynucleoside resistance (MDR) Q151M complex drug resistance (Shirasaka et al., 1995). An earlier study revealed that A62V confers a slight reduction in replication capacity (Dapp et al., 2013); however, it does not cause resistance (Maldonado and Mansky, 2018). Similarly, in this study, A62V displayed decreased replication capacity relative to the WT virus and also had the highest susceptibility to TDF. This observation confirmed the hypothesis that A62V does not

confer resistance to TDF on its own (Maldonado and Mansky, 2018). A62V has also been found to be associated with K65R (Margot et al., 2006a, Rhee et al., 2006, Rhee et al., 2017); however, we were unfortunately not able to do this analysis in this study.

The K65R mutation is found in the conserved IKKK domain comprising of 12 nucleotide bases (ranging from codon 63 to 66) found in the finger's subdomain of HIV-1 RT. The substitution of lysine (K) to arginine (R) at position 65 is said to be influenced by the movement resulting from the competition between the catalytic protein, the natural substrates and the RT inhibitors. Consequently, the correct substrate interaction or the successful phosphodiester bond generation of RT may be altered (Huang et al., 1998, Sarafianos et al., 1999b, Sarafianos et al., 2009). The mutant K65R has been reported to exhibit broad resistance to other NRTIs except for AZT (Deval et al., 2004, Margot et al., 2002, Wensing et al., 2017). In this study, K65R displayed a decrease in replication capacity (0.88) compared to the WT virus. This observation is similar to a previous report (Svarovskaia et al., 2008), while other studies have reported an even lower replication capacity relative to the WT virus compared to the value obtained in this study (Xu et al., 2015, Xu et al., 2009, Cong et al., 2007, Dapp et al., 2013). However, two studies have observed that there are no differences between the replication capacity of K65R and the wild-type virus (García-Lerma et al., 2003, Gu et al., 1994a).

In this investigation, K65R exhibited reduced susceptibility to TDF. This is in alignment with past findings (Kagan et al., 2007, Lanier et al., 2004, Vermeiren et al., 2007). However, other studies have reported high resistance to TDF as opposed to this study (Wainberg et al., 1999, Srinivas and Fridland, 1998, Rhee et al., 2006, Li et al., 2013b). Variations between assay techniques employed or the absence of other mutations not see in this recombinant mutant virus may give rise to these variations in the results obtained.

Clinical studies have reported the combination of K65R and S68N (Margot et al., 2006a, Margot et al., 2015, Rhee et al., 2006). In the present study, we explored the impact of K65R+S68N on replication capacity and TDF susceptibility. We observed that the K65R+S68N mutant had a considerably higher RC than the K65R mutant alone in the absence of TDF. However, there was no significant impact on susceptibility to TDF between K65R+S68N and K65R alone. This was consistent with previous studies (Margot et al., 2006b, Roge et al., 2003, Svarovskaia et al., 2008). Our investigation confirmed the hypothesis that S68N may have a similar compensatory function as S68G regarding the replication capacity of the K65R mutant (Margot et al., 2015, Margot et al., 2006b, Svarovskaia et al., 2008). Although clinical studies have reported a higher FC to TDF (2.8 to 3.0) (Margot et al., 2006a, Margot et al., 2015, Rhee et al., 2006) compared to the FC (1.8) obtained in this study, the differences may be as a result of clinical isolates harbour other mutations not found in our recombinant viruses. K65R has been observed in

association with S68D in a few patients receiving TDF based treatment, although there is currently very little data available (Margot et al., 2016, Rhee et al., 2017, Shafer, 2017).

The S68D mutation is found in the finger region of the flexible β3-β4 chain near to the polymerase hotspot of HIV-1 RT (Huang et al., 1998) and it is believed to be polymorphic. The current study was the first to investigate the impact of S68D on replication capacity and TDF susceptibility. We showed that the S68D mutant displayed similar replication capacity as that of the wildtype B virus and was highly susceptible to TDF as anticipated of a natural polymorphism (García-Lerma et al., 2000).

The Y115F mutation is situated in the polymerase catalytic site of the HIV-1 RT (Huang et al., 1998, Sarafianos et al., 2009) and the presence of Y115F mutation has been linked to alterations to the hydrophobic interactions in the RT functional position that affects the proper functioning of the polymerase (Ray et al., 2002). In our analysis, Y115F showed impaired replication capacity and slightly reduced susceptibility to TDF. This was consistent with past studies (Harrigan et al., 2000, Miller et al., 2000, Stone et al., 2004, Vermeiren et al., 2007, Lanier et al., 2004). This further supports the statement that Y115F has little impact on TDF susceptibility. However, another study did report reduced susceptibility to TDF (Ross et al., 2006). Difference between assay protocols used, the presence of other mutations found in clinical isolate or the effect of cART used in clinical isolates in contrast to the recombinant virus used in this study could have resulted in these variations of the findings.

4.2   Conclusion

In this study, we described the impact of TDF- selected mutations on replication capacity and TDF susceptibility. Most of the viruses displayed diminished replicative fitness in the absence of TDF and were susceptible to TDF, except for K65R and K65+S68N which showed reduced susceptibility. Our findings support the hypothesis that TDF-selected mutations only confer a moderate decrease in TDF susceptibility (Shafer, 2017). Hence, TDF may still be used for treatment in individuals harbouring these mutations alone, as its antiretroviral activity is still intact.

4.3   Study limitation

The inability to generate mutant viruses with single mutations in combination with K65R did not allow for the determination of the role of these as compensatory mutations in the presence of K65R.

4.4   Recommendation for future studies

- Further combinations of TDF drug-resistant HIV mutants need to be generated and their effect on replication capacity and drug susceptibility needs further investigation.

- Further study is required to determine how the number of nucleotide base changes (mutations) in the virus sequence can impact on viral fitness.

- The effect of amino acid variants at codon 68 of HIV-1 RT on viral fitness and drug susceptibility needs further examination.

Chapter 5

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Appendix A: Site directed mutagenesis primers

<u>**Mutation: A62V**</u>

Primer sequences:

Primer Name	Primer Sequence (5' to 3')
c68t_antisense	5'-tagtactgtccttcttttttatgacaaatactggagtgttatatgga-3'
c68t_	5'-tccatataacactccagtatttgtcataaaaaagaaggacagtacta-3'

Oligonucleotide information:

Primer Name	Length (nt.)	Tm	Duplex Energy at 68 °C	Energy Cost of Mismatches
c68t_antisense	47	78.10℃	-41.71 kcal/mole	9.70%
C68t_	47	78.10℃	-45.52 kcal/mole	9.58%

Primer-template duplexes:

Primer Name	Primer-Template Duplex
c68t_antisense	a a a t c c a t a t a a c a c t c c a g t a t t t g c c a t a a a a a a g a a g g a c a g t a c t a a g t |||||||||||||||||||||| ||||||||||||||||||||||| 3 ' - a g g t a t a t t g t g a g g t c a t a a a c a g t a t t t t t t c t t c c t g t c a t g a t - 5 '
C68t_	5 ' - t c c a t a t a a c a c t c c a g t a t t t g t c a t a a a a a a g a a g g a c a g t a c t a - 3 ' |||||||||||||||||||||| |||||||||||||||||||||||| t t t a g g t a t a t t g t g a g g t c a t a a a c g g t a t t t t t t c t t c c t g t c a t g a t t c a

<u>**Mutation: K65R**</u>

Primer sequences:

Primer Name	Primer Sequence (5' to 3')
a77g_ antisense	5'-cttagtactgtccttccttttttatggcaaatactggagtgttatat-3'
a77g_	5'-atataacactccagtatttgccataaaaaggaaggacagtactaag-3'

Oligonucleotide information:

Primer Name	Length (nt.)	Tm	Duplex Energy at 68 °C	Energy Cost of Mismatches
a77g_ antisense	46	78.02℃	-40.70 kcal/mole	.09%
a77g_	46	78.02℃	-45.61 kcal/mole	1.70%

Primer-template duplexes:

Primer Name	Primer-Template Duplex
a77g_ antisense	t c c a t a t a a c a c t c c a g t a t t t g c c a t a a a a a a g a a g g a c a g t a c t a a g t g g |||||||||||||||||||||||| |||||||||||||||| 3 ' - t a t a t t g t g a g g t c a t a a a c g g t a t t t t t c c t t c c t g t c a t g a t t c - 5 '
a77g_	5 ' - a t a t a a c a c t c c a g t a t t t g c c a t a a a a a g g a a g g a c a g t a c t a a g - 3 ' |||||||||||||||||||||||| |||||||||||||||| a g g t a t a t t g t g a g g t c a t a a a c g g t a t t t t t t c t t c c t g t c a t g a t t c a c c

Mutation: S68N

Primer sequences:

Primer Name	Primer Sequence (5' to 3')
g86a_antisense	5'-ctactaatttcctccacttagtattgtccttcttttttatggcaaat-3'
g86a___	5'-atttgccataaaaaagaaggacaatactaagtggaggaaattagtag-3'

Oligonucleotide information:

Primer Name	Length (nt.)	Tm	Duplex Energy at 68 °C	Energy Cost of Mismatches
g86a_antisense	47	78.10°C	-40.55 kcal/mole	6.88%
g86a___	47	78.10°C	-45.22 kcal/mole	4.58%

Primer-template duplexes:

Primer Name	Primer-Template Duplex
g86a_ antisense	a g t a t t t g c c a t a a a a a a g a a g g a c a g t a c t a a g t g g a g g a a a t t a g t a g a t t ||||||||||||||||||||||| ||||||||||||||||||||| 3 ' - t a a a c g g t a t t t t t t c t t c c t g t t a t g a t t c a c c t c c t t t a a t c a t c - 5 '
g86a_	5 ' - a t t t g c c a t a a a a a a g a a g g a c a a t a c t a a g t g g a g g a a a t t a g t a g - 3 ' |||||||||||||||||||||| |||||||||||||||||||| t c a t a a a c g g t a t t t t t t c t t c c t g t c a t g a t t c a c c t c c t t t a a t c a t c t a a

Mutation: S68G

Primer sequences:

Primer Name	Primer Sequence (5' to 3')
a85g_ antisense	5'-tactaatttcctccacttagtaccgtccttctttttttatggcaaata-3'
a85g_	5'-tatttgccataaaaaagaaggacggtactaagtggaggaaattagta-3'

Oligonucleotide information:

Primer Name	Length (nt.)	Tm	Duplex Energy at 68 °C	Energy Cost of Mismatches
a85g_ antisense	47	78.10°C	-42.02 kcal/mole	3.70%
a85g_	47	78.10°C	-45.75 kcal/mole	3.70%

Primer-template duplexes:

Primer Name	Primer-Template Duplex
a85g_ antisense	c a g t a t t t g c c a t a a a a a a g a a g g a c a g t a c t a a g t g g a g g a a a t t a g t a g a t |||||||||||||||||||| ||||||||||||||||||||||| 3 ' - a t a a a c g g t a t t t t t t c t t c c t g c c a t g a t t c a c c t c c t t t a a t c a t - 5 '
a85g_	5 ' - t a t t t g c c a t a a a a a a g a a g g a c g g t a c t a a g t g g a g g a a a t t a g t a - 3 ' ||||||||||||||||||||| ||||||||||||||||||||||| g t c a t a a a c g g t a t t t t t t c t t c c t g t c a t g a t t c a c c t c c t t t a a t c a t c t a

Mutation: S68D

Primer sequences:

Primer Name	Primer Sequence (5' to 3')
a85g_g86a_antisense	5'-aatctactaatttcctccacttagtatcgtccttcttttttatggcaaatactg-3'
a85g_g86a_	5'-cagtatttgccataaaaaagaaggacgatactaagtggaggaaattagtagatt-3'

Oligonucleotide information:

Primer Name	Length (nt.)	Tm	Duplex Energy at 68 °C	Energy Cost of Mismatches
a85g_g86a_antisense	54	78.20°C	-46.88 kcal/mole	7.98%
a85g_g86a_	54	78.20°C	-51.33 kcal/mole	6.47%

Primer-template duplexes:

Primer Name	Primer-Template Duplex
a85g_g86a_antisense	c t c c a g t a t t t g c c a t a a a a a a g a a g g a c a g t a c t a a g t g g a g g a a a t t a g t a g a t t t c a ||||||||||||||||||||||| |||||||||||||||||||||||| 3 ' - g t c a t a a a c g g t a t t t t t t c t t c c t g c t a t g a t t c a c c t c c t t t a a t c a t c t a a - 5 '
a85g_g86a_	5 ' - c a g t a t t t g c c a t a a a a a a g a a g g a c g a t a c t a a g t g g a g g a a a t t a g t a g a t t - 3 ' |||||||||||||||||||||||| |||||||||||||||||||||||| g a g g t c a t a a a c g g t a t t t t t t c t t c c t g t c a t g a t t c a c c t c c t t t a a t c a t c t a a a g t

Mutation: Y115F

Primer sequences:

Primer Name	Primer Sequence (5' to 3')
a227t_antisense	5'-catctaaaggaaccgaaaaaaatgcatcccccacatctaat-3'
a227t_	5'-attagatgtgggggatgcattttttcggttcctttagatg-3'

Oligonucleotide information:

Primer Name	Length (nt.)	Tm	Duplex Energy at 68 °C	Energy Cost of Mismatches
a227t_antisense	41	78.60℃	-47.67 kcal/mole	.37%
a227t_	41	78.60℃	-45.52 kcal/mole	5.51%

Primer-template duplexes:

Primer Name	Primer-Template Duplex
a227t_ antisense	a g t a t t a g a t g t g g g g g a t g c a t a t t t t t c g g t t c c t t t a g a t g a a a ||||||||||||||||||| ||||||||||||||||||||| 3 ' - t a a t c t a c a c c c c c t a c g t a a a a a a a g c c a a g g a a a t c t a c - 5 '
a227t_	5 ' - a t t a g a t g t g g g g g a t g c a t t t t t t t c g g t t c c t t t a g a t g - 3 ' ||||||||||||||||||| ||||||||||||||||||||| t c a t a a t c t a c a c c c c c t a c g t a t a a a a a g c c a a g g a a a t c t a c t t t

Appendix B: Ethics clearance certificate

UNIVERSITY OF KWAZULU-NATAL

INYUVESI YAKWAZULU-NATALI

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09 November 2018

Mrs CO Didamson (217079876)
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Dear Mrs Didamson

Protocol: The Differential influence of HIV-1 subtype C, nucleoside analog resistance mutations: K65R, A62V, S68N, and Y115F on tenofovir susceptibility.   Degree: MMedSC
BREC Ref No: BE600/17

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 30 October 2018
Expiration of Ethical Approval: 29 October 2019

I wish to advise you that your application for Recertification received on 02 November 2018 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 11 December 2018.

Yours sincerely

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc postgraduate administrator: dudhraihp@ukzn.ac.za
cc supervisor: Tarinm@ukzn.ac.za

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13 August 2019

Mrs CO Didamson (217079876)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
christyuzoka@gmail.com

Dear Mrs Didamson

Protocol: The Differential influence of HIV-1 subtype C, nucleoside analog resistance mutations:
K65R, A62V, S68N, and Y115F on tenofovir susceptibility.
Degree: MMedSC
BREC Ref No: BE600/17
NEW TITLE: The Differential influence of HIV-1 subtype C, nucleoside analogue resistance
mutations: K65R, A62V, S68D/G/N and Y115F on Tenofovir susceptibility.

We wish to advise you that your application for Amendments received on 05 August 2019 to change the title to the above for the above study has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee.

The committee will be advised of the above at the next meeting to be held on 10 September 2019.

Yours sincerely

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc postgraduate administrator: codl:ajl.o@ukzn.ac.za
cc supervisor: Tarin m@ukzn.ac.za